MITOCHONDRIAL DYSFUNCTION AND NEURODEGENERATION

EDITED BY: Victor Tapias, Pier Giorgio Mastroberardino and Roberto Di Maio

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MITOCHONDRIAL DYSFUNCTION AND NEURODEGENERATION

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EDITORIAL
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Editorial: Mitochondrial Dysfunction and Neurodegeneration

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Keywords: mitochondrial dysfunction, quality control, neurodegenerative diseases, Parkinson's disease, Alzheimer's disease

Editorial on the Research Topic

Mitochondrial Dysfunction and Neurodegeneration

Neurodegenerative diseases are incurable and inexorably progressive conditions that affect the central nervous system and result in a selective pattern of neuronal death. Parkinson's disease (PD) and Alzheimer's disease (AD) are the most common neurodegenerative diseases. While most cases are idiopathic, studies have confirmed that genetic factors contribute to the pathogenesis of both PD and AD. PD is characterized by loss of dopamine (DA)-producing neurons of the substantia nigra, and as a consequence of a reduction in striatal DA content. A neuropathological hallmark is the presence of Lewy body inclusions in many of the remaining neurons and Lewy-neurite pathology in the neuropil. The classic histopathological hallmarks of AD are the extracellular accumulation of amyloid-β (Aβ) plaques and intracellular deposition of hyperphosphorylated tau into neurofibrillary tangles. Despite distinct clinical and pathological features, the formation of misfolded protein aggregates is a common feature of neurodegenerative diseases, which can be mainly classified into synucleinopathies, tauopathies, and amyloidopathies. Neurodegenerative diseases share critical processes, such as mitochondrial anomalies, oxidative damage, and inflammation that are implicated in the gradual loss of neuronal function and cell death.

A plethora of reports indicate that mitochondrial dysfunction is a central factor in the pathophysiology of neurodegenerative diseases (Lin and Beal, 2006; Tapias et al., 2017, 2018, 2019). Elevated oxidative stress can damage the mitochondrial respiratory chain. Mitochondrial complexes I and III and the mitochondrially located monoamine oxidase (MAO) B are the main source of reactive oxygen and nitrogen species. A region-dependent regulation of MAO has been reported in PD and AD (Tong et al., 2017; Quartey et al.). Furthermore, perturbations in mitochondrial dynamics, mitochondrial transport within axons, mitophagy, and accumulation of somatic mtDNA mutations are associated with impaired mitochondrial function. Compromised mitochondrial quality control mechanisms may lead to the accumulation of defective mitochondria and concomitant oxidative damage, defective calcium (Ca^{2+}) homeostasis and signaling, synaptic pathology, and ferroptotic neuronal death. The present Research Topic aims to critically evaluate the current literature on molecular mechanisms associated with neurodegenerative diseases and it provides novel insights into disturbances in mitochondrial function, which occur during neurodegeneration. This topic also suggests that the development of novel mitochondria-targeted therapeutic strategies may be useful in the treatment of neurodegenerative diseases.

Mechanisms for the maintenance of mitochondrial integrity and functionality are crucial for neuronal survival. Mitochondrial dynamics play a key role in ensuring mitochondrial quality control and are tightly regulated by the fusion/fission machinery, which allows the formation or degradation of a mitochondrial syncytium. The molecular process of fusion is driven by the GTPases Opa1 and Mitofusin-1 (Mfn1) and Mfn2 while dynamin-related protein (Drp1) interacts with the mitochondrial fission 1 protein (Fis1),

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mitochondrial fission factor (Mff) and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/51) to mediate mitochondrial fission.It has been recently shown that mitofilin (Mic60), a component of the MICOS complex that plays a key role in the maintenance of mitochondrial structure and function,can regulate mitochondrial dynamics (Li et al., 2016; Van Laar et al., 2016; Van Laar et al.). Axonal transport, a cellular mechanism responsible for the active trafficking of lipids, proteins, neurotransmitters, and organelles, is essential for neuronal network function and viability. Anterograde transport carries new synthesized material from the cell body to distal axons and is mediated by kinesin motor proteins. Dynein-driven retrograde transport is required for efficient distribution of cargoes from the axon terminals toward the soma. Mitochondrial movement along both microtubule and actin filaments is regulated by a motor adaptor complex that attaches the anterograde kinesin-1 motor and retrograde dynein motor to the outer mitochondrial membrane, in a process mediated by the membrane-anchored Miro (RhoT1/2) and Milton (Trak1/2) proteins (Schwarz, 2013). Decreased mitochondrial trafficking within axons accompanied by inhibited neurite outgrowth was found in cultures of dorsal root ganglia sensory neurons overexpressing the muscarinic acetylcholine type 1 receptor (Sabbir et al.). There is growing evidence of a crosstalk between fusion-fission and axonal flux mitochondrial dynamics and axonal transport integrity (Misgeld and Schwarz, 2017; Tapias et al., 2017; Franco-Iborra et al.; Perez et al.).

Mitophagy is a specialized type of autophagy that mediates the clearance of damaged mitochondria by lysosomes. Mitochondrial autophagy is inextricably linked to protein import since the translocation of the PTEN-induced putative kinase 1 (Pink1) into the mitochondrial inner membrane via the Tim/Tom complex plays a pivotal role in regulating Pink1/Parkin-mediated mitophagy (Poole et al., 2008; Geisler et al., 2010; Vives-Bauza et al., 2010). Moreover, impaired lysosomal degradation can impact mitochondria by causing mitophagy deficits; aminochrome, a product of DA oxidation and the precursor of neuromelanin, induces mitochondrial dysfunction by blocking the selective clearance of damaged mitochondria by autophagy (Segura-Aguilar and Huenchuguala). Protein post-translational modifications such as enzymatic glycosylation and non-enzymatic glycation together with a disruption of the mitochondrial quality control system, result in defective mitophagy and excessive accumulation of dysfunctional proteins (Videira and Castro-Caldas). Altered autophagy phenotypes have recently been associated with optineurin, a multifunctional cargo adaptor protein observed in diverse brain regions of rats after exposure to rotenone (Wise et al.). Mitochondria contribute to aging, mitochondrial-related diseases, and neurodegeneration through the accumulation of somatic mtDNA mutations—point mutations and large-scale deletions (Simon et al., 2001; Dolle et al., 2016; Hoekstra et al., 2016; Chinnery and Gomez-Duran; Emperador et al.). Point mutations are likely to arise from an inefficient base excision repair system while mtDNA deletions and rearrangements may result from errors in replication and/or double-strand break repair (Krishnan et al., 2008). Although the precise mechanism by which mtDNA damage contributes to both aging phenotypes and neurodegeneration remains unclear, a direct relationship between age-related oxidative damage to mtDNA and oxidation of glutathione has been reported in the brains of mice and rats (de la Asuncion et al., 1996).

It has also been reported that there is a link between impaired mitochondrial function and depression (Bansal Kuhad and Kuhad, 2016; Allen et al.). Patients suffering from depression show reduced glucose metabolism in different regions of the brain (Baxter et al., 1989; Gardner et al., 2003). Hypothalamic-pituitary-adrenal axis hyperactivity has been implicated in the upregulation of glucocorticoid synthesis in depression, which plays a pivotal biphasic role in modulating mitochondrial functions. Indeed, following acute and chronic immobilization-induced stress, glucocorticoid receptors regulated the expression of several mitochondrial genes in the rat hippocampus (Hunter et al., 2016).

Sustained synaptic release of glutamate, the primary excitatory neurotransmitter in the mammalian central nervous system and the metabolic precursor for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA), results in the overactivation of the N-methyl-D-aspartate (NMDA) receptors and the subsequent loss of ionic homeostasis and excessive influx of Ca^{2+} into the cell, which causes excitotoxicity. Ca^{2+} is the most important signaling entity in neurons and its levels are tightly regulated by organelles such as mitochondria and the ER and by buffering through Ca^{2+}-binding proteins, such as calmodulin, calbindin, and parvalbumin. As shown in some manuscripts of this Research Topic, disruption of the processes underlying Ca^{2+} homeostasis and signaling have been consistently observed in neurodegenerative diseases and glaucoma (Cheung et al.; Muller et al.; Verma et al.; Barodia et al., 2019; Schrank et al., 2019). The acidic C-terminus of α-synuclein (α-syn) contains a Ca^{2+}-binding domain and a transient increase in free intracellular Ca^{2+} can accelerate α-syn aggregation (Nath et al., 2011; Follett et al., 2013). Oligomeric forms of α-syn can exacerbate the intracellular concentration of Ca^{2+} by forming pore-like structures in the plasma membrane (Pacheco et al., 2015). α-Syn can interact with calmodulin in a Ca^{2+}-dependent manner, resulting in an increased rate of α-syn fibrillation (Martinez et al., 2003). α-Syn causes sustained elevations of cytosolic Ca^{2+} and it initiates a toxic calmodulin–calcineurin cascade, which contributes to DA neuronal death (Caraveo et al., 2014; Luo et al., 2014). Disturbances in Ca^{2+} homeostasis promote Aβ formation and tau hyperphosphorylation (Buxbaum et al., 1994; LaFerla, 2002; Mattson and Chan, 2003). There are deleterious effects of presenilin 1 and synthetic Aβ oligomers in producing Ca^{2+} dysregulation, which can induce a rapid Ca^{2+} release mediated by the ryanodine and inositol triphosphate receptors (Mattson et al., 1992; Stutzmann et al., 2003; Demuro et al., 2005). *In vivo* experiments have shown that Aβ plaque deposition promotes Ca^{2+} overload and calcineurin activation, which leads to downstream synaptic and dendritic spine pathology (Kuchibhotla et al., 2008; Wu et al., 2010). Age-dependent alterations in mitochondrial Ca^{2+} efflux accelerate memory deficits and increase both amyloidosis and tau hyperphosphorylation in 3xTg-AD mice (Jadiya et al., 2019). Rescue of the expression of NCLX (a critical component of the mitochondrial Na^{+}/Ca^{2+} exchange) in these mice restored cognitive function and attenuated hippocampal

neuronal degeneration. Elevated Ca^{2+} influx plays a key role in promoting pathological tau phosphorylation via modulation of Ca^{2+}-binding proteins and/or dysregulation of the enzymatic activity of kinases and phosphatases (Zempel et al., 2010; Mairet-Coello et al., 2013).

Two main pathways cell death have been distinguished, namely apoptosis (programmed cell death) and necrosis (accidental cell death). Ferroptosis, a term coined in 2012, is a form of regulated cell death induced by erastin which is characterized by the iron-dependent accumulation of lipid hydroperoxides with a genetic, morphological, and biochemical profile different from apoptosis and necrosis (Dixon et al., 2012). Several biological processes determine the sensitivity to ferroptosis, such as the metabolism of amino acids, polyunsaturated fatty acids, and iron as well as the biosynthesis of glutathione, NADPH, coenzyme Q10, selenium, and phospholipids (Stockwell et al., 2017). Evidence supporting an involvement of ferroptosis in the pathogenesis of neurodegenerative diseases include iron accumulation, lipid peroxidation, depletion of GSH, and mutations in the transferrin and cerulopasmin encoding gene (Guiney et al., 2017). Deficient regulation of ferroptosis has been described in PD. Toxin-mediated ferroptotic activation was observed in LUHMES cells, MPTP-treated mice, and organotypic slice cultures (Do Van et al., 2016). The conversion of arachidonic acid—one of the main substrates of lipid peroxidation for ferroptosis—to polar degradation products was substantially accelerated in the hippocampus of different transgenic mouse models of AD as well as in post-mortem hippocampal tissue form patients with AD (Furman et al., 2016). Ferroptotic cell death can be triggered through diverse mechanisms. Upregulation of the selenoenzyme glutathione peroxidase 4 activity or treatment with ferroptosis inhibitors can confer neuroprotection in different cellular and animal models of PD and AD (Friedmann Angeli et al., 2014; Do Van et al., 2016; Guiney et al., 2017; Hambright et al., 2017). The nuclear factor erythroid-2-related factor 2 (Nrf2) transcriptionally regulates numerous genes involved in both oxidative damage and inflammation, which are implicated in ferroptosis. It indirectly controls the lipid content that is a critical determinant of sensitivity to ferroptotic cell death (Doll et al., 2017). Therefore, it has been suggested that compounds which target Nrf2 may counteract ferroptotic-mediated neuronal loss and exert beneficial effects in the treatment of neurogenerative diseases (Abdalkader et al.). Although pathologically-related aggregate species of α-syn, Aβ and tau regulate lipid peroxidation, glutathione levels, and iron homeostasis, as yet no studies have explored their potential role in ferroptotic cell death.

In conclusion, this special issue provides scientists and clinicians with new insights into the molecular mechanisms underlying the role of mitochondrial dysfunction in the pathophysiology of neurodegenerative diseases such as PD and AD. Furthermore, it may provide further rationale for the development of effective therapeutic interventions targeting mitochondria to treat these devastating illnesses.

AUTHOR CONTRIBUTIONS

VT confirms being the sole contributor of this work and has approved it for publication.

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Targeted Delivery of Mitochondrial Calcium Channel Regulators: The Future of Glaucoma Treatment?

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Keywords: calcium, mitochondria, glaucoma, mitochondrial drug delivery system, calcium channel blockers

INTRODUCTION

Glaucoma is a multifactorial neurodegenerative disease affecting 64.3 million people worldwide (Tham et al., 2014). Despite vigorous research on new treatments, those that reduce intraocular pressure (IOP) remain the gold standard. However, their effectiveness has been questioned as they only slow down degeneration without significantly reversing or stopping the disease (Osborne et al., 2016b). Recent studies have, therefore, investigated the causative roles of other processes, including glutamate toxicity, glial overactivation, etc., (Mann et al., 2005; Chong and Martin, 2015; Lopez Sanchez et al., 2016; Vecino et al., 2016).

Mitochondrial dysfunction is another widely studied causal process in the development of glaucoma and has also been investigated as a potential drug target. For example, red light therapy, manipulation of the mammalian target of rapamycin (mTOR) pathway, and nicotinamide treatment are three recently investigated clinical therapies for glaucoma-related mitochondrial dysfunction (Osborne et al., 2016a,b; Williams et al., 2017). Mitochondrial activity is intimately linked to oxidative metabolism and reactive oxygen species (ROS) formation (Schieke et al., 2006). ROS production is known to cause retinal ganglion cell (RGC) apoptosis and subsequent vision loss. Furthermore, while mitochondrial function is regulated by multiple pathways, calcium signaling likely plays a key role (Vosler et al., 2008; Hurst et al., 2017). In fact, plasma membrane calcium channel inhibitors were recently found to arrest acute axonal degeneration and improve regeneration after optic nerve crush (Ribas et al., 2017). A different combination of calcium permeability inhibitors also preserved optokinetic reflex following partial optic nerve transection (Savigni et al., 2013). While the inhibitors utilized in these studies targeted calcium channels in the plasma membrane, their effects indicate that ROS generation and calcium signaling, which are significantly regulated by the mitochondria, are critical during glaucoma pathogenesis.

Recently, a mitochondrial-specific drug delivery system was shown to be effective in increasing drug concentration in mitochondria in hepatic injuries and drug-resistant cancer cells (Yamada and Harashima, 2017; Yamada et al., 2017). However, the full potential of this system (and other similar systems) has not been fully evaluated with regards to calcium regulation in the diseased retina. In this opinion article, we provide a brief discussion concerning the role of mitochondrial calcium regulation during glaucoma pathogenesis as well as insight concerning the potential use of mitochondrial-specific drug delivery during disease treatment. We believe that the extensive research and overlap in the fields of glaucoma and mitochondrial disease/aging (including calcium signaling dysfunction) ultimately lead to the therapeutic utilization of mitochondrial-specific delivery of calcium channel regulators during glaucoma and other retinal/neurodegenerative diseases (**Figure 1**).

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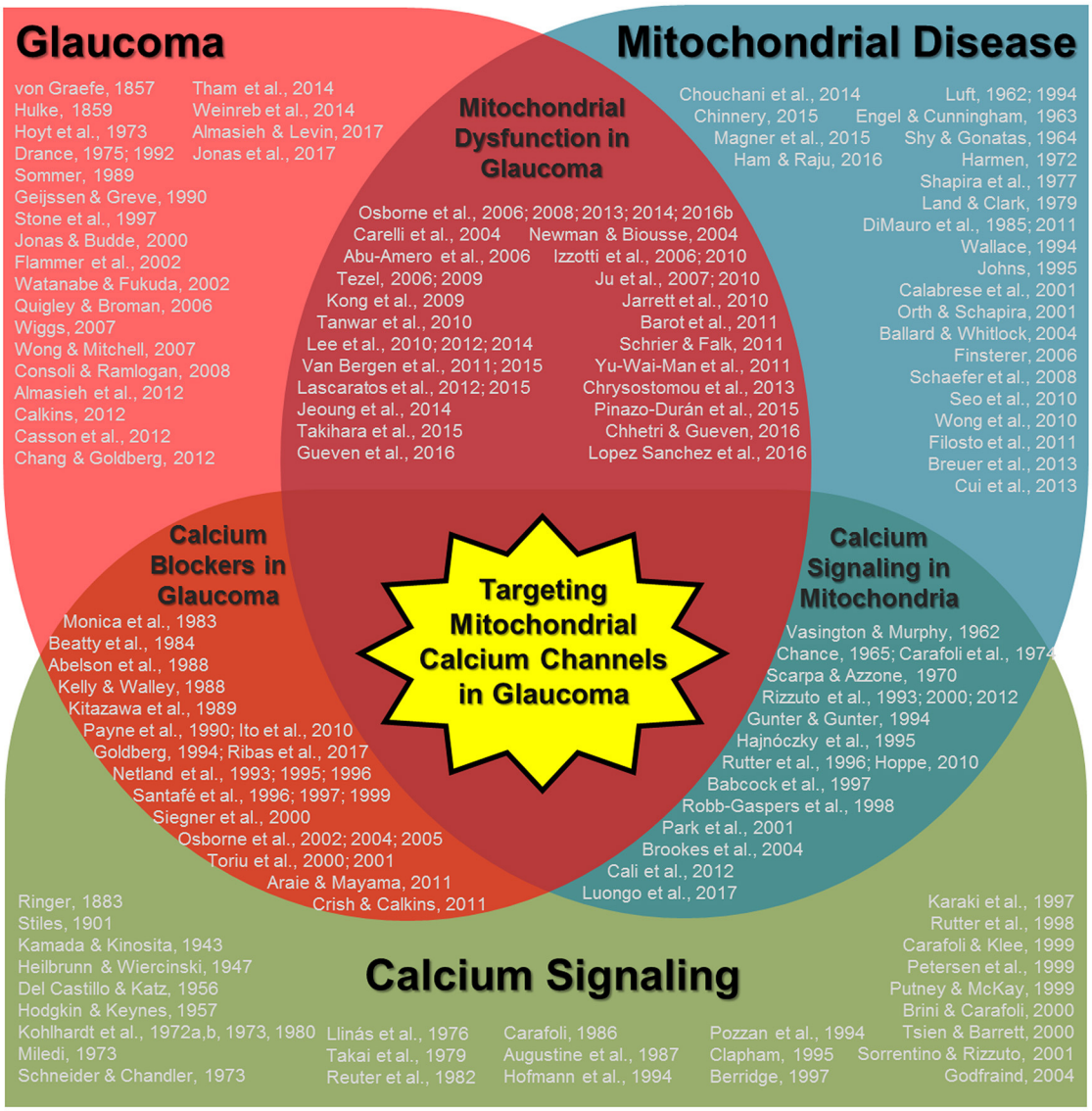


FIGURE 1 | Schematic diagram highlighting the relationships between glaucoma, mitochondrial disease/aging, and calcium signaling along with multiple keystone studies and reviews from prominent research groups. Some of the earliest published research concerning disease pathology and mechanisms are listed for each respective field as well as in areas of overlap (e.g., mitochondrial dysfunction in glaucoma, calcium channel treatment in glaucoma, and calcium signaling in mitochondria). While it is not possible to list all of the influential research published in each field, those listed include some of the key historical publications, with particular emphasis on relationships with the ocular environment or neurodegeneration when applicable. The cumulative research reported in these publications (and those cited within) in each respective field as well as other disease contexts has led to the development of multiple mitochondria-specific drug delivery systems, listed in the bottom panel. Their validation in parallel with the continued investigation of mitochondrial calcium signaling during disease pathogenesis indicate that targeting mitochondrial calcium channels during glaucoma could be a powerful therapeutic tool.

GLAUCOMA PATHOPHYSIOLOGY

Glaucoma is a two phase degenerative disease. The first phase involves a primary insult to the RGCs (Levkovitch-Verbin et al., 2003). Confirmed risk factors/insults for glaucoma include high IOP, ischemia, and aging. While these direct insults have classically been investigated as the cause of glaucoma-related vision loss, recent evidence indicates that damage to the visual cortex and/or optic nerve (i.e., distal axonopathy), which is then propagated to the retina following stress on axonal transport systems, may play a significant role in the initiation of the disease (Calkins and Horner, 2012; Crish and Calkins, 2015). Ultimately, all of these insults disrupt oxygen supply and alter retinal function. Furthermore, mitochondrial oxidative phosphorylation is significantly less effective in the affected RGCs, and energy production depends more on glycolysis and the tricarboxylic acid cycle. This change in energy supply causes oxidative stress and reduced ROS consumption, leading to mitochondrial damage and further ROS accumulation (Nguyen et al., 2011). While it has been hypothesized that RGCs can still function normally in this reduced energy state (Osborne et al., 2016b), they are more susceptible to secondary insults.

Secondary affronts to the RGCs can come in various forms. For example, primary insult-induced activation of retinal microglia and astrocytes as well as altered Müller cell function have detrimental secondary consequences related to the release of pro-inflammatory markers as well as other cytotoxic substances, including glutamate, nitrogen oxide, etc., in the extracellular space surrounding the RGCs. Furthermore, aged/dysfunctional mitochondria within the RGCs can also act as secondary stressors. In aged mitochondria, the initial increase in oxidative stress and reduced ROS consumption is amplified, resulting in a vicious positive feedback loop involving ROS along with damage to mitochondrial and nuclear DNA (Nguyen et al., 2011). This damage is largely irreversible as the repair mechanisms are often impaired in aged mitochondria. ATP production in cells with damaged mitochondria also becomes increasingly more difficult, ultimately leading to calcium dysregulation. As calcium is a known trigger for glutamate release (Neher and Sakaba, 2008), disrupted calcium signaling in aged mitochondria can further exacerbate primary insult-induced glutamate toxicity. Interestingly, increased glutamate concentration also mediates calcium influx (Wojda et al., 2008), indicating multiple points of crosstalk between mitochondrial calcium signaling and neuronal function.

MITOCHONDRIAL CALCIUM AS A KEY PLAYER IN GLAUCOMA

Mitochondria have two membrane layers. At the outer membrane, calcium influx is largely mediated through voltage-dependent anion channels (VDACs) (Cali et al., 2012; Rizzuto et al., 2012). Some reports suggest that open-state VDACs facilitate metabolite flow and prevent cytochrome C release, while closed-state VDACs mediate the opposite (Tan and Colombini, 2007; Hoppe, 2010; Williams et al., 2013). Further, elevated intracellular calcium concentrations appear to increase VDAC1 oligomerization and downstream apoptosis (Keinan et al., 2013). This increase in oligomerization has been demonstrated to be mediated specifically by mitochondrial, rather than cytosolic, calcium, providing a direct link between mitochondrial calcium and apoptosis.

At the inner membrane, calcium influx from the intermembrane space into the matrix is largely regulated by calcium-activated mitochondrial calcium uniporters (MCUs) (Cali et al., 2012). Although MCU calcium affinity is low, their effect on calcium concentration is significant as they mediate calcium inflow in response to the negative membrane potential/calcium gradient created by pumping protons across the membrane during oxidative phosphorylation. Thus, MCUs are functionally dependent on both intracellular calcium concentration and energy demand (Tsai et al., 2017). The links between calcium and energy are further strengthened by the calcium-dependent activation of three metabolic enzymes, pyruvate, α-ketoglutarate, and isocitrate dehydrogenases, all of which function in the tricarboxylic acid cycle (Cali et al., 2012). MCU function also depends on the proximity of the mitochondria to other calcium regulating organelles, including the endoplasmic reticulum, sarcoplasmic reticulum, and plasma membrane, which can alter the local calcium concentration (Kirichok et al., 2004; Rizzuto et al., 2012).

Cellular calcium homeostasis, whereby nanomolar levels of free calcium are found in the cytosol, is maintained, at least in part, via effective buffering mechanisms, including pH and phosphate/adenosine availability. Additional intermitochondrial buffering mechanisms involve calcium efflux via electrogenic $Ca^{2+}/3Na^{+}$ exchangers (mNCXs) and/or electroneutral $Ca^{2+}/2H^{+}$ exchangers (mHCXs) located on the inner mitochondrial membrane. mNCXs are the main efflux channels in excitable tissues, including RGCs, while mHCXs are found mainly in non-excitable tissues (Hoppe, 2010). Interestingly, these efflux systems appear to change into influx pathways during glaucoma (Wojda et al., 2008). In aged neurons, the expression of calcium buffering proteins, including calbindin-D28k, calretinin, and parvalbumin, is also reduced (Bu et al., 2003). Together, these changes in efflux levels and calcium buffering protein expression significantly alter calcium gradients, cytosolic calcium levels (Williams et al., 2013), and mitochondrial membrane polarization (Wojda et al., 2008), resulting in altered/inefficient oxidative phosphorylation, ROS accumulation, downstream changes in mitochondrial function, and neuronal cell survival.

In retinal neurons, intracellular free calcium overload triggers calpain activation which can subsequently initiate apoptotic cascades (Sharma and Rohrer, 2004; Huang et al., 2010; Kar et al., 2010). Calpain is a calcium-dependent cysteine protease that, once activated, cleaves pro-apoptotic B-cell lymphoma (Bcl)-2 family members as well as apoptosis-inducing factor (AIF) and inner membrane mNCXs (Vosler et al., 2008). Calpain activation is also related to the formation and opening of mitochondrial permeability transition pores (mPTPs) (Cali et al., 2012; Bernardi and Di Lisa, 2015). mPTPs are multi-protein complexes that facilitate calcium efflux. However, unlike mNCXs, once the mPTPs are opened, the inner membrane is

irreversibly permeabilized, resulting in uncontrolled dissipation of the electrochemical gradient, ATP depletion, ROS production, cytochrome C efflux, and mitochondrial swelling (Rasheed et al., 2017). Notably, increased cytoplasmic cytochrome C levels not only facilitate additional calpain activation, but the coupling of cytochrome C with apoptosis protease-activating factor (APAF)-1 results in apoptosome formation. Apoptosomes recruit and activate caspase-9 and downstream caspase-mediated apoptosis. Interestingly, activation of various caspases also feeds back into the process to further activate pro-apoptotic Bcl-2 proteins family members and increase mitochondrial permeability.

CALCIUM-RELATED DRUG TREATMENTS IN THE OCULAR ENVIRONMENT

Various calcium regulating therapies have been investigated for their use in treating visual neurodegeneration (Kamel et al., 2017). Indeed, in the dorsal lateral geniculate nucleus and superior colliculus as well as RGCs, lomerizine, a well-known plasma membrane calcium channel blocker, has been used to manage neuronal degeneration (Ito et al., 2010; Selt et al., 2010). Various combinations of calcium channel inhibitors, including the L-/N-type channel blocker amlodipine, T-type channel blocker amiloride, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blockers, and purinergic receptor blockers, also increase RGC survival, reduce axonal degeneration, and increase axonal regeneration in both partial optic nerve transection (Savigni et al., 2013) and crush models (Ribas et al., 2017). However, the effects on visual function preservation were not significant.

In glaucoma and retinitis pigmentosa models, inhibition of calpain signaling has also been demonstrated to be beneficial for RGC and photoreceptor survival, respectively. This is not surprising as the detrimental effects of calcium overload are mediated largely through calpain activation. Latanoprost, an ocular anti-hypertension drug, for example, modulates its neuroprotective effects in this calpain-mediated manner (Yamamoto et al., 2017).

Unfortunately, beyond these studies using agents against calcium signaling in the plasma membrane, other mitochondria-specific calcium channel regulators to block detrimental calcium changes have not been intensively studied and none have been investigated as a glaucoma treatment option. Notably, calcium can be transported across the outer and inner mitochondrial membranes via VDACs and MCUs, respectively, as well as during unregulated diffusion through mPTPs, making each of these protein complexes a potential target for calcium regulation during disease. While a number of agents have been used to block mPTPs (Kajitani et al., 2007; Halestrap and Richardson, 2015), treatment with these agents leaves the causative upstream changes in calcium concentration and channel function largely unchecked. Thus, targeting VDACs and/or MCUs and avoiding mPTP formation altogether would potentially be more advantageous. For example, an anti-VDAC antibody has been shown to reduce cytochrome C release from mitochondria (Madesh and Hajnóczky, 2001). Unfortunately, directly altering VDAC function in this manner can also manipulate the transport of other essential metabolites (Camara et al., 2017). In cancer, blocking VDACs results in apoptosis (Shoshan-Barmatz et al., 2017), which is counterproductive to the cellular rescue required during glaucoma treatment. Alternatively, an MCU blocker, Ru360, was demonstrated to alter ion transport through these channels as well as block iron overload and has the advantage of having minimal effects on other cellular functions (Sripetchwandee et al., 2013). This same blocker was also previously shown to prevent the accumulation of mitochondrial free calcium despite high cytosolic free calcium concentrations in post-ischaemic rat heart cells (de Jesús García-Rivas et al., 2005). Lastly, this drug also maintains normal oxidative phosphorylation levels and prevents mPTP opening, while other organelles and cellular processes are unaffected, making it a drug of interest for glaucoma therapy.

MITOCHONDRIAL-SPECIFIC DRUG DELIVERY AS A MEANS TO TREAT GLAUCOMA

Organelle-specific drug targeting itself is not novel, being reviewed in multiple excellent publications (Sakhrani and Padh, 2013; Zhang and Zhang, 2016). While mitochondrial-specific drug targeting has not been applied to glaucoma, researchers have been actively proposing new mitochondrial delivery/transporter systems to target this organelle in other diseases. For example, a liposomal-based carrier was recently described that uses octaarginine modification, electrostatic attraction, and membrane fusion to promote mitochondrial uptake (Yamada and Harashima, 2017). This style of "MITO-porter" was then used to deliver coenzyme Q10 in mice with hepatic ischemic/reperfusion injuries and mediated a significant decrease in serum alanine aminotransferase (ALT) (Yamada et al., 2015). Another study utilized a MITO-porter system to target doxorubicin to the mitochondria of drug-resistant cancer cells, successfully destroying these cells (Yamada et al., 2017). Other nanotechnology techniques have also been employed, including a recent hybrid of polylactide-co-glycolide nanoparticles and mitochondria-penetrating particles (Selmin et al., 2017; **Figure 1**, bottom panel). Taken together, the evidence emerging from these investigations provides a solid foundation for the continued study of these delivery systems in other cellular contexts.

Delivery of agents used to modulate channel function along with the expression of other essential compounds (e.g., cytochrome C, ATP, etc.,) in concentrated amounts directly to the mitochondria during glaucoma using these systems would allow some of the downstream detrimental changes to be managed before vision loss. While some current (e.g., Ru360) and future drugs targeting mitochondrial calcium channels already innately target the mitochondria, the use of MITO-porters and similar delivery systems would not only allow higher concentrations to be delivered, but would also avoid any unknown effects on other organelles. Furthermore, delivery systems could also be used to package multiple drugs/compounds together in order

to have the greatest therapeutic effect. Ultimately, these drugs would collectively reduce calcium efflux and restore calcium homeostasis as well as prevent mPTP formation, ATP depletion, ROS production, and cytochrome C dissipation. Doing so would prevent the second wave of apoptosis, allowing the cells to function normally even after the initial insult. While these drug delivery systems are not currently used as an ocular disease treatment, their potential to transport drugs to the retina and/or optic nerve/visual cortex that will subsequently manipulate mitochondrial function is a promising research avenue for novel treatment development for glaucoma as well as other retinal pathologies.

CONCLUSIONS

Mitochondrial dysfunction and the associated changes in calcium homeostasis, ROS production, and energy supply are intimately related to RGC death/dysfunction during glaucoma, making it an attractive treatment target. Mitochondrial-targeting drug delivery systems, which have been developed and validated in other cellular environments, could potentially avoid these issues by packaging multiple drugs and delivering them at high concentrations directly to the mitochondria. In discussing the recent advances in these techniques within the context of mitochondrial calcium regulation during glaucoma for the first time, we pose the question: Is this the future of glaucoma treatment? The relationships highlighted in multiple keystone studies investigating glaucoma and mitochondrial disease/aging in addition to the essential role of calcium signaling in these processes indicate an affirmative answer. Thus, while the full potential of these systems has yet to be fully established, we believe that mitochondrial-specific delivery of calcium channel regulators could effectively change how glaucoma and other neurodegenerative diseases affecting the retina are treated.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication

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frontiers in Neuroscience

CASE REPORT
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The Decrease in Mitochondrial DNA Mutation Load Parallels Visual Recovery in a Leber Hereditary Optic Neuropathy Patient

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The onset of Leber hereditary optic neuropathy is relatively rare in childhood and, interestingly, the rate of spontaneous visual recovery is very high in this group of patients. Here, we report a child harboring a rare pathological mitochondrial DNA mutation, present in heteroplasmy, associated with the disease. A patient follow-up showed a rapid recovery of the vision accompanied by a decrease of the percentage of mutated mtDNA. A retrospective study on the age of recovery of all childhood-onset Leber hereditary optic neuropathy patients reported in the literature suggested that this process was probably related with pubertal changes.

Keywords: LHON, childhood-onset disease, visual recovery, mtDNA, heteroplasmic mutation

INTRODUCTION

Leber hereditary optic neuropathy (LHON) is a type of blindness, usually characterized by severe central vision loss in one eye soon followed by the fellow eye, associated dense scotomas and impaired color vision. Ninety percent of the patients with this disease have been associated with one of the following point mutations within the mitochondrial DNA (mtDNA): m.3460G>A, m.11778G>A, and m.14484T>C. A plethora of rare mutations is responsible for the remaining 10% and some of them have been previously associated to other mitochondrial phenotypes.

LHON mainly affects young adult men. The peak age of onset is in the second and third decades of life (Majander et al., 2017). The onset of the disease in childhood is relatively rare. Thus, it has been reported that <10% of patients were 12 year-old or younger at the time of diagnosis (Majander et al., 2017). Notably, in this patient population, the rate of spontaneous visual recovery is very high (Majander et al., 2017). However, the reason for such spontaneous recovery is unknown and there is hardly any information on either biochemical nor lifestyle of the patients around the recovery period.

Hereby in the present paper, we report a case of a child suffering LHON who presents a rare mtDNA mutation. Biochemical, cellular and genetic studies performed on patient' fibroblasts and cybrids show that this mutation is pathological. Interestingly enough, the patient rapidly recovered

his vision. We also discuss a potential correlation of the age of recovery of childhood-onset LHON patients with pubertal changes since two thirds of these patients recovered sight before the age of 13.

MATERIALS AND METHODS

Case Report

A 10 year-old male came to our department referring bilateral and painless visual loss. Upon examination, visual acuity (VA) was 20/400 (1.3 logarithm of the minimum angle resolution-LogMAR) right eye (RE) and 20/100 (0.7 LogMAR) left eye (LE), Ishihara Test: 0/20 RE and 5/20 LE. Pupils were slightly anisocoric (RE > LE), with normal responses to light. Anterior segment was normal in both eyes, and ocular fundus showed temporal optic disc pallor RE and a congestive disc with telangiectasies LE (**Figure 1A**). Brain magnetic resonance was performed with normal results. The electroretinogram study was normal and visual evoked potential study showed a bilateral delay of conduction through the visual pathways suggestive of bilateral optic neuropathy (**Figures 1B,C**). After 6 months the RE showed a favorable evolution, with VA of 20/25 (0.1 LogMAR) but VA in the LE stayed at 20/100 (0.7 LogMAR). Ishihara test was 18/20 in RE and 1/20 in LE, and a slight relative afferent pupillary defect was observed in the LE. The visual fields showed a paracentral scotoma in the RE and a cecocentral scotoma in the LE. Fundoscopy revealed bilateral marked optic disc pallor (LE > RE) with an associate vascular attenuation (**Figure 1D**). There was a stabilization of the clinical picture over the following 2 years. Ultimately, the patient has experienced a favorable evolution after 4 years from the beginning of the clinical symptoms. His VA has presently improved to 20/20 RE (0.0 LogMAR) and 20/40 LE (0.3 LogMAR) with an improvement of the Ishihara test to 20/20 RE and 20/20 LE. Visual fields show minimal defects in the RE and a 5 degree central scotoma in the LE. However, bilateral pallor of the optic discs remains in the ocular fundus (**Figures 1E,F**). This study was approved by and carried out in accordance to the recommendations of Institutional Review Board from the Government of Aragón (CEICA CP-12/2014). The patient's mother written informed consent was obtained for their participation in the study, in accordance with the Declaration of Helsinki, and for publication of the case report.

Molecular-Genetic Analyses

Total DNA was extracted by standard methods. Screening for the three primary LHON mutations was performed by polymerase chain reaction (PCR)/restriction fragment length polymorphism (RFLP) (Supplementary Table 1). The complete mtDNA was amplified and sequenced according to previously described protocols (Supplementary Table 2; Gomez-Duran et al., 2010). The percentage of m.13094T>C transition was analyzed by PCR/RFLP by using protocols described elsewhere (Valente et al., 2009). The mtDNA content was measured, in triplicate in three independent experiments, by the real-time quantitative reverse transcription-PCR (RT-qPCR) method using an Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block, as described elsewhere (Andreu et al., 2009).

Production of Transmitochondrial Cell Lines and Cell Culture

To homogenize nuclear and environmental factors, we produced transmitochondrial cell lines (cytoplasmic hybrids or cybrids) with the osteosarcoma 143B rho^0 nuclear background using patient and control platelets (Chomyn, 1996). These cybrids, as well as patient and control fibroblasts, were grown in Dulbecco's modified eagle medium with no antibiotics and containing glucose (4.5 g/l), pyruvate (0.11 g/l) and 5 or 10% of fetal bovine serum (FBS), respectively.

Biochemical Investigations

The analyses of oxygen consumption, ATP, mitochondrial inner membrane potential (MIMP) and H_2O_2 levels were performed in triplicate in 3 independent experiments according to previously described protocols (Gomez-Duran et al., 2010; Llobet et al., 2015). The activity of several mitochondrial complexes was analyzed using BN-PAGE *in-gel* activity technique (Wittig et al., 2007). Western blots in cellular lysates were performed using Total OXPHOS Human WB Antibody Cocktail (1:1,000, ab110411, ABCAM), anti-LC3B (1:1,000, L7543, Sigma) and anti-actin (1:2,000, A2066, Sigma) as primary antibodies (Lopez-Gallardo et al., 2009).

Molecular Modeling

The three-dimensional structure of the bovine p.MT-ND5 (PDB 5LNK) was obtained with the RasMol 2.6 program (http://www.rasmol.org).

Statistical Analysis

The statistical package StatView 6.0 was used to perform all the statistics. Data for mean and standard deviation are presented. The unpaired two-tailed *t*-test was used to compare parameters. *P*-values lower than 0.05 were considered statistically significant.

RESULTS

The three most common LHON mutations (m.3460G>A, m.11778G>A, and m.14484T>C) are responsible for approximately 90% of all LHON cases (Majander et al., 2017). Therefore, we tested their presence by PCR-RFLP and ruled them out. Next, we sequenced the whole mtDNA from blood cells and assigned it to mitochondrial haplogroup H1 (Van Oven and Kayser, 2009). This mtDNA harbored 4 private mutations (GenBank MG386502): m.11113T>C synonymous and homoplasmic mutation in the *MT-ND4* gene; m.13094T>C in the *MT-ND5* gene; m.15527C>T in the *MT-CYB* gene; and the highly frequent and homoplasmic m.16295C>T mutation in the *MT-CR* control region. One of the two non-synonymous variants, the m.15527C>T transition, is a homoplasmic mutation (Supplementary Figure 1A) which has not been reported in 37,545 published human

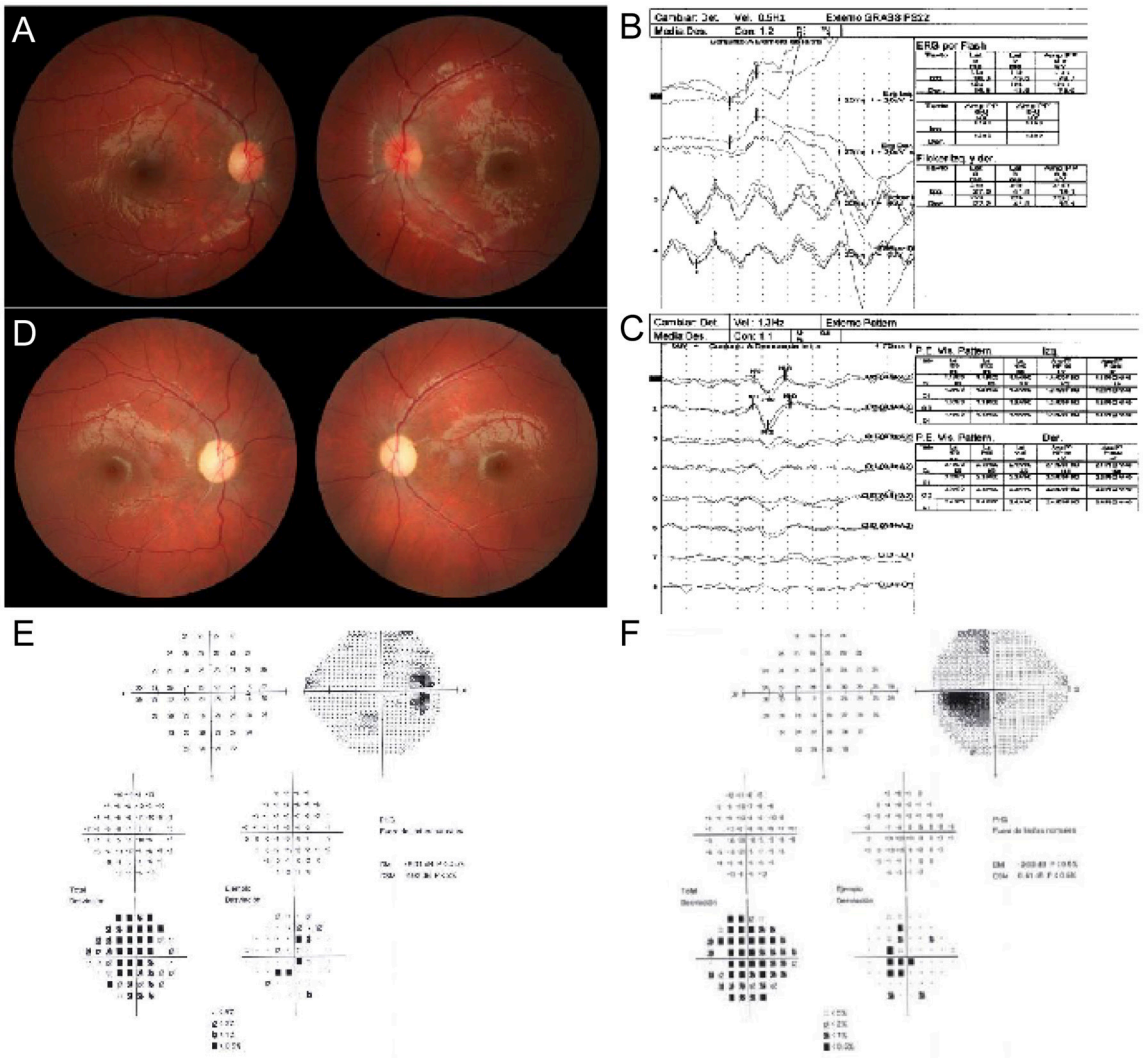


FIGURE 1 | Ocular exam. **(A)** Fundus photography. RE shows temporal optic disc pallor. LE shows a congestive disc with telangiectasies. **(B)** Electroretinogram. **(C)** Visual evoked potential pattern. Bilateral delay of conduction through the visual pathways is observed. **(D)** Fundus photography. Bilateral pallor of the optic discs remains. **(E,F)** Visual fields analysis. RE and LE show a paracentral and a cecocentral scotoma, respectively.

mtDNA sequences (GenBank, November 3, 2017), although it was present in homoplasmy in mother's blood and urine (Supplementary Figure 1B). This mutation causes a proline to serine substitution in p.MT-CYB position 261. This proline is conserved in 94.7% of 4,988 eukaryotic (from protists to mammals) p.MT-CYB sequences (Martin-Navarro et al., 2017). Pathogenicity predictors, such as MutPred (Pereira et al., 2011), PolyPhen-2 and Mitoclass.1 (Martin-Navarro et al., 2017), consider this amino acid substitution as a pathogenic mutation. The m.13094T>C mutation has not been reported in these 37,545 human sequences. This is a heteroplasmic mutation (**Figure 2A**) and its percentage varies between patient tissues (**Figures 2B,C**). Said mutation was not found in blood or urine of his younger brother nor in his mother's blood but it was present, in a low percentage (10%), in the urine of his mother (**Figure 2B**). This m.13094T>C transition provokes a valine to alanine change in p.MT-ND5 position 253 (**Figure 2D**). The valine is conserved in 99.7% of 5,159 eukaryotic p.MT-ND5 sequences (Martin-Navarro et al., 2017). This Val253 is located in the p.MT-ND5 transmembrane helix 8 (TMH8), following a serine pair (Ser249 and Ser250) that distorts TMH8 (Zhu et al., 2016), and a key His248 sit on a flexible loop of the discontinuous TMH8. This mid-membrane break/loop lend flexibility to key protonable residues (Fiedorczuk et al., 2016; **Figure 2D**). PolyPhen-2 and Mitoclass.1 consider this amino acid substitution as a pathogenic one, but as for MutPred its pathogenicity is low.

Along 3 years, four different blood analysis were performed and the percentage of m.13094T>C mutation decreased from 50% to undetectable (**Figure 2E**), but the m.15527C>T

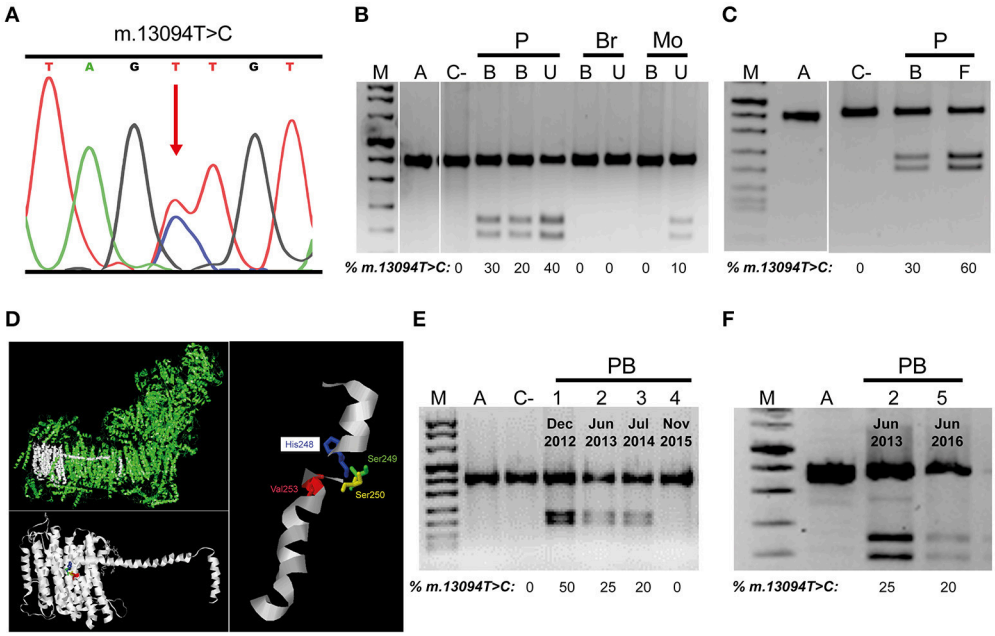


FIGURE 2 | mtDNA m.13094T>C mutation. **(A)** Electropherogram showing the presence of the heteroplasmic mutation in the patient. **(B,C)** Mutation load in different members of the family. **(D)** Molecular model of p.MT-ND5 subunit. Respiratory complex I (CI), p.MT-ND5 polypeptide and transmembrane helix 8 (TMH8) showing the affected amino acid. **(E,F)** Mutation load variation through time in patient's blood. M, DNA molecular weight marker VIII (Roche); A, amplicon; C-, negative control; P, patient; Br, brother; Mo, mother; B, U, F, patient blood, urine and fibroblasts; PB, 1-5, patient blood indicating the dates. Uncropped images for gels in **B,C** can be found in Supplementary Figure 2A,B, respectively.

transition stayed homoplasmic (Supplementary Figure 1C). Simultaneously, there was an improvement in visual acuities to an eventually almost complete visual recovery. However, another blood sample, 8 months later, showed a 15–20% of the m.13094T>C mutation (**Figure 2F**), although the vision was not altered. mtDNA copy numbers in blood from different ages [Patient Blood 2 (PB2), 78.8 copies/cell ± 4.71 (2); PB3, 35.0 copies/cell ± 0.22 (2); PB4, 55.5 copies/cell ± 2.75 (2); PB5, 69.3 copies/cell ± 0.30 (2)] were not correlated with the percentage of mutation. As LHON affected cells are the retinal ganglion cells (RGCs), this visual recovery with reduction in blood mutation load was probably accompanied by a decline in the RGCs mutation load. The onset of LHON is relatively rare in childhood, but their prognosis is more favorable (Majander et al., 2017). Thus, from 14 childhood-onset LHON patients (age of onset, 2–12 years) who showed visual recovery (exactly dated), 10 have got it before the age of 13 (Mackey and Howell, 1992; Kawasaki and Borruat, 2005; Sharkawi et al., 2012; Majander et al., 2017).

Next, we checked cellular effects of these mtDNA mutations in the patient's fibroblasts. Basal and uncoupled respiration and ATP levels were significantly decreased in patient's vs. control's fibroblasts (**Figures 3A,B**). There was no difference in the levels of hydrogen peroxide (**Figure 3B**). On the other hand, mitochondrial inner membrane potential was significantly increased in patient's fibroblasts (**Figure 3B**). An *in gel* activity analysis showed an important decrease in complex I (CI) and V (CV) activities (**Figure 3C**). The mtDNA amount was also significantly lower in the patient's fibroblasts (**Figure 3D**).

In the patient's vs control's fibroblasts comparison, culture conditions are homogenized, but nuclear DNA (nDNA) and mtDNA genetic backgrounds differ. On the contrary, in cybrids the nDNA is also homogeneous and they only differ in the mtDNA genotype. Therefore, we used osteosarcoma 143B rho^0 cells to build mutant and control cybrids (**Figure 4A** and Supplementary Figure 1D). The short terminal repeats (STR) markers reported in the American Type Culture Collection (ATCC) for the osteosarcoma 143B cell line did not differ from those of the cell lines used in this work, and they are the same than those previously reported in other osteosarcoma 143B cybrids (Lopez-Gallardo et al., 2014), thus confirming the nDNA homogeneity. Basal and uncoupled oxygen consumptions were significantly lower in mutant cells (**Figure 4B**), and this was accompanied by lesser cell ATP amount. The ATP levels were higher in cybrids with lower percentages of m.13094T>C mutation, although they were homoplasmic for the m.15527C>T transition (**Figure 4C** and Supplementary Figure 1D). Surprisingly, we found an inverse relationship between the percentage of m.13094T>C mutation and the mtDNA copy numbers [0%, 950 copies/cell ± 1 (1); 20%, 737 copies/cell ± 159 (8); 50%, 541 copies/cell ± 41 (3)]. Although levels of nDNA (CI-20kDa, CII-30kDa, CIII-Core2, and CV-F1a) and mtDNA (CIV-p.MT-CO2) subunits did not differ (**Figure 4D**), the *in gel* activity analysis showed an important decrease in CI and CIV activities, and CV

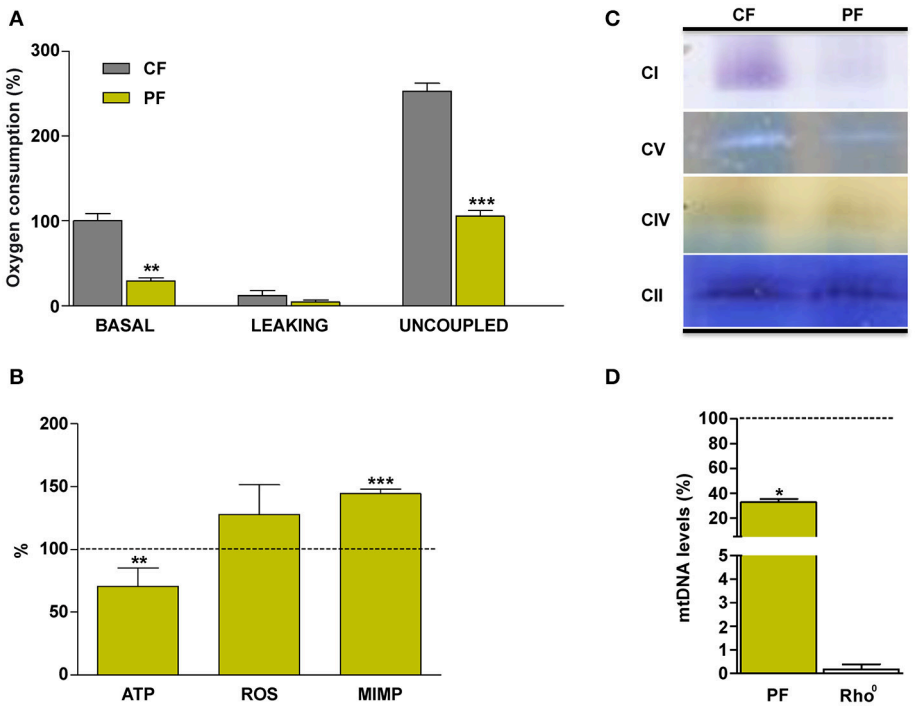


FIGURE 3 | Mitochondrial biochemical characterization of the patient's fibroblasts. **(A)** Oxygen consumption. **(B)** ATP and reactive oxygen species (ROS) levels and mitochondrial inner membrane potential (MIMP). **(C)** *In gel* complexes activity. **(D)** mtDNA quantity. CF, PF, CI, CII, CIV, CV, and Rho^0 code for control fibroblasts, patient fibroblasts, complex I, complex II, complex IV, complex V, and cells without mtDNA, respectively. Dashed line represents the control fibroblast values. *, **, *** indicate < 0.05, 0.01, and 0.001 *p*-values, respectively.

sub-complexes were also observed (**Figure 4E**). Autophagy, determined as LC3B-II/Actin levels, was significantly increased in mutant cybrids (**Figure 4F**).

DISCUSSION

We found two potential mtDNA pathologic mutations in the patient. The change in ATP levels of mutant cybrids, despite all of them harboring a homoplasmic m.15527C>T transition, along with other considerations make us rule out this mutation as the pathologic one for this patient. However, the m.13094T>C heteroplasmic mutation had been previously associated to two other patients. The first was a 7 year-old child suffering from Ataxia and Progressive External Ophthalmoplegia (PEO). The skeletal muscle biopsy was morphologically normal. Muscle and fibroblast were also biochemically normal. However, mutant fibroblasts had a clear reduction in CI *in gel* activity and CI-sub-complexes in a 2D-BNGE WB analysis. In osteosarcoma 143B cybrids, the CI/CS ratio negatively correlated with the percentage of mutation and the 80% mutant cybrid showed CI sub-complexes. Mutant and control CI amount was comparable in fibroblasts and also in cybrids (Valente et al., 2009). This heteroplasmic mutation was also found in a 34 year-old woman with Kearns-Sayre syndrome (Lax et al., 2012a although, in Lax et al., 2012b, this patient is referred as suffering from mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS)/Leigh syndrome (LS), myoclonus and fatigue). A vascular smooth muscle cell loss was observed along with thinning of the vascular smooth muscle cell layer and 50% of neurons were lost from the olivo-cerebelum (Lax et al., 2012a,b). On the other hand, besides m.13094T>C, several p.MT-ND5 mutations have been also associated with LHON or LHON-like phenotypes, and some of them were previously associated to other phenotypes, such as MELAS or LS (www.mitomap.org). Our evidences, along with those from these two other articles, strongly support the m.13094T>C transition as a pathologic mutation for PEO, MELAS/LS (KSS), or LHON. Probably, tissue distribution and percentage of the mutation are responsible for these different phenotypes. Interestingly, this mutation seemed to trigger a decrease in mtDNA amount that could be responsible for the OXPHOS multienzymatic deficit in patient fibroblasts and cybrids.

The decrease in mutation levels is probably responsible for the patient's recovery. A reduction in the mutation load in proliferative tissues has been observed in some longitudinal studies for LHON mutations (Howell et al., 2000; Jacobi et al., 2001; Puomila et al., 2002; Kaplanova et al., 2004). However, the decrease is mostly moderate. Only 2 patients and 2 carriers harboring the m.3460G>A LHON mutation showed a reduction $\geq$10% (Supplementary Table 3). One of the patients, who recovered the vision, showed a reduction in the mutation load from 46 to 35% over the following 5 years. Our patient, who also recovered vision, showed a 50% decrease in the blood mutation

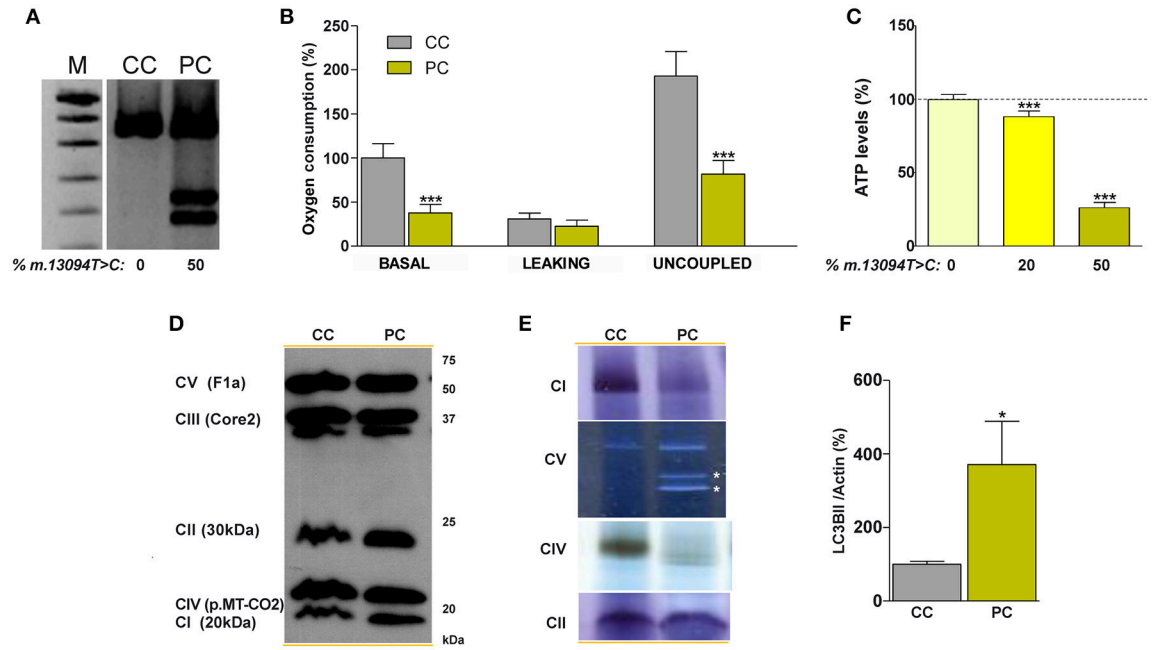


FIGURE 4 | Biochemical characterization of the normal and 50% mutation cybrid cell lines. **(A)** Gel showing the percentage of the m.13094T>C mutation. **(B)** oxygen consumption. **(C)** ATP levels in cybrids harboring different (0, 20, and 50%) m.13094T>C mutation load. **(D)** Levels of different OXPHOS subunits (in brackets) in cellular lysates. **(E)** *In gel* complexes activity. White asterisks denote CV subcomplexes. **(F)** Western blot quantification of the LC3BII autophagy marker, normalized to actin, in cellular lysates. M, DNA molecular weight marker VIII (Roche); CC and PC denote control and patient (50% mutation) cybrids, respectively. CI, CII, CIII, CIV, and CV code for complex I, complex II, complex III, complex IV and complex V, respectively. Dashed line represents the control cybrid values. *, *** indicate < 0.05 and 0.001 *p*-values, respectively. Uncropped image for gel in **(A)** can be found in Supplementary Figure 2C. Results for **(F)** can be found in Supplementary Figure 2D.

load. Blood is not the affected tissue in LHON patients, but RGCs is. These cells are mostly inaccessible for genetic tests. Then, why should the blood mutation load be related to the visual health? Despite tissue-specific directional selection for different mtDNA genotypes has been reported (Jenuth et al., 1997), it was also observed that a high amount of mutated mtDNA in leukocytes was also correlated to a high proportion in other tissues (Juvonen et al., 1997).

We have not been able to note any particular changes in the patient's life style as a potential cause of this decrease in the mutation load and vision recovery. We had previously found higher mtDNA content in peripheral blood cells of unaffected heteroplasmic mutation carriers with respect to the affected ones (Bianco et al., 2017). Moreover, many patients showing visual recovery are homoplasmic individuals. In these cases, their improvement cannot be associated to a reduction in the mutation load. Visual recovery of these individuals may also be accompanied by an increase in their RGCs mtDNA levels mirrored in their blood mtDNA levels. In fact, mtDNA content in peripheral blood cells is higher in unaffected homoplasmic mutation carriers with respect to the affected ones (Bianco et al., 2016). Remarkably, more than two thirds of unaffected homoplasmic carriers are female (Bianco et al., 2016), and it has been observed that oestradiol increases mtDNA content, which it could explain the lower LHON prevalence in females (Giordano et al., 2011). LHON is also less prevalent in prepubertal girls (Majander et al., 2017). Interestingly, oestradiol values are higher in prepubertal girls than in prepubertal boys and these levels increase with age and pubertal stage in both sexes (Ikegami et al., 2001; Janfaza et al., 2006). This rise in prepubertal oestradiol levels could be responsible for the high spontaneous visual recovery rate of childhood LHON patients (Majander et al., 2017). In this case, prepubertal oestradiol concentration might be a biomarker for childhood LHON. More importantly, transiently increasing oestradiol concentrations perhaps avoided the blindness or accelerated the recovery.

AUTHOR CONTRIBUTIONS

SE, CH-A, CR-R, DW, EL-G, and MB-B performed research, designed experiments, collected and analyzed data, revised paper; MV, AM-B, JA, and RA collected and analyzed data and revised paper; JM and ER-P directed the project, designed experiments, analyzed data, wrote and revised paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00061/full#supplementary-material

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frontiers in Neuroscience

OPINION
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Aminochrome Induces Irreversible Mitochondrial Dysfunction by Inducing Autophagy Dysfunction in Parkinson's Disease

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MITOCHONDRIAL DYSFUNCTION IN PARKINSON'S DISEASE

In Parkinson's disease, mitochondrial complex I activity is diminished, and mitochondrial deoxyribonucleic acid (DNA) mutations accumulate (Zhang, 2013). The first evidence that mitochondrial dysfunction was involved in the pathogenesis of Parkinson's disease came from parkinsonism induced by the accidental exposure of drug users to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), an inhibitor of the mitochondrial complex I of the electron transport chain (Langton et al., 1983; Esteves et al., 2011). Parker et al. (1989) found a significant reduction in the activity of complex I in platelet mitochondria purified from patients with idiopathic Parkinson's disease (Esteves et al., 2011). Further evidence for mitochondrial dysfunction in Parkinson's disease arose from studies developed in the *substantia nigra* of postmortem brains of patients with the disease, which showed deficiency of complex-I activity (Shapira et al., 1990; Esteves et al., 2011). Genes associated with familial form of the disease has been reported such as pink1, parkin, DJ-1, and CHCHD2 (Kazlauskaite and Muqit, 2015; Meng et al., 2017).

AUTOPHAGY

Macroautophagy is the most widely studied type of autophagy, where vacuoles with double membranes form, surround cellular elements (such as proteins, lipids, and organelles), and fuse with lysosomes, the enzymes of which degrade the autophagic cargo. Autophagy is controlled by proteins that are encoded by autophagy-related genes (ATGs), ATG1–ATG35. These proteins are organized into complexes that mediate the following steps in the autophagic process: initiation, elongation, maturation, and fusion and degradation (Tan et al., 2014). The pathways that control autophagy are primarily mTOR-dependent and mTOR-independent (mTOR: the mechanistic target of rapamycin, a serine/threonine kinase). mTOR is primarily an inhibitory signal, which participates upstream of the ATG proteins. In the mTOR-independent pathway the autophagy can be directly activated by AMPK [adenosine monophosphate (AMP)-activated protein kinase], leading to direct phosphorylation of ULK1 (serine-threonine-protein kinase that is encoded by the ULK1 gene) and beclin-1 (Tan et al., 2014).

MITOPHAGY

The degradation of mitochondria damaged by the autophagic pathway is known as mitophagy and constitutes one of the main mechanisms of cellular homeostasis (Zhang, 2013; Brady and Brady, 2016). Mitochondrial damage causes a decrease in mitochondrial membrane potential or an increase in mitochondrial fission, and both situations activate mitophagy (Brady and Brady, 2016). There are multiple mechanisms by which mitochondria are targeted for degradation in autophagosomes, but the best understood are the pathways of mitophagy induced by PINK/Parkin and BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3), and NIX-dependent mitophagy (Nix: also known as BNIP3L, a BH3-only protein of the BCL-2 pro-apoptotic family). The mitochondrial protein PINK1 [phosphatase and tensin homolog (PTEN)-induced putative kinase 1], a serine-threonine kinase, is unstable due to presenilin-associated rhomboid-like protease activities (PARL). The decrease in mitochondrial membrane potential inhibits PINK1 degradation by PARL. In response to mitochondrial depolarization, PINK stabilizes, and accumulates in the outer mitochondrial membrane (OMM), where it phosphorylates ubiquitin in mitochondrial proteins to recruit autophagic cargo adapters, such as OPTN (Optineurin) and NDP52 (Nuclear dot protein 52 kDa), which directly bind to light chain 3 (LC3) in the autophagosome leading to degradation of mitochondria within autophagolysosomes (Springer and Macleod, 2016). PINK1 also recruits the E3 ubiquitin ligase Parkin and ubiquitin-specific substrates in the OMM, including VDAC (voltage-dependent anion-selective channel), Miro, and Mitofusin-2 to amplify the signal initiated by PINK (Springer and Macleod, 2016). Mutations in PARK2 (Parkin) and PARK6 (PINK1) have been independently linked to familial cases of Parkinson's disease, associating defects in mitophagy with the degeneration of dopaminergic neurons, a major feature of Parkinson's disease (Pikrell and Youle, 2015; Springer and Macleod, 2016).

The clearance of mitochondria damaged by mitophagy prevents the accumulation of dysfunctional mitochondria and can also induce mitochondrial biogenesis, increasing cell survival. On the contrary, the decrease in mitophagy occurring during aging, for example, prevents both the removal of damaged mitochondria and alters mitochondrial biogenesis, which causes the progressive accumulation of dysfunctional mitochondria (Zhang, 2013; Palikaras et al., 2015).

The dysfunction of the autophagic/lysosomal pathway is associated with mitochondrial dysfunction, which may be due to the decrease in the autophagic degradation of the dysfunctional mitochondria. For example, Wu et al. (2009) reported that deletion of an ATG7 autophagic protein in mice causes mitochondrial dysfunction, characterized by decreased mitochondrial oxygen uptake rate and increased levels of reactive oxygen species in muscle fibers and pancreatic beta cells. On the other hand, lysosomal storage disorders are associated with mitochondrial dysfunctions, which include changes in mitochondrial morphology, decreased mitochondrial membrane potential, decreased ATP production, and increased generation of reactive oxygen species (De la Mata et al., 2016; Plotegher and Dushen, 2017). The loss of lysosomal glucocerebrosidase enzyme activity causes lysosomal dysfunction (Bae et al., 2015). In turn, glucocerebrosidase deficiency is associated with mitochondrial dysfunction (Gregg and Schapira, 2016). Mitochondrial dysfunction may also induce lysosomal dysfunction, resulting in a vicious circle (Plotegher and Dushen, 2017). In the context of Parkinson's disease, mitochondrial dysfunction and lysosomal dysfunction may be connected by the oxidation of dopamine. In fact, it has recently been reported that dopamine oxidation mediates mitochondrial and lysosomal dysfunction in both sporadic and familial Parkinson's disease (Burbulla et al., 2017).

DOPAMINE OXIDATION TO NEUROMELANIN

Dopamine oxidation to neuromelanin is a sequential pathway where several ortho(*o*)-quinones are formed: dopamine→dopamine *o*-quinone→aminochrome→5,6-indolequinone→neuromelanin. Dopamine *o*-quinone is converted to aminochrome with a constant rate of 0.15 $^{s-1}$ at physiological pH (Tse et al., 1976). Oxidation of dopamine with tyrosinase to dopamine *o*-quinone in the presence of mitochondria induce the formation adduct with a long list of proteins but in the same study incubation of SH-SY5Y cells with dopamine only few adducts were detected, questioning the feasibility of dopamine *o*-quinone to be responsible for dopamine neurotoxicity (Van Laar et al., 2009). Dopaminochrome, which structure has not be determined, induces adducts with alpha-synuclein (Norris et al., 2005) and 5,6-indolequinone induced adducts with alpha synuclein (Bisaglia et al., 2007). Aminochrome is the most stable *o*-quinone.

AMINOCHROME AND MITOCHONDRIAL DYSFUNCTION

For a long time, it has been accepted that mitochondrial dysfunction is involved in the degeneration of the nigrostriatal neurons containing neuromelanin in Parkinson's disease, but the question of what induces mitochondrial dysfunction inside of dopaminergic neurons containing neuromelanin remains. A possible candidate is aminochrome, an *o*-quinone formed during dopamine oxidation to neuromelanin (Segura-Aguilar et al., 2014, 2016; Herrera et al., 2017; Segura-Aguilar, 2017a,b). Aminochrome is an endogenous neurotoxin that induces mitochondrial dysfunction by inhibiting complex I and decreasing ATP levels in different cell lines (Arriagada et al., 2004; Aguirre et al., 2012; Huenchuguala et al., 2017). Aminochrome induces mitochondrial dysfunction in rat brain, resulting in a significant decrease in ATP levels, which explains a significant the decrease in dopamine release and amount of synaptic vesicles at the synaptic cleft. Both the axonal transport of neurotransmitter vesicles to the terminals and dopamine release require ATP (Herrera et al., 2016). Interestingly, aminochrome also induces other mechanisms related to the degeneration of dopaminergic

neurons containing neuromelanin, such as protein degradation dysfunction of both lysosomal and proteasomal systems (Zafar et al., 2006; Huenchuguala et al., 2014); aggregation of alpha-synuclein to neurotoxic oligomers (Muñoz et al., 2015); neuroinflammation (Santos et al., 2017); oxidative (Arriagada et al., 2004); and endoplasmic reticulum stress (Xiong et al., 2014). The oxidation of dopamine to neuromelanin is a normal pathway, because healthy seniors have intact dopaminergic neurons containing neuromelanin (Zecca et al., 2002; Zucca et al., 2017). A relationship between neuromelanin content and loss of dopaminergic neurons containing neuromelanin has been reported. The level of neuromelanin in substantia nigra pars compacta was 10 times higher than in ventral tegmental area of control subjects. The loss of dopaminergic neurons containing neuromelanin in Parkinson's disease patients was found to be 47% in comparison 0% in ventral tegmental area (Schwarz et al., 2017). The 10-fold lower amount of neuromelanin in ventral tegmental area results in lower amount of aminochrome, explaining why these neurons were intact in this study (Schwarz et al., 2017). Neuromelanin formation depends on VMAT-2 expression because dopamine is complete stable inside of monoaminergic vesicles where the pH is around 5.3 due to dopamine uptake is coupled to an ATPase proton pump (Sulzer et al., 2000; Herrera et al., 2017; Segura-Aguilar, 2017a,b). An inverse relationship between VMAT-2 expression level and neuromelanin content in human midbrain dopamine neurons has been reported (Liang et al., 2014). The reason why aminochrome is not neurotoxic in dopaminergic neurons containing neuromelanin in healthy seniors is because the enzymes DT-diaphorase and glutathione transferase M2-2 (GSTM2) prevent aminochrome neurotoxicity (Lozano et al., 2010; Huenchuguala et al., 2014, 2016; Segura-Aguilar, 2015, 2017a; Herrera-Soto et al., 2017; Muñoz and Segura-Aguilar, 2017) (**Figure 1**). DT-diaphorase is expressed both in dopaminergic neurons and astrocytes, but GSTM2 is only expressed in astrocytes. However, GSTM2 protects both astrocytes and dopaminergic neurons against aminochrome neurotoxicity because astrocytes secrete GSTM2 and dopaminergic neurons internalize GSTM2 into the cytosol (Cuevas et al., 2015; Segura-Aguilar et al., 2016; Segura-Aguilar, 2017b,c). The question is why GSTM2 and DT-diaphorase are not protecting dopaminergic neurons containing

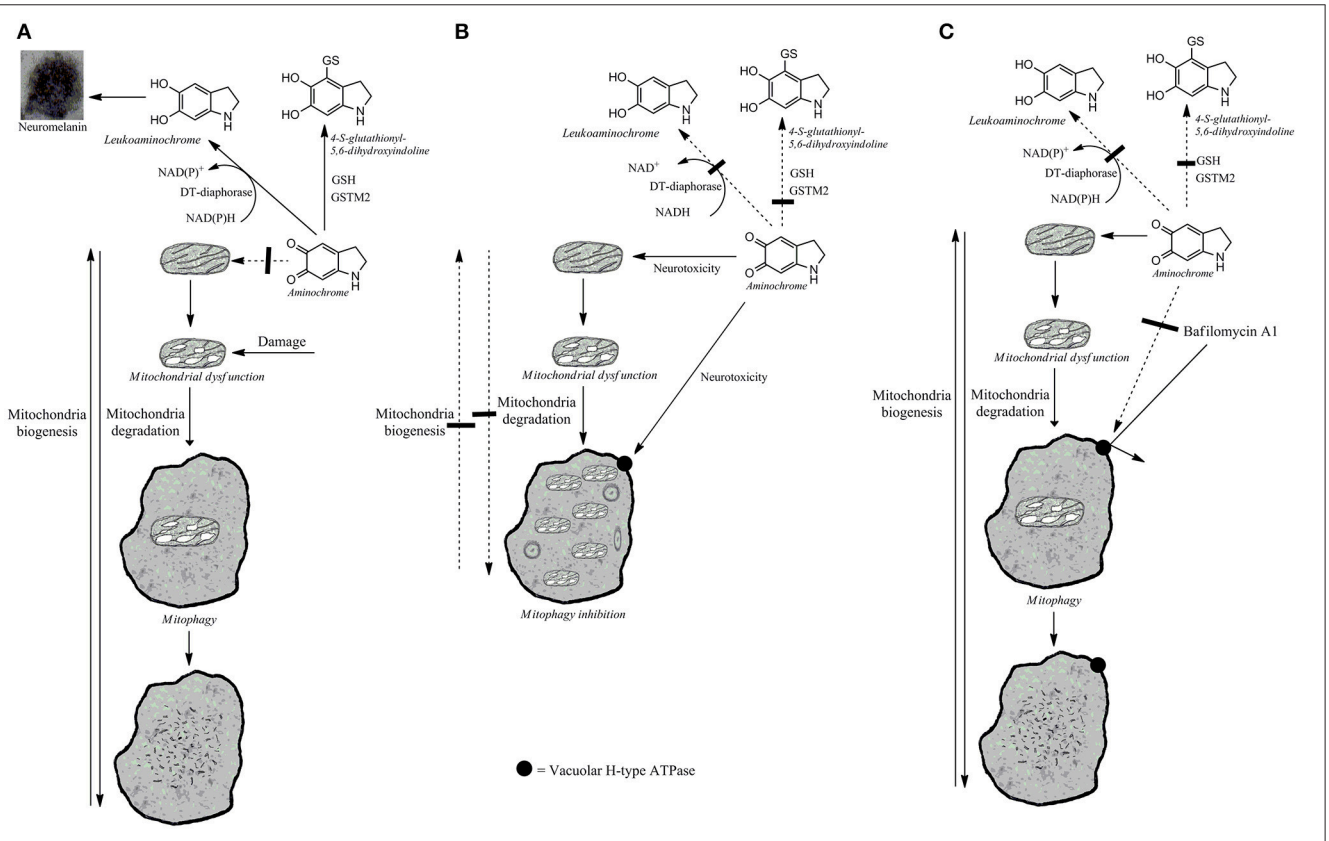


FIGURE 1 | Possible aminochrome effects on mitophagy. **(A)** Damaged mitochondria are recycled by mitophagy when the protective enzymes, DT-diaphorase, and GSTM2 prevent aminochrome-induced mitochondrial and lysosomal dysfunction. The two-electron reduction of aminochrome by DT-diaphorase to leukoaminochrome prevents aminochrome neurotoxicity (Lozano et al., 2010). Aminochrome conjugation to 4-S-glutathionyl-5,6-dihydroxyindoline catalyzed by GSTM2 prevent aminochrome neurotoxicity (Huenchuguala et al., 2014). **(B)** Aminochrome induces both mitochondrial and lysosomal protein degradation dysfunction when DT-diaphorase or GSTM2 enzymes are inhibited. Damaged mitochondria cannot be recycled by mitophagy resulting in a permanent mitochondrial dysfunction. **(C)** The reversible binding of bafilomycin to vacuolar H-type ATPase localized in lysosome membrane prevents aminochrome-induced lysosomal dysfunction, allowing the recycling of aminochrome-damaged mitochondria.

neuromelanin in Parkinson's disease patients. A possible explanation is that an over production of aminochrome surpass the enzyme capacity (Km) to prevent its neurotoxic effects or for some unknown reason these enzyme are down regulated or inhibited.

THE IMPORTANCE OF MITOPHAGY IN PREVENTING MITOCHONDRIAL DYSFUNCTION

In general, mitochondrial dysfunction activates mitophagy as a defense mechanism to remove damaged mitochondria (Brady and Brady, 2016). Mitophagy seems to play a key homeostatic role in mitochondrial quality control (Tan and Wong, 2017). It has been proposed that age-dependent deterioration of mitophagy both inhibits the removal of damaged mitochondria and impairs mitochondrial biogenesis (Palikaras et al., 2017). The problem is when mitochondrial dysfunction is induced by a neurotoxin that induces both mitochondrial and autophagy dysfunction. Aminochrome-induced mitochondrial dysfunction is irreversible because aminochrome also induces autophagy dysfunction by preventing the fusion between autophagy vacuoles and lysosome dysfunction by increasing their pH (Huenchuguala et al., 2014). Recently, we have demonstrated that mitophagy plays a key role in reversing aminochrome-induced mitochondrial dysfunction (Huenchuguala et al., 2017). The pre-incubation of cells with bafilomycin A1, a reversible inhibitor of lysosomal vacuolar-type H^+-ATPase, before the incubation with aminochrome restores ATP levels, mitochondrial membrane potential, and mitophagy, and decreases cell death (Huenchuguala et al., 2017). Aminochrome cannot form adduct with vacuolar-type H^+-ATPase because bafilomycin A1 pre-incubated with the cells prevents it. Then after a while, bafilomycin dissociates from ATPase but aminochrome is not as stable to form adducts with ATPase after bafilomycin has dissociated. When aminochrome is prevented from binding to lysosomal vacuolar-type H^+-ATPase it can bind other proteins or can be reduced by flavoenzymes. These experiments support (i) the enormous importance of mitophagy in preventing mitochondrial dysfunction and (ii) the irreversible neurotoxic action of aminochrome, as a consequence of its ability to be neurotoxic by inducing both mitochondria and mitophagy dysfunction (**Figure 1**).

CONCLUSION

Mitochondrial dysfunction seems to play an important role in the loss of dopaminergic neurons containing neuromelanin in the nigrostriatal system in Parkinson's disease. The accurate removal of dysfunctional mitochondria by mitophagy is essential for keeping control over normal mitochondrial function in neurons. The endogenous neurotoxin aminochrome induces an irreversible mitochondrial dysfunction because it induces both mitochondrial and lysosomal protein degradation dysfunction. However, when bafilomycin A1 prevented aminochrome-dependent lysosomal dysfunction, the normal mitochondrial function was recovered, highlighting the essential role of the lysosomal protein degradation system in the prevention of mitochondrial dysfunction in Parkinson's disease.

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JS-A: design and write the paper; SH: write a part of the paper.

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Autophagy Disruptions Associated With Altered Optineurin Expression in Extranigral Regions in a Rotenone Model of Parkinson's Disease

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The motor features of Parkinson's disease (PD) primarily result from a lesion to the nigrostriatal dopamine system. Numerous non-motor symptoms occur in PD, many of which are postulated to stem from pathology outside of the nigrostriatal dopamine system. Perturbations to protein trafficking, disruption of mitochondrial integrity, and impaired autophagy have repeatedly been implicated in dopaminergic neuron cell death. Previously, we demonstrated that multiple markers of autophagy are disrupted in a rotenone model of PD, with alterations occurring prior to an overt lesion to the nigrostriatal dopamine system. Whether these events occur in extra-nigral nuclei in PD and when relative to a lesion in the nigrostriatal dopamine system are generally unknown. The primary goal of these studies was to determine whether autophagy disruptions, in non-dopaminergic neuronal populations occur in an environmental model of PD utilizing a mitochondrial toxin. Here, we utilized the rat rotenone PD model, with sampling time-points before and after an overt lesion to the nigrostriatal dopamine system. In analyzing autophagy changes, we focused on optineurin (OPTN) and the autophagy marker, LC3. OPTN is an autophagy cargo adapter protein genetically linked to amyotrophic lateral sclerosis and glaucoma. In the present study, we observed OPTN enrichment in all PD-relevant brain regions examined. Further, alterations in OPTN and LC3 expression and colocalized puncta suggest specific impairments to autophagy that will inform future mechanistic studies. Thus, our data suggest that autophagy disruptions may be critical to PD pathogenesis in non-dopaminergic neurons and the onset of non-motor symptoms.

Keywords: Parkinson's disease, rotenone, autophagy, optineurin, LC3

INTRODUCTION

Parkinson's disease is a progressive debilitating neurological disease, where only a small proportion of cases have a known genetic link (<10%). The vast majority of cases have an unknown sporadic incidence which are thought to be caused by gene-environment interactions (McCulloch et al., 2008; Gao and Hong, 2011; Myers et al., 2013). Currently, clinical diagnosis relies on the evidence of several motor symptoms with attenuation by levodopa treatment; including a resting tremor, bradykinesia, stooped posture, and increased muscle tone, among others (Postuma et al., 2015). While motor symptoms primarily result from the loss of dopaminergic neurons and signaling

from the nigrostriatal dopamine system, non-motor deficits are thought to arise from pathology to several other brain regions and the peripheral nervous system (Wolters, 2009). Non-motor symptoms often precede a clinical diagnosis by years to decades and are often just as debilitating as the motor symptoms. Prominent non-motor symptoms may include REM sleep disorder, olfactory dysfunction, delayed gastrointestinal motility (typically resulting in constipation), and some visual dysfunctions (e.g., blurred vision, diplopia; Schapira et al., 2017). PD is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta, with ∼60% of this population lost by the time of clinical diagnosis (Bernheimer et al., 1973; Gelb et al., 1999).

A significant amount of PD research has focused on why the dopaminergic neurons of the substantia nigra pars compacta are selectively vulnerable. For example, dopaminergic neurons of the immediately adjacent ventral tegmental area are relatively spared and neurodegeneration is less severe in other affected regions (McRitchie et al., 1997; Braak and Braak, 2000). Much of the research points to the energetic stress of the nigral dopamine neurons; these neurons exhibit poorly myelinated axons, large dendritic processes, and a limited mitochondrial population (Double, 2012; Brichta and Greengard, 2014; Sanders et al., 2014a; Haddad and Nakamura, 2015; Pacelli et al., 2015). Further research in these neurons has revealed multiple coalescent dysfunctional pathways that contribute to their deterioration, including but not limited to; impairments in vesicle trafficking, iron accumulation, impaired oxidative stress management, mitochondrial dysfunction, Golgi fragmentation, misfolded and aggregated proteins, and impaired pathways for protein clearance (Bindoff et al., 1989; Anglade et al., 1997; Baba et al., 1998; Fujita et al., 2006; Hauser and Hastings, 2013; Hwang, 2013). However, relatively little research has considered such dysfunction in the extranigral regions that are affected. Clearly, many other brain regions are important in PD. For example, Braak et al. have demonstrated Lewy body pathology (LBP) in many extranigral regions and have proposed this pathology progresses up the brainstem, starting in the dorsal motor vagal nucleus and reaching the entire neocortex (Braak et al., 2003). Importantly, many of these regions affected are closely linked to nonmotor symptoms associated with PD and some regions also show signs of neurodegeneration (Braak et al., 1996; Wolters, 2009).

Autophagy is a catabolic process that serves as a clearance pathway for protein aggregates, cytoplasmic components, and subcellular organelles (e.g., mitochondria; Glick et al., 2010). **Figure 1** provides a schematic overview of the autophagy pathway, with critical points of dysfunction numbered for discussion. Beclin-1 interacts with ULK1 to induce autophagy. Specifically, beclin-1 is important for recruiting autophagy proteins to the pre-autophagosomal structure, during nucleation (Kang et al., 2011). Furthermore, GABARAP serves as a scaffolding protein to recruit ULK1 and beclin-1 to the site of nucleation. Upon induction of autophagy, microtubule associated protein light chain I (LC3-I) is cleaved into LC3-II and initiates the formation of a phagophore. The phagophore enters an elongation phase as cargo are recruited for degradation via cargo adaptor proteins (e.g., p62, optineurin). LC3 is the most studied autophagy protein and has been shown to interact with a variety of cargo adaptor proteins (e.g., p62, OPTN, FYCO1, NBR1). GABARAP also interacts with various cargo adaptors for cargo recruitment to the autophagosome during elongation (Schaaf et al., 2016). During elongation, GABARAP interacts with the cargo adaptors ALFY or NBR1 to recruit dysfunctional mitochondria and interacts with FYCO1 to recruit protein aggregates to the developing autophagosome. Due to this apparent overlap in function, LC3 and GABARAP have been studied to understand their critical functions in autophagy. Cells deficient in either protein show impaired autophagosome formation; GABARAP deficiency results in larger autophagosomes, while LC3 deficiency results in smaller autophagosomes. The membrane then fuses to form a mature autophagosome (a double-membraned structure marked by LC3-II on the inner and outer membranes). Finally, the mature autophagosome fuses with a lysosome, resulting in the lysosomal degradation of the inner membrane and the enclosed cargo (Klionsky et al., 2016). Autophagy is known to be impaired in PD in postmortem patient brains, in genetic and environmental *in vivo* and *in vitro* models, and has been suggested as a peripheral biomarker of PD (Anglade et al., 1997; Zhu et al., 2003; Higashi et al., 2011). However, whether such impairment occurs outside the substantia nigra is poorly understood. Currently it is unclear exactly how autophagy is impaired. Here, literature from various models points to impairments in induction, cargo recruitment, and autophagosome-lysosome fusion (Schapira, 2008; Sanchez-Perez et al., 2012). In support of the critical importance for autophagy in PD, a recent paper demonstrated PD-like behavior and pathology in the brains of mice with autophagy deficiency specific to dopaminergic neurons (Sato et al., 2018). Here, the authors reported significantly increased foot slips in autophagy-deficient mice on a runway test, deposition of alpha-synuclein aggregates in dopaminergic neurites, aggregates immuno-positive for p62 and ubiquitin in these neurons, decreased TH^+ neurons in the SNpc, and decreased dopamine and dopamine metabolites in the dorsal striatum.

OPTN is a cytoplasmic protein that serves many different roles in the cell (Ying and Yue, 2012; Slowicka et al., 2016). OPTN mutations are linked to amyotrophic lateral sclerosis, frontotemporal lobar degeneration, and normal-tension glaucoma; but it is also observed in protein inclusions present in Alzheimer's disease, Huntington's disease, and PD (Osawa et al., 2011; Ying and Yue, 2012). One of the most well studied roles of OPTN is its function as a cargo adaptor in autophagy; OPTN exhibits an LC3 interacting region near its N-terminus, and cargo recognition domains at its C-terminus (ubiquitin binding domain and coiled-coils) (Korac et al., 2013; Rogov et al., 2013; Ying and Yue, 2016). Point mutations and OPTN truncations impair its cargo adaptor function, impairing autophagy and contributing to cell death. Intriguingly, the glaucomatic $OPTN^{M98K}$ mutation was found to be a risk factor for PD in a recent genome wide association study, and PD patients exhibit a higher prevalence of developing glaucoma (Lill et al., 2012; Nucci et al., 2015; Sirohi et al., 2015). We previously demonstrated OPTN is robustly expressed in nigral

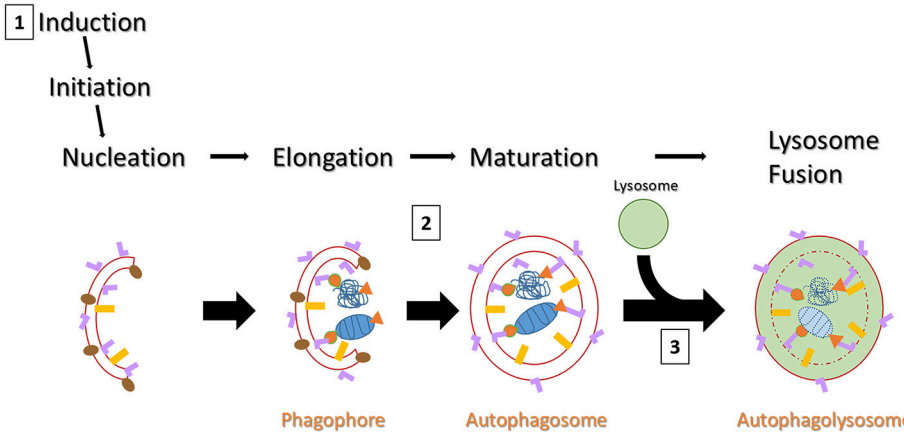


FIGURE 1 | Schematic overview of autophagy. Autophagy can be induced by a chemical stimulus or by amino acid starvation. Upon induction of autophagy, LC3-I is cleaved into LC3-II (pink "L") and an LC3-II positive membrane structure begins to form (usually on or near the endoplasmic reticulum). Meanwhile, GABARAP (gold rectangles) is expressed on the pre-autophagosomal structure during nucleation. GABARAP serves to recruit ULK1 (not pictured) and Beclin-1 (brown ovals). Beclin-1 serves as a scaffold protein to recruit more autophagy proteins and plays a key role in regulating autophagosome development. GABARAP then serves a similar role as LC3-II for cargo recruitment, but interacts with different cargo adaptors (not pictured) than LC3-II. As the membrane grows, various cargo are brought to the interior via cargo adaptor proteins (e.g., p62 or OPTN). Here, cargo is represented by a mitochondrion and a protein aggregate (both in blue) with orange p62 (triangles) and OPTN (tear drops) acting as cargo adaptors. The membranes fuse, forming a double-membraned autophagosome, marked by LC3-II on both inner and outer fuses with a lysosome and begins membranes. After maturity, the autophagosome lysosomal degradation of the inner membrane and all its constituents. There are three likely points of dysfunction that could result in detectable pathology *in vivo*: **(1)** failed induction would results in fewer autophagosomes being formed but little to no impact on expression and puncta formation of cargo adaptor proteins; **(2)** failed or delayed binding of LC3-II with cargo adaptor proteins would result in increased LC3 and cargo adaptor puncta, but decreased colocalized puncta; **(3)** failed lysosome fusion would result in all puncta being increased.

dopamine neurons, and its expression and interaction with LC3 is elevated after rotenone exposure in rats (Wise and Cannon, 2016). However, expression in other brain nuclei and changes in such expression in PD models are unknown. Our aim here was to address the key gaps in the literature with respect to brain-wide autophagy changes through testing the hypothesis that autophagy disruption would occur in extra-nigral nuclei important in PD and that such changes would precede a lesion to the nigrostriatal dopamine system.

MATERIALS AND METHODS

Overall Experimental Design Relative to Previous Studies

Detailed methodology is described below. However, it is worth noting that the exact animals utilized in this study have been well-characterized for lesion development in the nigrostriatal dopamine system and autophagic disruption in dopaminergic neurons (Wise and Cannon, 2016). None of the analyses in that publication overlap with this report, which has a different set of experimental goals. The main goal here was to examine autophagic disruptions in brain nuclei linked to non-motor PD symptoms in response to treatment with rotenone, a known mitochondrial toxin used to model PD. With the temporal development of the lesion already published in these animals and also extensively characterized in other studies (Cannon et al., 2009; Wise and Cannon, 2016), we are able to determine if changes in other brain regions precede an overt lesion to the nigrostriatal dopamine system.

Animals

All animals were male wild-type Lewis rats purchased from Harlan (now Envigo; Indianapolis, IN) and were between the ages of 31–42 weeks when euthanized. Rats were housed under standard 12 h light cycle, with free access to water and fed *ad libitum*. This study was carried out in accordance with the recommendations of United States Department of Agriculture (USDA) and the United States Public Health Service (USPHS) in accordance with the Animal Welfare Act and Purdue's Animal Welfare Assurance The protocol was approved by the Purdue University Animal Care and Use Committee.

Chemicals and Reagents

Primary antibodies for rabbit anti-OPTN (ab23666) and rabbit anti-beclin-1 (ab62557) were purchased from abcam (Cambridge, MA, USA); mouse anti-LC3 was purchased from MBL International Corporation (M152-3, Woburn, MA, USA); mouse anti-alpha-synuclein was purchased from BD Transduction Labs (610786, San Jose, CA, USA); chicken anti-MAP2 (AB5543), sheep anti-tyrosine hydroxylase (AB1542), anti-tryptophan hydroxylase (AB1541), and anti-choline acetyltransferase (AB1582) were purchased from EMD Millipore (Billerica, MA, USA). Normal donkey serum (017-000-121) and secondary antibodies, Alexa Fluor® 647 anti-sheep (713-605-147), Alexa Fluor® 488 anti-rabbit (711-545-152), and Cy™3 anti-mouse (715-165-151) were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Rotenone (R8875) and glycerol (G5516) were purchased

from Sigma-Aldrich (St. Louis, MO, USA). Triton X-100 was purchased from Fisher BioReagents (BP151-500, Fair Lawn, NJ).

Rotenone Administration

Rotenone was administered at 3.0 mg/kg/day by intraperitoneal injection as previously described (Cannon et al., 2009). This model has been extensively characterized in terms of the temporal development of behavioral, neurochemical, and neuropathological alterations in the nigrostriatal dopamine system that are relevant to PD (Cannon et al., 2009, 2013; Cannon and Greenamyre, 2010; Tapias et al., 2010, 2014; Zharikov et al., 2015). Of note, the dose and/or time of exposure can be adjusted to produce a preclinical model, where early-stage pathogenesis can be studied, prior to overt cell death (Drolet et al., 2009; Sanders et al., 2014a,b). In the present study, we chose to sample animals at preclinical time-points, where overt motor phenotypes were not yet present and at end-stage, where postural instability, rigidity, and bradykinesia were present. These deficits have been well described in the rotenone model at this dose (Cannon et al., 2009). Thus, animals were euthanized after 24 hours ($n = 3$), 5 days ($n = 3$), or at end-stage ($n = 3$) (typically at 9–12 days). In this model, overt behavioral deficits are associated with a lesion to the nigrostriatal dopamine system (Betarbet et al., 2000; Cannon et al., 2009), while sampling at 5 days or before is prior to the development of an detectable lesion (Sanders et al., 2014a,b). Of note, the temporal development of a nigrostriatal dopamine system has been characterized in the animals used in this report (Wise and Cannon, 2016).

Brain Regions Analyzed and Markers Chosen for Analysis

We analyzed changes to expression and punctate forms of OPTN and LC3 in four nuclei implicated in Braak's hypothesis of PD: the dorsal motor vagal nucleus (10N), magnocellular raphe (RMg), locus coeruleus (LC), pedunculopontine tegmentum nucleus (PTg). We also considered changes to expression of alpha-synuclein and beclin-1 in the substantia nigra pars compacta (SNpc) because we previously showed that OPTN and LC3 expression and puncta were altered in this brain region (Wise and Cannon, 2016). These regions were chosen because they are implicated in Braak's hypothesis of Lewy body progression in PD and they are linked to many PD nonmotor symptoms (Braak et al., 2003; Wolters, 2009). For example, constipation and REM sleep behavior disorder have been linked to pathology in the 10N and the LC, respectively (Cersosimo and Benarroch, 2012; García-Lorenzo et al., 2013). Specific details of processing and analyses are given below.

Immunohistochemistry

Rats were placed under deep anesthesia with pentobarbital (>50 mg/kg) (Beuthanasia-D Special, Schering-Plow Animal Health Corp, Union, NJ, USA), then transcardially perfused with 100–150 mL PBS, followed by 250–300 mL 4% buffered paraformaldehyde (PFA). Brains were surgically removed, post-fixed in 4% PFA for ∼24 h, and then saturated with 30% sucrose at 4°C for at least 5–7 days until sinking. Each brain was coronally sectioned on a frozen sliding microtome (Microm HM 450, Thermo Scientific) at a 35 μm thickness and stored in cryoprotectant at −20°C until used for staining. Brain sections containing the desired regions were randomly selected, rinsed in 10x PBS for 10 min, six times, at room temperature (RT) on an open-air platform shaker; blocked in 10% normal donkey serum (NDS, cat. # 017-000-121) in PBS with 0.3% Triton X-100 (PBS-T) for 90 min at RT; incubated with primary antibodies for ∼48 h in PBS-T with 1% NDS at 4°C. The sections were then rinsed three times with 10x PBS at RT for 10 min each time; incubated with secondary antibodies in PBS-T with 1% NDS for 90 min at RT; and rinsed six times with 10x PBS at RT for 10 min each time before being mounted on slides and coverslipped as a wet mount using 50/50 glycerol:PBS solution. Primary antibody dilutions were: mouse anti-LC3 (1:1000); rabbit anti-OPTN (1:5000); sheep anti-tyrosine hydroxylase (1:1000); sheep anti-tryptophan hydroxylase (1:4000); sheep anti-choline acetyltransferase (1:1500). Secondary antibody dilutions were: Alexa Fluor® 647 anti-sheep, Alexa Fluor® 488 anti-rabbit, or Cy™3 anti-mouse (1:500). In pilot studies, we optimized antibody titers using graded dilutions and columetric staining; then we performed staining for MAP2A (a pan-neuronal marker) + staining for autophagy markers to verify that the majority of total puncta were observed within neurons and not extracellular (we expect the few outside the MAP2A stain were present in glia).

Microscopy and Image Analysis

Confocal images were captured on an inverted Nikon Eclipse TE 2000-U microscope with an EZ-C1 confocal, equipped with 10x, 20x, and 60x Plan Fluor objectives. Region of interest (ROI) analysis was then conducted on images using the NIS-Elements software ver. 4.30 to measure signal intensity. High magnification images (60x) were analyzed for puncta analysis; puncta were considered bright circular dots with diameters ranging from 0.5 to 2.0 μm, as has been previously described for autophagosome diameters (Mizushima et al., 2002). Changes in the % of colocalized puncta determined as # colocalized/#OPTN or # colocalized/#LC3; and were interpreted as described in **Table 1**, with the rationale for such conclusions addressed in the Discussion.

Statistical Analysis

Statistical analysis was conducted using GraphPad PRISM, ver. 6. Data for intensity and puncta analyses were found not to have a Gaussian distribution by the D'Agostino & Pearson omnibus normality test. Thus, nonparametric analysis was conducted using Kruskal-Wallis nonparametric ANOVA, followed by *post-hoc* analysis using the Dunn's test. $p < 0.05$ deemed significant for all tests.

RESULTS

Dorsal Motor Vagal Nucleus (10N)

ROI analysis was used to evaluate changes in OPTN and LC3 expression across stages of PD in our rat models: control (145 neurons), 24 h (123 neurons), 5 d (91 neurons), and end-stage (186 neurons); representative images are shown in **Figure 2A**.

TABLE 1 | Assessment of autophagy function or dysfunction by immunohistochemistry.

	Expected outcome		
Expression data	**LC3**	**OPTN**	
Induced, flux not impaired	↑	↓	
Impaired induction	↓	↓	
Impaired interaction	↓	↑	
Failed autophagosome-lysosome fusion	↑	↑	
OPTN failure or exhaustion	Unknown	↓	
Autophagosome failure or exhaustion	↓	↑	
Puncta data	**LC3**	**OPTN**	Colocalized
Induced, flux not impaired	↑	↓ or no change	No change
Impaired induction	↓	↓ or no change	↓
Impaired interaction	↓ or no change	↑	↓
Failed autophagosome-lysosome fusion	↑	↑	↑
OPTN failure or exhaustion	Unknown	↓	↓
Autophagosome failure or exhaustion	↓	↑	↓
% Colocalized data	**LC3**	**OPTN**	
Induced, flux not impaired	No change	No change	
Impaired induction	No change	↓	
Impaired interaction	↓	↓	
Failed autophagosome-lysosome fusion	↑	↑	
OPTN failure or exhaustion	↓	↑	
Autophagosome failure or exhaustion	↑	↓	

The rationale for these conclusions is addressed in the Discussion.

Figures 2B,C show changes in OPTN and LC3 expression; mean OPTN expression (relative to control) exhibited significantly decreased expression after 24 h rotenone (86.73 ± 1.615% of control, $p < 0.001$), while 5 d and end-stage expression were significantly increased (149.02 ± 2.53% and 112.33 ± 1.79%, respectively; $p < 0.001$ and < 0.01, respectively). Mean LC3 expression (relative to control) was significantly increased across all stages (128.89 ± 3.82%, 141.12 ± 2.39%, and 110.09 ± 1.50%, respectively; $p < 0.001$ in all stages). Punctate expression of OPTN, LC3 and colocalized puncta were also quantified; overtly identifiable puncta with a diameter of 0.5-2 um were counted. **Figures 2D–F** show the mean number of puncta per cell for OPTN, LC3, and colocalized puncta. There were no statistically significant differences detected across stages for OPTN or LC3 puncta. However, a statistically significant decrease in colocalized puncta was detected after 24 h rotenone. Here, the control mean was 9.2 ± 0.61 puncta per cell and 24 h mean was 5.4 ± 0.36 puncta per cell ($p < 0.001$). We then considered changes in the percent of OPTN or LC3 puncta that were colocalized by PD stage. Results show decreased percent of OPTN puncta and LC3 puncta were found colocalized only after 24 h rotenone when compared to control; 13.7 ± 0.8% and 10.8 ± 0.9% for OPTN ($p < 0.01$), 33.5 ± 2.0% and 23.1 ± 1.7% for LC3 ($p < 0.001$), respectively (**Figure 6A,B**).

Magnocellular Raphe (RMg)

Representative images of RMg staining are shown in **Figure 3A**. For ROI analyses we evaluated 161, 153, 124, and 110 neurons for control, 24 h, 5 d, and end-stage rats, respectively. **Figure 3B** shows mean OPTN expression, where we found statistically significant increases across all stages (117.8 ± 3.25%, 143 ± 4.23%, and 126.7 ± 4.02% for 24 h, 5 d, and end-stage, respectively, $p < 0.001$). **Figure 3C** shows mean LC3 expression; here, we found a significant increase after 24 h rotenone (118.2 ± 3.07%, $p < 0.001$), a significantly decreased expression after 5 d rotenone (78.16 ± 1.87%, $p < 0.001$) and in end-stage rats (88.33 ± 2.17%, $p < 0.05$). Analysis of OPTN puncta (**Figure 3D**) exhibited increased mean puncta per cel l across all stages; 67.77 ± 2.5, 91.9 ± 3.16, 120 ± 4.4, and 126.2 ± 4.3 ($p < 0.001$), for control, 24 h, 5 d, and end-stage, respectively. Mean LC3 puncta were increased across all stages, but only reached significance after 24 h rotenone (50.9 ± 2.1, $p < 0.001$ for 24 h; 42.8 ± 2.4, and 38.6 ± 1.5 for 5 d and end-stage, respectively) when compared to control (35.6 ± 1.2). Colocalized puncta per cell exhibited no differences after 24 h (23.5 ± 1.0), but significantly reduced number of puncta at 5 d and end-stage (17.3 ± 1.2 and 16.8 ± 1.1, respectively) when compared to control (22.5 ± 1.0; **Figures 3E,F**). We then considered changes in the percent of OPTN or LC3 puncta that were colocalized by PD stage. Results show decreased percent of OPTN puncta and LC3 puncta were found across all stages when compared to control; 40.8 ± 2.3%, 28.2 ± 1.3% ($p < 0.01$), 14.3 ± 0.8% ($p < 0.001$) and 13.9 ± 0.9% ($p < 0.001$) for OPTN, 65.8 ± 2.2%, 48.3 ± 1.7%, 41.7 ± 1.9%, and 43.7 ± 2.2% for LC3 ($p < 0.001$ for all), respectively (**Figures 6C,D**).

Locus Coeruleus (LC)

Representative images of LC staining are shown in **Figure 4A**. For ROI analyses we evaluated 108, 123, 149, and 140 neurons for control, 24 h, 5 d, and end-stage rats, respectively. **Figure 4B** shows mean OPTN expression, where we observed a trend for increasing OPTN expression after rotenone; 91.18 ± 1.6%, 108.65 ± 2.27% ($p < 0.05$), and 124.19 ± 2.65% ($p < 0.001$) in 24 h, 5 d, and end-stage models, respectively. **Figure 4C** shows mean LC3 expression; here, we found an initial significant decrease after 24 h rotenone (84.64 ± 1.52%, $p < 0.001$), and significantly increased expression after 5 d rotenone (108.91 ± 1.97%, $p < 0.01$) and in end-stage rats (131.61 ± 2.98%, $p < 0.001$). Analysis of OPTN puncta (**Figure 4D**) exhibited increased mean puncta per cell across all PD model stages, but gradually decreasing mean puncta per cell with stage; 25 ± 1.1, 56 ± 3.0, 44 ± 1.7, and 42 ± 1.5 ($p < 0.001$ for all PD model stages) for control, 24 h, 5 d, and end-stage, respectively. **Figure 4E** shows mean LC3 puncta per cell exhibited little to no change in preclinical PD model stages when compared to control (19 ± 0.9, 18 ± 0.8, 20 ± 0.8 puncta per cell for control, 24 h, and 5 d), but showed a statistically significant increase in the end-stage PD model (22 ± 0.9 puncta

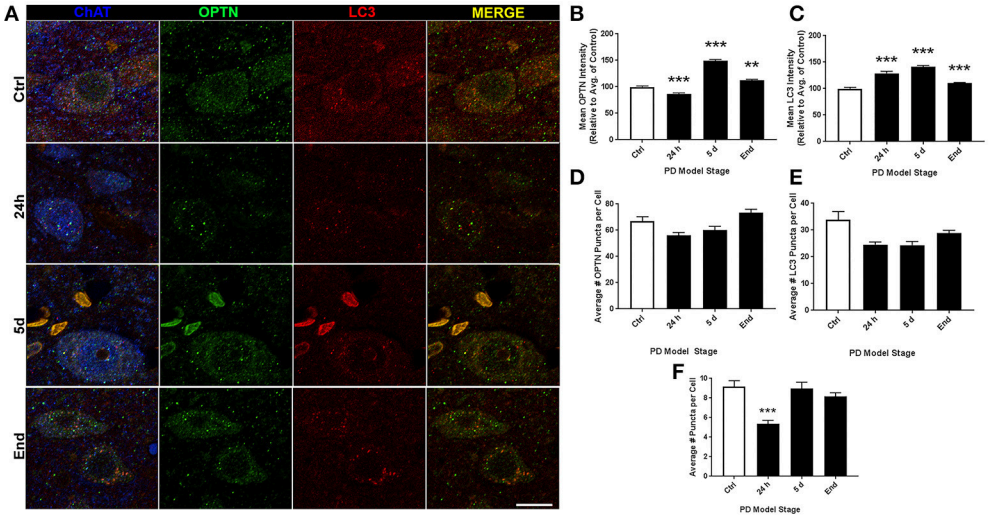


FIGURE 2 | 10N altered expression and puncta formation of OPTN and LC3 across PD stages. **(A)** Representative images of vagal cholinergic neurons co-stained with OPTN, LC3 and ChAT from animals treated as control(top), 24 h (second from top), 5 d (second from bottom), or end-stage (bottom); ChAT in blue, OPTN in green, LC3 in red (scale bar = 10 μm). **(B,C)** Quantitative analysis of OPTN and LC3 expression, respectively, measured by relative intensity of total cellular expression (relative to control); **(D–F)** mean number of puncta per neuron for OPTN, LC3 or colocalized puncta, respectively, across stages; data are presented as mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

per cell, $p < 0.05$). **Figure 4F** shows mean colocalized puncta per cell; here, we observed a trend for decreasing mean colocalized puncta per cell in the preclinical PD stages but a return to control levels in the end-stage model. Mean colocalized puncta were 13 ± 0.8, 10 ± 0.7 ($p < 0.01$), 5 ± 0.4 ($p < 0.001$), and 11 ± 0.6 for control, 24 h, 5 d, and end-stage, respectively. We then considered changes in the percent of OPTN or LC3 puncta that were colocalized by PD stage. Results show decreased percent of OPTN puncta and LC3 puncta across all stages when compared to control; 40.8 ± 2.3%, 25.1 ± 1.7% ($p < 0.001$), 12.6 ± 0.9% ($p < 0.001$) and 28.3 ± 2.2% ($p < 0.01$) for OPTN, 65.8 ± 2.1%, 51.7 ± 1.9%, 25.9 ± 1.4% and 48.4 ± 2.0% for LC3 ($p < 0.001$ for all), respectively (**Figures 6E,F**).

Pedunculopontine Tegmental Nucleus (PTg)

Representative images of PTg staining are shown in **Figure 5A**. For ROI analyses we evaluated 162, 121, 161, and 97 neurons for control, 24 h, 5 d, and end-stage rats, respectively. **Figure 5B** shows mean OPTN expression, where we observed significantly decreased OPTN expression after rotenone; 82.56 ± 1.8% ($p < 0.001$) and 84.37 ± 1.63% ($p < 0.001$) respectively; while OPTN expression was increased after 5 d rotenone (118.6 ± 2.15%, $p < 0.001$). **Figure 5C** shows mean LC3 expression; we found an initial significant increase after 24 h and 5d rotenone (119.1 ±2 .26% and 110.1 ± 1.84%, $p < 0.001$, respectively) and significantly decreased expression in end-stage rats (66.91 ± 1.83%, $p < 0.001$). Analysis of OPTN puncta (**Figure 5D**) exhibited increased mean puncta per cell after 5 d rotenone and end-stage rats; 53.3 ± 1.6, 53.9 ± 2.0, 65.2 ± 1.7, and 64.9 ± 2.9 ($p < 0.001$) for control, 24 h, 5 d, and end-stage, respectively. **Figure 5E** shows mean LC3 puncta per cell exhibited significantly increased LC3 puncta after 24 h rotenone when compared to control (33.6 ± 1.2 and 50.9 ± 2.1 puncta per cell in control and 24 h, respectively; $p < 0.001$). Slightly increased values were observed in 5 d and end-stage PD models when compared to control, but were not statistically significant (42.8 ± 2.4 and 38.6 ± 1.5, respectively). **Figure 5F** shows mean colocalized puncta per cell; here, we observed significantly reduced mean colocalized puncta per cell after 5 d rotenone and in end-stage rats. Mean colocalized puncta were 22.5 ± 1.0, 23.5 ± 1.1, 17.3 ± 1.2 ($p < 0.001$), and 16.8 ± 1.0 ($p < 0.001$) for control, 24 h, 5 d, and end-stage, respectively. We then considered changes in the percent of OPTN or LC3 puncta that were colocalized by PD stage. Results show decreased percent of OPTN puncta only after 5 d rotenone when compared to control: 28.3 ± 1.1% and 17.8 ± 0.7% ($p < 0.001$), respectively. Results show decreased percent of LC3 puncta colocalized after 24h and 5 d rotenone, but increased at end-stage: 38.5 ± 1.8%, 30.9 ± 1.8% ($p < 0.05$), 30.5 ± 1.3% ($p < 0.01$) and 42.8 ± 1.7% ($p < 0.05$) for LC3, respectively (**Figures 6G,H**).

Beclin-1 and Alpha-Synuclein Expression in SNpc

Representative images of SNpc staining are shown in **Figure 7A**. For ROI analyses we evaluated 158, 193, 99, and 160 neurons for control, 24 h, 5 d, and end-stage rats, respectively. **Figure 7B** shows mean beclin-1 expression, where we observed significantly decreased beclin-1 expression after 5 d rotenone and in end-stage rats; 59.16 ± 3.93% ($p < 0.001$) and 73.68 ± 2.85% ($p < 0.001$) respectively. **Figure 7C** shows mean alpha-synuclein expression; here, we found an initial significant increase after 24 h and

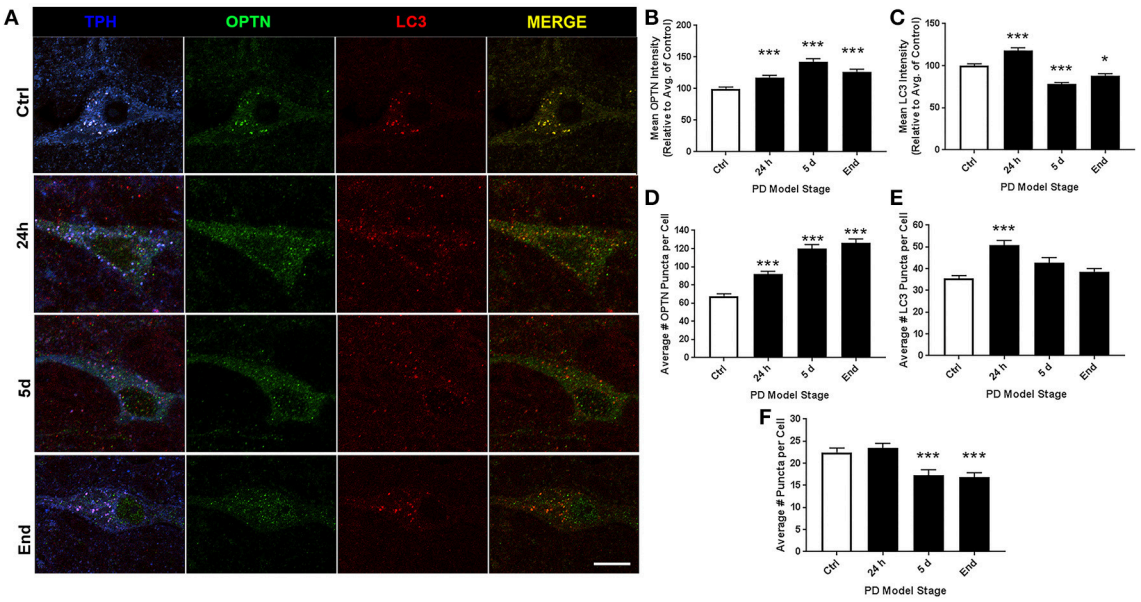


FIGURE 3 | RMg altered expression and puncta formation of OPTN and LC3 across PD stages. **(A)** Representative images of raphe serotonergic neurons co-stained with OPTN, LC3 and TPH from animals treated as control(top), 24 h (second from top), 5 d (second from bottom), or end-stage (bottom); ChAT in blue, OPTN in green, LC3 in red (scale bar = 10 μm). **(B,C)** Quantitative analysis of OPTN and LC3 expression, respectively, measured by relative intensity of total cellular expression (relative to control); **(D–F)** mean number of puncta per neuron for OPTN, LC3 or colocalized puncta, respectively, across stages; data are presented as mean ± SEM; $^{*}p < 0.05$, $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

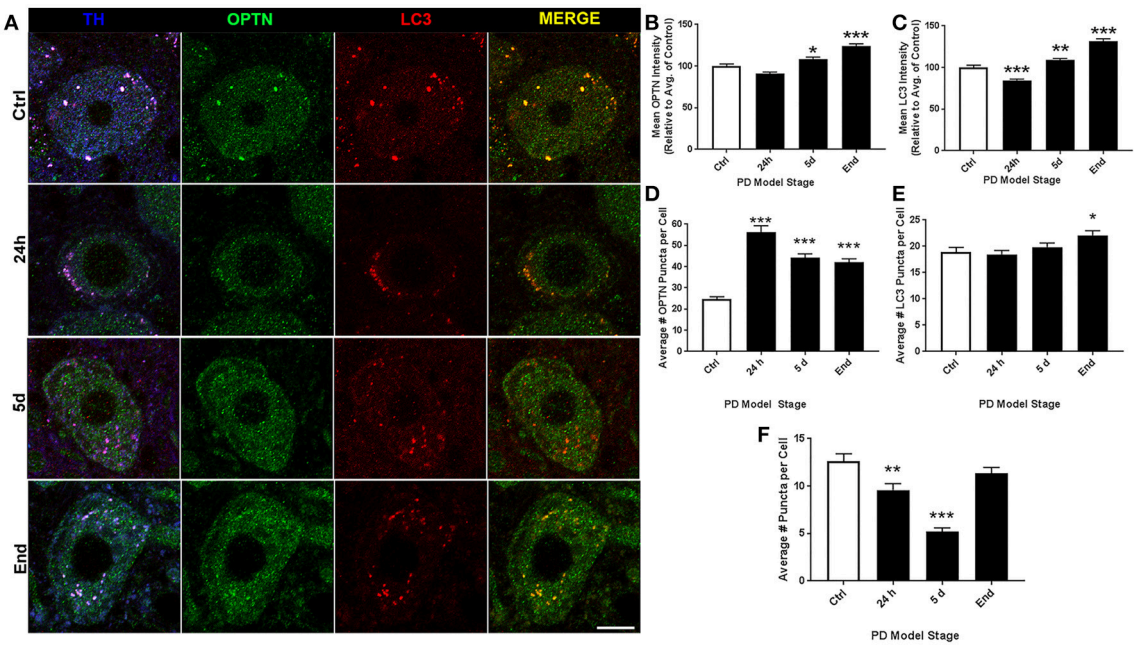


FIGURE 4 | LC altered expression and puncta formation of OPTN and LC3 across PD stages. **(A)** Representative images of coerular dopaminergic neurons co-stained with OPTN, LC3, and TH from animals treated as control(top), 24 h (second from top), 5 d (second from bottom), or end-stage (bottom); ChAT in blue, OPTN in green, LC3 in red (scale bar = 10 μm). **(B,C)** Quantitative analysis of OPTN and LC3 expression, respectively, measured by relative intensity of total cellular expression (relative to control); **(D–F)** mean number of puncta per neuron for OPTN, LC3 or colocalized puncta, respectively, across stages; data are presented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

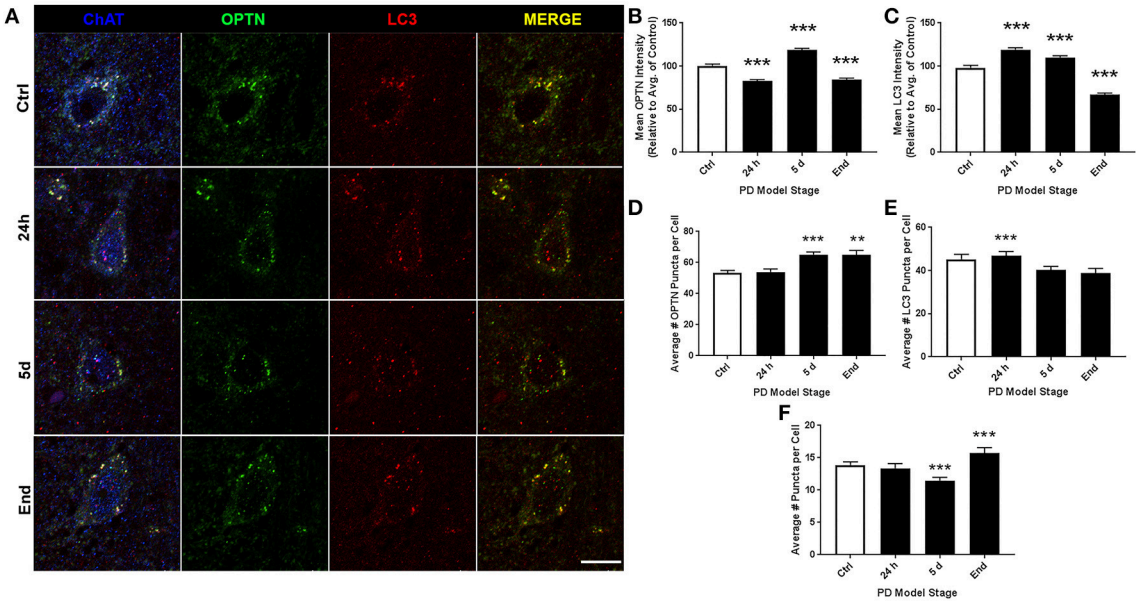


FIGURE 5 | PTg altered expression and puncta formation of OPTN and LC3 across PD stages. **(A)** Representative images of pontotegmental cholinergic neurons co-stained with OPTN, LC3, and TPH from animals treated as control(top), 24 h (second from top), 5 d (second from bottom), or end-stage (bottom); ChAT in blue, OPTN in green, LC3 in red (scale bar = 10 μm). **(B,C)** Quantitative analysis of OPTN and LC3 expression, respectively, measured by relative intensity of total cellular expression (relative to control); **(D–F)** mean number of puncta per neuron for OPTN, LC3 or colocalized puncta, respectively, across stages; data are presented as mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

5d rotenone (206.4 ± 8.5% and 152.3 ± 8.73%, $p < 0.001$, respectively), and slightly decreased expression in end-stage rats (89.8 ± 5.49%, $p < 0.001$).

Basal Autophagic Activity in Brainstem Regions

Finally, we compared the mean OPTN and LC3 puncta in control animals across all brainstem regions considered here (**Figure 8**). We also considered data from our previous report to compare basal autophagic activity to SNpc as well. Our data suggest the dopaminergic neurons in LC and SNpc exhibit significantly lower autophagic activity than other regions. Average numbers of LC3 and OPTN puncta were significantly lower when compared to 10N, RMg, or PTg. Furthermore, average number of OPTN and LC3 puncta in dopaminergic neurons of SNpc were even lower than those of LC.

DISCUSSION

Autophagy disruptions have been repeatedly implicated in the pathogenesis of PD and other neurodegenerative diseases. However, the temporal development of autophagic changes is poorly characterized with respect to phenotype onset. Further, there is a lack of data on how brain regions outside the substantia nigra may be affected. In this study, we considered OPTN's autophagic role in four extranigral regions during PD pathogenesis by analyzing the expression and interaction of OPTN and LC3 using a rat rotenone model of PD. Collectively, we show that autophagy is affected in multiple brain nuclei important in PD, and that changes in these regions precede a lesion to the nigrostriatal dopamine system. Taking these data together, we can assess impairments to autophagy that may result in autophagic stress. While autophagy is ideally assessed dynamically (e.g., live-cell imaging), our approach to assess multiple endpoints and assessing populations of neurons improves our ability to assess its activity at a single time point. Finally, our data suggest dopaminergic neurons exhibit lowered basal autophagic activity, and may be limited in their autophagic capacity—especially those in the SNpc (see **Figure 8**). Such a limitation may render these neurons prone to cellular stress and neurodegeneration when autophagy is required for neuroprotection.

In this study, we consider the role of OPTN in autophagy as we believe it is the autophagy cargo adaptor most likely to contribute to PD. Thus far only two studies have considered OPTN in PD; a paper from Osawa and colleagues who immunostained for OPTN in patients with PD and our previous report considering OPTN expression, puncta, and colocalization with LC3 in the same rat rotenone model we used here (Osawa et al., 2011; Wise and Cannon, 2016). The report by Osawa et al. is very limited for its investigation of OPTN in PD; only two high magnification images are shown of what the authors report as an OPTN-positive Lewy body and Lewy neurite, but no comparison to a control patient was provided and no validation of the criteria for Lewy bodies and neurites (e.g., alpha-synuclein immunostain; Osawa et al., 2011). OPTN was previously demonstrated to be an essential cargo adaptor for mitophagy, and mitophagy is critical for clearing dysfunctional mitochondria from neuronal

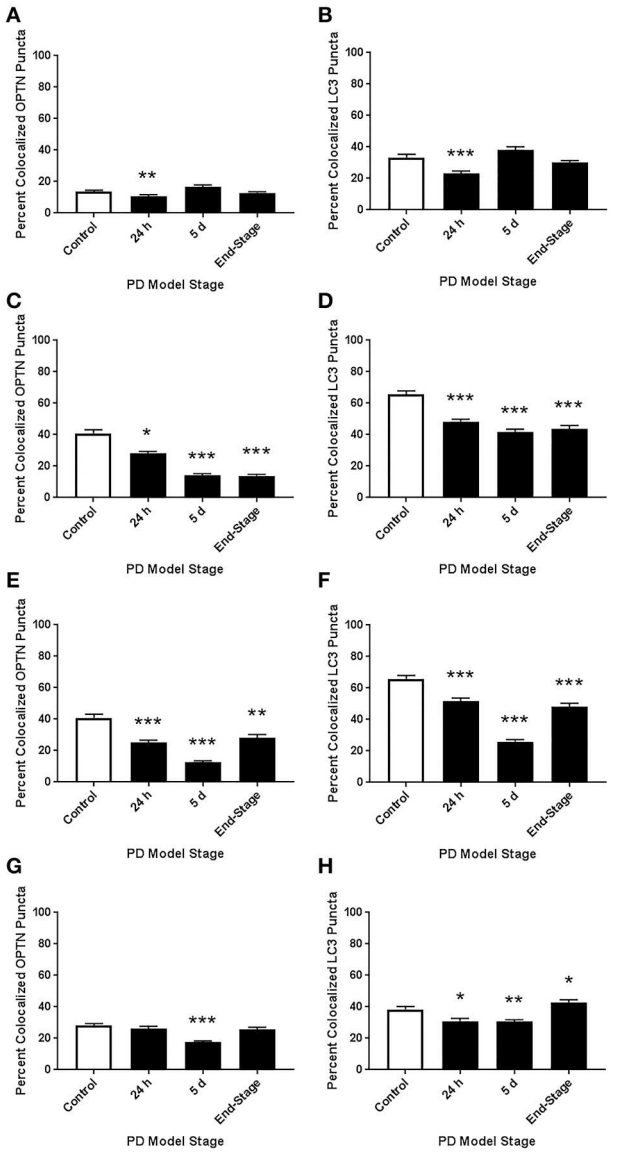


FIGURE 6 | Altered colocalization of OPTN and LC3 across brainstem regions and PD stages. Here, we assessed changes in the colocalization of OPTN and LC3 in each region across all PD stages with our rotenone model. We quantified changes in the percent of OPTN puncta that were colocalized and changes in the percent of LC3 puncta that were colocalized in 10N **(A,B)**, RMg **(C,D)**, LC **(E,F)**, and PTg **(G,H)**. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

populations (Wong and Holzbaur, 2014). Here, we used rotenone as a PD model; rotenone is a mitochondrial complex I inhibitor, and is known to induce mitophagy in neuronal populations (Pan et al., 2009; Gao et al., 2012; Chu et al., 2013). Thus, it is very likely that the changes we see in OPTN expression and puncta formation are linked to mitochondrial dysfunction and mitophagy activity. Furthermore, OPTN is the most likely candidate because from a recent genome-wide association study, only the $OPTN^{M98K}$ mutation was found to significantly increase risk of PD when all known autophagy cargo adaptors were considered (Lill et al., 2012). Importantly, we show OPTN is expressed in multiple extranigral regions that are implicated in Braak's hypothesis of PD progression (10N, RMg, LC, PTg). In each region, OPTN expression was more robust than surrounding nuclei, further implying a potential role for OPTN in PD pathogenesis (Braak and Braak, 2000; Braak et al., 2003).

Autophagy is a complex process that requires considerable cellular resources to execute; e.g., cell signaling for induction, recruitment of membranes from other organelles, complex signaling to identify and recruit cargo and to encourage autophagosome-lysosome fusion (Behrends et al., 2010; Glick et al., 2010; Damme et al., 2015). Despite the many complexities required to complete autophagy from induction to cargo degradation, there appear to be relatively few pathological endpoints that can be observed *in vivo*: autophagy is induced but unaffected (exhibiting increase LC3 expression and puncta, with decreased OPTN), impaired induction (reducing LC3 expression and number of autophagosomes), impaired cargo recruitment (e.g., OPTN-LC3 binding), or impaired autophagosome-lysosome fusion (resulting in increased LC3 expression and number of autophagosomes). Another potential impairment in autophagy lies in the activity of cargo adaptors (e.g., OPTN, p62, NBR1), though this is much more difficult to detect *in vivo* as many of these proteins require post-translational modifications to encourage binding to cargo or LC3 (Rogov et al., 2014). Based on this literature, we present a means of detecting impairment of cargo adaptor-LC3 interaction by quantifying the percent of LC3 or cargo adaptor puncta that are colocalized. With this method of analysis, we can evaluate potential impairments to autophagy induction as: (1) a decrease in the percent of colocalized OPTN puncta, without changes in colocalized LC3 puncta as indication of fewer autophagosomes to bind OPTN puncta, but interaction is unchanged; (2) impaired OPTN-LC3 interaction as decreased percent of colocalized puncta for both OPTN and LC3; or (3) impaired autophagosome-lysosome fusion as an increase in both. We can also evaluate changes in OPTN's activity as a cargo adaptor as: (1) no change or a decrease in the percent of colocalized OPTN puncta coinciding with increased percent of LC3 colocalized puncta; or (2) the availability of autophagosomes (i.e., autophagosome depletion) as no change or an increase in the percent of LC3 puncta coinciding with a decrease in the percent of colocalized OPTN puncta.

PD is well characterized as a systemic disorder, with extensive pathology beyond that in nigral dopaminergic neurons. Despite this, the vast majority of PD research still focuses on nigral pathology, dopaminergic dysfunction, or dopaminergic neuroprotection. Previous studies have shown histological evidence of alpha-synuclein aggregation in dopamine neurons of the substantia nigra of rats treated with the rotenone dose used in this study (Betarbet et al., 2000; Cannon et al., 2009). Of note, these studies showed qualitative evidence of the formation of Lewy body-like aggregates, whereas, here we conducted whole-cell ROI analysis. Interestingly, we found whole-cell alpha-synuclein levels to increase acutely and then return near baseline (**Figure 7C**). Indeed, while whole-cell synuclein levels decrease during the course of treatment, aggregate formation

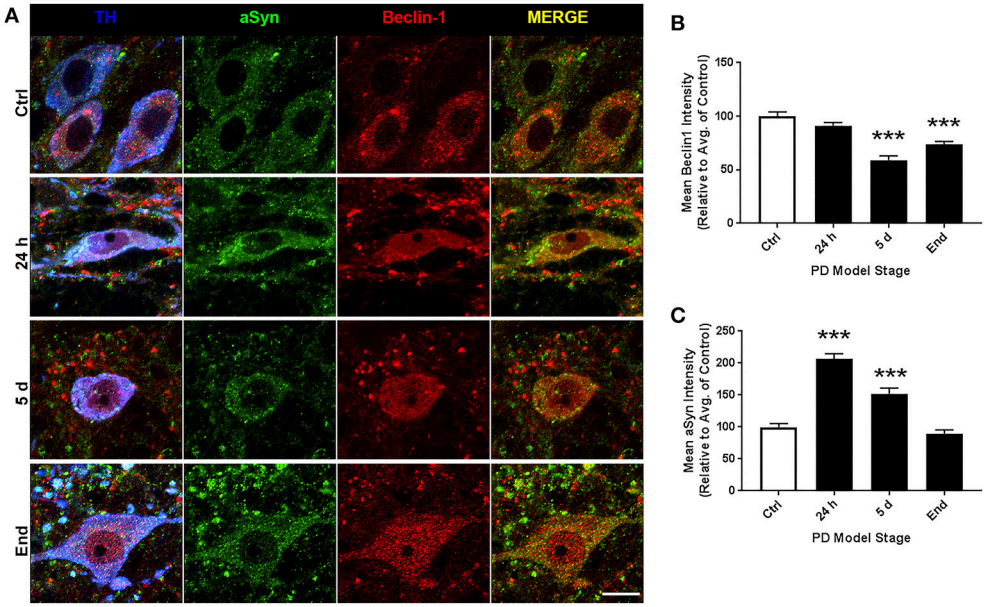


FIGURE 7 | SN altered expression of Beclin-1 and alpha-synuclein across PD stages. **(A)** Representative images of nigral dopaminergic neurons co-stained with alpha-synuclein, beclin-1, and TH from animals treated as control(top), 24 h (second from top), 5 d (second from bottom), or end-stage (bottom); TH in blue, alpha-synuclein in green, beclin-1 in red (scale bar = 10 um). **(B,C)** Quantitative analysis of beclin-1 and alpha-synuclein expression, respectively, measured by relative intensity of total cellular expression (relative to control); data are presented as mean ± SEM; $^{***}p < 0.001$ from control, Dunn's multiple comparison test after significant Kruskal-Wallis test.

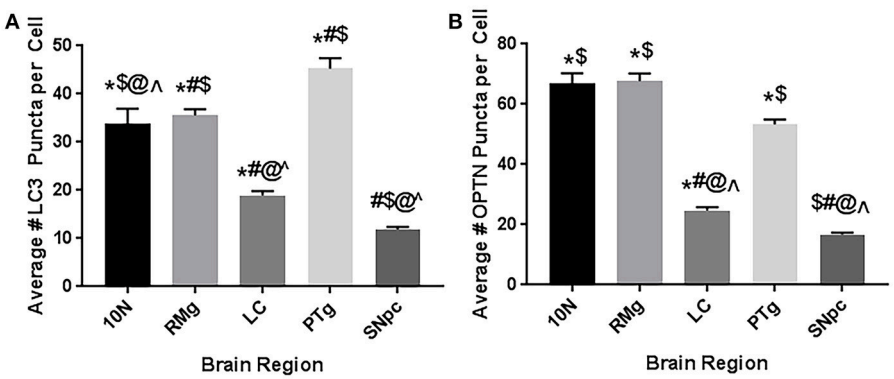


FIGURE 8 | Basal autophagic activity in brainstem regions implicated in Braak's hypothesis. Data represents the mean number of **(A)** LC3 puncta (i.e., autophagosomes) or **(B)** OPTN puncta per cell in 10N, RMg, LC, PTg, and SNpc. Data suggest the dopaminergic neurons (LC and SNpc) exhibit less basal autophagic activity than other regions. $^{*}p < 0.05$ vs. SNpc; $^{\#}p < 0.05$ vs. 10N; $^{\$}p < 0.05$ vs. LC; $^{@}p < 0.05$ vs. RMg; $^{\wedge}p < 0.05$ vs. PTg.

is also apparent in our data (**Figure 7A**). This result may be indicative of dynamic redistribution during aggregate formation that has been described in cell culture experiments for PD and other neurodegenerative diseases (Arrasate et al., 2004; Opazo et al., 2008). While the etiology of PD remains elusive, Braak et al. have published a substantial amount of work showing progression of LBP through distinct synaptic tracts in the PD brain, but how LBP progresses from one region to another remains unclear. Given that large protein aggregates are normally cleared via autophagy, we proposed autophagic dysfunction might follow a similar pattern and precede LBP. Intriguingly, we observed decreasing beclin-1 expression coinciding with alpha-synuclein expression redistribution from cytosolic expression to more punctate expression (**Figure 7**). Indeed, beclin-1 has been shown to aid in autophagic clearance of alpha-synuclein aggregates and its dysfunction may have a pivotal role in autophagic dysfunction (Spencer et al., 2009). Considering the autophagic pathway (see **Figure 1**), we defined three possible outcomes of impairment that could be detected *in vivo* using our immunohistochemical staining: (1) impaired induction, (2)

impaired OPTN-LC3 binding, and (3) impaired autophagosome-lysosome fusion. We also considered the possibilities of OPTN failing to serve as a cargo adaptor protein and of depleted autophagosome pool. Interestingly, our results suggest different types of impairment in different regions (see **Table 2**). Most of our data suggest impairments in OPTN-LC3 binding (perhaps due to impaired phosphorylation of OPTN by TBK1) or impaired autophagosome-lysosome fusion. Specifically, results from the 10N suggest early disruptions (after 24 h rotenone) in LC3-OPTN binding (evidenced by significantly decreased colocalized puncta in **Figure 2F** and significantly decreased percent of colocalized puncta in **Figures 6A,B**), and later impairment of autophagy induction (based on decreased number of LC3 puncta in **Figure 2E**). The RMg largely exhibited pathology that suggested impaired LC3-OPTN interaction and autophagosome-lysosome fusion across all stages (based on consistently decreased percent of colocalized puncta seen in **Figures 6C,D**), and likely exhaustion of the autophagosome pool at end-stage PD (especially when considering the decreasing number of LC3 puncta coinciding with increasing OPTN puncta in **Figures 3D,E**). Data from LC suggest autophagy induction is initially impaired (based on 24 h expression data in **Figures 4B,C**) with impaired LC3-OPTN interactions (see **Figure 4F**), but then shifts entirely to impaired binding between LC3 and OPTN (based on puncta analyses in **Figures 4F**, **6E,F**). There also seems to be potential impairment of autophagosome-lysosome fusion after 5 d rotenone, especially when considering the accumulation of LC3 and colocalized puncta (**Figure 4F**) and percent of colocalized puncta (**Figures 6E,F**), where we see increases in these values between 5 d and end-stage, reversing the apparent trend of decreasing values between control, 24 h, and 5d. Data from the PTg collectively showed the least amount of autophagic impairments. Importantly, we see especially little pathology after 24 h rotenone, where we saw signs of autophagic dysfunction in lower brainstem regions. We began to see signs of autophagic dysfunction in the PTg after 5 d rotenone, where there appears to be impaired LC3-OPTN binding (significantly decreased number of colocalized puncta in **Figure 5F** and percent of colocalized LC3 and OPTN puncta in **Figures 6G,H**) and/or autophagosome-lysosome fusion (significantly increased colocalized puncta in **Figure 5F**). Alternatively, these data could indicate autophagosome depletion, where we see decreasing number of autophagosomes with increasing PD severity (**Figure 5E**) and significantly increased percent of colocalized LC3 puncta but no change in percent of colocalized OPTN puncta in end-stage animals (**Figures 6G,H**). While these data do not indicate if autophagic dysfunction is occurring ahead of LB pathology progression, the data do suggest a similar pattern—with lower brainstem regions (10N, RMg, LC) exhibiting signs of autophagic dysfunction earlier than the PTg which is the brainstem region highest in the brain and last affected of the regions considered in this study.

Given that the nigral dopaminergic neurons exhibit the most severe deterioration in PD, much research has considered what makes this population selectively vulnerable. Collectively, the literature points to limitations in the energetic capacity of these neurons. Nigral dopamine neurons are characterized by dense and extensive dendritic processes, with each neuron exhibiting thousands of synaptic connections (Matsuda et al., 2009). This, combined with poorly myelinated processes, puts a significant amount of stress on the energy demand of the neurons (Braak et al., 2003; Matsuda et al., 2009). Compounding these high-energy demands, dopaminergic neurons of the SNpc were reported to have relatively low mitochondrial populations and energetic capacities per neuron (Pacelli et al., 2015). Our results suggest yet another reason why these neurons might be selectively vulnerable; when we consider the mean number of LC3 puncta per neuron in control animals across all the regions considered, SNpc dopaminergic neurons exhibited the fewest, while the cholinergic neurons of the PTg exhibited the highest number of LC3 puncta. If these neurons exhibit limited autophagic capacity, they would be severely limited in their ability to clear pathogenic protein aggregates such as Lewy bodies and dysfunctional mitochondria that leak reactive oxygen species. Such a limitation could create a negative feedback loop, with increased oxidative stress from dysfunctional mitochondria impairing the already limited autophagic capacity of these neurons and thus prolonging the damage incurred. This hypothesis is further supported by Sato et al. who reported their results from mice with selective autophagy-deficiency in dopaminergic neurons in mice. In their report, these autophagy-deficient mice exhibited behavior and pathology in LC and SNpc akin to other PD models (Sato et al., 2018).

Assessing autophagic dysfunction *in vivo* has been extremely limited due to its dynamic and microscopic process (Klionsky et al., 2016). *In vivo* assessment of autophagy through immunohistochemistry is an area of autophagy research that is severely limited and lacking standardized analyses (Klionsky et al., 2016). Here, we presented a new manner of considering autophagic dysfunction *in vivo* using a multivariate analysis approach: (1) quantifying expression of LC3 and OPTN (a known autophagy cargo-adaptor), (2) quantifying puncta formation of each (also considering colocalized puncta), and (3) by assessing changes to the percent of total LC3 and OPTN puncta that are colocalized. This novel approach to *in vivo* autophagy analysis enables the evaluation of several critical steps in autophagy flux. Here, we use this approach to asses changes in autophagy induction, interaction between LC3 and OPTN (an autophagy cargo adaptor), autophagosome-lysosome fusion, and exhaustion or depletion of OPTN serving a cargo adaptor function or availability of autophagosomes. Given that autophagy is a complex process, the best approach to analyze its function or dysfunction utilizes live-cell imaging and pharmacological inhibition of specific junctions (e.g., induction or autophagosome-lysosome fusion). To address the challenge of autophagy changes over time and to assess autophagic activity changes over the course of PD progression, we considered these endpoints using a thoroughly characterized dosing scheme for the PD neurotoxin rotenone. Our results demonstrate variable types of impairment in extranigral regions implicated in preclinical PD, which supports our hypothesis that autophagic dysfunction may precede the spread of Lewy bodies through the PD brain. Further investigation is needed to understand the complexity of autophagic dysfunction in these regions and

TABLE 2 | Overall summary of autophagic changes: *Results for OPTN and LC3*.

	Braak region:	**10N**			**RMg**			**LC**			**PTg**		
	PD model stage:	**24 h**	**5d**	**End**	**24 h**	**5d**	**End**	**24 h**	**5d**	**End**	**24 h**	**5d**	**End**
	Expected outcome												
Autophagy Induced, flux unimpaired	Expression: Increased LC3, OPTN decreased or no change	X									X		
	# Puncta: LC3 increased, OPTN decreased or no change in colocalized	/									X		
	% Colocalized Puncta: No changes												
(1) Impaired Induction	Expression: Decreased LC3, OPTN decreased or no change							X					X
	# Puncta: OPTN increased or no change, LC3 and colocalized decreased	/	X									/	
	% Colocalized Puncta: % colocalized OPTN decreased, no change in % colocalized LC3												
(2) Impaired OPTN-LC3 Binding	Expression: Decreased LC3, increased OPTN												
	# Puncta: OPTN increased, LC3 decreased or no change, colocalized decreased			/		/		X	X	/		/	
	% Colocalized Puncta: Decreased LC3 and OPTN	X			X	X	X	X	X	X	/	X	
(3) Impaired Autophagosome-Lysosome Fusion	Expression: Increased LC3 and OPTN		X	X	X	/	/		X	X		X	
	# Puncta: All increased (colocalized increased substantially)				X	/				/			/
	% Colocalized Puncta: Increased LC3 and OPTN												
OPTN Failure or Exhaustion	Expression: Decreased OPTN, unknown effect on LC3	/									/		
	# Puncta: Decreased OPTN, unknown effect on LC3, decreased colocalized	/											
	% Colocalized Puncta: Decreased LC3, increased OPTN												
Autophagosomeexhaustion	Expression: Decreased LC3, increased OPTN					/	/						
	# Puncta: Increased OPTN, decreased LC3 and colocalized			/			X					/	/
	% Colocalized Puncta: Increased LC3, decreased OPTN												/

We identified four possible endpoints to assess autophagy impairments in vivo when considering our data points: autophagy is induced but unaffected (i.e., autophagy is functioning normally), autophagy induction is impaired, OPTN-LC3 binding is impaired, or autophagosome-lysosome fusion are impaired. "X" indicates all criteria were met, whereas "/" indicates criteria were partially met.

how it contributes to PD progression. Importantly, if autophagic dysfunction precedes LBP, it may become an important target for future PD therapeutics to help slow the progression of the disease.

AUTHOR CONTRIBUTIONS

JW Designed, performed, and analyzed experiments; wrote the 1st draft, and subsequent drafts. CP Performed and analyzed experiments. JA Performed and analyzed experiments. JC Responsible for all aspects of the research design, execution and interpretation; review and refinement of initial and subsequent manuscript drafts. JC also funded the work through internal and extramural awards.

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REVIEW
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Mitochondrial Quality Control in Neurodegenerative Diseases: Focus on Parkinson's Disease and Huntington's Disease

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In recent years, several important advances have been made in our understanding of the pathways that lead to cell dysfunction and death in Parkinson's disease (PD) and Huntington's disease (HD). Despite distinct clinical and pathological features, these two neurodegenerative diseases share critical processes, such as the presence of misfolded and/or aggregated proteins, oxidative stress, and mitochondrial anomalies. Even though the mitochondria are commonly regarded as the "powerhouses" of the cell, they are involved in a multitude of cellular events such as heme metabolism, calcium homeostasis, and apoptosis. Disruption of mitochondrial homeostasis and subsequent mitochondrial dysfunction play a key role in the pathophysiology of neurodegenerative diseases, further highlighting the importance of these organelles, especially in neurons. The maintenance of mitochondrial integrity through different surveillance mechanisms is thus critical for neuron survival. Mitochondria display a wide range of quality control mechanisms, from the molecular to the organellar level. Interestingly, many of these lines of defense have been found to be altered in neurodegenerative diseases such as PD and HD. Current knowledge and further elucidation of the novel pathways that protect the cell through mitochondrial quality control may offer unique opportunities for disease therapy in situations where ongoing mitochondrial damage occurs. In this review, we discuss the involvement of mitochondrial dysfunction in neurodegeneration with a special focus on the recent findings regarding mitochondrial quality control pathways, beyond the classical effects of increased production of reactive oxygen species (ROS) and bioenergetic alterations. We also discuss how disturbances in these processes underlie the pathophysiology of neurodegenerative disorders such as PD and HD.

Keywords: Parkinson's disease, Huntington's disease, mitochondrial quality control, mitochondrial dysfunction, neurodegeneration

MITOCHONDRIAL DYSFUNCTION: A COMMON FEATURE IN NEURODEGENERATIVE DISORDERS

Despite distinct clinical and pathological features, neurodegenerative diseases share critical processes such as (i) the presence of misfolded and/or aggregated proteins; (ii) neuroinflammation; (iii) impairment of autophagy; (iv) oxidative stress; and (v) mitochondrial anomalies. Neurons are highly dependent on mitochondrial function to maintain energy-intensive functions such as membrane excitability, neurotransmission, and plasticity: the brain consumes 20% of the total oxygen and about a quarter of the total glucose used by the body for energy supply.

Parkinson's Disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder, affecting 1–3% of the population over 65 years of age. Most PD patients are diagnosed as sporadic patients with idiopathic PD, occurring from complex interactions between environmental and genetic factors. The main features of this pathology are motor alterations such as resting tremor, postural instability, rigidity and bradykinesia, and non-motor symptoms like fatigue, depression, anxiety, sleep and autonomic disturbances, decreased motivation, apathy, decline in cognition and dementia. The neuropathological hallmarks of PD include loss of dopaminergic neurons in the midbrain substantia nigra pars compacta (SNpc) and accumulation of Lewy bodies (LBs) containing α-synuclein.

The first evidence linking mitochondrial dysfunction and PD was the discovery that MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), an inhibitor of complex I (NADH/ubiquinone oxidoreductase) of the mitochondrial electron transport chain, causes parkinsonism in humans (Langston et al., 1983). Later, sporadic PD patients were reported to present reduced complex I activity, not only in the SNpc (Schapira et al., 1990) but also in other brain areas and peripheral tissues (Parker et al., 1989, 2008; Krige et al., 1992; Blin et al., 1994; Haas et al., 1995), as well as in cytoplasmic hybrid (cybrid) cells (Swerdlow et al., 1996) derived from PD patients (**Figure 1**). Besides the mitochondrial complex I defect, genetic evidence suggests that mutations in mitochondrial DNA (mtDNA) also play a role in the pathogenesis of PD. Haplogroups J and K have a protective effect against PD in certain European populations (van der Walt et al., 2003; Fachal et al., 2015), whereas haplogroup HV has been reported to increase the risk of PD in Caucasians (Hudson et al., 2013). Alterations in mtDNA have long been hypothesized to play a pathogenic role in PD. In particular, multiple mtDNA deletions have been observed in SNpc dopaminergic neurons from post-mortem human brains of both aged individuals and patients with idiopathic PD (Bender et al., 2006; Kraytsberg et al., 2006). Mutations in mitochondrial DNA polymerase gamma (*POLG*), which result in the accumulation of multiple mtDNA deletions in muscle, have been associated with levodopa-responsive parkinsonism with severe SNpc dopaminergic neuronal loss, usually as part of a more complex syndrome (Luoma et al., 2004; Davidzon et al., 2006). Our group and others showed that despite high levels of mtDNA deletions, SNpc dopaminergic neurons from *POLG* mutant mice did not exhibit gross mitochondrial dysfunction or degeneration (Perier et al., 2013; Dai et al., 2014), indicating that a high level of mtDNA deletions is not sufficient *per se* to trigger cell death in SNpc dopaminergic neurons. In contrast, partial depletion of mtDNA in mice by a conditional disruption in dopaminergic neurons of mitochondrial transcription factor A (TFAM), which regulates mtDNA transcription, leads to a decrease in mtDNA content and cytochrome c oxidase enzymatic activity, which is associated with a progressive parkinsonism phenotype (Ekstrand et al., 2007). These findings strongly support a role of respiratory chain and mitochondrial dysfunction in the pathogenesis of PD.

A primary role of mitochondrial dysfunction in this process was boosted by the identification of genes related to familial forms of PD: α-synuclein (*SNCA*), leucine-rich repeat kinase 2 (*LRRK2*), PTEN-induced putative kinase 1 (*PINK1*), parkin RBR E3 ubiquitin protein ligase, also known as Parkin, (*PRKN*), DJ-1 (*PARK7*), and vacuolar sorting protein 35 (*VPS35*; **Figure 1**). Mutations in *SNCA*, *LRRK2,* and *VPS35* are associated with autosomal dominant PD. Several missense mutations have been linked to α-synuclein (A53T, A30P, E46K, H50Q, and A53E) and some families also present duplications and triplications of the *SNCA* wild-type gene (Polymeropoulos et al., 1997; Krüger et al., 1998; Singleton et al., 2003; Zarranz et al., 2004; Appel-Cresswell et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014). *SNCA* mutations have been linked to increased mtDNA damage, mitophagy, and mitochondrial fission, among others (Wong and Krainc, 2017). For example, the A53T mutation leads to increased mitochondrial fission (Xie and Chung, 2012; Pozo Devoto et al., 2017) and mitophagy (Chinta et al., 2010; Choubey et al., 2011; Chen et al., 2015). *LRRK2* mutations are the major cause of familial PD (Paisán-Ruiz et al., 2004; Zimprich et al., 2004), with more than 50 mutations identified thus far, the most common of which affects the kinase activity of the protein (Nuytemans et al., 2010). *LRRK2* mutations in *Caenorhabditis elegans* and neurons derived from induced pluripotent stem cells are associated with mitochondrial dysfunction and altered mitochondrial dynamics (Saha et al., 2009; Wang et al., 2012). Mutations in *VPS35* have been recently described (Vilariño-Güell et al., 2011; Zimprich et al., 2011), and may cause abnormal trafficking of cargo by mitochondrial-derived vesicles (MDVs) (Wang et al., 2015), these being cargo-selective vesicles that bud off mitochondria independently from the mitochondrial fission machinery (Neuspiel et al., 2008).

PINK1, a serine/threonine kinase, and Parkin, an E3 ubiquitin ligase, work together in a common pathway leading to the clearance of dysfunctional mitochondria (Pickrell and Youle, 2015). Mutations in *PINK1* and Parkin gene are associated with recessive PD (Kitada et al., 1998; Valente et al., 2004). Parkin loss in mice and Drosophila flies leads to decreased complex I and complex IV activities (Palacino et al., 2004; Casarejos et al., 2006), with Drosophila flies exhibiting locomotor defects, reduced lifespan, mitochondrial abnormalities and dopaminergic neuron degeneration (Greene et al., 2003). In cell lines, loss of PINK1 or mutations therein lead to decreased mitochondrial

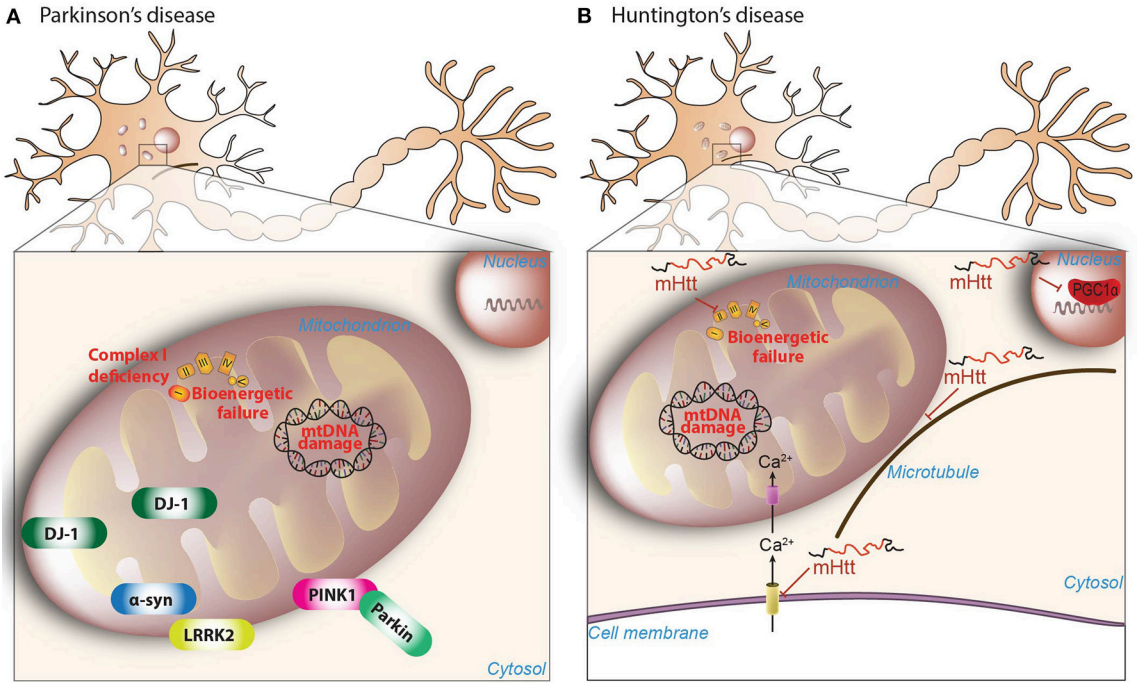


FIGURE 1 | Mitochondrial dysfunction in PD and HD. **(A)** Reduced complex I activity is a classical hallmark of the pathogenesis of PD, with a subsequent increased production of mitochondrial-derived ROS. Increased ROS is accompanied by mtDNA damage and bioenergetic failure. Further highlighting the role of mitochondrial dysfunction in PD, many of the mutated nuclear genes linked to familial forms of the disease, including PINK1 (phosphatase, and tensin homolog-induced kinase 1), Parkin, α-synuclein, DJ-1, and LRRK2 (leucine-rich-repeat kinase 2), have been shown to affect many of these mitochondrial features (see main text for details). **(B)** Mutant huntingtin (mHtt) inhibits the activity of succinate dehydrogenase enzyme of complex-II of mitochondria, which may account for the bioenergetic deficit present in HD. Moreover, mHtt protein directly impairs the ability of PGC-1α to activate downstream target genes involved in mitochondrial biogenesis and normal mitochondrial function. Further amplifying the damage, mHtt is able to promote calcium-dependent mitochondrial depolarization and mitochondrial swelling. Mitochondrial transport is also impaired due to the ablity of mHtt to combines specifically with the beta subunit of tubulin.

respiration and decreased ATP synthesis (Liu et al., 2009). DJ-1 mutations also cause autosomal recessive PD (Thomas and Beal, 2007) leading to impaired mitochondrial respiration, reduced membrane potential, increased ROS levels and altered mitochondrial morphology (Krebiehl et al., 2010).

Huntington's Disease

Huntington's disease (HD) is a devastating neurodegenerative disease affecting 10.6–13.7 individuals per 100,000 people in Western populations (Bates et al., 2015). It is an autosomal dominant neurodegenerative disorder, characterized by the abnormal expansion of the cytosine, adenine, and guanine (CAG) triplet repeats in the polyglutamine region of the huntingtin (*HTT*) gene. Clinically, HD is characterized by motor dysfunction, cognitive decline, and psychiatric disturbances caused by the preferential atrophy of GABAergic medium spiny neurons in the striatum as well as in other regions such as the cerebral cortex (McColgan and Tabrizi, 2018). How the mutant huntingtin protein elicits its toxic effects remains elusive, but several lines of evidence suggest involvement of transcriptional dysregulation and impaired mitochondrial function in the pathogenesis of HD (Labbadia and Morimoto, 2013; **Figure 1**).

Bioenergetics has been a classical field of study in HD research since the discovery of glucose hypometabolism in the caudate nuclei of presymptomatic HD subjects (Grafton et al., 1992). Both HD patients and presymptomatic carriers present increased lactate levels in brain areas linked with the disease (Jenkins et al., 1993), which is indicative of impaired energy metabolism. Interestingly, the presence of metabolic alterations in presymptomatic carriers argues in favor of mitochondrial dysfunction as an early appearing defect in the disease. As for PD and complex I, HD is linked to reduced complex II and complex III activity in the striatum, with complex IV also being affected to a lesser extent (Brennan et al., 1985; Gu et al., 1996). Accidental intoxication is also capable of recapitulating some of the clinical and pathologic phenotypes of HD in humans (Ludolph et al., 1991). Indeed, intoxication with mildewed sugar cane contaminated with 3-nitropropionic acid (3-NPA), an inhibitor of the succinate dehydrogenase enzyme of complex II, is able to induce HD symptoms in rodents and non-human primates (Brouillet et al., 2005). Studies have also suggested the involvement of mtDNA mutations in HD pathogenesis, as an increased frequency of mtDNA lesions and mtDNA depletion are also found in the HD striatum and skin fibroblasts (Siddiqui et al., 2012). How mitochondrial dysfunction and bioenergetic defects in HD occur is still a matter of debate. Nonetheless, mutant huntingtin is capable of directly interacting with the outer mitochondrial membrane

(OMM) (Gutekunst et al., 1998; Choo et al., 2004; Orr et al., 2008), reinforcing the notion that mutant huntingtin directly impairs mitochondrial function. Further linking mitochondrial dysfunction and HD is the ability of mutant huntingtin to inhibit peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) expression (**Figure 1**). PGC-1α is a transcriptional coactivator that regulates several metabolic processes such as mitochondrial biogenesis, thermogenesis or oxidative phosphorylation (OXPHOS) (Puigserver and Spiegelman, 2003). Mutant huntingtin interferes with the PGC-1α transcriptional pathway, impairing its ability to activate downstream genes, while PGC-1α ectopic expression provides neuroprotection in transgenic HD mice and in the 3-NPA mouse model (Cui et al., 2006; Weydt et al., 2006).

Beyond bioenergetics, mitochondria also regulate calcium homeostasis. Panov et al. (2002) demonstrated that lymphoblast mitochondria from HD patients have a lower membrane potential and depolarize at lower calcium loads compared to control mitochondria. This defect was also present in brain mitochondria from a HD transgenic mouse model (Panov et al., 2002). Cyclosporine treatment, which inhibits mitochondrial permeability transition pore (mPTP) opening, was able to delay this effect (Quintanilla et al., 2013), supporting a role for dysfunctional mitochondrial calcium handling in the pathogenesis of HD.

Considering the important role that mitochondrial function plays in neuronal homeostasis, some endogenous mechanisms have evolved to maintain mitochondrial integrity and functionality. These mechanisms can be divided into different categories, and although they respond to similar cues, the degree of damage determines their sequential activation. Upon excessive production of reactive oxygen and nitrogen species, for example, the first level of defense takes place with a coordinated detoxifying network. The second level of defense maintains the integrity of the mitochondrial proteome, while the third level controls organellar shape and number through interconnected processes such as mitochondrial fusion, fission and mitophagy. In this review article, we focus attention on the role of mitochondrial quality control processes that are responsible for maintaining mitochondrial integrity and functionality, and how these mechanisms may be functionally disturbed in PD and HD.

MITOCHONDRIA, THE SOURCE, AND TARGET OF ROS: MECHANISMS FOR KEEPING THEM HEALTHY

The term oxidative stress describes conditions that result from the imbalance between free radical generation, their detoxification and elimination. Mitochondria are the main source of cellular reactive oxygen species (ROS) as well as having the highest antioxidant capacity (**Figure 2**). The harmful effects of ROS involve damage to macromolecules such as proteins, lipids, polysaccharides, or nucleic acids. The intrinsic properties of neurons, i.e., (i) high metabolic rates; (ii) a rich composition of fatty acids prone to peroxidation; (iii) high intracellular concentrations of transition metals, capable of catalyzing the formation of reactive hydroxyl radicals; (iv) low levels of antioxidants; and (v) reduced capability to regenerate, make them highly vulnerable to the detrimental effects of ROS. Under normal conditions, small amounts of molecular oxygen in the mitochondria are reduced to ROS rather than being converted into water. Because of its high metabolic rate, the brain consumes high levels of oxygen, leading to increased radical formation. Thus, the presence of antioxidant defenses to reduce ROS levels below a toxic threshold is fundamental. The superoxide radical ($^{\bullet}O_2^-$) is the first radical that appears with the reduction of molecular oxygen. It can be transformed into hydrogen peroxide (H_2O_2) by the action of the enzyme superoxide dismutase (SOD). Hydrogen peroxide is poorly reactive with most biomolecules, but it can react with Fe^{2+} (in the Fenton reaction) to give rise to the hydroxyl radical ($OH^{\bullet}$), probably the most reactive free radical found *in vivo* (Halliwell and Gutteridge, 1984; Liochev, 2013), which can react with virtually all biomolecules. To maintain a balanced level of reactive species, cells have developed various antioxidant defenses, the first being SOD (superoxide dismutase), which accelerates the dismutation of two molecules of superoxide into one molecule of H_2O_2 and one molecule of O_2, thus, reducing the potentially harmful effects of $^{\bullet}O_2^-$. Three SOD isoenzymes have been identified in humans: copper and zinc-containing SOD (CuZnSOD/SOD1), mainly located in the cytosol and nuclear compartments; manganese-containing SOD (MnSOD/SOD2), a mitochondrial enzyme; and SOD3, a MnSOD located extracellularly (Perry et al., 2010). Even though H_2O_2 is a radical with low reactivity, it must be eliminated by the action of enzymes such as catalase, glutathione peroxidases (GPXs), the thioredoxin system, peroxiredoxins (PRDX), and glutaredoxins. GPX couples the reduction of H_2O_2 (and other peroxides) to H_2O (or to corresponding alcohols) with the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG), which can be converted back to GSH by glutathione reductase (GR). PRDX also couples the reduction of H_2O_2 with a cycle of peroxide-dependent oxidation and thiol-dependent reduction of cystein residues, the latter catalyzed by thioredoxin and thioredoxin reductase. Glutaredoxins have the same function as the thioredoxin system (Chance et al., 1979; Rhee et al., 2005). Glutathione S-transferases participate in the detoxification of electrophilic compounds by their conjugation with GSH (Hayes et al., 2005). Another group of non-enzymatic antioxidants such as glutathione, ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotenoids, or flavonoids, which are mainly ingested with the diet, act by scavenging free radicals (Forman et al., 2014). The last antioxidant defense layer is the sequestration of "free" transition metals, such as iron or copper, which are capable of stimulating free radical formation and also the direct oxidation of macromolecules. Within cells, iron is stored in the form of ferritin and copper as metallothionein (Rutherford and Bird, 2004).

Oxidative Stress and Parkinson's Disease

It is now clear that complex I inhibition-induced mitochondrial dysfunction causes the chronic production of ROS and is instrumental in the demise of dopaminergic neurons. Some catalytic subunits of complex I have been found to be oxidized in

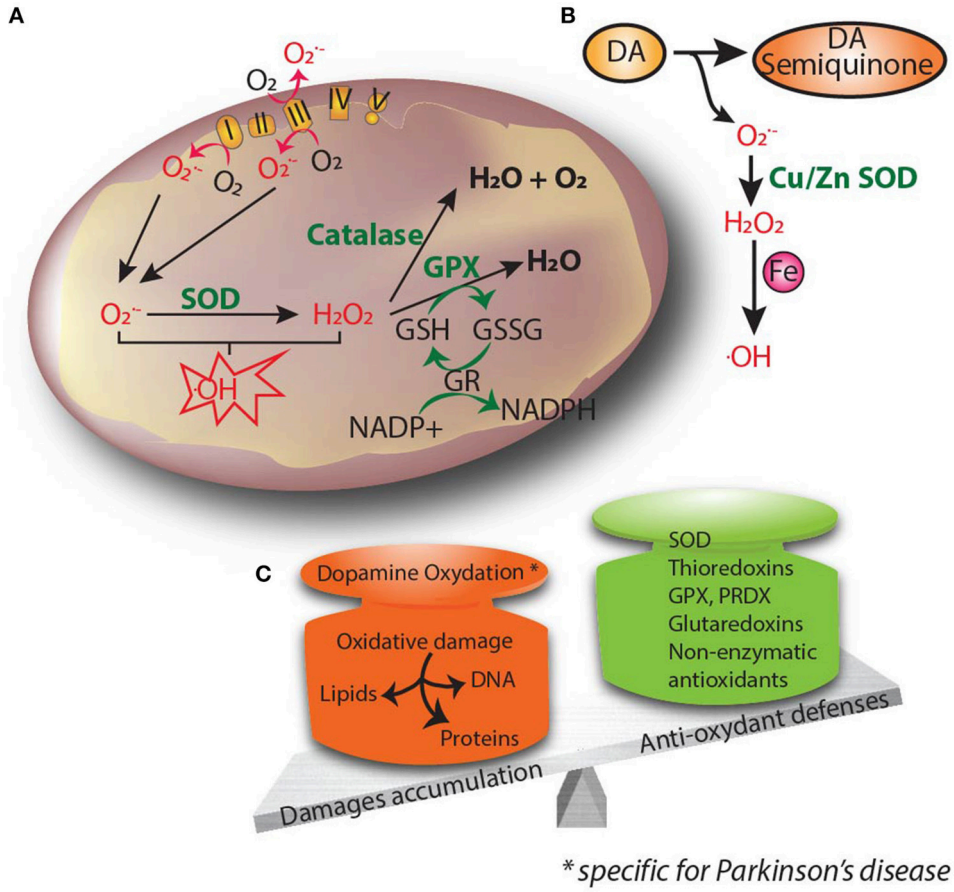


FIGURE 2 | Oxidative stress is thought to be the common underlying mechanism that leads to cellular dysfunction and eventual cell death. **(A)** Oxidants and superoxide radicals are produced as by-products of oxidative phosphorylation, making mitochondria the main site of reactive oxygen species (ROS) generation within the cell. **(B)** Dopamine can be oxidized to reactive dopamine quinones, contributing to increased levels of ROS. **(C)** Oxidative stress occurs when there is an imbalance between ROS production and cellular antioxidant activity. Excessive ROS causes lipid peroxidation, DNA damage and protein oxidation. Accumulation of free radicals causes mitochondrial dysfunction and contributes to the propagation of stress related to neuronal pathophysiology.

PD brain (Keeney et al., 2006), which represents a feed-forward cycle in which complex I inhibition produces ROS, which in turn impacts on complex I activity. Oxidative damage to proteins, lipids and DNA has been observed in post-mortem brain samples from PD patients (Dauer and Przedborski, 2003). Supporting a pathogenic role for mitochondria-derived ROS in the context of PD, transgenic mice overexpressing human catalase (an antioxidant enzyme normally localized in the peroxisome) specifically targeted to the mitochondria exhibit an attenuation of MPTP-induced mitochondrial ROS and reduced dopaminergic cell death (Perier et al., 2010). Moreover, oxidation-mediated damage to mitochondrial components, such as optic atrophy 1 (OPA1), contributes to PD neurodegeneration by remodeling the mitochondrial structure and cristae organization. Such cristae remodeling associated with cardiolipin peroxidation induces cytochrome c release and subsequent activation of apoptosis (Perier et al., 2005; Ramonet et al., 2013).

The SNpc is particularly sensitive to oxidative stress, probably because of its unique dopaminergic environment. Dopamine is vulnerable to auto- and/or enzymatic oxidation, producing reactive dopamine quinone, and ROS (**Figure 2**; Graham et al., 1978; Maker et al., 1981). Dopamine-induced toxicity has been observed in several *in vitro* and *in vivo* models (Hastings et al., 1996), and has been reported to affect several proteins, among them complex I (Ben-Shachar et al., 2004). Recently, it was shown that dopaminergic neurons derived from idiopathic and familial PD patients had decreased basal respiration and accumulation of oxidized dopamine. Interestingly, the treatment of those neurons with isradipine, a Cav1 channel antagonist, or a calcineurin inhibitor was able to decrease oxidized dopamine levels (Burbulla et al., 2017), highlighting the important role of calcium in dopamine oxidation. Besides, the expression of constitutively open Cav1.3 channels has been suggested to render nigrostriatal neurons more vulnerable to excitotoxic cell death. SNpc neurons are able to generate action potentials in the absence of synaptic input (Romo and Schultz, 1990). For their pacemaking activity, SNpc neurons not only engage monovalent cation channels but also L-type calcium channels with a distinctive Cav1.3, that

allow calcium entry into the cytoplasm (Ping and Shepard, 1996). The engagement of Cav1.3 calcium channels comes at a metabolic cost for the neurons, since they have to pump calcium back through ATP-dependent pumps (Wilson and Callaway, 2000). A key paper by Guzman et al. (2010) showed how L-type calcium channels induce oxidative stress specific to SNpc dopaminergic neurons, which exhibit higher levels of basal oxidation compared to dopaminergic ventral tegmental area (VTA) neurons. Antagonizing L-type calcium channels lowered the oxidation state of SNpc dopaminergic neurons, but not VTA neurons. Moreover, blocking calcium entry into the mitochondria also decreased oxidation (Guzman et al., 2010). Interestingly, oxidative stress is amplified in SNpc dopaminergic neurons from DJ-1 KO mice. These mice exhibit decreased mRNA levels of two UCPs (uncoupling protein), Ucp4 and Ucp5, in the SNpc leading to a blunted UCP-mediated mitochondrial uncoupling, which could explain the increased oxidation state (Guzman et al., 2010). Taken together, this work suggests that SNpc dopaminergic neurons have higher basal oxidation states, which is a consequence of the activation of L-type calcium channels during autonomous pacemaking. Additionally, SNpc dopaminergic neurons have also a relatively large axonal arborization (Matsuda et al., 2009), increasing their bioenergetic demands and rendering them more susceptible to ROS production. Pacelli et al. (2015) showed that SNpc neurons have a higher oxygen consumption rate and a lower maximal capacity together with increased ROS levels compared to VTA neurons, due to their larger axonal arborization. Neurons with smaller arborization size were less susceptible to die from the toxic effects of complex I inhibitors such as MPP^+ or rotenone (Pacelli et al., 2015).

Not only are SNpc dopaminergic neurons more vulnerable to oxidative stress, but PD neuropathogenesis is associated with alterations in the antioxidant defense system (**Figure 2**). SOD2 activity in PD post-mortem tissue is increased, highlighting the mitochondria as one of the main sites of ROS production (Saggu et al., 1989; Poirier et al., 1994). SOD1 and α-synuclein co-deposition with Lewy pathology has also been reported (Nishiyama et al., 1995), together with an upregulation of SOD1 (Marttila et al., 1988). Degenerating neurons exhibit reduced intracellular copper levels (Davies et al., 2014), which renders the SOD1 metal-deficient isoform more prone to aggregate. This vicious circle is then fed with reduced copper metallation of SOD1, decreasing its enzymatic function on the one hand and upregulating oxidative stress-induced SOD1 on the other. As a result, SOD1 aggregates increase, rendering SNpc neurons more vulnerable to oxidative stress (Trist et al., 2017). Additionally, GSH content is decreased in the SNpc post-mortem tissue of PD patients (Sian et al., 1994). Metal homeostasis is also dysregulated in PD patients, who display a higher content of iron in the SNpc compared to age-matched controls (Dexter et al., 1989). Increased levels of iron can stimulate free radical formation through the Fenton reaction, leading to the generation of highly reactive $OH^{\bullet}$ radicals. Interestingly, iron chelation was shown to be neuroprotective against MPTP-induced dopaminergic cell death (Kaur et al., 2003).

Oxidative Stress and Huntington's Disease

Several markers of oxidative stress were found in post-mortem tissue of HD patients; these include (i) lipofuscin accumulation, (ii) presence of the classical marker of oxidative modification in proteins (protein carbonyls) and lipids (malondialdehyde and 4-hydroxynonenal), and (iii) markers of oxidative damage to nuclear DNA, such as 8-hydroxydeoxyguanosine (Browne et al., 1997; Stack et al., 2008).

HD neuropathogenesis is also associated with alterations in the antioxidant defense system (**Figure 2**). Glutathione is decreased in the cerebral cortex from HD patients (Beal et al., 1992), whereas antioxidant enzymes, such as peroxiredoxin and GPX, together with the activity of SOD2 and catalase, were reported to be increased in the post-mortem human HD striatum (Sorolla et al., 2008). In general, the enhanced activity of antioxidants reduces neuronal oxidative stress by maintaining ROS at nanomolar levels, indicating that only neurons with higher antioxidant capacity are able to survive. Impairment of antioxidant defenses renders proteins, among other biomolecules, more prone to oxidation. In HD, the enzyme aconitase is one of the most affected enzymes of the tricarboxylic acid cycle (TCA), probably due to its Fe-S clusters. Aconitase oxidation is accompanied by decreased activity in the HD caudate, putamen and cerebral cortex (Tabrizi et al., 1999). Importantly, aconitase inactivation directly correlates with the generation of superoxide produced by excitotoxicity (Patel et al., 1996). Moreover, mitochondrial creatine kinase, citrate synthase, and ATP synthase are also oxidized in the striatum of HD patients, leading to a reduced catalytic activity (Sorolla et al., 2010) and providing a link between oxidative stress and the characteristic bioenergetic deficit present in HD.

Several neuroimaging studies suggest an increased iron signal in the basal ganglia of HD patients (Muller and Leavitt, 2014), which was confirmed by biochemical and histochemical evidence (Dexter et al., 1991), although the findings have some limitations. Another study identified that not only iron but also copper increases in R6/2 transgenic mice (expressing truncated N-terminal fragments of mutant huntingtin with 144 CAG repeats), which would contribute to creating a toxic microenvironment in HD pathology (Fox et al., 2007). Iron-containing proteins also become altered in HD (Muller and Leavitt, 2014). Succinate dehydrogenase, the main component of complex II, displayed decrease expression of its Fe-S subunit Ip and subunit Fp in human post-mortem tissue and in a HD cellular model. Reduced expression of those subunits was accompanied by a decrease in succinate dehydrogenase catalytic activity (Benchoua et al., 2006).

Taken together, these data highlight that antioxidant defense mechanisms are hampered in the context of PD and HD, with specific enzymes being altered in each pathology, but always leading to a scenario in which the balance between ROS production and scavenging is altered. This breakdown can have important implications in the pathology of neurodegenerative disorders, amplifying the detrimental cascade and generating new defects.

MITOCHONDRIAL PROTEIN QUALITY CONTROL: THE GUARANTEE OF MITOCHONDRIAL HEALTH

The endosymbiotic hypothesis argues that the mitochondrion evolved from a bacterial ancestor within the phylum *Alphaproteobacteria* through a symbiotic process within a eukaryotic host cell (Margulis, 1970). Mammalian mitochondria have a ∼16-kb genome encoding for 13 protein subunits of the mitochondrial electron transport chain and ATP synthase, as well as the ribosomal RNA (rRNA) and transfer RNA (tRNA) components of the mitochondrial translation system (Anderson et al., 1981). This means that most of the mitochondrial proteome is encoded in the nucleus, synthesized in the cytosol, and imported into the organelle. Most nuclear-encoded mitochondrial proteins are synthesized as precursors and maintained in an unfolded conformation to make them translocation-competent. Precursor proteins in the cytosol must exist in complexes with cytosolic chaperones, such as HSP70 and HSP90, to avoid their degradation and aggregation (Young et al., 2003). Moreover, those precursors need to contain information that directs them to the mitochondria and into the correct mitochondrial compartment. The most common signal is the N-terminal targeting sequence called mitochondrial-targeting sequence (MTS), which consists of 10–80 amino acid residues with no sequence identity, but with some characteristic physicochemical properties, such as its potential to form amphipathic helices with one hydrophobic and one positively charged face (Roise et al., 1986; Pfanner et al., 2000) The MTS is normally cleaved in the matrix leading to the mature polypeptide. There are also C-terminal targeting sequences and internal signals whose purpose remains elusive (Gordon et al., 2000). Most mitochondrial proteins are located within the matrix; for these proteins to be imported, there needs to be cooperation between the two main mitochondrial translocases—the translocase of the outer membrane (TOM) complex on the outer membrane, formed by seven components (Tom70, Tom22, Tom20, Tom40, Tom5, 6, and 7) (Model et al., 2008; Bausewein et al., 2017), and the translocase of the inner membrane (TIM23) complex (Tim50, Tim17, Tim21, Tim23, Tim44, Pam 17, Tim16/Pam16, Tim14/Pam18, mtHSP70, and Mge1) (Mokranjac and Neupert, 2010). The TOM complex mediates the translocation of nearly all of the mitochondrial proteome, whereas the TIM23 complex translocates pre-proteins through the inner mitochondrial membrane. Translocation through the TIM23 complex requires an intact mitochondrial membrane potential and the hydrolysis of ATP. The inner mitochondrial membrane harbors a variety of proteins, including the mitochondrial electron transport chain complexes and ATP synthase. There are three different routes for the import of inner mitochondrial membrane proteins once they have been translocated by the TOM complex: (1) through the TIM22 complex located in the inner mitochondrial membrane, (2) through a lateral insertion by the TIM23, complex, and (3) in an export-like route from the matrix to the inner membrane (Wiedemann and Pfanner, 2017).

Changes in the stoichiometric balance between nuclear- and mitochondrial-encoded proteins due to improper mitochondrial protein import and folding, as well as mutations in mtDNA, affect organelle proteostasis (Houtkooper et al., 2013). This is sufficient to disrupt the integrity and functionality of mitochondria. Mitochondrial protein import is thus under the surveillance of molecular chaperones (proteins that stabilize or assist the acquisition of the active conformation of other proteins without being part of their final structure) in order to avoid misfolding or aggregation of the newly transported proteins (Kim et al., 2013). mtHSP70 (mitochondrial heat shock 70 kDa protein), also called mortalin, is a chaperone crucial for the trafficking of proteins into the mitochondrial matrix, since it forms the core of the import motor between the TIM23 complex and the matrix. Mortalin also interacts with HSP60 (heat shock 60 kDa protein), one of the most important components of the protein folding machinery in the matrix, helping with the proper folding of newly imported proteins (Deocaris et al., 2006). The HSP60 and HSP10 (heat shock 10 kDa protein) machineries form two stacked rings in the mitochondrial matrix that allow accommodation of the unfolded polypeptide following the hydrolysis of ATP, assisting henceforth its proper folding (Walter, 2002; Okamoto et al., 2017). TRAP1 (tumor necrosis factor receptor-associated protein 1), another molecular chaperone present in the matrix, was first identified as a member of the HSP90 (heat shock 90 kDa protein) family because of its high degree of sequence and structural similarity. HSP90 and TRAP1 both have an N-terminal targeting sequence and an ATP-binding domain, but TRAP1 displays different functional characteristics (Song et al., 1995) and is suggested to play an important role in preventing ROS-dependent cell death (Gesualdi et al., 2007). In the intermembrane space, the small TIM chaperones assist the transfer of polypeptides through this compartment (Webb et al., 2006).

Another critical component of mitochondrial quality control relies on proteolytic systems located in different mitochondrial compartments. Together with chaperones, these systems participate in the import and trafficking of proteins from the cytosol into the mitochondria. Mitochondrial processing peptidase (MPP) is the main peptidase responsible for cleaving the targeting signals of preproteins. After MPP processing, other peptidases can act, such Oct 1 (octapeptidyl aminopeptidase 1) which removes an octapeptide (Gakh et al., 2002) and Icp55 (intermediate cleaving peptidase of 55 kDa) which removes a unique amino acid to stabilize the mitochondrial proteins (Vögtle et al., 2009). Besides their role in protein trafficking through the mitochondria, mitochondrial proteases are required to degrade misfolded, damaged, and unfolded proteins that are no longer capable of being refolded by chaperones, as well as process proteins that have been imported into the mitochondria. ATP-dependent proteases hydrolyse ATP to disassemble, unfold and sequester their substrates in compartments where they are degraded. Both iAAA and mAAA (ATPases associated with diverse cellular activities) proteases are inserted into the inner mitochondrial membrane, with iAAA facing the intermembrane space and mAAA facing the matrix; these participate in OXPHOS chain subunits' quality control and

turnover (Gerdes et al., 2012). LONP1 (Lon protease) and CLPP (Clp protease proteolytic subunit) are both serine peptidases acting in the mitochondrial matrix (Haynes et al., 2007; Quirós et al., 2014). LONP1 homozygous deletion in mouse causes embryonic lethality, underscoring its crucial role in cellular homeostasis (Quirós et al., 2014). Once the ATP-dependent proteases act, oligopeptidases further process the substrates. The ATP-independent proteases HTRA2 (high temperature requirement protein A2) and ATP23 are located in the intermembrane space and participate in the protein quality control of this compartment (Clausen et al., 2011; Quirós et al., 2015). These proteases are highly regulated and are able to modulate many biochemical activities such as protein importation, cardiolipin metabolism, mitochondrial gene expression, and mtDNA stability (Quirós et al., 2015). For instance, HTRA2 also has a role during apoptosis, since it can be released from the intermembrane space to relieve the inhibition of cytosolic caspases by binding to inhibitors of apoptotic proteins in the cytosol (Hegde et al., 2002; Verhagen et al., 2002). HTRA2 can also mediate caspase-independent cell death through its own protease activity (Hegde et al., 2002).

Mitochondrial Protein Quality Control and Parkinson's Disease

Several studies have linked a deficit of mitochondrial import with the accumulation of α-synuclein within the mitochondria. Most α-synuclein is soluble and resides in the cytoplasm. However, some years ago, Devi et al. (2008) reported the presence of a "cryptic" N-terminus MTS in the human α-synuclein protein sequence. They also observed an increased accumulation of α-synuclein in mitochondrial fractions from post-mortem SNpc of PD patients although which protein of the import machinery interacts with α-synuclein is still under investigation. Authors showed that α-synuclein can be translocated into the mitochondria using physiological import machinery, particularly the TOM complex (Devi et al., 2008). Another study suggested the interaction of α-synuclein with Tom40 and SAM50 (McFarland et al., 2008), while Bender et al. (2013) reported decreased Tom40 protein levels in post-mortem human midbrain samples and in the α-synuclein transgenic mouse model. Moreover, the overexpression of Tom40 in α-synuclein-accumulating cells and in the α-synuclein transgenic mouse model resulted in decreased α-synuclein levels. However, no mechanism was elucidated and import was not measured (Bender et al., 2013). On the other hand, another study recently linked α-synuclein with Tom20; the authors described an α-synuclein-specific interaction with Tom20 in nigrostriatal dopaminergic neurons of different genetic and pharmacological PD models and in human post-mortem tissue. This interaction was prevented by knocking down α-synuclein. Using *in vitro* models, oligomeric, dopamine-modified, and phosphomimetic mutant α-synuclein were able to impair protein import and induce mitochondrial dysfunction. Moreover, α-synuclein disrupts the normal Tom20-Tom22 interaction, possibly by binding to the MTS receptor site in Tom20 (Di Maio et al., 2016; **Figure 3**). These results tie in two pathogenic characteristics of PD: α-synuclein accumulation and mitochondrial dysfunction. However, very few reports have linked PD with mitochondrial protein import, and they seem to contradict each other, making it necessary to clarify the exact role that α-synuclein plays under normal and pathogenic conditions, and whether complex I inhibition can also impair the translocation of proteins into mitochondria.

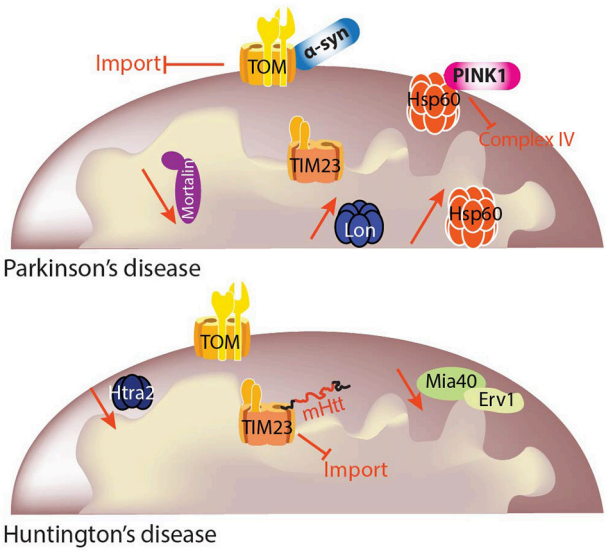


FIGURE 3 | Mitochondrial protein quality control (MQC) in PD and HD. Mitochondrial function and cellular metabolism are dependent on maintenance of the mitochondrial proteome. It is critical to ensure that the mitochondrial proteome is correctly regulated depending on cellular demands, and this is achieved by protein import and protein quality control. Different import pathways and machineries exist for precursor translocation into the mitochondria. Mitochondrial precursor proteins begin their journey in the cytosol and are delivered to the translocase of the outer membrane (TOM) complex. Precursor proteins containing a positively charged N-terminal presequence are then delivered to the translocase of the inner membrane 23 (TIM23) complex for their translocation into or across the inner mitochondrial membrane in a membrane potential ($\Delta\psi$)-dependent manner. Each mitochondrial compartment has its own quality control machinery. The link between MQC and neurodegenerative diseases is still under investigation, but some studies already have shown specific deficits linked to particular diseases. Although mitochondrial protein import and quality control may have been traditionally investigated independently, the overlap between the two is now clear and mitochondrial protein homeostasis cannot be sustained without both of these processes.

One of the first clues linking PD to molecular chaperones was the observation of decreased mortalin levels in the SN of PD patients. Using proteomics, Jin et al. (2006) reported a significant decrease of mortalin levels in mitochondria-enriched fractions from the SN of PD patients compared to age-matched controls (Jin et al., 2006; **Figure 3**). Three variants in the mortalin gene (*HSP9*), two missense (R126W and P509S) and a 17 kb insertion in intron 8, were found in 3 patients from a Spanish cohort but in none of the controls, suggesting a genetic link between mortalin and PD. The missense variants consisted of an amino acid change in the actin-like ATPase domain and in

the HSP peptide binding domain of the protein, both amino acids being highly conserved between HSP70 members from different species (De Mena et al., 2009). A German cohort was also screened for mortalin variants, with one variant, A476T, suggested to act as a risk factor for PD together with the previously reported variants. Cells overexpressing PD-associated variants of mortalin showed increased ROS levels after proteolytic stress compared to cells expressing wild-type mortalin. Moreover, mitochondrial dysfunction induced by knock-down of mortalin could only be rescued by wild-type mortalin but not by PD-associated variants (Burbulla et al., 2010). Some of these variants were mimicked in yeast, thus unraveling the presence of a wide range of mitochondrial alterations and further reinforcing mortalin involvement as a susceptibility factor for PD (Goswami et al., 2012). Mortalin has been found to interact with several proteins linked to genetic PD, such as DJ-1 (Jin et al., 2007; Burbulla et al., 2010), α-synuclein (Jin et al., 2007), or Parkin (Davison et al., 2009). Interestingly, PINK1 or Parkin overexpression in mortalin-knock down cells rescued the mitochondrial phenotype, thus suggesting that mortalin might also participate in the PINK1-Parkin pathway (Yang et al., 2011; Burbulla et al., 2014). However, in recent years, mortalin's role in the pathogenesis of PD has been questioned. In the screening of a cohort of 139 early-onset PD patients, only one missense change in the PD group and another in the control group were found, suggesting that mortalin variants may not be a major determinant of early-onset PD (Freimann et al., 2013). Another study of 500 PD patients and 500 controls from a Korean population revealed no significant association of *HSP9* variants with PD (Chung et al., 2017).

While the most accepted PINK1 substrates are ubiquitin and Parkin, TRAP1 was the first *in vivo* target identified for PINK1, linking mitochondrial protein quality control and PD. PD-linked PINK1 mutations (G309D, L347P, and W437X) abolished basal and oxidative stress-induced TRAP1 phosphorylation *in vitro*, thus impairing PINK1's cytoprotective effect against oxidative stress (Pridgeon et al., 2007). The authors revealed that PINK1 overexpression protects cells against oxidative stress-induced apoptosis by suppressing mitochondrial cytochrome c release; this protective action depends on the PINK1-dependent phosphorylation of TRAP1 (Pridgeon et al., 2007). Moreover, Drosophila TRAP1-null mutants present some features similar to PINK1 and Parkin null mutants; TRAP1 is able to partially rescue mitochondrial impairment in Parkin mutant flies and vice versa, suggesting that TRAP1 might work in parallel with Parkin in Drosophila (Costa et al., 2013). In human SH-SY5Y cells, TRAP1 was able to rescue mitochondrial dysfunction upon siRNA-induced silencing of PINK1 but not of Parkin (Zhang et al., 2013). Taken together, these data suggest that TRAP1 works within the PINK1-Parkin pathway to maintain mitochondrial integrity, and is also altered in the presence of PINK1 or Parkin loss-of-function mutations. TRAP1 was also decreased in A53T α-synuclein-expressing flies, resulting in an increased sensitivity to oxidative stress and motor alterations that were rescued after TRAP1 overexpression, thus providing a link between PINK1 and α-synuclein through TRAP1 (Butler et al., 2012). PINK1 also interacts with HSP60 (Rakovic et al., 2011) and its protein levels are decreased in PINK1 null cells, which was associated with complex IV deficit (Kim et al., 2012). The protease LONP1 is responsible for the degradation of oxidized proteins. MPTP-intoxicated mice displayed increase expression of LONP1 in the ventral mesencephalon concomitantly with the presence of oxidized proteins and dopaminergic cell loss. Moreover, LONP1 activity was inactivated by increased ROS levels, raising the possibility that LONP1 dysfunction represents an early event in the pathogenesis of PD (Bulteau et al., 2017). Mutations in the gene encoding HTRA2, another protease, were reported in PD patients. These mutations do not affect the localization of the protein, but instead affect its proteolytic activity, resulting in increased mitochondrial susceptibility (Strauss et al., 2005). Mice lacking HTRA2 expression develop a specific neurodegeneration of striatal neurons together with a parkinsonian phenotype (akinetic and rigid syndrome, showing a lack of coordination, decreased mobility and tremor) leading to early death, thus challenging the notion that HTRA2 is a major regulator of apoptotic cell death (Martins et al., 2004). HTRA2 phosphorylation increases its proteolytic activity in a PINK1-dependent manner, suggesting that these proteins might be part of the same stress-sensing pathway (Plun-Favreau et al., 2007). In the same way, drosophila HTRA2 mutants develop a similar phenotype to those of PINK1 and Parkin mutants, and HTRA2 was able to rescue the PINK1 phenotype, also suggesting that HTRA2 and PINK1 might act in the same pathway (Tain et al., 2009), but independently from Parkin (Whitworth et al., 2008). TRAP1 might work as a downstream effector in this pathway since HTRA2 and TRAP1 can interact with each other; however, this interaction does not involve the protease activity of HTRA2 (Fitzgerald et al., 2017). Another protease, rhomboid-7, was found to act upstream of PINK1 and HTRA2 by cleaving their precursor forms (Whitworth et al., 2008). Other studies, however, reported no association of HTRA2 variants with PD (Simón-Sánchez and Singleton, 2008; Krüger et al., 2011).

Mitochondrial Protein Quality Control and Huntington's Disease

Huntingtin fragments have been reported to be in close apposition to mitochondria in cellular and animal models of HD (Gutekunst et al., 1998; Choo et al., 2004; Orr et al., 2008) as well as in brain mitochondria of the caudate nucleus of HD patients (Yano et al., 2014) suggesting a direct role of mutant huntingtin in mitochondrial dysfunction. Localization of mutant huntingtin to brain mitochondria from mouse has been reported to impair protein import through the interaction with TIM23 complex. Importantly, mutant huntingtin association with TIM23 complex was not present with wild-type huntingtin, suggesting a role for polyglutamine domains in the interaction (**Figure 3**). Interestingly, import deficiency is present in highly purified synaptosomal mitochondria from presymptomatic R6/2 mice, thus indicating that the deficiency represents an early deficit in a subset of mitochondria where high energetic demands are placed, suggesting that they are more sensitive to these

mutations. The rescue of mitochondrial protein import by lentiviral delivery of Tim23, Tim50, and Tim17a, prevented mitochondrial dysfunction and cell death in mutant huntingtin-expressing neurons (Yano et al., 2014). The presence of mutant huntingtin with 111 polyglutamine repeats also seems to impair the mitochondrial disulfide relay system (MDRS) in mouse striatal cells. Specifically, Napoli et al. (2013) reported a decrease in FAD-linked sulfhydryl oxidase augmenter of liver regeneration, also called Erv1, and Mia40 (mitochondrial intermembrane space import and assembly protein 40), together with one of its downstream substrates, Cox17, in total cell lysates. This deficit was concomitant with the presence of a disrupted mitochondrial morphology, lower mtDNA copy number and increased deletions, lower complex I, IV, and V activities, decreased ATP production and increased oxidative stress (Napoli et al., 2013). Importantly, MDRS is in charge of the translocation of some complex I and complex IV proteins (Mesecke et al., 2005) and small TIM chaperones (Tim8, 9, 10, and 13) (Lutz et al., 2003), some of which are part of TIM22 complex, which, in turn, is responsible for the insertion of other translocases. This suggests that an initial deficit in MDRS could lead to an altered downstream import pathway (**Figure 3**). However, the reported study presents some limitations such as the use of only *in vitro* models and the absence of any mechanistic description. It is true, though, that an impairment of the mitochondrial import system can hamper the translocation of proteins involved in the TCA cycle, OXPHOS, and antioxidant defenses, among others, giving rise to decreases in ATP production and ROS levels, both classical hallmarks of HD.

A missense mutation (S276C) in the protease domain of HTRA2 was discovered to be the cause of motor neuron degeneration 2, a spontaneous recessively inherited mutation that appeared in the C57BL/6J inbred background. Interestingly, this mutation leads to specific degeneration of striatal neurons (Jones et al., 2003), suggesting that those neurons are particularly vulnerable to HTRA2 deficits and indicating that this protein could play a role in HD. In a study comparing the gene expression profiles of three types of neurons expressing wild-type or mutant huntingtin, HTRA2 mRNA was specifically downregulated only in striatal neurons with mutant huntingtin (Tagawa et al., 2007). HTRA2 is downregulated in primary striatal neurons and in striatal neurons of R6/2 mutant huntingtin-transgenic mice at the presymptomatic stage, but not in the cortex or cerebellum. Moreover, the overexpression of HTRA2 in primary neurons protected against mutant huntingtin-induced cell death, while the suppression of HTRA2 renders those neurons susceptible to mutant huntingtin. These data suggest that the selective reduction of HTRA2 in striatal neurons could be linked to their selective vulnerability in HD pathology (Inagaki et al., 2008).

While it is clear that proteostasis is altered in HD, it remains uncertain whether the aggregates present are the main mediators of neuronal dysfunction. Those aggregates can have a dual role, on one hand sequestering the toxic protein, but on the other, acting as a hub to incorporate several proteins. While molecular chaperones are the first line of defense against protein aggregates, expression of expanded polyglutamine peptides leads to impaired protein folding capacity. However, no specific alteration in mitochondrial molecular chaperones has been reported so far in the context of HD.

MtUPR: A New Piece in the Neurodegeneration Puzzle?

As discussed above, the mitochondrial proteome is encoded by both mitochondrial and nuclear DNA. Proteostatic stress can arise for a variety of reasons: (i) when there is a high input of nuclear-encoded mitochondrial proteins that need to be folded, (ii) imbalance between the nuclear and the mitochondrial genome, or (iii) oxidative stress that modifies endogenous mitochondrial proteins. Mitochondria have developed their own mechanisms (such as the one present in the endoplasmic reticulum, ER) to respond to this proteostatic stress, called the mitochondrial Unfolded Protein Response (mtUPR). This response is a mechanism of mitochondrial to nucleus communication (Martinus et al., 1996) to activate a transcriptional program for mitochondrial homeostasis (Zhao et al., 2002).

The first characterization was made in mammalian cells and uncovered a transcriptional response characterized by the upregulation of nuclear genes encoding for mitochondrial chaperones (HSP60, HSP10, mtDnaJ) and CLPP protease (Zhao et al., 2002). However, the following studies have used *C. elegans* to further analyze the mtUPR. One of the suggested models for mtUPR activation in *C. elegans* is based on Clpp-1 (*C. elegans* homolog of CLPP protease) dependent degradation, which generates small peptides that are pumped out through the HAF-1 transporter [HAlF transporter (PGP related) family member] and contribute to the downstream signaling of mtUPR by triggering the relocalization of transcription factor DVE-1 (defective proventriculus in Drosophila homolog family member) and UBL-5 (ubiquitin-like protein 5) to the nucleus (Haynes et al., 2007, 2010; Haynes and Ron, 2010). HAF-1 is also suggested to work by modulating the stress activated transcription factor ATFS-1 import. ATFS-1 is a bZIP transcription factor normally imported into mitochondria and degraded by Lon protease. ATFS-1 has also a nuclear localization signal and upon mtUPR activation, its trafficking to mitochondria is impaired, leading to its translocation into the nucleus and subsequent transcriptional activation of several genes that are protective against mitochondrial dysfunction (Nargund et al., 2012). Therefore, ATFS-1 functions as a sensor of mitochondrial import efficiency, implying that any condition that hampers mitochondrial protein import activity could potentially activate mtUPR (Chacinska et al., 2009; Nargund et al., 2012).

While the mtUPR has been extensively investigated in *C. elegans*, it is not clear how this response occurs in mammals. In cell lines, different interventions activate CHOP (CCAAT-enhancer-binding protein homologous protein) and C/EBPβ (CCAAT/enhancer binding protein β) transcription factors, which heterodimerize, bind to target promoters on a CHOP binding site, and activate the expression of mtUPR genes (Aldridge et al., 2007). Recently, ATF5 (cyclic AMP-dependent transcription factor 5), a bZIP transcription factor, was proposed to act like ATFS-1 in *C. elegans* (Fiorese et al., 2016). Importantly,

in response to mitochondrial stress in both *C. elegans* and mammals, there is an increased phosphorylation of eIF2α (eukaryotic translation initiation factor 2 α) resulting in a reduction of protein synthesis (Baker et al., 2012; Martínez-Reyes et al., 2012; Michel et al., 2015; for mtUPR review see Schulz and Haynes, 2015; Fiorese and Haynes, 2017). In summary, mtUPR is emerging as a stress response pathway, which coordinates two different compartments, the nuclear and the mitochondrial, to promote mitochondrial health.

Several pieces of evidence have linked mtUPR with PD, but they are mostly circumstantial since mtUPR regulation in mammals is not fully understood. What is clear is that parkinsonian toxins such as rotenone or MPP^+ (complex I inhibitors), as well as paraquat (a ROS inducer), are potent mtUPR inducers, indicating that PD pathogenesis shares common alterations with mtUPR induction, thus raising the possibility that the mtUPR response is impaired in PD and worsens mitochondrial dysfunction (de Castro et al., 2010, 2011). A reduction in protein synthesis, which is one of the consequences of mtUPR activation, rescues many of the defects in flies lacking PINK1 (Liu and Lu, 2010), while Drosophila flies overexpressing a mutant OTC (ornithine transcarbamylase) protein prone to aggregation develop mitochondrial dysfunction phenotypes similar to PINK1 and Parkin mutants (Pimenta de Castro et al., 2012), further confirming the pathogenic role of protein misfolding in PD. To this end, analysis of post-mortem brains from PD patients carrying PINK1 mutations revealed enhanced levels of misfolded components of the mitochondrial respiratory chain as well as increased levels of the mtUPR marker of activation HSP60 (Pimenta de Castro et al., 2012). Another link between mtUPR and PD is mediated by the association of HTRA2 with PD. In mice, deletion of HTRA2 results in the accumulation of unfolded proteins in the mitochondria and leads to neurodegeneration (Moisoi et al., 2009). However, the role of HTRA2 mutations in familial PD remains controversial.

The mtUPR pathway is generally regarded as beneficial for cellular homeostasis, especially in response to genetic or environmental challenges; however, some reports have started to challenge this view, indicating that the mtUPR pathway could be detrimental to the cell under some circumstances. Recently, Martinez et al. (2017) studied the mtUPR pathway in a *C. elegans* model of PD, through the expression of different forms of α-synuclein. α-synuclein PD-associated variants (A53T and A30P) were able to induce mtUPR machinery, and this induction was sustained over time. Importantly, overexpression and overactivation of ATFS-1 over time, which lead to mtUPR signaling, was found to be detrimental and to induce neurodegeneration in *C. elegans* dopaminergic neurons. In addition, the co-expression of α-synuclein and overactivation of the mtUPR potentiate α-synuclein toxicity (Martinez et al., 2017). Taken together, while these observations challenge the mainstream view of mtUPR as a protective pathway, more studies are needed to fully understand the role of mtUPR in the context of PD.

So far there are no data linking HD with mtUPR. However, given the discovery that mutant huntingtin is able to block mitochondrial protein import, which is thought to be, at least in *C. elegans*, an important sensor of mitochondrial homeostasis, it is plausible that mtUPR could play a role in HD.

REGULATION OF THE MITOCHONDRIAL NETWORK: AT THE CROSSROADS OF MITOCHONDRIAL DYNAMICS, MITOPHAGY, MITOCHONDRIAL BIOGENESIS, AND MITOCHONDRIAL TRANSPORT

Mitochondria are dynamic organelles whose structure varies constantly from a tubular network to individual mitochondria. This mitochondrial network is controlled by the balance between various regulated processes: mitochondrial dynamics that control mitochondrial fusion and fission, *de novo* mitochondrial biogenesis, and the elimination of unwanted mitochondria by mitophagy (Ploumi et al., 2017; **Figure 4**). While mitochondrial fusion is believed to favor mitochondrial biogenesis by the exchange of new proteins and mtDNA between the merging organelles, mitochondrial fission is considered to be a process that isolates dysfunctional mitochondria so that they can be cleared by mitophagy.

Mitochondrial Fusion

Mitofusin 1 and mitofusin 2 (Mfn) are dynamin-like GTPases that control outer membrane fusion, whereas dynamin-like 120 kDa protein, encoded by the OPA1 gene is the dynamin-like GTPase in charge of inner membrane fusion (Mishra and Chan, 2014; **Figure 4**). Both Mfn have redundant roles but with certain features: (i) Mfn1, but not Mfn2, is needed for OPA1-mediated inner membrane fusion (Cipolat et al., 2004); (ii) Mfn2 mutations cause Charcot-Marie-Tooth disease type 2A, a peripheral neuropathy characterized by progressive degeneration of the peripheral nerves (Züchner et al., 2004); and (iii) in addition to its role in mitochondrial fusion, Mfn2 plays also a key role in mitochondria-ER tethering (de Brito and Scorrano, 2008). For the complete fusion of mitochondria, the inner mitochondrial membrane must fuse as well. OPA1 is a dynamin-like GTPase protein anchored to the inner mitochondrial membrane that exposes the bulk of the protein to the intermembrane space. Different OPA1 variants, determined by alternative-splicing and proteolytic cleavage, regulate the balance between fusion and fission. The processing of OPA1 by OMA1 (overlapping activity with m-AAA protease) and the i-AAA protease YME1 is central to the regulation of OPA1 activity, while the balance between long-OPA1 (L-OPA1) and short-OPA1 (S-OPA1) maintains normal mitochondrial morphology. While L-OPA1 is sufficient to mediate complete mitochondrial fusion, the activation of OMA1-dependent processing of OPA1 in the absence of YME1 leads to mitochondrial fission (Anand et al., 2014). Upon apoptotic stimuli, a complete conversion of L-OPA1 to S-OPA1 takes place, inhibiting mitochondrial fusion (Song et al., 2007). Hence, OMA1-mediated degradation of OPA1 is considered a general cellular stress response.

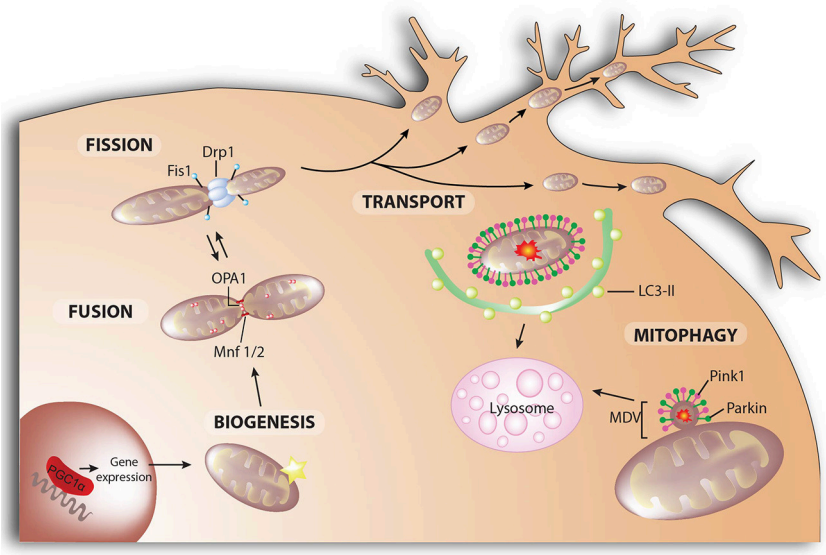


FIGURE 4 | Mitochondrial dynamics. Mitochondrial fusion and fission control mitochondrial number and size. Fission is mediated by dynamin-related protein 1 (Drp1) and mitochondrial fission-1 protein (Fis1). Mitofusins (Mnf) 1 and 2 are involved in the fusion of the outer membrane, whereas protein optic atrophy type 1 (OPA1) regulates the fusion of the inner membrane. Mitochondrial biogenesis is the process by which cells increase their individual mitochondrial mass to increase the production of ATP as a response to greater energy demand. In neurons, mitochondria are recruited to subcellular compartments distant from the cell body, such axons and dendrites, by active transport along microtubules and actin filaments. Selective autophagic degradation of mitochondria (i.e., mitophagy) involves the recruitment of damaged mitochondria into a pre-autophagosome structure via a PINK1/Parkin-dependent process. When mitochondrial deterioration is mild and without global mitochondrial depolarization, local removal of mitochondrial content can be achieved by the generation of mitochondrial-derived vesicles (MDV) that bud-off from the mitochondria and contain matrix components. Both MDVs and PINK1/Parkin-targeted mitochondria are then delivered to lysosomes for degradation.

Mitochondrial Fission

Mitochondrial fission facilitates the segregation of damaged mitochondria from a healthy network and mitochondrial transport through neuronal processes. Dynamin-related protein 1 (Drp1) is a cytosolic protein that is recruited to the OMM, where it oligomerizes to form ring-like structures, which upon GTP hydrolysis facilitate membrane constriction (Koirala et al., 2013; **Figure 4**). Additional adaptors are required for Drp1 recruitment to the mitochondrial surface, such as mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff) and the 49 and 51 kDa mitochondrial dynamics proteins (MiD49 and MiD51) (Losón et al., 2013). Mitochondrial fission occurs at ER-mitochondrial contact sites, where ER tubules mediate constriction before Drp1 recruitment, indicating a role for ER tubules in defining division sites (Friedman et al., 2011). Less-well understood is the fission of the inner mitochondrial membrane and whether Drp1-mediated outer membrane constriction can also lead to inner membrane scission is still unknown. However, S-OPA1 fragment accumulation does favor mitochondrial fission. This discovery opens a new area of study in the field of mitochondrial fission, since further investigation is needed to understand the precise role of S-OPA1, together with its processing-peptidases, in regulating mitochondrial dynamics.

Mitophagy

The accumulation of dysfunctional mitochondria is suggested to play a key role in the pathology of neurodegenerative diseases. Impaired mitochondria can be selectively targeted and eliminated through a process termed mitophagy. Mitophagy is a highly specialized type of autophagy that consists of three steps: recognition of the mitochondrion that needs to be cleared, formation of the autophagic membrane that surrounds the organelle, and fusion of the mito-autophagosome with the lysosome. The classical pathway to flag mitochondria for degradation involves PINK1 and Parkin and has been reviewed elsewhere (Pickrell and Youle, 2015; Martinez-Vicente, 2017; McWilliams and Muqit, 2017; Misgeld and Schwarz, 2017; **Figure 4**). Briefly, PINK1 is constitutively imported inside healthy mitochondria, cleaved (Whitworth et al., 2008; Jin et al., 2010; Meissner et al., 2011; Greene et al., 2012) and degraded by the proteasome (Yamano and Youle, 2013), so upon mitochondrial protein import impairment, PINK1 accumulates in the outer membrane of the dysfunctional mitochondrion and directly phosphorylates ubiquitin chains linked to the outer membrane proteins at Ser65. The presence of phospho-ubiquitins stimulates the recruitment and activation of Parkin, which is phosphorylated as well by PINK1 leading to its complete activation (Kondapalli et al., 2012; Shiba-Fukushima et al., 2012; Kane et al., 2014; Kazlauskaite et al., 2014; Koyano et al., 2014). As an E3-ubiquitin ligase, Parkin ubiquitinates many substrates with K48- and K63-linked ubiquitin chains (Ordureau et al., 2015). K63-chains recruit autophagy adaptor receptors such as sequestrome 1 (SQSTM1, also known as p62), NBR1 (neighbor of BRCA1 gene 1), OPTN (optineurin), or NDP52 (nuclear dot protein 52), while K48-chains might lead to the degradation of some outer mitochondrial proteins, which

helps to control mitochondrial motility and dynamics during the process (Komander and Rape, 2012; Lazarou et al., 2015). Mitophagy receptors connect the mitochondria to be eliminated with LC3-II (microtubule-associated protein 1A/1B light chain 3B), the main component of the autophagosomal membrane (Wild et al., 2014).

Mitophagy extends well beyond the so-called PINK1-Parkin pathway. In fact, proteins in the OMM can act as mitophagy adapters under certain conditions. Nix (NIP1-like protein X, also known as BNIP3L), is induced upon hypoxia together with Bcl2/E1B 19 kDa-interacting protein 3 (BNIP3); this triggers opening of the mitochondrial permeability transition pore, depolarization, and LC3/GABARAP (GABAA receptor-associated protein) recruitment for the autophagosome formation (Zhang and Ney, 2009; Novak et al., 2010). FUNDC1 (Fun14 Domain Containing 1), an OMM-protein, has also been implicated in hypoxia-induced mitophagy. FUNDC1 phosphorylation regulates the affinity of the FUNDC1-LC3 interaction, where dephosphorylation increases this interaction and leads to mitophagy (Liu et al., 2012a). Other mitochondrial proteins have been described to act as mitophagy receptors by recruiting the autophagosomal machinery (Martinez-Vicente, 2017).

Mitochondrial Biogenesis

The need to degrade dysfunctional mitochondria must be balanced with the generation of *de novo* mitochondria to keep a healthy network (**Figure 4**). Mitochondrial biogenesis requires a complex coordination of the nuclear and mitochondrial expression programs. PGC-1α is the master regulator of mitochondrial biogenesis; it interacts with and coactivates several transcription factors such as nuclear respiratory factors 1 and 2 (NRF1 and NRF2), estrogen-related receptor alpha (ERRα), ying and yang 1 (YY1), myocyte enhancer factor 2C (MEF2C), peroxisome proliferator-activated receptors (PPAR), and many others coordinating mitochondrial biogenesis and oxidative metabolism (Wu et al., 1999; Scarpulla, 2006, 2011). NRF1 and NRF2 frequently work together to up-regulate the transcription of several nuclear-encoded genes with essential mitochondrial functions, and to induce the expression of mitochondrial transcription factor A (TFAM) (Virbasius et al., 1993; Scarpulla, 2008), which initiates the transcription and replication of mtDNA given its ability to bind and bend DNA without sequence specificity (Ekstrand et al., 2004). Through the regulation of TFAM levels, PGC-1α can regulate the expression of mitochondrial genes (Scarpulla, 2008).

Mitochondrial Transport

Mitochondrial transport is crucial for the distribution of mitochondria throughout the neuron, from the cell body to the presynaptic terminals, and to accomplish the resupply of newly synthesized mitochondria and mitochondrial proteins (Saxton and Hollenbeck, 2012; Schwarz, 2013; Misgeld and Schwarz, 2017; **Figure 4**). Large numbers of mitochondria localize at the presynaptic terminals, probably reflecting the elevated levels of ATP required for synaptic transmission and the need to regulate Ca^{2+} homeostasis during intense synaptic activity (Keating, 2007). Mitochondrial transport relies on microtubule-based motors—the anterograde kinesin-1 motor (Kif5B) and the retrograde dynein motor—attached to the mitochondria by the complex formed by Miro and a mitochondrion-kinesin linker protein, Milton (Wang and Schwarz, 2009). Stationary mitochondria are held in place by anchoring proteins, such as syntaphilin, through their interaction with microtubules (Kang et al., 2008).

Organellar Quality Control and PD

Alterations in the mitochondrial fusion/fission balance have not yet been directly demonstrated in PD. However, our group and others have shown that the parkinsonian neurotoxins 6-OHDA (6-hydroxydopamine), rotenone and MPP^+ induce mitochondrial fragmentation (fission) and cell death in neuronal cultures (Barsoum et al., 2006; Meuer et al., 2007; Gomez-Lazaro et al., 2008; Ramonet et al., 2013). Supporting a pathogenic role for mitochondrial fission, genetic inhibition of pro-fission Drp1 or pro-fusion Mfn1 or OPA1 overexpression are able to prevent cell death induced by these neurotoxins.

The potential importance of mitochondrial dynamics in PD was revealed in part by the identification of different autosomal recessive and autosomal dominant genes linked to PD. α-synuclein overexpression leads to mitochondrial fragmentation in several models, ranging from *C. elegans* to neuroblastoma cell lines (Kamp et al., 2010; Nakamura et al., 2011; Butler et al., 2012; Xie and Chung, 2012; O'Donnell et al., 2014), but no direct mechanism has been elucidated so far. It was suggested that binding of α-synuclein to sodium dodecyl sulfate micelles induces a reduction in the membrane curvature (Perlmutter et al., 2009). This observation reinforces the notion that α-synuclein-dependent mitochondrial fragmentation may be exerted through direct association with the mitochondria. Mice overexpressing A53T α-synuclein present reductions in Mfn1, Mfn2, and Drp1 protein levels in the spinal cord, which correlated with decreased mitochondrial size (Xie and Chung, 2012). However, upon Drp1 loss in mice, mitochondrial fragmentation induced by α-synuclein still occurs (Nakamura et al., 2011; Guardia-Laguarta et al., 2014). Furthermore, N-terminal disruption of α-synuclein in human induced pluripotent stem cells led to mitochondrial elongation in neurons (Pozo Devoto et al., 2017), suggesting a possible physiological role for α-synuclein in regulating mitochondrial size through its direct interaction with the fusion-fission process rather than with fusion-fission machinery.

LRRK2 protein has also been linked to mitochondrial dynamics, although its exact role remains to be clarified. Enlarged mitochondria were reported in G2019S mutant LRRK2 overexpression in Drosophila (Ng et al., 2012) and in skin biopsies from LRRK2 G2019S mutation carriers (Mortiboys et al., 2010). In Drosophila, increased mitochondrial length can be rescued by Parkin overexpression (Ng et al., 2012), suggesting that both LRRK2 and Parkin may function in the same pathway to promote fusion. LRRK2 has also been found to interact with mitofusins and OPA1 in mitochondrial membranes, while S-OPA1 levels, but not L-OPA1, are reduced in G2019S PD brains in contrast to idiopathic PD brains, where no changes in S-OPA1 are observed (Stafa et al., 2014). However, others

have shown that wild-type LRRK2 interacts with Drp1 and that this interaction was exacerbated by the expression of PD-associated mutants. Moreover, LRRK2 wild-type or G2019S variant overexpression leads to mitochondrial fragmentation and clearance through the recruitment of Drp1 (Niu et al., 2012; Wang et al., 2012). Treatment with an LRRK2 inhibitor of cybrid cell lines from sporadic and LRRK2 patients (G2019S) leads to increased mitochondrial elongation and reduced phospho-Drp1 levels (Esteves et al., 2015), further supporting a role for LRRK2 in Drp1-mediated mitochondrial fission.

The PD-related proteins PINK1 and Parkin appear to control mitochondrial morphology by regulating mitochondrial fusion-fission events. However, whereas PINK1 and Parkin seem to promote mitochondrial fission and/or inhibit fusion in Drosophila, their role in mammals is more controversial (Guo, 2012; Scarffe et al., 2014). While PINK1 and Parkin functions in mitophagy have been studied extensively, their contribution to neuronal mitophagy has been challenged recently. Given that PINK1 and Parkin have been observed to be mutated in familial forms of PD, one would expect that impaired mitophagy might be a general theme in the pathogenesis of PD; however PINK1- and Parkin-knockout mice do not display neurodegeneration (Palacino et al., 2004; Gautier et al., 2008). Moreover, some groups have failed to observe Parkin recruitment in neurons following mitochondrial depolarization (Van Laar et al., 2011), or reported failure of endogenous Parkin to mediate mitophagy in neurons and cultured cells (Rakovic et al., 2013). Nonetheless, Cai et al. (2012) reported Parkin-dependent mitochondrial clearance in the somatodendritic region of primary cortical neurons upon CCCP (carbonyl cyanide m-chlorophenyl hydrazone) treatment (Cai et al., 2012). Besides, Suzuki et al. (2017) reported dysfunctional mitophagy in dopaminergic neurons derived from induced pluripotent stem cells from Parkin-mutated patients (Suzuki et al., 2017). In the MitoPark mouse model, in which there are clear mitochondrial alterations such as mitochondrial fragmentation and mitochondrial-derived aggregates, no evidence of Parkin recruitment to the defective mitochondria was observed, nor was there any effect of Parkin loss on the progression of neurodegeneration (Sterky et al., 2011). Conversely, the absence of Parkin in the Mutator mice (mice homozygous for a proofreading deficiency in DNA polymerase γ) led to a robust dopaminergic neuronal loss (Pickrell and Youle, 2015), suggesting that, at least in some cases, a basal level of mitochondrial dysfunction is needed in order to detect the detrimental effects of Parkin loss. What might be the role of PINK1-Parkin-dependent neuronal mitophagy *in vivo* is therefore still a matter of debate since; (i) most studies use proliferating cell lines, which poorly reflect the post-mitotic state of degenerating neurons; (ii) to induce mitophagy, a potent uncoupler (CCCP) is used, which inducesd a severe mitochondrial depolarization that rarely happens *in vivo*; and (iii) PINK1 and Parkin must be overexpressed in order to detect robust mitophagy. Interestingly, two *in vivo* mouse models, the mito-Keima and the mito-QC, have been recently developed (Sun et al., 2015; McWilliams et al., 2016). These models, which are based on the use of fluorescent reporter proteins, enable the visualization of mitophagy *in vivo* and might help to finally understand the *in vivo* role of PINK1-parkin-dependent mitophagy. Of note, loss of function of glucocerebrosidase and SREBF1, both proteins linked to or associated with PD, have been shown to be linked to mitophagy defects (Osellame et al., 2013; Ivatt et al., 2014).

PINK1 and Parkin have also been linked to the biogenesis of a population of MDV. Importantly, this process takes place faster than mitophagy, suggesting that such a mechanism may help to preserve the integrity of the organelle while damaged components are extracted. Parkin seems to colocalize with MDVs in a PINK1-dependent fashion upon oxidative stress, and MDVs are targeted to lysosomes for degradation independently of "typical" mitophagy mechanisms (McLelland et al., 2014). MDVs transport specific oxidized mitochondrial cargo, including subunits of the OXPHOS chain such as COXI, a transmembrane component of complex IV of the electron transport chain (McLelland et al., 2014; Sugiura et al., 2014). Interestingly, VPS35, which is linked to late-onset autosomal dominant PD, participates in the generation of some MDVs (Braschi et al., 2010).

Important advances emphasizing the contribution of impaired mitochondrial biogenesis to PD have recently been made. For downregulation of nuclear-encoded complex I genes was found associated with decreased expression of mitobiogenesis factors in PD frontal cortex, pointing at defects in mitochondrial biogenesis as another player in mitochondrial dysfunction (Thomas et al., 2012). Parkin also plays a role in the mitochondrial biogenesis pathway through the ubiquitination of Parkin Interacting Substrate (PARIS), leading to its ubiquitin-dependent degradation. PARIS is a major transcriptional repressor of PGC-1α expression, with a loss of Parkin in adult mice leading to PGC-1α downregulation due to PARIS upregulation, and dopaminergic neurodegeneration (Shin et al., 2011). The aforementioned study is particularly interesting since earlier reports failed to detect neurodegeneration in Parkin germline knock-out models. Here, there is a conditional knock-out in adult mice, which may suggest that whole body knock-out since birth may result in the development of compensatory mechanisms. The authors argue that similar compensatory mechanisms might occur in PD, accounting for the age-dependence of neurodegeneration observed in PD. PGC-1α and NRF-1 mRNAs are downregulated in the SN and striatum of PD patients, with no changes in mRNA levels of PARIS or Parkin, indicating that the upregulation of PARIS protein levels responds to a loss in the E3 ubiquitin ligase activity of Parkin (Shin et al., 2011). Decreased PGC-1α protein levels are associated with a loss in mitochondrial markers (Jiang et al., 2016). Further supporting the role of PGC-1α in PD, PGC-1α overexpression in adult conditional Parkin knock-out mice prevents dopaminergic neurodegeneration (Shin et al., 2011). Moreover, upon Parkin loss, mice present reduced mitochondrial size and number, together with structural abnormalities. These mitochondrial alterations depend on PARIS accumulation, since its loss rescues the mitochondrial mass (Stevens et al., 2015). This

data further reinforces the role of the Parkin-PARIS-PGC-1α axis in mitochondrial biogenesis and homeostasis, and provide more evidence for the key role of Parkin in the regulation of mitochondrial quality control. Recently, it was shown that PINK1 cooperates with Parkin by mediating PARIS phosphorylation to control its ubiquitination and clearance by Parkin (Lee et al., 2017). PGC-1α conditional deletion in adult mice leads to dopaminergic loss in the SNpc together with a reduction of dopamine in the striatum, highlighting the role of PGC-1α in neuronal viability in adult mice (Jiang et al., 2016). PGC-1α polymorphisms are associated with increased risk and age of onset of PD (Clark et al., 2011). The tandem PINK1-Parkin also participates in mitochondrial transport regulation by mediating Miro phosphorylation and ubiquitination, which targets the protein for proteasomal degradation (Wang et al., 2011; Liu et al., 2012b). Since PINK1 stabilization in the OMM occurs in damaged mitochondria, this mechanism is thought to arrest dysfunctional mitochondria, potentially decreasing their capacity to fuse with healthy ones. PINK1 and/or Parkin loss would facilitate the trafficking of dysfunctional mitochondria toward the axon terminals, where they could hamper energy production. Recent work has linked the α-synuclein A53T mutation with an altered balance in anterograde and retrograde transport (Pozo Devoto et al., 2017), while the LRRK2 R1441C variant is associated with impaired neuritic mitochondrial transport in SH-SY5Y cells (Thomas et al., 2017).

Organellar Quality Control and Huntington's Disease

In HD, the balance between fusion and fission is aberrantly shifted toward fission, which is associated with increased levels of Drp1 and Fis1 mRNA and decreased mitofusins in striatal and cortical regions (Kim et al., 2010; Shirendeb et al., 2011). These changes in key proteins controlling the mitochondrial fission/fusion balance are translated into mitochondrial fragmentation in HD human tissue and mouse models (Liot et al., 2009; Shirendeb et al., 2011). The mechanism leading to exacerbated mitochondrial fission in the presence of mutant huntingtin seems to involve a direct interaction with Drp1. In human post-mortem samples and in transgenic mice lines, mutant huntingtin interacts with Drp1 with a higher affinity than that of the wild-type huntingtin. This interaction leads to increased Drp1 enzymatic activity and mitochondrial fragmentation. The transfection of a dominant-negative Drp1 mutant (Drp1 K38A) leads to elongated and uniformly distributed mitochondria and protects against ATP loss and cell death, further supporting a role for Drp1 in mitochondrial fragmentation (Wang et al., 2009; Song et al., 2011; Shirendeb et al., 2012). Consistent with this hypothesis, Drp1 GTPase activity was increased in the cortex of HD patients (Shirendeb et al., 2012). Furthermore, blocking the interaction of Drp1 with Fis1, which inhibits mitochondrial fission, rescues striatal neuronal loss, improves motor activity and reduces mortality in R6/2 HD mice (Guo et al., 2013), suggesting that shifting the balance toward mitochondrial fusion could have a positive impact on disease progression. Brains of HD patients exhibit increased levels of S-nitrosylated Drp1 (Haun et al., 2013), a post-translational modification reported to enhance its GTPase activity (Cho et al., 2009). Since mutant huntingtin increases nitric oxide production, it is hypothesized that mutant huntingtin's interaction with Drp1 enhances its nitrosylation, thereby increasing mitochondrial fragmentation in HD (Haun et al., 2013).

Huntingtin protein plays an important role in the cargo recognition step in selective autophagy (Ochaba et al., 2014); the presence of the polyglutamine tract can affect the efficiency of this step and thus lead to impaired clearance of damaged mitochondria by mitophagy. Initial work by Martínez-Vicente et al. described the presence of "empty autophagosomes" in HD cells, suggesting that mutant huntingtin somehow impairs the cargo sequestration step, leading to inefficient cargo loading with lipid droplets and mitochondria being excluded from autophagic vacuoles (Martinez-Vicente et al., 2010). Huntingtin acts as a scaffold to bring together different proteins needed for autophagy to take place, such as p62 and ULK1 (unc-51 like autophagy activating kinase 1), which is normally inhibited by its association with mTORC1 (mammalian target of ramapycin complex 1). Upon exposure to different stresses, UKL1 is released from mTOR inhibition and interacts with huntingtin in a kinase-active form (Rui et al., 2015). It is likely, therefore, that the polyglutamine tract affects huntingtin's ability to associate with cargo receptors and autophagy machinery. While some studies have suggested that the presence of undigested mitochondria could stem from disturbances in autophagosomal transport, which is required for lysosomal fusion to autophagosomes (Wong and Holzbaur, 2014), others reported decreased LC3-mitochondria colocalization, arguing in favor of decreased mitophagy due to an impairment in the targeting of defective mitochondria to autophagosomes (Khalil et al., 2015). Interestingly, PINK1 overexpression could partially rescue the mitophagy defect in mouse striatal cells expressing mutant huntingtin (Khalil et al., 2015). Recent work has also linked mutant huntingtin with the impairement of a new form of micro-mitophagy mediated by GAPDH (glyceraldehydes-3-phosphate dehydrogenase) association with damaged mitochondria upon oxidative stress (Hwang et al., 2015). All in all, the ability of mutant huntingtin to interfere with different quality control process at the level of the whole mitochondria organelle prevents the correct elimination of damaged mitochondria, which potentially amplifies the deleterious cascade.

Studies on axonal transport in several *in vitro* models of HD reported impaired mitochondrial trafficking (Trushina et al., 2004; Chang et al., 2006; Orr et al., 2008). Wild-type huntingtin is involved in fast axonal trafficking in mammals, and this function has been attributed to its association with huntingtin-associated protein-1 (HAP1) (Gutekunst et al., 1998). HAP1 is an essential neuronal protein that interacts with dynactin p150 (Engelender et al., 1997) and kinesin light chain (McGuire et al., 2006). In contrast, mutant huntingtin leads to

trafficking abnormalities (Gunawardena et al., 2003), but the exact mechanism for this remains unknown. One possibility is that mutant huntingtin leads to abnormal interactions with HAP1, leading to impaired vesicular and organellar trafficking. Moreover, data from mouse neurons and human HD-affected brain suggests that large mutant huntingtin aggregates impair neuronal trafficking by sequestering wild-type huntingtin and motor proteins from soluble pools (Trushina et al., 2004). This trafficking defect also affects mitochondrial motility and appears to correlate with glutamine length (Trushina et al., 2004), leading mitochondria to accumulate adjacent to aggregates and become immobilized (Chang et al., 2006). Moreover, some polyglutamine-containing N-terminal huntingtin fragments, caused by proteolytic cleavage, can associate with mitochondria and, *in vitro*, interfere in its association with microtubule-based transport proteins, thus illustrating another mechanism by which mutant huntingtin can impair mitochondrial trafficking (Orr et al., 2008). More recently, mitochondrial trafficking was studied in primary hippocampal neurons from bacterial artificial chromosome mouse expressing full length human mutant huntingtin (BACHD mice) (Shirendeb et al., 2012). The authors found a decreased number of mitochondria moving anterogradely, together with increased numbers of mitochondria in the cell soma. Neuronal processes were, moreover, devoid of mitochondria, thus further documenting the aberrant mitochondrial transport associated with mutant huntingtin expression.

As discussed above, early-stage HD patients present decreased PGC-1α mRNA levels in the striatum but not in the hippocampus or cerebellum. Expression data from HD caudate tissue showed reduced expression of 24 out of 26 PGC-1α target genes (Cui et al., 2006; Weydt et al., 2006), while a splice variant of PGC-1α, which leads to a 38 kDa protein that complements, overlaps or prolongs PGC-1α full length action, has been found to be severely altered in human HD brain and myoblasts, as well as in mouse and cellular HD models (Johri et al., 2011; Török et al., 2015). Recently, a coding variant in PGC-1α was found to be associated with the age of onset of motor symptoms in men carrying the HD mutation (Weydt et al., 2014). Moreover, a correlation exists between PGC-1α reductions

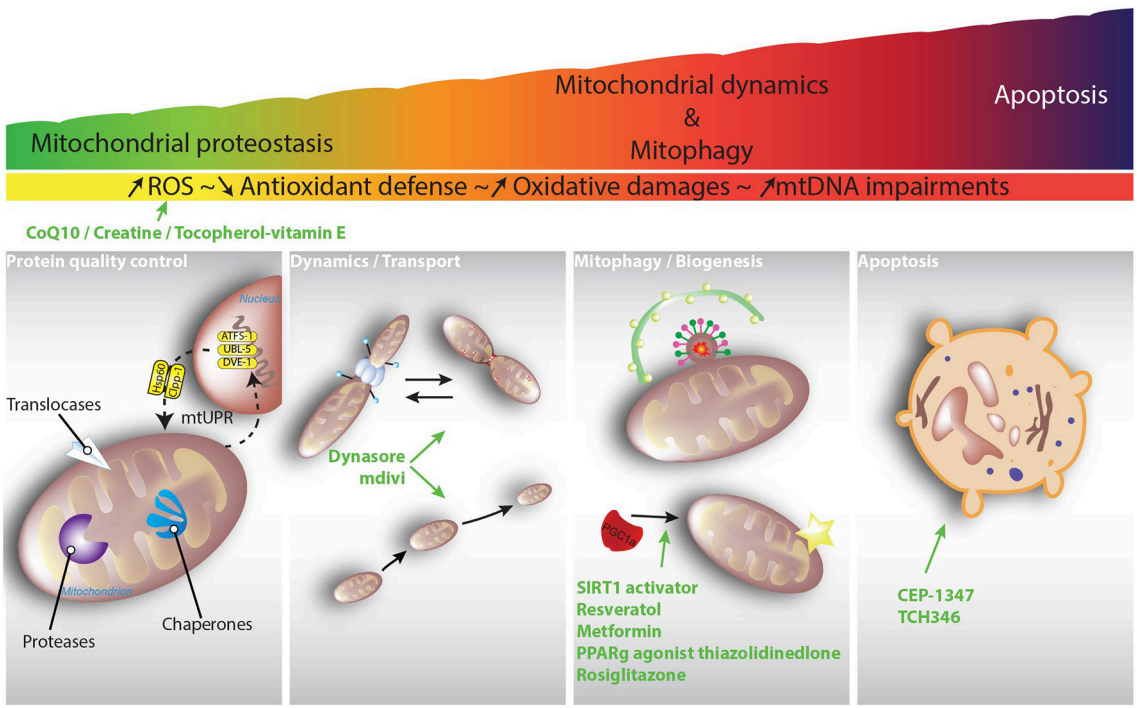


FIGURE 5 | Schematic diagram depicting an hypothesized scenario responsible for mitochondrial dysfunction in PD and HD. ROS are continuously produced *in vivo* by all body tissues. The presence of mitochondrial translocases, chaperones and proteases within the mitochondrial matrix and intermembrane space acts as a first line of defense against unfolded and oxidized soluble proteins. Mitochondria unfolded response (mtUPR) is believed to be activated by cytosolic transcription factors (ATFS-1, UBL-5, DVE-1). Once inside the nucleus, these transcription factors promote the upregulation of genes involved in mitochondrial proteostasis. To maintain a healthy network, mitochondrial are dynamic and can fuse, divide, and move. Once the accumulation of mitochondrial defects exceeds a threshold, patches of mitochondria can be removed through the generation of mitochondrial-derived vesicles (MDVs), which transit to the lysosome. Upon complete mitochondrial dysfunction, the entire organelle can be targeted to the autophagosome via so-called mitophagy. When none of the rescue strategies are able to restore mitochondrial function, the cell enters its ultimate destiny, apoptosis. Clinical trials using general caspase inhibitors such as CEP-1347 and TCH346 failed, maybe because it was too late in the process to intervene. Other compounds were tested in clinical trials, from fission inhibition using mdivi to PPARγ activation, but none were able to show clear treatment benefits. Interestingly, the role of mitochondrial quality control (MQC) in neuronal health is a recently growing subject for investigation, indicating that MQC might be central to maintain healthy mitochondria. However, until now, no novel therapeutic strategy specifically targeting MQC has been developed. It is clear that future research focusing on understanding MQC is needed to develop better pharmacological interventions.

and mitochondrial reductions in HD post-mortem brain (Kim et al., 2010). In line with this, PGC-1α null mice develop a neurological phenotype consistent with neurodegeneration, together with spongiform lesions predominantly in the striatum (Lin et al., 2004). Pharmacologic activation of PGC-1α expression resulted in improved behavior, improved survival and reduced brain, muscle, and brown adipose tissue pathology in R6/2 transgenic and full-length huntingtin mouse models of HD (Johri et al., 2012; Chandra et al., 2016). These observations provide further support of the key role that alterations in the PGC-1α pathway play in mitochondrial dysfunction in HD. cAMP signaling is a key activator in PGC-1α transcription, promoting the binding of cAMP response element (CRE)-binding protein (CREB) or activating transcription factor-2 to a conserved DNA response element in the PGC-1α promoter. Mutant huntingtin interferes with the CREB/TAF4 transcriptional pathway in striatal neurons, thus repressing the CRE-mediated transcription of PGC-1α (Cui et al., 2006; Weydt et al., 2006). Levels of TORC (Transducers of Regulated CREB Activity), a CREB coactivator, were decreased in the striatum of HD patients, mice and cellular HD models (Chaturvedi et al., 2012).

CONCLUSION

Mitochondria are essential organelles for the maintenance of neuronal homeostasis. The importance of functional mitochondria to neurons is highlighted by the fact that situations leading to mitochondrial dysfunction are often associated with neurodegenerative diseases. Many of these diseases manifest later in life, where mitochondria seem to be less functional (Grimm and Eckert, 2017). Mitochondria are at the center of the free radical theory of aging by being both a source and target of ROS. As discussed in this review, the imbalance between ROS production and antioxidant defense in neurodegenerative diseases leads to protein, lipid and DNA oxidation, which in turn affect mitochondrial function. It was previously considered that when ROS levels exceeded a pathological threshold, this may trigger cell death by apoptosis (**Figure 5**). However, recent developments highlight the increasing potential of mitochondria to defend themselves against various threats (Perier et al., 2010, 2013). Different levels of quality control co-exist within mitochondria to detect and repair defects that affect mitochondrial performance, before the point of inescapable cell death is reached: (i) novel, critical roles of mitochondrial proteases have emerged in neural physiology and pathology and (ii) mitochondrial dynamics play an important role in maintaining a healthy organelle population. Thus, impairments in these quality control systems may lead to the accumulation of defective mitochondria, as well as inefficient mitochondrial transport and distribution, leading to synaptic and neuronal degeneration. However, the more our understanding of protein quality control activities in mitochondria continues to expand, the more questions that are raised. How are all the mitochondrial quality control mechanisms, at the protein and the organelle levels, inter-connected? As mechanisms that flag defective mitochondria are thought to be similar, are these pathways temporarily interlinked and is there crosstalk between them to coordinate mitochondrial homeostasis?

Despite the many therapeutic advances in PD and HD, no agent has yet been established that has neuroprotective effects in either disease. Although apoptosis is potentially a terminal pathway relevant to both PD and HD, therapeutic targeting of executioner caspases, despite initially high expectations due to the availability of pharmacological caspase inhibitors, has failed to prevent neurodegeneration in clinical trials (Parkinson Study Group PRECEPT Investigators, 2007; Huntington Study Group DOMINO Investigators, 2010; **Figure 5**). Acting before the terminal phase is reached might represent an effective neuroprotective therapy that slows or stops disease progression and prevents the development of cumulative disability (Bové et al., 2014). Some compounds, which target mitochondrial dysfunction and mitochondrial quality control, have shown beneficial effects in mouse models of neurodegenerative diseases and show great promise for treating patients (**Figure 5**). Our current knowledge regarding mitochondrial quality control continues to evolve, opening novel and exciting research paths that will likely help to develop clinically effective therapeutic applications to prevent or combat neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

SF-I and CP wrote the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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REVIEW
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Mitochondria and Mood: Mitochondrial Dysfunction as a Key Player in the Manifestation of Depression

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Human and animal studies suggest an intriguing link between mitochondrial diseases and depression. Although depression has historically been linked to alterations in monoaminergic pharmacology and adult hippocampal neurogenesis, new data increasingly implicate broader forms of dampened plasticity, including plasticity within the cell. Mitochondria are the cellular powerhouse of eukaryotic cells, and they also regulate brain function through oxidative stress and apoptosis. In this paper, we make the case that mitochondrial dysfunction could play an important role in the pathophysiology of depression. Alterations in mitochondrial functions such as oxidative phosphorylation (OXPHOS) and membrane polarity, which increase oxidative stress and apoptosis, may precede the development of depressive symptoms. However, the data in relation to antidepressant drug effects are contradictory: some studies reveal they have no effect on mitochondrial function or even potentiate dysfunction, whereas other studies show more beneficial effects. Overall, the data suggest an intriguing link between mitochondrial function and depression that warrants further investigation. Mitochondria could be targeted in the development of novel antidepressant drugs, and specific forms of mitochondrial dysfunction could be identified as biomarkers to personalize treatment and aid in early diagnosis by differentiating between disorders with overlapping symptoms.

Keywords: depression, behavior, reelin, mitochondria, oxidative phosphorylation, antidepressants

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MITOCHONDRIA

Mitochondria are the main energy factories of eukaryotic cells. The brain is particularly dependent on mitochondrial activity due to both its high levels of energy use and its inability to store large amounts of energy reserves in the form of glycogen. As a result of the their roles in energy production, mitochondria also generate reactive oxygen species (ROS) that may have a toxic effects in cells. In addition, mitochondria also play a prominent role in the regulation of apoptotic cell death (for examples, see Davidson and Hardison, 1984; Herrmann and Neupert, 2000; Calabrese et al., 2001; Chan, 2006; Chipuk et al., 2006; Fattal et al., 2006; McBride et al., 2006; Youle and van der Bliek, 2012; Tobe, 2013; Bansal and Kuhad, 2016).

The focus of this review is the link between mitochondrial dysfunction and major depression. Depression has historically been considered a disorder of altered pharmacology and altered hippocampal neurogenesis. However, recent evidence has opened the door to an expanded notion of the neurobiology of depression, such that a reduction in ATP levels, enhancement of oxidative stress, and acceleration of apoptosis are now considered to be important events (reviewed in Rezin et al., 2008a). In this review, we summarize some of the latest knowledge on mitochondrial dysregulation in major depression (depicted in **Figure 1**) and also discuss how mitochondrial dysfunction could instigate downstream changes in extracellular matrix proteins such as reelin, neuronal nitric oxide (nNOS), oxidative stress, and inflammation, and finally adult hippocampal neurogenesis. Uncovering how all these factors influence one another could lead to new vistas in the development of novel therapeutics for the treatment of this problematic disorder.

HYPOTHESES ABOUT THE NEUROBIOLOGICAL BASIS OF DEPRESSION

Depression is a common neuropsychiatric disorder, affecting up to 20% of the population (Kessler et al., 1994). The presence and severity of symptoms vary among individuals, and can include low mood and anhedonia, decreased energy, altered appetite and weight, irritability, sleep disturbances, and cognitive deficits (Nemeroff, 1998). Patients with depression have a higher rate of other physical illnesses (i.e., comorbidities with cardiovascular disorders, stroke, etc.), decreased social functioning, and a high mortality rate (Nemeroff, 1998). The complexity of this disorder is further compounded by the fact that it often co-occurs with other psychiatric conditions. For example, about 50% of depression patients also suffer from anxiety disorders (Kessler et al., 1996), which can indicate a more severe form of the disease, with delayed recovery, increased risk of relapse, greater disability, and increased suicide attempts (Hirschfeld, 2001). It is generally thought that a combination of environmental and genetic factors influences the development of depression (Nestler et al., 2002; Kalia, 2005). However, despite extensive clinical and preclinical research efforts, there is still a fundamental lack of understanding about the specific biological changes that give rise to depressive symptoms.

For more than 50 years, the dominant theory for the pathogenesis of depression was the monoamine hypothesis (Schildkraut, 1965), which arose from observations that antidepressant drugs work by inhibiting the reuptake of monoamines such as serotonin and norepinephrine. However, this theory has largely fallen out of favor due to a number of discrepancies, such as the fact that the therapeutic effects of antidepressants take weeks to develop even though monoamine levels are elevated within hours of administration, and the fact that only about 40% of patients respond satisfactorily to treatment (Trivedi et al., 2006). More recent theories about the neurobiological basis of depression have focused on the neurogenesis theory, which posits that stress-induced decreases in hippocampal neurogenesis could be a causal factor in depression (Jacobs et al., 2000). This hypothesis is supported by observations of decreased hippocampal volume in patients with depression (MacQueen et al., 2003; Campbell et al., 2004), decreased cell proliferation and survival in preclinical animal models of depression (Brummelte and Galea, 2010; Schoenfeld and Cameron, 2015), and increased cell proliferation and survival after antidepressant treatment (Santarelli et al., 2003; Fenton et al., 2015). Our laboratory has contributed to this literature using a well-validated preclinical model of depression in which rats or mice are subjected to daily injections of the stress hormone corticosterone (CORT) for several weeks, followed by behavioral testing and then tissue collection for further analyses (Gregus et al., 2005; Johnson et al., 2006; Sterner and Kalynchuk, 2010). Using this approach, we showed that the time course for the onset of depression-like behavior in rats is paralleled by dampened hippocampal neurogenesis and neuronal maturation (Lussier et al., 2013). Importantly, this work also implicated the extracellular matrix protein reelin in the pathogenesis of depression (see Caruncho et al., 2016). There is evidence that reelin can regulate adult hippocampal neurogenesis and dendritic spine plasticity (Pujadas et al., 2010), and our data show that rats subjected to repeated CORT injections have dampened reelin expression selectively in the proliferative subgranular zone of the dentate gyrus (Lussier et al., 2009) and also that antidepressant treatment normalizes depression-like behavior, hippocampal neurogenesis, and hippocampal reelin expression in tandem (Fenton et al., 2015). The reelin link is relevant to mitochondrial dysfunction because reelin in the periphery interacts with the immune system and a loss of reelin can magnify markers of inflammation that influence mitochondria (Green-Johnson et al., 1995). This issue is discussed in more detail in the section below on reelin and inflammation.

It is important to note that the neurogenesis hypothesis of depression is somewhat controversial because depression-like symptoms can occur even when cell proliferation is not decreased and the behavioral actions of antidepressants do not always coincide with increases in the number of hippocampal neurons (Surget et al., 2008; Bessa et al., 2009; David et al., 2009). Instead, some data suggest that depression and the therapeutic actions of antidepressants may be more related to alterations in dendritic complexity and neuronal remodeling than cell number *per se* (Bessa et al., 2009; Lussier et al., 2013). The neurogenesis hypothesis of depression may therefore be better described as the neuroplasticity hypothesis of depression – a broadening of concept that would include plasticity within the cell, such as mitochondrial activity.

Because the brain has high aerobic activity, requiring about 20 times more energy than the rest of the body by weight (Kety, 1950), it is highly vulnerable to conditions stemming from impaired energy production. A resting cortical neuron consumes 4.7 billion ATP molecules every second (Zhu et al., 2012). Evidence from post-mortem (Kato et al., 1997; Konradi et al., 2004; Iwamoto et al., 2005; Munakata et al., 2005), imaging (Frey et al., 2007), genetic (Kato et al., 1997; Kato and Kato, 2000; Iwamoto et al., 2005; Benes et al., 2006),

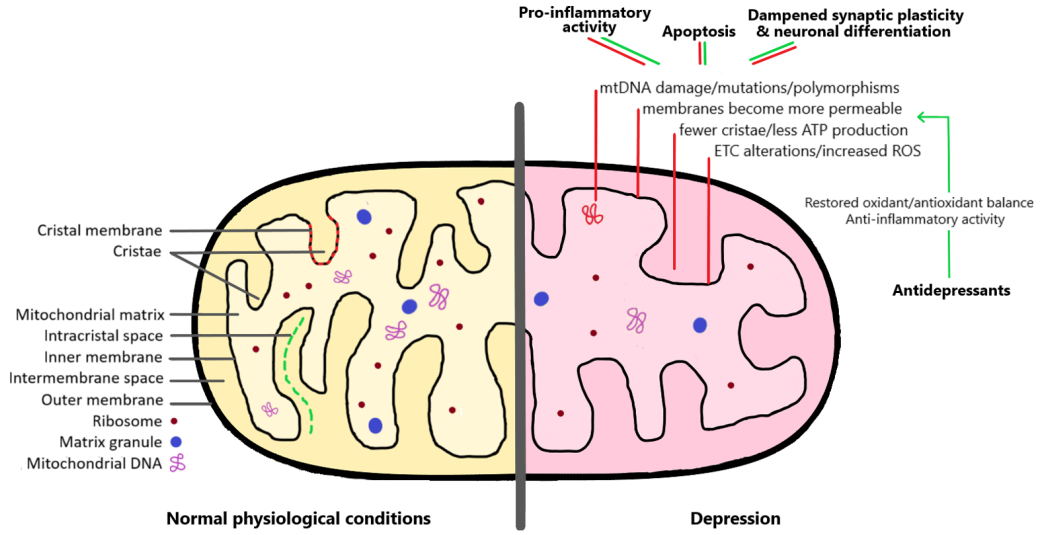


FIGURE 1 | The mitochondrion under normal physiological conditions and in the depression brain. As detailed in the right side of the image, there are a series of mitochondrial alterations that have been observed both in depressed patients and in animal models of depression (red lines). These include changes affecting mitochondrial DNA, membrane permeability, and increased formation of reactive oxygen species (ROS). As a consequence, these alterations lead to pro-inflammatory activity, increased apoptosis, and dampened synaptic plasticity and neuronal differentiation. Interestingly, antidepressant medication can restore the mitochondrial oxidant/antioxidant balance, and therefore help to rescue the negative effects of mitochondrial dysregulation (green lines). See the text for more detailed explanations.

and cellular (Cataldo et al., 2010) studies is plentiful in showing the involvement of mitochondrial dysfunction in bipolar disorder and schizophrenia. Studies are also emerging providing evidence that mitochondria-mediated mechanisms are related to depressive symptoms (reviewed in Castren, 2005; Tobe, 2013; Shimamoto and Rappeneau, 2017; Petschner et al., 2018), that mitochondrial mutations are witnessed in individuals diagnosed with depression (Munakata et al., 2007; Ben-Shachar and Karry, 2008) and that the two diseases are often comorbid (Koene et al., 2009; Morava et al., 2010).

Mitochondria could play a role in the dampened plasticity associated with depression. Depression is associated with abnormalities in intracellular second messenger signal transduction cascades resulting from 5HT and NE receptor activation (Perez et al., 2000; Popoli et al., 2000) and dysregulated and desensitized monoamine receptors (Hamon and Blier, 2013). These observations can be related to mitochondrial dysfunction because ATP is needed for the activation of downstream signaling following the binding of neurotransmitters to receptors (Moretti et al., 2003). ATP is also necessary to attend to the energy demands of vesicle transport and neurotransmitter release (reviewed in Vos et al., 2010; and more recently in Devine and Kittler, 2018). Furthermore, patients with mitochondrial diseases or mitochondrial DNA (mtDNA) mutations and polymorphisms often present symptoms characteristic of mood disorders (Suomalainen et al., 1992; Onishi et al., 1997; Kato et al., 2001; Fattal et al., 2006; Morava et al., 2010). Higher rates of mitochondrial biogenesis are needed for neuronal differentiation (Calingasan et al., 2008) and therefore, dysfunctional mitochondria could result in impaired neuroplasticity in depressed patients.

GENETICS

There have been many observations of links between mtDNA and depression. As mentioned above, depression is a heterogeneous disorder, with several different symptom profiles, and genetic background contributes to its development (Lesch, 2004). Prevalence rates for depression are as high as 54% in patients with mitochondrial diseases (Fattal et al., 2007). However, not all patients who have the same mitochondrial gene mutations develop depressive symptoms, indicating a genetic and non-genetic interplay of factors (Koene et al., 2009). Mitochondrial disorders may result from mutations in nuclear DNA or mtDNA, with the amount of mtDNA mutations possibly correlating with disease severity (Chinnery et al., 2000). In one interesting study, long-PCR revealed that 68% of patients with depression have mtDNA deletions, compared to 36% of control subjects (Gardner et al., 2003). Similarly, in leukocytes of depressed patients, mtDNA copy number variates were significantly lower than in control subjects, and mtDNA oxidative damage was increased (Chang et al., 2015). Interestingly, oxidized mtDNA activates pro-inflammatory cytokines (Adzic et al., 2016) and increased inflammation is known to play a role in the development of depressive symptoms (e.g., Brymer et al., 2018; Wang et al., 2018). Variations in mtDNA have also been shown to cause cognitive impairments in mice (Sharpley et al., 2012) and in humans (Inczedy-Farkas et al., 2014; Petschner et al., 2018), and cognitive deficits are a common symptom associated with depression.

Beyond these general linkages between mtDNA and depression, recent research has implicated a number of specific mitochondrial genes in depression. This topic has been reviewed in detail recently (Petschner et al., 2018), so only

a few examples will be mentioned here. A recent systematic assessment using mitochondrial PCR array profiling identified 16 genes that were differentially expressed in the dorsolateral prefrontal cortex of post-mortem brains from depressed patients compared to control subjects (Wang and Dwivedi, 2017). The identified genes are ones known to govern oxidative stress and neuronal ATP levels, suggesting for the first time that mitochondrial genes might be altered in tissue samples from human patients. Similarly, the mitochondrial ATP6V1B2 gene has been implicated in depression, possibly through its effects on neurotransmission and receptor-mediated endocytosis (Petschner et al., 2018). Less direct evidence comes from observations that mice with mutations of the POLG gene, which encodes a subunit of mtDNA polymerase, exhibit depression-like symptoms (Kasahara et al., 2006), and that polymorphisms of genes that code for mitochondrial enzymes, such as MTHFD1L, are associated with negative rumination, which is a precursor to depression (Eszlari et al., 2016). This polymorphism is also associated with high levels of homocysteine, which has been related to hippocampal volume and depression (Moustafa et al., 2014).

These examples point to an intriguing link between mtDNA or gene expression and depression, though more work needs to be done in this area, particularly to identify gene alterations in tissue from human patients.

PROTEOMIC STUDIES

There have been many studies indicating the involvement of the oxidative phosphorylation (OXPHOS) pathway in depression. Proteomic studies conducted on post-mortem brains from depressed patients suggest that about 21% of dysregulated proteins are also commonly dysregulated in patients with schizophrenia and bipolar disorder (Saia-Cereda et al., 2017; Villa et al., 2017). In a mutant mouse model of depression, a dysregulated OXPHOS pathway was seen in the hippocampus (Zubenko et al., 2014), which is a key region of dampened plasticity in both human depression and rodent models (Sheline et al., 2003; Frodl et al., 2006; Sterner and Kalynchuk, 2010). In addition, proteomic studies using different animal models of depression have revealed alterations in specific proteins involved in OXPHOS and also confirmed the effect of antidepressant treatment in the expression of these proteins (reviewed in Carboni, 2015).

In depressed patients, most of the differentially expressed proteins are involved in cellular assembly, organization, function, and maintenance, as well as cardiovascular system development and function, but they are mainly related to deregulation of energy metabolism pathways (Martins-de-Souza et al., 2012). Twenty different subunits of the OXPHOS complex were increased in post-mortem brains from depressed patients (Martins-de-Souza et al., 2012), whereas the opposite effect has been seen in brains from patients with schizophrenia (Martins-de-Souza et al., 2011). A proteomics approach also revealed that the SSRI fluoxetine upregulated and downregulated 23 and 60 cytosolic mitochondrial-related proteins, respectively (Filipović et al., 2017). In addition, 60 non-synaptic mitochondrial-related proteins were upregulated whereas three were downregulated. These effects were largely confirmed in a subsequent study (Głombik et al., 2017). When looking at samples from the dorsolateral prefrontal cortex, which shows reduced activation and volume in patients with depression (Drevets et al., 1998; Halari et al., 2009), a shotgun label-free approach revealed that 32% of differentially expressed proteins associated with depression were involved in metabolic/energy pathways (Martins-de-Souza et al., 2012). Two other proteomic studies showed that several proteins involved in energy metabolism, such as carbonic anhydrase and aldolase C, were increased in the frontal cortex (Johnston-Wilson et al., 2000) and anterior cingulate cortex (Beasley et al., 2006) of depressed patients. These results are consistent with PET findings of a reduction in cerebral glucose metabolism in the brains of depressed patients (Baxter et al., 1989), which was reversed by 6 weeks of treatment with the SSRI paroxetine (Kennedy et al., 2001). Similarly, PET studies also revealed that depressed patients had reduced blood flow and bioenergetic metabolism in the prefrontal cortex (Drevets et al., 1997; Mayberg et al., 1999; Moretti et al., 2003), cingulate gyrus, and basal ganglia (Videbech, 2000).

Psychiatric disorders almost always have overlapping symptoms, which might reflect common mechanisms when compared against controls. An interesting study addressing this issue showed that patients with major depression that included psychosis had more differentially expressed proteins associated with energy metabolism, whereas patients with depression without psychosis had changes in proteins associated with cell growth and maintenance, although 53.7% of the altered proteins overlapped (Martins-de-Souza et al., 2012). Subtle differences in proteome fingerprints may become useful biomarkers that could be used to stratify patients with different symptoms profiles and to formulate effective personalized treatment plans. Proteomic studies support the view that mitochondrial dysfunction is one of many important factors involved in depression, and may identify novel pathogenic mechanisms of psychiatric disorders.

That alterations in mitochondria bioenergetics pathways contributed to the pathophysiology of depression also raise the possibility of developing mitochondrial biomarkers that can illustrate a better therapeutic approach to the treatment of depression. However, this field is still undeveloped and additional studies are needed characterizing specific mitochondrial dysfunctions in depression in relation to therapeutic response to antidepressants, or to evaluate the possibility of identifying mitochondrial drug targets that could be used to develop novel antidepressant drugs (Klinedinst and Regenold, 2015).

DECREASED ATP PRODUCTION

The production of ATP through OXPHOS is a key method by which mitochondria provide energy to the cell. Several lines of research have confirmed that depression is associated with lower than normal levels of ATP production. For example, brain levels

of ATP are generally lower in the brains of depressed patients compared to control subjects (Moretti et al., 2003; Martins-de-Souza et al., 2012). This may be related to the dampened neuronal plasticity and impaired hippocampal neurogenesis thought to be operative in depression (Caruncho et al., 2016), as neurogenesis is a metabolically demanding process. Other researchers have found changes in ATP in depression in areas outside the brain. Gardner et al. (2003) found that mitochondrial ATP production rates and mitochondrial enzyme ratios in electron transport chain (ETC) complexes I–IV were significantly decreased in the muscles of patients with depression compared to controls. A correlation was found between altered biochemistry and depression rating scale scores further evidencing the relationship between mitochondrial dysfunction and psychopathology. In peripheral blood mononuclear cells, ATP turnover-related respiration was lowered in depressed patients compared to age-matched controls, as well as routine and uncoupled respiration and coupling efficiency (Karabatsiakis et al., 2014). Moreover, ATP-binding cassette transporters, which utilize the energy of ATP, are also altered in depressed patients and single nucleotide polymorphisms in the gene that codes for these transporters may be indicators of severity of the disorder and patient responsiveness to antidepressants (Lin et al., 2011).

Decreased ATP production has also been observed in preclinical animal models of depression. Female rats displaying anhedonia (i.e., decreased preference for sucrose) after 40 days of mild stress also had decreased hippocampal NA+, K+-ATPase activity (Gamaro et al., 2003). Fluoxetine reversed the effects of stress on enzymatic activity suggesting that NA+, K+-ATPase activity may be involved in the depression-like phenotype. In another study, fluoxetine restored sucrose preference, and normalized ATP synthesis rate and mitochondrial respiratory control in the raphe nucleus after 18 days of chronic unpredictable stress (Wen et al., 2014). Another study using the chronic mild stress paradigm revealed that mice with decreased sucrose preference and increased immobility in the tail suspension test (i.e., learned helplessness) also showed damaged mitochondrial ultrastructure, impaired respiration rates, and altered membrane potentials in the hippocampus, hypothalamus, and the cortex (Gong et al., 2011).

OXIDATIVE STRESS

Mitochondria are the primary source of ROS, which under normal conditions play important roles in cell signaling and homeostasis. ROS are produced in the OXPHOS pathway; however, in normal physiological conditions, mitochondria create protective factors that can neutralize harmful free radicals (Petschner et al., 2018). For example, there is a mitochondrial matrix thiol system that has an important role in antioxidant protection (Murphy, 2012). In the ETC, complexes donate electrons to oxygen producing radicals like superoxide and peroxidases, and high levels of such radicals and oxidative stress cause damage to lipids, enhance DNA breaks, and oxidize nuclear and mtDNA (Tobe, 2013; Czarny et al., 2015). Lower levels of ROS also play a role in normal cellular functioning, such as differentiation of cells, tissue regeneration, redox biology, and promoting adaptation to environmental changes (Vakifahmetoglu-Norberg et al., 2017). The production of highly reactive free radicals is increased when premature leakage of electrons to oxygen occurs in the ETC, increasing oxidative stress. Superoxide is a precursor for ROS, and complex I and III are mainly responsible for its production (Vakifahmetoglu-Norberg et al., 2017). Oxidative stress could be the cause or consequence of damage to mitochondria and mtDNA (Xie et al., 2017). Martins-de-Souza et al. (2012) speculated that a reduction in ATP could be due to oxidative stress and that the increased levels of subunits of OXPHOS complexes were compensatory. In fact, ATP reduction and its relation to oxidative stress have been linked not only to depression (detailed below), but also to psychotic disorders (see Chouinard et al., 2017), autism (Rose et al., 2014), anxiety (Kumar and Chanana, 2017), Alzheimer's disease (recently reviewed in Tramutola et al., 2017), and Huntington disease (Quintanilla et al., 2017).

Several papers have reported links between oxidative stress and depression. Ben-Shachar and Karry (2008) reported an increase in oxidative damage and alterations in ETC complex I in the prefrontal cortex of depressed patients. Other researchers noted decreased levels of antioxidants and antioxidant enzymes in depression and related these changes to deficits in cognition (Anderson, 2018). In an immobilization stress preclinical model of depression, in which animals were restrained for 6 h a day, levels of the cellular antioxidant glutathione were reduced by 36.7% after 21 days, while lipid peroxidation increased (Madrigal et al., 2001). The authors speculated that lipid peroxidation could cause mitochondrial dysfunction by damaging membranes and causing excitotoxicity, which could be potentiated by increased production of reactive molecules (Braughler and Hall, 1989) or decreased antioxidant levels. In the olfactory bulbectomy model of depression, glutathione levels were also decreased whereas ROS superoxide, nitric oxide (NO), and lipid hydroperoxide levels were increased in mice (Holzmann et al., 2015) and rats (Almeida et al., 2017).

As previously mentioned, the effect of 21 days of environmental stress (i.e., restraint) on mitochondrial dysfunction in rats was investigated (Madrigal et al., 2001). The authors reported that mitochondrial activity of ETC complexes I–III were significantly decreased after just 7 days of restraint stress (6 h per day); however, there was no difference in complex IV and no stress-induced decreases in oxygen consumption throughout the 21-day period. They speculated that mitochondrial dysfunction was a result of overproduction of NO, as an accumulation of NO metabolites was found in the brain tissue. Similarly, 40 days of chronic variable stress decreased sucrose preference as well as inhibited ETC complexes I, II, and IV in the cerebral cortex and cerebellum of rats (Rezin et al., 2008b). Interestingly, increased expression of proteins related to mitochondrial import and transport in the OXPHOS pathway was also seen in a preclinical mouse model of anxiety, a disorder that is highly comorbid with depression (Filiou et al., 2011). Moreover, in this model, the expression of enzymes involved in catalyzing glycolysis pathway reactions was also dysregulated.

The mechanisms by which environmental stress negatively impacts the brain are still not fully understood. However, there is evidence that free-radicals such as NO cause rapid damage to certain cell macromolecules that are involved in the ETC system, which in turn will decrease production of ATP and may be implicated in cytotoxic effects in the central nervous system (Cleeter et al., 1994; Lizasoain et al., 1996). Madrigal et al. (2001) did not find changes in ATP levels, which provides further evidence that a threshold of ETC complex dysfunction may have to be reached before the capability of mitochondria to maintain homeostasis diminishes (Davey and Clark, 1996).

Antidepressant treatment improves oxidative stress parameters in patients with depression. For example, a higher serum total oxidant status and a lower serum total antioxidant capacity in depressed patients were normalized after 42 days of antidepressant treatment (Cumurcu et al., 2009). Similar findings have been reported in animal models of depression. Venlafaxine increased expression of antioxidant mitochondrial genes in the mouse brain, which reduced levels of hydrogen peroxide and peroxynitrite (Goemaere and Knoops, 2012; Tamási et al., 2014). Furthermore, in the chronic mild stress paradigm, lamotrigine, aripiprazole, and escitalopram all normalized glutathione and glutathione peroxidase activity in rat cortical regions (Eren et al., 2007a). Lipid peroxidation in the cortex and plasma was increased by chronic mild stress, but also reversed by the same three treatments. A similar study revealed that venlafaxine can reverse chronic mild stress-induced decreases in glutathione peroxidase activity and vitamin C, and increases in lipid peroxidation and NO in the rat cortex (Eren et al., 2007b). Moreover, unpredictable stress in mice resulted in increased open field test exploration along with decreased liver glutathione, superoxide dismutase, and total antioxidant capability, which was reversed by the traditional Chinese medicine, Shudihuang, in a dose-dependent manner (Zhang et al., 2009).

REELIN, OXIDATIVE STRESS, INFLAMMATION, AND DEPRESSION

A further link between ROS and depression has been suggested by recent work focused on the extracellular matrix protein reelin. Reelin has been linked to depression in preclinical models of depression: A decline in reelin expression in the hippocampal subgranular zone is associated with the emergence of depression-like behavior, and heterozygous reeler mice (HRM) with 50% of the normal levels of reelin are highly susceptible to the depressogenic effects of stress hormones (Lussier et al., 2011, 2013). Interestingly, a subpopulation of reelin containing cells also coexpress nNOS, and the percentage of neurons coexpressing both markers is specifically decreased in the subgranular zone and molecular layer of the dentate gyrus in HRM (Romay-Tallon et al., 2010).

As reelin secretion by neurons in the subgranular zone may be involved in regulating the maturation of adult hippocampal newborn neurons (Lussier et al., 2009), and as deficits in adult hippocampal neurogenesis have been proposed to be a key event underlying the development of a depressive phenotype (detailed above in section "Hypotheses About the Neurobiological Basis of Depression"), we recently examined the effects of repeated CORT injections on coexpression of reelin and nNOS across hippocampal subregions in brains from HRM and wildtype mice. We found that repeated CORT (administered at a dose that induces depression-like behavior in HRM; Lussier et al., 2011) creates an imbalance between reelin and nNOS expression in the proliferative subgranular zone of the dentate gyrus, with CORT inducing a decrease in colocalization of reelin and nNOS in wildtype mice but a significant increase in colocalization of these markers in HRM. We interpreted these results as being indicative of profound excitotoxicity in dentate gyrus neurons after chronic exposure to stress hormones to a degree that produces depression-like behavior (Romay-Tallon et al., 2010, 2015).

Nitric oxide and other ROS inhibit mitochondrial 2-oxoglutarate dehydrogenase giving rise to increased levels of glutamate, which eventually leads to glutamate excitotoxicity and cell death (Weidinger et al., 2017). The reelin–nNOS connection should receive more experimental attention, as a number of reports indicate that alterations in reelin expression within the dentate gyrus may result in deficient maturation of newborn granule neurons and dampened hippocampal plasticity, and may represent a key event in the pathophysiology of depression (reviewed in Caruncho et al., 2016).

There is also a key link with inflammation to consider in the context of these experiments. Many studies support the idea that inflammatory processes are involved in depression, and in fact targeting of inflammatory cytokines to reduce depression symptoms is a very active area of research (recently reviewed by Shariq et al., 2018). Studies have shown that pro-inflammatory cytokines alter ETC complexes and complex associated enzymes (Samavati et al., 2008), and that they activate pro-apoptotic proteins and the caspase cascade (Bansal and Kuhad, 2016). In mice, the injection of lipopolysaccharide, which induces strong immune responses and secretion of pro-inflammatory cytokines, significantly increased depression-like behavior in the sucrose preference and forced swim tests, and decreased ATP levels and mitochondrial membrane potential in the hippocampus (Chen et al., 2017).

Alterations in several components of the immune system, and in inflammatory markers, have also been observed in animals with low or null reelin expression (Green-Johnson et al., 1995). We have reported that these animals not only are quite susceptible to the depressogenic effects of repeated CORT (Lussier et al., 2011), but also show alterations in the clustering of specific membrane proteins in lymphocytes (Rivera-Baltanás et al., 2010) which prompted us to investigate membrane protein clustering in lymphocytes in depression patients, and to propose that the pattern of clustering of specific proteins along the plasma membrane of lymphocytes could be a putative biomarker of depression, and perhaps underlie some of the inflammatory events observed in depression patients (Rivera-Baltanas et al., 2012, 2014, 2015). In fact, alterations in oxidative stress in lymphocytes have been clearly demonstrated in depression (Szuster-Ciesielska et al., 2008; Czarny et al., 2018). Following up this line of thought, we have recently

demonstrated that peripheral injections of the anti-inflammatory drug etanercept (which is unable to cross the blood–brain–barrier) not only rescues the depression-like behavior induced by repeated CORT but also normalizes the neurochemical phenotype of reelin expressing cells in the hippocampal dentate gyrus. We speculated that both peripheral and secondary central actions may be operative in the antidepressant effects of etanercept injections (Brymer et al., 2018). It seems clear that additional studies would be required to determine the connection between reelin, oxidative stress, and inflammation in depression, not only to determine how these factors may be an important component of the pathophysiology of depression, but also to evaluate them as possible targets to develop novel antidepressant drugs.

APOPTOSIS

Mitochondria have a clear role in cell metabolism, and evidence suggests that mitochondrial morphology also affects metabolic enzymes through fusion and fission (Chen et al., 2005). The formation and morphology of cristae on the inner membrane, which regulate mitochondrial metabolism, may require fusion machinery as a loss of such machinery results in decreased metabolism (McBride et al., 2006). Abnormal cell structure and function could result in alterations in synaptic signaling and neural circuits and vice versa (Kaidanovich-Beilin et al., 2012). Excessive glutamatergic activation of NMDA receptors was shown to increase ROS levels and alter mitochondrial membrane polarity, which led to elevated apoptosis rates in cardiomyocytes, possibly as a result of increased calcium ion influx (Gao et al., 2007). Mitochondria are present at synapses and responsive to synaptic stimulation (McBride et al., 2006). As the hippocampus is highly vulnerable to the depressogenic effects of chronic stress, it is likely that hippocampal mitochondria behave abnormally in depressed patients. For example, the clustering of mitochondria in dendritic spines in response to neural activity may be altered (Li et al., 2004). Glucocorticoid receptors (GRs) are also highly prevalent in the hippocampus. These receptors are activated when stress hormone levels are high, such as during periods of chronic stress. GRs coordinate OXPHOS enzyme biosynthesis (Simoes et al., 2012) and regulate mitochondrial gene transcription such as cytochrome oxidase 1 and 3, the activity of which correlates with levels of ATP (Adzic et al., 2013). In the hippocampus, chronic stress altered the phosphorylation of mitochondrial GRs, whereas in the prefrontal cortex, chronic stress significantly increased mitochondrial GR levels (Adzic et al., 2013). In the gut, stress increased serum CORT levels, which activated GR recruitment to instigate decreased ETC complex I activity, hyper-fission, and accumulation of ROS inducing apoptosis (De et al., 2017). This is the intrinsic pathway of apoptosis, which is affected by oxidative stress, elevated Ca2+ levels, and damaged DNA (Green and Kroemer, 1998; Kroemer et al., 2007).

Mitochondria are also involved in apoptosis through the extrinsic pathway, in which the death-inducing signaling complex is formed, leading to activation of caspase-8 and then downstream caspases that target substrates leading to programmed cell death (Vakifahmetoglu-Norberg et al., 2017). Other proteins such as K-Ras or BH3 interacting domain death agonist can also induce cell death when activated by caspases by translocating to mitochondria, where they trigger the release of the executioner caspases (Bivona et al., 2006; Bansal and Kuhad, 2016).

It is important to note that the effect of stress on mitochondrial function may depend on the nature of the stressor or period of chronicity. Although chronic stress and high levels of circulating stress hormones are a clear risk factor for depression (Gibbons and McHugh, 1962; Holsboer, 2001; Parker et al., 2003), low levels of stress hormones can be beneficial. For example, the effects of the stress hormone CORT on depression-like behavior in rodent models depend on the dose and time period of administration: higher doses and longer periods of administration produce robust increases in depression-like behavior but low doses or high doses given for short periods do not (Johnson et al., 2006; Lussier et al., 2013). This is consistent with the effects of stress hormones on mitochondria. Glucocorticoids can translocate to mitochondria, where they inhibit the release of cytochrome c and decrease apoptosis (Du et al., 2009). However, this is dependent on the level of glucocorticoids present in the tissue. Du et al. (2009) revealed that low doses of CORT were neuroprotective through regulation of mitochondria, but high doses were neurotoxic. Similarly, inhibiting mitochondrial protein synthesis completely impairs neuronal differentiation, but inhibiting ATP synthetase alone does not affect neurogenesis (Vayssiere et al., 1992). It would be of interest to map the dose-dependent effects of glucocorticoids on markers of mitochondrial function along with depression-like behavior to further confirm these relationships.

THE EFFECT OF ANTIDEPRESSANTS ON MITOCHONDRIA

There has been quite a bit of work done to try to understand the effect of antidepressant drugs on mitochondrial function. Most antidepressants work by increasing synaptic levels of serotonin and/or norepinephrine, and adverse side effects are commonly reported. Much of the research done to examine links between antidepressant drugs and mitochondrial function have used the SSRI fluoxetine, which may either inhibit or trigger mitochondrial apoptosis and alter activity of the ETC, depending on the cell type (de Oliveira, 2016). In the rat liver, fluoxetine administered *in vitro* inhibited state 3 of mitochondrial respiration for α-ketoglutarate and succinate oxidation, stimulated state 4 for succinate, and decreased the respiratory control ratio for both oxidizable substrates (Souza et al., 1994). The same effects were found in a later study on the rat brain, with fluoxetine decreasing the rate of ATP synthesis (Curti et al., 1999), and a study on the pig brain, showing that fluoxetine can inhibit mitochondrial function (Hroudová and Fišar, 2012). These results indicate that high doses of fluoxetine have negative effects on mitochondria.

Fluoxetine crosses mitochondrial membranes with ease, and it is possible that fluoxetine could interfere with membrane-bound proteins causing pro-apoptotic events (de Oliveira, 2016).

In vivo studies reveal a slightly more complex scenario, in that fluoxetine has both beneficial and detrimental effects on mitochondria when given systemically. After a single injection of fluoxetine (25 mg/kg), activity of the Krebs cycle enzyme citrate synthase was increased in the striatum, but not in the prefrontal cortex or hippocampus, and the striatal increase was no longer evident after 28 days of treatment (Agostinho et al., 2011a). Fluoxetine at the same dose also increased activity of ETC complex I in the hippocampus after one injection, but not in the prefrontal cortex or striatum (Agostinho et al., 2011b). However, after 28 days of daily injections, complex IV activity was decreased in the hippocampus. In another study, 21 days of low dose fluoxetine injections (5 mg/kg) increased expression of cytochrome oxidase 1 and cytochrome oxidase 3 mRNA in the prefrontal cortex in female rats, but not male rats, and decreased cytochrome oxidase 1 and cytochrome oxidase 3 mRNA in the hippocampus of male rats but not female rats (Adzic et al., 2013). These results suggest sex and region specific effects of systemic fluoxetine on mitochondrial function.

There has been some work done to examine the effect of other antidepressants. For example, chronic treatment with the tricyclic antidepressant imipramine as well as electroconvulsive shocks increased levels of cytochrome b mRNA in the rat cortex but not in the hippocampus, cerebellum or liver (Huang et al., 1997). Cytochrome b mRNA translates a protein that is involved in ETC complex III functioning. In addition, the SNRI venlafaxine actually had detrimental effects on complex IV of the ETC, although it increased expression of anti-apoptotic and antioxidant mitochondrial genes (Tamási et al., 2014). Finally, fluoxetine and desipramine enhanced cytochrome oxidase and glutamate dehydrogenase in presynaptic mitochondria located in the rat hippocampus (Villa et al., 2017). These data highlight the importance of antidepressants at a subcellular level and suggest that mitochondrial energy metabolism could be a mechanism of antidepressant drug action.

GENDER DIFFERENCES IN DEPRESSION AND MITOCHONDRIA

Women are more than twice as likely to suffer from depression than men, but it is not yet clear why this occurs and whether or not it has a biological basis. There is evidence that gender differences might arise due to decreased levels of circulating estrogens (Bloch et al., 2003), which is reinforced by observations that ovariectomy increases depression-like behavior in mice subjected to a chronic unpredictable stress paradigm (Lagunas et al., 2010). Furthermore, administering estradiol alleviates depression-like symptoms in ovariectomized rats (Rachman et al., 1998) and may accelerate antidepressant effects in humans (Rasgon et al., 2007). The evidence linking mitochondria to estradiol and depression is sparse, but emerging. Some studies have indicated a protective role of estradiol in mitochondria, showing that it can inhibit the passage of ROS into mitochondria as well as preventing mitochondrial collapse and increasing the rate of ATP synthesis (Wang et al., 2003; Shimamoto and Rappeneau, 2017). Mitochondria are known to express estrogen and GRs in lung tissue, suggesting that mitochondria are responsive to fluctuating levels of stress hormones and estradiol (Walf and Frye, 2006). It seems that mitochondrial estrogen and GRs in lung tissue are involved in the biosynthesis of OXPHOS enzymes, which will affect other mitochondrial functions such as apoptosis and ROS production (Simoes et al., 2012). It would be quite interesting to follow these studies with an investigation of brain mitochondria and estrogen receptors to determine whether sex steroid hormones in the brain might be involved in the gender differences seen in the prevalence of depression.

CONCLUSION

The specific biological mechanisms underlying major depression have yet to be elucidated. This review highlights the potential importance of mitochondrial function in depression. This is an area that has received relatively little experimental attention, but the data that have been published to date are promising and should be pursued. Although one must be cautious in extrapolating findings from preclinical animal models to the human condition, there is evidence that chronic stress-induced inhibition of ETC complexes in the inner membrane of mitochondria is a contributing factor in the pathophysiology of depression. Dysfunctional mitochondria decrease the pool of available ATP, which could have detrimental effects on signal transduction pathways, dampening activity in neuronal circuits, and interfering with mitochondrial fusion and fission. This negative cascade would ultimately increase oxidative stress, inflammatory responses, and pro-apoptotic events, some of which are known to be involved in the pathogenesis of depression. Viewed this way, it seems logical that reversing the early stages of mitochondrial dysfunction could provide a novel target for therapeutic intervention.

AUTHOR CONTRIBUTIONS

All authors contributed to the writing of this manuscript. JA wrote the first draft. RR-T, KB, HC, and LK edited the draft. JA constructed the figure. LK finalized the manuscript.

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Linking Glycation and Glycosylation With Inflammation and Mitochondrial Dysfunction in Parkinson's Disease

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Parkinson's disease (PD) is the second most common neurodegenerative disorder, affecting about 6.3 million people worldwide. PD is characterized by the progressive degeneration of dopaminergic neurons in the *Substantia nigra pars compacta*, resulting into severe motor symptoms. The cellular mechanisms underlying dopaminergic cell death in PD are still not fully understood, but mitochondrial dysfunction, oxidative stress and inflammation are strongly implicated in the pathogenesis of both familial and sporadic PD cases. Aberrant post-translational modifications, namely glycation and glycosylation, together with age-dependent insufficient endogenous scavengers and quality control systems, lead to cellular overload of dysfunctional proteins. Such injuries accumulate with time and may lead to mitochondrial dysfunction and exacerbated inflammatory responses, culminating in neuronal cell death. Here, we will discuss how PD-linked protein mutations, aging, impaired quality control mechanisms and sugar metabolism lead to up-regulated abnormal post-translational modifications in proteins. Abnormal glycation and glycosylation seem to be more common than previously thought in PD and may underlie mitochondria-induced oxidative stress and inflammation in a feed-forward mechanism. Moreover, the stress-induced post-translational modifications that directly affect parkin and/or its substrates, deeply impairing its ability to regulate mitochondrial dynamics or to suppress inflammation will also be discussed. Together, these represent still unexplored deleterious mechanisms implicated in neurodegeneration in PD, which may be used for a more in-depth knowledge of the pathogenic mechanisms, or as biomarkers of the disease.

Keywords: Parkinson's disease, mitochondrial dysfunction, inflammation, glycation, glycosylation, aging

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PARKINSON'S DISEASE

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease, clinically characterized by typical motor symptoms such as resting tremor, rigidity, bradykinesia, gait, and balance dysfunction that result in near total immobility and strongly impair patients' quality of life (Chaudhuri et al., 2006; Thomas and Beal, 2007; Jankovic, 2008). Currently, it is well accepted that several non-motor symptoms are also a key component of PD. These symptoms include hyposmia, constipation, hallucinations, depression, anxiety, sleep dysfunction, apathy, and dementia, and some of them may even arise in the pre-motor phase of the disease (Zis et al., 2015; Poewe et al., 2017).

Aging is the main risk factor for developing PD, a disease that affects about 1% of the population above 60 years (Tysnes and Storstein, 2017). There is yet no objective test or biomarker for PD, so the ultimate diagnosis of PD is *post-mortem*. The main neuropathological hallmarks are the progressive degeneration of pigmented dopaminergic neurons within the *Substantia nigra pars compacta* (SNpc) resulting into dramatic reduction of striatal dopamine, and α-synuclein-containing inclusions, called Lewy bodies, in the surviving neurons (Dauer and Przedborski, 2003; Tysnes and Storstein, 2017).

Epidemiological studies revealed that approximately 90% of PD cases have a sporadic origin, which may be caused by unknown environmental factors together with genetic susceptibility. The remaining 10% of PD cases represent familial forms of the disease (Thomas and Beal, 2007). Out of the six gene mutations responsible for monogenic PD, two are accountable for autosomal dominant (AD) PD forms (*SNCA* and *LRRK2*) and the remaining four for autosomal recessive (AR) PD (*PARK2*, *PINK1*, *DJ-1,* and *ATP13A2*) (Klein and Westenberger, 2012). The main mutations involved in familial forms of PD are summarized in **Table 1**.

Evidence for Mitochondrial Dysfunction in Parkinson's Disease

Mammalian mitochondria contain 2 to 10 molecules of mtDNA, a double-stranded circular genome of about 16.6 kb that encodes 22 transfer RNAs (tRNA), 2 ribosomal RNAs (rRNA), and 13 polypeptides (Schapira, 1994). However, it still depends on nuclear enzymes to replicate and translate its 13 polypeptides encoding genes, all of which generate a small proportion of subunits of the respiratory chain complexes. For example, complex I is composed of about 40 protein subunits but only seven of those are encoded by mtDNA (Schapira, 1994). The remaining subunits of this multimeric complex are nuclear encoded and must be imported to mitochondria, and properly assembled in the inner mitochondrial membrane. The mitochondrial respiratory chain constitutes the site of oxidative phosphorylation responsible for NADH and FADH2 oxidation, concomitantly with the translocation of protons from the matrix to the intermembrane space, establishing an electrochemical gradient commonly known as mitochondrial membrane potential ($\Delta\Psi m$). The electrochemical gradient of protons drives the action of ATP synthase, reducing molecular oxygen and synthesizing ATP. This step is fundamental in aerobic metabolism, and constitutes the main provider of ATP at the final stage of cellular respiration (Schapira, 1994). During oxidative phosphorylation electrons may leak from the electron transport chain and may react with oxygen generating reactive oxygen species (ROS), which under normal circumstances are removed by antioxidant agents in the mitochondria (Keane et al., 2011). The absence of a protective envelope favors mtDNA damage by ROS due to the proximity to the main source of production of these reactive molecules. Additionally, mtDNA is highly vulnerable to damages due to inefficient DNA repair mechanisms and to an absence of a protective histone coating (Schapira et al., 1990; Schapira, 1994). This vulnerability increases with aging when the endogenous antioxidant defense mechanisms tend to be down-regulated. Such oxidative injury to mitochondria and other cellular structures in the brain accumulates with time leading to several deleterious effects associated with age-related neurodegenerative disorders like PD (Perry et al., 2009; Schapira, 2012). This highly reactive, reduced species of oxygen are responsible for the oxidative damage of lipids, proteins and nucleic acids including the mitochondrial components themselves predisposing to apoptotic cell death.

Mitochondria are highly dynamic organelles, that continually fuse and divide, and the balance between fusion and fission determines the size, shape, number and function of these organelles (Bose and Beal, 2016). Damaged and/or obsolete mitochondria are selectively removed by a quality control mechanism known as mitochondrial autophagy or mitophagy. Coordination between clearance of damaged mitochondria and mitochondrial biogenesis is essential in the maintenance of a healthy mitochondrial pool and cellular homeostasis. Thus, mitophagy is essential in mitochondrial turnover regulation, by adjusting the amount of organelles accordingly to metabolic requirements of cells. It is generally accepted that mitochondrial biogenesis depends upon fusion events, while fission is assumed to isolate damaged organelles that can then be targeted for degradation.

Importantly, the fine-tuned regulation of mitophagy seems to be impaired in PD, which is not an odd observation, since this mechanism is regulated by parkin and by the serine/threonine protein phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), and mutations on these proteins are known to be related with rare familial forms of PD.

Parkin, PINK1, and α-Synuclein in Parkinson's Disease

Half of familial PD cases are associated with a wide variety of loss-of-function mutations in *PARK2* gene, encoding for

Abbreviations: AD, Alzheimer's disease; ADTIQ, 1-acetyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; APC, antigen presenting cell; ATF6, activating transcription factor-6; AGE, advanced glycation end-product; BBB, blood–brain barrier; CHOP, C/EBP-homologous protein; CNS, central nervous system; COX-2, cyclooxygenase-2; CREB, cAMP response element-binding factor; CSF, cerebrospinal fluid; DAT, dopamine transporter; ER, endoplasmic reticulum; ERAD, ER-associated degradation; GAG, glycosaminoglycan; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GPI, glycophosphatidylinositol; GRP78, glucose-regulated protein-78; GSLs, glycosphingolipids; HMGB1, high mobility group box 1; IL-1β, interleukin-1 beta; IFN-γ, interferon-gamma; IgG, immunoglobulin G; iNOS, inducible nitric oxide synthase; IRE1, inositol requiring enzyme-1; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MGO, methylglyoxal; MHC, major histocompatibility complex; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mtDNA, mitochondrial DNA; NKT, natural killer T; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor-kappa B; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; 6-OHDA, 6-hydroxydopamine; PARIS, parkin interacting substrate; PERK, protein kinase RNA-like ER kinase; PD, Parkinson's disease; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1α; PINK 1, protein phosphatase and tensin homolog (PTEN)-induced putative kinase 1; RAGEs, receptor for advanced glycation end products; ROS, reactive oxygen species; Siglec, sialic-acid-binding immunoglobulin superfamily; SNpc, *Substantia nigra pars compacta*; TNF-α, tumor necrosis factor alpha; TREM2, triggering receptor expressed on myeloid cells 2; UPR, unfolded protein response; UPS, ubiquitin–proteasome system; VMAT, vesicular monoamine transporter.

TABLE 1 | Genes and loci associated with autosomal recessive (AR) or autosomal dominant (AD) Parkinsonism.

PARK locus	Gene	Full gene name	Inheritance	Disease onset
PARK1/4	*SNCA*	Alpha-synuclein	AD	Early-onset parkinsonism with common dementia
PARK2	*PARKIN*	Parkin	AR	Early-onset parkinsonism, slowly progressive
PARK3	Unknown	Unknown	AD	Late onset parkinsonism
PARK5	*UCHL1*	Ubiquitin C-terminal hydrolase	AD	Early- and late-onset parkinsonism
PARK6	*PINK1*	PTEN induced putative kinase 1	AR	Early-onset, slowly progressive parkinsonism
PARK7	*DJ-1*	DJ-1 (parkinsonism associated deglycase)	AR	Early-onset parkinsonism
PARK8	*LRRK2*	Leucine rich repeat kinase 2	AD	Late-onset parkinsonism
PARK9	*ATP13A2*	ATPase 13A2	AR	Early-onset parkinsonism with Kufor-Rakeb syndrome
PARK10	Unknown	Unknown	Unclear	Late-onset parkinsonism
PARK11	*GIGYF2*	GRB10 interacting GYF protein 2	AD	Late-onset parkinsonism
PARK12	Unknown	Unknown	X-linked inheritance	Late-onset parkinsonism
PARK13	*HTRA2*	HtrA serine peptidase 2	AD	Unclear
PARK14	*PLA2G6*	Phospholipase A2 group VI	AR	Early-onset dystonia parkinsonism
PARK15	*FBX07*	F-box protein 7	AR	Early-onset parkinsonism with pyramidal syndrome
PARK16	Unknown	Unknown	Unclear	Late-onset parkinsonism
PARK17	*VPS35*	VPS35, retromer complex component	AD	Late-onset parkinsonism
PARK18	*EIF4G1*	Eukaryotic translation initiation factor 4 gamma 1	AD	Late-onset parkinsonism
PARK19	*DNAJC6*	DNAJ heat shock protein family (Hsp40) member C6	AR	Early-onset parkinsonism
PARK20	*SYNJ1*	Synaptojanin 1	AR	Early-onset parkinsonism
PARK21	*TMEM230*	Transmembrane protein 230	AD	Unclear
PARK22	*CHCHD2*	Coiled-coil-helix-coiled-coil-helix domain containing 2	AD	Unclear
Gaucher's locus	*GBA*	Glucocerebrosidase	AD	Late-onset parkinsonism

parkin, a constituent of a E3 ubiquitin ligase complex. These mutations also account for about 20% of the cases with early onset, indicating loss-of-function pathogenic mechanisms in PD (Thomas and Beal, 2007; Martin et al., 2011; Klein and Westenberger, 2012). Parkin is an interesting protein involved in the modulation of different aspects of mitochondrial turnover. In fact, parkin, together with PINK1, regulates mitophagy and maintains mitochondrial homeostasis (Martin et al., 2011; Song et al., 2013). Importantly, parkin also regulates mitochondrial biogenesis by modulating the levels of parkin interacting substrate (PARIS), a transcriptional repressor of PGC-1α (Shin et al., 2011). peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) is an important co-activator of mitochondrial transcription factors and thus a master regulator of mitochondria biogenesis (Shin et al., 2011). Accordingly, deletion of PGC-1α gene renders dopaminergic neurons more vulnerable, whereas its over-expression is neuroprotective in experimental models of PD (Zheng et al., 2010; Jiang et al., 2016). Mutations in *PINK1* gene also cause autosomal recessive PD with early onset, being the first gene identified that suggested that impaired mitochondrial function was involved in PD pathogenesis (Silvestri et al., 2005). The majority of the mutations identified in *PINK1* are non-sense or missense mutations that affect the serine/threonine kinase domain, suggesting that loss of kinase function may be an important part of PINK1-induced pathogenesis in PD (Klein and Westenberger, 2012; Song et al., 2013). Parkin and/or PINK1 loss of function may lead to the accumulation of injured mitochondria, which will contribute to increased production of oxidative stress that may possibly underlie PD pathogenesis.

On the other hand, only a small number of mutations have been reported for *SNCA* gene, including missense mutations (e.g., A53T, A30P, A53E, and E46K mutations), duplications and triplications, all of them implicated in familial PD (Nuytemans et al., 2010). The *SNCA* gene encodes the α-synuclein protein, whose normal function of is still not fully understood, although evidence indicates that it is a pre-synaptic protein involved in neurotransmitter release (Burre et al., 2010; Nemani et al., 2010). However, over-expression of this protein and its gain-of-function pathological mutations promote the formation of oligomeric species and fibrils that are considered the main toxic species triggering deleterious mechanisms in PD (Conway et al., 2000; Outeiro et al., 2009). In fact, aggregated α-synuclein is the primary fibrillary component of Lewy bodies (Lee and Trojanowski, 2006) and oxidative and nitrosative stresses promote its aggregation, which in turn can damage mitochondria, contributing to further oxidative stress and neuron degeneration.

Oxidative Stress in Parkinson's Disease

The etiology of PD still remains unknown, although several mechanisms leading to the neurodegenerative process, associated with dopaminergic neuron loss have been proposed. These include mitochondrial complex I dysfunction, impairment of ATP production, oxidative stress, neuroinflammation, endoplasmic reticulum (ER) stress and aberrant proteolytic degradation. Among these, mitochondrial dysfunction seems to play a key role in PD, since it may lead to over-production of ROS, inflammatory responses, and activation of cell death pathways (Mizuno et al., 1989; Przedborski et al., 2004).

Indeed, mitochondrial impairment caused by mutations in genes linked to familial PD, together with data from human *post-mortem* tissue indicate that impaired mitochondrial function, increased oxidative stress and deficient anti-oxidant capacity are common pathological mechanisms implicated in the etiology of both familial and sporadic PD cases (Dauer and Przedborski, 2003; Perry et al., 2009). Accordingly, *post-mortem* observations of PD patients' brains revealed a decreased activity of mitochondrial complex I in the SNpc (Mizuno et al., 1989; Schapira et al., 1990; Moore et al., 2005). Moreover, additional studies also revealed mitochondrial complex I deficits in platelets and skeletal muscle of PD patients (Yoshino et al., 1992; Dexter and Jenner, 2013). Mitochondrial complex I inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and its toxic metabolite (1-methyl-4-phenylpyridinium, MPP^+) induce a cascade of events, leading to neuropathological features of the disease in humans and animals (Chun et al., 2001; Nicotra and Parvez, 2002; Dauer and Przedborski, 2003; Castro-Caldas et al., 2012), further reinforcing the involvement of mitochondrial dysfunction in PD pathogenesis.

Studies using PD brain mitochondria indicate that oxidation of complex I catalytic subunits is the mechanism responsible for its reduced function and disassembly (Keeney et al., 2006). Interestingly, oxidative and nitrosative stress can also inactivate parkin by inducing post-translational protein modifications and misfolding with the subsequent accumulation of PARIS, and may also accelerate α-synuclein aggregation, which together may account for mitochondrial dysfunction (West and Maidment, 2004; Dawson and Dawson, 2010; Chakraborty et al., 2017). Additionally, several evidences support the idea that ATP depletion and ROS overproduction occurs soon after injection of the mitochondrial complex I inhibitor MPTP in mice (Dauer and Przedborski, 2003; Castro-Caldas et al., 2012; Moreira et al., 2017). Although complex I inhibition elicited by MPP^+ reduces ATP production and increases ROS production within dopaminergic neurons, mounting evidence indicates that rather than killing the cells, these changes are triggering the activation of cell death molecular pathways, which underlie the demise of damaged neurons (Cleeter et al., 1992; Przedborski et al., 2004). These damages are thought to be irreversible and can induce neurodegeneration of the nigrostriatal dopaminergic system. In parallel, it has also been described an impaired antioxidant ability of SN neurons in PD in part due to lower levels of reduced glutathione (Dexter and Jenner, 2013).

Protein Quality Control Mechanisms in Parkinson's Disease

Protein quality control mechanisms is the way by which cell monitors proteins to ensure that they are appropriately folded, and prevents overload of aberrant proteins, and formation of toxic aggregates, allowing cells to cope with environmental stresses. The ubiquitin-proteasome system (UPS) is the major non-lysosomal protein degradation pathway within cells (Ciechanover and Kwon, 2017). The UPS is critical for degradation of misfolded intracellular proteins, through the regulation of protein turnover and cellular response to stress (Ciechanover and Kwon, 2017). Impairment of UPS has already been reported in patients with familial PD, namely with mutations in genes linked to quality control mechanisms, such as parkin and ubiquitin C-terminal hydrolase-L1 (Kitada et al., 1998; Leroy et al., 1998; Dawson and Dawson, 2003; Martin et al., 2011). Sporadic PD patients also display impaired proteasomal activity in the SNpc (McNaught and Jenner, 2001). Impairment of UPS in PD triggers a cycle of cell-damaging events including accumulation of misfolded proteins, aggregation of α-synuclein and mitochondrial dysfunction.

Another important quality control pathway is autophagy. In light of results published in the literature, there is still controversy about the role of autophagy in PD. On one hand, it has been shown that inhibition of the autophagic flux by MPTP underlies the effect of this toxin on neuronal survival (Liu et al., 2013). Indeed, an efficient activation of autophagy/mitophagy as a response to mitochondrial damage or neuronal injury has a clear pro-survival role, and this process has been reported as being deregulated in some PD models (Zhu et al., 2007; Chu, 2011; Menzies et al., 2015; Rosa et al., 2017). On the other hand, the active metabolite of MPTP, MPP^+, in culture cells may be an inducer of autophagy as a cell death pathway (Zhu et al., 2007; Wong et al., 2011). Nonetheless, fine tune regulation of autophagy seems to be impaired in PD, contributing to the accumulation of toxic species and misfolded proteins. The disproportional increase in the concentration of misfolded proteins and toxic species has critical consequences on the normal functioning of cells, in particular of organelles such as mitochondria and ER.

Although several ER quality control mechanisms exist, chronic cellular stress may disrupt the mechanisms of protein folding with concomitant increase of misfolded proteins. Accumulation of these abnormal proteins in the lumen of the ER activates the adaptive signaling pathway designated by unfolded protein response (UPR). This mechanism is controlled by the ER resident chaperone glucose-regulated protein-78 (GRP78) that regulates the activity of 3 important sensors of stress: protein kinase RNA-like ER kinase (PERK), inositol requiring enzyme-1 (IRE1), and activating transcription factor-6 (ATF6) (Mercado et al., 2013; Tsujii et al., 2015). UPR works primarily to restore homeostasis and lessen ER stress by arresting protein translation, increasing folding ability, enlargement of ER membrane and initiation of ER-associated degradation (ERAD) pathway (Mercado et al., 2013; Tsujii et al., 2015). Interestingly, ER stress has been gaining increasing importance

in PD research (Mercado et al., 2013; Tsujii et al., 2015). In fact, it was demonstrated activation of the PERK pathway in SN of *post-mortem* PD brains (Hoozemans et al., 2007). Accordingly, a downstream partner of the PERK pathway, the C/EBP-homologous protein (CHOP), is activated upon 6-hydroxydopamine (6-OHDA) administration (Holtz and O'Malley, 2003), while ablation of CHOP is protective against 6-OHDA toxicity (Silva et al., 2005). Besides PERK, the IRE1 and ATF6 branches of the UPR have also been implicated in experimental models of PD (Mercado et al., 2013). Accordingly, *in vivo* and *in vitro* studies demonstrated that toxins that induce PD also induce ER stress, and *vice versa*. Interestingly, recently it was shown that stereotaxic injection at the level of SN of the potent ER stressor tunicamycin, an inhibitor of the first steps of the *N*-glycosylation pathway, can be used as a stress rodent model for PD (Cóppola-Segovia et al., 2017). These data collectively suggest that in PD the UPR is up-regulated, and this may account for the accumulation of misfolded proteins and aggregation of α-synuclein and subsequently to neuronal loss, possibly linking aberrant glycosylation with neuropathological events.

Immune System and Parkinson's Disease

Besides mitochondrial impairment and oxidative stress, activation of glial cells and neuroinflammation also play an important role in the progression of the neurodegenerative process in PD (Hirsch et al., 2003; Hald and Lotharius, 2005). Glia play a key role in brain homeostasis, continuously monitoring the neuronal microenvironment, contributing to the endogenous antioxidant defense mechanisms, as well as in supplying trophic factors to neurons (McGeer and McGeer, 2008).

Microglia are brain-resident myeloid cell population, normally present in the central nervous system (CNS) in a surveillant non-polarized state but rapidly become activated in the presence of neuronal injuries (Hald and Lotharius, 2005; McGeer and McGeer, 2008). Activation of microglia generally comprises morphological alterations, expression of cell surface markers release of pro-inflammatory cytokines, up-regulation of pro-inflammatory enzymes, particularly cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), leading to the synthesis of prostaglandins and nitric oxide (NO), and phagocytosis of degenerating neurons and debris (Vijitruth et al., 2006; Pattarini et al., 2007). Thus, once activated by injured neurons microglia can itself become a donor source of toxic factors causing injuries to nearby neurons, in a process designated by neuroinflammation. These neuronal injuries, in turn, will induce additional microglial activation and may be an important process that drives and exacerbates the progressive neurodegeneration after the initial stimulus.

Hence, chronic inflammation seems to play a substantial role in the neurodegenerative pathway underlying PD as well as in different PD models (Hebert et al., 2003; Hirsch et al., 2003; Vijitruth et al., 2006; Pattarini et al., 2007). Microglia are thought to present a cell protective anti-inflammatory phenotype in early stages of the disease, however, due to the chronic nature of PD they tend to switch to an activated pro-inflammatory subtype at latter stages of disease progression (Tang and Le, 2016). In these circumstances where the inflammatory process is sustained and when the levels of inflammatory challenge exceed a threshold, the death of neurons and the neuroinflammation potentiate each other in a vicious manner (Gao et al., 2011; Hirsch et al., 2013).

The involvement of the immune system in the pathogenesis of PD, has been reinforced by observation of reactive microglia, as well as pro-inflammatory soluble mediators and enzymes in PD patients' SNpc (Mogi et al., 1994; Mosley et al., 2012; Kannarkat et al., 2013). We and others have also showed that a sustained inflammatory reaction by activated microglia is also recapitulated in genetic and toxin-based experimental models of the disease (Hebert et al., 2003; Vijitruth et al., 2006; Pattarini et al., 2007; Rosa et al., 2018). Accordingly, increased expression of anti-inflammatory mediators, including interleukin 1 beta (IL-1β) and COX-2, and decreased expression of inflammation resolution proteins such as annexin-A1, were found in MPTP treated mice brain (Hebert et al., 2003; Vijitruth et al., 2006; Pattarini et al., 2007; Rosa et al., 2018). The beneficial effects of non-steroid anti-inflammatory medication, such as ibuprofen and aspirin, in PD further reinforce the contribution of neuroinflammation in dopaminergic neuron cell death and disease progression (Wang Y.D. et al., 2016; Tentillier et al., 2016; Naeem et al., 2017).

However, controversy still exists regarding the onset of neuroinflammation in PD. On one hand, some authors suggest that glial cells are effectively involved in the early stages of PD and their activation status is maintained throughout the course of disease progression (Halliday and Stevens, 2011). On the other hand, others claim that it is unlikely that glia activation initiates neuron loss in PD, but quite possibly exacerbates and sustains the neurodegenerative process. In turn, animal models indicate microglia activation in early stages, while specific their specific inhibition led to a significant protection against MPTP toxicity (Wu et al., 2002; Halliday and Stevens, 2011). Nonetheless, even if these are early events in the degenerative process, a sustained glial activation occurs in parallel with dopaminergic cell loss and is still evident in long-term treatment with MPTP and in the SNpc of human PD (Hebert et al., 2003; Hirsch et al., 2003; Vijitruth et al., 2006; Pattarini et al., 2007).

Interestingly, recent studies have shown that dopaminergic neurons display major histocompatibility complex (MHC) class I molecules and these are involved in presentation of intracellular digested proteins on the neuronal membrane that are recognized by $CD8^+$ cytotoxic T lymphocytes (Cebrián et al., 2014). Remarkably, MHC class I molecules expressed at dopaminergic neurons surface are up-regulated in presence of activated microglia or other pro-inflammatory markers, such as interferon-gamma (IFN-γ) related to unusually high levels of oxidative stress (Cebrián et al., 2014). Up-regulation of MHC class I may initiate T cell mediated responses leading to neurotoxicity. Recently, it was suggested that parkin and PINK1 could mediate mitochondrial antigen presentation, repressing this pathway in dysfunctional organelles (Matheoud et al., 2016). In PD decreased parkin activity leads to increased accumulation of dysfunctional mitochondria, as well as increased antigen presentation at the surface of these organelles, triggering the recruitment and activation of microglia and peripheral infiltrating inflammatory cells. Mitochondria antigen presentation pathway dependent

on PINK1 and parkin, provides an elegant way to connect mitochondrial dynamics and immunologic pathways in PD.

On the other hand, MHC II molecules at the surface of microglia and T lymphocytes were found to be deeply implicated in the inflammatory process in PD. Accordingly, abundant MHC class II positive microglia and $CD4^+$, $CD8^+$ T cells were found in the SN of PD patients, and microglia activity was confirmed to be related to the degeneration severity as well as PD progression (Cebrián et al., 2014; Martin et al., 2016). The contribution of immune mechanisms in PD is also corroborated by the observation that ablation of MHC class II renders mice more resistant to MPTP toxicity (Martin et al., 2016).

The infiltration of T cells and direct access to dopaminergic neurons is facilitated by a lymphatic system in the CNS that may be responsible for the transport of periphery immune cells into the brain (Louveau et al., 2015). Additionally, blood brain barrier (BBB) dysfunctions, and increased permeability have already been shown in PD patients, allowing circulating inflammatory T cells to access the brain (Kortekaas et al., 2005; Mosley et al., 2012; Sweeney et al., 2018). Concomitantly with the neurodegenerative process, reactive microglia secrete pro-inflammatory mediators, such as tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6, or present antigens to $CD4^+$ T cells by the MHC-II pathway (More et al., 2013; Cebrián et al., 2014). Interestingly, abnormal populations of T cells were not only found in CNS but also in peripheral blood from PD patients (Chen et al., 2016). Together these immune reactions triggered by different cell types result in increased secretion of pro-inflammatory cytokines in cerebrospinal fluid (CSF) or blood (More et al., 2013; Santiago and Potashkin, 2014). Thus, CNS infiltration of immune cells associate the peripheral immune system with the progression of PD (Mosley et al., 2012; Kannarkat et al., 2013). Additionally, PD patients showed increased levels of oxidative stress, decreased healthy mitochondrial population and accumulation of damaged mitochondria in circulating neutrophils (Vitte et al., 2004), favoring the hypothesis of systemic mitochondrial defects in PD, and indicating a possible link between mitochondrial dysfunction and inflammation. The observation of periphery immune cells in CNS of PD patients goes beyond the local involvement of microglia and neuroinflammation, but also associates the whole immune system in the progression of this disease.

Together these observations indicate the importance of central and peripheral immunity reactions in PD, keeping the current question whether inflammation is involved in triggering the disease, or occurs instead as a consequence. Recently, autoimmune diseases or peripheral infections are considered by some authors as a risk factor for the development of PD (Witoelar et al., 2017), in a current view of the disease as being a multisystem pathology and not only confined to the CNS.

GLYCATION IN PARKINSON'S DISEASE

AGE and RAGE in Parkinson's Disease

The fact that the vast majority of PD cases are sporadic suggests that several environmental factors trigger the onset and progression of this disease. In fact, epidemiological studies indicate that PD incidence is related with exposure to pesticides, herbicides, and heavy metals. Interestingly, he risk for developing PD can also be associated with the quality of diet and metabolism. On one hand, a diet rich in fresh fruit, vegetables and fish is associated with low risk to develop PD (Gao et al., 2007). On the other hand, although some controversy still exists, it is thought that diabetes and altered glucose metabolism are strongly associated with PD, and major risk factors for the disease (Becker et al., 2008; Driver et al., 2008; Lu et al., 2014). A major consequence of diabetes is glucose metabolism imbalance and subsequent hyperglycemia, that lead to biochemical abnormalities that may trigger and/or aggravate PD progression (Vicente Miranda et al., 2016). In fact, it was demonstrated that patients that develop diabetes before PD onset tend to present more severe motor and non-motor symptoms and faster disease progression (Schwab, 1960; Arvanitakis et al., 2007; Cereda et al., 2012). Accordingly, abnormal glucose tolerance and hyperglycemia have been detected in the majority of PD cases (Lipman et al., 1974; Sandyk, 1993). Glucose and its by-products have the ability to react with amino groups, following the Maillard reaction ultimately giving rise to advanced glycation end-products (AGEs) that severely impact the function of target proteins (Yamagishi et al., 2015) (**Figure 1**). This reaction is designated by glycation, that is the glycosylation of proteins, lipids, and nucleic acids, that occurs spontaneously without being catalyzed by any enzyme. Due to its deleterious effects on biochemical targets, glycation is an important player in cellular aging (Ulrich and Cerami, 2001; Li et al., 2012; Salahuddin et al., 2014). Low amounts of AGEs are constitutively formed under normal conditions but their production is significantly augmented under conditions of hyperglycemia and oxidative stress. Accordingly, a glucose rich diet can increase up to 34-fold the generation of AGEs in the brain, particularly in the SNpc (Uchiki et al., 2012). In fact, it is now well accepted that the glycolytic pathway is not harmless reaction sequence, and its partial inhibition has been linked to increased life span in many animal experiments (Fontana et al., 2010). Methylglyoxal (MGO) is a by-product of glycolysis but can also be formed by the catabolism of serine and threonine or by lipid peroxidation. Interestingly, PD patients CSF seems to have a specific metabolomic signature that reflects alterations in glycation or glycosylation, oxidative stress and innate immunity and that may be explored as new early-stage disease biomarkers (Trezzi et al., 2017). Specifically, elevated fructose levels were found in the CSF of PD patients, which like other reducing sugars, can react with proteins through the Maillard reaction (glycation). Thus, fructose may also indicate an early event of pathological accumulation of AGEs in PD (Trezzi et al., 2017). However, both these monosaccharides may also act as ROS scavengers shifting oxidative phosphorylation to glycolysis, as a protective mechanism activated in early stages of the disease.

Advanced glycation end-products generation is a time-dependent irreversible process that leads to the accumulation and/or aggregation of proteins due to cross-linking between AGE-modified peptides. Interestingly, AGE-modified cell proteins and abnormal glycated mitochondrial proteins are implicated in mitochondria-induced oxidative stress and

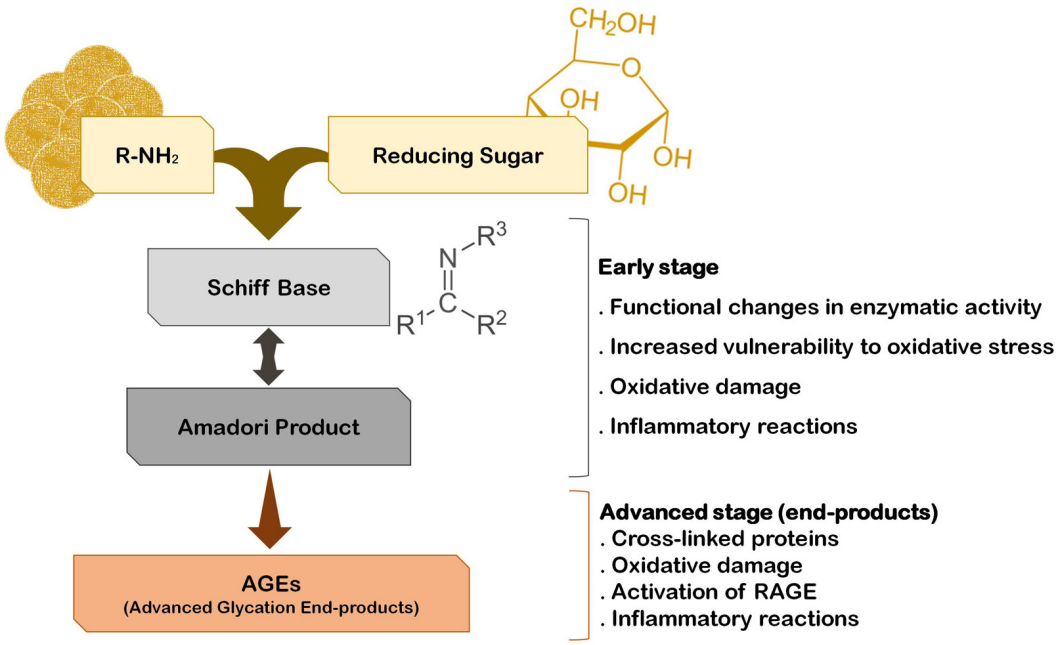


FIGURE 1 | The pathway of protein glycation and AGE formation. Endogenous glycating agents include glucose, fructose, galactose, mannose, ribose, and reactive triose intermediates of energy metabolism, glyoxal and MGO. The formation of AGEs occurs spontaneously by non-enzymatic reactions in a process designated by Maillard reaction. The early stage of this reaction usually occurs rapidly (hours to days), while the advanced stage of AGE formation is thought to take weeks to months to occur.

inflammation (Rosca et al., 2005; Rabbani and Thornalley, 2008), whereas oxidative stress seems to exacerbate the formation of AGEs (Baynes and Thorpe, 2000), eliciting a positive loop of oxidative damage in the brain. Together these AGE-dependent mechanisms may underlie, at least in part, the neurodegenerative process in PD (**Figure 1**). This is sustained by several studies indicating the presence of AGEs in the periphery of Lewy bodies, SNpc, cerebral cortex and amygdala of PD patients' brains, suggesting that protein glycation is part of PD pathogenic mechanisms (Dalfó et al., 2005; Kurz et al., 2011). Importantly, it has even been suggested that AGEs might trigger Lewy body formation prior to the onset of PD, since they were found in Lewy bodies in incidental Lewy body disease patients' brains (Münch et al., 2000; Dalfó et al., 2005). Glycation was also reported in several models of parkinsonism, namely the MPTP model of PD (Teismann et al., 2012; Viana et al., 2016; Vicente Miranda et al., 2017). Interestingly, in toxin-based animal models of PD the depletion of dopaminergic neurons in the SNpc is aggravated in animals that were concomitantly submitted to a high fat diet regimen (Bousquet et al., 2012; Ma et al., 2015), suggesting that this diet may increase the susceptibility of animal to PD-inducing drugs. Moreover, mice expressing A30P mutant α-synuclein fed with a high-fat diet showed earlier onset of the motor symptoms and α-synuclein aggregation (Rotermund et al., 2014), further corroborating the contribution of AGEs for PD development and/or progression.

Besides their direct effects on proteins, AGEs may trigger a cell response through binding to the receptor for advanced glycation end products (RAGEs). RAGE is a multi-ligand receptor of the immunoglobulin superfamily of cell surface molecules with a crucial role in the CNS in neuroinflammation, oxidative stress and neurotoxicity (Ding and Keller, 2005). In the CNS RAGE is found in neurons, microglia, astrocytes, and brain endothelial cells (Ding and Keller, 2005; Deane et al., 2012; Teismann et al., 2012; Gasparotto et al., 2017). Typically, after ligand binding RAGE induces the activation of the transcription factor nuclear factor-kappa B (NF-κB) (Bierhaus et al., 2001; Angelo et al., 2014; Tóbon-Velasco et al., 2014) (**Figure 2**). RAGE is expressed as both full-length membrane localized (mRAGE), as well as a soluble (sRAGE) isoform that lacks the transmembrane domain. Whereas mRAGE is the signaling isoform, sRAGE circulates in blood and body fluids. However, sRAGE maintains the ability to bind circulating ligands, but since it lacks the intracellular signaling domain, it does not trigger any cellular reactions (Ding and Keller, 2005; Alexiou et al., 2010; Yan et al., 2010; Kalea et al., 2011). Therefore, competing for the ligands with mRAGE acting as a decoy for ligands, sRAGE has cytoprotective effects (Yan et al., 2010; Aldini et al., 2013) (**Figure 2**). By controlling the levels of circulating AGEs, sRAGE regulates RAGE intracellular signaling, thereby avoiding over-expression of inflammatory mediators (Ding and Keller, 2005). Therefore, in PD the scenario may involve accumulation of RAGE ligands due to environmental cell conditions that favor up-regulated biosynthesis and/or impaired endogenous clearance systems.

Additionally, RAGE expression may be up-regulated by its own ligands, since the human RAGE gene has two NF-κB responsive elements on its promoter. Thus, ligand-induced RAGE activation triggers its own up-regulation and perpetuates

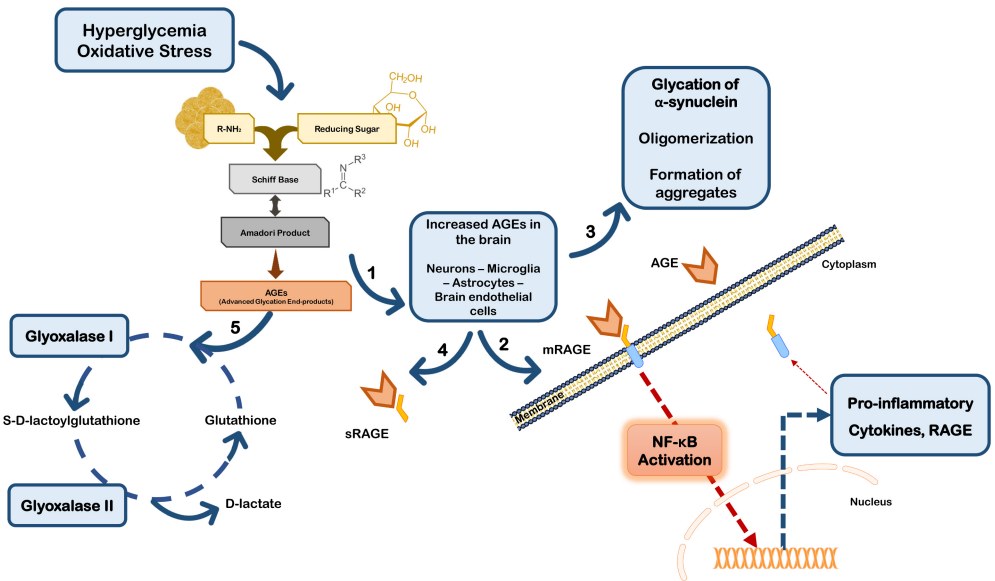


FIGURE 2 | Glycation in Parkinson's disease. Hyperglycemia and oxidative stress may up-regulate the Maillard reaction with increased formation of AGEs in brain cells (1). AGEs can either bind to membrane RAGE (mRAGE) at the cell surface of microglia and/or neurons, triggering NF-κB activation and subsequent expression of pro-inflammatory mediators and RAGE (2). Glycation can also directly affect the function of proteins, namely α-synuclein favoring its accumulation and aggregation (3). On the other hand, circulating AGEs may bind to soluble RAGE receptors (sRAGE) that do not control any intracellular pathway, acting as a neuroprotective strategy (4). Additionally, cells are endowed with efficient endogenous systems for detoxication against AGEs, namely glyoxalase-I and -II that constitute the main systems responsible for the metabolization of AGEs in a pathway dependent on glutathione (5).

inflammation and neurodegeneration (Ding and Keller, 2005; Tóbon-Velasco et al., 2014). In fact, RAGE activation may induce increased secretion of TNF-α which in turn, can to up-regulate cellular RAGE expression via NF-κB (Tanaka et al., 2000; Sriram et al., 2004). Interestingly, strong evidence indicates that TNF-α is involved in early and late stages in the immune pathophysiology of PD (McCoy et al., 2011), further suggesting that RAGE-inducing inflammation through NF-κB activation is implicated in PD progression. Conflicting findings on RAGE biology subsist in PD studies, however, it has been described that PD patients' brains have increased expression of RAGE paralleled with AGEs accumulation, and that RAGE activation was linked to oxidative stress (Dalfó et al., 2005; Ding and Keller, 2005; Sathe et al., 2012). Additionally, RAGE up-regulation and activation was also demonstrated in sub-acute MPTP, rotenone and 6-OHDA mice models of PD (Teismann et al., 2012; Abdelsalam and Safar, 2015; Gasparotto et al., 2017). On the other hand, ablation of RAGE protects primary dopaminergic neurons against MPP^+ induced toxicity (Teismann et al., 2012), and selective inhibition of RAGE prevented dopaminergic denervation and locomotory and exploratory deficits induced by 6-OHDA in rats (Gasparotto et al., 2017). Importantly, RAGE polymorphisms were associated increased susceptibility or protection against PD in Chinese Han population (Gao et al., 2014). Together these data strongly implicate RAGE expression and activation in PD, and are corroborated by the findings in several experimental models of the disease, further reinforcing the involvement of this receptor in PD pathologic mechanisms.

The intracellular signaling pathways induced upon RAGE activation depend on the ligands that bind to it and the on the cell type where it is expressed. Besides NF-κB, RAGE induces the activation of multiple intracellular pathways including mitogen-activated protein kinases (MAPKs) (Xu et al., 2010; Kim et al., 2011), nuclear factor of activated T-cells (NF-AT) (Cho et al., 2009), and the cAMP response element-binding factor (CREB) (Huttunen et al., 2002), though activation of NF-κB seems to be the main pathway in neurodegenerative conditions. This diversity suggests that there are multiple modes of RAGE activation by different ligands. Indeed, in the brain pathogenic mechanism have been attributed to different RAGE ligands, besides AGEs, including S100 proteins, amyloid peptide, lipopolysaccharide (LPS) and high mobility group box 1 (HMGB1) (Gasparotto et al., 2017).

Receptor for advanced glycation end product has indeed affinity for a large number of proteins, and new putative RAGE ligands are reported continuously. Among those, S100/calgranulin family of proinflammatory cytokine like mediators is a major inducer of RAGE activation (Hofmann et al., 1999; Donato, 2001), already reported in neurodegenerative diseases. Interestingly, S100B protein levels are increased in SNpc and CSF from PD patients (Sathe et al., 2012), and in striata from mice submitted to MPTP treatment (Viana et al., 2016). Accordingly, lack of S100B resulted in decreased expression of both RAGE and TNF-α with consequent reduced microglia activation and neuroprotection (Sathe et al., 2012), further implicating S100B/RAGE axis in the neurodegenerative process in PD. Nonetheless, S100B protein levels were unaltered in PD

patients' serum (Schaf et al., 2005), indicating that this may not be useful as a biomarker for the disease.

Another important ligand for RAGE is HMGB1, a protein with different roles during neural development and neurodegeneration. While during early brain development, HMGB1 is involved in neurite outgrowth and cell migration, during adulthood HMGB1 mediates neuroinflammation after injury (Fang et al., 2012). In PD, and other neurodegenerative diseases, HMGB1 expression is considered as an important risk factor for chronic neurodegeneration, due to its role as a pro-inflammatory mediator involved in the progression of neuroinflammation in the brain (Kim et al., 2011; Takata et al., 2012; Santoro et al., 2016). Accordingly, HMGB1 was found to be up-regulated in the cytosol of dopaminergic neurons from PD patients tissue, but not in healthy age-matched individuals (Santoro et al., 2016). This is reinforced by the detection of HMGB1 in the neuronal cytoplasm from MPTP-treated mice, together with observations that the deleterious effects induced by MPTP were partially prevented by blocking HMGB1 (Santoro et al., 2016; Sasaki et al., 2016). Due to its ability to activate RAGE, once again neutralization of HMGB1 protected dopaminergic neurons from MPTP-induced cell death by decreasing the levels of self-induced RAGE and TNF-α release (Sasaki et al., 2016). Together, these observations indicate that the downstream signaling cascades of RAGE, namely the activation of NF-κB, and the self-perpetuating expression of RAGE, contribute to the damaging effect of HMGB1 in PD.

Glycation of α-Synuclein

As already mentioned, the major hallmark of PD is the development of Lewy bodies composed of aggregated α-synuclein (Lee and Trojanowski, 2006). Although the mechanisms underlying α-synuclein aggregation and toxicity are not fully elucidated, it is clear that its aggregation is linked with the pathogenesis of PD (Conway et al., 2000; Outeiro et al., 2009). Interestingly, α-synuclein may undergo numerous post-translational modifications, including glycation and glycosylation among others, and interacts with several endogenous and exogenous macromolecules, proteins, metals, hormones, neurotransmitters and drugs that can all interfere with its ability to form aggregates. Indeed, α-synuclein is one of the most predominantly glycated proteins in the context of PD (Vicente Miranda et al., 2017). The glycation of α-synuclein, in one of its 15 lysine residues, influences the initial formation of aggregates and induces its oligomerization, by stabilizing the formed oligomers (Padmaraju et al., 2011). As expected, AGEs were found to co-localize with α-synuclein and to accelerate its aggregation by inducing complex protein cross-links (Padmaraju et al., 2011; Guerrero et al., 2013). Oligomerization of α-synuclein is an important pathological modification, since oligomeric species are now considered to be highly toxic (Guerrero et al., 2013).

Additionally, monomeric species of glycated α-synuclein can directly interact with DNA increasing genome damage, and oligomeric and monomeric species of the glycated α-synuclein further promote the formation of ROS (Padmaraju et al., 2011; Guerrero et al., 2013). Moreover, such α-synuclein oligomers can directly affect membrane permeability by forming pores, with consequent loss of cell homeostasis and neuronal cell dysfunction (Guerrero et al., 2013). Importantly, oligomers composed of glycated α-synuclein due to protein cross-links can be resistant to degradation by the proteasome system or by other quality control systems, and thus accumulate and cause neuronal cell death. Finally, the deleterious effects of glycated α-synuclein may also arise from its ability to trigger neuroinflammation, not only by efficiently activating microglia, but also by interacting with RAGE and activating the downstream NF-κB transcription factor (Salahuddin et al., 2014). Thus, increased cell surface expression of RAGE receptors in PD allows the binding of more glycated α-synuclein, fueling a feedback loop, which sustains inflammation, α-synuclein accumulation and neuronal cell death.

MGO in Parkinson's Disease

The discovery that MGO can react with dopamine to forming 1-acetyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (ADTIQ), allows the establishment of another relationship between glycation and PD. This metabolite, which structure resembles that of MPTP, is found in human brain, including the SNpc, and its levels are increased in PD (Deng et al., 2012; Song et al., 2014). Therefore, up-regulated glycation in SNpc increases the probability of ADTIQ formation due to reaction with dopamine and can promote specific dopaminergic degeneration. Consistently, ADTIQ levels were increased in mouse models of familial PD, and this molecule also acted as an endogenous neurotoxin in SH-SY5Y neuroblastoma cells (Xie et al., 2015). Interestingly, the levels of ADTIQ and MGO were increased in the striatum of diabetic brains and in rat models of diabetes (Song et al., 2014; Xie et al., 2015), linking together the onset of these two pathologies.

Additionally, MGO may also interfere with the UPS, which in the context of PD, plays a major role in the clearance of misfolded α-synuclein (Ciechanover and Kwon, 2017). The failure of the UPS may arise due to the fact that ubiquitin is a target for MGO, which impairs its ability to conjugate with its target proteins (Uchiki et al., 2012). Again, this promotes a self-perpetuating reaction further contributing to α-synuclein aggregation and impairment of proteasome function, probably contributing to the deleterious effects of glycation in PD.

MGO can also directly react with mitochondria inducing the dysfunction of these organelles with concomitant increased ROS production and oxidative stress (Rosca et al., 2005; Rabbani and Thornalley, 2008; Wang et al., 2009). Interestingly, under these stress conditions cells tend to increase the rate of glycolysis in order to maintain a sufficient ATP synthesis, but as already mentioned this metabolic shift exacerbates MGO production. This mechanism may also be involved in the deleterious effects of mutated or dysfunctional PD-linked proteins like parkin and PINK1, which strongly affect mitochondrial function, ATP production and consequently energy metabolism. In fact, it has been described that loss of function mutations in parkin and PINK1 stimulate glycolysis (Vincent et al., 2012; Requejo-Aguilar et al., 2014), and therefore the propensity for an increased formation of MGO.

Glyoxalase System

Although the formation of AGE occurs spontaneously, endogenous active mechanisms exist in cells to revert AGEing of proteins and to overcome glycation toxicity. For example, MGO is detoxified by the glyoxalase system (glyoxalase-I and -II) and by aldose reductases (Thornalley, 1993; Rabbani and Thornalley, 2012) (**Figure 2**). Glyoxalase-I is an important endogenous anti-glycation agent, that has been found to decrease with age in the human brain (Kuhla et al., 2006). Notably, another gene linked to familial PD, *PARK7*, that codes for DJ-1, has been associated with the metabolism of AGEs, through several anti-glycation activities. Although the function of DJ-1 is still not fully understood, amongst its multiple functions it was shown to modulate the activity of nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of the cellular anti-oxidant response (Clements et al., 2006; Xue et al., 2012). Importantly, Nrf2 is an upstream key regulator of glyoxalase-I expression (Clements et al., 2006; Lee et al., 2012; Xue et al., 2012). Additionally, DJ-1 was also recently considered as a protein deglycase that restores proteins that were MGO-glycated (Richarme et al., 2015). However, controversy still exists regarding this putative direct effect of DJ-1 on MGO (Pfaff et al., 2017), instead this effect, independent of Nrf2, may be related to the ability of DJ-1 to repress glycolysis (Requejo-Aguilar et al., 2015). Additionally, Hsp-31 a member of the DJ-1 superfamily is also described as having deglycase activity (Mihoub et al., 2015). Nonetheless, loss of function mutations of DJ-1, or stress-induced DJ-1 dysfunction appear to be associated with mitochondrial impairment and increased levels of MGO, although the exact mechanism still needs to be fully understood.

In summary, increased expression of RAGE as well as RAGE activation were proposed to be involved in dopaminergic degeneration in human PD and experimental models of the disease. Moreover, several studies using experimental models of PD suggest that RAGE blocking should be beneficial in human PD. Thus, research should be conducted in order to understand the balance between the expression of sRAGE and mRAGE, in healthy individuals and PD patients, and the way the putative shifts in the expression levels of both will affect RAGE signaling. Interestingly, a decreased plasma level of sRAGE has been reported in patients with Alzheimer's disease (AD) (Emanuele et al., 2005; Xu et al., 2017), but to our knowledge this information is lacking in PD. Nonetheless, glycation and the underlying mechanisms of RAGE activation seem to represent important therapeutic targets in PD.

GLYCOSYLATION IN PARKINSON'S DISEASE

Structural Diversity of Glycans

All cells and a significant number of macromolecules in our body display covalently attached sugars, named "glycans." These glycans present a great structural variability, according to the involved building blocks (the monosaccharides) and the bond-type. In addition, alterations of the physiological condition of the cells and differentiation stage can easily alter their structures (Lowe and Marth, 2003; Haltiwanger and Lowe, 2004). The major classes are typically defined according to the nature and linkage to the conjugate, i.e., a protein or lipid (glycoprotein or glycolipid).

Among the various post-translational modifications that a protein may suffer, glycosylation is the most common and complex type. It is predicted that about 50–60% of human proteins, including almost all cell surface and all secreted proteins, are glycosylated (Apweiler et al., 1999; Hagglund et al., 2004; Kameyama et al., 2006). A glycoprotein usual has multiple oligosaccharide attachment sites, and each glycosylation site in turn may be altered with a variety of oligosaccharide chains (**Figure 3**).

Typically, the two major types of protein glycosylation are categorized as either *N*-glycosylation or *O*-glycosylation (**Figure 1**). *N*-glycosylation is by far the most common (Spiro, 2002; Stanley et al., 2009), involving an *N*-glycosidic bound that links the nitrogen of an asparagine residue (Asn) amide group to GlcNAc of a glycan (Nalivaeva and Turner, 2001). This Asn must belong to the consensus amino acid sequence Asn-X-Thr (X is any amino acid, excluding proline). *O*-glycosylation in humans often occurs via an N-acetylgalactosamine (GalNAc) attached to the hydroxyl group of serine or threonine residues (Spiro, 2002). The *N*-and *O*-glycosylated proteins are synthesized essentially in the ER and Golgi through sequential reactions involving several enzymes, namely sugar nucleotide synthases, transporters, glycosyltransferases, glycosidases, and other sugar-modifying enzymes. Conversely to what happens in *N*-glycosylation, *O*-glycosylation usually starts in the lumen of the ER and the assembly step is not proceeded by a remodeling step (**Figure 3**).

A distinct *O*-glycosylation is the *O*-linked N-acetylglucosamine (GlcNAc), occurring independently of the ER-Golgi pathway, and consisting in the single addition of a monosaccharide GlcNAc to nucleus and cytoplasm proteins (**Figure 3**).

Included within glycoproteins are also the proteoglycans, which are proteins that contain, through a serine residue, one or more glycosaminoglycans (GAG) chains attached. Another important glycoconjugate is the glycophosphatidylinositol (GPI) anchor, which is actually a glycan link between phosphatidylinositol and a phosphoethanolamine that is bound to the carboxy terminal of a protein. Usually this arrangement is the only anchor that attaches these type of proteins to the lipid bilayer membrane (**Figure 3**).

A glycolipid, usually designated by glycosphingolipid, is a glycan linked through glucose or galactose to the terminal primary hydroxyl group of the lipid moiety ceramide. As these ceramides consist of a long chain base (sphingosine) and a fatty acid, the glycolipids are also named glycosphingolipids (GSLs). A ganglioside, in turn, consists of an anionic glycolipid that contains one or several residues of the monosaccharide sialic acid.

Implications of Glycosylation in Parkinson's Disease

Glycosylation influences not only the native conformation of the protein, but also specific properties such as solubility, antigenicity and half-life, and its subcellular location. It has also a role

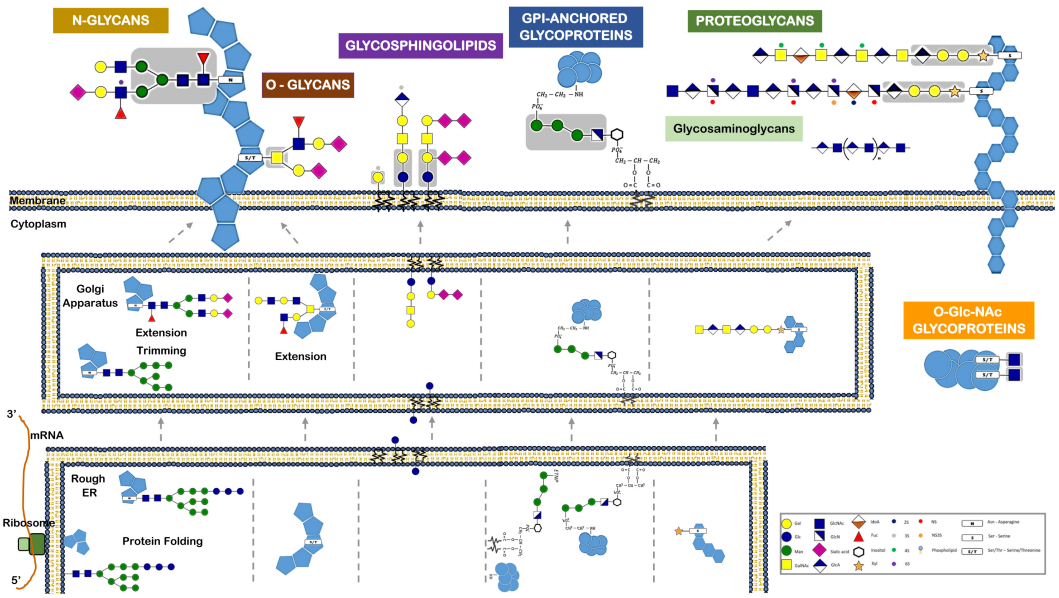


FIGURE 3 | Different classes of glycans modify proteins and lipids and have distinct biosynthetic pathways. This figure refers the major glycan classes expressed by human cells and that are described in the text. It also depicts their different biosynthetic steps and their localization for initiation, trimming, and elongation within the cells. Glycans can be attached to proteins via *N*-linkage to Asparagine (N). The *N*-glycans share a common pentasaccharide core region (highlighted in the figure in a gray box) that can be further diversified. *N*-glycans are initiated by the en-bloc transfer of a large preformed precursor glycan to a newly synthesized glycoprotein. Glycans can be attached to proteins via *O*-glycosylation. Common *O*-glycans are initiated by GalNAc *O*-linked to S/T in a polypeptide chain. *O*-glycans are further extended with the sequential addition of other monosaccharides. Glycans linked to a lipid moiety ceramide are named glycolipids or glycosphingolipids. These are initiated by the addition of glucose to ceramide on the outer face of the ER-Golgi compartments, and the glycan is then flipped into the lumen to be extended. The glycosylphosphatidylinositol (GPI)-anchored proteins are glycoproteins linked to a phosphatidylinositol. Like *N*-glycans, GPI-anchored proteins are initiated by the en-bloc transfer of a large preformed precursor glycan to a newly synthesized glycoprotein. Proteoglycans are glycoproteins where glycosaminoglycans are attached to the proteins. Glycosaminoglycans are linear polymers containing disaccharide unit repeats that can be attached to proteins or exist in free form. Intracellular proteins can also be modified with *O*-GlcNAc. *O*-GlcNAcylation is the covalent link of a GlcNAc to a serine or threonine (S/T). It is distinct from all other common forms of protein glycosylation, since it occurs exclusively within the nucleus and cytoplasm and it is not further elongated or modified.

in cell to cell communication, intracellular molecular signaling pathways, and in particular the modulation of the immune response.

Taking into account the importance of proper glycosylation in the overall functioning of proteins, it is conceivable that aberrant glycosylation may contribute to the appearance of several human pathologies. Indeed, shifts in the human serum glycoform profile in health and disease have the potential to be used as biomarkers for cancer, such as hepatocellular (Nishimura, 2011; Kamiyama et al., 2013), pancreatic (Nouso et al., 2013), renal (Hatakeyama et al., 2014), and prostate cancers (Ishibashi et al., 2014), and for other diseases, including neurodegenerative diseases (Gizaw et al., 2016). In the scope of neurodegenerative diseases, it has been recently described that Huntington's disease transgenic mice show significantly different major glycoforms and expression levels of total glycans (Gizaw et al., 2015). Moreover, major *N*- and GSL-glycans from human AD brain, serum and CSF samples were characterized, and were shown to be different from healthy controls (Gizaw et al., 2016). Moreover, protein glycosylation was shown to be altered in human Creutzfeldt–Jakob neurodegenerative disease (Silveyra et al., 2006; Xiao et al., 2013). In the context of PD, these approaches have recently begun to emerge and are still scarce. However, the results are promising regarding the use of serum or CSF glycome profiling as a biomarker of the disease (Russell et al., 2017), as an indicator of disease stages or for defining therapeutic treatments.

Glycosylation is a major regulator of cell-to-cell and cell-environment interactions, and since the immune response relies upon countless of these contacts, it is not surprising that glycans play a major role in the immune communication and that alterations affect the patient immune status. Surprisingly, this is yet a relatively understudied and unraveled subject of glycoimmunology. Cell-surface glycans arrangements are recognized by lectins that are carbohydrate-binding receptors (Ohtsubo and Marth, 2006). These molecular recognitions between lectins and their ligands mediates complex cell-to-cell interactions, including between cells from the immune systems (van Kooyk and Rabinovich, 2008).

As mentioned above, in the CNS, microglia are the immune cells that in the presence of a stimulus may manifest either pro-inflammatory or anti-inflammatory phenotypes. Microglia activation is highly regulated by carbohydrate-binding receptors such as sialic-acid-binding immunoglobulin superfamily (Siglec) and their carbohydrate ligands (Linnartz et al., 2012). Most Siglecs contain an immunoreceptor tyrosine-based inhibition motif and its signaling leads to the termination of activation signals. Thus, pro-inflammatory immune responses such as those

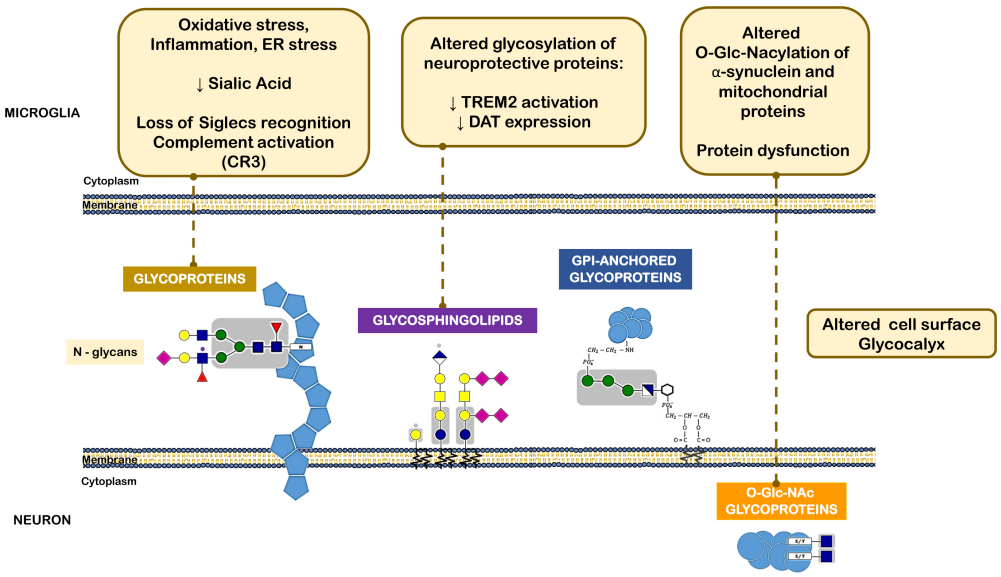


FIGURE 4 | Different classes of glycans implicated in the pathogenesis of Parkinson's disease. The oxidative stress, inflammatory milieu and ER stress reduce sialic acid content within neuronal *N*-glycans. This leads to loss of microglia Siglec receptors recognition and also complement activation. Altered glycolipids content and glycosylation of neuroprotective proteins, such as TREM2 and DAT can lead to decreased activation or expression of such proteins. *O*-GlcNAcylation of α-synuclein and other mitochondrial proteins was also observed in PD that leads to protein dysfunction.

triggered by Toll-like receptors can be turned down in microglia by inhibitory Siglec signaling (Linnartz-Gerlach et al., 2014). The terminal monosaccharides on the cell surface and extracellular proteins are frequently sialic acids. These represent the first interaction site in leukocyte communication and determine the overall immune response (Crespo et al., 2013). Sialic acid is a ligand for a number of inhibitory receptors such as Siglecs, helps masking ligands expressed by host cells from pathogen recognition and avoids autoimmune responses by inhibiting complement deposition. Consequently, alterations in sialic acid expression or in glycans decorated by sialic acids play an important role in the development of immune responses (Crespo et al., 2013). Some defects in glycosylation and, in particular, sialic acid shortage seem to exacerbate immune cell activation (Videira et al., 2008; Cabral et al., 2013; Silva et al., 2016). Pathological conditions such as oxidative stress can lead to shortage of cell surface sialic acid, probably by acidosis. In this scenario, the interactions with protective Siglecs are loss and complement components bind to cell surface and induce a complement-mediated proinflammatory cascade (**Figure 4**). Interestingly, animal models of sialic acid containing GSL (ganglioside) deficiency display some PD-like symptoms that are alleviated by administration of L-DOPA or cell-permeable ganglioside mimetics, and gangliosides may be reduced in PD patients (Wu et al., 2011). This is in agreement with a series of reports that ganglioside GM1 can alleviate symptoms in models of PD and inhibit aggregation of α-synuclein (Schneider et al., 1995; Martinez et al., 2007).

As another example, a recent report showed that altered glycosylation is also evident in peripheral immunoglobulin (IgG) glycome, with patients carrying a more pro-inflammatory fraction of IgG (Russell et al., 2017). These pro-inflammatory modifications in the peripheral IgG glycome may sustain a low-grade inflammation, triggering a positive feed-back loop that may maintain and spread the inflammation. In the context of a progressive degenerative disease associated with neuroinflammation like PD, this loop may lead to BBB disruption (Kortekaas et al., 2005; Mosley et al., 2012; Sweeney et al., 2018), enabling molecular transfer from peripheral blood. This could further explain the relationship between the peripheral alterations in the glycome and exacerbation and spreading of CNS pathogenic mechanisms.

Additionally, changes in glycosylation forms and regulation of the amounts of glycans expressed in cells, either in CNS or periphery, can be influenced by the accumulation α-synuclein. Interestingly, α-synuclein has been found in body fluids, including blood and CSF, and is likely produced by both peripheral tissues and the CNS (Sui et al., 2014). Exchange of α-synuclein between the brain and peripheral tissues is mainly due to increased BBB permeability and can have important pathophysiologic implications, such as modulation of glycans assembly. Additionally, in the course of PD progression RAGE levels at the BBB are increased, as well as AGE and RAGE levels in CNS cells (Dalfó et al., 2005; Ding and Keller, 2005; Kurz et al., 2011; Sathe et al., 2012), with subsequent increased neuronal death and abnormal sugar metabolism. This can decrease the concentration of brain type specific *N*-glycans and gangliosides, linking the deleterious effects of glycation and aberrant glycosylation.

We still know very little about the role of glycosylation of specific proteins in PD, although it appears that the glycome may be different between patients and age-matched healthy

individuals (Russell et al., 2017). Interestingly, two putative *N*-glycosylation sites were found in the V-type ligand domain of mature RAGE protein (Neeper et al., 1992; Kislinger et al., 1999). While the structural/functional basis underlying the ability of RAGE to bind a multitude of ligands was not revealed yet, one possibility is that these *N*-glycosylation sites may influence RAGE signaling, by modulating its binding to different molecules, and thus modifying the intracellular pathways activated by this receptor in different cellular contexts.

Another example of a PD-related protein that may be subjected to impaired glycosylation is the surface receptor triggering receptor expressed on myeloid cells 2 (TREM2), a type I membrane protein expressed on myeloid cells including microglia. TREM2 has multiple ligands, including Apolipoprotein E (APOE), lipids, and seems to transduce its signal through interaction with DNAX-activating protein. Activation of TREM2 was suggested to have an anti-inflammatory function after ligand binding in various diseases, including PD (Rayaprolu et al., 2013). Interestingly, *N*-glycans including sialylated and/or fucosylated complex-type glycans can decorate TREM2, and alterations in a portion of these *N*-glycans can dictate the conformation or trafficking of TREM2, leading to altered protein stability and impaired antioxidant potential (Park et al., 2015) (**Figure 4**).

Shimura et al. (2001) found post translationally modified form of human α-synuclein containing *O*-linked sugars. Interestingly, whereas normal parkin recognizes and binds to glycosylated α-synuclein, they showed that in PD mutant parkin was not able to bind the *O*-glycosylated α-synuclein. Therefore, this suggests that this mechanism is involved in the accumulation of ubiquitinated α-synuclein in Lewy bodies in familiar but also in idiopathic PD, where loss of parkin function was already described (Shimura et al., 2001).

It has been then shown that α-synuclein, similarly to several aggregation-prone proteins that directly contribute to neurodegeneration are modified by a specific *O*-glycan, the *O*-GlcNAcylation (Alfaro et al., 2012). *O*-GlcNAcylation has been shown to affect the phosphorylation of α-synuclein and block the toxicity of α-synuclein, suggesting that increasing *O*-GlcNAcylation may prevent protein aggregation (Marotta et al., 2015) (**Figure 4**).

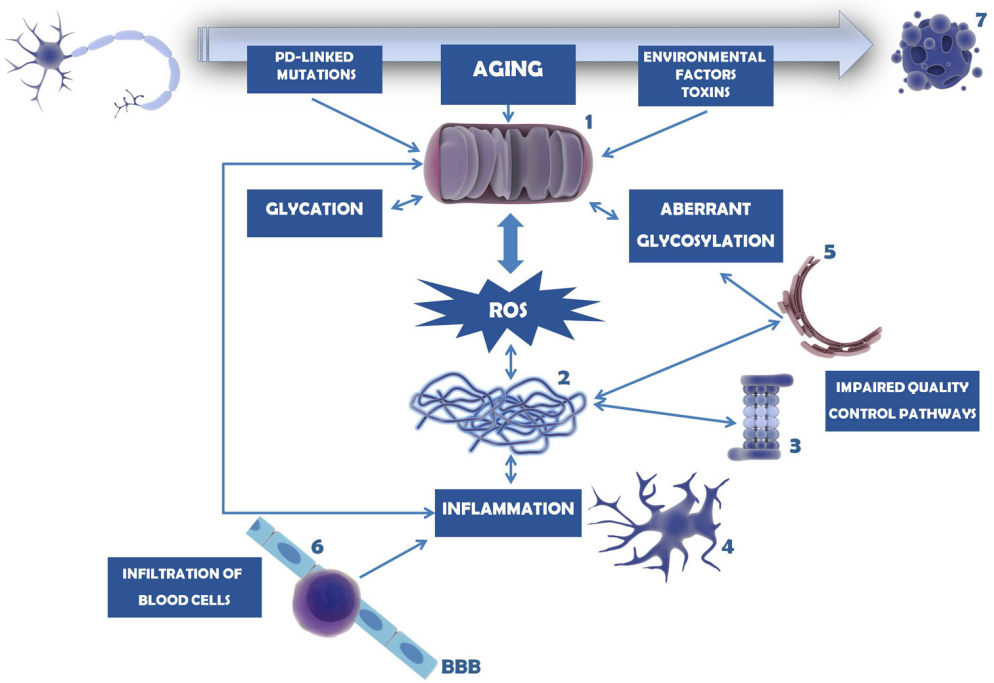


FIGURE 5 | Mechanisms involved in dopaminergic neuron cell death in PD. Mitochondria dysfunction in PD can be caused by mutations in PD-linked proteins (e.g., parkin, PINK1, DJ-1), environmental factors and toxins (e.g., MPTP, 6-OHDA, pesticides, herbicides) or age-induced depletion of endogenous antioxidant defense mechanisms (1). As a consequence, ROS levels and oxidative stress increase and accumulate, overcoming the endogenous antioxidant capacity. Mitochondria dysfunction and oxidative stress may trigger glycation that in turn will affect several proteins, including mitochondrial proteins. Under these circumstances, proteins, in particular α-synuclein, tend to be misfolded and to form insoluble aggregates (2). This is further potentiated by impairment of several quality control pathways in PD, namely proteasome-dependent protein degradation (3), UPR, UPS, and autophagy. Protein aggregates and oxidative stress activate microglia and trigger the neuroinflammatory response within the brain (4). Inflammation, malfunction of ER resident enzymes responsible for glycosylation of target proteins (5), among other causes, may induce aberrant protein glycosylation. Impairment of this mechanism may underlie improper protein function as well as abnormal cell–cell contacts. With PD progression BBB permeability increases and peripheral T cells invade the CNS (6), contributing to the accumulation of pro-inflammatory mediators and further activating microglia. In turn, (neuro)inflammation may damage mitochondria, entering a vicious cycle of deleterious effects that ultimately lead to dopaminergic cell death (7).

As discussed above, mitochondria dysfunction plays a central role in the neurodegenerative process in PD. However, although the glycosylation process involved in secreted, membrane and nucleocytosolic proteins is substantially well-understood, this is not yet the case for mitochondrial proteins. So far there are only a scarce studies on glycosylated mitochondrial proteins (Levrat et al., 1989; Chandra et al., 1998; Hu et al., 2009; Kung et al., 2009), but together suggest that mitochondrial glycome may dictate mitochondrial function and cell fate. A number of mitochondrial proteins are predicted as a target of glycosylation, and these glycosylated isoforms, could be a still unexplored mechanism for regulating mitochondrial metabolic functions even if they represent just a small fraction of the total expressed proteins in these organelles (Burnham-Marusich and Berninsone, 2012). Accordingly, Zhao et al. (2014) showed that enhanced *O*-GlcNAcylation of mitochondrial proteins might protect from oxidative stress and increase mitochondrial respiration rate in aged retina. Whereas others indicate that *O*-GlcNAcylation, might contribute to impaired mitochondrial function. Although the relationship between *O*-GlcNAcylation and mitochondria is not yet completely characterized, it is suggested that *O*-GlcNAcylation could mediate the link between mitochondrial motility and availability of mitochondrial substrates in neurons (Pekkurnaz et al., 2014). Importantly, the master regulator of mitochondrial biogenesis PGC1-α (Wang X. et al., 2016) and ATP synthase α-subunit (Kung et al., 2009) were shown to be *O*-GlcNAcylated, however, the physiological role is not very clear yet. Therefore, one may extrapolate that these mechanisms could also be recapitulated in PD or other neurodegenerative diseases, and that abnormal mitochondrial glycome could trigger the acceleration of mitochondrial dysfunction and neuronal death. In fact, *O*-GlcNAcylation in lysates from the *post-mortem* temporal cortex of PD patients was shown to be detrimental to neurons by inhibition of autophagy and with consequent α-synuclein accumulation (Wani et al., 2017).

Although the causes of idiopathic PD are still unknown, dopamine metabolism may be an important source of ROS in dopaminergic cells. The cytosolic levels of dopamine depend on its biosynthesis, and on two transporters, namely the cell membrane dopamine transporter (DAT) and vesicular monoamine transporter (VMAT). Dopamine uptake from the synaptic cleft is undertaken by DAT, a glycoprotein with 12-transmembrane domains, which interestingly is also required for the uptake of neurotoxins like MPTP that are similar to dopamine (Storch et al., 2004). The human DAT is a heavily glycosylated protein containing three putative *N*-linked glycosylation sites in the second extracellular (Li et al., 2004). Notably, the *N*-linked glycosylation status of DAT influences its cell surface expression and transporter activity (Hastrup et al., 2001; Torres et al., 2003). In fact, the expression of DAT on the membranes can be efficiently inhibited by tunicamycin or mutations of its *N*-linked glycosylation sites (Hastrup et al., 2001). Additionally, glycosylation is also crucial for DAT activity, since dopamine is more efficiently transported by glycosylated DAT than by the non-glycosylated form of the transporter (Torres et al., 2003; Li et al., 2004).

Thus, an aberrant glycosylation may contribute to a decrease in DAT membrane expression, as well as an imbalance between the functional vs. dysfunctional (or less efficient) receptor populations. Both processes result in an accumulation of dopamine in the extramembrane space, thus contributing to an increase in ROS formation in the vicinity of dopaminergic neurons. Interestingly, parkin can significantly enhance the ubiquitination of misfolded DAT that results from by *N*-glycosylation inhibition by tunicamycin, consequently attenuating the damaging effects of aberrantly glycosylated DAT on dopamine uptake (Jiang et al., 2004). Accordingly to what was found for α-synuclein, normal parkin, but not its PD-linked T240R mutant form, can recover DAT membrane expression and transporter activity even in the presence of an abnormal glycosylated form of the protein (Jiang et al., 2004). These results highlight the neuroprotective role of native parkin, through a less well-known mechanism that involves its ability to maintain proper dopamine uptake. Parkin, in fact, ubiquitinates and targets for degradation misfolded abnormally glycosylated DAT, and favors the proper membrane localization of this transporter, contributing to an efficient dopamine recycling and reduction of dopamine-associated toxicity toward neighboring cells.

In summary, about one-third of the cellular proteins are synthesized in the ER where they gain proper folding and undergo posttranslational modifications to become mature functional peptides, and then move on to their target organelle or are secreted. Amongst all the proteins that are synthesized most of them are glycosylated at the ER, in a cascade of reactions that involve an orchestrated activation of several different enzymes. Since ER stress is one of the identified pathological mechanisms in PD (Mercado et al., 2013; Tsujii et al., 2015), aberrant glycosylation might indeed be due to an overload of the ER with underglycosylated proteins. Additionally, oxidative stress and inflammation may also trigger abnormal glycosylation in PD. The above mentioned mechanisms are depicted in **Figure 4**.

CONCLUSION

Here, we focused on the established concepts of neuroinflammation and abnormal glucose metabolism in PD, linking them to mitochondrial dysfunction and emerging fields of research that take into account current discoveries suggesting that glycation and abnormal glycosylation may underlie the development and/or progression of this disease.

Mitochondria are key organelles in the development and progression of PD. Not only they are the major source or ROS within the cells, as they are also very susceptible to ROS-induced effects. Several different causes and pathways may affect mitochondria integrity and function, with severe consequences in the assembly of proteins, and induction of (neuro)inflammation. These mechanisms seem to be linked to each other in a circular way, since more oxidative stress generates more inflammation and *vice versa*, as briefly schematized in **Figure 5**.

Understanding the glycosylation pattern of glycoproteins which are affected by either genetic or environmental cellular

stressors in PD, can be a promising approach for the discovery of novel biomarkers to assist an easy prognosis. However, only a few glyco-PD studies have been performed, and our knowledge of glycan functions in the context of PD is still limited. The characterization of the general *N*-glycome as well as specific glycosylated membrane proteins in peripheral blood cells from PD patients and healthy controls will reveal the potential contribution of aberrant glycosylation in the cellular dysfunctions leading to neurodegeneration in PD. Certainly, the large-scale serum glycomics of a variety of stages of PD patients could accelerate the discovery of novel class of biomarkers and molecular targets toward the development of the diagnostic and therapeutic agents for this disease.

AUTHOR CONTRIBUTIONS

PV and MC-C wrote the manuscript, and approved the submitted version.

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Muscarinic Acetylcholine Type 1 Receptor Activity Constrains Neurite Outgrowth by Inhibiting Microtubule Polymerization and Mitochondrial Trafficking in Adult Sensory Neurons

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The muscarinic acetylcholine type 1 receptor (M_1R) is a metabotropic G protein-coupled receptor. Knockout of M_1R or exposure to selective or specific receptor antagonists elevates neurite outgrowth in adult sensory neurons and is therapeutic in diverse models of peripheral neuropathy. We tested the hypothesis that endogenous M_1R activation constrained neurite outgrowth via a negative impact on the cytoskeleton and subsequent mitochondrial trafficking. We overexpressed M_1R in primary cultures of adult rat sensory neurons and cell lines and studied the physiological and molecular consequences related to regulation of cytoskeletal/mitochondrial dynamics and neurite outgrowth. In adult primary neurons, overexpression of M_1R caused disruption of the tubulin, but not actin, cytoskeleton and significantly reduced neurite outgrowth. Over-expression of a M_1R-DREADD mutant comparatively increased neurite outgrowth suggesting that acetylcholine released from cultured neurons interacts with M_1R to suppress neurite outgrowth. M_1R-dependent constraint on neurite outgrowth was removed by selective (pirenzepine) or specific (muscarinic toxin 7) M_1R antagonists. M_1R-dependent disruption of the cytoskeleton also diminished mitochondrial abundance and trafficking in distal neurites, a disorder that was also rescued by pirenzepine or muscarinic toxin 7. M_1R activation modulated cytoskeletal dynamics through activation of the G protein ($G\alpha13$) that inhibited tubulin polymerization and thus reduced neurite outgrowth. Our study provides a novel mechanism of M_1R control of $G\alpha13$ protein-dependent modulation of the tubulin cytoskeleton, mitochondrial trafficking and neurite outgrowth in axons of adult sensory neurons. This novel pathway could be harnessed to treat dying-back neuropathies since anti-muscarinic drugs are currently utilized for other clinical conditions.

Keywords: muscarinic receptors, mitochondria, antagonist, G proteins, cytoskeleton dynamics, mitochondrial trafficking, neurodegeneration, tubulin

INTRODUCTION

Muscarinic acetylcholine receptors constitute a sub-family of G protein-coupled receptors (GPCRs) that act as metabotropic activators of the neurotransmitter acetylcholine (ACh). Five distinct subtypes have been identified (M1-M5), based on their G-protein coupling preferences (Wess, 1996). Downstream pathways activated include phospholipase C, inositol triphosphate (IP_3), cyclic adenosine monophosphate (cAMP) and altered calcium homeostasis (Eglen, 2005; Wess et al., 2007; Kruse et al., 2014). In addition, these GPCRs modulate the cytoskeleton through trimeric G protein signaling (Kapitein and Hoogenraad, 2015). For example, α and βγ subunits of heterotrimeric G proteins modulate microtubule assembly (Roychowdhury and Rasenick, 2008; Schappi et al., 2014). Activated Gα, acts as a GTPase activating protein (GAP) and increases microtubule disassembly by activating the intrinsic GTPase activity of tubulin (Roychowdhury et al., 1999).

The muscarinic acetylcholine type 1 receptor (M_1R) is widely expressed in the central nervous system (CNS) (Levey, 1993; Wess et al., 2003; Jiang et al., 2014) and peripheral nervous system (PNS) (Bernardini et al., 1999; Tata et al., 2000b). Membranes isolated from hippocampus and cortex of M_1R knockout (KO) mice showed a significant decrease in GTPγ-S loading to the Gα-q/11 G protein upon agonist stimulation (Felder et al., 2001). In cortical neuron cultures obtained from M_1R KO mice, carbachol-stimulated phosphoinositide hydrolysis was reduced by 60% compared with wild type (Bymaster et al., 2003). In addition, phosphorylation of extracellular signal-regulated kinase (ERK) was eliminated in pyramidal neurons of hippocampal slices or cortical cultures derived from M_1R KO mice (Berkeley et al., 2001; Hamilton and Nathanson, 2001).

In sympathetic neurons, ACh activation of M_1R mobilizes internal Ca^{2+} stores leading to closure of M-type K^+ channels (Kv7 subtypes) and enhancement of slow depolarization and discharge (Delmas and Brown, 2005; Brown and Passmore, 2009). In embryonic neurons, ACh modulates neurite outgrowth in a positive or negative manner based upon context (Tata et al., 2000a, 2003; Bernardini et al., 2004; Yang and Kunes, 2004). Furthermore, both adult sensory dorsal root ganglia (DRG) neurons and epidermal keratinocytes synthesize and secrete ACh (Bernardini et al., 1999; Khan et al., 2002; Nguyen et al., 2004; Grando et al., 2006; Schlereth et al., 2006; Corsetti et al., 2012). Adult rat sensory neurons of the DRG express a peripheral form of ChAT (pChAT), exhibit ChAT activity, have low AChE activity and express multiple muscarinic receptors including M_1R (Bernardini et al., 1999; Tata et al., 2000b; Bellier and Kimura, 2007; Hanada et al., 2013).

We have recently reported that selective or specific antagonists of M_1R elevated neurite outgrowth and augmented mitochondrial function in adult sensory neurons (Calcutt et al., 2017). These drugs also afforded protection against several different forms of peripheral neuropathy. However, the mechanism of M_1R antagonist-driven neurite outgrowth and neuroprotection has not been studied in detail. Mitochondrial oxidative phosphorylation is the main mechanism providing ATP to power neuronal activities such as production of presynaptic action potentials, neurotransmitter release, postsynaptic currents and postsynaptic action potentials (Hall et al., 2012). Mitochondria are known to concentrate in regions of active signaling and high metabolic demand (Chen and Chan, 2006; Mironov, 2007; Verburg and Hollenbeck, 2008). This substantial energy demand at the nerve ending or synapse implies that neurons must have a mechanism to maintain microtubules to augment mitochondrial trafficking upon demand (Sheng and Cai, 2012; Schwarz, 2013).

In the present study we manipulated M_1R expression/function in adult DRG sensory neurons and related cell lines and studied the cellular phenotypes and molecular consequences. Specifically, we tested the hypothesis that the M_1R regulates the tubulin cytoskeleton, G-protein recruitment (Gα13 subtype) and mitochondrial trafficking. We identified that excessive cholinergic signaling triggered tubulin destabilization through over-activation of Gα13 proteins. Further, we studied the ability of specific (muscarinic toxin 7: MT7) or selective (pirenzepine) M_1R antagonists to ameliorate the endogenous and M_1R overexpression-induced neuronal phenotypes that primarily result in a constraint on neurite outgrowth.

MATERIALS AND METHODS

All animal procedures followed guidelines of University of Manitoba Animal Care Committee using Canadian Council of Animal Care rules or of the Institutional Animal Care and Use Committee at UCSD.

Cell Culture

Dorsal root ganglia from adult male Sprague-Dawley rats were dissected and dissociated using previously described methods (Akude et al., 2011; Roy Chowdhury et al., 2012; Saleh et al., 2013). All animal protocols carefully followed the Canadian Committee on Animal Care (CCAC) guidelines. Neurons were cultured in defined Hams F12 media containing 10 mM D-Glucose (N4888, Sigma, St. Louis, MO, United States) supplemented with modified Bottenstein's N2 additives without insulin (0.1 mg/ml transferrin, 20 nM progesterone, 100 μM putrescine, 30 nM sodium selenite, 0.1 mg/ml BSA; all additives were from Sigma, St. Louis, MO, United States). In all experiments, the media was also supplemented with 0.146 g/L L-glutamine, a low dose or high dose cocktail of neurotrophic factors (Low dose = 0.1 ng/ml NGF, 1.0 ng/ml GDNF and 1 ng/ml NT-3, High dose = 1 ng/ml NGF, 10 ng/ml GDNF, 10 ng/ml NT3 – all from Promega, Madison, WI, United States), 0.1 nM insulin and 1X antibiotic antimycotic solution (A5955, Sigma). Cultures were treated with 100 nM MT7 (M-200, Alomone Labs, Jerusalem, Israel) or 1 μM pirenzepine (P7412, Sigma).

HEK293 and HTLA cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated FBS. The β-arrestin null (ARRB1 and ARRB2) and Gα12/13 (GNA12 and GNA13) null HEK293 cells were obtained from the laboratory of Dr. Asuka Inoue, Tohoku University, Japan. HTLA cells were provided by Prof Bryan Roth, University of North Carolina, United States.

Cloning, Transfection and siRNA Based Gene Silencing

Total mRNA was extracted from adult rat DRGs using Trizol reagent and used for amplifying the full length M_1R cDNA using the following primer sets: F: 5′-ATGAACACCT CAGTGCCCCCTGC-3′ and R: 5′-TTAGCATTGGCGGGAG GGGGTG-3′. The cDNA was cloned in the pEGFP-C1 vector (Clontech, now Takara Bio United States, Inc., Mountain View, CA, United States) in the XhoI and SacII restriction sites. In addition, the cDNA was also cloned in the pHTN Halo-Tag CMV-neo Vector (Promega, Madison, WI, United States) at the SacII and Not1 restriction sites. The plasmids were transiently transfected in freshly dissociated sensory neurons using the rat neuron nucleofection kit (VPG-1003, Amaxa, Lonza Inc., Allendale, NJ, United States) and Amaxa nucleofector-II apparatus (program 0-003) and cultured in poly L-Ornithine (P8638, Sigma) and laminin coated μ-Plate-24 well (Ibidi United States, Inc., Madison, WI, United States). The human M1-DREADD construct was obtained from Dr. Arthur Christopoulos, Monash University, Australia and sub-cloned in pEGFP-C1 vector (Abdul-Ridha et al., 2013). The rat Gα13(GNA13) was knocked down using a cocktail of three siRNAs targeted to exon 2 (AGTATCTTCCTGCTATAAGAGCC) and exon 4 (CTACAT TCCGTCACAGCAAGATA and CATCAAAGACTATTTCCTA GAAT), respectively. The siRNAs were transfected in to primary sensory neurons using Amaxa transfection reagent.

Quantification of Neurite Outgrowth

The transgene transfected neurons were cultured for 48 h and then cells were fixed in 4% paraformaldehyde for 10 min and immunostained using monoclonal anti-β-tubulin III antibody. The neurons were also stained with Hoechst for nuclear staining. The neurons were imaged in an unbiased manner using a Cellomics Arrayscan-VTI high content screening (HCS) Reader (Thermo Fisher Scientific, Waltham, MA United States) and total neurite outgrowth per neuron was measured by Neuronal Profiling V4.1 software. The automated HCS reader provided a bias-free objective analysis of neurite outgrowth.

Confocal Microscopic Image Acquisition and Analysis to Determine Mitochondrial Volume and Trafficking

Mitochondrial trafficking in GFP or GFP-M_1R overexpressing neurons was monitored using LSM510 confocal live cell imaging and involved co-transfection of sensory neurons with GFP/GFP-M_1R and DsRed2Mito7 plasmids (Addgene plasmid #55838, a gift from Dr. Michael W. Davidson, Florida State University), respectively. The DsRed2Mito7 consists of a mitochondrial targeting sequence from subunit VIII of human cytochrome C oxidase which is placed before the Red fluorescence protein and the resultant fusion protein selectively accumulates inside the mitochondria (Van Kuilenburg et al., 1988). The transfected cells were live imaged at 10 s interval for 80 time frames (∼13 min). The time lapse images were used to generate kymographs using the ImageJ make kymograph plugin (Schneider et al., 2012). In each kymograph, the *x*-axis represents the position along the length of the axon and the *y*-axis represents time. Vertical lines indicate stationary mitochondria with no displacement during the time elapsed and diagonal lines represent moving mitochondria and their direction. Their velocity is reflected in the slope of the line. In addition we used Fiji (Schindelin et al., 2012) based MTrackJ to determine mitochondrial velocity (μm/sec) in the neurites (Meijering et al., 2012). The volume of mitochondria in the neurites was calculated by using Image J analyze particles plugin and expressed in μm per neurite length (Schindelin et al., 2012).

Western Blotting and Immune-Detection

Relative quantification of proteins was done by SDS-PAGE separation of total proteins followed by transfer to nitrocellulose membrane and immunoblotting based detection using HRP-conjugated secondary antibodies. The immunoblots were imaged in Bio-Rad Chemidoc system (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). **Table 1** summarizes all the primary antibodies used in this study. The cell lysates were prepared in 1X RIPA lysis and extraction buffer (Cat No: 89900, Thermo Fisher Scientific) supplemented with 1X Halt protease and phosphatase inhibitor cocktail (Cat No: 78441, Thermo Fisher Scientific).

Polymerized Tubulin Quantification

The polymerized tubulin in M_1R overexpressed cells (40–50% transfection efficiency) was quantified by methods described previously (Butcher et al., 2016). Briefly, the soluble fraction of tubulin was first removed by lysing the cells in a microtubule stabilizing buffer (MSB) containing 50% glycerol, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.3 mM guanosine triphosphate (grade II-S, Sigma Chemical Co.), and 10 mM sodium phosphate, pH 6.8

TABLE 1 | List of antibodies.

Name of the antibody; clone number	Catalog number	Host species and type	Vendor
Anti-porin (B-6)	Sc-390996	Mouse monoclonal	SCBT
Anti-β-actin (C-4)	Sc-47728	Mouse monoclonal	SCBT
Anti GAPDH (0411)	Sc-25778	Mouse monoclonal	SCBT
Anti-α-tubulin (TU-02)	Sc-8035 (TU-02)	Mouse monoclonal	SCBT
Anti-GFP	Sc-9996	Mouse monoclonal	SCBT
Anti-M_1R	Sc-365966	Mouse monoclonal	SCBT
Anti-Gγ2/3/4/7	Sc-166419	Mouse monoclonal	SCBT
Anti-Gα12/13	Sc-28588	Mouse monoclonal	SCBT
Anti-β-tubulin III	T8578	Mouse monoclonal	Sigma

SCBT, Santa Cruz Biotechnology.

(Beertsen et al., 1982). Cells were harvested with a rubber scraper in MSB, homogenized, and centrifuged at 26,000 × *g* in a Sorvall RC2-B centrifuge (17,000 rpm in rotor SS-34 in 1.0 ml tubes; Dupont Instruments-Sorvall Biomedical Div., Dupont Co., Newtown, CT, United States) at 20°C for 30 min. Supernatants containing the soluble tubulin fraction were removed and the pellet containing polymerized tubulin and other cytoskeletal protein was assessed by immunoblotting using anti-actin and anti-β tubulin III antibodies.

Halo-M_1R Pull Down and Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

SH-SY5Y human neuroblastoma cells (provided by Dr. Jun-Feng Wang, University of Manitoba) were grown in dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher). Halo-M_1R plasmid was transiently expressed in SH-SY5Y cells that were treated with 100 nM MT7 or 1 μM pirenzepine for 1 h. Halo-M_1R was then pulled down using Halo-link resin by overnight incubation at 4°C (Promega Corporation, Madison, WI, United States) as per manufacturer's instruction and the pull-down product was cleaved using TEV-protease (Promega) overnight on a constant rotating shaking platform. The cleaved fraction was resolved in SDS-PAGE and immunoblotted using anti-M_1R, anti Gγ2/3/4/7 and anti-Gα12/13 antibodies. The BN-PAGE was performed as described previously (Sabbir et al., 2016). Polymeric tubulins in Gα12/13 null and native HEK293 cells were separated by BN-PAGE using microtubule stabilizing native cell lysis buffer containing 20 mM Bis-tris (pH7.0), 500 mM ε-aminocaproic acid, 20 mM NaCl, 10% Glycerol, 5 mM $MgCl_2$, and 0.3 mM GTP (Beertsen et al., 1982).

Isoelectric Focusing

Fifty microgram of total cellular protein was precipitated by acetone and dissolved in rehydration buffer containing 8 M Urea, 2% CHAPS, 50 mM dithiothreitol (DTT) and 0.2% Bio-Lyte ampholytes pH3-10. The dissolved proteins were incubated in Zoom IPG-strip 3-10 non-linear (NL) (Thermo Fisher) for 1 h and then focused at 175 volt (V) for 15 min, 175–2000 V ramp for 45 min and 2000 V for 30 min. After the run, the strips were alkylated and resolved on 2D SDS-PAGE and immunoblotted using antibodies previously described.

Statistical Analysis

Statistical analysis was performed using Prism version 7.00 (GraphPad Software). The mean of two or more groups were compared using one-way ANOVA followed by multiple comparison tests (Siegel, 1956; Dunn, 1964). The mean of multiple experimental groups were compared with the control group by Dunnett's *post hoc* multiple comparison test, whereas, the mean difference between two experimental groups were compared by Sidak's *post hoc* multiple comparison test (Dunn, 1964). Comparisons between two groups were performed using Student's *t*-test (unpaired). Differences were considered significant at $P < 0.05$. Neurite outgrowth, neurite width and mitochondrial quantification data were plotted as box and whisker plot where the end of the box represents the upper and lower quartiles and the median is marked by a horizontal line inside the box. The whiskers represent the highest and lowest values excluding the outliers. In some figures the individual data points of outliers are also indicated.

RESULTS

MT7 and Pirenzepine Significantly Augmented Neurite Outgrowth in Cultured Primary Sensory Neurons

Pirenzepine is a selective M_1R antagonist whereas MT7 is the only specific antagonist of this receptor (Birdsall et al., 1983; Max et al., 1993a). Our previous study demonstrated that both antagonists enhanced neurite outgrowth from adult sensory neurons (Calcutt et al., 2017). In order to confirm that MT7 and pirenzepine had growth promoting effects under the current conditions, we measured total neurite outgrowth from primary sensory neurons derived from adult rats and cultured in defined media containing a cocktail comprising of low or high concentrations of GDNF, NGF, and NT3 growth factors (LGF and HGF, respectively) and either 100 nM MT7 or 1 μM pirenzepine (**Figures 1A–D**). The growth factor concentrations in the LGF cocktail reflects concentrations of growth factors that are sub-saturating and induce small but significant increases in neurite outgrowth (Calcutt et al., 2017). The HGF cocktail contained 10-fold higher concentrations to allow us to determine whether the neuritogenic effects of MT7 and pirenzepine were also effective when growth factors were present in excess and, also to see if increased tyrosine kinase signaling masked the effect of antagonist-M_1R signaling mediated growth. In the absence of muscarinic antagonists, the HGF promoted significantly ($p < 0.0001$) more neurite outgrowth than LGF, as assessed using unbiased automated high content imaging combined with data analysis (**Figures 1A,B**). Both MT7 and pirenzepine significantly increased neurite outgrowth in LGF and HGF conditions within 48 h of treatment (**Figures 1A,B**). In addition, binning of the entire data set of 1249 (LGF) and 1517 (HGF) neurons for their total neurite length revealed that the neuritogenic effect of pirenzepine and MT7 evenly affected neurite outgrowth of the majority of the population of neurons (**Figures 1C,D**). Further, pirenzepine treatment elicited significantly higher neurite outgrowth under the LGF conditions when compared to MT7, whereas under the HGF condition, the primacy was reversed (**Figures 1A,B**). The exact reason for the difference between the drugs in terms of synergistic effect on growth at HGF condition is not known. The difference in the chemical nature of these drugs may be responsible. MT7 is a cell impermeable 7.4 kDa protein (Krajewski et al., 2001) which binds allosterically to M_1R (Max et al., 1993b; Karlsson et al., 2000) whereas pirenzepine is a cell-permeable orthosteric antagonist molecule (Caulfield and Birdsall, 1998). We used LGF conditions in subsequent experiments.

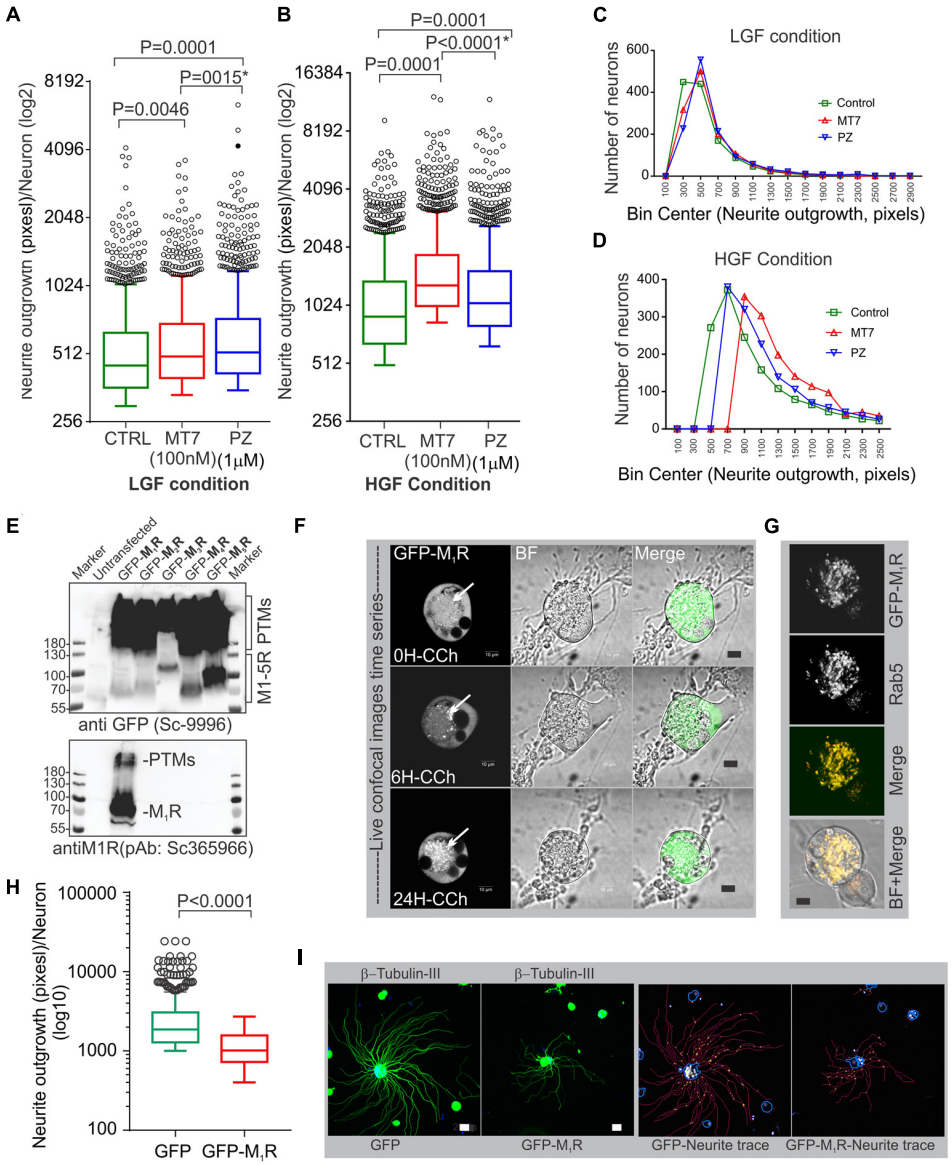


FIGURE 1 | M$_1$R antagonists, MT7 and pirenzepine, augment neurite outgrowth in primary sensory neurons and M$_1$R overexpression inhibits neurite outgrowth. **(A,B)** Whiskers box (Tukey) showing total neurite outgrowth per neuron. Neurons were grown for 48 h in defined media containing LGF **(A)** or HGF **(B)** condition and treated with 100 nM MT7 or 1 μM pirenzepine (PZ), respectively. *N* = 1249 (LGF) and 1517 (HGF), respectively. *P*-values were calculated by one-way ANOVA followed by *post hoc* multiple comparison tests. Dunnett's multiple comparisons test was used to compare the MT7 and PZ treatment groups with the control group and Sidak's multiple comparisons test was used to compare between the MT7 and PZ treatment groups; * indicates the *p*-value obtained by Sidak's multiple comparisons test. **(C,D)** Binning of the entire data set presented in **(A,B)**. **(E)** Immunoblot showing GFP-tagged muscarinic receptors (M$_1$R to M$_5$R) and GFP expression in transfected adult rat DRG neurons. pEGFP-C1-(M$_1$R-M$_5$R) plasmids were transfected in to DRG neurons and the lysate was resolved in SDS page and subsequently immunoblotted with anti-M$_1$R (bottom panel) and anti-GFP (top panel) antibodies. **(F)** Time lapse confocal images showing increasing internalization (white arrows) of the GFP-M$_1$R following treatment with carbachol (10 μM). Scale bar: 10 μm. **(G)** Immunofluorescence images showing colocalization of 24h CCh treated GFP-M1R with endosomal marker Rab5. Scale bar: 10 μm. **(H)** Whiskers box (Tukey) showing total neurite outgrowth per neuron, *N* = 634 (GFP), and *N* = 553 (GFP-M$_1$R), neurons, respectively. *P*-value was calculated by *t*-test (unpaired). **(I)** Immunofluorescence images showing β-tubulin III staining and corresponding neurite trace (red lines) images in GFP and GFP-M$_1$R overexpressed neurons. The total neurite outgrowth measurement was performed in Cellomics ArrayScan HCS Reader using neuronal profiling software. Scale bar: 10 μm.

M$_1$R Overexpression Induced Significant Reduction in Neurite Outgrowth

The muscarinic receptor subtypes (M1–M5) show considerable heterogeneity of expression in sensory neurons (Bernardini et al., 1999; Chiu et al., 2014). In order to understand the biological function of M$_1$R in sensory neurons, we overexpressed GFP-tagged M$_1$R in sensory neurons and measured the impact on neurite outgrowth. M$_1$R overexpression

significantly reduced neurite outgrowth when compared to GFP-expressing neurons (**Figures 1H,I**). The GFP-M_1R transgene-induced protein production was verified in the transfected DRG neurons by immunoblotting of the expressed recombinant proteins (GFP-M1-5R) using both anti-M_1R and anti-GFP antibodies (**Figure 1E**). The biological functionality of the GFP-M_1R recombinant protein was verified by treating GFP-M_1R overexpressing DRG neurons with the broad spectrum muscarinic agonist carbachol (10 μM) followed by live confocal imaging to monitor internalization of recombinant protein. Treatment with carbachol increased presence of the recombinant protein as internalized spots that were positive for the early endosomal marker Rab5 (Zerial and McBride, 2001) (**Figures 1F,G**). This indicates that GFP-tagged recombinant M_1R elicited agonist induced internalization response similar to that of naïve proteins. In addition, to assess functionality of the recombinant M_1R in overexpressed cells, we performed a β-arrestin recruitment assay [TANGO assay: (Kroeze et al., 2015), **Figure 2**]. In the TANGO assay, upon activation, β-arrestin is recruited to the C-terminus of the M_1R-TEV-tTA fusion protein at the TEV protease site and cleaves to release the tTA transcription factor, which after transport to

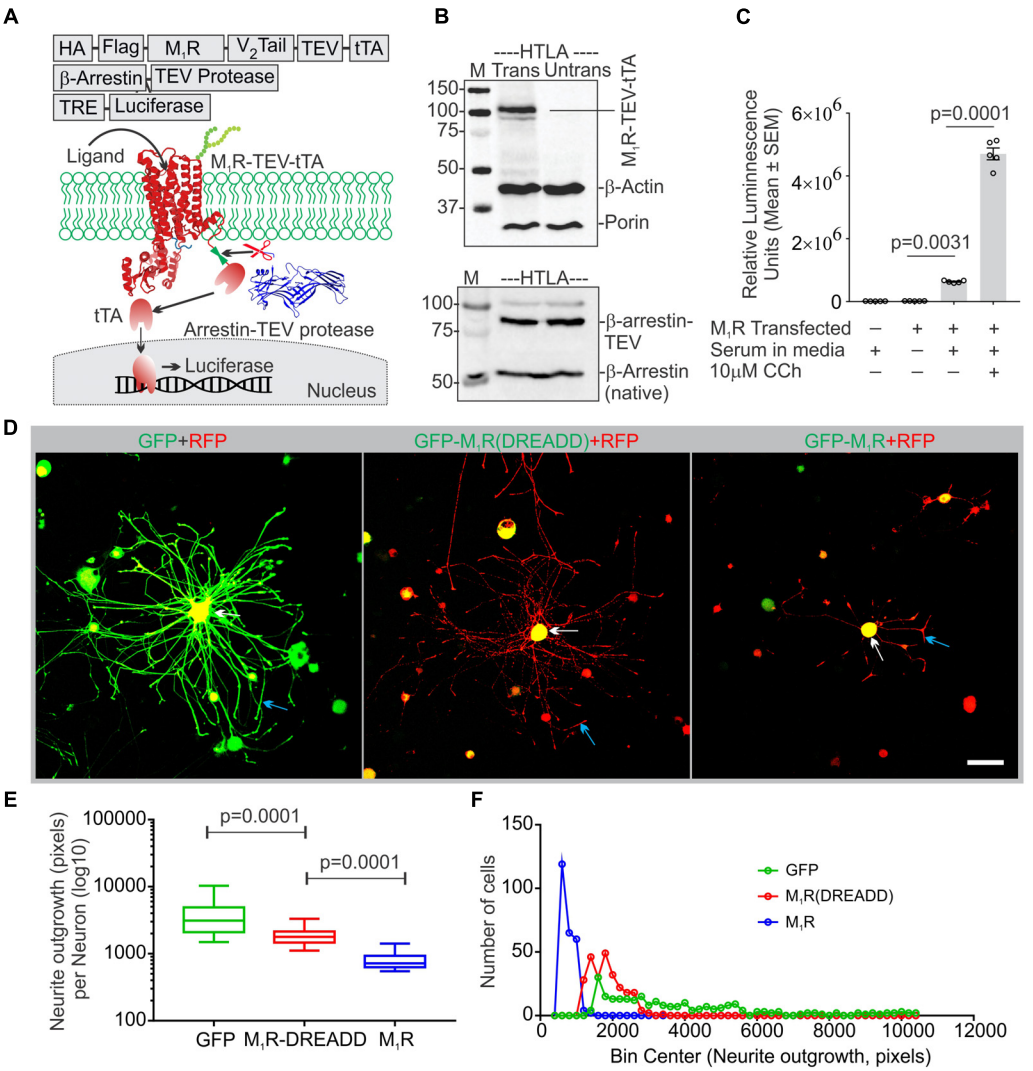


FIGURE 2 | Constitutive basal activity of M_1R and the effect of DREADD mutant on neurite outgrowth. **(A)** Graphical representation of the arrestin recruitment assay (TANGO) strategy, TRE-Tet response element. HA-cleavable signal sequence to promote membrane localization, FLAG-epitope tag, TEV- Tobacco Etch Virus cleavage site, V_2 tail: C-terminus of the V_2 vasopressin receptor (V_2 tail) to promote arrestin recruitment and tTA- tetracyclin transactivator. **(B)** Immunoblot showing expression of M_1R-TEV-tTA and β-arrestin-TEV protease transgene. **(C)** Scatter plot showing the RLU (Relative Luminescence Units) for the drug treated HTLA cells. $N = 5$ independent experiments. **(D)** Co-expression of GFP/GFP-M_1R/GFP-M_1R(DREADD) with mito7-RFP in DRG neurons following 48 h of growth. Neurite outgrowth suppression was comparatively less in M_1R-DREADD mutant over-expressing neurons. The white and blue arrows indicate that localization of GFP-M_1R was mainly restricted in the perikaryon where as GFP was localized both in perikaryon and neurites. LSM510 confocal images acquisition parameters were same for all images. Scale bar: 100 μm. **(E)** Whiskers box (Tukey) showing total neurite outgrowth per neuron. $N = 250$. p-value by one-way ANOVA followed by Dunnett's multiple comparisons test. **(F)** Binning of the entire data set presented in **(E)**.

the nucleus activates transcription of luciferase reporter gene (Kroeze et al., 2015) (**Figure 2A**). We verified expression of the M$_1$R-TEV-tTA and β-arrestin transgenes by immunoblotting (**Figure 2B**). We found significant recruitment of β-arrestin due to basal M$_1$R activation in presence of serum in the culture media in the HTLA cells (**Figure 2C**). This basal activation of M$_1$R occurred in the absence of exogenously supplied agonist. However, fetal bovine serum (FBS) used in the culture media is reported to contain acetylcholine (ACh) (Lau et al., 2013) and this may have constitutively acted upon the M$_1$R. Treatment with the muscarinic agonist, carbachol, significantly increased activation of the M$_1$R by up to ∼7.4-fold (**Figure 2C**).

Endogenous ACh Binds M$_1$R to Constrain Neurite Outgrowth

Adult sensory neurons secrete ACh into the extracellular media when grown in culture (Calcutt et al., 2017). The Tango Assay revealed that ACh present in the serum supplied to the culture media may also act upon M$_1$R and constitutively activate the receptor. We therefore hypothesized that overexpression of M$_1$R in sensory neurons may lead to increased constitutive activation of the receptor by recruitment of trimeric G-proteins, including the Gα12/13 subtype that promotes tubulin destabilization (Roychowdhury et al., 1999). In order to test this hypothesis we transfected DRG neurons with an M$_1$R-DREADD (designer receptors exclusively activated by designer drugs) mutant to eliminate the putative basal activity in M$_1$R-DREADD overexpressing neurons (**Figures 2D,E**). The M$_1$R-DREADD mutant contained two mutations in the conserved orthosteric site residues (Y106C and A196G in the M$_1$R) that minimize responsiveness to ACh (Abdul-Ridha et al., 2013). Overexpression of GFP-M$_1$R and M$_1$R-DREADD caused low levels of neurite outgrowth (**Figures 2D–F**). However, neurite outgrowth in M$_1$R-DREADD neurons was significantly higher than GFP-M$_1$R alone over 72 h of culture (**Figures 2D–F**). This indicates that endogenous ACh may interact with M$_1$R to limit neurite outgrowth.

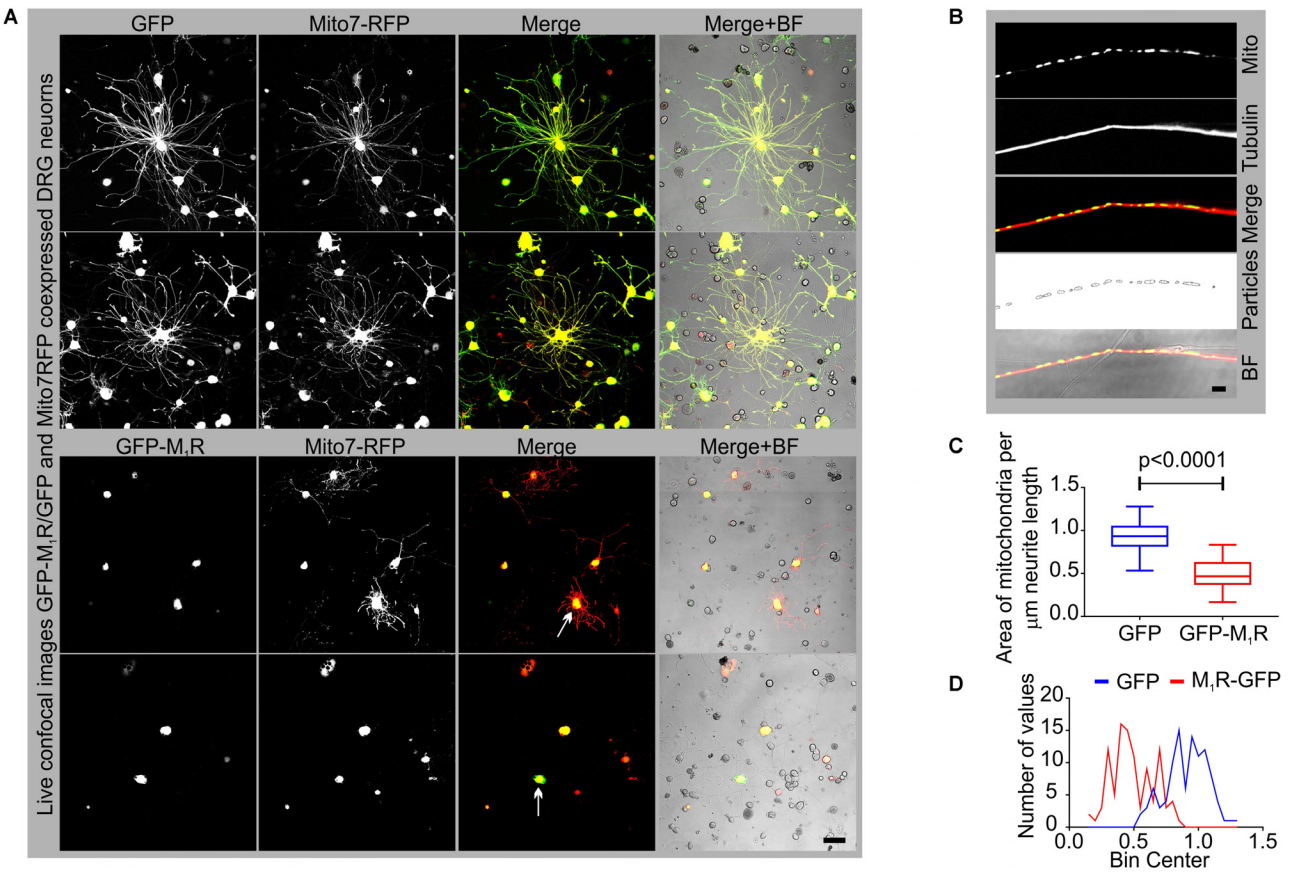


FIGURE 3 | GFP-M$_1$R over-expression in sensory neurons caused reduced mitochondrial abundance. **(A)** Representative images showing the signal of co-expression of GFP/GFP-M$_1$R and mito7-RFP in DRG neurons. BF, bright field. **(B)** Immunofluorescence images showing mito7-RFP and β-tubulin III stained neurites. The 4th panel image showing tracing of the mitochondrial volume used for determining the amount of mitochondria per unit length of the neurites in GFP/GFP-M$_1$R overexpressed DRG neurons. The tracing image was created using ImageJ particle counter plugin. Scale bar: 5 µm. **(C)** Whiskers box (min–max) showing amount of mitochondria in GFP/GFP-M$_1$R transfected neurons. *N* = 101 from three independent experiments, *p*-value by *t*-test (unpaired). **(D)** Binning of the entire data set presented in **(C)**.

Impact of M_1R Overexpression on Mitochondrial Abundance

In an attempt to understand the mechanism of the growth inhibitory effect of M_1R overexpression, we examined the abundance of mitochondria in the neurites by co-expressing DsRed2Mito7 plasmid (**Figure 3A**). The DsRed2Mito7 plasmid expresses DsRed protein tagged with mitochondrial targeting sequence from subunit VIII of human cytochrome C oxidase and therefore localizes specifically to mitochondria. We measured the volume of mitochondria present per unit length (μm) of neurites in the M_1R and GFP overexpressing DRG neurons (**Figure 3B**). M_1R overexpression significantly reduced the abundance of mitochondria in the neurites after 48 h of culture (**Figures 3C,D**). The mitochondria in both M_1R and GFP over-expressing neurons accumulated mitotracker CMXRos dye, indicating that functionality of the mitochondria was not impaired in M_1R overexpressed neurons despite reduced abundance (data not shown).

Reduced Mitochondrial Abundance Was Associated With Impaired Cytoskeletal Structure

To determine whether reduced mitochondrial abundance was related to a defect in the actin or tubulin cytoskeletons, we immunostained DRG neurons that overexpressed GFP/GFP-M_1R with phalloidin and anti-β-tubulin III antibodies. In the presence of M_1R over-expression, immunofluorescent imaging of phalloidin revealed the presence of abundant and continuous actin filaments in the neurites, although the neurite tips were notably thinner compared to those of GFP expressing neurites (**Figure 4A**). In contrast, the β-tubulin III associated cytoskeleton appeared less abundant and discontinuous (**Figure 4B**). Fragmentation of the tubulin cytoskeleton was confirmed using a polymerized tubulin quantification assay in which there was significantly less polymerized tubulin in the DRG neurons overexpressing M_1R compared to those that overexpressed only GFP (**Figures 4C,D**).

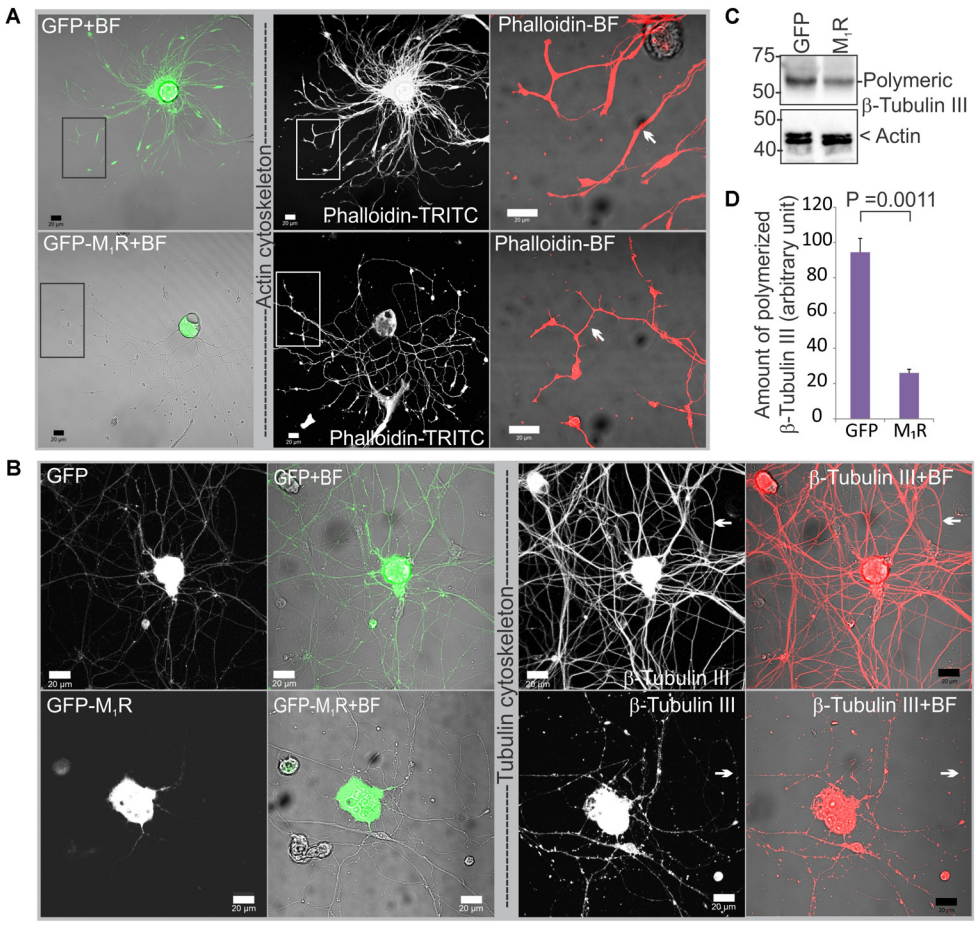


FIGURE 4 | Dorsal root ganglia (DRG) neurons over-expressing M_1R exhibited cytoskeletal defects. **(A)** Actin cytoskeleton in GFP-M_1R and GFP over-expressing DRG neurons. White arrow indicates narrower neurites in GFP-M_1R expressing neurons. **(B)** β-tubulin III associated cytoskeleton in GFP-M_1R and GFP over-expressing neurons. Black rectangular area is shown in a magnified view in the right panel; white arrow indicates discontinuous/continuous tubulin cytoskeleton. BF, bright field. Scale bar: 20 μm. **(C)** Immunoblot and **(D)** bar graph showing relative amount of polymerized tubulin in the M_1R-GFP and GFP over-expressed neurons. The data represent the mean ± SEM of three independent experiments. $P = 0.0011$ calculated by unpaired *t*-test.

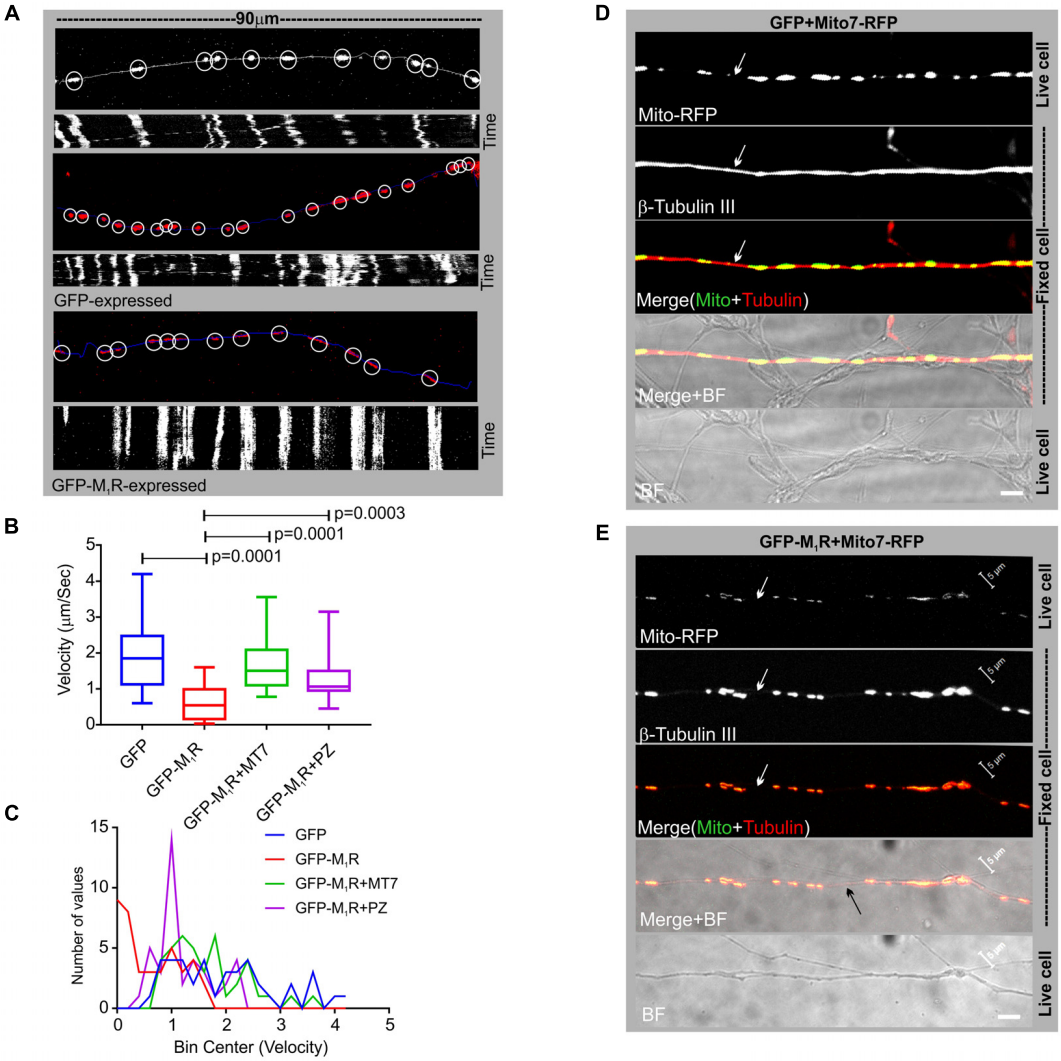


FIGURE 5 | Altered mitochondrial trafficking in M_1R overexpressing neurons was rescued by antagonist treatment. **(A)** Representative images (top panels) of the first frame of a series of live cell time-lapse images showing the expression of DsRed2Mito7 in the mitochondria of GFP/M_1R–GFP expressing neurons. The circles represent mitochondria identified by MTrackJ plugin, which then tracked migration through time in a series of time lapse images and calculated velocity of specific mitochondria. White/blue line represents neurite trace. The bottom panel represents Kymographs generated from live cell time-lapse images. The Kymograph was generated using ImageJ Kymograph plugin. The *X*-axis represents the physical location of mitochondria on the neurite, and the *Y*-axis represents the location of mitochondria in time. Streak of particles traversing the kymograph from left to right in angular lines indicates retrograde/anterograde mitochondrial motion. **(B)** Whisker plot showing mitochondrial velocity. DRG neurons were cultured in LGF media supplemented with 100 nM MT7 or 1 μM pirenzepine for 48 h following transfection. $N = 40$ from three independent experiments, p-value by one-way ANOVA followed by Dunnett's multiple comparisons test. **(C)** Binning of the entire data set presented in **(B)**. **(D,E)** Immunofluorescence images showing mito7-RFP and β-tubulin-III staining in the GFP/GFP-M_1R expressing neurites. White arrows indicate continuous/discontinuous tubulin cytoskeleton in GFP/GFP-M_1R expressing neurites, respectively. Scale bar: 5 μm.

Discontinuous Tubulin Cytoskeleton Was Associated With Reduced Mitochondrial Trafficking in Sensory Neurons Overexpressing M_1R

In order to determine whether the discontinuous β-tubulin cytoskeleton was associated with altered mitochondrial trafficking in growing neurites, we measured velocity of mitochondrial movement in the neurites of DRG neurons that co-expressed GFP/GFP-M_1R and DsRed2Mito7. Time-lapse images of the neurites at 10 s intervals were used to generate kymographs of mitochondrial trafficking (**Figure 5A**). The mean velocity of mitochondria was calculated as 1.9 μm/sec in GFP expressing neurons whereas in GFP-M_1R expressing neurons it was significantly lower at 0.62 μm/sec (**Figures 5B,C**). In addition, immunostaining of neurites with a similar appearance to those used in the mitochondrial velocity measurement revealed a continuous β-tubulin-III cytoskeleton in GFP over-expressed neurons and a discontinuous β-tubulin-III cytoskeleton in M_1R over-expressed neurons (**Figures 5D,E**).

Muscarinic Antagonists MT7 and Pirenzepine Rescued the Cytoskeletal Defect, Aberrant Mitochondrial Distribution and Trafficking in Neurites

We investigated whether pirenzepine or MT7 could overcome M_1R overexpression-induced cytoskeletal defects. M_1R over-expressing neurons were maintained for 48 h and then treated for 24 h with 100 nM MT7 or 1 μM pirenzepine and total neurite outgrowth and mitochondrial velocity (**Figure 5**) and abundance (**Figure 6**) quantified. MT7 and pirenzepine treatment significantly rescued the deficits in the mitochondrial velocity in M_1R overexpressed neurons, with mean mitochondrial velocities of 0.78/1.2 μm/s in MT7/pirenzepine treated neurons being significantly higher than untreated neurons (**Figures 5B,C**). Both MT7 and pirenzepine also caused considerable re-localization of M_1R from the perikarya to the neurites, as revealed by time lapse confocal live cell images over a period of 72 h. This may indicate increased vesicular transport of internalized M_1R (**Figure 6A**). Within the same neuron (shown in **Figure 6A**), following 24 h of drug treatment and upon fixation and immunostaining for β-tubulin III, there was continuity in the β-tubulin III associated cytoskeleton (right panel). MT7 or pirenzepine significantly increased total neurite outgrowth, reversed the reduced neurite caliber and increased mitochondrial abundance in DRG neurons that overexpressed GFP-M_1R (**Figures 6B–E**).

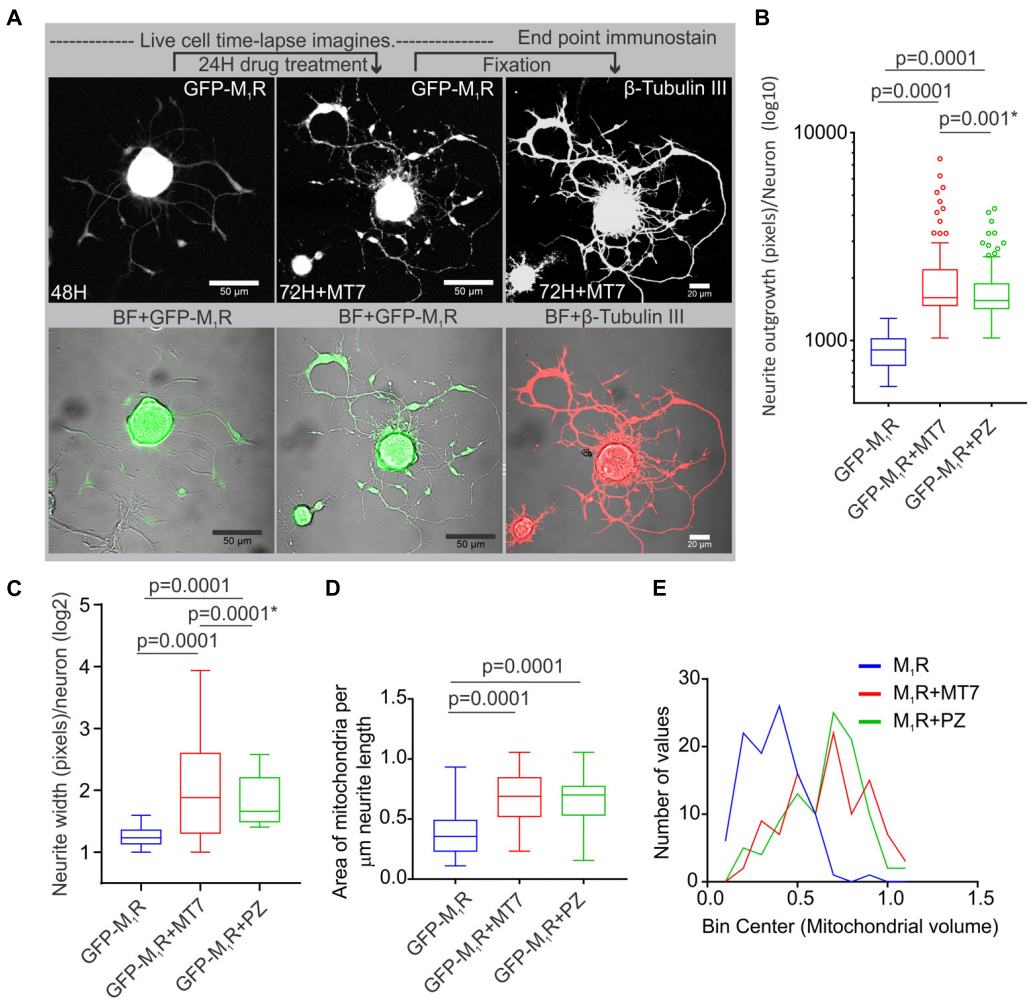


FIGURE 6 | Restoration of cytoskeleton, mitochondrial abundance and neurite outgrowth by M_1R antagonists MT7 and pirenzepine treatment. **(A)** Time-lapse live confocal images of GFP-M_1R over-expression in sensory neurons showing MT7 induced re-localization of M_1R from perikaryon to neurites. The overexpressed neurons were grown in defined media for 48 h and imaged (left panel). Neurons were then treated with 100 nM MT7 for 24 h and imaged (middle panel). Right panel: The same neuron depicted left was fixed and stained for β-tubulin III to show continuity of cytoskeleton. Scale bars: 50 μm. **(B,C)** Whiskers box (Tukey) showing total neurite outgrowth per neuron **(B)** and average neurite width per neuron **(C)**. *p*-value calculated by one-way ANOVA, *N* = 224, 230 and 198 cells, respectively, for **(A)** and *N* = 1250, 1268, 1280, and 1306 cells, respectively, for **(B)**. Asterisks indicate *p*-value calculated by unpaired *t*-test. **(D)** Whisker box plot showing amount of mitochondria in the M_1R expressing neurons treated with 100 nM MT7 or 1 μM pirenzepine. *N* = 101, *p*-values were calculated by one-way ANOVA followed by Dunnett's multiple comparisons tests. **(E)** Binning of the entire data set presented in **(D)**.

Knockdown of Gα13 in Sensory Neurons Reversed the M_1R Overexpression-Induced Tubulin Cytoskeleton Defect

The M_1R-DREADD mutant study raised the possibility that, in normal DRG neurons, basal M_1R activity resulting from binding of endogenous ACh release may destabilize the tubulin cytoskeleton through increased active G proteins. Gα12/13 proteins are known for their effect on cytoskeleton remodeling and the M_1R receptor activates Gα12/13 type G proteins leading to mobilization of the small GTP-binding protein Rho through activation of Rho-GEF (RhoGTPase nucleotide exchange factor) (Haga, 2013). We, therefore, measured the relative expression of Gα12 and Gα13 proteins in sensory neurons (**Figure 7A**). Immunoblots revealed that DRG neurons express significantly

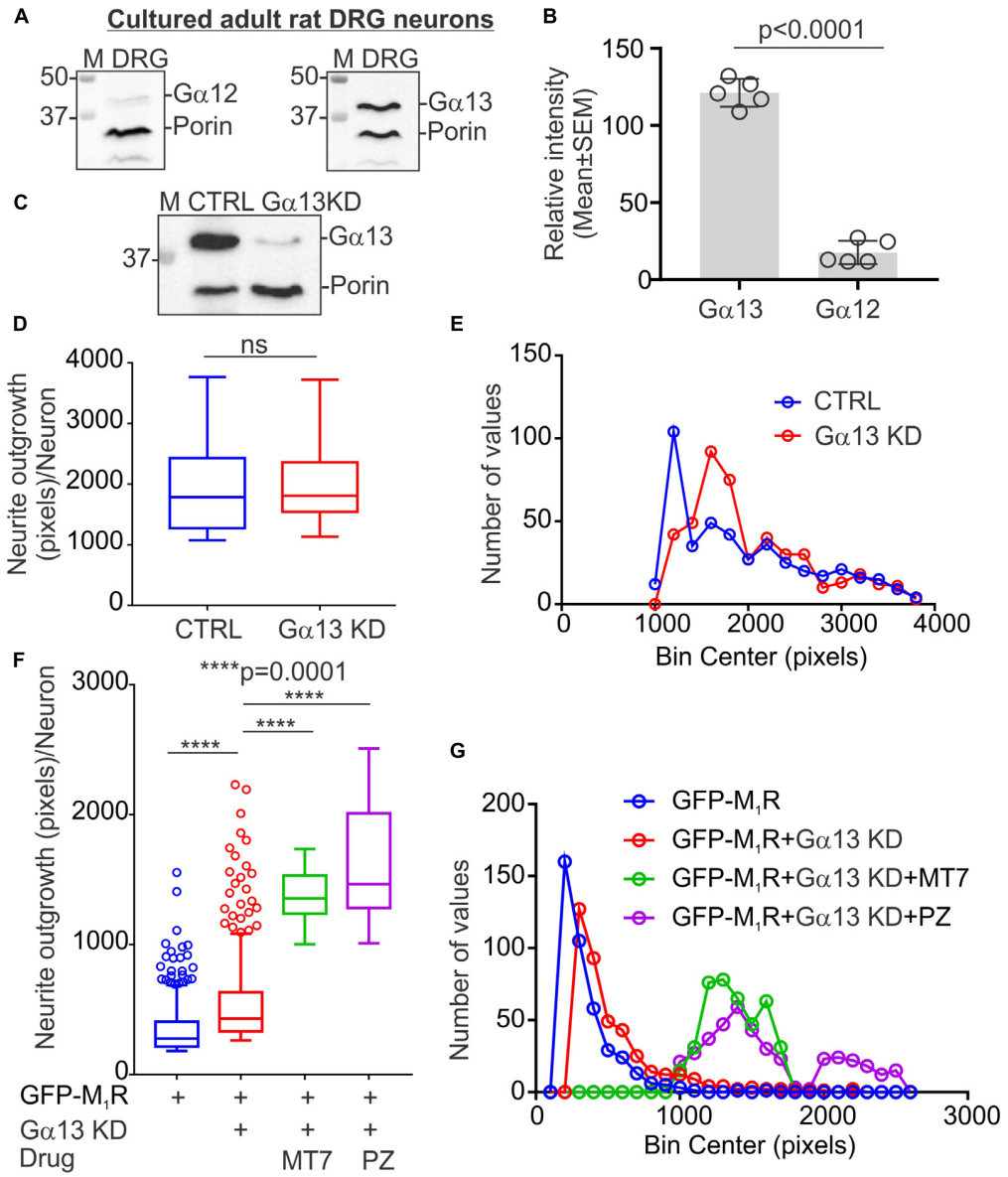


FIGURE 7 | Knockdown of Gα13 reversed M_1R overexpression-induced inhibition of neurite outgrowth. **(A)** Immunoblots showing relative expression of Gα12 and Gα13 proteins in cultured DRG neurons. **(B)** Scatter plot showing relative amount of Gα12 and Gα13 proteins in cultured sensory neurons. *N* = 5 independent experiments. *p*-value was calculated by unpaired *t*-test. **(C)** Immunoblots showing siRNA (cocktail of 3 siRNAs targeted to rat Gα) based knockdown of Gα13 protein in cultured adult rat DRG neurons. **(D,F)** Whisker box (Tukey) showing total neurite outgrowth per neuron. DRG neurons were transfected with a pEFGP-C1-M_1R plasmid and siRNA using Amaxa nucleofection reagent and allowed to grow for 48 h. Scrambled siRNAs were used for control. In drug treatment groups, neurons were cultured in media supplemented with 100 nM MT7 or 1 μM pirenzepine following transfection. The neurons were fixed after 48 h of culture, stained with β-tubulin III and imaged using Cellomics ArrayScan HCS Reader. *p*-value by unpaired *t*-test or one-way ANOVA test followed by Dunnett's multiple comparisons tests. *N* = 432/452 (in **D**) and 406/694 (in **F**). **(E,G)** Binning of the entire data set presented in **(D,F)**.

more Gα13 compared to Gα12 (**Figures 7A,B**). The relative levels of Gα12 and Gα13 expression were comparable to those of the human carcinoma cell line HEK293 (**Supplementary Figure S1A**). We used siRNA to knockdown Gα13 in DRG neurons and used a CRISPR/Cas9 based Gα12/13 null HEK293 cell line to study the effect of cholinergic signaling through Gα13 on the tubulin cytoskeleton (**Figure 7C** and **Supplementary Figure S1A**). The HEK293 cell line has been reported to exhibit a neuronal lineage phenotype and express neuronal proteins (Stepanenko and Dmitrenko, 2015). Therefore, we considered it suitable for this study. The siRNA based knockdown of Gα13 in DRG neurons had no significant effect on neurite outgrowth (**Figures 7D,E**). Interestingly, knockdown of Gα13 in M_1R overexpressed sensory neurons significantly reversed the suppressed neurite outgrowth (**Figures 7F,G**). In addition, treatment of the M_1R overexpressed and Gα13 knockdown neurons with 100 nM MT7 or 1 μM pirenzepine exhibited significantly increased neurite outgrowth (**Figures 7F,G**).

CRISPR/Cas9 Based Gα12/13 Null HEK293 Cells Showed Abundant Tubulin Cytoskeleton but Diminished Actin Stress Fibers

RNA sequencing data in the human protein atlas (HPA) indicates that HEK293 cells express very high levels of α-tubulin compared to β-tubulin III (Uhlen et al., 2005). The transcripts per million bases (TPM) value for α-tubulin (TUBA1B isoform) and β-tubulin III has been recorded as 1923.6 and 13.7, respectively[1]. β-tubulin III was undetectable by immunoblotting in HEK293 cells (data not shown). We, therefore, studied α-tubulin dynamics in CRSIPR/Cas9 based Gα12/13 knockout HEK293 cells. We performed immunofluorescent labeling using phalloidin and anti-α-tubulin specific antibodies to visualize the actin and tubulin based cytoskeletal structures in Gα12/13 knockout HEK293 cells (**Supplementary Figure S1E**). Phalloidin staining revealed that actin stress fibers were diminished in Gα12/13 null cells, with appearance of abundant distinct punctate actin rich focal adhesion points (**Supplementary Figure S1E**). Further, the tubulin cytoskeleton appeared more robust and organized in Gα12/13 null cells (**Supplementary Figures S1E, S2**). The BN-PAGE based microtubule fractionation assay and polymerized microtubule quantitative assay showed Gα12/13 null cells exhibited significantly more polymerized tubulin than wild type HEK293 cells (**Supplementary Figures S1B–D**). Overexpression of M_1R in Gα12/13 null cells and treatment with muscarinic agonist carbachol did not alter the tubulin networks as compared to wild type cells (**Supplementary Figures S2, S3**).

MT7 and Pirenzepine Modulate G Protein Interaction With the M_1R

Adult DRG cultures have very low cell yields, in the range of 250,000 per culture. To enable feasible pull down of protein complexes we used human neuroblastoma SH-SY5Y cultures that allow use of millions of cells. This cell line also exhibits a cholinergic phenotype with ACh secretion, expression of muscarinic receptors and AChE shedding (Yamada et al., 2011). We examined the recruitment of trimeric G proteins to M_1R by Halo-pull down assay and BN-PAGE analysis (**Figures 8**, **9**). The Halo-pull down assay permitted a focus on the over-expressed M_1R with no contamination from endogenous muscarinic receptors of mixed sub-type. The SH-SY5Y cells that overexpressed Halo-M_1R were treated with drugs and Halo-tagged M_1R was pulled down using Halo-linked resin. Subsequently, the pull down product was resolved in SDS-PAGE and immunoblotted with anti-M_1R, anti-Gα12/13 and anti-Gγ/2/3/4/7 antibodies (**Figures 8B–D**). The Halo-M_1R pull down fraction from the drug treated cells showed significantly elevated levels of Gγ/2/3/4/7 and Gα12/13 proteins when compared with untreated cells (**Figures 8E,F**).

The 2D BN-PAGE/SDS-PAGE analysis revealed existence of 2 protein complexes at ∼900 and ∼1200 kDa equivalent molecular weights in Halo-M_1R over-expressing SH-SY5Y cells (**Figures 9A,B**). Each complex was associated with a native ∼100 kDa Halo-tagged M_1R protein and >180 kDa fractions, the latter may be derived from different PTMs of the Halo-M_1R. Treatment with 100 nM MT7 caused a major shift of the ∼900 kDa protein complex to the ∼1200 kDa protein complex within 1 h of treatment, suggestive of recruitment of putative interacting proteins (**Figure 9**, blue and red dotted areas). In contrast, untreated cells did not show any shift in the ∼900 kDa protein complex to the ∼1200 kDa protein complex indicating less or no recruitment of interacting proteins (**Figure 9A**). Further, when the same blots were immunoblotted with anti-Gα12/13 antibodies, the Gα12/13 proteins appeared as spots on a vertical line corresponding to the ∼1200 kDa protein complex in the drug treated cells which suggests possible co-migration and association with M_1R (**Figures 9A,B**, bottom panel).

DISCUSSION

Our recent work has shown that sensory neurons derived from M_1R null mice exhibit enhanced neurite outgrowth (Calcutt et al., 2017). We now demonstrate that over-expression of M_1R inhibited neurite outgrowth, caused disruption of the tubulin cytoskeleton and blockade of mitochondrial trafficking in adult sensory neurons, all of which were rescued by exposure to selective or specific M_1R antagonists. We then used overexpression of GFP-M_1R to identify the molecular pathway components associated with specific M_1R-mediated cellular phenotypes. Based on our data, we propose that ACh mediated signaling via M_1R constrains neurite outgrowth via activation of Gα13 proteins, which in turn limits tubulin polymerization and mitochondrial trafficking within axons.

Overexpression of M_1R in sensory neurons has biological consequences that likely arise from the presence of neuron-derived ACh in the culture environment. Cultured sensory neurons secrete endogenous ACh into the extracellular media to generate a local concentration in the range of approximately 16 μM (Calcutt et al., 2017). This far exceeds the ACh K_d of 25–35 nM measured in several regions of rat brain tissue (Kellar

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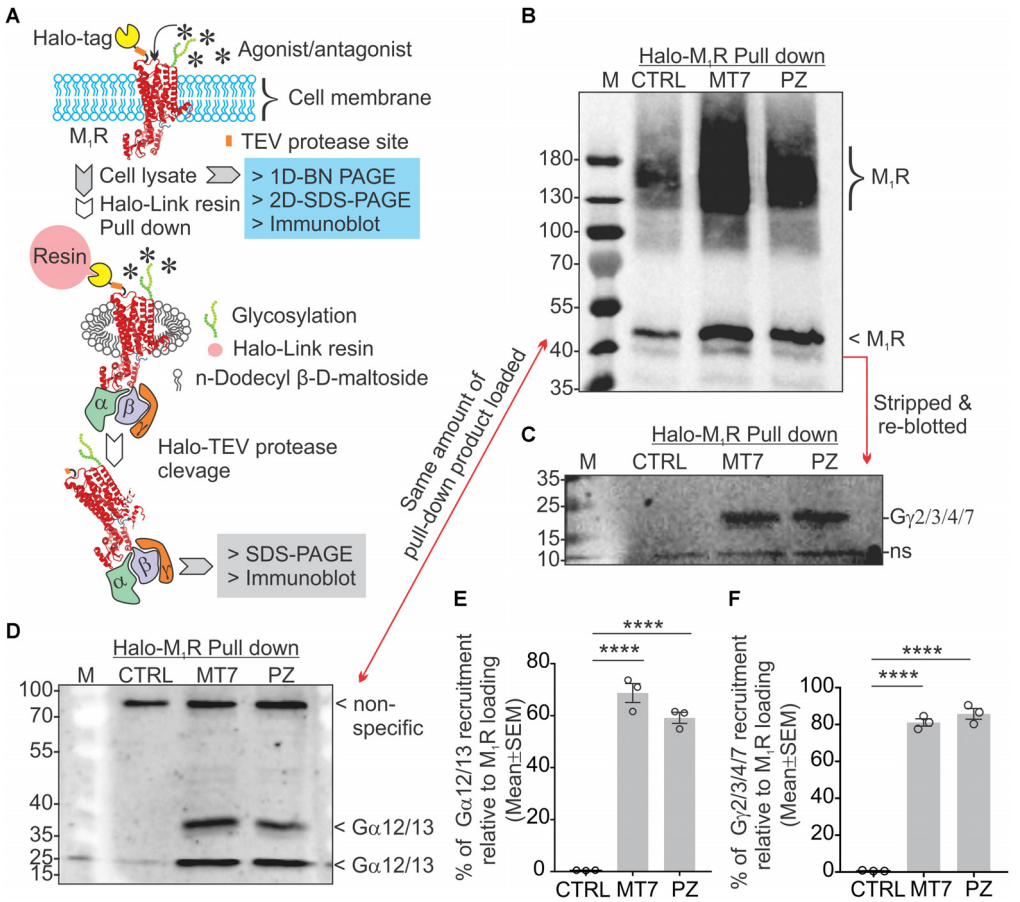


FIGURE 8 | MT7 and pirenzepine elevated sequestration of trimeric G proteins associated with M_1R in SH-SY5Y cells. **(A)** Diagrammatic representation of the experimental strategy. **(B–D)** Halo-tagged M_1R was expressed transiently in sensory neurons and then treated with 100 nM MT7 or 1 μM PZ for 1 h. Cells were then lysed and Halo-M_1R was pulled down using halo-linked resin. The halo tag was cleaved by TEV protease and the cleaved M_1R associated multiprotein complex (MPC) was resolved in denaturing SDS-PAGE and immunoblotted using **(B)** anti-M_1R, **(C)** anti-Gγ2/3/4/7, and **(D)** anti-Gα12/13 antibodies. **(E,F)** Scatter plot showing the relative amount of G proteins (Gα and Gγ, respectively) associated with M_1R following drug treatment. The data represent mean ± SEM of three independent experiments. *p*-values (**** <0.0001) were calculated by one-way ANOVA with Dunnett's *post hoc* multiple comparison test.

et al., 1985) or the K_d of 0.2–0.4 nM measured using rat brain neurons (Pavia et al., 1991; Bakker et al., 2015). In the presence of abundant extracellular ACh, overexpressed. M_1R will trigger activation of Gα13 proteins that leads to the dissociation of tubulin microtubules, as seen in **Figure 4**. The chemogenetically modified M_1R-DREADD mutant significantly reduced M_1R induced growth retardation (**Figure 2**). Thus, ACh-driven basal activity of M_1R is responsible for neurite outgrowth suppression under conditions of M_1R over-expression. In addition, we propose in normal un-transfected neurons that basal endogenous M_1R signaling tonically suppresses neurite outgrowth by restricting mitochondrial (and potentially vesicular) transport, thereby explaining the ability of antimuscarinic drugs to prevent/reverse this constraint.

Knockdown of Gα13 in M_1R-overexpressed neurons significantly reversed the M_1R-induced growth inhibitory effect and protected from tubulin destabilization in growth cones (**Figure 7**). Gα13 is more abundant than Gα12 in sensory neurons and M_1R was linked to activation of Gα12/13 type G proteins that leads to activation of small GTP binding protein Rho through mobilization of RhoGEF (Luo et al., 2001; Siehler, 2009; Haga, 2013). Activation of Gα12/13 leads to stimulation of the Rho/Rho Kinase pathway via a subgroup of Rho guanine nucleotide exchange factors (Fukuhara et al., 2001). Involvement of Rho and ROCK (Rho-associated coiled coil forming protein kinase) in agonist-induced neurite retraction and cell rounding has been reported in N1E-115 neuroblastoma cells (Hirose et al., 1998). Dominant-negative p160-ROCK completely abolished this neurite retraction suggesting a clear link between RhoA-ROCK signaling and cytoskeleton disassembly (Hirose et al., 1998). Activation of neuronal cannabinoid receptors linked to Gα12/13 proteins triggered rapid and reversible contraction of actinomyosin cytoskeleton through a Rho-GTPase and ROCK (Roland et al., 2014). Further, the CRISPR/Cas9 based knockout of Gα12/13 proteins in the HEK293 cell line augmented the abundance of polymerized tubulin (**Supplementary Figure S1**). HEK293 cells have been utilized as a model system for neuronal synapse formation (Biederer and Scheiffele, 2007) as they express

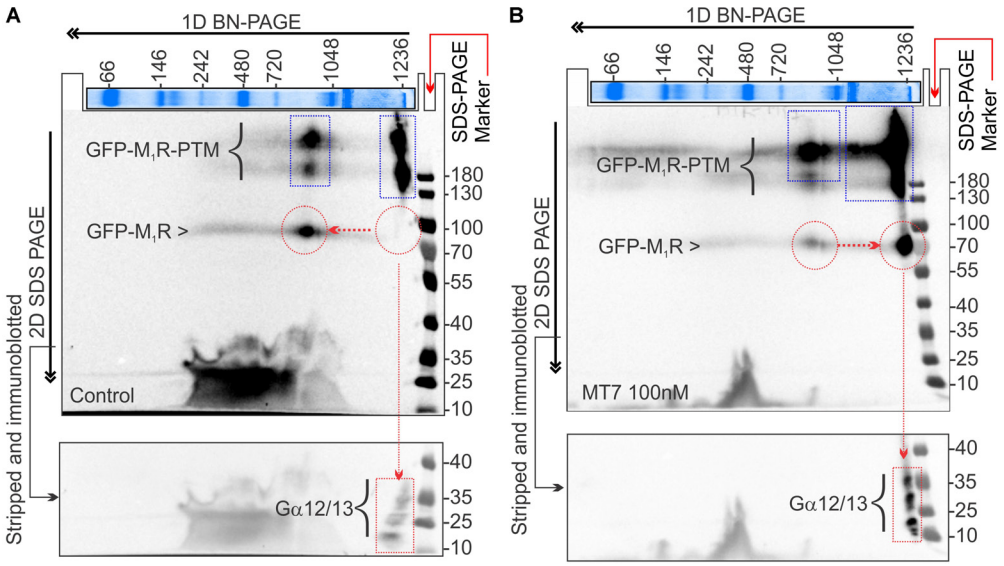


FIGURE 9 | MT7 and pirenzepine elevated sequestration of trimeric G proteins in a protein complex associated with M$_1$R in SH-SY5Y cells. **(A,B)** BN-PAGE analysis showing recruitment and sequestration of G proteins to M$_1$R following MT7 treatment. GFP-M$_1$R transfected cells were treated with 100 nM MT7 and incubated for 1 h. Cell lysates were then separated on 1D BN PAGE followed by 2D SDS-PAGE, **(A)** Control, **(B)** 100 nM MT7 treatment. Top panel: Coomassie stained gel piece showing 1D BN-PAGE separation of native page protein molecular weight marker. The red horizontal and vertical arrows indicate the direction of the 1D BN-PAGE and 2D SDS-PAGE, respectively. The blue rectangle shows PTMs of GFP-M$_1$R associated with 1000 kDa and >1200 kDa MPCs. The red circle indicates native form of the GFP-M$_1$R associated with the MPCs. The red arrow connecting the red circles indicates shift of molecular weight in MPC due to recruitment of G proteins following drug treatment. The red rectangle in the bottom panel shows the co-migration of possible interacting G proteins with the MPCs.

neuronal proteins and have neuronal cell-lineage (Shaw et al., 2002; Stepanenko and Dmitrenko, 2015). Using a NanoBIT split luciferase based RhoA biosensor that detects Gq-induced RhoA activation showed that the RhoA signal is completely lost in the Gα12/13 KO cell (Mercier et al., manuscript in revision, personal communication).

Activated G proteins regulate tubulin polymerization (Schappi et al., 2014) and tubulin binds directly to Gα or Gβγ subunits (Wang et al., 1990; Roychowdhury et al., 1999; Sarma et al., 2003). Activated GTP bound Gα promotes microtubule instability by increasing the intrinsic hydrolysis of GTP-tubulin to the less polymer stable GDP-tubulin (Roychowdhury et al., 1999, 2006). We have modelled this in **Figure 10**. Overexpression of $G\alpha_q$ in a rat pituitary cell line showed a 50% decrease in the ratio of soluble to polymerized tubulin (Ravindra et al., 1996). There is considerable cell type and isoform specificity in Gα mediated tubulin cytoskeleton dynamics (Sarma et al., 2015). We found that Gα12/13 and Gγ(2,3,4,7) were sequestered upon M$_1$R antagonist binding (**Figures 8**, **9**) and it is plausible that these factors also regulate tubulin polymerization in sensory neurons. In addition, some guanine nucleotide exchange factors (GEFs) for Rho GTPases, namely p115 RhoGEF (Kozasa et al., 1998), PDZ-RhoGEFs (Fukuhara et al., 1999), and LARG (Suzuki et al., 2003) can act as direct couplers of Gα12/13 proteins to small GTPases such as RhoA, Rac1, and CDC42, all of which are known to influence microtubule dynamics (Hall and Lalli, 2010). The Gα12/13-RhoGEF-RhoA pathway of GPCR has been implicated in many diseases (Siehler, 2009). Interestingly, while Gα promotes tubulin disassembly by increasing the tubulin GTPase activity, Gβγ subunits preferentially associate with GDP-bound tubulin to promote polymerization and stability of the microtubule (Roychowdhury and Rasenick, 1997; Popova and Rasenick, 2003; Roychowdhury et al., 2006). Our data from non-crosslinked halo-pull down (**Figure 8**) and BN-PAGE analyses (**Figure 9**) show augmented association and occupancy of trimeric G-proteins (Gα12/13) to M$_1$R during MT7 or PZ treatment, which indicates elevated sequestration of these proteins on M$_1$R. We propose that muscarinic antagonist-induced sequestration of trimeric G-proteins restricts their dissociation from the overexpressed M$_1$R associated protein complex and thereby limits their detrimental effect on tubulin polymerization (see **Figure 10**). Further, we posit that the same M$_1$R suppression pathway is occurring in normal un-transfected neurons to limit cytoskeleton formation in axons and is counteracted by these drugs. However, this does not exclude the possibility that other pathways may be involved. For example, Ca^{2+} signaling/homeostasis are known to be responsible for the maintenance of cytoskeletal integrity (Tsai et al., 2015). Cholinergic activation of M$_1$R coupled with the Gq/11 protein generates cytosolic calcium transients via phospholipase-C signaling pathway (Langmead et al., 2008). High Ca^{2+} may act to increase the intrinsic GTP hydrolysis of tubulin and directly destabilize growing microtubule ends without changing the effective concentration of tubulin (O'Brien et al., 1997). Therefore, it is possible that excessive cholinergic signaling may imbalance intracellular Ca^{2+} homeostasis and promotes tubulin destabilization. Antagonist

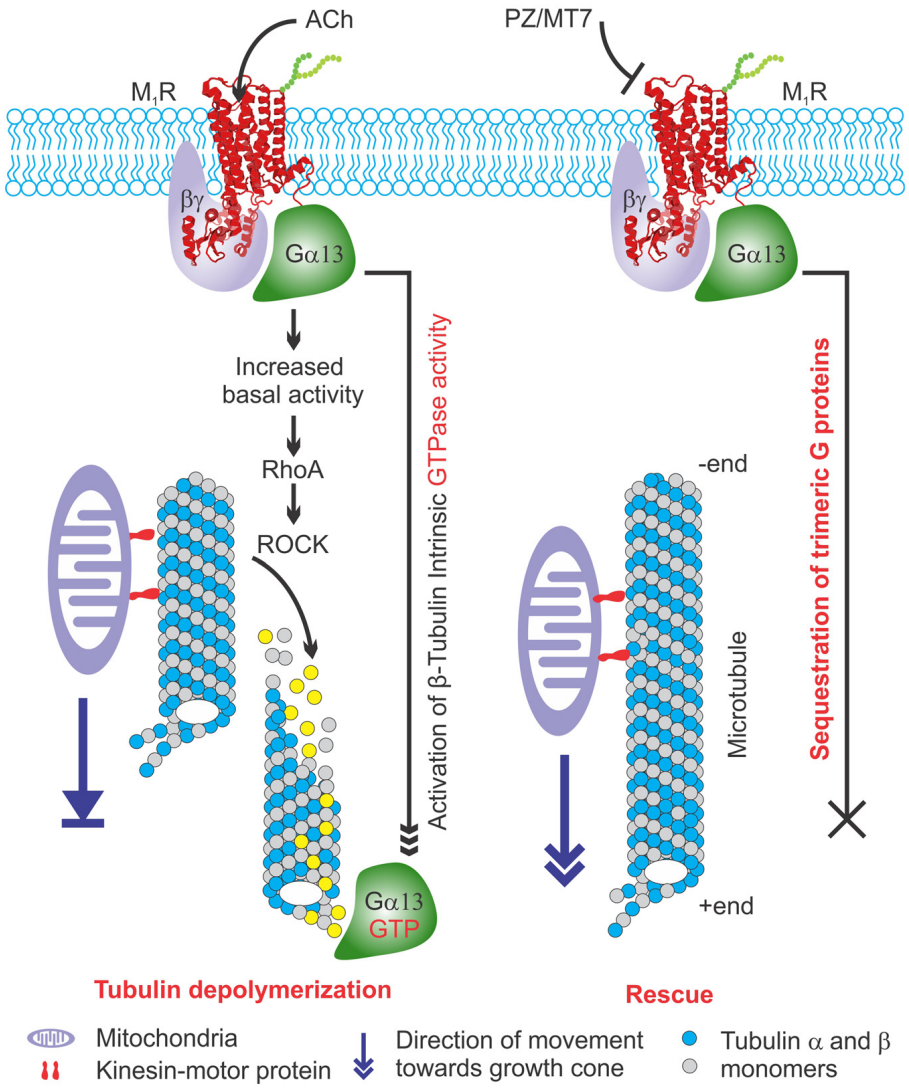


FIGURE 10 | Model explaining the effect of M_1R overexpression and antagonism on tubulin associated cytoskeleton and mitochondrial trafficking. **(Left)** M_1R overexpressing neurons have increased basal activity in response to secreted acetylcholine signaling through overexpressed receptor. This is turn causes recruitment of trimeric G proteins that destabilize tubulin polymers by increasing intrinsic GTPase activity of tubulin. The lack of tubulin cytoskeleton in M_1R overexpressing neuron leads to decreased mitochondrial trafficking and stagnation of mitochondria in the neurites that impairs outgrowth. **(Right)** M_1R overexpression in the presence of antagonists MT7 and pirenzepine (PZ) stabilizes tubulin polymerization. The antagonists bind to the M_1R and may stabilize a specific structural ensemble, which in turn recruit trimeric G proteins. However, the antagonist mediated M_1R structural ensemble may sequester the bound G proteins and makes them unavailable for exerting their effect on tubulin polymerization which in turn stabilizes microtubule cytoskeleton and promotes mitochondrial trafficking and neurite outgrowth.

mediated sequestration of G-proteins may limit this response and protect Ca^{2+} induced tubulin destabilization. However, further experimentation is required to prove this hypothesis. Insertion and site-directed mutagenesis based studies have revealed potential G-protein interaction sites in the i2 and i3 loops in mAChRs (Blin et al., 1995; Liu et al., 1996; Hu et al., 2010). Overexpression of mutated M_1R for disruptive Gα13 binding and subsequent reversal of cholinergic tubulin destabilization effect would provide another means of testing. The exact binding site for Gα13 protein in M_1R needs to be evaluated.

Overexpression of M_1R restricted the number of active mitochondria in neurites, presumably through diminished trafficking as a direct consequence of overexpressed M_1R-induced disruption of the cytoskeleton. The M_1R antagonists, pirenzepine and MT7, were able to protect mitochondrial transport. Pirenzepine and MT7 also enhance mitochondrial oxygen consumption rate and respiratory complex activities through activation of the AMP-activated protein kinase (AMPK)/peroxisome proliferator-activated receptor γ coactivator-1α signaling axis (Calcutt et al., 2017). Mitochondrial function was also enhanced in sensory neuron cultures derived

from M$_1$R null mice. Thus, ACh signaling through M$_1$R can negatively regulate mitochondrial phenotype at multiple levels in the neuron that include trafficking and positioning as well as fine regulation of activity of the respiratory complexes. Optimal regulation of mitochondrial function is critical in distal regions of sensory neurons which, in human peripheral nerve, can be up to a meter from the cell body (Schwarz, 2013). The architecture of sensory neurons poses an extreme cellular environment for mitochondrial distribution and a need to supply energy to the distal endings where energy demand is high (Bernstein and Bamburg, 2003; Chowdhury et al., 2013). Any defect in mitochondrial function is likely to have a profound influence on the axon. Recent *in vivo* studies of mitochondrial transport along the saphenous nerve of adult mice revealed elevated anterograde transport of this organelle in axons undergoing high rates of depolarization and impulse conduction (Sajic et al., 2013). Indeed, loss of function of mitochondrial proteins such as *bcl-w* or mitofusin-2 results in a length-dependent dying-back sensory neuropathy (Baloh et al., 2007; Misko et al., 2010; Courchesne et al., 2011). Genetic ablation of mitochondrial transport also leads to axonal growth failure following axotomy in mice (Zhou et al., 2016). In axons, approximately 30–40% of total mitochondria are constantly engaged in saltatory motion (Lovas and Wang, 2013). It is plausible that the microtubule disruption induced by overexpression of M$_1$R eliminated the basic framework for motor proteins to carry their mitochondrial cargo and potentially other vesicular cargos. As a result, the physical abundance of active mitochondria was diminished in the distal neuritis, which would be expected to deprive the neuronal growth cone of an essential supply of ATP, resulting in suppressed actin treadmilling and neurite outgrowth. This hypothesis, summarized in **Figure 10**, is further supported by reports that several microtubule-targeted chemotherapeutic agents, such as colchicine and vincristine, are known to induce a sensory neuropathy in which the distal aspect of the sensory axon gradually degenerates (Bennett et al., 2014).

Our pre-clinical studies have demonstrated that selective and specific M$_1$R antagonists promote neurite outgrowth in adult sensory neurons *in vitro* (Calcutt et al., 2017). MT7 and pirenzepine were also able to prevent or reverse the distal degenerative neuropathy characteristic of diabetes, chemotherapy-induced and HIV-induced peripheral neuropathy. In the present study, we have reinforced the finding that M$_1$R antagonists are neuronal growth promoters using an unbiased automated high throughput neurite outgrowth measurement technique in a large cohort of sensory neurons. In addition, we have highlighted a molecular mechanism that indicates that treatment with these drugs stabilizes microtubules by sequestering Gα13 proteins and promotes microtubule-based axonal transport of mitochondria, which in turn augments neurite outgrowth. Peripheral neuropathy is a major cause of human morbidity with huge associated health care costs (Gordois et al., 2003; McInnes, 2012). One particularly encouraging implication of our identification of the endogenous M$_1$R-mediated suppression of axonal outgrowth in sensory neurons is that antimuscarinic drugs that can prevent/reverse this process have already been widely used as approved drugs for other indications (Siatkowski et al., 2008). Since small fiber axonal degeneration is an early feature of many peripheral neuropathies, the novel growth-regulating pathway we have identified could be mobilized to prevent or reverse distal neurodegeneration. Our studies support a previously unrecognized therapeutic potential for M$_1$R antagonists in the treatment of peripheral neuropathies and unravels a novel pathway of cholinergic signaling mediated via control of microtubule dynamics through Gα13 signaling.

AUTHOR CONTRIBUTIONS

MS conceptualized, designed and performed all the experiments, analyzed all data, prepared all figures, and wrote the manuscript. PF and NC obtained funding to support the work, aided in the design of experiments, and contributed to writing and editing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00402/full#supplementary-material

FIGURE S1 | Polymerized α-tubulin is augmented in Gα12/13 null HEK293 cells. **(A)** Immunoblots showing expression of Gα12, Gα13, and porin (VDAC1) in wild

type and Gα12/13 null HEK293 cell. **(B)** Immunoblots showing the relative amount of polymerized tubulin in wild type and Gα12/13 null HEK293 cells. **(C)** In BN-PAGE based assay, polymerized tubulin was stabilized in microtubule stabilization buffer and resolved in native gradient gel. The polymerized tubulin microtubules are trapped in the native page at the top (∼1200 kDa) whereas the dimers and monomers migrated to lower molecular weight regions. The first dimension gel was denatured and the proteins were further separated in 2nd dimension SDS-PAGE and immunoblotted. The spots at ∼146 and ∼66 kDa represent α-tubulin dimer and monomers. The red dotted circle represents the polymerized tubulin. Green and blue arrows indicate direction of protein movement. **(D)** Scatter plot showing polymerized tubulin in wild type and Gα12/13 null HEK293 cell as revealed by polymerized tubulin and BN-Page based assays. In the BN-PAGE assay the polymerized tubulin was quantified based on the relative intensity of the monomeric tubulin. *N* = 3/4 independent experiments. Data represented as mean ± SEM, *p*-value by unpaired *t*-test. **(E)** Immunofluorescent images showing actin (phalloidin) and (α-tubulin) in HEK293 (Wild type: WT) and GNAS12/13 knockout (ΔGNAS12/13) cells. Scale bar: 20 μm.

FIGURE S2 | Actin and tubulin cytoskeleton in wild type and G12/13 KO cells. **(A,B)** Confocal immunofluorescent images showing F-actin (phalloidin stained, top panel, **A**) and tubulin (α-tubulin immunolabelled, bottom panel, **B**) cytoskeleton in GFP-M_1R expressing wild type and G12/13 KO cells. Blue arrow indicates focal adhesions, yellow arrow indicates extended cytoplasmic processes enriched in tubulin. Scale bar: 20 μm.

FIGURE S3 | Effect of carbachol on tubulin cytoskeleton in M_1R expressed wild type and G12/13 KO cells. Confocal immunofluorescence images showing the tubulin cytoskeleton. Yellow arrow indicates crest of the cell with dense packing of tubulin. White and blue arrows indicate elongated cytoplasmic processes enriched in tubulin. Blue arrow indicates localization of GFP-M_1R in extended cytoplasmic processes. *Z* represents a particular optical slice in *Z*-stacked image series. Scale bar: 20 μm.

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Alzheimer Disease and Selected Risk Factors Disrupt a Co-regulation of Monoamine Oxidase-A/B in the Hippocampus, but Not in the Cortex

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Monoamine oxidase-A (MAO-A) and MAO-B have both been implicated in the pathology of Alzheimer disease (AD). We examined 60 autopsied control and AD donor brain samples to determine how well MAO function aligned with two major risk factors for AD, namely sex and *APOE* ε4 status. MAO-A activity was increased in AD cortical, but not hippocampal, samples. In contrast, MAO-B activity was increased in both regions (with a strong input from female donors) whether sample means were compared based on: (a) diagnosis alone; (b) diagnosis-by-*APOE* ε4 status (i.e., carriers vs. non-carriers of the ε4 allele); or (c) *APOE* ε4 status alone (i.e., ignoring 'diagnosis' as a variable). Sample means strictly based on the donor's sex did not reveal any difference in either MAO-A or MAO-B activity. Unexpectedly, we found that cortical MAO-A and MAO-B activities were highly correlated in both males and females (if focussing strictly on the donor's sex), while in the hippocampus, any correlation was lost in female samples. Stratifying for sex-by-*APOE* ε4 status revealed a strong correlation between cortical MAO-A and MAO-B activities in both non-carriers and carriers of the allele, but any correlation in hippocampal samples was lost in carriers of the allele. A diagnosis of AD disrupted the correlation between MAO-A and MAO-B activities in the hippocampus, but not the cortex. We observed a novel region-dependent co-regulation of *MAO-A* and *MAO-B* mRNAs (but not proteins), while a lack of correlation between MAO activities and the respective proteins corroborated previous reports. Overexpression of human APOE4 increased MAO activity (but not mRNA/protein) in C6 and in HT-22 cell cultures. We identified a novel co-regulation of MAO-A and MAO-B activities that is spared from any influence of risk factors for AD or AD itself in the cortex, but vulnerable to these same factors in the hippocampus. Sex- and region-dependent abilities to buffer influences on brain MAO activities could have significant bearing on ambiguous outcomes when monoaminergic systems are targeted in clinical populations.

Keywords: Alzheimer disease, depression, monoamine oxidase, APOE4, sex/gender risk

INTRODUCTION

The two isoforms of monoamine oxidase [amine: oxygen oxidoreductase (deaminating) (flavin containing), EC 1.4.3.4, monoamine oxidase (MAO)], i.e., MAO-A and MAO-B, are expressed primarily on the mitochondria. Altered function of either isoform or any associated disruptions in the degradation of biogenic amine neurotransmitter substrates such as serotonin, dopamine, and noradrenaline, have been associated with disorders as varied as depression, cancers, and neurodegeneration (e.g., Parkinson's disease and Alzheimer disease/AD) (Mousseau and Baker, 2012). MAO may also be a factor in neuropathology because of the generation of hydrogen peroxide as a by-product of the deamination reaction. The ensuing oxidative stress and potential for cell death –invariably involving the mitochondria– would be exacerbated when antioxidant systems are compromised, such as during aging (Zhu et al., 2006) and particularly in AD (Crack et al., 2006).

The loss of MAO-A-immunoreactive cells is exacerbated in brainstem monoaminergic nuclei and other regions in late-stage cognitive decline (Chan-Palay et al., 1993). MAO-B is primarily expressed in glia (Riederer et al., 1987) and its role in neurodegeneration has also been widely studied (Inaba-Hasegawa et al., 2017; Riederer and Muller, 2017). MAO-A/-B-associated change in aminergic neurotransmitter levels in AD (Nazarali and Reynolds, 1992; Sjogren et al., 1998; Lai et al., 2002) likely contributes to the neurobiology of a range of neuropsychiatric symptoms observed in AD populations (Li et al., 2014; Vermeiren et al., 2014; Rosenberg et al., 2015). For example, depression, which has been historically associated with monoaminergic dysfunction, has been proposed to represent a prodrome for AD-related dementia in certain vulnerable cohorts (Geerlings et al., 2008; Caraci et al., 2010; Wuwongse et al., 2010), while anxiety and aggression in individuals with mild cognitive impairment might indicate imminent conversion to AD (Gallagher et al., 2011). Furthermore, changes in levels of the MAO-mediated acid metabolites of serotonin and dopamine – i.e., 5-hydroxyindole-3-acetic acid (5-HIAA) and homovanillic acid (HVA), respectively– have long been associated with cognitive deficits and dementia (Gottfries et al., 1969a,b; Nazarali and Reynolds, 1992; Vermeiren et al., 2015) and are observed in diverse mouse models of AD-related pathology (Ash et al., 2010; Wei et al., 2012). The effect of monoamines in AD might extend beyond a contribution to neuropsychiatric symptoms; for example, the cleavage of the Amyloid Protein Precursor (APP: which yields the toxic β-amyloid (Aβ) peptide in AD) is sensitive to 5-HT *via* activation of the 5-HT2a, 5-HT2c, and 5-HT4 receptors (Nitsch et al., 1996; Cochet et al., 2013). In addition, levels of monoamine acid metabolites have been positively correlated with cerebrospinal levels of Aβ (Stuerenburg et al., 2004), while MAO-B-positive astrocytes are detected in the vicinity of amyloid plaques, a hallmark of AD neuropathology (Saura et al., 1994). This latter association has been re-confirmed recently using two-photon imaging in the 5xFAD mouse model of AD (Kim et al., 2016). Reversible inhibitors of MAO-A, such as moclobemide, have shown modest results in elderly individuals, including those presenting with cognitive deficits (Rosenzweig et al., 1998; Gareri et al., 2000), whereas inhibitors of MAO-B, such as l-deprenyl, might provide benefit in the early stages of clinical neurodegenerative diseases, such as Parkinson's disease (Magyar et al., 2004; Youdim et al., 2006) and mild AD-type dementia (Riederer et al., 2004).

Female sex is a risk for AD and estrogen has been shown to reduce *MAO-A* mRNA in the macaque dorsal raphé (Gundlah et al., 2002) and several regions of the rat brain (Holschneider et al., 1998), while progesterone affects platelet MAO (i.e., MAO-B) activity (Klaiber et al., 1996). Regional brain MAO-B activity is increased in AD (Adolfsson et al., 1980; Oreland and Gottfries, 1986) and mean platelet MAO-B activity is increased in female AD patients (Robinson et al., 1971; Veral et al., 1997), yet it is not clear how much of this change might rely on the patient's biological sex or on other factors such as the widely-acknowledged genetic risk for late-onset AD, i.e., the *APOE* ε4 allele (Poirier et al., 1993). A single ε4 allele can impart significant risk in women, with little effect in men (Payami et al., 1996; Farrer et al., 1997; Bretsky et al., 1999). Furthermore, female carriers (but not males) might be more likely to have been depressed *prior* to developing AD (Delano-Wood et al., 2008) with the interaction between ε4 genotype and depression increasing the risk of incident dementia (Meng and D'Arcy, 2013). Although a larger cohort study has not supported the *APOE* ε4 allele as a risk factor for depression as a prodrome in AD (Locke et al., 2013), another study has found an interaction between *APOE* ε4 genotype and depression, as well as a higher risk of incident mild cognitive impairment in male depressed patients (Geda et al., 2006). Despite evidence such as this, most clinical research views male and female *APOE* ε4 carriers as having equal risk (Altmann et al., 2014) and persist in pooling data from males and females, which has likely biased outcomes and any extrapolation of data generated in these contexts.

The fact that monoaminergic systems are affected in early stages of AD is clear. What remains unclear is whether monoaminergic function is more closely aligned with risk or with diagnosis of AD. Previous studies have associated the *APOE* ε4 allele with decreased *mao-A* mRNA expression in C6 glioblastoma cells (Liu et al., 2012), while risk in a Brazilian cohort of 128 late-onset AD patients was associated with combined *MAO-A* polymorphism (allele 1; lower transcription efficiency), the short variant of the serotonin transporter promoter, and a positive *APOE* ε4 status (Nishimura et al., 2005). Our work differs from previous reports in that we used several stratification approaches that would allow us to draw some conclusions as to the degree to which factors such as brain region, sex, and *APOE* ε4 status could be influencing any AD-related, MAO-associated neurochemical phenotype. Our results strongly align changes in MAO (particularly MAO-B) function with a diagnosis of AD and with the *APOE* ε4 risk factor. As importantly, or perhaps more so, a closer examination of the data suggests that MAO-A and MAO-B activities in the cortex are tightly co-regulated and that this co-regulation is not overtly affected by the donor's sex or *APOE* ε4 status, or by a diagnosis of AD. This is in contrast to the hippocampus where the co-regulation is far less robust in the female and where this co-regulation appears to be

vulnerable in carriers of the *APOE* ε4 allele and in individuals with a diagnosis of AD. This could explain some of the region-dependent pathology associated with AD and, possibly, some of the variable response to MAO inhibitors in clinical trials.

MATERIALS AND METHODS

Human Brain Samples

Sixty cortical samples were obtained from the Douglas–Bell Canada Brain Bank (McGill University, Montréal, QC, Canada). These included 16 male and female (M/F) early-onset AD (EOAD: 7M/9F), 18 late-onset/sporadic AD (LOAD: 8M/10F), and 26 controls (CTL: 12M/14F) matched as closely as possible for age and sex (see section "*Donor Statistics*," Supplementary Table 1). Cortical samples were randomly chosen from left and right hemispheres, and represent a mix of superior and middle frontal cortices (Brodmann Areas 9/46, respectively). These areas are associated with executive function and cognition, and are a target of relative hypoperfusion in AD patients, particularly those with a co-morbid depression (Levy-Cooperman et al., 2008). Diagnoses were histopathologically confirmed by on-site pathologists (using CERAD criteria). Wherever available, we examined the corresponding hippocampal samples from each donor. Our 60-sample sets were randomly assigned at source, without any information available regarding the donors' *APOE* ε4 status.

De-identified samples were assayed under the University of Saskatchewan's Research Ethics Office Certificate of Approval 'Bio 06-124' (held by DM).

Reagents and Antibodies

The anti-GAPDH antibody (14C10: #2118) was purchased from Cell Signaling Technology (Whitby, ON, Canada). [2-^{14}C]-5-HT binoxalate ([^{14}C]-5-HT: NEC-225) and β-[ethyl-1-^{14}C]-PEA hydrochloride ([^{14}C]-PEA: NEC-502) were purchased from PerkinElmer Life Sciences (Waltham, MA, United States). The MAO-A (H-70) and MAO-B (C-17) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States). The anti-Apolipoprotein E antibody (D6E10; ab1906) was purchased from Abcam. IgG-HRP conjugates were from Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada). All other reagents were obtained from commercial sources.

Monoamine Oxidase (MAO) Catalytic Activity

MAO-A and MAO-B catalytic activities were estimated using 250 μM [^{14}C]-5-HT and 50 μM [^{14}C]-PEA, respectively, and 100 μg total cell protein *per* reaction in oxygenated potassium phosphate buffer (0.2 M, pH 7.8) (Cao et al., 2009b). The 10-min reaction was terminated by addition of 25 μL of HCl, after which the radiolabeled reaction products were extracted into 1 mL of water-saturated ethyl acetate/toluene. Samples were centrifuged and 700 μL of the organic phase was used to determine radioactive content using scintillation spectrometry. Individual sample means represent the average of 3–5 replicates.

Immunodetection

Standard SDS-PAGE denaturing conditions were used to detect expression of target proteins in cleared (12,000 × *g*, 10 min, 4°C) lysates (15 μg/lane) (Cao et al., 2009b; Wei et al., 2012). Detection relied on enhanced chemiluminescence and ImageJ 1.32j[1] was used for densitometric analyses of scanned blots.

Quantitative Real-Time PCR

Total RNA was isolated using an RNeasy® Mini Kit (Qiagen; Mississauga, ON, Canada) and reverse-transcribed to cDNA using iScript Select cDNA Synthesis Kit from Bio-Rad (Cat # 170-8897). Gene expression was quantified using Taqman® primers, specifically (Hs00165140_m1, spans Ex6-7 boundary of *MAO-A*) and (Hs01106246_m1, spans Ex2-3 boundary of *MAO-B*) from Applied Biosystems (Foster City, CA, United States). Triplicate reactions were performed using the Taqman Universal Master Mix, FAM-labeled Taqman Gene Expression assays for the target gene, VIC-labeled Taqman Endogenous Control GAPDH, and 500 ng of cDNA and thermocycling parameters as described previously (Wei et al., 2012).

High Pressure Liquid Chromatography (HPLC)

Levels of MAO-mediated acid metabolites of serotonin and dopamine, i.e., 5-HIAA and HVA, respectively, were determined by HPLC with electrochemical detection as described before and based on comparing peak heights of the analytes to those of a set of authentic standards processed in parallel (Wei et al., 2012). Note that HVA, rather than DOPAC, is the primary acid metabolite of dopamine in the human brain (Ebinger et al., 1987; Vermeiren et al., 2015). In addition, levels of DOPAC were not consistently detected across samples. As such DOPAC was excluded from any analyses.

APOE Genotyping

The APOE2, APOE3, and APOE4 variants differ by arginine (Arg) and/or cysteine (Cys) substitutions, i.e., Cys/Cys (E2), Cys/Arg (E3), and Arg/Arg (E4), at positions 112 and 158, respectively. *APOE* restriction isotyping for the two single nucleotide polymorphisms encoding for these substitutions was done as described fully elsewhere (Nyarko et al., 2018). Briefly, PCR amplification was performed on 500 ng of genomic DNA and the resulting 226 bp amplicon was restricted with *Afl*III and *Hae*II. The fragments were resolved on a 10% non-denaturing, polyacrylamide gel and visualized by staining with GelRed (Biotium). Genotyping identified ε2, ε3, and ε4 homo- and heterozygotes, with the frequency of ε4 carriers across cases in our sample set supporting those reported in the literature (Poirier et al., 1993).

[1] http://rsb.info.nih.gov/ij/

Expression of Human APOE Variants in Immortalized Neuronal and Glial Cell Cultures

Human *APOE* cDNA was cloned from an *APOE* ε3/ε3 donor sample as well as from an *APOE* ε4/ε4 donor sample. Primers used were: sense 5′-*Hind*III-APOE: tgc aag ctt atg aag gtt ctg tgg gct gcg ttg; and antisense 5′-*BamH*I-APOE: agt gga tcc tca gtg att gtc gct ggg cac ag. The fragments were subcloned into pcDNA3.1/Hygro(+) and confirmed by sequencing. The *APOE* ε3 cDNA was used to generate an Arg158Cys substitution by mutating the cgc (Arg) codon to a ***tgc*** (Cys) codon using the sense-5′ primer: at gac ctg cag aag ***tgc*** ctg gca gtg tac cag, and complementary primer: tg gta cac tgc cag ***gca*** ctt ctg cag gtc atc. The resulting *APOE* ε2 cDNA was confirmed by sequencing.

Rat C6 glioblastoma cells (ATCC: CCL-107) and mouse immortalized HT-22 hippocampal cells (Maher and Davis, 1996) were transfected with the individual *APOE* ε2, ε3, or ε4 expression plasmids. Cell pellets were collected 24 h later for mao-A and mao-B catalytic activity assays, for Western blotting, and for *mao-A* and *mao-B* mRNA levels.

Statistical Analyses

Any possibility of bias using our autopsy-derived data was minimized by having some individuals assay de-identified samples and others perform the analysis. For example, for Western blotting, one individual prepares, resolves, and probes the protein blot, another individual scans the blot and performs the densitometric analysis, and another individual analyzes the data. This, in principle, effectively dilutes the possibility of any single observer bias. Data were analyzed using either the Mann–Whitney *U* test or ANOVA (Kruskal–Wallis) with *post hoc* multiple comparisons using Dunn's test. Significance was set at $P < 0.05$, but values that fell between 0.051 and 0.10 were discussed as *tendencies*. Only those *P*-values associated with a significant change are included (*note*, a non-significant *P*-value might be included to support a pivotal conclusion). Data are represented as scatter plots with the lines representing the sampling mean ± standard deviation. Correlation statistics were based on Pearson's correlation coefficient (*r*). Note that we will use the term 'correlated' to mean 'positively correlated,' unless otherwise stated. We acknowledge that a limitation in the interpretation of our data is that our 60-sample set was not sufficiently powered to undertake statistically relevant three-way stratification, i.e., sex-by-diagnosis-by-genotype.

RESULTS

This report identifies changes in monoaminergic profiles that align strongly with the donor's sex and *APOE* ε4 status (i.e., as carrier, or not, of an ε4 allele), and also identifies a regionally dependent co-regulation of MAO-A and MAO-B function. In the cortex, this co-regulation is spared from any influence of risk factors for AD, such as sex or the *APOE* ε4 allele. In the hippocampus, this co-regulation is disrupted by these same risk factors.

Donor Statistics

De-identified information on the basic metrics of the donors – i.e., sex and age at time of death, and the post-mortem interval and brain weight– are summarized in Supplementary Table 1. Briefly, there was the expected difference in the age of the donors based on diagnosis (control: 70.7 ± 12.5; EOAD: 58.1 ± 7.8, $P < 0.05$; LOAD: 83.2 ± 5.8, $P < 0.01$). There was a difference in average brain weight (g) based on diagnosis (control: 1232 ± 137; EOAD: 1016 ± 200, $P < 0.01$; LOAD: 1036 ± 122, $P < 0.001$). There was no difference in post-mortem interval between the groups.

In general, we did not observe any significant correlation between the activity of either isoform in cortical samples and (a) post-mortem interval, (b) age at time of death, or (c) brain weight. In contrast, we did observe a few significant correlations in the hippocampal sample sets. These included a negative correlation ($P = 0.0021$) between MAO-A activity and age at time of death in the LOAD sample set; a positive correlation ($P = 0.0053$) between MAO-A activity and brain weight in the control sample set; and a negative correlation ($P = 0.0160$) between MAO-B activity and brain weight in the EOAD sample set.

MAO-A and MAO-B Parameters Based on the Donor's Diagnosis

MAO-A activity was increased in cortical AD samples ($P = 0.0528$), but not in hippocampal AD samples ($P = 0.3614$). In contrast, MAO-B activity was increased in AD samples in both cortex ($P = 0.0009$) and hippocampus ($P = 0.0005$), with contributions from both sexes (cortex: males, $P = 0.0631$ and females, $P = 0.0218$; hippocampus: males, $P = 0.0331$ and females, $P = 0.0052$) (**Figure 1**). The MAO-B protein was selectively increased in cortex ($P = 0.0254$) and in hippocampus ($P = 0.0031$), and aligned primarily with changes in female samples (**Figure 2**). Representative Western blots for MAO-A and MAO-B in male as well as female cortical and hippocampal samples are included in **Figure 2**.

MAO-A and MAO-B Activities Based on Diagnosis and *APOE* ε4 Status

We recently reported on the frequency of ε4 carriers across cases in our sample set, which includes ε3/ε3 and ε4/ε4 homozygotes as well as ε2/ε3, ε2/ε4, and ε3/ε4 heterozygotes (Nyarko et al., 2018). A limitation of our sample set is that it does not allow for stratification based on individual APOE allele heterozygosity and homozygosity. Thus, *APOE* ε4 status was used as a dichotomous nominal variable, i.e., a donor was identified as either a carrier (having at least one ε4 allele) or a non-carrier.

Any change in MAO-A activity that we observed in our cortical samples (**Figure 1** and noted above) was lost when the data were stratified by diagnosis-by-*APOE* ε4 status (**Figure 3**). In contrast, MAO-B activities were increased in cortex ($P = 0.0165$) and in hippocampus ($P = 0.0412$) in AD donors who carried the ε4 allele. We did observe a trend toward a significant interaction between diagnosis and ε4 status on MAO-B activity in non-carriers in cortex ($P = 0.1008$), but not in hippocampus ($P = 0.3301$).

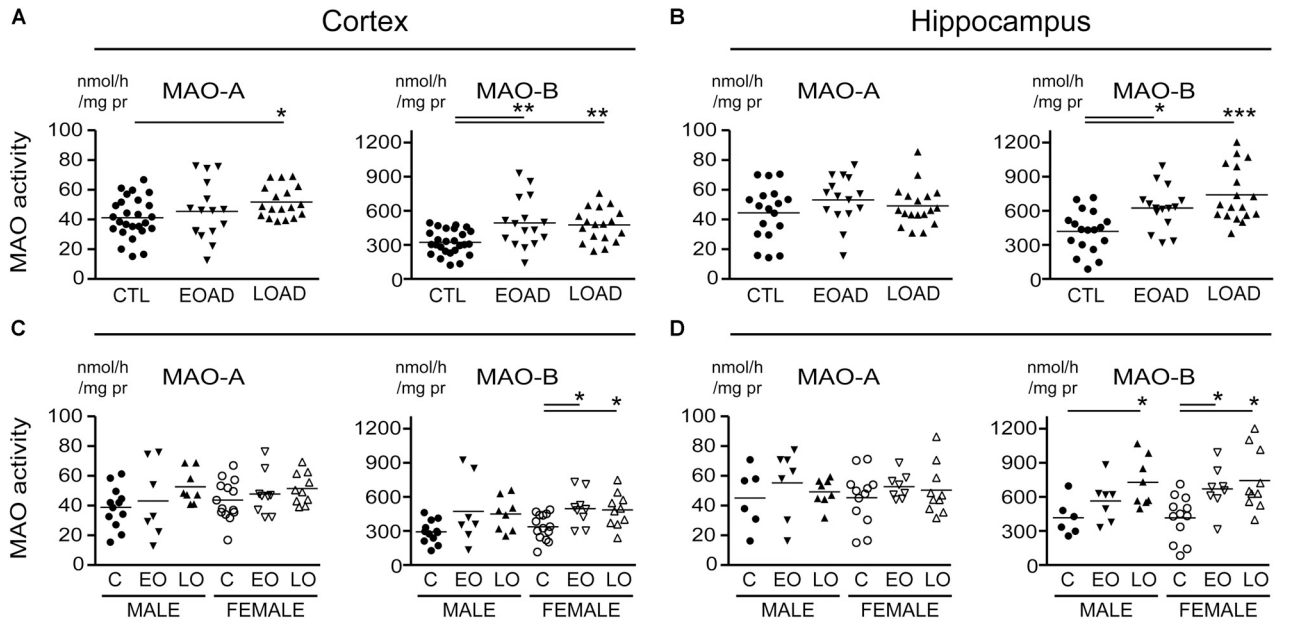


FIGURE 1 | Monoamine oxidase (MAO) activites in human brain: data analyzed based on the donor's diagnosis. MAO-A and MAO-B activites were measured in **(A)** the cortex and **(B)** the hippocampus of control (CTL, C), Early-Onset AD (EOAD, EO), and Late-Onset AD (LOAD, LO) samples by radioenzymatic assay. **(C,D)** The same samples re-analyzed based on the donor's sex. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ between indicated groups.

MAO-A and MAO-B Activities Based on *APOE* ε4 Status Alone

Our recent report suggested that *APOE* ε4 might be exerting effects independently of any diagnosis of AD (Nyarko et al., 2018). Based on that observation, we re-evaluated our data based strictly on the donor's *APOE* ε4 status (i.e., excluding 'diagnosis' as a variable in our analyses). This revealed an increase in MAO-B that was limited to hippocampal samples from carriers of the ε4 allele ($P = 0.0033$), with contributions from males ($P = 0.0524$) as well as females ($P = 0.0262$) (**Figure 4**). Interestingly, we also observed a significant increase in hippocampal MAO-A activity in male carriers of the ε4 allele ($P = 0.0292$). The regional changes in MAO-B activities in carriers of the ε4 allele corresponded to changes in MAO-B protein in the hippocampus ($P = 0.0524$), but not in cortex ($P = 0.1148$) (**Figure 5**).

MAO-A and MAO-B Activities Based Strictly on the Donor's Sex

There is evidence that platelet MAO activity (i.e., MAO-B) increases in women as they age (Robinson et al., 1971; Veral et al., 1997). As such, we examined the regional MAO activities based strictly on the donor's sex. Comparing sample means between males and females did not reveal any significant difference in MAO-A or MAO-B activities or MAO proteins, except for a significantly higher level of MAO-A protein in female cortical samples ($P = 0.0019$) (**Figure 6**).

Up to this point, our analyses were based on comparing samples means. We did observe parallel increases in MAO-A and MAO-B activity in some of our data sets. For example, we observed increases in both MAO-A and MAO-B activities in the AD cortex (**Figure 1**) and increases in both MAO-A and MAO-B activities in the hippocampus of ε4-positive males (**Figure 4**). We wondered whether any of these changes might be correlated, and whether any potential correlation was influenced by the stratification model used. The summaries of our correlational analyses are provided in Supplementary Tables 2–7. We provide the significant highlights of these analyses below.

Cortical MAO-A and MAO-B Activities Are Highly Correlated, but This Is Not True for the Hippocampus

Beginning with the simplest of models, i.e., based strictly on the donor's sex, we found that cortical MAO-A and MAO-B activities were significantly correlated in males ($P < 0.0001$, $r = 0.730$) and females ($P = 0.0004$, $r = 0.581$). While hippocampal MAO-A and MAO-B activities were significantly correlated in males ($P = 0.0252$, $r = 0.487$), they were not correlated in females ($P = 0.1352$) (**Figures 7A,D**). Focussing on *APOE* ε4 status revealed that MAO-A and MAO-B activities in the cortical samples were correlated in non-carriers of the ε4 allele (males, $P = 0.0005$, $r = 0.846$; females, $P = 0.0082$, 0.622) as well as in carriers (males, $P = 0.0058$, $r = 0.654$; females, $P = 0.0848$, $r = 0.460$). In contrast, hippocampal MAO-A and MAO-B activities were correlated in non-carriers of the ε4 allele (males, $P = 0.0403$, $r = 0.776$; females, $P = 0.0154$, $r = 0.612$), but not in those donors that did carry the allele (males, $P = 0.7079$; females, $P = 0.4963$) (**Figures 7B,E**). Finally, stratifying for 'diagnosis' (i.e., control *versus* EOAD or LOAD) revealed that MAO-A and MAO-B activities were correlated in cortical control ($P < 0.0001$, $r = 0.784$), EOAD ($P = 0.0027$, $r = 0.696$), and LOAD ($P = 0.0419$,

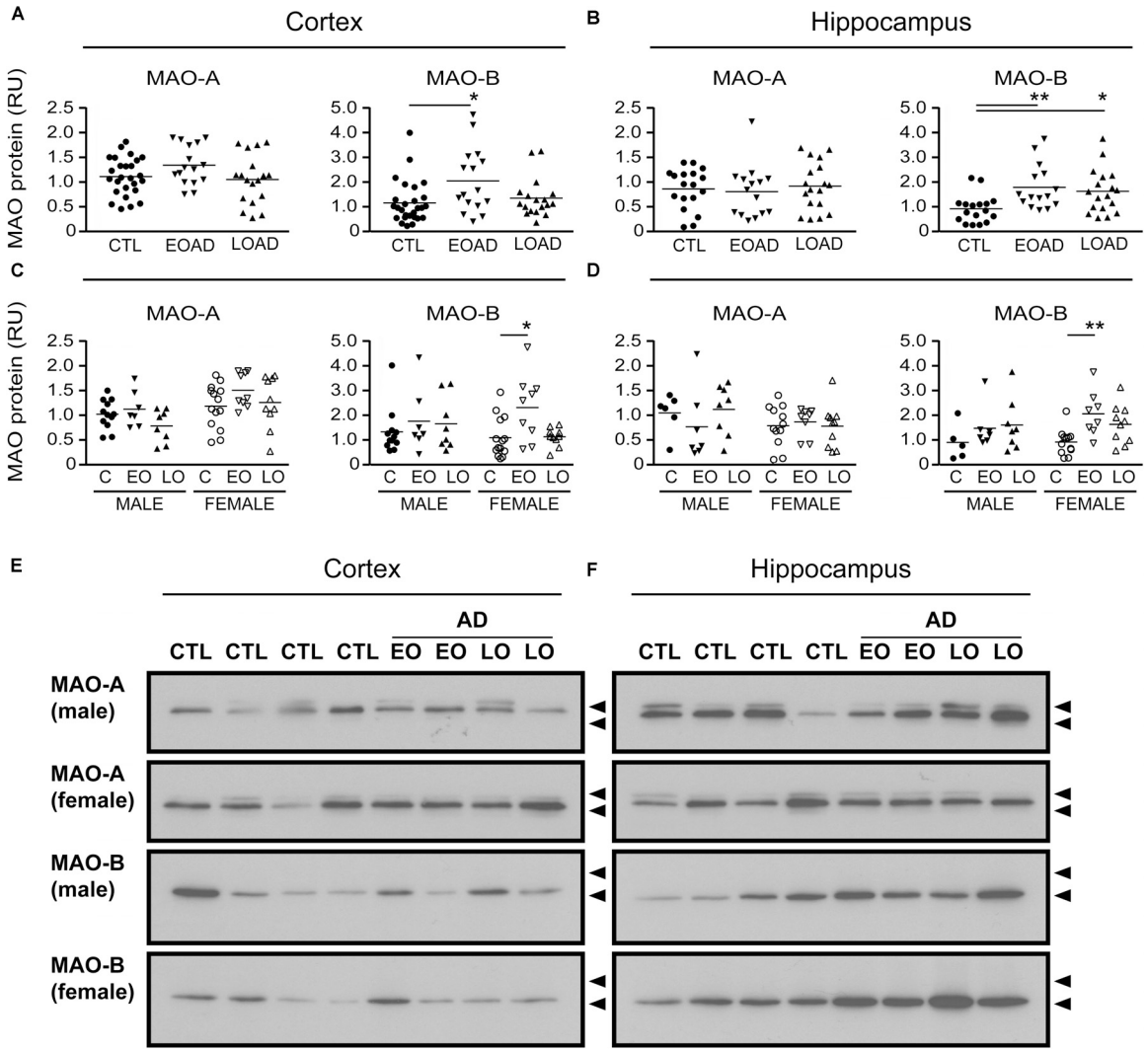


FIGURE 2 | MAO proteins in human brain: data analyzed by diagnosis. Western blots were probed for MAO-A and MAO-B proteins. Scans were analyzed by densitometry in **(A)** the cortex and **(B)** the hippocampus of control (CTL, C), Early-Onset AD (EOAD, EO), and Late-Onset AD (LOAD, LO) samples. **(C,D)** The samples were separated based on the donor's sex. $^{*}P < 0.05$; $^{**}P < 0.01$ between indicated groups. **(E,F)** Representative immunoblots show the MAO-A and MAO-B protein bands in **(E)** cortical and **(F)** hippocampal extracts from male and female donors. Lower arrowhead (55 kDa) and upper arrowhead (72 kDa) represent protein standard markers.

$r = 0.484$) samples. Once again, we observed a different pattern in hippocampal MAO-A and MAO-B activities; indeed, they were correlated in control samples ($P = 0.0012$, $r = 0.702$), less so in EOAD samples ($P = 0.0827$, $r = 0.462$), and not at all in LOAD samples ($P = 0.7255$) (**Figures 7C,F**).

MAO-A and MAO-B Proteins Are Generally Not Correlated, Regardless of the Sex, *APOE* ε4 Status, or Diagnosis of the Donor

A tendency for correlation in the cortex (males: $P = 0.0822$; females: $P = 0.0571$) (if the data are stratified by sex alone, **Figure 8A**) and in cortical samples from male non-carriers of the ε4 allele ($P = 0.0299$) (**Figure 8B**) were observed. Aside from these few exceptions, MAO-A and MAO-B protein expression levels did not correlate, regardless of the stratification model used or the region being tested (**Figures 8C–F**).

MAO-A and *MAO-B* mRNA Transcript Levels Are Correlated in the Cortex, but Not in the Hippocampus

MAO-A and *MAO-B* mRNA transcript levels were compared in the simplest models, i.e., based strictly on the donor's sex. *MAO-A* and *MAO-B* mRNAs were significantly correlated in males ($P = 0.0001$, $r = 0.687$) and females ($P = 0.0083$, $r = 0.466$) in the cortex (**Figure 9A**). However, there was no correlation in the corresponding male hippocampal samples

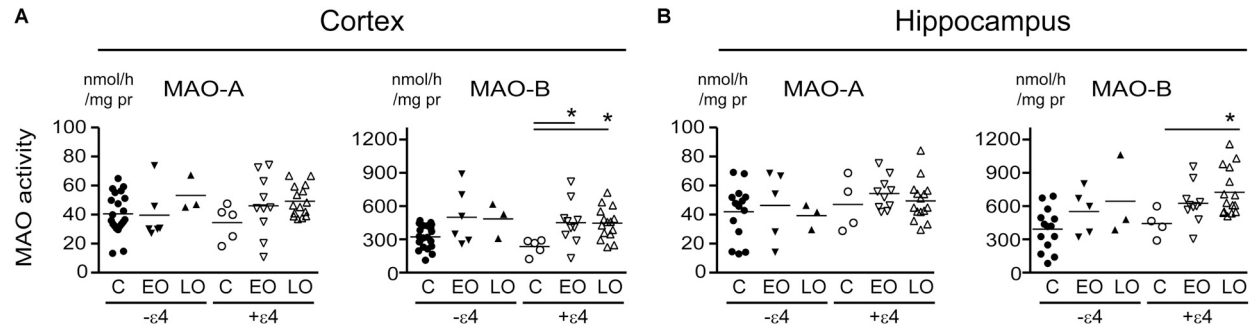


FIGURE 3 | MAO activites in human brain: data stratified by diagnosis-by-*APOE* ε4 status. MAO-A and MAO-B activites were measured in **(A)** cortical and **(B)** the hippocampal samples. Donors with a diagnosis of either Early-Onset AD (EO) or Late-Onset AD (LO), or controls (C) were separated based on their *APOE* ε4 status, i.e., carrier (+ε4) or not (–ε4) of at least one allele. $^{*}P < 0.05$ between indicated groups.

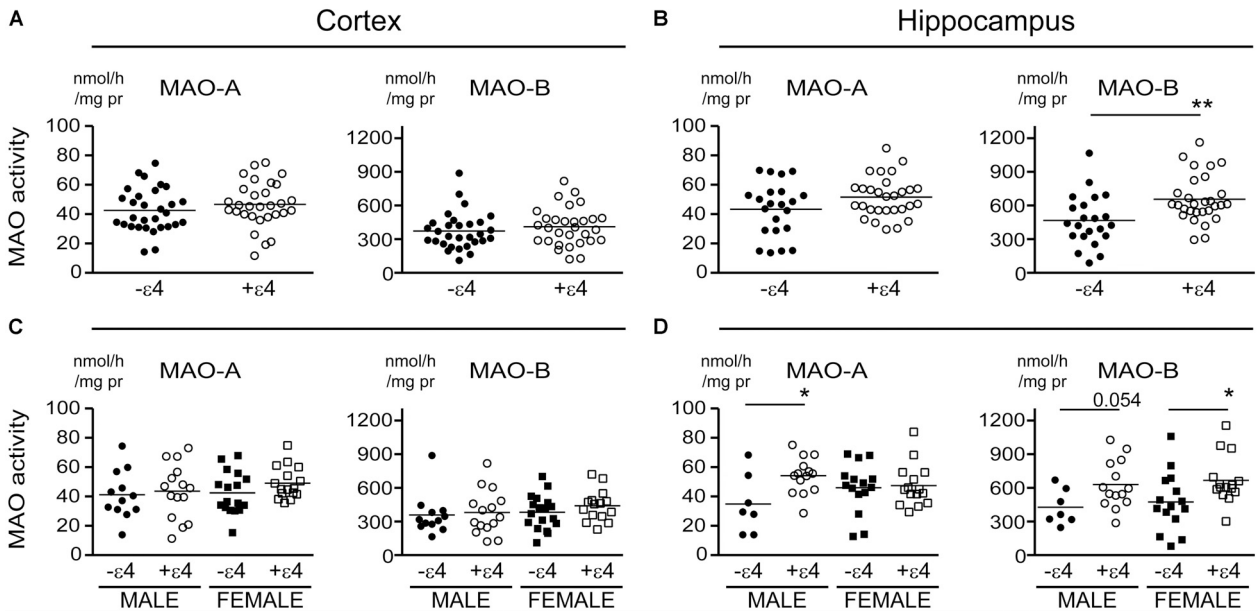


FIGURE 4 | MAO activites in human brain: data stratified by *APOE* ε4 status alone. MAO-A and MAO-B activites were measured in **(A)** the cortex and **(B)** the hippocampus of donors that were carriers (+ε4) or not (–ε4) of an *APOE* ε4 allele. **(C,D)** The same samples were re-analyzed based on the donor's sex. $^{*}P < 0.05$; $^{**}P < 0.01$ between indicated groups.

(P = 0.1992; **Figure 9D**), while a tendency for a correlation was observed for female hippocampal samples (P = 0.0650, r = 0.353) (**Figure 9B**). Focussing on *APOE* ε4 status revealed that cortical *MAO-A* and *MAO-B* mRNAs were correlated in non-carriers of the ε4 allele (males, P = 0.0045, r = 0.754; females, P = 0.0198, r = 0.575) and in male (P = 0.0126, r = 0.646), but not female (P = 0.1636), carriers of the allele (**Figure 9B**). In contrast, *MAO-A* and *MAO-B* mRNAs were not correlated in the corresponding hippocampal samples when stratified for *APOE* ε4 status (**Figure 9E**). Finally, stratifying for 'diagnosis' revealed that *MAO-A* and *MAO-B* mRNAs were correlated in cortical control samples ($P < 0.0001$, r = 0.691), but not in EOAD (P = 0.3924) and LOAD (P = 0.6147) samples (**Figure 9C**). Hippocampal *MAO-A* and *MAO-B* mRNAs were not correlated in any diagnosis using this stratification model (**Figure 9F**).

MAO Activities and Proteins Are Generally Not Correlated

We did observe a correlation between hippocampal MAO-B activity and protein expression in males (P = 0.0579) and females (P = 0.0455) and a negative correlation between MAO-A activity and protein expression in male cortical samples (P = 0.0558) (**Figure 10A**). When stratifying for *APOE* ε4 status, we did observe a strong correlation between MAO-B activity and protein expression in hippocampal samples from female non-carriers of the ε4 allele (P = 0.0094) (**Figure 10B**). Finally, there

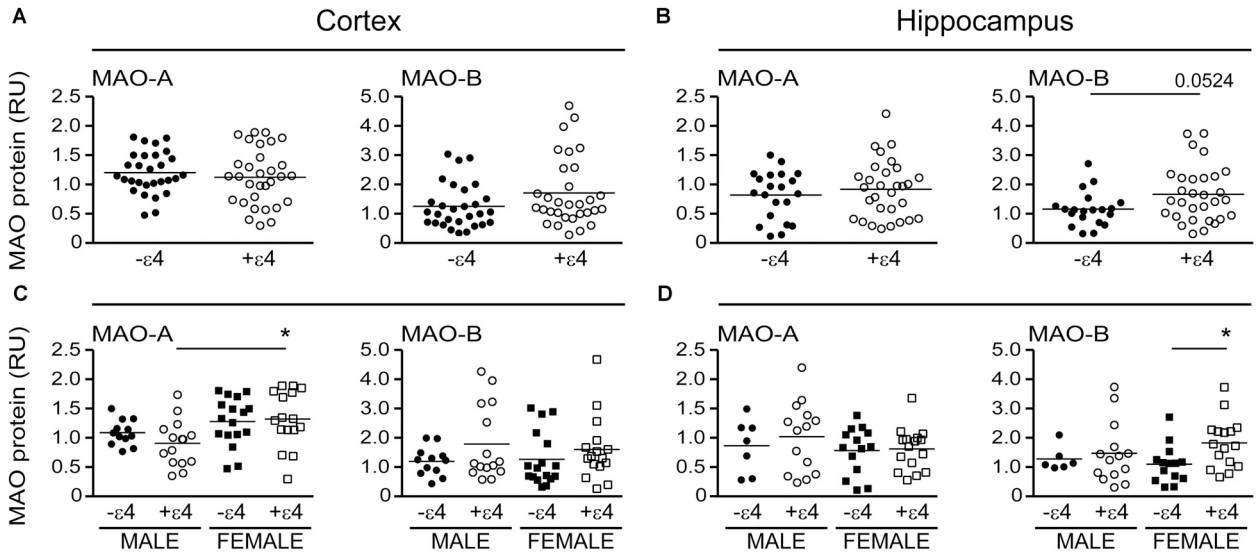


FIGURE 5 | MAO proteins in human brain: data stratified by *APOE* ε4 status alone. MAO-A and MAO-B proteins were quantified by densitometry in **(A)** cortical and **(B)** hippocampal lysates from donors that were carriers (+ε4) or not (–ε4) of an *APOE* ε4 allele. **(C,D)** The same protein densitometries were analyzed based on the donor's sex.*$P < 0.05$ between indicated groups.

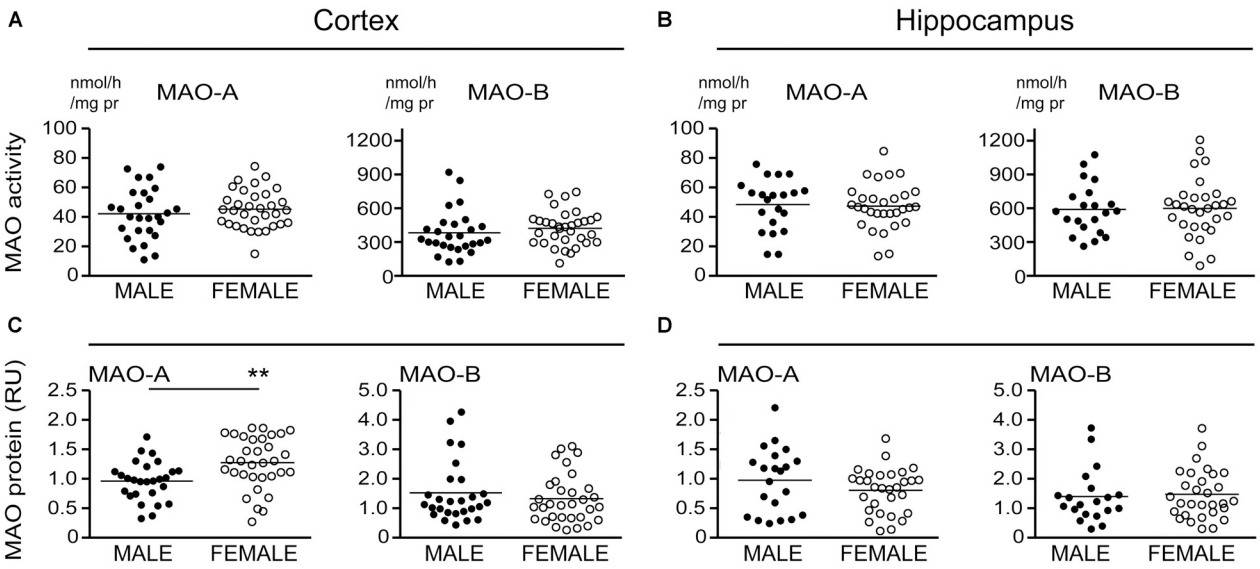


FIGURE 6 | MAO activites in human brain: data stratified by the donor's sex. **(A)** Cortical and **(B)** hippocampal MAO-A and MAO-B activites were compared based strictly on the donor's sex. **(C,D)** The corresponding protein densitometries were also analyzed based on the donor's sex. **$P < 0.01$ between indicated groups.

was a correlation between MAO-A activity and protein expression in control hippocampal samples ($P = 0.0214$) and a negative correlation between MAO-A activity and protein expression in cortical LOAD samples ($P = 0.0244$) (**Figure 10C**). Aside from these exceptions, all other regression analyses did not reveal any significant patterns. We were unable to demonstrate any correlation between MAO-A and MAO-B protein expression and respective mRNA transcript levels (*in the interest of space, these data are not shown*).

Overexpression of Human APOE4 Increases MAO-A and MAO-B Activities in C6 and HT-22 Cultures

C6 glial cells and hippocampal HT-22 cells were transfected with cDNA coding for human APOE2, APOE3, and APOE4 for 24 h. There was a significant increase in MAO-A activity in C6 cells overexpressing APOE3, while APOE4 expression led to increases in both MAO-A and MAO-B activities in both cell lines (**Figure 11**). The overexpressed APOE variants did not affect

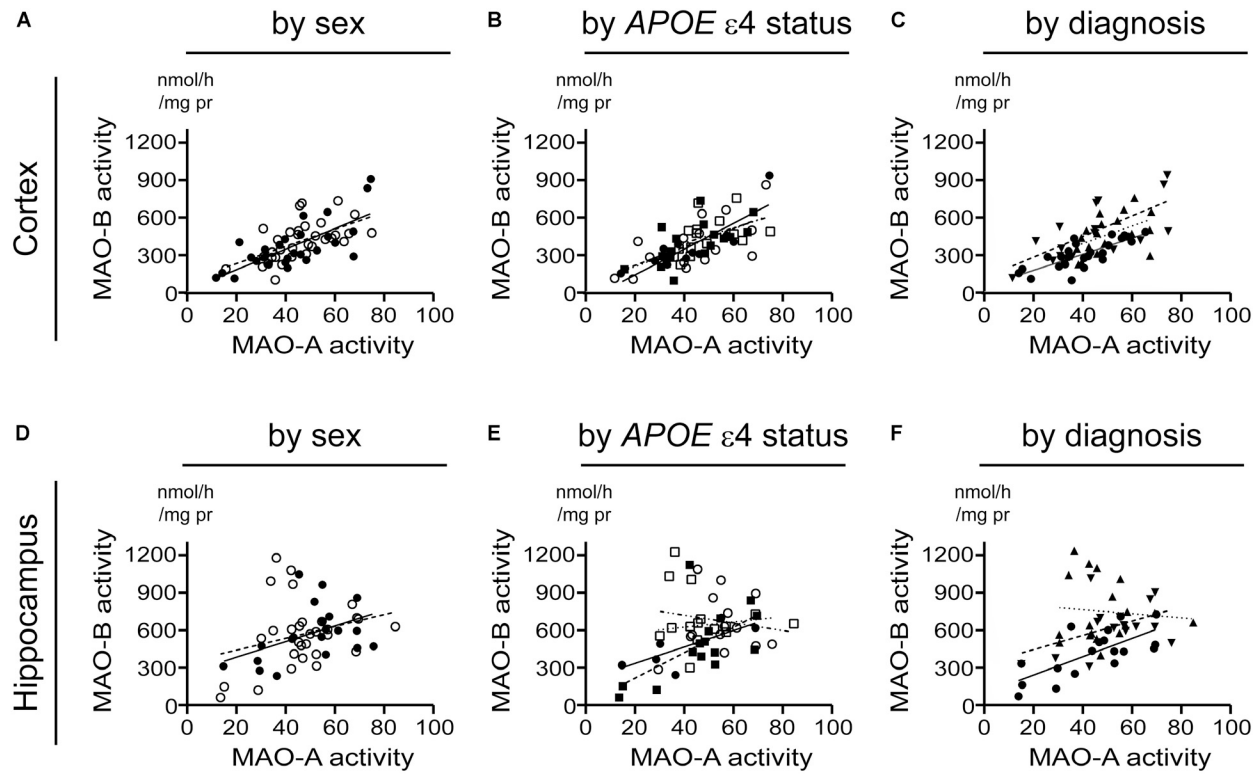


FIGURE 7 | A putative correlation between MAO-A and MAO-B activities in cortex, but not hippocampus, is spared from influence by risk factors for AD. Data are presented as scatter plots and analyses are shown as regression lines (using Pearson's coefficient). The strength of any potential association between MAO-A and MAO-B activites in our sample set was tested using the three stratifications used for comparing sample means in the previous figures. Cortical data were stratified by **(A)** sex alone (male: ●, solid line; female: ○, dashed line); **(B)** by *APOE* ε4 status and sex (male/-ε4: ●, solid line; male/+ε4: ○, dotted line; female/-ε4: ■, dashed line; female/+ε4: □, dotdashed line); and **(C)** by diagnosis (control: ●, solid line; early-onset AD: ▼, dashed line; late-onset AD: ▲ dotted line). The corresponding analyses based on hippocampal data (symbols and line types as for cortical data) are presented by **(D)** sex alone, **(E)** *APOE* ε4-by-sex, and **(F)** diagnosis.

endogenous MAO protein or mRNA expression in either cell line (**Figure 11**).

Levels of MAO-Mediated Metabolites and Substrates

Aside from a tendency for an increase in hippocampal HVA levels in females with a diagnosis of EOAD/LOAD (vs. control females) (P = 0.0824), significantly more HVA in the cortex of female (vs. male) carriers of the ε4 allele (P = 0.0564) and significantly more 5-HIAA in the hippocampus of female (vs. male) carriers of the ε4 allele (P = 0.0194). There were generally no differences in the levels of either 5-HT or dopamine, or their respective metabolites 5-HIAA, or HVA (*data not shown*).

DISCUSSION

One of the strengths of this study was our ability to compare data generated from cortical and corresponding hippocampal samples from a given donor; this allowed us to assess whether region-dependent differences existed in any of the patterns we observed for a given MAO parameter.

MAO Indices and the Risk of AD

The stratification based on the donor's diagnosis (i.e., control vs. AD) revealed the anticipated regional changes in MAO activities. Increased MAO-A activity in the AD brain has been associated with prodromal and co-morbid neuropsychiatric symptoms (Mousseau and Baker, 2012) and with neurodegeneration (Naoi et al., 2012). Similarly, region-specific changes in MAO-B activity in the AD brain have been observed (Adolfsson et al., 1980; Oreland and Gottfries, 1986) and MAO-B-positive astrocytes have been localized to the amyloid plaque in human brain (Saura et al., 1994) as well as in the brain of a mouse model of AD (Kim et al., 2016). Some of these changes are region-dependent, which could be reflecting different roles attributed to MAO-A in a given disease. For example, changes in cortical MAO-A binding in humans have been implicated in depression (Meyer et al., 2006), whereas changes in hippocampal MAO-A (based on animal models) have been associated with changes in cognition (Steckler et al., 2001) and even motor activity (Morishima et al., 2006), which is more often associated with the dopaminergic system. In fact, cerebrospinal fluid levels of the MAO-mediated dopamine metabolite, i.e., HVA, have been used to propose different subtypes of AD (Sjogren et al., 2002). Our current data suggest that HVA changes might be

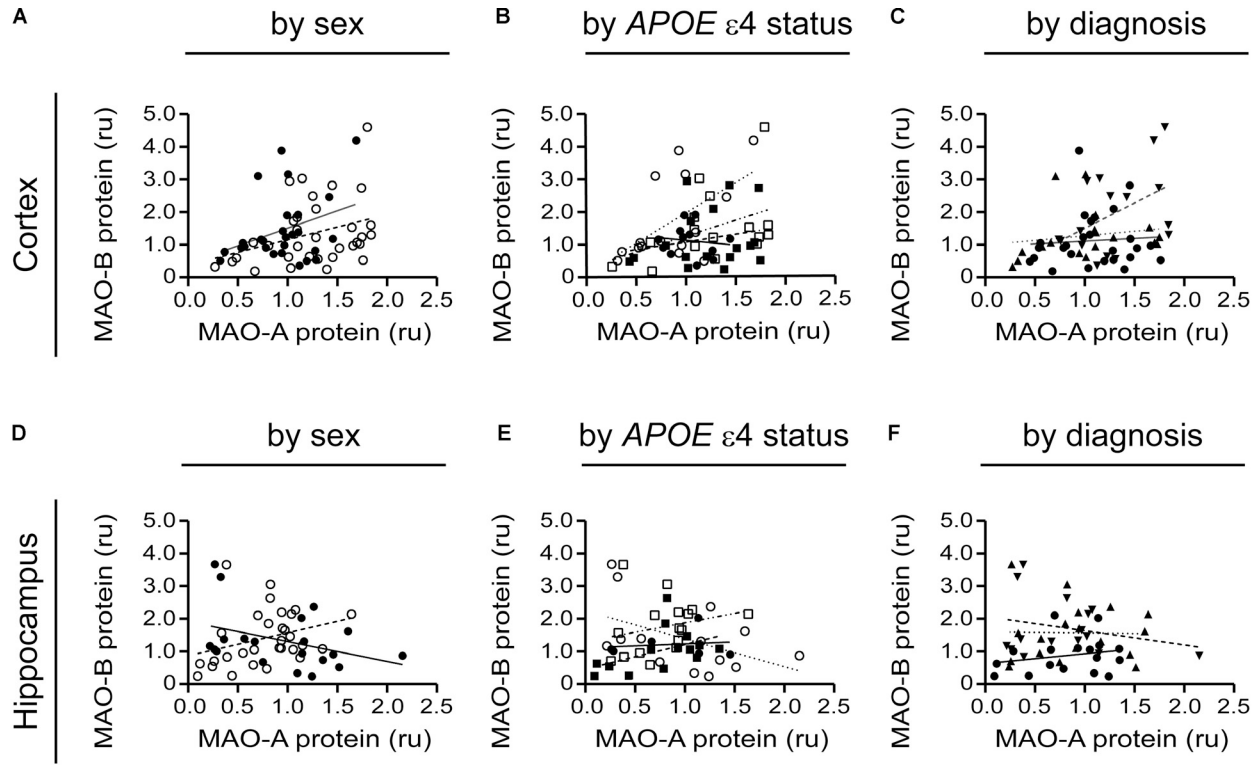


FIGURE 8 | MAO-A and MAO-B proteins are generally not correlated in either the cortex or hippocampus. Cortical data were stratified by **(A)** sex alone (male: ●, solid line; female: ○, dashed line); **(B)** by *APOE* ε4 status and sex (male/-ε4: ●, solid line; male/+ε4: ○, dotted line; female/-ε4: ■, dashed line; female/+ε4: □, dotdashed line); and **(C)** by diagnosis (control: ●, solid line; early-onset AD: ▼, dashed line; late-onset AD: ▲ dotted line). The corresponding analyses revealed a similar lack of correlation in hippocampal data (symbols and line types as for cortical data) whether analyzed by **(D)** sex alone, **(E)** *APOE* ε4-by-sex, or **(F)** diagnosis.

more evident in the female (vs. male) hippocampus (whether stratified by diagnosis or by *APOE* ε4 status). MAO-B appears to contribute to oxidative stress and motor deficits associated with AD, parkinsonism, and aging (Fowler et al., 1980; Riederer and Jellinger, 1982; Oreland and Gottfries, 1986; Danielczyk et al., 1988). In addition, MAO-B inhibitors might reduce the generation of the Aβ peptide from the APP precursor (Huang et al., 2012) by increasing non-amyloidogenic APP processing *via* α-secretase (Yang et al., 2009). MAO function, however, is complex (Mousseau and Baker, 2012) and our inability to correlate MAO-A (or -B) activity with MAO protein expression (or with mRNA expression) in our brain sample set, while perplexing, is not unique. Indeed, mean platelet MAO-B activity has been shown to correlate with MAO-B mRNA and protein expression (Zellner et al., 2012) and to increase in female AD patients (Robinson et al., 1971; Veral et al., 1997), while MAO-A or MAO-B activities tend to correlate with the respective proteins or binding densities (reviewed in (Tong et al., 2013)). Yet, in contrast to these observations, *MAO-A* genotypes that influence transcriptional activity (Fowler et al., 2007) and [^{11}C]-harmane/MAO-A autoradiography in autopsied human brain (Tong et al., 2013) do not reflect levels of MAO-A activity. We now know that some of these discrepancies might be reflecting possible post-translational modifications based on, for example, phosphorylation (Cao et al., 2009b), calcium-binding (Kosenko et al., 2003; Cao et al., 2007, 2009a), aberrant subcellular localization (Gujrati et al., 1996), and/or a direct interaction with a binding partner, including the presenilin protein (Pennington et al., 2011; Wei et al., 2012; Schedin-Weiss et al., 2017) that is central to Aβ/AD-related changes in γ-secretase activity (Roychaudhuri et al., 2009).

While the stratification based on diagnosis corroborated previous reports, we were far more intrigued by what other stratifications –based on putative risk factors for AD– revealed about MAO-A and MAO-B function. Given that the *APOE* ε4 allele has been linked to AD in a Brazilian cohort *via* co-segregation with a *MAO-A* polymorphism and an allelic variant of the serotonin transporter (Nishimura et al., 2005) and that a multiplex protein biochip for screening for AD –based on the ε4 allele and MAO-B– has been proposed (Veitinger et al., 2014), there is surprisingly very little published on the interaction between *APOE* risk alleles and MAO.

The most robust findings that we observed were the significant increases in MAO-B activities in the cortex and hippocampus of carriers of the *APOE* ε4 allele. Yet, tendencies for an increase were also observed in non-carriers of the allele (although the small sample size in these groups might have mitigated statistical significance). When 'diagnosis' was excluded as a nominal variable, we observed a male-specific increase in hippocampal

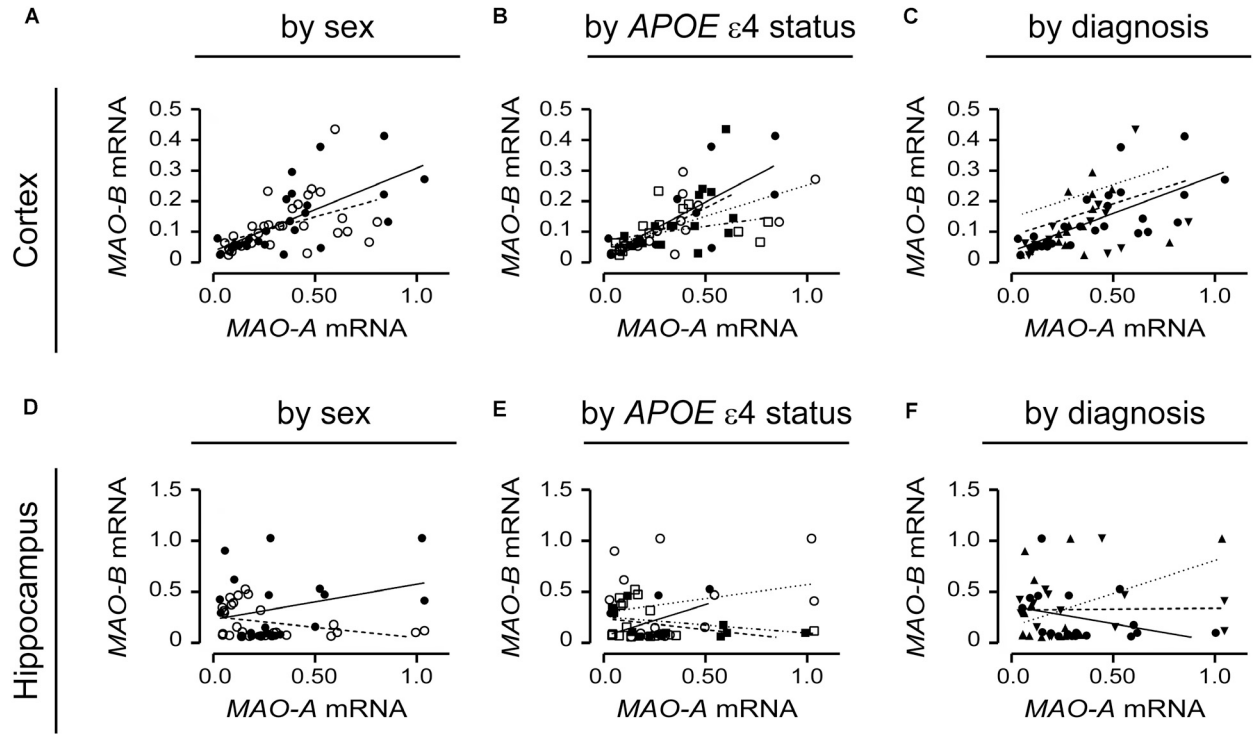


FIGURE 9 | *MAO-A* and *MAO-B* mRNA transcript levels are correlated in the cortex. Cortical data were stratified by **(A)** sex alone (male: ●, solid line; female: ○, dashed line); **(B)** by *APOE* ε4 status and sex (male/-ε4: ●, solid line; male/+ε4: ○, dotted line; female/-ε4: ■, dashed line; female/+ε4: □, dotdashed line); and **(C)** by diagnosis (control: ●, solid line; early-onset AD: ▼, dashed line; late-onset AD: ▲ dotted line). The corresponding analyses of hippocampal data (symbols and line types as for cortical data) presented by **(D)** sex alone, **(E)** *APOE* ε4-by-sex, and **(F)** diagnosis revealed a different pattern.

MAO-A activity and an increase in hippocampal MAO-B activity in both males and females. This is surprising given the female-specific risk associated with the ε4 allele in pathologies such as AD (Poirier et al., 1993) and depression (Delano-Wood et al., 2008). We are unsure what this observation implies, but perhaps it is revealing a more subtle role for the APOE4 protein in males as already shown for sexual dimorphism in neurogenesis (Rijpma et al., 2013), intelligence (Mondadori et al., 2007), or enhanced damage due to stroke (DeCarli et al., 1999). In our C6 and HT-22 cell cultures, overexpression of the human APOE4 protein led to increases in mao-A and mao-B activities (without any concurrent change in MAO protein), which suggest that APOE4 might exert a post-translational influence on MAO function and/or associated pathologies or potentially in very specific populations of AD patients, for example, the Brazilian cohort (Nishimura et al., 2005). This certainly deserves further attention.

A Putative Co-regulation of MAO-A and MAO-B Is Vulnerable to AD Risk Factors in the Hippocampus, but Not in the Cortex

The outcomes discussed in the previous section, based on comparing sample means, generally confirmed what has been reported in the literature. We have recently reported that variability in regional Aβ levels in carriers of the *APOE* ε4 allele correlated with the variability in the expression of catalytic enzymes implicated in the generation of the Aβ peptide (Nyarko et al., 2018). We re-examined our data so as to determine whether any of the parallel increases observed in MAO-A and MAO-B parameters quantified up to this point might be indicating a more subtle relation and, if so, whether any potential correlation was influenced by risk factors or AD itself. While there have been plenty of studies looking at the correlation between MAO activity and physiological correlates such as age or with behavioral correlates such as risk-seeking, drug dependence and/or criminality (Mousseau and Baker, 2012), or specifically between platelet MAO activity and protein expression (Zellner et al., 2012), we were surprised, upon review of the literature, that there have only been a few reports suggesting that the function of the two MAO isoforms might be correlated. An earlier study had found a correlation between MAO-A and MAO-B activity in surgical resection samples (Young et al., 1986), while a more recent study found that MAO-A and MAO-B activities were correlated in the prefrontal cortex of controls and marginally less-so in AD samples (Kennedy et al., 2003).

We began with the 'simplest' stratification, i.e., focussing on biological sex, and progressed toward the more complex stratifications, e.g., including *APOE* ε4 status or 'diagnosis.' We observed that the activities of MAO-A and MAO-B in the cortex were highly correlated in males and females, and that this correlation generally held even when the data were stratified

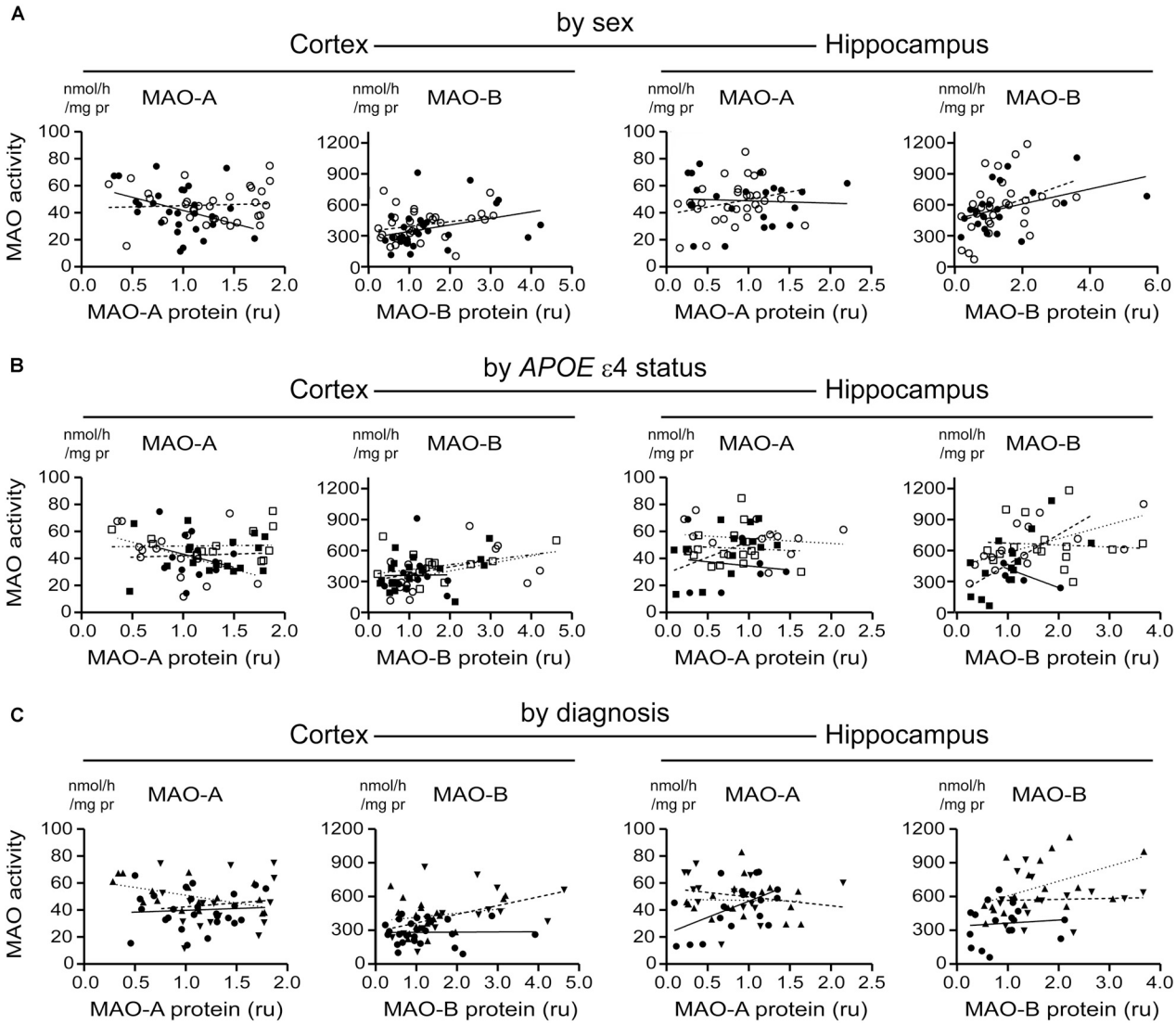


FIGURE 10 | MAO-A and MAO-B activities do not correlate with their corresponding proteins in either the cortex or the hippocampus. Cortical and hippocampal data were stratified by **(A)** sex alone (male: ●, solid line; female: ○, dashed line); **(B)** by *APOE* ε4 status and sex (male/-ε4: ●, solid line; male/+ε4: ○, dotted line; female/-e4: ■, dashed line; female/+ε4: □, dotdashed line); and **(C)** by diagnosis (control: ●, solid line; early-onset AD: ▼, dashed line; late-onset AD: ▲ dotted line). In general, there was no correlation between activities and respective proteins.

for sex-by-*APOE* ε4 status, or for a diagnosis of EOAD or LOAD. Interestingly, any correlation in the hippocampal data was limited to males (if stratifying for sex alone), to individuals who did not carry an ε4 allele (regardless of sex), or to individuals who did not have a diagnosis of EOAD or LOAD. In other words the co-regulation of MAO-A and MAO-B activities was maintained in the cortex regardless of risk or diagnosis of AD, but was disrupted in the hippocampus by these same risk factors for AD, i.e., sex (female) or *APOE* ε4 allele, or in donors with a confirmed diagnosis of AD. Parenthetically, it would be interesting to determine how much of the blockade of MAO-A activity that is observed following chronic MAO-B inhibition is due to any disruption of this co-regulatory mechanism rather than to changes in substrate specificity or an off-target effect of the drug, as often surmised (Inaba-Hasegawa et al., 2012; Bartl et al., 2014).

The fact that the MAO-A and MAO-B proteins did not appear to correlate at all (regardless of region or stratification) is perhaps not surprising given that region-dependent factors such as post-translational modifications, availability of Ca^{2+}-binding proteins, and physical interactions with other cellular proteins all could be regulating protein activity and/or availability at any given time (discussed above). The fact that *MAO-A* and *MAO-B* mRNA were highly co-regulated in cortical, but not hippocampal, samples is certainly more intriguing. We do not have an explanation for this phenomenon at the moment, but could speculate on region-dependent influences, perhaps involving sex-dependent differences in hormonal signaling

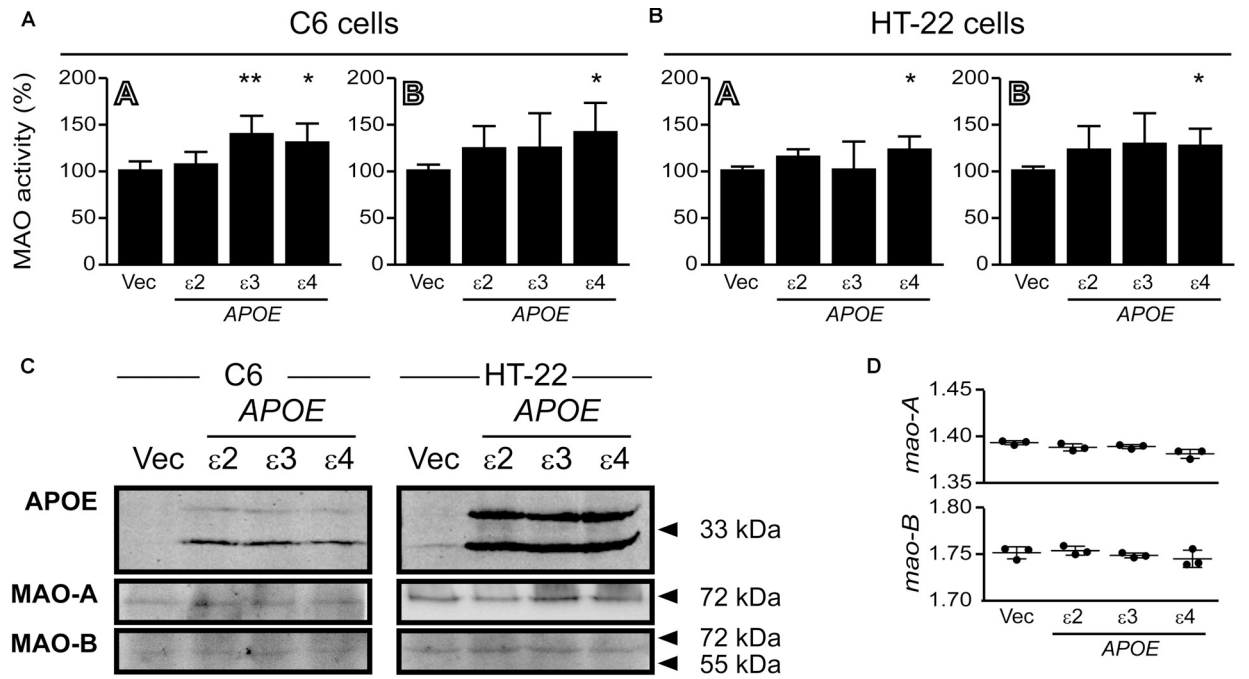


FIGURE 11 | Overexpressed APOE4 influences MAO activity in cell cultures. C6 (glial) and HT-22 (neuronal) cells were transfected with cDNA coding for the human *APOE* ε2, ε3, or ε4 allele. Overexpressed APOE4 induced MAO-A and MAO-B activity in **(A)** C6 cultures and in **(B)** HT-22 cultures. The APOE3 variant induced MAO-A activity specifically in C6 cells. **(C)** Representative blots of the overexpressed APOE variants and MAO-A and MAO-B. **(D)** The APOE variants did not elicit any change in C6 *mao-A* or *mao-B* mRNA transcript levels. $^{*}P < 0.05$; $^{**}P < 0.01$ relative to the Vector (Vec) transfected control group.

pathways (Barth et al., 2015; Bethea et al., 2015), epigenetics (direct or indirect) *via* clock genes, microRNA (e.g., miR-142) or differential methylation of the *MAO-A* and *MAO-B* genes (Hampp et al., 2008; Shumay et al., 2012; Chaudhuri et al., 2013; Tiili et al., 2017), or even the possibility of transcriptional *trans*-regulation. The latter is not an unreasonable scenario given that we observed that the overexpression of human MAO-A is able to reduce *mao-B* –but not *mao-A*– mRNA transcript levels in C6 cells (preliminary observations; data not shown). The possibility of a co-regulation of *MAO-A* and *MAO-B* transcription by any of these factors, or combinations thereof, will add to our understanding of the promoter activities of these two genes (Bach et al., 1988; Zhu et al., 1992).

Monoamines play a significant role in cognition, which likely reflects their anatomical association with those brain areas regulating memory and learning (King et al., 2008). Aberrant monoaminergic (i.e., serotoninergic) signaling usually accompanies cholinergic deficits (Grailhe et al., 2009), which supports early-stage changes in monoaminergic tone, compounded by cholinergic deficits, as contributing factors to the cognitive decline in AD (Robinson, 1983; Richter-Levin and Segal, 1993). Selective changes in monoaminergic function could be a major contributor to non-cognitive symptoms, including depression (Ritchie and Lovestone, 2002), which could account for prodromal depression, as mentioned above. Some of these earlier stages of AD are often associated with non-cognitive, neuropsychiatric symptoms including depression, irritability, aggressive outbursts, and delusions (Ritchie and Lovestone, 2002). It is well known that these disorders reflect region-specific noradrenergic and serotoninergic insults. Thus, MAO-A and -B dysfunction as well as a monoaminergic insult could be contributing to a spectrum of symptoms and pathology spanning earlier –and sustained– AD progression.

We acknowledge that correlation does not mean causation; yet, given that MAO-B inhibitors have been shown to reduce the amyloidogenic processing of the APP molecule (Yang et al., 2009; Huang et al., 2012), this would imply that an increase in MAO-B function would promote amyloidogenic processing of APP. This could explain the well-documented increase in Aβ levels and plaque burden in the AD hippocampus, where an increase in MAO-B has been observed. Unfortunately, the role for MAO in the context of AD progression is not likely to be as straightforward as anticipated given that Roche recently terminated a clinical trial centered on sembragiline, a highly selective MAO-B inhibitor, because of a lack of amelioration of the Alzheimer's Disease Assessment Scale-Cognitive Behavior Subscale[2].

Any increase in MAO function would lead to the generation of hydrogen peroxide as a by-product of the deamination reaction, and the ensuing oxidative stress and potential for cell death –invariably involving the mitochondria– would be exacerbated when antioxidant systems are compromised, such as during aging (Zhu et al., 2006) and particularly in AD (Crack et al., 2006).

[2]http://www.alzforum.org/therapeutics/sembragiline

CONCLUSION

Our study, based on post-mortem brain tissue, highlights a region-dependent MAO (co)regulation. While we could not fully characterize the role of this co-regulation in the male and female brain, the findings do provide insight into the role of MAOs – and the monoaminergic neurotransmitters that they regulate– and support sex– and genetic-specific responses to risk of AD and the pathology associated with disease progression.

Our observations confirm some of what is already known, but certainly expand on the reported literature. For instance, the increases in MAO-B activity that appear to align much more strongly with carriers of the *APOE* ε4 allele is novel and given the implied role of MAO-B in neurodegeneration and the gender-risk of AD associated with the *APOE* ε4 allele, it is not unreasonable to infer that their contributions to AD reflect an overlapping mechanism. What our data also provide is a side-by-side comparison of two brain regions, namely the cortex and the hippocampus, from the same donor. Again, the fact that many of the changes in MAO-B (and MAO-A) are occurring in the hippocampus –a region particularly vulnerable during the course of AD– is supportive of a contribution of these enzymes to disease progression. What our data are also revealing is that it is important to test for activity, protein expression, and mRNA expression before one can truly determine a role for MAOs in any pathological (or physiological) state. As importantly, or perhaps more so, our observations also highlight the limitation(s) of only examining one or the other MAO isoform, and/or using sample means to compare between test groups; indeed, something as obvious as a co-regulation of the two isoforms has been inadvertently overlooked. The fact that this co-regulation of MAO-A and MAO-B is sex-dependent and apparently invariant in the cortex, but so vulnerable to risk factors for AD in the hippocampus (observed using samples from the same donors), strongly suggests a contribution to AD progression. Whether this co-regulation contributes to other neuropathologies such as Parkinson's disease, depression, autism –or any other disorder associated with monoaminergic defects– remains to be determined, but could certainly explain some of the ambiguity in neurochemical underpinnings and treatment responses associated with these various diseases in the clinic. This new-found knowledge relating to two enzymes that are also important to oxidative stress and mitochondria-associated processes in the central nervous system as well as in the periphery will provide fundamental and critically important insight into their implied roles in the research and clinical contexts.

AUTHOR CONTRIBUTIONS

MQ, JN, PP, RH, and PK: data collation. MQ, JN, GB, and DM: experimental design, and manuscript preparation and editing (all authors).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00419/full#supplementary-material

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REVIEW
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Contribution of Tau Pathology to Mitochondrial Impairment in Neurodegeneration

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Tau is an essential protein that physiologically promotes the assembly and stabilization of microtubules, and participates in neuronal development, axonal transport, and neuronal polarity. However, in a number of neurodegenerative diseases, including Alzheimer's disease (AD), tau undergoes pathological modifications in which soluble tau assembles into insoluble filaments, leading to synaptic failure and neurodegeneration. Mitochondria are responsible for energy supply, detoxification, and communication in brain cells, and important evidence suggests that mitochondrial failure could have a pivotal role in the pathogenesis of AD. In this context, our group and others investigated the negative effects of tau pathology on specific neuronal functions. In particular, we observed that the presence of these tau forms could affect mitochondrial function at three different levels: (i) mitochondrial transport, (ii) morphology, and (iii) bioenergetics. Therefore, mitochondrial dysfunction mediated by anomalous tau modifications represents a novel mechanism by which these forms contribute to the pathogenesis of AD. In this review, we will discuss the main results reported on pathological tau modifications and their effects on mitochondrial function and their importance for the synaptic communication and neurodegeneration.

Keywords: tau, mitochondria, Alzheimer's disease, synapse neurodegeneration, synapsis

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INTRODUCTION

Tau protein, which belongs to the family of microtubule-associated proteins (MAPs), was first discovered in 1975 and identified as a molecule that physiologically promotes the assembly and stabilization of microtubules (Weingarten et al., 1975). Tau is mainly expressed in neurons and is present in great extent in axons controlling neuronal development (Ding et al., 2006; McMillan et al., 2011; Kolarova et al., 2012), promoting the vesicular and axonal transport (Dolan and Johnson, 2010; Rodriguez-Martin et al., 2013) and is critical in defining the polarity of neurons (Avila et al., 2016). In a number of human diseases called tauopathies, including Progressive Supranuclear Palsy (PSP), Pick's disease (PiD), Down's syndrome (DS), and Frontotemporal Dementia and Parkinsonism linked to chromosome 17 (FTDP-17), soluble tau assembles into insoluble filaments, leading to synaptic failure and neurodegeneration (Spillantini and Goedert, 2013). Moreover, in AD, one of the most common forms of dementia in elderly, tau undergoes specific pathological modifications that are the principal components of neurofibrillary tangles (NFTs), one of the main neuropathological hallmarks of AD (Kosik et al., 1986).

Mitochondria are responsible for energy supply, detoxification, and communication in brain cells, and several investigations suggest that mitochondria failure could have a role in the pathogenesis of Parkinson's disease, Huntington disease, and AD (Cabezas-Opazo et al., 2015). On the other hand, several studies suggest that mitochondrial dysfunction is an early event in the pathogenesis of AD and is involved in other tauopathies (Gibson and Shi, 2010; Cabezas-Opazo et al., 2015). In particular, the presence or accumulation of tau pathology can affect mitochondrial function at three specific points: (i) mitochondrial transport (**Figure 1**), (ii) dynamics (morphology) (**Figure 2**), and (iii) bioenergetics (**Figure 3**). Therefore, in this review, we discuss the major results reported on tau pathological modifications and their effects on mitochondrial function and its implications for the pathogenesis of AD.

TAU PROTEIN IN HEALTH AND DISEASE

In the human genome, tau is encoded by a single gene, which is located on the long arm of chromosome 17 (Neve et al., 1986; Avila et al., 2004, 2016; Kolarova et al., 2012; Medina and Avila, 2014). When tau is routinely studied, it is often treated as if it was a single protein, whereas, in fact, it exists in six protein isoforms and several phosphorylated species whose functions are not entirely understood (McMillan et al., 2011; Andreadis, 2012; Kolarova et al., 2012; Medina and Avila, 2014; Derisbourg et al., 2015). The tau gene contains 16 exons and the different isoforms arise from the alternative splicing of exons 2, 3, and 10. Exons 2 and 3 are alternatively spliced, and adult human tau can contain exon 2 (one N-terminal insert or 1N), exons 2 and 3 (2N), or neither (0N) (Avila et al., 2004; Crespo-Biel et al., 2012; Kolarova et al., 2012; Medina and Avila, 2014). The N-terminal is projected from the microtubule surface, where it is believed to interact with cytoskeletal elements and the plasma membrane (Gong et al., 2005). The remainder protein contains the four microtubule-binding domains, encoded from exons 9, 10, 11, and 12. From these, only exon 10 is alternatively spliced and can either be spliced in (4R) or out (3R) (Goedert and Crowther, 1989; Goedert et al., 1989; Ittner et al., 2010; Martin et al., 2011; Kolarova et al., 2012; Pedersen and Sigurdsson, 2015). In addition, the C-terminal region also contains projection domains, suggesting that the different tau species must interact with specific subsets of proteins and execute specific cellular functions (Kolarova et al., 2012; Kadavath et al., 2015).

In physiological conditions, tau could be phosphorylated or dephosphorylated depending on the equilibrium between the actions of kinases (such as CDK5/p35 and GSK3β) and phosphatases (including PP1, PP2A, PP2B, and PP2C), respectively (Arrasate et al., 2000; Maas et al., 2000; Maccioni et al., 2001; Kolarova et al., 2012; Hernandez et al., 2013). This balance in the phosphorylation state of tau determines binding and stability of tubulin polymerization in neuronal cells, which is necessary for maintaining the structure of the axon and dendrites (Wolfe, 2012; Liu S.L. et al., 2016). In several neurodegenerative disorders, tau becomes hyperphosphorylated which alters its secondary structure and leads to conformational and functional alterations (Hanger and Wray, 2010). The loss of the binding ability of tau to microtubules due to hyperphosphorylation also facilitates the formation of paired helical filaments (PHFs), which leads to NFT formation in AD (de Calignon et al., 2010; Crespo-Biel et al., 2012). Those alterations cause destabilization of the cytoskeleton network and disruption of axonal transport (Alonso et al., 2001; Kolarova et al., 2012), which can also lead to irreversible changes in microtubule dynamics, neuronal dysfunction, synaptic damage, and ultimately cell death (Liu et al., 2007; Kolarova et al., 2012; Dorostkar et al., 2015).

Although phosphorylation is usually considered as one of the most important modifications of tau in AD, evidence suggests that the proteolysis impairment may play a significant role as an early step in neurodegeneration (Johnson, 2006). Tau protein is a substrate of several endogenous proteases (Wang et al., 2010), and among them, caspases and calpain have been investigated most intensively (Wang et al., 2010). Studies in cellular and animal models show that proteolysis processing of tau increases its propensity for aggregation, an event that was found to be relevant in the formation of NFTs which are composed of insoluble PHFs and accumulate inside neurons (Hanger et al., 2009). At the same time, caspase activation is related to the toxic effects mediated by amyloid β-peptide (Aβ) in AD (Rissman et al., 2004; Hanger and Wray, 2010). In that context, it has been found that tau can be cleaved at several residues of the carboxy-terminal region (Gamblin et al., 2003), preferentially by caspase-3 cleaving at aspartic acid 421 (Asp 421) (Ding et al., 2006; Quintanilla et al., 2012). On the other hand, the digestion of native PHFs leaves a filament fragment that contains only a 12 kDa tau protein truncated at glutamic acid 391 (Glu391) (Garcia-Sierra et al., 2008). This C-terminal proteolytic process induces self-aggregation and increases the rate of polymerization of tau, which later promotes formation and assembly of NFTs (Gamblin et al., 2003; Ding et al., 2006; de Calignon et al., 2010; Hanger and Wray, 2010; Kolarova et al., 2012), which leads to dendritic spine loss, synaptic impairment, and memory deficits (D'Amelio et al., 2011; Zhao et al., 2015). Another truncation of tau occurs in the N-terminal region by the action of caspase-6 (Wang et al., 2010; Kolarova et al., 2012). N-terminal tau fragments have been found in several *in vitro* and *in vivo* models, such as in primary neuronal cultures undergoing apoptosis (Canu et al., 1998; Park and Ferreira, 2005), in the cerebrospinal fluid (CSF) of rats after traumatic brain injury (TBI), in transient forebrain ischemia (Siman et al., 2004), and in brain tissue of AD patients (Rohn et al., 2002). Further reports have demonstrated that a significant proportion of 20–22 kDa N-terminal tau fragments (NH2hTau) is preferentially located in the mitochondria-rich synapses from AD hippocampus and frontal cortex. In addition, this NH2hTau fragment is associated with neurofibrillary degeneration and synaptic impairment in human AD brains (Amadoro et al., 2010). Although this is not an early event in AD, these findings suggest that N-terminal tau truncation contributes to the progression of the disease and is a critical step in the toxic cascade leading to neuronal death, similar to what has been proposed for the C-terminal cleavage of tau by caspases (Fasulo et al., 2000, 2005).

It is clear that even under normal physiological conditions tau may undergo different posttranslational modifications,

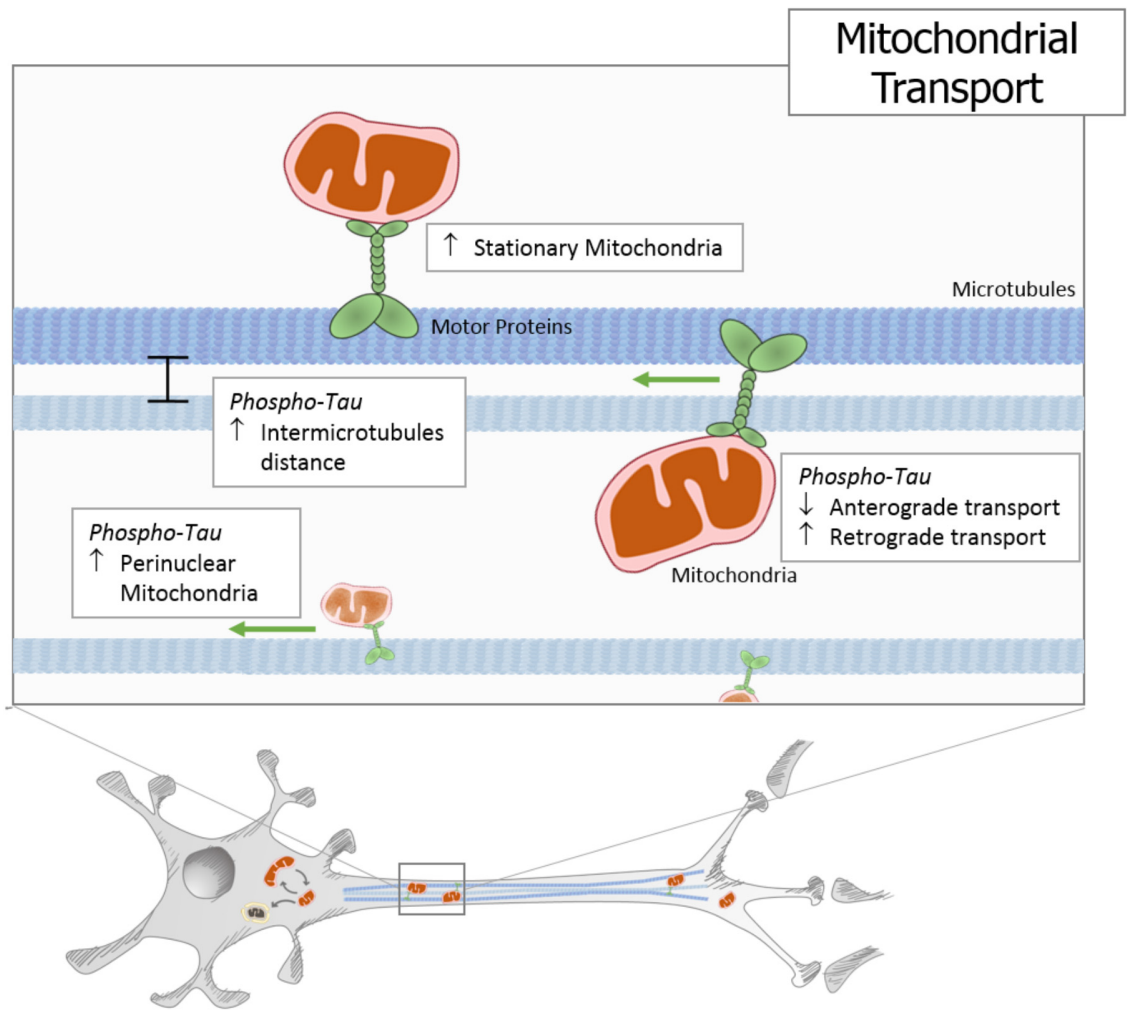


FIGURE 1 | Tau pathology impairs mitochondrial transport. In different cells and animal models, it has been described that hyperphosphorylated and truncated tau generate an increase in stationary mitochondria. Moreover, studies using different tau pathology models show an increase in the intermicrotubular distance that could be responsible for the mitochondrial movement reduction. At the same time, neuronal models that express pathological forms of tau show a decrease in anterograde transport and an increase in retrograde transport, which finally leads to an increase in perinuclear mitochondria.

such as phosphorylation, acetylation, glycation, ubiquitination, nitration, truncations (proteolytic cleavage), and abnormal conformational changes (Hanger and Wray, 2010; Pritchard et al., 2011; Kolarova et al., 2012; Kumar et al., 2014; Tenreiro et al., 2014). To this date, it is unknown when and how these posttranslational modifications affect tau functions and triggering different pathological conditions (Bodea et al., 2016). However, these abnormal tau conformations generate serious alterations in neuronal activity, causing a loss in its ability to transmit synaptic signals, and contribute to dendritic spine loss (Dorostkar et al., 2015). Interestingly, in the last years, it was hypothesized that abnormalities in tau function may also accelerate the development of several signs of neurotoxicity or become neurons more vulnerable to insults, which includes oxidative stress, calcium dysregulation, inflammation, mitochondrial impairment, and excitotoxicity (Gendron and Petrucelli, 2009). This suggests a direct participation of tau as an intermediary in these processes.

In that context, several studies using tau knockout (KO) mice have shown a protection from neurotoxicity induced by Aβ treatment compared to wild-type (WT) mice (Rapoport et al., 2002; Roberson et al., 2007). Furthermore, Roberson and collaborators described that reducing the endogenous tau levels prevented behavioral deficits caused by Aβ and protected against excitotoxicity (Roberson et al., 2007). The morphological analysis shows that WT neurons degenerate in the presence of Aβ, while tau-depleted neurons show no signs of degeneration in those conditions (Rapoport et al., 2002). In a similar fashion, blocking tau expression with an antisense oligonucleotide completely blocks Aβ toxicity in differentiated primary neurons (Liu et al., 2004). These results provide direct evidence supporting a key role for tau in the mechanisms leading to Aβ-induced neurodegeneration in the

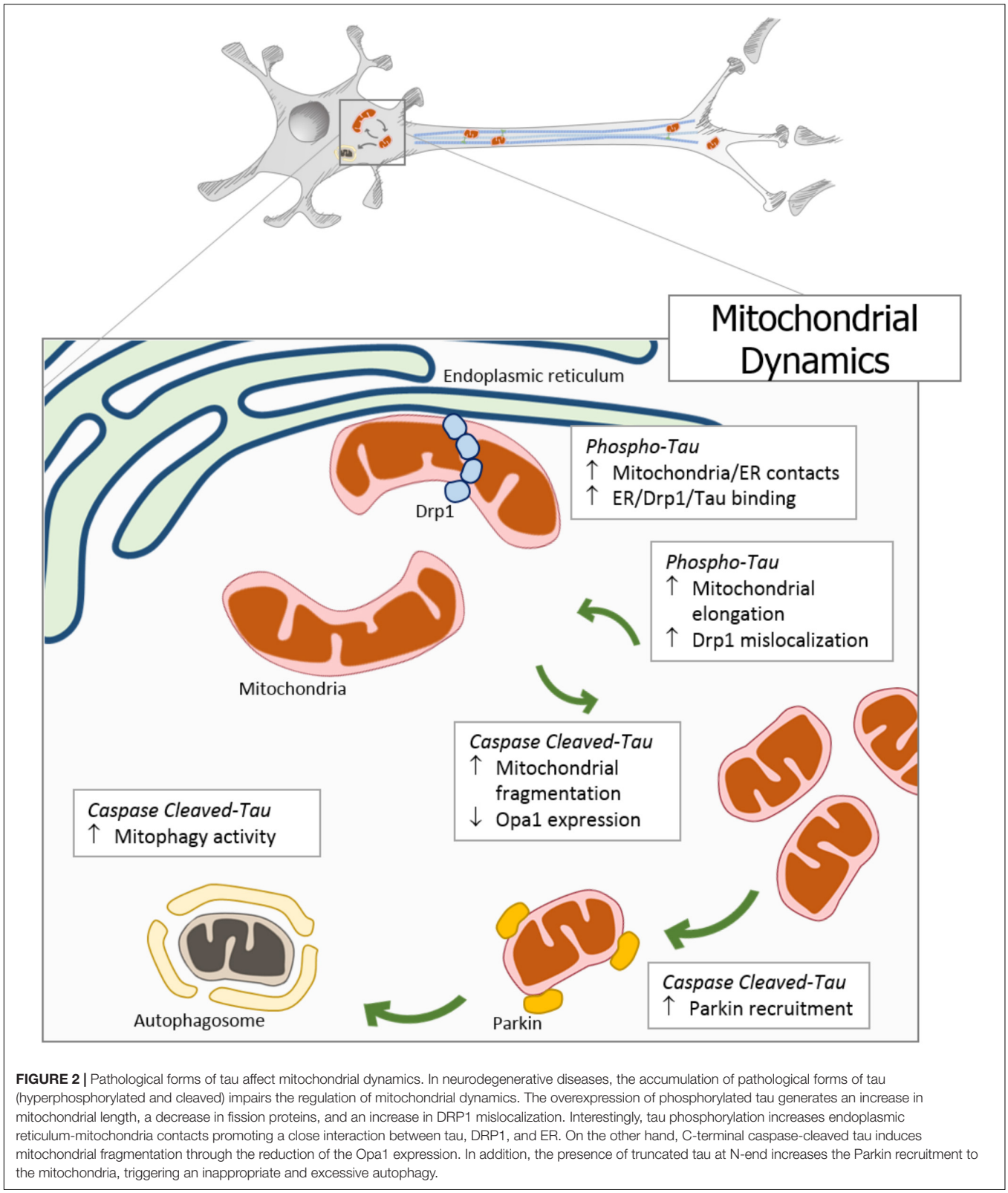


FIGURE 2 | Pathological forms of tau affect mitochondrial dynamics. In neurodegenerative diseases, the accumulation of pathological forms of tau (hyperphosphorylated and cleaved) impairs the regulation of mitochondrial dynamics. The overexpression of phosphorylated tau generates an increase in mitochondrial length, a decrease in fission proteins, and an increase in DRP1 mislocalization. Interestingly, tau phosphorylation increases endoplasmic reticulum-mitochondria contacts promoting a close interaction between tau, DRP1, and ER. On the other hand, C-terminal caspase-cleaved tau induces mitochondrial fragmentation through the reduction of the Opa1 expression. In addition, the presence of truncated tau at N-end increases the Parkin recruitment to the mitochondria, triggering an inappropriate and excessive autophagy.

central nervous system (Gendron and Petrucelli, 2009) and predict that only cells containing appreciable levels of tau are susceptible to Aβ toxicity (Rapoport et al., 2002). On the other hand, it was described that tau KO mice are not only protected from Aβ neurotoxicity but also against the effects of neurologic stress. For example, Lopes and colleagues describe

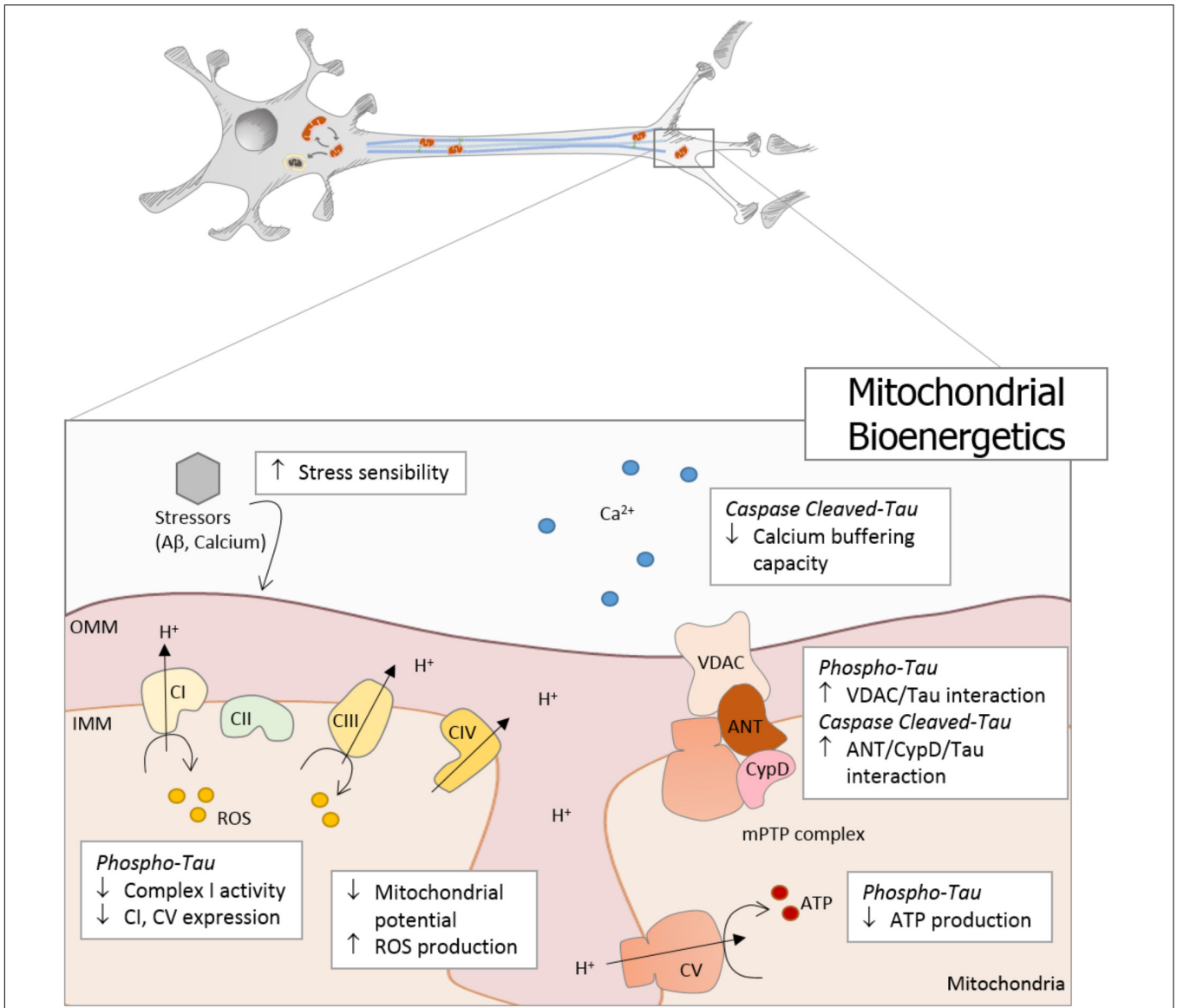


FIGURE 3 | Tau pathology affects mitochondrial bioenergetics in AD. Mitochondrial bioenergetics is significantly affected by tau pathology in AD. Phosphorylated and truncated tau severely affects mitochondrial membrane potential (MMP) and induces oxidative stress, which finally increases the mitochondrial sensibility to different stressors, such as Aβ and calcium. Moreover, phosphorylated tau decreases the expression of complex I and V, with a concomitant reduction in complex I activity and ATP production. In addition, caspase-cleaved tau impairs mitochondrial function affecting the calcium buffering capacity. Both pathological forms of tau present dysfunctional characteristics that suggest the involvement of the mitochondrial permeability transition pore (mPTP) in the bioenergetics failure. Phosphorylated tau induces an increase in VDAC/Tau interaction, and N-end truncated tau promotes an increase in ANT/CypD/Tau binding.

that the reduction of tau expression protects from working memory impairments, dendritic spine loss, and synaptic failure induced in the prefrontal cortex (PFC) of a chronic stress mouse model (Lopes et al., 2016). Interestingly, this study suggests that stress-induced neuronal damage and cognitive decline depend on an interaction between tau and several mitochondrial proteins that affects mitochondrial localization at the synapses. Therefore, it is highly plausible that the ablation of tau expression prevents mitochondria motility impairment leading to a protection of dendrites and synapses against stress (Lopes et al., 2016).

Interestingly, the relationship between the reduction of tau expression and the improvement of mitochondrial health has been previously suggested (Vossel et al., 2010). Vossel and collaborators describe that neurons from WT animals present an impaired axonal motility of mitochondria in the presence of Aβ, an effect that was stronger for anterograde than retrograde transport. However, the complete or partial reduction of tau expression prevents these defects without affecting the axonal transport baseline (Vossel et al., 2010). Other groups describe that neurons from tau KO mice are also resistant to Aβ-induced mitochondrial damage compared to neurons obtained from WT

mice (Pallo and Johnson, 2015). However, tau KO neurons show a more pronounced cytosolic calcium elevation in response to Aβ, suggesting that tau may facilitate Aβ-induced mitochondrial damage in a manner that is independent of cytosolic calcium increase (Pallo and Johnson, 2015). Complementary studies show that tau protein interacts either directly or indirectly with more than 100 proteins in physiological conditions. Almost half of them were grouped as membrane-bound proteins, and mitochondrial proteins composed the largest fraction (40.4%) of this group. In addition, this study identified several proteins with a preferential binding to distinct isoforms of tau, and it was described that the specific 2N tau isoforms have preferential binding to proteins involved in ATP biosynthesis and synaptic transmission. Interestingly, these proteins are abundant in several neurodegenerative diseases, dementia, and tauopathies, suggesting a tight relationship between tau, mitochondria, and synaptic transmission (Liu C. et al., 2016).

Regarding the relationship between mitochondria and the neuronal damage, it seems logical to think that defects in mitochondria trafficking could result in a decrease in local ATP levels which could impair synapses (Gendron and Petrucelli, 2009). Also, mitochondrial dysfunction can generate an imbalance in the calcium buffering capacity and the consequent release of presynaptic glutamate or impair the clearance of glutamate from the synapse, thus leading to high levels of extracellular glutamate thereby inducing neurotoxicity (Di Monte et al., 1999; Bobba et al., 2004). In this context, it is necessary to investigate the relationship between tau protein, its pathological modifications, mitochondrial function, and neuronal damage.

MITOCHONDRIAL TRANSPORT AND TAU PATHOLOGY

As we already mentioned, microtubule-associated proteins such as MAPs regulate the organization, polymerization, and stability of microtubules. Tau localizes predominantly in the axons where it participates in the regulation of the stability and assembly of microtubules (Gendron and Petrucelli, 2009). Microtubules, one of the principal components of the cytoskeleton, are involved in the maintenance of neuronal morphology and the formation of axonal and dendritic processes, and they play a vital role in cellular trafficking (Cabezas-Opazo et al., 2015). The transport of cargo proteins to different parts of neurons is critical for their synaptic functions, as motor proteins like kinesin and dynein travel along the microtubules to and from pre- and postsynaptic sites. These actions allow the movement of mitochondria, synaptic vesicles, ion channels, receptors, and scaffolding proteins through neurons (Hollenbeck and Saxton, 2005; Bereiter-Hahn and Jendrach, 2010). Synapses are highly vulnerable to the impairment in microtubule transport; therefore, any perturbation in this communication system could cause neurotransmission impairment and lead to synaptic degeneration (Gendron and Petrucelli, 2009).

It has been proposed, that pathological forms of tau could disrupt axonal transport and cause synaptic damage by several mechanisms. Tau overexpression increases the pausing frequency of mitochondria movement by 16% in neurons (Shahpasand et al., 2012). In addition, overexpression of tau reduces anterograde movement of mitochondria, suggesting that tau itself inhibits mitochondrial transport independent of its posttranscriptional modification state (Shahpasand et al., 2012). Therefore, this indicates that overload of tau binding to microtubules results in a transport inhibition essentially by blocking the movement of the motor proteins (Stamer et al., 2002).

Overexpression of tau modifications can also destabilize microtubules leading to microtubule disassembly, thus impairing the microtubule tracks needed for the transport of molecular motors and their cargo (Alonso et al., 1994). In an *in vitro* model of overexpression of pathological tau forms, it was shown that tau hyperphosphorylated at the AT8 sites (Ser199/Ser202/Thr205) inhibits mitochondrial transport to a greater degree than WT tau by increasing the inter-microtubule distance (Shahpasand et al., 2012). On the other hand, SH-SY5Y cells stably overexpressing either human WT tau or the tau carrying the P301L FTDP-17 mutation present a significant decrease in mitochondrial movement, causing a destabilization of the microtubule network, which leads to a perinuclear localization of the mitochondria (Schulz et al., 2012). In addition, in rTg4510 mice, a mouse model that overexpresses the P301L mutation and develops a robust NFT-like pathology at 4–5 months of age, an aberrant mitochondrial distribution is evident in neurites regardless of whether or not aggregated tau is present in the form of neutropil threads (Kopeikina et al., 2011). The somatic distribution is less affected, as only cells with somatic accumulation of misfolded tau show indications of perinuclear mitochondrial clumping, and this phenotype is worsening with age (Kopeikina et al., 2011). Interestingly, this work suggests tau-induced anterograde transport deficits as the general mechanism by which mitochondria become improperly distributed (Kopeikina et al., 2011). Therefore, it is possible that the ability of tau to impair axonal transport does not necessarily involve microtubule dysfunction. That is because, in most models, retrograde transport is not impaired, and for that, it seems unlikely that the inhibition of anterograde axonal transport resulting from tau overexpression is caused only by altered microtubule dynamics (Gendron and Petrucelli, 2009). The alternative is that tau itself could be interacting with kinesin (Utton et al., 2005; Cuchillo-Ibanez et al., 2008; Dubey et al., 2008), raising the possibility that high levels of tau may compete with potential kinesin cargo and thus prevent their translocation to the synapse.

Furthermore, in cortical neurons from the knockin (KI) P301L mice, expression of mutated tau at physiological levels also suggests mitochondrial transport defects (Rodriguez-Martin et al., 2016). In this model, a reduced number of axonal mitochondria was found, without differences in kinetic parameters of anterograde and retrograde transport (Rodriguez-Martin et al., 2016). Furthermore, neurons that were expressing the KI-P301L tau mutation showed a negative alteration in the angle that defines the orientation of the mitochondria in the axon. Such change in the orientation could lead to a temporary blockage of axonal mitochondria that could

increase the mitochondrial fusion and decrease the number of mitochondria in the axon (Rodriguez-Martin et al., 2016). On the other hand, tau could also be involved in the trafficking deficits elicited by MDMA (commonly known as ecstasy), as confirmed by the partial reversion of mitochondrial movement deficits in tau KO neurons treated with this drug (Barbosa et al., 2014). MDMA treatment increases tau hyperphosphorylation mediated by GSK3β, and the blocking of this activity reduces MDMA-induced mitochondrial trafficking alterations (Barbosa et al., 2014). These results together suggest that physiological levels of modified tau protein could also alter mitochondrial transport.

Interestingly, other work has shown that an increase in phosphorylated tau is correlated with an increase in mitochondrial movement from cytoplasm to synapses in hippocampal neurons of a chronic stress mouse model (Zhang et al., 2012). Consistent with the *in vivo* model, the mitochondrial transport was decreased in cultured primary hippocampal neurons when hyperphosphorylated tau was inhibited by lithium (Zhang et al., 2012). On the other hand, overexpression of GSK3β in cultured neurons results in an increase in the number of motile axonal mitochondria (Llorens-Martin et al., 2011). This effect was completely abolished in tau KO mice, indicating that the effects of this kinase are mediated by its action on tau, as an overexpression of GSK3β results in an increase in tau hyperphosphorylation and a decrease of tau binding to microtubules (Llorens-Martin et al., 2011). These different approaches have shown that an increase in phosphorylated tau is not always related with a decrease in mitochondrial movement. However, it is a fact that changes in tau levels and their posttranslational modifications directly influence the mitochondrial transport in neurons and finally affect the synaptic process (**Figure 1**).

EFFECTS OF TAU PATHOLOGY ON MITOCHONDRIAL DYNAMICS AND MITOPHAGY

Mitochondria are the powerhouse of the cell. Apart from the energy production, they play important roles in many cellular activities, such as metabolism, aging, and cell death (Nunnari and Suomalainen, 2012). Because of the high demand of energy and the characteristics mitochondrial metabolism, neurons contain through the cytoplasm and axons many mitochondria are maintained as short tubular structures with high dynamic actions (Kageyama et al., 2011). Fission and fusion proteins, that regulate organelle size, number, and shape and contribute to the correct function of the organelle, coordinate the dynamic interactions among mitochondria (Itoh et al., 2013). The soluble cytosolic protein that assembles into spiral filaments around mitochondrial tubules, dynamin-related GTPase (Drp1), mediates mitochondrial fission (Kageyama et al., 2011; Tamura et al., 2011). Drp1 regulates its interactions with mitochondria trough several posttranslational modifications, including phosphorylation, ubiquitination, and sumoylation (Chang and Blackstone, 2010). Also, this protein can interact with other outer membrane proteins, including Mff, Fis1, and the two homologous proteins Mid49 and Mid51, that contribute to the mitochondrial division (Kageyama et al., 2011). On the other hand, mitochondrial fusion is mediated by the dynamin-related GTPases mitofusin 1 and 2 (Mfn1/2) and optic dominant atrophy 1 (Opa1) (Tamura et al., 2011; Wilson et al., 2013). Mfns are located in the outer membrane and are subjected to ubiquitination and proteasomal degradation (Gegg et al., 2010; Tanaka, 2010), while Opa1 is located in the inner mitochondrial membrane where it is proteolytically regulated by different mitochondrial proteases (Ishihara et al., 2006; Quiros et al., 2012). Opa1 and Mfns interact to form mitochondrial intermembrane complexes that promote the fusion of outer and inner mitochondrial membranes (Song et al., 2009). An imbalance in one of these proteins leads to a similar mitochondrial fragmentation phenotype, suggesting that both outer and inner membrane fusion processes are affected (Itoh et al., 2013).

Many age-related neurodegenerative diseases are associated with alterations in the fission and fusion of mitochondria (Cho et al., 2010). For example, in brain studies of higher order animals it was reported important changes in mitochondrial morphology from aged rhesus macaque (RM), including numerous mitochondria with different size profiles, related with an unfinished fission by Drp1 and Fis1 proteins (Morozov et al., 2017). Despite that changes in RM brain were most similar to healthy elderly humans than AD pathology (Cramer et al., 2018); those studies suggest that alterations in mitochondrial morphology in normal brain aging may contribute to cognitive decline. Remains to be determinate the contribution of tau protein in those processes.

Overexpression of human tau (hTau) in different cell types (HEK 293, primary neurons, and neuronal cultures from hTau transgenic mice) not only enhances retrograde mitochondrial transport rate but also mitochondrial fusion, which may explain the perinuclear mitochondrial accumulation. The fusion proteins Mfns and Opa1 are significantly increased and fission proteins are not changed (Li et al., 2016). Reduced polyubiquitinated Mfn2 was also found, suggesting that an impaired ubiquitination may underlie Mfn accumulation. Interestingly, in the same studies, the downregulation of Mfn2 prevented the hTau-induced mitochondrial injury and the cell viability loss (Li et al., 2016). Related with this specific effect, mitochondria from WT tau expressing cells show an orthodox mitochondrial state with small intracristal spaces, contrary to the globular structure of cristae and dense matrix that is present in mitochondria of cells bearing the P301L mutation (Li et al., 2016). Furthermore, the P301L tau mutation leads to an impairment of mitochondrial fission and fusion generated by a reduced expression of the fusion factors Mfn1 and Opa1 and all fission factors (Schulz et al., 2012).

Complementary studies show that expression of hTau results in the elongation of mitochondria in both Drosophila and mouse neurons, an event that is enhanced by the expression of tau R406W (a human mutation that enhances toxicity in the aging brain) (DuBoff et al., 2012). In addition, a greater mitochondrial elongation was triggered by expression of a more toxic, pseudo-hyperphosphorylated form of tau (tau E14) (DuBoff et al., 2012). This event seems to be related with a mislocalization of Drp1 with a subsequent failure of normal mitochondria dynamics

control. In addition, mitochondrial elongation is accompanied by the excessive production of reactive oxygen species (ROS) and cell cycle-mediated death, which can be rescued *in vivo* by genetically restoring the proper balance of mitochondrial fission and fusion (DuBoff et al., 2012). More important, in postmortem brain of AD patients and brain tissues from APP, APP/PS1, and 3xTgAD mice, it was found that phosphorylated tau interacts with Drp1 and that this interaction occurs mainly at a late stage of disease progression (Manczak and Reddy, 2012a). Those effects are accompanied by an increase in GTPase activity and it appears that the interaction between Drp1 and hyperphosphorylated tau exacerbates mitochondrial and synaptic deficiencies, ultimately leading to neuronal damage and cognitive decline in AD (Manczak and Reddy, 2012a). Interestingly, it has been suggested that prior to the assembly of Drp1 filaments into the mitochondria the endoplasmic reticulum (ER) wraps around the organelle at an early stage of division (Friedman et al., 2011). These ER-mitochondria contacts may help Drp1 to assemble and, after fission of the mitochondria, Drp1 to disassemble from ER-mitochondria contacts for future rounds of mitochondrial fission (Friedman et al., 2011). In motor neurons of JNPL3 mice overexpressing tau P301L, an increase in the numerous contacts between ER and mitochondria compared to WT mice was shown (Friedman et al., 2011). In addition, tau immunogold labeling indicates that this increased number of contacts might result from the preferential association of tau with ER membranes. Interestingly, this association pattern was shown in AD brains and indicates an imbalance of mitochondrial fission mediated by Drp1 (Perreault et al., 2009).

It is important to remember that phosphorylation is not the only important modification of tau as it was suggested that tau truncation could be an early step in neurodegeneration (Johnson, 2006). Results from our group show that expression of caspase-cleaved tau in a neuronal cell model results in mitochondrial impairment (Quintanilla et al., 2012, 2014). We have shown that truncated tau alone induces an increase in mitochondrial fragmentation in neurons. In addition, when transfected cells are treated with Aβ at sublethal concentrations, there is an increase in the stationary mitochondrial population (Quintanilla et al., 2012, 2014). Also, we have recently described that this impairment in mitochondrial morphology is mediated by a decrease in Opa1 levels in neuronal cells (Perez et al., 2017). These effects are likely to affect mitochondrial bioenergetics and neuronal function since the presence of truncated tau enhances mitochondrial damage and cell viability loss induced by Aβ (Perez et al., 2017).

Interestingly, it has been widely described that the regulation of mitochondrial morphology by fission is important for the clearance of this organelle by mitophagy (Shutt et al., 2011). During this autophagy-mediated degradation of mitochondria, mitochondrial fission is enhanced partly due to the proteasomal degradation of Mfns and the proteolytic processing of Opa1 (Gegg et al., 2010; Tanaka, 2010; Ziviani and Whitworth, 2010). Whereas an increase in mitochondrial division facilitates the mitophagy of dysfunctional mitochondria, fission is downregulated by a Drp1 phosphorylation mechanism during starvation-induced autophagy, resulting in elongated mitochondrial networks that are protected against degradation (Itoh et al., 2013). After the fragmentation process, Parkin, an E3 ubiquitin ligase, is recruited to dysfunctional mitochondria and ubiquitinates mitochondrial proteins for proteasomal degradation and promotes the engulfment of mitochondria by autophagosomes (Shutt et al., 2011).

In that context, intracellular accumulation of WT hTau results in mitophagy deficits, as the increase in hTau may block the transport of autophagosomes (Hu et al., 2016). In addition, Parkin levels are reduced in the mitochondrial fraction of hTau transfected cells, and tau directly inserts into the outer membrane fraction, which could be reduce the interaction between Parkin and mitochondria (Hu et al., 2016). Taken together these events suggest that overexpression of hTau itself generates a massive accumulation of dysfunctional mitochondria in somatodendritic compartments of neurons. In the case of cleaved tau, specifically the NH2hTau fragment, an impairment in its selective autophagic clearance and the mitochondrial dynamics was described. Fragmentation and perinuclear mislocalization of mitochondria with smaller size and density are early found in dying NH2hTau-expressing neurons (Amadoro et al., 2014). This effect could be related with a reduction in the general Parkin-mediated remodeling of the proteosome membrane, an increase in the colocalization of mitochondria with autophagic markers, bioenergetics deficits, and *in vitro* synaptic pathology (Amadoro et al., 2014). Interestingly, a later work by the same group shows that NH2hTau generates an imbalance in Parkin-mediated mitophagy favoring cell death in a neuronal model. The NH2hTau fragment modifies the quality control of neuronal mitochondria by facilitating subcellular trafficking and/or recruitment of both Parkin and UCHL-1 to these organelles compelling them to inappropriate, excessive, and deleterious elimination via selective autophagy. Moreover, inhibition of excessive mitophagy in NH2hTau neurons partially restores the mitochondrial content but does not completely prevent the mitochondrial damage resulting in a modest but significant protection against cell death (Corsetti et al., 2015). All this evidence suggests that mitochondrial fragmentation, mitophagy, and neuronal death represent a mechanism of response to a mitochondrial bioenergetics damage generated by an overexpression of tau and/or an increase in its pathological processing (**Figure 2**).

MITOCHONDRIAL BIOENERGETICS FAILURE AND TAU PATHOLOGY

The main function of mitochondria is to convert the energy derived from nutrients into heat and ATP, but it is also a major contributor to calcium regulation, ROS production, cell metabolism, and cell death (Nunnari and Suomalainen, 2012). Under normal conditions, mitochondria can buffer substantial amounts of calcium during neurotransmission, and finely control oxidative stress in the brain (Friberg et al., 2002). In pathological conditions, mitochondrial bioenergetics dysfunction can occur, leading to neuronal degeneration and cell death (Mattson et al., 2008). In that context, it is currently controversial if overexpression of human tau produces mitochondrial damage in neuronal cell lines. Li et al. (2016) describe that hTau

overexpression in primary culture decreases ATP levels and the ratio ATP/ADP as well as inhibits complex I activity. On the other hand, overexpression of WT tau in SH-SY5Y cells improves mitochondrial function through an increase in complex I activity, resulting in a hyperpolarized mitochondrial membrane potential (MMP), higher ATP levels, and an increased metabolic activity (Schulz et al., 2012). The exact reasons for the discrepancy are currently not clear, but the different cell types and transfection protocols may be one of them.

Despite that, it seems to be a common conclusion that overexpression of the P301L tau mutation leads to mitochondrial dysfunction. In neuronal models, a reduction in ATP levels, a slight depolarization of the MMP as well as decreased metabolic activity induced by a pronounced reduction in complex I activity have been described (Schulz et al., 2012). Moreover, studies in P301L tau mice show a downregulation of complexes I and V, accompanied by a significant reduction in MMP levels of mitochondria from 12-month-old P301L tau mice after treatment with the complex I inhibitor rotenone and the complex V inhibitor oligomycin (David et al., 2005). In addition, these animals show a decrease in complex I activity without affecting the basal respiration and the MMP which suggests a compensatory effect of other mitochondrial respiratory chain complexes. However, when the tau pathology is worsened during aging, this effect is not sufficient, and mitochondria from 24-month-old mice exhibit an impaired mitochondrial respiratory activity, diminished capacity in electron transport, and a significant reduction in ATP levels (David et al., 2005). All these events are accompanied by an increase in cytosolic H_2O_2 and superoxide anion radicals in 24-month-old P301L mice (David et al., 2005).

Another interesting study compares brains from mice strains with tau pathology (tripleAD; TauP301L, line pR5) in the presence (tripleAD; APP/PS2) or absence (TauP301L; WT) of Aβ production. The study demonstrates that one third of the proteins deregulated by tau pathology have functions in mitochondria and confirms differences in the expression of complex I and IV (Rhein et al., 2009). Furthermore, it shows that at 8 months, complex I activity is only decreased in pR5 mice. Interestingly, during aging, the tripleAD mice show an increase in the mitochondrial respiratory capacity compared to pR5 and APP/PS2, suggesting a synergistic effect of tau and Aβ on mitochondria (Rhein et al., 2009). Moreover, this synergistic property of Aβ seems to be related to the toxicity of different $A\beta_{42}$ conformations. It has been shown that both oligomeric and fibril, but not monomeric $A\beta_{42}$ cause a decrease in MMP levels in cortical neurons obtained from P301L mice (Eckert et al., 2010). In addition, mitochondrial preparations extracts from P301L mice brains show a reduction in mitochondrial respiration, respiratory control ratio, and uncoupled respiration after the treatment with oligomeric or fibril Aβ peptide (Eckert et al., 2010).

Furthermore, other tauopathies that involve an increase in tau deregulation also present mitochondrial bioenergetic dysfunction. In a segmental trisomy 16 mouse model for Down Syndrome, Ts1Cje, that presents significant tau hyperphosphorylation, decreases of ATP production and MMP as well as increases in ROS levels were shown (Esteras et al., 2017). These alterations were not related to NFT formation or APP metabolism but seem to be connected with an increase in GSK3β and JNK/SAPK activities (Shukkur et al., 2006). On the other hand, induced-pluripotent stem cells (iPSC)-derived neurons carrying the 10 + 16 MAPT mutation (inducing altered tau splicing that causes FTD) present hyperpolarization of the mitochondria, which is partially maintained by the complex V working in reverse mode, leading to an increase in ROS production, oxidative stress, and cell death (Esteras et al., 2017). Moreover, complex I respiration is inhibited, causing a decrease in the ATP production by oxidative phosphorylation that is compensated by an increase in glycolysis. Interestingly, these cells present an increase in cell death as a result of the increased rate of ROS production linked to the hyperpolarization of the mitochondria and not related with the impairment of complex I (Esteras et al., 2017).

Regarding the truncated forms of tau, our group has described that mitochondria in neuronal models expressing Asp421-cleaved tau by caspase-3 present a fragmented morphology, high levels of ROS, a significant reduction in the calcium-buffering capacity, and a significant decrease in MMP and mitochondrial membrane integrity (Quintanilla et al., 2009). Also, primary cortical neurons that express Asp421-cleaved tau enhance Aβ-induced mitochondrial failure. These observations indicate that Asp421-cleaved tau and Aβ cooperate to impair mitochondria, which likely contributes to the neuronal dysfunction in AD (Quintanilla et al., 2012). Further studies show that only aged cortical neurons expressing tau pseudo-phosphorylated at S396/404 present mitochondrial depolarization and an increase in superoxide production when these neurons are treated with Aβ (Quintanilla et al., 2014). In contrast, neurons that express Asp421-cleaved tau show a significant mitochondrial depolarization in young and aged neuronal cultures. This indicates that truncated but not phosphorylated tau may contribute to the early mitochondrial impairment reported in brain samples and neuronal cell models of AD (Quintanilla et al., 2014). Interestingly our group has described that the classic mitochondrial permeability transition pore (mPTP) inhibitor cyclosporin A (CsA) was effective in partially preventing the mitochondrial fragmentation and decrease in MMP observed in cells that express truncated tau (Quintanilla et al., 2009). Moreover, pretreatment with CsA attenuates mitochondrial membrane integrity loss after calcium overload induced by thapsigargin in immortalized cortical neurons that express Asp421-cleaved tau (Quintanilla et al., 2009).

The mPTP is a mitochondrial channel whose opening generates a non-specific increase in the permeability to ions and small solutes (Haworth and Hunter, 1979; Hunter and Haworth, 1979a,b). The original mPTP model suggests that this channel is formed by three principal proteins: cyclophilin D (CyPD), located in the mitochondrial matrix; the adenine nucleotide translocator (ANT), found in the inner membrane; and the voltage-dependent anion channel (VDAC) in the outer membrane (Rao et al., 2014). However, recently it was proposed that ATP synthase is also a major component of the mPTP, for review see Perez et al., 2017). In the most common neurological disorders, the impairment

of the calcium regulation and increased ROS levels are potent inducers of an mPTP opening (Du and Yan, 2010). The formation and the consequent opening of the mPTP is a key factor in mitochondrial dysfunction and mitochondria-driven cell death, as this process involves a failure in MMP, a decrease in ATP production, release of mitochondrial content, and finally cell death (Kroemer and Blomgren, 2007; Bonora et al., 2013; Bernardi and Di Lisa, 2015; Bernardi et al., 2015; Jonas et al., 2015).

Interestingly, Asp421-cleaved tau is not the only pathological form that affects mitochondria through the mPTP opening. Amadoro and colleagues overexpressed some N-terminal derived fragments of tau located around different protease(s)-cleavage consensus sites in the tau NH2 domain. They show that tau N-terminal fragments lacking the first 25 amino acids induce neurodegeneration, cell death, and synaptic failure (Amadoro et al., 2006). More importantly, this NH2-26-44 tau fragment can impair oxidative phosphorylation due to the non-competitive inhibition of the mitochondrial ANT, an ADP/ATP exchanger (Atlante et al., 2008). In addition, Amadoro and colleagues found that the NH2-derived tau fragment preferentially interacts with Aβ peptide in human AD tissues in association with mitochondrial ANT and CypD. These interactions between the tau fragments and Aβ exacerbate the ANT impairment, thereby potentiating ANT dysfunction and further decrease the ATP production (Amadoro et al., 2012). Moreover, they describe that the addition of the VDAC inhibitor DIDS reduces the levels of the mitochondrial superoxide anions produced in these cells, which is caused by a dysfunctional complex I activity (Amadoro et al., 2012). Finally, superoxide levels increases, that are generated by the presence of this tau fragment, modify the active site of mitochondrial ANT, thereby directly influencing the opening of the mPTP (Bobba et al., 2013). On the other hand, the mPTP component VDAC was also analyzed in postmortem brains of AD patients and brain samples from APP, APP/PS1, and tripleAD mice (Manczak and Reddy, 2012b). These studies show progressively increased levels of VDAC in the cortical tissues from AD brains compared to control subjects (Manczak and Reddy, 2012b). It was also found that VDAC1 interacts with Aβ and phosphorylated tau in the brain of these AD mice models suggesting that this interaction may block mitochondrial pores, leading to defects in oxidative phosphorylation and mitochondrial dysfunction (Manczak and Reddy, 2012b). Although several proteins and bioenergetics deregulation mediated by tau pathology could be associated with the opening of the mPTP (**Figure 3**), further investigation is necessary to unravel this pending issue.

FINAL REMARKS

Even under normal physiological conditions, tau can undergo different posttranslational modifications that play various roles in the onset and progression of AD. Several of these modifications may have converging mechanisms of toxicity, but to date, it is unknown how these posttranslational modifications affect neuronal function and trigger different pathological conditions. Here, we showed that the presence of tau pathology could affect mitochondria function, which it seems to explain better the neuronal dysfunction observed in AD. We described evidence indicating that the presence of pathological forms of tau negatively affects the mitochondria trafficking, morphology, and bioenergetics. These actions compromise mitochondrial function generating: (i) a decrease in local ATP levels, which impairs normal neurotransmission, and (ii) an imbalance in the calcium buffering capacity with subsequent neurotoxicity. Both mechanisms can directly affect the neuronal metabolism and further brain functions. While many questions remain, a better understanding of the early events in tau-mediated neurotoxicity is particularly important as it may lead to the development of new therapeutic strategies that prevent the impairment of mitochondrial function and eventually decrease the pathological neuronal events that initiate neurodegeneration.

AUTHOR CONTRIBUTIONS

MP performed the research, wrote the paper, drew the figures, and revised the paper. CJ performed the research and wrote the paper. RQ directed the project, supported the research, and revised the paper.

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Targeting Nrf2 to Suppress Ferroptosis and Mitochondrial Dysfunction in Neurodegeneration

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Ferroptosis is a newly described form of regulated cell death, distinct from apoptosis, necroptosis and other forms of cell death. Ferroptosis is induced by disruption of glutathione synthesis or inhibition of glutathione peroxidase 4, exacerbated by iron, and prevented by radical scavengers such as ferrostatin-1, liproxstatin-1, and endogenous vitamin E. Ferroptosis terminates with mitochondrial dysfunction and toxic lipid peroxidation. Although conclusive identification of ferroptosis *in vivo* is challenging, several salient and very well established features of neurodegenerative diseases are consistent with ferroptosis, including lipid peroxidation, mitochondrial disruption and iron dysregulation. Accordingly, interest in the role of ferroptosis in neurodegeneration is escalating and specific evidence is rapidly emerging. One aspect that has thus far received little attention is the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). This transcription factor regulates hundreds of genes, of which many are either directly or indirectly involved in modulating ferroptosis, including metabolism of glutathione, iron and lipids, and mitochondrial function. This potentially positions Nrf2 as a key deterministic component modulating the onset and outcomes of ferroptotic stress. The minimal direct evidence currently available is consistent with this and indicates that Nrf2 may be critical for protection against ferroptosis. In contrast, abundant evidence demonstrates that enhancing Nrf2 signaling is potently neuroprotective in models of neurodegeneration, although the exact mechanism by which this is achieved is unclear. Further studies are required to determine to extent to which the neuroprotective effects of Nrf2 activation involve the prevention of ferroptosis.

Keywords: Alzheimer's disease, Parkinson's disease, Huntington's disease, motor neuron disease, RSL3, erastin, Keap1, system x_c^-

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FERROPTOSIS; AN IRON-DEPENDENT NON-APOPTOTIC FORM OF REGULATED CELL DEATH

The last few decades have witnessed a surge in the discovery of new forms of regulated cell death that have immense implications for both health and disease (Galluzzi et al., 2018). Ferroptosis is a recently described form of non-apoptotic regulated cell death caused by uncontrolled iron-dependent lipid peroxidation that is distinct in its morphological, biochemical, and genetic profile from other cell death mechanisms (Dixon et al., 2012; Galluzzi et al., 2018). Cells undergoing

ferroptosis show none of the classical morphological alterations associated with apoptosis, necroptosis, or autophagy (e.g., cell swelling, nuclear disruption, membrane blebbing, etc.), with the only discernable ultrastructural feature being distinctly altered mitochondrial morphology (Dixon et al., 2012; Stockwell et al., 2017).

A discriminating feature of ferroptosis is the potent capacity of lipid peroxide scavengers (such as ferrostatin-1, liproxstatin-1, and vitamin E) to prevent ferroptotic cell death (Dixon et al., 2012; Friedmann Angeli et al., 2014; Yang et al., 2014). This explicit requirement for lipid peroxidation is a core feature of ferroptosis that distinguishes it from other forms of cell death: ferroptosis inhibitors cannot prevent other forms of cell death (Dixon et al., 2012; Friedmann Angeli et al., 2014; Yang et al., 2014), and conversely classic inhibitors of necrosis, apoptosis, and autophagy do not modulate ferroptosis (Dixon et al., 2012), with the exception of the necroptosis inhibitor, necrostatin-1 (RIPK1 inhibitor), which can inhibit ferroptosis in a necroptosis/RIPK1-independent manner (Friedmann Angeli et al., 2014). The central endogenous suppressor of ferroptosis is the selenoenzyme glutathione peroxidase 4 (Gpx4). Gpx4 detoxifies membrane lipid hydroperoxides, preventing unchecked toxic lipid peroxidation. Gpx4 requires the major cellular antioxidant glutathione as a substrate, and hence ferroptosis is also dependent on glutathione levels (Friedmann Angeli et al., 2014). The precise role of iron in ferroptosis is ironically unclear (see below), however, its involvement is unequivocally indicated by the strong inhibition of cell death associated with iron chelation or limiting iron availability (**Figure 1**).

Ferroptosis appears to require the presence of specific highly oxidisable phosphatidylethanolamine phospholipids containing the polyunsaturated fatty acids (PUFAs) arachidonic acid and adrenic acid. Acyl-CoA synthetase long-chain family member 4 (ACSL4) is important for the synthesis of phospholipids from these PUFAs, while lysophosphatidylcholine acyltransferase 3 (LPCAT3) is important for their insertion into membrane phospholipids (Kagan et al., 2017). Pharmacological or genetic inhibition of either ACSL4 or LPCAT3 suppresses ferroptosis specifically over other forms of cell death (Dixon et al., 2015; Yuan et al., 2016b; Doll et al., 2017; Kagan et al., 2017). Lipoxygenases can catalyze the formation of lipid hydroperoxides from PUFAs, instigating toxic lipid peroxidation (Yang et al., 2016), although this can occur independently from lipoxygenase activity (Shah et al., 2017).

As mentioned above, the precise role of iron in ferroptosis remains unclear. The ability of the iron chelators including deferoxamine and deferiprone to salvage cells from ferroptotic death in a variety of models underscores the role of iron in triggering ferroptosis (Dixon et al., 2012; Friedmann Angeli et al., 2014; Yang et al., 2014; Do Van et al., 2016). The lipoxygenases involved in generating toxic lipid hydroperoxides require catalytic iron in their active sites (Abeysinghe et al., 1996), hence the protective effect of iron chelation has been proposed to involve inhibition of lipoxygenase activity by removal of the essential catalytic iron from these enzymes. Alternatively, iron has been demonstrated to potentiate ferroptosis in a free radical-mediated process independent from lipoxygenase activity (Shah et al., 2017).

Ferroptosis can be experimentally induced by direct inhibition of Gpx4 via Ras-selective lethal small molecule 3 (RSL3) or by genetic knockdown or deletion of Gpx4 (Friedmann Angeli et al., 2014; Yang et al., 2014). As Gpx4 requires glutathione as a substrate, ferroptosis is also induced by disruption of glutathione supply via inhibition of glutathione synthesis (e.g., buthionine sulfoximine) or inhibiting the supply of cysteine required for glutathione synthesis via inhibition of the cystine-glutamate antiporter system x_c^- (via erastin, sulfasalazine, or sorafenib) (Dixon et al., 2012; Friedmann Angeli et al., 2014) (**Figure 1**).

Mitochondria play key roles in regulated cell death (Galluzzi et al., 2018), and ferroptosis is no exception. Cells undergoing ferroptosis exhibit specific mitochondrial morphology. Early studies using the system x_c^- inhibitor erastin show ferroptosis results in smaller mitochondria with increased mitochondrial membrane density (Yagoda et al., 2007; Dixon et al., 2012). Later studies employing pharmacological or genetic disruption of Gpx4 causes mitochondrial swelling, decreased cristae and outer membrane rupture (Friedmann Angeli et al., 2014; Doll et al., 2017; Maiorino et al., 2017; Ingold et al., 2018). Toxicity induced by inhibition of mitochondrial complex I can be rescued by ferroptosis inhibitors (Basit et al., 2017). Furthermore, lack of ACSL4 prevents the RSL3-mediated rupture of mitochondrial outer membrane (Doll et al., 2017), while knockdown of mitochondrial acyl-CoA synthetase family member 2 (ACSF2; involved in fatty acid metabolism) prevents erastin toxicity, suggesting ACSF2 generates a mitochondrial-specific lipid necessary for ferroptosis (Dixon et al., 2012).

Despite these observations, evidence for mitochondrial lipid peroxidation during ferroptosis is mixed. Ferroptosis induced by erastin does not appear to be accompanied by mitochondrial lipid peroxidation *in vitro* (Dixon et al., 2012; Do Van et al., 2016) or *in vivo* (Wang et al., 2016). In contrast, disruption of Gpx4 results in mitochondrial lipid peroxidation *in vitro* and in kidneys (Friedmann Angeli et al., 2014). This discrepancy suggests that the inducing stimuli may be critical for the subcellular localization of lipid peroxidation.

Although mitochondria are clearly impaired in ferroptosis, evidence suggests that they are not driving the cell death process. Cells deficient in mitochondria remain sensitive to ferroptosis (Gaschler et al., 2018). Furthermore, extramitochondrial lipid peroxidation temporally precedes mitochondrial lipid peroxidation, and mitochondrial damage including rupture of the outer mitochondrial membrane is a late event, closely preceding cell lysis (Friedmann Angeli et al., 2014; Jelinek et al., 2018).

Reports on targeting antioxidants to mitochondria are mixed. MitoQ rescues neuronal cells from RSL3 toxicity (Jelinek et al., 2018). However, when compared their non-mitochondrial analogs, mitochondrially targeted radical scavengers are opposingly reported as being less effective (Friedmann Angeli et al., 2014) or more effective (Krainz et al., 2016).

Mitochondrial iron is also implicated in ferroptosis. MitoNEET, also known as CISD1, is an iron-containing outer mitochondrial membrane protein involved in iron

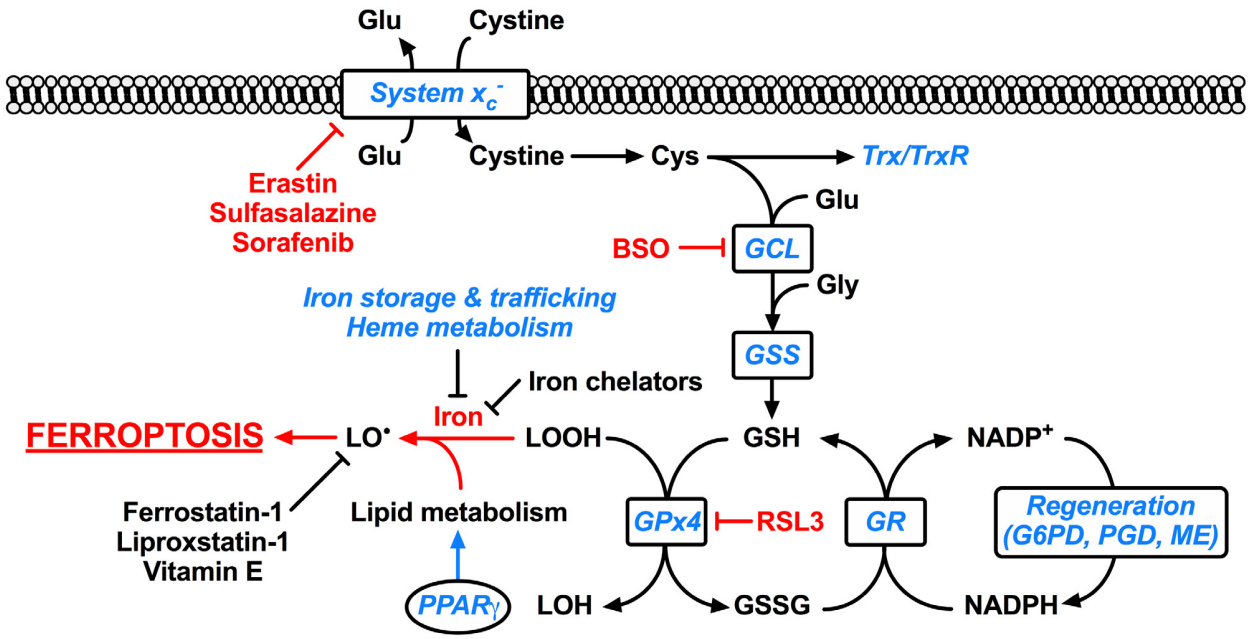


FIGURE 1 | Ferroptosis and its molecular regulation by Nrf2. Glutathione peroxidase 4 (Gpx4) utilizes the major cellular antioxidant glutathione (GSH) as a substrate to reduce lipid hydroperoxides (LOOH). Oxidized glutathione (GSSG) generated by Gpx4 is reduced back to glutathione by glutathione reductase (GR) in a reaction requiring NADPH, which can be regenerated by glucose-6-phosphate dehydrogenase (G6PD) and phosphogluconate dehydrogenase (PGD) of the pentose-phosphate pathway, and malic enzyme (ME). The tripeptide glutathione is synthesized by the consecutive action of glutamate-cysteine ligase (GCL) and glutathione synthetase (GSS), where the ligation of cysteine (Cys) and glutamate (Glu) by glutamate-cysteine ligase is the rate-limiting step of glutathione synthesis. The cellular import of cystine by the glutamate-cystine antiporter system x_c^- constitutes an significant route of cysteine supply for glutathione synthesis. Cysteine is also required for other important cellular antioxidants including thioredoxin (Trx) and thioredoxin reductase (TrxR). Ferroptosis occurs when the Gpx4-catalyzed reduction of lipid hydroperoxides is insufficient to prevent the iron-mediated generation of lipid radicals (LO•). This leads to the propagation of lipid peroxidation and culminates in ferroptosis. Ferroptosis can be experimentally induced by inhibiting Gpx4 via the small molecule inhibitor RSL3, or by limiting glutathione supply to Gpx4. The latter is induced by direct [e.g., buthionine sulfoximine (BSO)] or indirect (e.g., by limiting cysteine availability) inhibition of glutathione synthesis. Cysteine supply is disrupted by inhibitors of system x_c^- including erastin, sulfasalazine, and sorafenib. Ferroptosis can also be inhibited by iron chelators such as deferoxamine and deferiprone, and lipid radical scavengers such as ferrostatin-1, liproxstatin-1, and vitamin E. Factors transcriptionally regulated by Nrf2 are indicated in blue italics, whereas factors promoting ferroptosis are indicated in red. Clearly Nrf2 signaling is likely to have an integral and pervasive impact on the manifestation of ferroptosis.

export from mitochondria (Mittler et al., 2018). Knockdown of mitoNEET exacerbates erastin toxicity and increases mitochondrial iron content and lipid peroxidation, whereas stabilization of mitoNEET attenuates erastin toxicity and decreases mitochondrial lipid peroxidation (Yuan et al., 2016a). Alternatively, safely sequestering iron within mitochondria via overexpression of mitochondrial ferritin is able to curb erastin-induced cell death, both *in vitro* and *in vivo* (Wang et al., 2016).

EVIDENCE FOR FERROPTOSIS IN NEURODEGENERATION

Explicitly identifying ferroptosis *in vivo* is hampered by the lack of specific biomarkers. Nevertheless, considerable evidence exists that implicates ferroptosis in neurodegeneration. The association between oxidative stress, lipid peroxidation and neurodegeneration has long been appreciated. Notably, elevated levels of lipid peroxidation are reliably detected in brain tissues and body fluids of Alzheimer's, Parkinson's, Huntington's disease, motor neuron disease and multiple sclerosis patients (Adibhatla and Hatcher, 2010; Shichiri, 2014; Sugiyama and Sun, 2014; Wang et al., 2014; Bradley-Whitman and Lovell, 2015). Iron accumulation is a consistent feature of neurodegeneration (Belaidi and Bush, 2016). The level of iron in brains of individuals with mild cognitive impairment and Alzheimer's disease correlates with disease progression (Smith et al., 2010; Ayton et al., 2017). Elevated iron is a cardinal feature of Parkinson's disease substantia nigra (Ayton and Lei, 2014), and increased iron is detected in affected brain regions of patients with motor neuron disease, multiple sclerosis, Huntington's disease and Friedreich ataxia (Kwan et al., 2012; Li and Reichmann, 2016; Sheykhansari et al., 2018). Reducing brain iron via the chelators deferiprone or deferoxamine is efficacious in clinical trials of Parkinson's (Devos et al., 2014) and Alzheimer's patients (Crapper McLachlan et al., 1991), respectively, indicating iron is contributing to the disease process. Further indirect evidence, including diminished glutathione and insufficient Nrf2 signaling (see below), is consistent with the presence of ferroptosis in neurodegeneration (Liddell, 2017; Liddell and White, 2017). Moreover, impaired mitochondrial function is

common to many neurodegenerative diseases (Carri et al., 2017; Liddell and White, 2017; Liot et al., 2017; Swerdlow, 2017). Morphologically, mitochondria in brains of mice modeling Huntington's disease exhibit disrupted cristae (Lee et al., 2011), while those in motor neuron disease human postmortem tissue and model mice feature swollen and vacuolated mitochondria (Jaarsma et al., 2000; Cozzolino and Carri, 2012) reminiscent of the mitochondrial changes evident in ferroptosis.

Since its original characterisation in cancer cells, the concept of ferroptosis has instigated growing efforts to explicitly detect and measure its footprint in neurodegeneration (Guiney et al., 2017; Morris et al., 2018). In this regard, more direct evidence for the role of ferroptosis in neurodegeneration has recently been generated. Neuronal cells are sensitive to erastin and RSL3 toxicity *in vitro*. This toxicity is associated with mitochondrial impairments, and is rescued by ferroptosis inhibitors or a mitochondrially targeted ROS scavenger (Neitemeier et al., 2017; Jelinek et al., 2018). Genetic models demonstrate the substantial reliance of neurons on Gpx4 to prevent toxic lipid peroxidation. Whereas global deletion of Gpx4 is embryonic lethal (Imai et al., 2003), mice with targeted mutation of Gpx4 selenocysteine to cysteine (sensitive to inactivation), or knockout of Gpx4 specifically in neurons are viable but exhibit selective loss of CA3 hippocampal interneurons, resulting in seizures and early death (Seiler et al., 2008; Ingold et al., 2018). Targeting Gpx4 knockout to photoreceptor neurons results in death of these cells within 21 days of birth (Ueta et al., 2012). Post-development, conditional knockout of Gpx4 in adult mice results in loss of CA1 hippocampal neurons and rapid death, indicating neurons are specifically sensitive to Gpx4 deletion (Yoo et al., 2012). Conditional ablation of Gpx4 targeted to neurons results in dramatic degeneration of motor neurons that rapidly progresses to paralysis and death (Chen et al., 2015). Targeting conditional knockout of Gpx4 to forebrain neurons of adult mice causes cognitive impairments and hippocampal degeneration reminiscent of Alzheimer's disease (Hambright et al., 2017). These models are all accompanied by lipid peroxidation and mitochondrial impairments, consistent with ferroptosis. Conditional Gpx4 knockout can partially rescued by ferroptosis inhibitors, indicating the involvement of ferroptosis (Chen et al., 2015; Hambright et al., 2017).

Ferroptosis inhibitors have also been investigated in explicit models of neurodegneration. Ferroptosis inhibitors prevent the toxicity of Huntington's disease-associated mutated Htt in brain slice cultures (Skouta et al., 2014). Ferroptosis inhibitors are also protective in dopaminergic cultured cells, organotypic slice cultures, and in an MPTP mouse model of Parkinson's disease (Do Van et al., 2016). Furthermore, ferroptosis is implicated in hemorrhagic and ischemic stroke based on the ability of ferroptosis inhibitors to protect against neuronal death in both *in vivo* and *in vitro* models (Li et al., 2017; Tuo et al., 2017; Zille et al., 2017). Ischemic stroke is also strongly modulated by brain iron levels (Tuo et al., 2017). Knockout of glutathione synthesis accelerates the disease phenotype in motor neuron disease-model mice, and ultrastructural analysis of spinal cord tissue reveals mitochondrial swelling, rupture and decreased cristae, all consistent with ferroptosis (Vargas et al., 2011).

Ferroptosis also appears to mechanistically overlap with other cell death modalities in neurodegeneration. Consistent with a distinct form of cell death, it was initially reported that ferroptosis induced by erastin does not involve mitochondrial genes implicated in other cell death pathways, nor the release of cytochrome c (Dixon et al., 2012). However, recent studies show that ferroptosis induces the pro-apoptotic translocation of BH3 interacting-domain death agonist (BID) to mitochondria in neuronal cells (Neitemeier et al., 2017; Jelinek et al., 2018). Oxytosis is described as glutamate-induced inhibition of cystine uptake via system x_c^- leading to glutathione depletion and subsequent lipoxygenase-dependent toxic lipid peroxidation (Tan et al., 2001). This is clearly very similar to ferroptosis and has led to the recent proposal that perhaps ferroptosis and oxytosis are in fact the same pathway (Lewerenz et al., 2018). Accordingly, oxytosis in neurons causes mitochondrial morphological changes similar to ferroptosis (Lee et al., 2011), and the toxicity of glutamate mirrors that of erastin or RSL3 in neuronal cells, and are all amenable to prevention by ferroptosis inhibitors (Liu et al., 2015; Neitemeier et al., 2017; Jelinek et al., 2018) or necrostatin-1 (Xu et al., 2007). Furthermore, oxytosis/ferroptosis in neurons involves the translocation of apoptosis inducing factor (AIF) from mitochondria to nucleus, prevention of which alleviates cell death (Xu et al., 2010; Neitemeier et al., 2017; Jelinek et al., 2018). To further complicate the mechanisms of cell death, mitochondrial AIF release is the penultimate stage of parthanatos, a poly ADP-ribose polymerase 1 (PARP-1)-mediated form of cell death (Fricker et al., 2018; Galluzzi et al., 2018). Parthanatos can be induced by oxidative stress, and is also implicated in neurodegeneration (Fricker et al., 2018; Galluzzi et al., 2018). Hence both initiating events and late stages are shared by ferroptosis/oxytosis and parthanatos.

Together these studies show that many features facilitating ferroptosis are present in neurodegeneration, and mounting evidence indicates that targeting ferroptosis with specific inhibitors is a valid therapeutic strategy. However, overlapping mechanisms highlight the need for more comprehensive delineation of the cellular mechanisms of cell death, and the potential for new or repurposed treatments. An alternate approach to attenuate ferroptosis is to augment the endogenous anti-ferroptotic mechanisms within cells. To this end, the role of the transcription factor Nrf2 will now be discussed.

IS Nrf2 AN ANTI-FERROPTOSIS TRANSCRIPTION FACTOR?

In terms of endogenous cellular mechanisms preventing ferroptosis, the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is exquisitely positioned to modulate the onset and outcomes of ferroptosis. Nrf2 is responsible for regulating hundreds of antioxidant genes (Gao et al., 2014). Under normal conditions, Nrf2 resides in the cytosol bound to its negative regulator Kelch-like ECH-associated protein 1 (Keap1). Keap1 constitutively targets Nrf2 for ubiquitination and proteasomal degradation, thereby maintaining Nrf2 signaling capacity at a low level. However,

TABLE 1 | Ferroptosis-related genes that are transcriptionally regulated by Nrf2.

Gene symbol	Gene name	Reference
Glutathione-related		
GCLM	Glutamate-cysteine ligase modifier subunit	Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003
GCLC	Glutamate-cysteine ligase catalytic subunit	Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Wu et al., 2011
GSS	Glutathione synthetase	Thimmulappa et al., 2002; Wu et al., 2011
SLC7A11	System x_c^-	Sasaki et al., 2002
GPX4	Glutathione peroxidase 4	Lee et al., 2003; Salazar et al., 2006; Wu et al., 2011
GSR	Glutathione reductase	Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Wu et al., 2011
TXN1	Thioredoxin 1	Kwak et al., 2003; Lee et al., 2003
TXNRD1	Thioredoxin reductase 1	Kwak et al., 2003; Lee et al., 2003; Wu et al., 2011
Iron-related		
FTH1	Ferritin heavy chain 1	Lee et al., 2003; Pietsch et al., 2003; Wu et al., 2011
FTL	Ferritin light chain	Thimmulappa et al., 2002; Lee et al., 2003; Pietsch et al., 2003; Wu et al., 2011
TFRC	Transferrin receptor 1	Lee et al., 2003
FPN1 (SLC40A1)	Ferroportin	Marro et al., 2010
MT1G	Metallothionein 1G	Sun et al., 2016a
HMOX1	Heme oxygenase 1	Alam et al., 1999; Lee et al., 2003
BLVRA	Biliverdin reductase A	Agyeman et al., 2012
BLVRB	Biliverdin reductase B	Wu et al., 2011; Agyeman et al., 2012
FECH	Ferrochelatase	Wu et al., 2011; Campbell et al., 2013
ABCB6	ATP binding cassette subfamily B member 6	Campbell et al., 2013
HRG1	Heme-response gene 1	Campbell et al., 2013
NAPDH regeneration		
G6PD	Glucose-6-phosphate dehydrogenase	Thimmulappa et al., 2002; Lee et al., 2003; Wu et al., 2011
PGD	Phosphogluconate dehydrogenase	Thimmulappa et al., 2002; Wu et al., 2011
ME1	Malic enzyme 1	Thimmulappa et al., 2002; Kwak et al., 2003; Lee et al., 2003; Wu et al., 2011
Transcription factors		
PPARG	Peroxisome proliferator-activated receptor gamma	Cho et al., 2010
PPARGC1A	Peroxisome proliferator-activated receptor gamma coactivator 1α	Neymotin et al., 2011

states of increased oxidative stress facilitate the dissociation of Nrf2 from Keap1, and promote the nuclear translocation of Nrf2. In the nucleus, Nrf2 interacts with antioxidant response elements (AREs) in the promoter region of target genes resulting in their transcriptional activation (Yamamoto et al., 2018).

Importantly in the context of ferroptosis, almost all genes thus far implicated in ferroptosis are transcriptionally regulated by Nrf2 (**Table 1**). These include genes for glutathione regulation (synthesis, cysteine supply via system x_c^-, glutathione reductase, glutathione peroxidase 4), NADPH regeneration which is critical for Gpx4 activity (glucose 6-phosphate dehydrogenase, phosphogluconate dehydrogenase, malic enzyme), and iron regulation (including iron export and storage, heme synthesis and catabolism) (Sasaki et al., 2002; Lee et al., 2003; Wu et al., 2011; Kerins and Ooi, 2017). In addition, Nrf2 is involved in the regulation of lipids via the ligand-mediated transcription factor peroxisome proliferator-activated receptor gamma (PPARγ). Nrf2 and PPARγ are reciprocally regulated, with activation of either upregulating the other (Cai et al., 2017; Lee, 2017). PPARγ is a major regulator of lipid metabolism (Cai et al., 2017), and can be activated by oxidized lipids relevant to the initiation of ferroptosis (Itoh et al., 2008). Hence Nrf2 indirectly modulates the lipids whose abundance contributes to the sensitivity to ferroptosis (Doll et al., 2017) (**Figure 1**).

Nrf2 also plays important roles in modulating mitochondrial function. Nrf2 can be physically bound to mitochondria and thus monitor and respond to changes in mitochondrial function (Lo and Hannink, 2008). Nrf2 also regulates mitochondrial dynamics, including biogenesis (Piantadosi et al., 2008; Merry and Ristow, 2016) via interaction with peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) (Dinkova-Kostova and Abramov, 2015; Navarro et al., 2017), and mitophagy via a P62-dependent, PINK1/Parkin-independent mechanism (East et al., 2014). Mitochondria in Nrf2 knockout mice have impaired function, whereas activation of Nrf2 enhances mitochondrial function and resistance to stressors (Greco and Fiskum, 2010; Neymotin et al., 2011; Holmstrom et al., 2016).

Therefore, by virtue of its direct and indirect regulation of many genes central to ferroptosis and its regulation of mitochondrial function, Nrf2 is positioned to be a key player in ferroptosis. Despite this, the role of Nrf2 in ferroptosis has received very little attention thus far, limited mainly to *in vitro* studies using cancer cells. As expected, Nrf2 activation confers resistance to ferroptosis in cancer cells (Sun et al., 2016b; Chen et al., 2017a,b; Roh et al., 2017). Genetic modulation of Nrf2 expression, including knockdown and overexpression, can fine-tune the sensitivity of glioma cells to the ferroptosis inducers erastin and RSL3 (Fan et al., 2017). This may contribute to the

cancer-promoting and chemoresistance effects of elevated Nrf2 present in many cancers (Fan et al., 2017; Taguchi and Yamamoto, 2017).

Nrf2 activation is sensitive to shifts in cellular redox status, hence it follows that the lipid peroxidation accompanying ferroptosis should activate Nrf2. Indeed, the ferroptosis inducers erastin and sorafenib are sufficient to activate Nrf2 in hepatocellular carcinoma cells (Houessinon et al., 2016; Sun et al., 2016b). Furthermore, the protective action of the mitochondrially targeted antioxidant, MitoQ, involves Nrf2 activation (Hu et al., 2018).

Given the pervasive lipid peroxidation evident in neurodegeneration, it would be expected that Nrf2 would be elevated in neurodegenerative diseases. While there is some evidence for Nrf2 activation in neurodegeneration, it appears to be relatively mild and clearly insufficient to prevent neuronal dysfunction (Liddell, 2017). In some cases, particularly for motor neuron disease, Nrf2 signaling appears to be impaired (Moujalled et al., 2017). This is in contrast to the strong activation of Nrf2 evident when genetic or pharmacological Nrf2 inducers are applied to models of neurodegeneration (Liddell, 2017). These treatments are robustly neuroprotective in animal models of disease. While several drugs targeting Nrf2 are currently under clinical investigation, to date, dimethyl fumarate (Tecfidera) remains the only clinically approved drug for the treatment of a neurodegenerative disease (relapsing-remitting multiple sclerosis) in which Nrf2 activation clearly contributes to its mechanism of action (Havrdova et al., 2017).

The above mentioned studies in cancer cells provide empirical *in vitro* support for the anti-ferroptotic action of Nrf2. However, direct *in vivo* evidence for an anti-ferroptotic effect of Nrf2 induction does not yet exist, and it is currently unknown whether and to what extent the demonstrated neuroprotective efficacy of Nrf2 activation in models of neurodegeneration involve attenuation of ferroptosis. Evaluation of ferroptosis *in vivo* is currently hindered by the lack of specific markers of ferroptosis. Studies examining the effect of Nrf2 activation in explicit *in vivo* models of ferroptosis (e.g., Gpx4 knockout) would provide some insight.

CONCLUSION AND FUTURE PERSPECTIVES

Ferroptosis is an iron-dependent form of regulated cell death instigated by impaired glutathione metabolism, culminating in mitochondrial failure and toxic lipid peroxidation. Emerging evidence implicates ferroptosis in neurodegeneration, both in the molecular and biochemical signatures of neurodegeneration, and in terms of functional abrogation of neuron death via specific ferroptosis inhibitors. Elucidating how ferroptosis provokes neurodegeneration will expose new therapeutic opportunities to treat these diseases. To this end, targeting the antioxidant transcription factor Nrf2 is an attractive option. Nrf2 signaling is involved in regulating mitochondrial function and impacts almost all identified molecular aspects of ferroptosis. Treatments targeting Nrf2 have been demonstrated to exert anti-ferroptotic effects in the context of cancer cells, and are beneficial in many models of neurodegeneration. The protective mechanism of Nrf2 activation in these models may involve attenuating ferroptosis via upregulation of the endogenous anti-ferroptotic machinery, however, direct evidence for this is currently lacking. The myriad of Nrf2 actions described here suggest that targeting Nrf2 is an exciting therapeutic option to attenuate ferroptosis.

Several questions remain unanswered. The discovery of bonafide biomarkers of ferroptosis will be invaluable to unequivocally probe its involvement in neurodegeneration and facilitate the development of therapeutic treatments targeting ferroptosis. The continued evaluation of ferroptosis inhibitors and comparison to inhibitors of other cell death modalities in further models of neurodegeneration will help elucidate the key pathways involved. Finally, whether Nrf2 activation is directly alleviating ferroptotic stress.

Taken together, the arguments presented in this review elucidate a coherent network that links Nrf2 signaling to mitochondrial function and ferroptotic cell death, and proposes the targeting of Nrf2 as a rational line of therapy for ferroptotic neurodegeneration.

AUTHOR CONTRIBUTIONS

JL conceived the review. MA prepared the first draft. RL, KK, and TM reviewed literature and contributed to writing. JL prepared the final manuscript.

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frontiers in Neuroscience

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Mitochondria and Calcium Regulation as Basis of Neurodegeneration Associated With Aging

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Age is the main risk factor for the onset of neurodegenerative diseases. A decline of mitochondrial function has been observed in several age-dependent neurodegenerative diseases and may be a major contributing factor in their progression. Recent findings have shown that mitochondrial fitness is tightly regulated by Ca^{2+} signals, which are altered long before the onset of measurable histopathology hallmarks or cognitive deficits in several neurodegenerative diseases including Alzheimer's disease (AD), the most frequent cause of dementia. The transfer of Ca^{2+} from the endoplasmic reticulum (ER) to the mitochondria, facilitated by the presence of mitochondria-associated membranes (MAMs), is essential for several physiological mitochondrial functions such as respiration. Ca^{2+} transfer to mitochondria must be finely regulated because excess Ca^{2+} will disturb oxidative phosphorylation (OXPHOS), thereby increasing the generation of reactive oxygen species (ROS) that leads to cellular damage observed in both aging and neurodegenerative diseases. In addition, excess Ca^{2+} and ROS trigger the opening of the mitochondrial transition pore mPTP, leading to loss of mitochondrial function and cell death. mPTP opening probably increases with age and its activity has been associated with several neurodegenerative diseases. As Ca^{2+} seems to be the initiator of the mitochondrial failure that contributes to the synaptic deficit observed during aging and neurodegeneration, in this review, we aim to look at current evidence for mitochondrial dysfunction caused by Ca^{2+} miscommunication in neuronal models of neurodegenerative disorders related to aging, with special emphasis on AD.

Keywords: mitochondria, MAMS, calcium, neurodegeneration, ROS, MPTP, aging, endoplasmic reticulum

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INTRODUCTION

In the last century, the population aged over 60 years old has rapidly increased around the world (Beard et al., 2016). Aging is the major risk factor for many chronic diseases such as cancer, diabetes, hypertension, and neurodegenerative disorders (Kennedy et al., 2014). In particular, aging has been correlated with the occurrence of several types of dementia, affecting 5–10% of people over 65, and

about 50% of people over 85 years old according to the Alzheimer's Disease (AD) International (Prince et al., 2015). AD, the most common and still incurable form of dementia, shares several similar cellular alterations with brain aging including mitochondrial dysfunction, oxidative stress, Ca^{2+} dysregulation, and impaired proteostasis (Leuner et al., 2007; Kern and Behl, 2009; Rodrigue et al., 2009; Martinez et al., 2017). Most cases of AD are sporadic (SAD) and characterized by a late onset of symptoms, such as a decline of intellectual and cognitive functions and irreversible memory loss as major features. Several genes have been found to increase the risk of SAD, with the gene for apolipoprotein E (APOE) being the most studied, specifically, the polymorphism that produces the ε4 allele of the APOE, APOE4 variant of the protein (Allen et al., 2012). In addition, nearly 1% of the cases of AD that are dominantly inherited present an early development known as familial AD (FAD) characterized by mutations in presenilin-1 (PS1) and -2 (PS2) or in the amyloid precursor protein (APP; Sherrington et al., 1995). Both SAD and FAD are characterized by neuronal cell death and assumed to be similar to some extent (Hardy and Selkoe, 2002), but the key events prior to cell death are still unclear.

Mitochondria are central organelles in neuronal physiology integrating several crucial functions such as cell respiration, energy metabolism, Ca^{2+} homeostasis, and reactive oxygen species (ROS) generation, all of which have been found to be dysregulated in aging, AD, and other neurodegenerative disorders such as Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS)/frontotemporal dementia (FTD) disease (Winklhofer and Haass, 2010; Schon and Przedborski, 2011; Itoh et al., 2013; Manfredi and Kawamata, 2016). Here we present an overview of selected findings regarding mitochondrial dysfunction in neurodegenerative disease and discuss their potential as therapeutic targets.

ER-MITOCHONDRIA COMMUNICATION AND CA^{2+} REGULATION IN AGE-ASSOCIATED NEURODEGENERATIVE DISEASES

Communication between organelles allows cells to function and adapt in a changing cellular environment. The endoplasmic reticulum (ER) and mitochondria couple at specific sites termed mitochondria-associated membranes (MAMs), which integrate and coordinate several cellular functions, including synthesis and exchange of phospholipid, apoptosis, mitochondrial dynamics, and Ca^{2+} signaling (Liu and Zhu, 2017; **Figure 1A**). Remarkably, all these processes are affected early during aging, AD pathogenesis, and other neurodegenerative conditions, suggesting a role for MAMs in the pathogenesis of these diseases (De Vos et al., 2012; Hedskog et al., 2013; Gautier et al., 2016; Area-Gomez et al., 2018). For example, the overexpression of both wild-type and familial ALS/FTD mutant TDP-43 in HEK293, CV-1, and NSC34 cell lines reduces ER–mitochondria associations and Ca^{2+} exchange between these two organelles (Stoica et al., 2014; **Figure 1C**). Likewise, loss of Sigma 1 receptor (which is responsible for some familial forms of ALS/FTD) has been shown to interfere with ER–mitochondria associations (Bernard-Marissal et al., 2015; **Figure 1C**). Conversely, an increase in the lipidic enzymatic function of MAMs and their inter-organelle extension has been described in fibroblasts from patients with SAD, in human SAD brains, and in AD mouse models (Area-Gomez et al., 2012; Hedskog et al., 2013). Remarkably, one of the most common and validated risk factors for SAD, the presence of APOE4 (Holtzman et al., 2012), has recently been associated with an increase in the ER–mitochondrial communication and MAM enzymatic activity (Tambini et al., 2016; **Figure 1B**). Furthermore, MAMs are highly enriched in PS1 and PS2 proteins (Area-Gomez et al., 2009) which when mutated, as in fibroblasts from patients with FAD, also increase the lipidic enzymatic function of the MAMs and ER–mitochondria communication (Area-Gomez et al., 2012), through a mechanism that involves an interaction between the mutated form of PS2 and mitofusin-2 (Mfn2; **Figure 1B**), a key protein in the formation of MAMs (Filadi et al., 2016).

Conditions that increase or decrease the extension of MAMs will affect the transfer of Ca^{2+} from the ER to mitochondria, resulting in either a mitochondrial Ca^{2+} overload, or a lack of Ca^{2+}. If the transfer of Ca^{2+} is excessive, cell death occurs (Schinder et al., 1996). If Ca^{2+} transfer to mitochondria is too low, a bioenergetics crisis occurs, also resulting in cell death (Cardenas et al., 2010). Importantly, Ca^{2+} is dysregulated in the aged brain and in AD (Landfield and Pitler, 1984; Gibson and Peterson, 1987; Khachaturian, 1987). Upregulation of Ca^{2+} levels can both initiate and accelerate several AD features, from amyloid deposition to synapse loss (Stutzmann et al., 2007). Several mechanisms have been proposed to explain the upregulation of cytoplasmic Ca^{2+} levels in AD including overexpression of the ryanodine receptor (RyR; Chakroborty et al., 2012), or β-amyloid (Aβ)-triggering release of Ca^{2+} from both extracellular and intracellular sources (Demuro and Parker, 2013). Another mechanism involves an increase of Ca^{2+} leak from the ER through sensitization of the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) Ca^{2+} channel by directed interaction with FAD-linked PS mutants (Cheung et al., 2008, 2010) or indirectly by interaction of FAD-linked PS mutants with the SERCA pump (Green et al., 2008). In agreement with the latter, it has been demonstrated that overexpression of the FAD-linked PS2 mutant leads to an increase in the generation of cytosolic Ca^{2+} hot spots, ER–mitochondria tethering, and mitochondrial Ca^{2+} uptake (Zampese et al., 2011). This in turn may result in mitochondrial Ca^{2+} overload and could explain the metabolic dysfunction and cell death observed in AD (**Figure 1B**). On the other hand, decreasing intracellular Ca^{2+} overload, specifically through a reduction of the $InsP_3R$ protein expression by 50%, normalizes FAD PS-associated Ca^{2+} signaling and rescues the biochemical, electrophysiological, and behavioral phenotypes observed in two different PS1-FAD animal models (Shilling et al., 2014; **Figure 1B**). Altogether, the above findings highlight the importance of MAMs and the transfer of Ca^{2+} from the ER to mitochondria in AD pathogenesis and their potential as a therapeutic target.

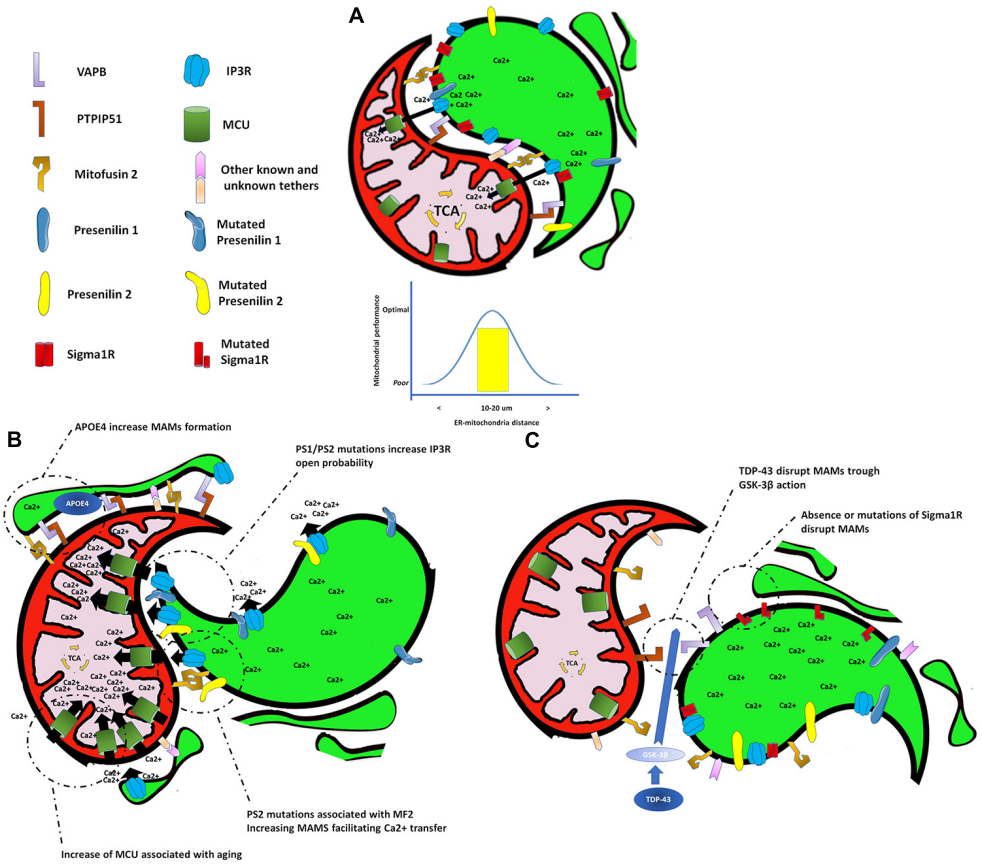


FIGURE 1 | Endoplasmic reticulum and mitochondria interactions under different scenarios. **(A)** Endoplasmic reticulum–mitochondrial interaction at a proper distance allows a correct transfer of calcium to the mitochondrial matrix to sustain bioenergetic function. **(B)** Shortening of the distance at the endoplasmic reticulum–mitochondrial interaction, or an increase in either the mitochondrial calcium uptake or the calcium release from the endoplasmic reticulum, causes an exaggerated transfer of calcium to mitochondria affecting its normal bioenergetic function that leads to cell death. **(C)** Separation of the endoplasmic reticulum and mitochondria causes a decrease in the calcium transfer to the mitochondrial matrix generating a drop in the bioenergetic and metabolic activity of the mitochondria.

The role of MAMs in aging has just begun to be unveiled. Similar to what has been observed in AD models, aging increases the ER to mitochondria Ca^{2+} transfer in long-term culture of hippocampal neurons, with correlates with an increase of the mitochondrial Ca^{2+} uniporter (MCU; Calvo-Rodriguez et al., 2016; **Figure 1B**). Interestingly, a decrease in Ca^{2+} transfer to mitochondria and a dissociation of the MAMs have been described in cardiomyocytes from old mice suggesting that the type of modification that MAMs undergo with aging might be cell specific (Fernandez-Sanz et al., 2014).

THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE (MPTP) FORMATION IN AGE-ASSOCIATED NEURODEGENERATIVE DISEASES

Under conditions of Ca^{2+} and/or ROS overload, formation of the mitochondrial permeability transition pore (mPTP) takes place, which corresponds to a non-selective channel formed by a protein complex spanning the outer and inner mitochondrial membranes (Bernardi et al., 2006). In physiological conditions, transient opening of the mPTP can regulate Ca^{2+} levels in the mitochondrial matrix (Ichas et al., 1997). However, dysregulated mPTP opening triggers the release of most matrix metabolites such as ROS, Ca^{2+}, and NAD^{+}, leading to loss of the mitochondrial membrane potential, inhibition of oxidative phosphorylation (OXPHOS), and mitochondrial swelling (Elrod and Molkentin, 2013; Rottenberg and Hoek, 2017). Even though several proteins are known to participate in mPTP formation [anion channel VDAC, adenine nucleotide translocator (ANT), mitochondrial ATP synthase (F0F1), phosphate carrier (PiC), and cyclophilin D (CypD; Bernardi et al., 2006; Rao et al., 2014), its detailed structural configuration is not yet entirely known.

The mPTP has been linked to neurodegeneration *in vitro* and *in vivo*. In neural progenitor cells, Aβ-amyloid exposure leads to mPTP opening and a decrease in mitochondrial membrane potential, release of cytochrome C, and cell death (Hou et al., 2014). In human AD brains, Aβ-amyloid binds CypD in mitochondria (Du and Yan, 2010), and CypD deficiency improves mitochondrial function, memory, and learning in an AD mouse model (Du et al., 2008). Aβ-induced neurotoxicity *in vitro* was

also attenuated pharmacologically by inhibition of the mPTP using cyclosporine A (CsA) on neural stem cells (Chen et al., 2016). Interestingly, it has been shown that CypD knock-out mice exhibit delayed axonal degeneration, a common feature of diverse neurodegenerative diseases (Barrientos et al., 2011; Catenaccio et al., 2017; Salvadores et al., 2017). Indeed, genetic deletion of CypD delays disease progression in other mouse models of neurodegenerative disorders, including ALS (Martin et al., 2009), PD (Thomas et al., 2012), and multiple sclerosis (Forte et al., 2007). Therefore, novel compounds that inhibit mPTP opening are currently been developed, including sanglifehrin A, N-Me-Ala-6-cyclosporin A, and antmanide (Rao et al., 2014).

Aging also modifies the opening probability of the mPTP (Rottenberg and Hoek, 2017). During aging, the probability of the mPTP opening increases due to higher expression levels of CypD and the CypD-activator p53 (Priami et al., 2015). Furthermore, the expression of HSP90, a chaperone that binds CypD to trigger its degradation, is decreased in aged cells (Lam et al., 2015), which could also increase the mPTP opening probability. This evidence is further supported by a faster Ca^{2+} -induced mitochondrial swelling in purified liver mitochondria obtained from aged mice (Goodell and Cortopassi, 1998). Interestingly, CypD is inactivated by the deacetylase SIRT3 (Hafner et al., 2010), a known modulator of longevity in diverse species (Jasper, 2013). The reported decline in SIRT3 activity during aging (Brown et al., 2013) may lead to a greater activation of the mPTP, underscoring the role of mitochondria in longevity and onset of age-dependent neurodegenerative diseases. Interestingly, several modulators of longevity, including metformin, mitochondrial UPR, and caloric restriction inhibit the activation of the mPTP (Bhamra et al., 2008; Altieri, 2013; Amigo et al., 2017), and may contribute to lifespan extension (Rottenberg and Hoek, 2017). A key role for mitochondria in age-related disorders has been associated to broad damaging events including increased ROS production and defects in the regulation of intracellular Ca^{2+} levels, which are directly associated to mPTP activation with profound negative consequences for cell survival. Therefore, mPTP emerges as a potential target for neuroprotection in age-related neurodegenerative conditions.

MITOCHONDRIA, ROS, AGING, AND NEURODEGENERATION

Reactive oxygen species are chemical species that are produced by most cell types. The group of molecules that fulfill the criteria for ROS includes hydrogen peroxide, and the highly reactive species superoxide anion and hydroxyl radical (Wilson et al., 2017). The production of ROS in cells is controlled by enzymatic or non-enzymatic mechanisms. The main source for ROS production in terms of quantitative production is the mitochondria (Holmstrom and Finkel, 2014). Mitochondria produces superoxide anion, a by-product of the inefficient transfer of electrons by the electron transport chain (ETC) during OXPHOS, that is quickly converted into hydrogen peroxide by the action of the superoxide dismutases 1–3 (SOD1–3; Quinlan et al., 2013). Of note, despite mitochondria being the main source of ROS in cells, hydrogen peroxide can be produced by more than 30 different enzymes (Go et al., 2015).

While a huge amount of work in the past focused on the deleterious roles for ROS species in cells and organisms, including the "free radical" or "oxidative stress" theory of aging (Harman, 1956) supported by many studies (Harman, 1992; Cadenas and Davies, 2000; Golden et al., 2002), there is growing evidence in the last decades that ROS may serve physiological functions (Zuo et al., 2015; Sies et al., 2017; Wilson et al., 2017). Related to aging, other studies show that unbalanced ROS production does not modify lifespan in mice under tightly controlled conditions (Van Remmen et al., 2003; Ran et al., 2007). Moreover, it was demonstrated that there is no increased oxidative damage with age (Barja and Herrero, 2000; Kauppila et al., 2017). Currently, it has been proposed that adaptive or hormetic production of ROS is required to maintain several cellular mechanisms including stem cell proliferation and fate determination in the brain (Sena and Chandel, 2012; Chaudhari et al., 2014).

In terms of neurodegeneration associated to aging, it has been reported in AD that the Aβ peptide interacts with the mitochondrial protein termed amyloid binding alcohol dehydrogenase (ABAD) in AD mouse models and in post-mortem samples derived from AD human patients. The functional consequence of such an interaction is an increase in ROS production due to abnormal mitochondrial membrane permeability (Lustbader et al., 2004). Altered OXPHOS increases the generation of ROS (Koopman et al., 2013) and is indeed a hallmark for early AD abnormalities in humans. In fact, samples from human subjects show that mitochondrial-encoded OXPHOS genes are altered in aging, mild cognitive impairment, and AD (Mastroeni et al., 2017). Similarly, AD mouse models have shown that both Aβ and tau protein can induce alterations in mitochondrial proteins involved in OXPHOS (Caspersen et al., 2005; Rhein et al., 2009; Eckert et al., 2010), causing an aberrant ROS generation leading to cellular damage. In addition, ROS are known to cause mitochondrial fragmentation (Wang et al., 2014), which reduces mitochondrial performance (Westermann, 2012) favoring the generation of more ROS and cellular damage associated to it.

MITOCHONDRIAL DYSFUNCTION AND SYNAPTIC DEFICITS

Synapses are neuronal structures in which mitochondria are fundamental (Li et al., 2004) by providing large amounts of ATP required to fuel synaptic vesicle physiology and by acting as a Ca^{2+} buffer modulating cytoplasmic Ca^{2+} signal and hence, neurotransmission (Ghosh and Greenberg, 1995; Verstreken et al., 2005; Gunter and Sheu, 2009; Wan et al., 2012). Synaptic mitochondria are more vulnerable to cumulative damage showing impaired Ca^{2+} uptake capacity and increased propensity to undergo mPTP compared to non-synaptic mitochondria (Scheff et al., 2006). Likewise, in an APP/PS1 AD mouse, synaptic mitochondrial function was significantly more affected than non-synaptic mitochondria (Dragicevic et al., 2010). Synaptic deficit is an early event in the

pathogenesis of several neurodegenerative disorders including AD and worsens with disease progression and age (Wilcox et al., 2011). The extent of cognitive decline in AD patients is tightly associated with the extent of synapse loss in specific brain regions including cortex and hippocampus (Scheff et al., 2006; Mattson, 2010). Post-mortem hippocampus from AD patients shows a considerable decrease in dendritic spine density (Ferrer et al., 1990) and transgenic mouse models of AD show age-dependent reduction in spine density before plaque deposition (Lanz et al., 2003). Along these lines, in aged synapses and from AD models, a decline in mitochondrial respiration and signs of mitochondrial damage such as reduced antioxidant contents and increased oxidative stress markers has been described (Du et al., 2010; Quiroz-Baez et al., 2013). Proteomic analysis of aged synaptic mitochondria reveals changes in ETC proteins, antioxidants, and proteins related to mitochondrial dynamics (Stauch et al., 2014). Just recently, through the use of cytoplasmic hybrid ("cybrid") technology, Yu et al. (2017) were able to recapitulate mitochondrial structural and functional changes observed in AD-affected brains. In this model, their findings demonstrate that AD-affected mitochondria elicited detrimental effects on synaptic development (Yu et al., 2017). How ER vesicles found in the synaptic region and the transfer of Ca^{2+} contribute to the impairment of mitochondria and synaptic formation remains to be explored, but given the dysregulation of Ca^{2+} observed during aging, AD, and other neurodegeneration, an important role is expected. Elucidating the factors that underlie early synaptic dysfunction will be key to prevent the widespread neurodegeneration associated with aging.

CONCLUSION

Aging continues to be the most relevant risk factor for AD, the most common form of dementia in the elderly, and other neurodegenerative diseases. Both aging and neurodegeneration are accompanied by a loss in the ability of the cells to adjust and rewire their metabolic networks to keep a tight balance between energy production and expenditure in an ever-changing environment. Mitochondria work as an adaptable metabolic control, a "rheostat," that integrates inputs from the intra and extracellular environment to generate functional outputs that adjust cell behavior and energy production and consumption. Several lines of evidence suggest that mitochondrial function deteriorates with increasing age and the progression of several neurodegenerative diseases. This supports the notion that aging and the neurodegenerative diseases such as AD may share a common root, the failure of the rheostat program. Since Ca^{2+} is also altered in both conditions and can either energize or overload the rheostat depending on the concentration, understanding how MAM formation is regulated is important. Identifying the players that participate in the regulation to assure a proper Ca^{2+} transfer to mitochondria is critical in order to determine the real potential of this intracellular signaling platform as an intervention candidate to improve aging and hinder the onset of neurodegenerative disease such as AD.

AUTHOR CONTRIBUTIONS

MM, UA-C, and CC designed and outlined the structure and contents of the review. MM, UA-C, MS, FC, CG-B, and CC contributed to the literature review, discussion, and writing of the manuscript. All authors contributed equally to the draft revisions and final approval of the version to be published.

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REVIEW
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Excitatory Dendritic Mitochondrial Calcium Toxicity: Implications for Parkinson's and Other Neurodegenerative Diseases

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Dysregulation of calcium homeostasis has been linked to multiple neurological diseases. In addition to excitotoxic neuronal cell death observed following stroke, a growing number of studies implicate excess excitatory neuronal activity in chronic neurodegenerative diseases. Mitochondria function to rapidly sequester large influxes of cytosolic calcium through the activity of the mitochondrial calcium uniporter (MCU) complex, followed by more gradual release via calcium antiporters, such as NCLX. Increased cytosolic calcium levels almost invariably result in increased mitochondrial calcium uptake. While this response may augment mitochondrial respiration, limiting classic excitotoxic injury in the short term, recent studies employing live calcium imaging and molecular manipulation of calcium transporter activities suggest that mitochondrial calcium overload plays a key role in Parkinson's disease (PD), Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and related dementias [PD with dementia (PDD), dementia with Lewy bodies (DLB), and frontotemporal dementia (FTD)]. Herein, we review the literature on increased excitatory input, mitochondrial calcium dysregulation, and the transcriptional or post-translational regulation of mitochondrial calcium transport proteins, with an emphasis on the PD-linked kinases *LRRK2* and *PINK1.* The impact on pathological dendrite remodeling and neuroprotective effects of manipulating MCU, NCLX, and LETM1 are reviewed. We propose that shortening and simplification of the dendritic arbor observed in neurodegenerative diseases occur through a process of excitatory mitochondrial toxicity (EMT), which triggers mitophagy and perisynaptic mitochondrial depletion, mechanisms that are distinct from classic excitotoxicity.

Keywords: mitochondrial calcium uniporter, PINK1, LRRK2, calcium overload, Parkinson Disease/Lewy body dementia, Alzheimer Disease, FTD-ALS, dendrite degeneration

INTRODUCTION

Neuronal function is dependent upon the formation, maintenance, and activity-regulated remodeling of multiple synaptic contacts supported by extensive axo-dendritic arborization. The primary excitatory neurotransmitter is glutamate. Glutamate binds to calcium-permeable ionotropic receptors that are also activated by *N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA). These NMDA receptors (NMDAR) and AMPA receptors (AMPAR) are present predominantly in dendritic spines, but also exist in perisynaptic regions. NMDA receptors are composed of NR1 and NR2 subunits; the four NR2A-NR2D subunits confer different kinetic properties, channel open probabilities, ion conductance, and effects on synaptic plasticity. Excitatory synaptic activity engages NMDAR subsets that contain the NR2A subunit, resulting in activation of Akt, ERK1/2, and CREB (Lau and Zukin, 2007). In addition to ligand-gated channels, L-type voltage gated calcium channels are also involved in activating ERK1/2 and CREB to regulate activity-dependent transcription (West et al., 2002). Transient activation of these signaling pathways is implicated in neuronal survival as well as in synaptic plasticity.

Due to its essential role in signaling both pre-synaptic and post-synaptic processes, as well as cellular processes of differentiation, cell death, vesicular transport, and cytoplasmic motility, calcium undergoes exquisitely precise regulation in neurons that allow simultaneous engagement of multiple spatially separated calcium-dependent processes. Dendritic spines function as physical compartments that isolate and concentrate calcium signals arising from synaptic activity (Koch et al., 1992). Following depolarization or ligand-stimulated calcium uptake, calcium signal recovery is mediated by channel inactivation, plasma membrane sodium-calcium exchangers (NCX) that extrude calcium, and sequestration of calcium into mitochondria, endoplasmic reticulum, and other intracellular stores. The mitochondrion plays a key role in rapid, post-stimulatory calcium recovery by taking up massive amounts of calcium into its matrix (White and Reynolds, 1997), while also fueling ATP-dependent pumps on other membranes (Budd and Nicholls, 1996).

While increased excitatory stimulation has been extensively studied in the context of acute neuronal injury and cell death, it has become clear in recent years that increased neuronal calcium handling may also play a pathogenic role in chronic neurodegenerative diseases. Shortening and simplification of the dendritic arbor and spine loss, often accompanied by loss of dendritic mitochondria (Cherra et al., 2013; Dagda et al., 2014) are observed in post-mortem studies of Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Hammer et al., 1979; Patt et al., 1991; Baloyannis et al., 2004; Stephens et al., 2005) or their experimental models (MacLeod et al., 2006; Wu et al., 2010; Dagda et al., 2014; Fogarty et al., 2016). Although inhibiting calcium uptake from the extracellular space is frequently neuroprotective (Ilijic et al., 2011; Cherra et al., 2013; Esposito et al., 2013; Plowey et al., 2014), the mechanism(s) by which sublethal increases in cytosolic calcium fluxes trigger dendritic retraction have been unclear. A series of recent studies discussed below implicate increased mitochondrial calcium stress as a key factor by which increased excitatory neuronal activity triggers mitochondrial depletion from and retraction of dendritic structures. Moreover, several mitochondrial calcium transporters are regulated by genes mutated in familial PD, causing functional changes that increase susceptibility to this neurodegenerative mechanism, which we have termed excitatory mitochondrial toxicity (EMT). Genetic and aging- or disease-related signaling alterations may also predispose to EMT in sporadic PD, AD, and the ALS-frontotemporal dementia (FTD) spectrum.

A BRIEF SUMMARY OF EXCITOTOXICITY

Over the past 40–50 years, it has become well recognized that excessive glutamatergic neurotransmission leads to neuronal cell death, which was first described by Olney (1971). While this has been studied most extensively in the context of brain ischemia from stroke or trauma, excitotoxic cell death has also been implicated in epilepsy and to a lesser extent in AD (Tannenberg et al., 2004), PD (Caudle and Zhang, 2009), and ALS (Shi et al., 2010).

In classic excitotoxicity, a transient episode of ischemia causes the extracellular concentrations of glutamate to rise. This results in widespread stimulation of both synaptic and extrasynaptic NMDARs, resulting in massive influx of sodium and calcium (**Figure 1A**). Ischemia induced neuronal damage is attenuated by pretreatment with an NMDAR antagonist, implicating glutamate toxicity (Simon et al., 1984). Apart from glutamate, earlier studies also implicated kainate and *N*-methyl-DL-aspartate in calcium dependent neuronal cell death (Berdichevsky et al., 1983). Whereas sodium may mediate the initial, reversible swelling of neurons, irreversible excitotoxic injury is believed to be mediated primarily by elevated calcium levels (Choi, 1995). The data suggest that transient elevations of intracellular calcium is tolerated by the cell and is reversible, whereas sustained calcium overload causes activation of intracellular enzymes (Choi, 1987) and a wave of mitochondrial collapse propagating to the cell body (Greenwood et al., 2007) to cause cell death. The initial glutamate stimulated calcium influx also triggers secondary increases in cytosolic calcium through other mechanisms, which are tightly correlated with neuronal cell death (Randall and Thayer, 1992). Classic excitotoxicity thus involves multiple calcium-dependent pathways initiated in the cytosolic compartment.

In addition to activating calcium-dependent degradative enzymes, such as calpains, phospholipases, and endonucleases, engagement of extrasynaptic NMDARs shuts off CREB signaling (Hardingham et al., 2002), while activating death associated protein kinase 1 (DAPK1) and neuronal nitric oxide synthase (nNOS) bound to the NR2B cytosolic tail (Tu et al., 2010; Martel et al., 2012). Calpain inhibitors confer dose-dependent protection from excitotoxic cell death, as well as preventing mitochondrial permeability transition and release of pro-death factors (Lankiewicz et al., 2000). In turn, inhibiting mitochondrial calcium uptake confers at least partial protection

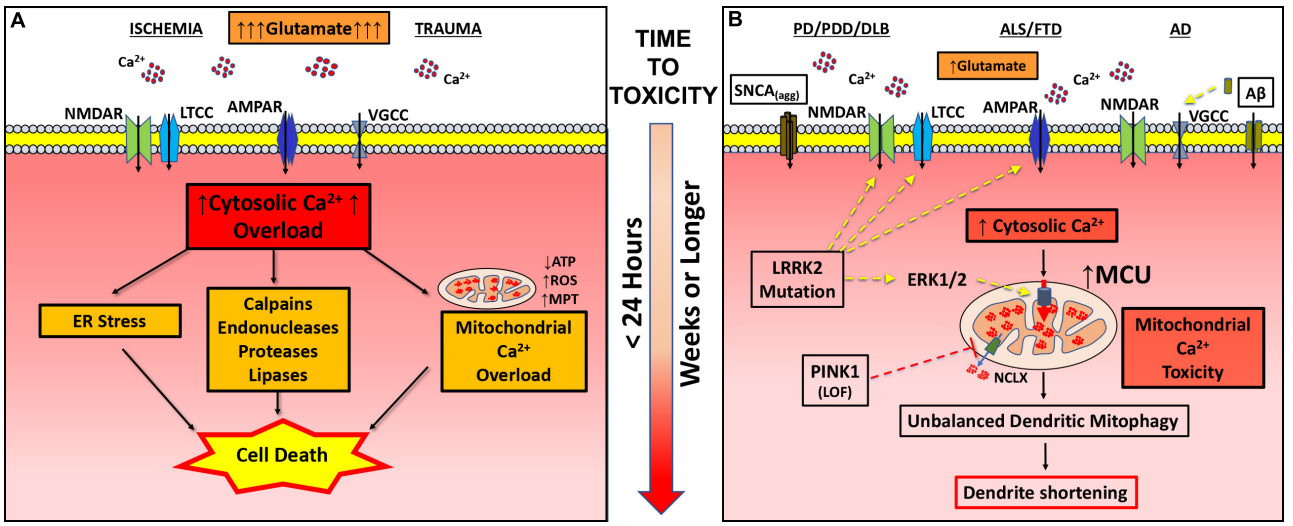


FIGURE 1 | Pathogenic mechanisms in excitotoxicity and dendritic EMT. **(A)** In classic excitotoxicity an insult, such as ischemia or trauma, causes increased release of excitatory neurotransmitters (glutamate) leading to post-synaptic uptake of calcium through channels (AMPAR, α-amino-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; LTCC, L-type calcium channel; NMDAR, *N*-methyl-D-aspartate receptor; VGCC, voltage gated calcium channel). Falling ATP levels impair calcium pumps, contributing to cytosolic calcium overload. Sustained intracellular calcium elevation activates a variety of degradative enzymes (calpains, endonucleases, proteases, and lipases), ER stress and mitochondrial permeability transition (MPT), leading to cell death within hours of initiation of excitotoxicity. **(B)** In neurodegenerative diseases (PD/PDD/DLB, ALS/FTD, and AD), various changes including enhanced glutamate neurotransmission contribute to increased cytosolic calcium flux. In PD/PDD/DLB, aggregated α-synuclein ($SNCA_{agg}$) can insert into the membrane forming calcium permeable pores. Mutations in the LRRK2 gene contribute to more frequent excitatory post-synaptic potentials and increased calcium influx via NMDAR, AMPAR, and/or LTCC. In AD, soluble amyloid beta (Aβ) can either directly stimulate VGCC or aggregates to form calcium permeable pores. Chronically elevated calcium transients, while insufficient in magnitude to trigger calcium-dependent cell death, results in greater calcium uptake by mitochondria. In addition to elevating cytosolic calcium, mutations in LRRK2 (R1441C/G2019S) transcriptionally upregulate MCU through activation of ERK1/2. On the other hand, loss of function (LOF) mutations in PINK1 result in impaired activation of NCLX-mediated calcium efflux from the mitochondria. Dysregulation of mitochondrial calcium handling, whether driven by increased excitatory cytosolic calcium uptake or changes in the function of mitochondrial calcium transporters, results in mitochondrial injury sufficient to trigger mitophagy. Depletion of dendritic mitochondria precedes subsequent shortening and simplification of the dendritic arbor. Inhibiting NMDAR, LTCC, MCU or autophagy/mitophagy, or stimulating the activity of NCLX, each confers protection against excitatory dendritic mitochondrial toxicity.

against cell death, depending on the severity of injury (Stout et al., 1998; Qiu et al., 2013), and mitochondrial permeability transition further amplifies calpain as well as caspase activation (Ferrand-Drake et al., 2003). However, adult neurons dying from ischemic/hypoxic injuries do not exhibit classic apoptotic morphology, most likely due to calpain-mediated inactivation of procaspase-9 (Volbracht et al., 2005). These data implicate m-calpain activation as a major factor in both mitochondrial and non-mitochondrial mechanisms of excitotoxic cell death.

CALCIUM DYSREGULATION IN CHRONIC NEURODEGENERATION

In recent years, it has become clear that calcium dysregulation also contributes to chronic neurodegeneration in relation to AD (Lopez et al., 2008; Anekonda and Quinn, 2011), ALS (Joo et al., 2007), and PD (Cali et al., 2014) and its related dementias: Parkinson disease with dementia (PDD) (Verma et al., 2017) and Dementia with Lewy Bodies (DLB) (Overk et al., 2014), which by convention are distinguished by the relative timing of cognitive and motor symptoms. A variety of clinical studies have converged on the possible neuroprotective role of calcium channel inhibitors for PD. In particular, both substantia nigra pars compacta neurons and cortical neurons express L-type voltage-gated channels (Guzman et al., 2009). Interestingly, L-type calcium channel inhibitors confer protection of SN and cortical neuron types in both toxic (Ilijic et al., 2011) and genetic (Cherra et al., 2013) models of PD. Moreover, use of centrally acting dihydropyridine L-type calcium channel blockers for hypertension treatment may reduce the risk of PD (Becker et al., 2008; Ritz et al., 2010). These studies emphasize the importance of understanding how neuronal calcium dysregulation contributes to structural and functional changes early in the neurodegenerative process.

In the remainder of this review, we summarize data that supports the concept of a new pathway of sublethal excitatory injury focused near sites of calcium entry, which contributes to dendrite retraction rather than propagating to the soma to cause cell death. We propose the term EMT, to emphasize the key role of mitochondrial calcium dysregulation in this pathway of neurodegeneration. Dysregulation of post-synaptic calcium handling may be triggered by several mechanisms involving proteins implicated in PD, ALS, or AD. The resultant elevations in cytosolic and mitochondrial calcium result in mitochondrial calcium injury, mitochondrial autophagy (mitophagy) and depletion of mitochondria from dendrites (**Figure 1B**).

In contrast to excitotoxicity, which is predominantly triggered by excessive extracellular glutamate release, this pathway may also be triggered by post-synaptic changes in excitability, calcium buffering/recovery, and mitochondrial calcium influx or efflux.

CALCIUM, MITOCHONDRIAL CONTENT/DISTRIBUTION, AND NEURONAL ARBORIZATION

Mitochondria play a key role in buffering and shaping cytosolic calcium transients, as well as a critical permissive role for neurite outgrowth, maintenance, and remodeling of axo-dendritic extensions. Before considering disease-linked alterations, basic mechanisms underlying these processes are briefly summarized below.

Regulation of Mitochondrial Calcium Handling

Mitochondrial act to buffer intracellular calcium levels through high capacity, low affinity uptake by the mitochondrial calcium uniporter (MCU) complex (Baughman et al., 2011; De Stefani et al., 2011). As such, disease-associated changes in excitatory activity or other sources of increased cytosolic calcium will invariably affect mitochondria. Fine-tuning of MCU function is mediated by accessory proteins MICU1 (Perocchi et al., 2010), MICU2 (Plovanich et al., 2013), EMRE (Sancak et al., 2013), MCUR1 (Mallilankaraman et al., 2012), and MCUb (Raffaello et al., 2013). Mitochondrial calcium uptake is balanced by the activity of sodium/calcium antiporters, such as NCLX (Palty et al., 2010), which release calcium back into the cytosol. Another mitochondrial inner membrane protein LETM1 may act to mediate calcium uptake in response to moderate increases in cytosolic calcium as well as acting in calcium extrusion from the matrix (Doonan et al., 2014), although this latter effect is controversial (De Marchi et al., 2014). Changes to the numbers or function of ER-mitochondrial contact sites may also affect mitochondrial calcium homeostasis (Raffaello et al., 2016), and this process may be regulated by Parkin (Cali et al., 2013), whose mutations cause recessive PD.

From a physiological perspective, calcium uptake into the mitochondrial matrix results in enhanced respiratory function, tuning mitochondrial function to synaptic activity (Bianchi et al., 2004; Vos et al., 2010). However, with massive or sustained calcium stress, this response may result in mitochondrial injury from calcium overload. Following classic excitotoxic glutamate stimulation, excess mitochondrial calcium uptake results in ROS production (Reynolds and Hastings, 1995), collapse of membrane potential and opening of the permeability transition pore (Li et al., 2009), and induction of neuronal cell death (Stout et al., 1998). Indeed, MCU overexpression exacerbates NMDAR-mediated mitochondrial depolarization and excitotoxic cell death (Qiu et al., 2013). As discussed below, calcium uptake via MCU may also contribute to sublethal pathways of mitochondrial injury sufficient to trigger mitophagy and subsequent dendritic remodeling.

Calcium, Mitochondria and Dendritic Remodeling

Mitochondria play a key role in the maintenance of dendritic integrity in neurons. Neurons are heavily dependent on the proper function and distribution of mitochondria to stay healthy (Gusdon and Chu, 2011). These accumulate or move toward regions of high energy demand, such as the growth cones of developing neurons (Morris and Hollenbeck, 1993) or regions of higher synaptic activity (Chang et al., 2006). The density and distribution of dendritic mitochondria regulates dendritic morphology, spinogenesis, and the plasticity of spines and synapses (Li et al., 2004). Moreover, in genetic models of neurodegeneration, loss of dendritic mitochondria precedes dendritic retraction (Cherra et al., 2013). This may relate to the requirement for sufficient mitochondrial densities not only to support synaptogenesis during development (Ishihara et al., 2009), but also for maintenance of dendritic arbors in mature neurons (Lopez-Domenech et al., 2016). Depletion of dendritic mitochondria may occur through reduced mitochondrial biogenesis, increased mitochondrial degradation, or alterations in mitochondrial transport.

Two important signals affect mitochondrial movement within a neuronal cell. (i) The energy status of the neuron modulates the transport of mitochondria, wherein high ATP levels increases mobility and high ADP concentration causes either slowing or total arrest of mitochondrial movement. Interestingly, mitochondrial velocity is also decreased in the close vicinity of a spine (Mironov, 2007). (ii) Changes in intracellular calcium levels regulate mitochondrial motility, wherein high cytosolic calcium levels decrease mitochondrial mobility. This may account for the tendency of mitochondria to accumulate near glutamate receptors, where they are situated to provide ATP and to buffer incoming intracellular calcium. Activity dependent mitochondrial movement was elegantly shown by Li et al. (2004), with the number of mitochondria in dendritic protrusions increased by repetitive KCl depolarization. Although the majority of mitochondria were present in the dendritic shaft, a small fraction of mitochondria was observed in the spine itself (Li et al., 2004). Interestingly, even under basal conditions, levels of mitochondria derived oxidative stress is higher in dendrites than in the soma, consistent with an increased bioenergetic demand associated with buffering calcium near a synapse (Dryanovski et al., 2013).

The stimulation of NMDA receptors leads to the activation of protein kinases, such as $Ca^{(2+)}$/calmodulin-dependent protein kinase (Ojuka, 2004), AMP kinase, and mitogen activated protein kinases, such as ERK1/2 (Yun et al., 1999). Whereas AMP kinase (Ojuka, 2004) and ERK1/2 (Wang et al., 2014) show opposite effects on mitochondrial biogenesis, activation of either signaling pathway serves to promote autophagy or mitophagy (Pattingre et al., 2003; Meijer and Codogno, 2007; Dagda et al., 2008; Bootman et al., 2018). The ability of neuronal cells to undergo mitochondrial biogenesis regulates the outcome of mitophagy

stimulation (Zhu et al., 2012). It is reasonable to surmise that an imbalance in the rates of mitochondrial degradation by mitophagy and replacement by biogenesis/transport will similarly determine the degree of mitochondrial depletion from dendrites.

SUBLETHAL EXCITATORY CALCIUM DYSREGULATION IN CHRONIC NEURODEGENERATION

In contrast to classic excitotoxicity, in which massive, acute elevations in glutamatergic neurotransmission results in both non-mitochondrial and mitochondrial pathways of cell death, functional impairment and shrinkage of the synaptic-dendritic arbor likely occur long before cell death in chronic neurodegenerative diseases. Interestingly, sublethal stimulation of NMDA receptors decreases dendrite outgrowth in immature neurons (Monnerie et al., 2003), but the impact on dendritic integrity in mature neurons is less understood. Nevertheless, there are a growing number of studies implicating chronic elevations in excitatory post-synaptic potentials and cytosolic calcium in models of neurodegenerative diseases, which may be due to either pre-synaptic or post-synaptic changes.

Parkinson's Disease

The movement symptoms that characterize PD result from degeneration of dopaminergic substantia nigra neurons in the midbrain, which project to the striatum. In addition, PD patients frequently experience olfactory and autonomic dysfunction, mood disorders, and cognitive/executive dysfunction. In addition to playing a key role in cortical neuron function, glutamate plays an important role in modulating dopaminergic neurotransmission, acting on both pre-synaptic and post-synaptic sides. Dopaminergic midbrain neurons express both synaptic and extrasynaptic glutamate receptors (Wild et al., 2015) and are susceptible to classic NMDA excitotoxicity (Kikuchi and Kim, 1993). Excitatory cortical input also modulates striatal neurotransmission in both direct and indirect basal ganglia pathways (Stocco, 2012). While dementia may represent a late stage development in some forms of PD, early cognitive-executive dysfunction represents the defining feature of DLB as well as in several forms of familial PD.

Mutations in the *LRRK2* gene, which encodes leucine-rich repeat kinase 2, represent the most frequent known cause of PD (Gandhi P.N. et al., 2009). Recent studies using cultured primary neurons transfected with disease-linked G2019S and R1441C mutations of *LRRK2* implicate increased excitatory neurotransmission as one of the earliest pathogenic changes, preceding subsequent dendritic degeneration (Plowey et al., 2014). EPSP frequency was elevated basally, and neurons showed enhanced responses to NMDA and AMPA. Interestingly, memantine, a partial NMDA antagonist conferred protection against subsequent dendritic simplification and loss, implicating an excitatory pathogenesis (Plowey et al., 2014). Increased post-synaptic excitatory neurotransmission was also observed in hippocampal slice cultures of LRRK2-G2019S transgenic mice (Sweet et al., 2015). Interestingly, LRRK2-G2019S knockin mice exhibit an early stage of hyperactivity, accompanied by increased striatal glutamate and dopamine neurotransmission (Volta et al., 2017). Thus, primary neuron cultures, slice cultures and *in vivo* studies all support an early role for increased excitatory synaptic activity in several mutant LRRK2 models.

Other mechanisms may also contribute to increased intracellular calcium in mutant LRRK2-expressing neurons. As mentioned above, L-type voltage-gated channels contribute to Ca^{2+} influx during an action potential. Expression of either the G2019S or the R1441C mutation in LRRK2 dysregulates intracellular calcium homeostasis in response to KCl depolarization (Cherra et al., 2013). Calcium chelators or inhibitors of L-type calcium channels confer protection in this system. Calcium release from lysosomal stores has also been implicated in mutant LRRK2 pathogenesis (Gomez-Suaga et al., 2012; Hockey et al., 2015).

Oligomeric α-synuclein, implicated in both dominant familial and sporadic PD/DLB, elicits increased cytosolic calcium uptake through effects on AMPARs (Huls et al., 2011). This creates increased susceptibility to MPP+ toxicity (Lieberman et al., 2017). Interestingly, α-synuclein oligomers can act to increase intracellular calcium levels by forming pores in the plasma membrane (Pacheco et al., 2015). Furthermore, the neurite retraction and increased intracellular calcium elicited by the A53T mutation in α-synuclein are exacerbated by concurrent expression of PINK1-W437X (Marongiu et al., 2009), implicating a mechanistic convergence between dominant and recessive forms of PD. It has been proposed that the reduced mitochondrial membrane potential often observed in PINK1 knockdown/knockout cells (Exner et al., 2007; Dagda et al., 2011, 2014; Huang et al., 2017) may serve to limit mitochondrial calcium uptake, exacerbating excitotoxic injury (Heeman et al., 2011). When post-synaptically expressed, Parkin participates in pruning excitatory synapses (Helton et al., 2008). This may represent another point of convergence between dominant and recessive PD, as either loss of Parkin function or dominant LRRK2 mutations would tend to increase excitatory synapses, conferring enhanced vulnerability to excitatory injury. Taken together, dysregulated neuronal calcium handling resulting in increased cytosolic levels forms a common theme in multiple forms of familial Parkinsonism.

Alzheimer's Disease

Alzheimer's disease is the most common age-related neurodegenerative disease, characterized by memory deficits and the pathological hallmarks of neuritic plaques and neurofibrillary tangles. Proteins that are pathologically implicated in AD include the amyloid beta peptides (Aβ) and the microtubule associated protein tau. Calcium mishandling has been implicated in AD and elevated serum calcium levels are well correlated with cognitive decline in aging (Lopez et al., 2008; Popugaeva et al., 2017). Oligomeric Aβ, proteolytic products of the amyloid precursor protein (APP) that is mutated in familial AD (fAD), are enriched

in the plaques that typify the disease and are primary culprits for initiating this calcium dysregulation. Neurons exposed to Aβ oligomers elicit elevations in somatic, dendritic, and synaptic calcium in neurons (Arbel-Ornath et al., 2017; Zhao et al., 2017) and contribute to excitotoxic neuron death (Mattson et al., 1992; Shankar et al., 2008). In mice engineered to co-express fAD mutations in APP_{swe} and Presenilin 1($PS1_{G384A}$), a gamma secretase that cleaves APP to generate Aβ peptides, hyperactive neurons are observed in the hippocampus and cortex of young animals, prior to formation of plaques (Busche et al., 2012). Mutations in presenilins alone have also been reported to elicit endoplasmic reticulum calcium overload, with post-translational modification of neuronal ryanodine receptors further promoting calcium leakage into the cytosol (Lacampagne et al., 2017; Popugaeva et al., 2017). Emerging theories suggest long-term Aβ-dependent calcium dysregulation may trigger a cascade of deficits in homeostatic machinery that result in loss in neural network activity (Frere and Slutsky, 2018). For example, elevated calcium plays a key role in promoting tau pathology (Zempel et al., 2010), a component of neurofibrillary tangles that characterize an intermediate step in AD progression, through activation of numerous kinases thought to mediate tau's effects (Mairet-Coello et al., 2013).

ALS-FTD

Amyotrophic lateral sclerosis is a debilitating disorder affecting upper and lower motor neurons in the cortex, brainstem, and spinal cord (Rowland and Shneider, 2001). Similar to other neurodegenerative diseases, most of the ALS cases are sporadic and 10% of the cases are familial. There is both genetic and pathological overlap between ALS and forms of FTD characterized by accumulations of TAR DNA binding protein-43 (TDP-43) and/or with mutations in C90rf72 (Ling et al., 2013). Motor neurons in ALS are vulnerable to excitotoxic injury as these neurons highly express AMPAR calcium channels (Williams et al., 1997; Corona and Tapia, 2007), accompanied by low expression of calcium buffering proteins (Alexianu et al., 1994; Jaiswal, 2013). In addition, mitochondrial dysfunctions have been reported in post-mortem brain tissues of ALS patients (Sasaki and Iwata, 1996; Kong and Xu, 1998) as well as in animal models of ALS (Nguyen et al., 2009; Santa-Cruz et al., 2016). Given the susceptibility of these neurons to calcium overload induced toxicity, mitochondria play important calcium buffering roles in these neurons (Smith et al., 2017). Excessive exposure to glutamate can lead to glutamate induced excitotoxicity (Stout et al., 1998). Increased glutamate toxicity could be due to enhanced synaptic activity (Milanese et al., 2011) or dysfunctional reuptake by neighboring glial cells (Fray et al., 1998), which can cause persistent activation of AMPAR and increased cytosolic calcium burden leading to mitochondrial calcium overload (Goodall and Morrison, 2006). Interestingly, increased excitatory activity and dendritic spine numbers are observed in early pre-symptomatic stages of the TDP-43(Q331K) model of ALS (Fogarty et al., 2016). Thus, genetic mouse models of all three diseases, PD, AD, and ALS indicate an early phase of excitatory hyperactivity.

EXCITATORY MITOCHONDRIAL TOXICITY (EMT) IN CHRONIC NEURODEGENERATIVE DISEASES

In this section, we discuss how PD-linked changes in mitochondrial calcium transport proteins act in concert with sublethal elevations in excitatory neurotransmission to elicit mitochondrial injury and mitochondrial depletion from dendrites. Mitochondrial depletion then contributes to retraction and simplification of the dendritic arbor. In contrast to excitotoxicity, which rapidly results in the classic red, dead neuron observed in stroke, calcium injury triggered autophagy/mitophagy plays a key role in dendritic simplification observed in several models of PD. Given that dendritic pathology is observed in post-mortem studies of PD (Patt et al., 1991), AD (Brizzee, 1987), and ALS (Genc et al., 2017), the review closes with a discussion of the potential implications of the EMT mechanism for sporadic PD and other neurodegenerative diseases.

EMT in the LRRK2 Model

Shrinkage of the dendritic arbor represents one of the most frequently reported phenotypes exhibited by neurons expressing disease-linked mutations in LRRK2 (MacLeod et al., 2006; Ramonet et al., 2011; Winner et al., 2011; Cherra et al., 2013; Reinhardt et al., 2013; Plowey et al., 2014; Verma et al., 2017). This may be related to effects on microtubule dynamics, endosomal trafficking and/or autophagy [Reviewed in Ref. (Verma et al., 2014)]. Among the earliest changes exhibited by primary cortical neurons transfected with LRRK2-G2019S or LRRK2-R1441C are increased activity-dependent calcium influx through glutamate receptors and L-type calcium channels (Cherra et al., 2013; Plowey et al., 2014). This is followed by loss of mitochondria specifically from the dendritic compartment, which precedes subsequent neuritic retraction (Cherra et al., 2013). The loss of mitochondria can be blocked by inhibiting autophagy or expressing a phosphomimicking mutation of the autophagy protein LC3 (Cherra et al., 2013), which is predicted to impair the cardiolipin pathway of mitophagy (Chu et al., 2014). Mitochondrial fission is often required for efficient mitophagy (Twig et al., 2008; Dagda et al., 2009). Interestingly, mutant LRRK2 regulates Drp1-dependent mitochondrial fission as well as activating ULK1 to mediate mitophagy (Zhu et al., 2012; Su and Qi, 2013). The mechanism that leads to mitophagy of dendritic mitochondria downstream of mutant LRRK2-induced cytosolic calcium uptake was recently delineated using primary neurons transfected with genetically encoded calcium sensors (Verma et al., 2017). As expected, LRRK2-G2019S and -R1441C increased intracellular calcium uptake in response to stimulation, and this was accompanied by increased mitochondrial calcium uptake in dendrites. The increased dendritic mitochondrial calcium uptake persisted even in permeabilized neurons exposed to the same calcium concentrations, implicating increased mitochondrial calcium transport capacity in dendrites of mutant-LRRK2 expressing neurons. Further investigation revealed that mutant LRRK2-transfected neurons, as well as fibroblasts from

PD patients with the G2019S and R1441C mutations, showed increased mRNA and protein expression of MCU and MICU1, with no changes in MICU2 or NCLX expression (Verma et al., 2017). Neurons treated with MCU inhibitors exhibited decreased mitophagy and were protected from dendritic simplification induced by mutant LRRK2. These data implicate calcium-dependent injury to mitochondria within dendrites, and their subsequent mitophagic elimination, as mechanisms linking increased excitatory input with dendritic simplification.

EMT in the PINK1 Model

The recessive PD-linked gene PINK1, which is targeted to mitochondria via a classic N-terminal mitochondrial targeting sequence, has also been implicated in regulation of mitochondrial calcium homeostasis. As mentioned above, decreases in mitochondrial membrane potential are likely to have multiple consequences including decreased cytosolic calcium buffering and the loss of mitochondria due to mitophagy. Indeed, PINK1-deficient systems exhibit impaired calcium recovery (Heeman et al., 2011) and elevated basal mitophagy in neuronal cells (Dagda et al., 2009; Chu, 2010) and in pancreatic beta cells *in vivo* (McWilliams et al., 2018), evidently through one of several PINK1- and Parkin-independent mechanisms (Chu et al., 2013; Strappazzon et al., 2015; Bhujabal et al., 2017). In particular, mitochondrial calcium overload has been implicated, as inhibiting mitochondrial calcium uptake, in cells co-expressing α-syn A53T and Pink1 W437X, restores $\Delta\Psi_m$ and rescues neurite outgrowth (Marongiu et al., 2009).

Like primary neurons expressing mutant LRRK2, neurons cultured from PINK1 knockout mice to model recessive PD pathogenesis exhibit reduced dendritic arbors (Dagda et al., 2014). Interestingly, PINK1 was shown to regulate calcium efflux via NCLX, with PINK1 deficiency causing mitochondrial calcium overload (Gandhi S. et al., 2009). Indeed, it has recently been shown that PINK1 promotes PKA-dependent phosphorylation and activation of NCLX (Kostic et al., 2015). PINK1-deficient neurons are susceptible to dopamine toxicity, and expression of the NCLX-S258D phosphomimic mutant restores mitochondrial calcium efflux and confers neuroprotection. LETM1, another mitochondrial calcium transporter, represents a direct phosphorylation target of PINK1 (Huang et al., 2017). As LETM1 mediates both calcium influx and efflux (Doonan et al., 2014), the effects of LETM1 activation or loss of function are difficult to predict. Nevertheless, impaired mitochondrial calcium efflux, in a parallel pathway to the effects of PINK1 deficiency on NCLX activity, appears to represent the key pathogenic factor.

Converging Mechanisms in Neuroprotection

Whereas episodic calcium entry into the mitochondrial matrix stimulates respiration to adjust mitochondrial output to bioenergetic needs, chronically elevated cytosolic calcium oscillations elicit mitochondrially derived ROS, elevated mitophagy and decreased basal mitochondrial content in dopaminergic substantia nigra neurons (Liang et al., 2007; Guzman et al., 2018). While this occurs under normal conditions for pacemaking cells, such as substantia nigra neurons (Guzman et al., 2018), the mitochondrial response to cytosolic calcium influx is exaggerated in disease states, triggering mitophagy and loss of dendritic mitochondria, followed by a delayed degeneration of dendritic processes (Cherra et al., 2013; Verma et al., 2017). Inhibiting cytosolic calcium influx through NMDA receptors (Plowey et al., 2014) or L-type calcium channels (Cherra et al., 2013; Guzman et al., 2018) prevents the elevated dendritic mitophagy and restores mitochondrial density and dendrite lengths. Inhibiting mitochondrial calcium uptake via MCU confers complete restoration of dendrite lengths (Verma et al., 2017), supporting a central role for mitochondrial calcium mishandling in EMT.

It is likely that increased mitochondrial calcium uptake in the mutant LRRK2 model represents, at least initially, a compensatory response to increased excitatory input. Indeed, MCU and MICU1 are transcriptionally upregulated in mutant LRRK2-expressing neurons and fibroblasts through activation of the ERK1/2 signaling pathway (Verma et al., 2017), which has been proposed to mediate several effects of mutant LRRK2 (Carballo-Carbajal et al., 2010; Reinhardt et al., 2013). Interestingly, a similar elevation in phospho-ERK2, MCU, and MICU1 expression is observed in cortical brain samples from patients with sporadic PD/PDD (Verma et al., 2017, suggesting that enhanced susceptibility to mitochondrial calcium overload could contribute to sporadic disease as well.

One of the models of sporadic disease involves complex I inhibitors, such as MPP+ and rotenone, as PD patients exhibit systemically decreased complex I activity (Greenamyre et al., 2001). Mitochondrial calcium overload has been implicated specifically in the substantia nigra, but not the relatively resistant ventral tegmental area, in the MPP+ model of parkinsonian complex I deficiency (Lieberman et al., 2017). Downregulating autophagy through PKA-mediated phosphorylation of LC3 confers protection against neurite retraction in this model (Cherra et al., 2010), although the potential protective role of modulating MCU or NCLX activities remains unexplored.

While stimulated mitochondrial calcium uptake is either unchanged (Kostic et al., 2015) or slightly decreased (Huang et al., 2017) in PINK1-deficient cells, future studies are needed to determine whether or not there may be concurrent disruption of mitochondrial efflux mechanisms in the mutant LRRK2 model. Irregardless, mutant forms of NCLX that mimic phosphorylation at the PINK1/PKA-regulated NCLX-S258 site (Kostic et al., 2015) confer protection from mutant LRRK2-mediated dendritic simplification to the same extent as inhibiting MCU (Verma et al., 2017). Likewise, inhibition of MCU is neuroprotective in a zebrafish model of PINK1 deficiency (Soman et al., 2017), indicating that reducing the likelihood of mitochondrial calcium overload through either the influx or efflux pathways may be effective irregardless of the original predisposing mechanism.

Implications for Other Diseases

It is known that inhibiting mitochondrial calcium uptake via MCU is beneficial in protecting against neuronal cell death after

stroke (Abramov and Duchen, 2008) or during NMDA induced excitotoxic neuronal cell death (Qiu et al., 2013). Recent studies have also shown neuroprotective effects of inhibiting of MCU on Aβ induced microglial cell death (Xie et al., 2017), loss of hippocampal neurons in pilocarpine induced status epilepticus (Wang et al., 2015) or ischemia/reperfusion injury (Zhao et al., 2013). Interestingly, inhibition of MCU protects against neuronal ischemia-reperfusion injury by inhibiting excess mitophagy (Yu et al., 2016), similar the mechanism described in the mutant LRRK2 model (Verma et al., 2017). However, the potential role of sublethal mitochondrial injuries in triggering EMT has been much less studied outside of PD.

In particular, it is unknown if changes in MCU or NCLX expression or post-translational modifications may contribute to sensitivity to excitatory injury in these other diseases. It would be important to delineate whether or not the activities of kinases that regulate mitochondrial calcium transporters are altered in susceptible neurons. In addition to being elevated in patient brains with familial LRRK2 mutation (Verma et al., 2017), elevated ERK1/2 is also observed in sporadic PD/PDD/DLB (Zhu et al., 2002; Verma et al., 2017), AD (Perry et al., 1999), hypoxia-ischemia (Wang et al., 2003), and in organotypic spinal cord culture models of ALS-related TDP-43 pathology (Ayala et al., 2011). As ERK1/2 drives the changes in MCU and MICU expression observed in familial PD patient cells and models, it is possible that EMT contributes to dendritic retraction and simplification in a spectrum of neurodegenerative conditions.

While experiments involving the inhibition of autophagy support the conclusion that mitophagy contributes to mitochondrial depletion from dendrites, another key question to be considered is to understand why aging or diseased neurons fail to replace the degraded mitochondria. These factors may include age- or disease-related decline in mitochondrial biogenesis as observed in PD (Zheng et al., 2010; Zhu et al., 2012) or alterations in mitochondrial transport in neuronal processes as implicated in AD (Calkins and Reddy, 2011) and ALS (Magrane et al., 2012). Mitochondrial depletion would persist only if mitophagy is not balanced by mechanisms to replace the degraded mitochondria. It is thus conceivable that therapies targeting mitochondrial biogenesis or transport may also rescue the ill effects of dendritic EMT.

CONCLUSION

Dendritic simplification is observed in mutant *LRRK2*-expressing neurons, in *PINK1* knockout neurons, in post-mortem sporadic PD patient neurons and in other neurodegenerative and neuropsychiatric conditions. We propose that dominant, recessive and sporadic contributions to altered mitochondrial calcium homeostasis converge on a process of EMT to mediate degeneration of the dendritic arbor observed in many neurodegenerative diseases. This may result from effects on the MCU complex itself, on mitochondrial calcium extrusion mechanisms and/or any change that result in greater cytosolic calcium levels. While an alteration in mitochondrial calcium handling on its own may be insufficient to cause neuronal injury, when combined with increased post-synaptic calcium fluxes that accompany excitatory synaptic activity, EMT leads to dendritic shortening and simplification by triggering unbalanced mitophagy and perisynaptic mitochondrial depletion, mechanisms that are distinct from classic excitotoxicity. Moreover, while dominant and recessive contributions to dendritic EMT occur through different mechanisms, interventions that either reduce mitochondrial calcium uptake via MCU or that target NLCX to enhance mitochondrial calcium release are reciprocally effective in both systems (Kostic et al., 2015; Soman et al., 2017; Verma et al., 2017). Future studies to determine whether increased excitatory activity observed in AD and ALS-FTD is also linked to dendritic simplification and spine loss via mitochondrial calcium overload will help determine whether or not therapies targeting EMT may have even broader applicability.

AUTHOR CONTRIBUTIONS

MV reviewed the literature, designed figures, and wrote sections of the manuscript. ZW contributed to concept development and edited the manuscript. CC developed the conceptual framework, wrote, and edited the manuscript.

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REVIEW
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Oldies but Goldies mtDNA Population Variants and Neurodegenerative Diseases

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mtDNA is transmitted through the maternal line and its sequence variability, which is population specific, is assumed to be phenotypically neutral. However, several studies have shown associations between the variants defining some genetic backgrounds and the susceptibility to several pathogenic phenotypes, including neurodegenerative diseases. Many of these studies have found that some of these variants impact many of these phenotypes, including the ones defining the Caucasian haplogroups H, J, and Uk, while others, such as the ones defining the T haplogroup, have phenotype specific associations. In this review, we will focus on those that have shown a pleiotropic effect in population studies in neurological diseases. We will also explore their bioenergetic and genomic characteristics in order to provide an insight into the role of these variants in disease. Given the importance of mitochondrial population variants in neurodegenerative diseases a deeper analysis of their effects might unravel new mechanisms of disease and help design new strategies for successful treatments.

Keywords: mtDNA, haplogroups, PD, LHON, neurodegenerative diseases

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MITOCHONDRIA AND OXPHOS

Mitochondria, from ancient Greek, *mito* (thread), and *chondros* (grain), are highly dynamic organelles in continuous communication with the rest of the cell, that mediate several key cellular functions (Wai and Langer, 2016). These include being the primary source of cellular energy, in the form of adenosine triphosphate (ATP), regulating levels of calcium (Grishanin et al., 1996), reactive oxygen species (ROS) (Boveris and Chance, 1973) and apoptosis (Green and Reed, 1998). Mitochondria play a central role in several fundamental metabolic pathways including the tricarboxylic acids cycle (TCA), fatty-acid β-oxidation, and the pyrimidine biosynthesis (Attardi and Schatz, 1988).

Mitochondria generate the vast majority of cellular energy through the oxidative phosphorylation system (OXPHOS), combining respiration with the synthesis of ATP. Cellular respiration is an ordered chain of redox reactions using reducing equivalents [Nicotinamide adenine dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2)] produced from the degradation of carbohydrates to convert oxygen into water. These reactions are carried out by four multi-protein enzymes: the complexes of the electron transport chain (ETC) I, II, III, and IV and two "shuttles": ubiquinone (coenzyme Q10) and cytochrome c (Saraste, 1999; Smeitink et al., 2000). The energy released in this process is used to pump protons (H^+) through complex I (4 H^+), III ($4H^+$), and IV ($2H^+$), into to the inter-membrane space,

generating a positive electrochemical gradient which drives the transport back to the matrix through the complex V or *ATP* synthase. This complex acts as a proton channel that returns the protons to the mitochondrial matrix. The proton flux provides the energy needed to bind adenosine di-phosphate (ADP) and inorganic phosphate into ATP (Mitchell, 1961).

MITOCHONDRIAL GENOME

One of the principal features of mitochondria is that they contain their own genetic system. In humans this is a double chain circular molecule, 16.6 Kb long, which codes for 13 proteins of the OXPHOS system, and the 22 tRNAs and 2 rRNAs required for their expression (Taanman, 1999). Seven of the 13 subunits contribute to complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), one to the complex III (CYB), three to the complex IV (CO1, CO2, and CO3), and two to the complex V (ATP6 and ATP8). mtDNA is composed of 2 chains, heavy (H) and light (L) with different density based on the G/C composition. Most of the genes are coded by the heavy chain including 2 rRNAs, 14 tRNAs and 12 polypeptides), while 8 tRNAs and only one polypeptide (ND6) are encoded by the light chain (Montoya et al., 1982).

mtDNA is polyplasmic, with each mitochondria containing several copies. A somatic cell can contain between hundreds to thousand mtDNA copies depending on the cell type (Robin and Wong, 1988). Usually all of the molecules are identical (homoplasmy). The presence of more than one type or allele of mtDNA in a cell is known as heteroplasmy (DiMauro et al., 1993). The proportion of a heteroplasmic mutation can vary from cell to cell and selective pressures can influence both of these processes (Taylor and Turnbull, 2005; Stewart and Chinnery, 2015; Burr et al., 2018).

Mitochondrial DNA is strictly maternally inherited (Giles et al., 1980; Pyle et al., 2015) and has a mutation rate, 5–10 times higher than the nuclear genome (nDNA) (Brown et al., 1995). These factors have led to the accumulation of a wide range of polymorphisms across the mtDNA sequence that are restricted to geographically isolated populations throughout the globe. Given that these genetic variants are inherited from mother to offspring without any recombination, they sequentially accumulate along the radiating maternal lineages (**Figure 1**). This had generated phylogenetically related haplotypes (**Figure 1**). The most common (>1% frequency in the population) are known as mitochondrial haplogroups (Wallace et al., 1999; Wallace, 2015).

ANCIENT POLYMORPHISMS MTDNA HAPLOGROUPS

The human mtDNA phylogeny clusters into a three with "unique" ancestor, known as the "Mitochondrial Eve," rooted in Africa about 150,000 years before present (YBP) (Cann et al., 1987). From this root, four lineages specific for sub-Saharan Africa: L0, L1, L2, and L3 were generated about 100,000 YBP. Then, 60,000 YBP the African haplogroup L3 diverged into the two

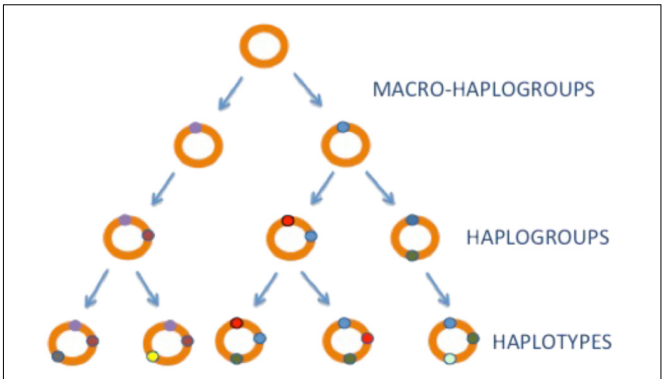


FIGURE 1 | mtDNA inheritance. mtDNA haplogroups. mtDNA is shown as a orange circle, mtDNA variants with little circles in colors.

recent macro-haplogroups M and N, which define populations which left Africa to populate the rest of the world (Soares et al., 2012). During this migration, the haplogroup N was directed to Eurasia and Asia and America, while the M went exclusively to Asia giving place to the haplogroups A, B, C, D, G, and F. In Europe, the haplogroup N 60,000 YBP gave rise to the haplogroup R (Gandini et al., 2016), which is the root of the "European" haplogroups U (60,000 YBP), J (40,000 YBP), T (20,000 YBP), H (30,000–5,000 YBP) (Achilli et al., 2004), and V (15,000 YBP) (Torroni et al., 1996) (**Figure 2**). With the implementation of the next generation sequencing techniques (NGS) there has been a huge increase in the number of mtDNA genomes sequenced and the branches of the tree have diverged into many sub-haplogroups (Brotherton et al., 2013). The current global mtDNA phylogenetic tree contains more than 4,000 different haplogroups (van Oven and Kayser, 2009).

In Europe, 90% of the population belongs to the macro-haplogroups HV, U, and JT (Macaulay et al., 1999; Torroni et al., 2000). The macro-haplogroup HV represents more than 50% of the population, it comprises the haplogroups H and V and HV*. Among them, the haplogroup H an extremely widely distributed and has the highest frequency reaching 45% in Europe, 20% in Turkey and the Caucasus, and around 10% in Gulf countries (Roostalu et al., 2007). It is formed by more than 90 sub-haplogroups (van Oven and Kayser, 2009), where the H1 is the major one having two peaks of high frequency in Scandinavian Peninsula and Southern Iberian Peninsula. The second most frequent haplogroup is the H3, commoner in South of Europe, particularly in France and Spain. The rest of the sub-haplogroups, as for example H2 and H6, are more frequent in the Caucasus and Eastern Europe (Pereira et al., 2005). On the other hand, haplogroup V is found in 4% of the population, principally in European populations, but also present in the north of Africa (Coudray et al., 2009).

The other two macro-haplogroups are the JT and U which account 40% of the European population. Haplogroup U is divided into several sub-haplogroups that make up 20% of the Caucasian population, whereas the subhaplogroups U5 and Uk comprise 9% (Montiel-Sosa et al., 2006). This clade is widely spread from Portugal to India and North of

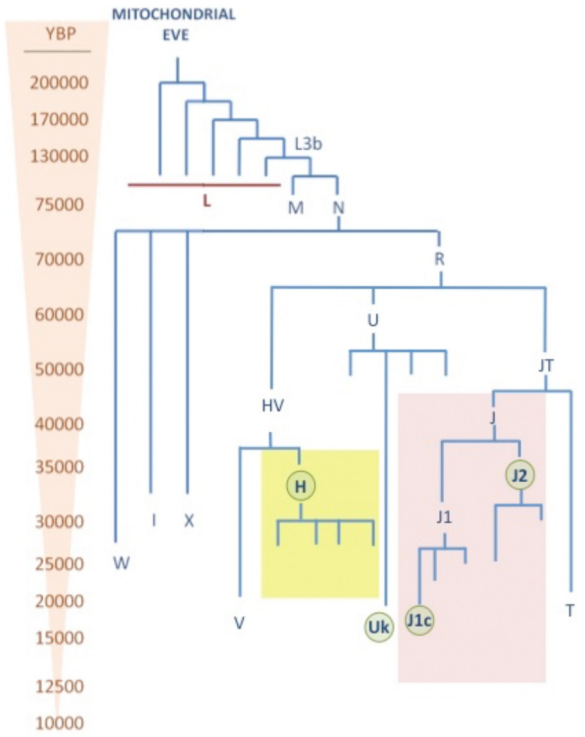


FIGURE 2 | mtDNA phylogenetic tree. This tree shows the age (YBP) of different mtDNA haplogroups. The major Caucasian haplogroups H, U, and J are highlighted in yellow, blue, and red, respectively. Sub-haplogroups which has shown pleiotropic effect in neurodegenerative diseases are highlighted with green circles.

Africa (Achilli et al., 2005). The macro-haplogroup JT includes haplogroups J and T (Ruiz-Pesini et al., 2004). Haplogroup T is divided also in several haplogroups and embody 8% of the population (SanGiovanni et al., 2009). Haplogroup J is found in 9% of Europeans, and is divided into 2 principal sub-haplogroups J1c and J2 (Carelli et al., 2006).

MTDNA POLYMORPHISMS AND NEUROLOGICAL DISEASES

Over the last 20 years, many studies have found associations between inherited mtDNA population variants and neurological diseases. Since the first association of Leber Hereditary Optic Neuropathy (LHON) with the mitochondrial haplogroup J in the late 90s (Torroni et al., 1996; Carelli et al., 1997; Hofmann et al., 1997; Lamminen et al., 1997), many other mitochondrial disorders along with classic neurodegenerative diseases like Parkinson (PD), Alzheimer (AD), Multiple Sclerosis (MS), and Amyotrophic Lateral Sclerosis (ALS).

Mitochondrial Diseases

Mitochondrial diseases are a large group of heterogeneous disorders caused by mutations in the mtDNA and the nuclear DNA (nDNA). Although, their phenotypes are hugely variable, some of them overlap with clinical phenotypes observed in neurodegenerative diseases (Betts et al., 2004; Swerdlow, 2009). For example, neurodegeneration in the cerebellar purkinje layer and cortical neurons in the occipital and parietal lobes has been observed in patients with Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) (Betts et al., 2004). Leber Hereditary Optic Neuropathy (LHON) disorder is characterized by neurodegeneration of the retinal ganglion cell (RGC) layer and optic nerve (Yu-Wai-Man et al., 2011). In addition, a small sub-set of LHON patients develop similar signs to multiple sclerosis, in a disease known as Harding's disease (Pfeffer et al., 2013; Bargiela and Chinnery, 2017) and signs of parkinsonism have also been observed in LHON pedigrees (Simon et al., 1999; Vital et al., 2015).

Leber Hereditary Optic Neuropathy

LHON is a mitochondrial neurodegenerative disorder characterized by RGC dysfunction and rapid visual loss. In Caucasian population, approximately 90% of LHON cases are caused by a mutation in the *MT-ND* genes subunits encoding for the mitochondrial complex I; m.11778G > A:*MT-ND4* (60%), m.3460G > A:*MT-ND1* (15%) and m.14484T > C:*MT-ND6* (15%). The remaining 10% of the LHON cases harbor rarer mutations (Achilli et al., 2012; Maresca et al., 2014; Caporali et al., 2018). Although the primary genetic cause of LHON is known, the presence of a mtDNA mutation is not enough on its own to cause the blindness. Many factors such as gender, with higher penetrance in males than females, genetic factors including the mitochondrial haplogroups, and other environmental factors have been related with the clinical penetrance of the disease (Kirkman et al., 2009; Caporali et al., 2017).

The role of mtDNA population variants in LHON has been widely studied. Initial studies described the variants m.4216C > T:*MT-ND1* and m.13708G > A:*MT-ND5*, m.15257G > A:*MT-CYB*, m.15812G > A:*MT-CYB* defining J and J2 haplogroup, respectively, as "secondary" LHON mutations (Johns and Berman, 1991; Johns and Neufeld, 1991; Huoponen et al., 1993; Oostra et al., 1994; Brown et al., 1995; Harding et al., 1995; Howell et al., 1995; Lodi et al., 2000), before the association was described with haplogroup J (Carelli et al., 1997; Hofmann et al., 1997; Lamminen et al., 1997; Torroni et al., 1997). Studies with bigger cohorts in Italian population (86 cases) narrowed down the associations to specific LHON mutations, where the m.11778G > A:*MT-ND4* mutation was over-represented in subhaplogroups J1c and J2b defined by variants in the *MT-CYB*; and m.14484T > C: *MT-ND6* was over-represented in haplogroup J1 (Carelli et al., 2006). The role of the *MT-CYB* variants was also confirmed in the most comprehensive study carried out to date. In a cohort counting with 3,613 individuals from 159 European LHON families, Hudson et al. confirmed mutation specific sub-haplogroup associations (Hudson et al., 2007). Individuals carrying the mutation m.3460G > A: *MT-ND1* had an increase of risk in the haplogroup Uk [OR = 2.37, p = 0.002, CI 95% (1.36–4.13)]. Individuals carrying m.11778G > A: *MT-ND4* had an increased risk in the J haplogroup [OR = 1.31, p = 0.02, CI 95% (1.03–1.65)], and a reduced risk on a haplogroup H background

[OR = 0.79, p = 0.04, CI 95% (0.63–0.98)] (Hudson et al., 2007).

Recent studies have shown the combinations of polymorphisms may lead to a reduced OXPHOS efficiency and be sufficient to trigger LHON (Achilli et al., 2004; Caporali et al., 2018). This is in keeping with our recent findings, where we showed that some mtDNA sequences appear to influence the probability of acquiring new pathological mutations in a population specific manner (Wei et al., 2017).

Other Mitochondrial Diseases

Independently from LHON studies, other mitochondrial disorders such as MELAS, neuropathy, ataxia, retinitis pigmentosa (NARP)/Maternally inherited Leigh's syndrome (MILS), and age-related hearing loss (ARHL) have been associated with certain mtDNA haplogroups, but these findings remain controversial.

In the case of MELAS, a study in 142 unrelated French families carrying the m.3243A > G mutation observed a statistically significant under-representation of the mutation in haplogroup J patients [OR = 0.26, p = 0.01, CI 95% (0.08–0.83)] (Pierron et al., 2008). Analysis of the same mutation in smaller sample from Spanish population did not find any association (Torroni et al., 2003).

Mitochondrial haplogroups do not appear to play a role in non-syndromic deafness caused by the m.1555G > A (Torroni et al., 1999), although an association with haplogroup H3 was described in one small cohort of Spanish mutation carriers (Achilli et al., 2004). The analysis of 912 ARHL patients found that haplogroup U was significantly associated with moderate to severe phenotype (OR 3.02; CI 95%: 1.30–6.99); and in patients aged from 50 to 59 years sub-haplogroup Uk was associated with severe ARHL only (OR 3.02; CI 95%: 1.30–6.99) (Manwaring et al., 2007). Although it is difficult to draw firm conclusions from these small single studies, in line with these findings, a study in transmitochondrial cell lines carrying the mutation m.8993T > G: *MT-ATP6* responsible NARP/MILS syndrome showed an significant increase severity of the OXPHOS defect in cell lines from the haplogroup U5b compared those belonging to haplogroup H (D'Aurelio et al., 2010).

Parkinson Disease

The association of mtDNA polymorphisms and Parkinson's disease pathogenesis has been controversial, although recent large studies have validated the original findings in independent cohorts. Many studies have shown association between PD and particular haplogroups (Moilanen et al., 2001; Ross et al., 2003; van der Walt et al., 2003; Otaegui et al., 2004a; Ghezzi et al., 2005; Huerta et al., 2005; Pyle et al., 2005; Gaweda-Walerych et al., 2008; Khusnutdinova et al., 2008; Latsoudis et al., 2008; Georgiou et al., 2017), while others could not directly replicate these findings (Simon et al., 2010; Fachal et al., 2015). Most of the studies showed population specific associations. Indeed, an early work in Finns found a reduced risk for PD in the supercluster HVKU compared to the supercluster JTIWX which was exclusively associated with the Uk cluster (Autere et al., 2004). Studies in a Polish population of 241 PD patients and 277 control subjects, didn't find differences between the haplogroups, however, after stratification by gender, they found that haplogroup J [OR = 0.19, p = 0.0014, CI 95% (0.069–0.53)] was associated with a lower PD risk in males (Gaweda-Walerych et al., 2008). Another study in an Italian population comprising of 620 idiopathic PD patients and about 2000 controls found a role of haplogroup Uk in decreasing the penetrance of PD [OR = 0.54, p = 0.048, CI 95% (0.35–0.83)] (Ghezzi et al., 2005), while in an study on a Spanish cohort of 271 PD patients and 230 healthy controls significant association was found for the polymorphism defining the haplogroup H5 mt.4336 T>C:$tRNA^{Gln}$ with a significantly increased frequency in PD compared to controls [OR = 4.45, p = 0.011, CI 95% (1.23–15.96)] but only in females (Huerta et al., 2005). Other study in a Tatar Russian population of 183 unrelated PD patients and 157 controls found that polymorphisms associated with haplogroup H mtDNAs increased PD risk (OR = 2.58, p = 0.0001), whereas those associated with haplogroup Uk were protective (OR = 0.38, p = 0.003) (Khusnutdinova et al., 2008). Conversely, the Irish study that included 90 Irish PD patients and 129 Irish controls, reported that haplogroups J and T increased PD risk (Ross et al., 2003).

The variability of results could have been affected by population stratification issues, small sample sizes and variations in the statistical approach used. However, three recent meta-analysis have shown evidence of the existence of both protective and risk haplogroup alleles associated with PD. The first study counted with 3,074 PD cases and 5,659 ethnically matched controls followed by meta-analysis of 6,140 PD cases and 13,280 controls. They found that two variants, m.2158T > C:*MT-RNR2* and m.11251A > G:*MT-ND4*, which are phylogenetically linked and define the mitochondrial superhaplogroup JT and sub-haplogroup J1b, respectively, were associated with a reduced risk of PD [OR = 0.87, CI 95% (0.77–0.97)] (Hudson et al., 2013). In line with this finding, a second study from the same group, including 2,197 patients compared with three independent control groups genotyped on the same platforms (C1 = 2997, C2 = 2897, and C3 = 5841 control samples), also showed a protective effect for the variant m.10398A > G:*MT-ND3*, homoplastic (recurrent in several branches of the mtDNA phylogenetic tree) on haplogroups J and Uk, which was previously associated with reduced risk to PD (Ghezzi et al., 2005; Huerta et al., 2005; Hudson et al., 2014). Finally, a meta-analysis from 13 different studies combining n = 9243 patients and n = 17,999 controls found that individuals mtDNA haplogroup Uk [OR = 0.839, p = 0.004, CI 95% (0.744–0.945)], haplogroup T [OR = 0.857, p = 0.014, CI 95% (0.757–0.969)] and haplogroup J [OR = 0.876, p = 0.011, CI 95% (0.79–0.971)] had a significantly reduced risk of developing PD, while the macro-haplogroup HV act as a increased risk factor [OR = 1.091, p = 0.038, CI 95% (1.005–1.184)] (Marom et al., 2017).

Alzheimer Disease

The implication of mtDNA variants in AD remains contentious. Several studies have reported haplogroup associations (Chagnon et al., 1999; Carrieri et al., 2001; Fesahat et al., 2007; Santoro et al.,

2010; Maruszak et al., 2011; Coskun et al., 2012; Ridge et al., 2012) but the results have been inconsistent, and others found no evidence of an association (Chinnery et al., 2000; Hudson et al., 2012; Fachal et al., 2015; Pyle et al., 2015) (for extensive review of the data see Ridge and Kauwe, 2018). The largest study for mtDNA carried with 3,250 AD patients and 1,221 controls found no significant association between mtDNA variants and AD (Hudson et al., 2012). However, a more recent meta-analysis involving several studies did report an increased risk of AD in people belonging to haplogroup H [OR = 1.283, p = 0.016, 95% CI (1.047–1.574)] (Marom et al., 2017), but larger studies are warranted to validate these findings which only just reach conventional statistical significance.

In addition to the genetic analysis, a study from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database including 175 controls and 154 AD patients found that individuals from U5b1 and Uk1a1b haplogroups had grater rates of temporal pole atrophy, an endophenotype of AD risk (Ridge et al., 2013).

Multiple Sclerosis

Following the trend of the previous disorders, the data on associations between mitochondrial haplogroups and MS is contradictory. While some small studies have seen an increase in the risk associated with the haplogroup Uk in different populations (Kalman et al., 1999; Hassani-Kumleh et al., 2006; Vyshkina et al., 2008), others have shown a consistent association with haplogroup J (Kalman et al., 1999; Houshmand et al., 2005; Mihailova et al., 2007), and others failed to find any association in United Kingdom (Ban et al., 2008) and Basque population (Otaegui et al., 2004b).

Larger studies have, however, found consistent associations, mainly with the macro-haplogroup JT as a risk factor in European populations. The first meta-analysis studying more than 2500 MS samples and the same number of controls found that the variant m.13708G > A: *MT-ND5*, defining the haplogroup J, was significantly associated with an increased risk to MS [OR = 1.71, P = 0.0002, CI 95% (1.29–2.27)] (Yu et al., 2008). Similarly, another recent meta-analysis involving 7,391 and 14,568 controls confirmed the association between haplogroup J (OR = 1.11, p = 0.03, CI 95% (1.01–1.22)] and haplogroup T carriers [OR = 1.17, p = 0.002, CI 95% (1.06–1.29)]. In the same study, only 3 populations (Italy, United Kingdom, and Germany) showed an association between primary progressive (PP) PPMS and haplogroup J [OR = 1.49, p = 0.009, CI 95% (1.10–2.01)], but a smaller cohort with 3,720 cases and 879 controls from US did not show the same association (Tranah et al., 2015). These findings could be due to differences in the frequency of sub-haplogroups in the populations studied (Andalib et al., 2015).

Amyotrophic Lateral Sclerosis

The larger study carried out in ALS in a cohort of 700 patients and 462 controls from two European populations did not find any association between mtDNA haplogroups and ALS (Ingram et al., 2012). Similar results were previously obtained in a cohort from UK population of 504 ALS patients and 493 controls (Chinnery et al., 2007). Conversely, in a cohort from Italian population with 222 patients with sporadic ALS (sALS) and 151 matched controls, the haplogroup I demonstrated to be associated a higher risk of ALS when compared to the common haplogroup, H [OR: 0.08, p < 0.01, CI 95% (0.04–0.4)] (Mancuso et al., 2004). Overall, studies of mtDNA haplogroups in ALS remain much smaller than other neurodegenerative diseases.

FROM EPIDEMIOLOGY TO BIOENERGETICS

Despite earlier contention, there is now clear evidence that mtDNA variants within haplogroups are associated with specific neurodegenerative disorders, with the most consistent evidence for Parkinson disease and LHON. This raises the question: how do these genotypes mediate their pleiotropic effects? Functional studies are technically challenging because the biochemical effect of common polymorphisms is likely to be subtle. In addition, given that many of the associated variants are found on genomes containing other polymorphisms, several linked alleles may be interacting. Unfortunately, the double mitochondrial membrane precludes site directed mutagenesis at present, so functional studies are limited to mtDNA harvested from human cells. The generation of transmitochondrial cybrids present one approach to address some of these issues (Chomyn et al., 1994). A cybrid gives the opportunity of studying the effect of a determined mtDNA in a fixed nuclear background under the same ambient conditions. Briefly, a rho0 cell line is generated by removing mtDNA (King and Attardi, 1996; Miller et al., 1996), can be fused with enucleated fibroblasts (King and Attardi, 1989) or with platelets containing the mtDNA of interest (Chomyn et al., 1994; **Figure 3**).

Cybrid technology has been widely used for the study of phenotypical effect of inherited pathogenic mutations in the mtDNA (Hayashi et al., 1991; Trounce et al., 1994; Vergani et al., 1995; Brown et al., 2001; D'Aurelio et al., 2001), but the model itself raises several concerns, including aneuploidy and an unstable nuclear background in a cancer derived cell line, the use of mutagenic agents for the generation of rho0, and biochemical properties of the original tumor (for further information see Iglesias et al., 2012). However, given the benefits shown when using this approach to elucidate disease mechanisms for mitochondrial diseases, the technique has been extensively applied for the study of the mtDNA population variants to look for subtler biochemical effects of defined genetic variants.

Cybrids from haplogroup H contain higher mtDNA levels and mtDNA encoded mRNA levels, growing faster, have a higher membrane potential, and consume more oxygen per ETC unit than cybrids from haplogroup Uk individuals (Gómez-Durán et al., 2010). Similar differences were found when characterizing cybrids from haplogroup H and T in HEK293 cells background, where H cybrids showed higher respiration capacity per molecule of mtDNA, compared to HEK293 cybrids, but lower growth rate (Mueller et al., 2012). Contrarily, in another study, Caucasian haplogroups H and T showed no differences in the mitochondrial membrane potential and the oxygen consumption per cell. Other analysis didn't find differences between the "artic" (A, C, and D)

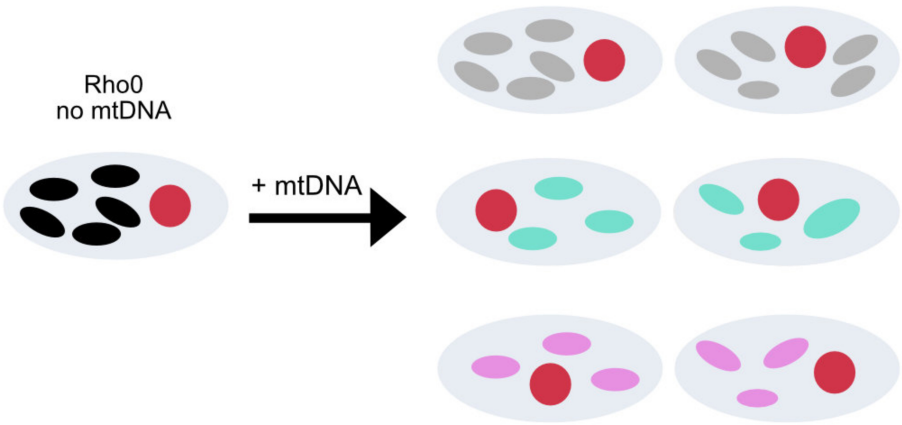


FIGURE 3 | Transmitochondrial cybrid model. Construction of cybrid cell lines containing the same nuclear DNA, but mtDNA from healthy individuals with different mtDNA haplogroups. The oval circles inside the cell with colors white, gray, blue, pink and black represent no mtDNA and mtDNA from with different mtDNA, respectively.

and the "tropical" haplogroups (L1, L2, and L3) or the European haplogroups H and T (Amo and Brand, 2007; Amo et al., 2008).

Haplogroup J has been widely linked to several diseases, and therefore deeply characterized using different nuclear genetic backgrounds. Similarly, to the epidemiological studies, initial work with a small number of samples showed no functional differences between the haplogroup H, J, and T (Carelli et al., 2002). However, larger studies showed that haplogroup J cell lines have slower rate of assembly of the mitochondrial complexes (Pello et al., 2008) on a nuclear 143B background. In studies in Wal-2A cell line haplogroup J1b revealed higher mtDNA levels and TFAM binding than a cell lines from haplogroup H (Suissa et al., 2009). However, using ARPE-19 cells, the same group did not find differences in the mtDNA levels between haplogroups H and J, but found lower levels in ROS production and ATP levels in haplogroup J (Kenney et al., 2013). Similarly, J cybrids on a 143B background showed less lower ATP and ROS production than haplogroup H cybrids (Bellizzi et al., 2012). This contrasts with another study of 9 cybrid lines from the haplogroup J, which were compared to 5 from the haplogroup H, and did not find any difference in manganese superoxide dismutase (MnSOD) expression, a marker of reactive oxidative species (ROS) production. This study did, however, confirmed the previous findings of lower oxygen consumption and low total ATP levels in the haplogroup J cell lines (Gómez-Durán et al., 2012).

Besides the discrepancies among the studies, it seems that cell lines carrying the mitochondrial haplogroup J have less OXPHOS capacity and ATP levels than those from the haplogroup H. In keeping with this, *in vivo* studies of individuals showed that individuals from the J haplogroup were shown to have lower maximal oxygen uptake (VO2max) (Marcuello et al., 2009) and haplogroup H higher (Martinez-Redondo et al., 2010) in an Spanish cohort.

The variation observed in all studies between the haplogroups carried *in vitro* could be due to 2 factors; the effect in the nuclear background uses and/or to the differences between the sub-haplogroups of each haplogroup (Chen et al., 2012). Indeed, while in our studies we studied cells carrying mtDNA from the subhaplogroups of the J; J1b1 (1), J1c (4), J2 (4), H1 (3), H5 (1), and H1 (3) (Gómez-Durán et al., 2012), Suissa et al. and the Kenney et al. included J1 (1), J1b2 (1), H1 (1), H6 (2) and H*(2) and J1c (2), J1d1a (1), and H subhaplogroups that were not stated, respectively, (Suissa et al., 2009; Kenney et al., 2013). Thus, the observed inconsistency could be due to the variants in the younger branches of the phylogenetic tree that define the sub-haplogroups (Hudson et al., 2014). Altogether, this could also affect nDNA-mtDNA retrograde response signaling in a haplogroup dependent manner.

MITOCHONDRIAL SIGNALING IN MTDNA POPULATION VARIANTS

Mitochondrial nuclear crosstalk was first described in yeast after the depletion of mtDNA which induce the expression of transcription factors (Butow and Avadhani, 2004; Picard et al., 2013). Mitochondrial dysfunction also induces retrograde responses (Chae et al., 2013). The biochemical disruption alter many downstream including immune signaling (Picard et al., 2015), mTORC1 (Khan et al., 2017), AMP-activated protein kinase (AMPK) (Zheng et al., 2016) and the transcription factors ATF4 (Quiros et al., 2017) and/or ATF5 (Fiorese et al., 2016; Suomalainen and Battersby, 2017).

mtDNA population variants also have been shown to affect a variety of signaling pathways. Cybrids in 143B osteosarcoma background from haplogroup J showed higher expression levels of IL-1β and TNFR2 than the ones from haplogroup H (Bellizzi et al., 2006). In the same cell lines J haplogroup showed an increase expression of the methionine adenosyltransferase 1A (*MAT1A*) gene, and therefore an increase in the global methylation compared to the cells from the H haplogroup (Bellizzi et al., 2012). In ARPE-19, cybrids cell lines from the haplogroup J have reduced Complement factor H (*CFH*),

Complement component 3 (*C3)* and expression levels than those from haplogroup H (Kenney et al., 2013) and increase in apoptotic genes like *RAR* (Kenney et al., 2014). Another study in 143B cells showed a significantly increased expression of BBC3 in H cybrids compared with J cybrids (Fernández-Moreno et al., 2017). We have seen an increase in the expression of phosphofructokinase in the cell lines from the haplogroup J and Uk when compared to cells from the haplogroup H (Gómez-Durán et al., 2010, 2012).

In addition, a recent study has shown that the variant defining haplogroup J m.13708G > A: *MT-ND5* is 2 bases upstream of a methylation RNA site at the gene *MT-ND5* (13710) which severely reduces the capacity of *MT-ND5* to be methylated and its predicted to have phenotypical implications (Safra et al., 2017).

FUTURE PERSPECTIVES

In summary, the large number of studies on mtDNA population variants over the last 25 years have clearly shown that mtDNA polymorphisms defining the mitochondrial haplogroups are certainly not phenotypically neutral as previously assumed. Extensive work has shown their role in evolution, disease, bioenergetics and cell signaling. However, understanding how the same variant could be advantageous or detrimental in different contexts, and their effect in mtDNA-nDNA communication needs to be further understood. An important caveat is that many of the published studies could be false positive results, in part related to multiple significance testing. To date, there has been limited or no attempt to independently replicate the original findings. Confirmatory replication has been possible in some instances, most notably Parkinson's disease (Hudson et al., 2013; Marom et al., 2017), but there is a need for large well-powered discovery and replication studies to validate many of the findings discussed above, accounting for multiple significance testing. Another challenge is the phylogenetically related nature of the mtDNA which makes difficult to determine how the dependence between the variants influences the associations. The recent increase of available data in NGS for whole genome sequence studies (including mtDNA) in combination with accessible public data from other "omic" technologies such as proteomics, metabolomics and transcriptomics will definitely make a difference helping understanding which ("goldies") variants are responsible for the associations. Given the complexity of these pathologies, a combination of efforts between multidisciplinary teams combining genomics studies on patient samples in combination with wet lab studies on cell biology and biochemistry will pave the way to elucidate the mechanisms of their role in cellular metabolism and dysfunction. This will lead to enhanced understanding of mtDNA heritability in neurodegenerative diseases and open new avenues for effective treatments.

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REVIEW
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Potential Role of Mic60/Mitofilin in Parkinson's Disease

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There are currently no treatments that hinder or halt the inexorable progression of Parkinson's disease (PD). While the etiology of PD remains elusive, evidence suggests that early dysfunction of mitochondrial respiration and homeostasis play a major role in PD pathogenesis. The mitochondrial structural protein Mic60, also known as mitofilin, is critical for maintaining mitochondrial architecture and function. Loss of Mic60 is associated with detrimental effects on mitochondrial homeostasis. Growing evidence now implicates Mic60 in the pathogenesis of PD. In this review, we discuss the data supporting a role of Mic60 and mitochondrial dysfunction in PD. We will also consider the potential of Mic60 as a therapeutic target for treating neurological disorders.

Keywords: Mic60/mitofilin, mitochondria, Parkinson's disease, neurodegeneration, mitochondrial dynamics

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INTRODUCTION

Parkinson's disease (PD), the most common neurodegenerative movement disorder, was first described in 1817 by James Parkinson in "An Essay on the Shaking Palsy." (Parkinson, 1817) In the 200 years that have passed since recognition of this neurological disorder, great strides have been made to characterize disease pathology, distinguish clinical symptoms, and develop a therapeutic treatment. However, the causes of PD neurodegeneration are still unknown, *and there is no cure nor are there any available neuroprotective therapies to hinder disease progression*. Identifying and understanding the etiology of PD progression is key to the development of new therapeutics for disease treatment.

Epidemiological studies and laboratory research have long sought to find potential causes of this prevalent disease (Khandhar and Marks, 2007; Delamarre and Meissner, 2017). Though the underlying mechanism of PD pathogenesis remains elusive, current ideology suggests that a combination of environmental exposure and genetic predisposition are responsible for most cases of PD (Horowitz and Greenamyre, 2010; Ritz et al., 2016). Genetic and epidemiological studies have identified multiple biological pathways that promote PD pathogenesis, many of which converge on the function of the mitochondria, the "powerhouses" of the cell (Schapira, 2008; Cieri et al., 2017; Ammal Kaidery and Thomas, 2018; Zanon et al., 2018).

Mitochondrial dysfunction is a known contributor to PD pathophysiology, with impaired mitochondrial respiration, morphology, and fission/fusion/transport dynamics all associated with PD (Van Laar and Berman, 2013; Bose and Beal, 2016). The connection between PD and mitochondria is reinforced by heritable forms of the disease, wherein monogenetic PD-causing

mutations in nuclear-expressed proteins such as PINK1, Parkin, LRRK2, and alpha-synuclein have all been shown to affect mitochondrial function (Narendra et al., 2010; Sanders et al., 2014; Di Maio et al., 2016; Verma et al., 2017). In recent years, independent studies from multiple labs have associated the inner mitochondrial membrane protein Mic60, also known as mitofilin, with PD pathogenesis (Van Laar et al., 2008, 2009, 2016; Akabane et al., 2016; Tsai et al., 2018). Mic60 is a core component of the mitochondrial contact site and cristae junction organizing system (MICOS) (Zerbes et al., 2012b; Pfanner et al., 2014; Kozjak-Pavlovic, 2017). The MICOS is a large, multi-protein complex of the mitochondrial inner membrane that maintains cristae structure, forms inner-outer mitochondrial membrane contact sites, organizes respiratory complexes, and regulates protein import (Bohnert et al., 2012; Zerbes et al., 2012a; Harner et al., 2014; Pfanner et al., 2014; Friedman et al., 2015; Horvath et al., 2015; Kozjak-Pavlovic, 2017; **Figure 1A**). Growing evidence places the MICOS complex, and in particular the protein Mic60, in a central role in regulating PD-relevant mitochondrial processes. Here, we will review the role of Mic60 in mitochondrial function and will review evidence for a role for Mic60 in PD neurodegeneration and as a potential therapeutic target in PD.

MITOCHONDRIAL DYSFUNCTION, MITOCHONDRIAL DYNAMICS, AND OXIDATIVE STRESS IN PD

PD is a progressive neurodegenerative disorder. A pathological hallmark of PD is a loss of the dopamine (DA) neurons of the nigrostriatal pathway, though other populations throughout the midbrain, basal ganglia, and cortex degenerate as well (Braak et al., 2003, 2004). Despite years of study, the cause of this neurodegeneration is still unknown, but growing evidence implicates mitochondrial respiratory dysfunction, oxidative stress, and dysregulation of mitochondrial dynamics in the neuropathogenesis of PD, as has been thoroughly reviewed elsewhere (Murphy et al., 1999; Toescu et al., 2000; Friberg and Wieloch, 2002; Beal, 2007; Schapira, 2008; Van Laar and Berman, 2009; Exner et al., 2012; Cieri et al., 2017; Ammal Kaidery and Thomas, 2018). To briefly summarize, many studies have identified protein oxidation, DNA damage, phospholipid oxidation, and decreased function of mitochondrial respiration in brain tissue from PD patients (Dexter et al., 1989; Schapira et al., 1990; Alam et al., 1997a,b; Good et al., 1998; Sanders and Greenamyre, 2013). A low-grade deficiency in mitochondrial electron transport chain (ETC) Complex I (NADH dehydrogenase) activity has been found not only in PD brain, but also other non-neuronal tissues throughout the body (Parker et al., 1989, 2008; Schapira et al., 1989; Krige et al., 1992; Yoshino et al., 1992; Barroso et al., 1993; Mann et al., 1994; Taylor et al., 1994; Haas et al., 1995; Penn et al., 1995; Blandini et al., 1998; Keeney et al., 2006), suggesting a systemic mitochondrial respiration deficit associated with PD. The discovery of the DA neuron-specific toxicant N-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) in the 1980's further implicated mitochondria in PD (Davis et al., 1979; Langston and Ballard, 1983; Langston et al., 1983). MPTP toxicity is elicited through its metabolite MPP+, which is selectively imported into DA neurons and acts as a Complex I inhibitor (Nicklas et al., 1985; Ramsay et al., 1986; Mizuno et al., 1987). MPP+ results in increased production of ROS (Rossetti et al., 1988; Adams et al., 1993; Smith and Bennett, 1997) and a loss of nigrostriatal DA neurons (Langston et al., 1999), suggesting that environmental toxins affecting mitochondrial function could also contribute to PD. These discoveries ultimately led to the seminal finding that systemic administration of low doses of rotenone, also a Complex I inhibitor, leads to PD-specific neurodegeneration and pathology, despite rotenone freely crossing all cell membranes (Betarbet et al., 2000; Sherer et al., 2003; Cannon et al., 2009).

Mitochondrial Complex I is known to be the major site for production of reactive oxygen species (ROS). Complex I dysfunction or inhibition results in increased ROS production (Lenaz, 2001; Votyakova and Reynolds, 2001), implicating mitochondrially produced oxidative stress in PD pathogenesis. A role for ROS in pathogenesis is bolstered by the fact that many of the most susceptible neurons in PD contain dopamine (DA), which produces ROS through its metabolism and through oxidation to reactive quinones (Bindoli et al., 1992; Monks et al., 1992; Hastings et al., 1996; Hastings and Berman, 2000). DA quinones bind to sulfhydryl groups on free cysteine, glutathione, and protein cysteinyl residues in the cell, and DA oxidation products have been shown to alter mitochondrial respiration and permeability transition pore opening (Hastings et al., 1996; Berman and Hastings, 1999; Gluck et al., 2002; Gluck and Zeevalk, 2004).

In addition to mitochondrial respiratory deficiencies and oxidative stress, PD pathogenesis is associated with defects in the dynamic properties of mitochondria that maintain mitochondrial homeostasis (mitochondrial fission, fusion, transport, biogenesis, and degradation) and are necessary for maintaining bioenergetic function (for review, see (Chen and Chan, 2009; Van Laar and Berman, 2009; McCoy and Cookson, 2012; Van Laar and Berman, 2013; Bose and Beal, 2016). This evidence arises not only from studies of *in vitro* toxicant models of PD, but also from PD patient-derived cell lines, and actions of familial PD-causing gene products (Exner et al., 2007; Poole et al., 2008; Arnold et al., 2011; Bose and Beal, 2016). Perhaps the most well studied is the shared pathway of two PD-associated proteins PINK1 and Parkin, in which evidence suggests that Parkin works downstream of PINK1 to signal damaged mitochondria for autophagic degradation (Narendra et al., 2010; Pickrell et al., 2015).

The evidence suggests that regulation of mitochondrial respiratory, morphologic, and maintenance functions plays a critical role in PD pathogenesis. Proteins that integrate these various and interrelated mitochondrial structural and homeostatic functions are therefore uniquely positioned to play an important role in PD-relevant mitochondrial dysfunction. As we will detail below, Mic60 is emerging as central to these integrated mitochondrial functions and, importantly, in PD pathogenesis. Mic60 is integral in the maintenance of both structural dynamics and respiratory function of mitochondria and interacts with PD gene products. These functions place

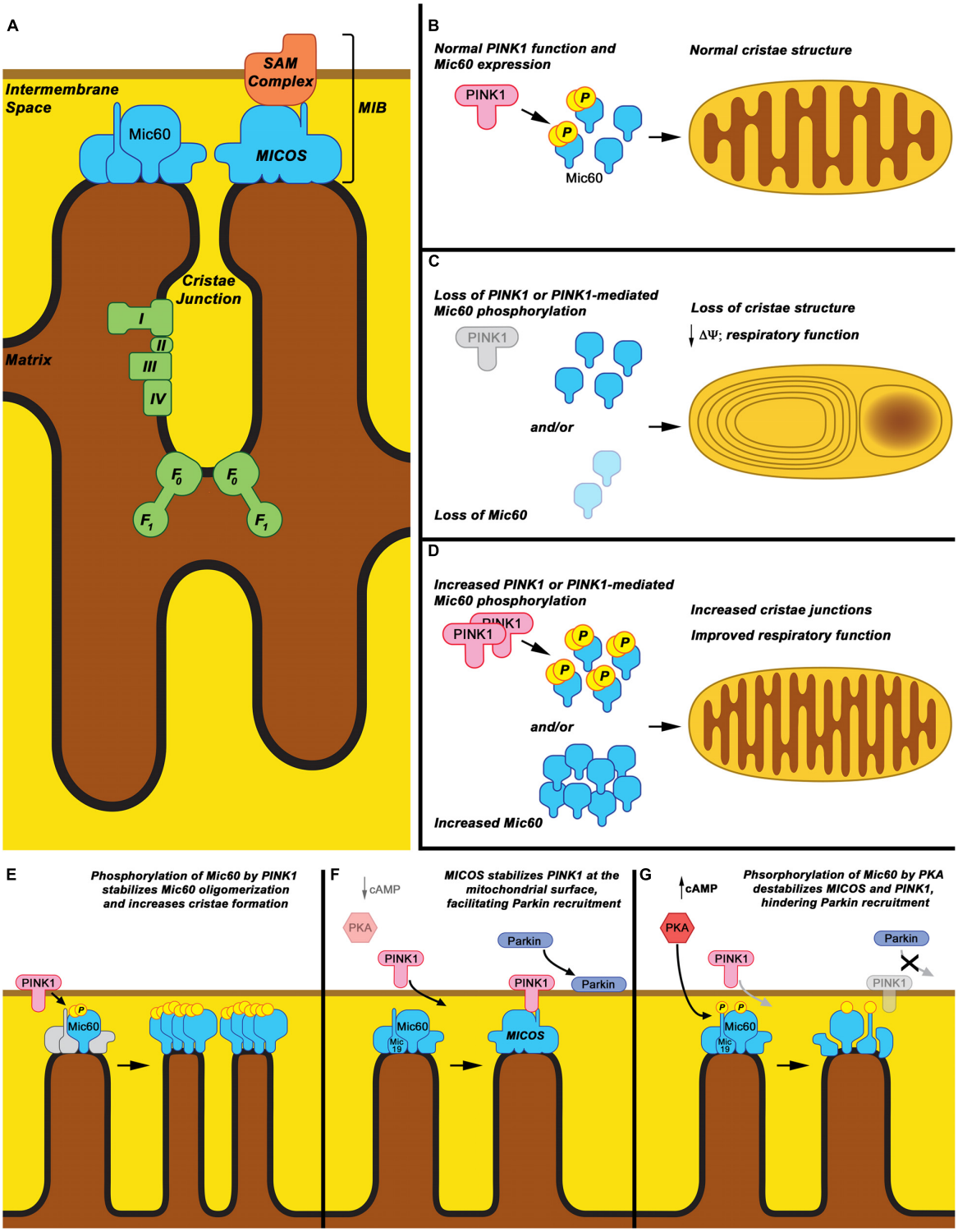


FIGURE 1 | Mic60: Mitochondrial cristae structure maintenance and proposed phospho-regulation. **(A)** Mic60 is an inner mitochondrial membrane protein and key component of the MICOS and MIB protein complexes, which bridge inner membrane cristae junctions and inner-outer membrane contact sites, respectively. Maintenance of cristae structure by MICOS also regulates function of the mitochondrial Electron Transport Chain Complexes (I, II, III, and IV) and ATP synthase (F_0-F_1). **(B)** It is proposed that under normal conditions, Mic60 interacts with and is phosphorylated by PINK1 at a basal rate, thus regulating its ability to maintain normal cristae structure. **(C)** Under conditions in which PINK1 expression is decreased, PINK1 kinase function is inactivated, or Mic60 expression is reduced, the mitochondrial cristae structure is not maintained. This leads to mitochondria exhibiting characteristic onion ring-like whorls of the inner membrane or formation of

(Continued)

FIGURE 1 | Continued
large vacuoles within the mitochondrion. This is accompanied by decreased membrane potential ($\Delta\psi$) and respiratory function. **(D)** Under conditions in which PINK1 phosphorylation of Mic60 is increased or Mic60 expression is increased, mitochondria can exhibit increased numbers of inner membrane cristae structures and cristae junctions. This is accompanied by highly coupled respiratory function. **(E)** One proposed mechanism of PINK1's effects on Mic60 is that phosphorylation increases the ability of Mic60 to oligomerize with itself and, presumably, the MICOS complex, leading to increased inner membrane structural integrity. **(F)** In a cellular state in which cAMP levels and PKA activation are low, PINK1 can also interact with Mic60 and be stabilized on the surface of mitochondria, aiding in its function to recruit Parkin to damaged mitochondria. **(G)** However, in apparent opposition to the PINK1-Mic60 interaction, PKA activation and phosphorylation of Mic60 destabilizes the MICOS complex and decreases the ability of PINK1 to stabilize on the mitochondrial surface, preventing the recruitment of Parkin to damaged mitochondria.

Mic60 in a unique position to regulate mitochondrial response to stress, particularly in mitochondria-dependent neurons, and increasing evidence, as detailed below, links Mic60 to PD pathogenesis.

Mic60, A PROTEIN AT THE INTERSECTION OF REGULATION OF MITOCHONDRIAL FUNCTION AND STRUCTURE

Mic60 was first identified as "HMP," heart muscle protein, due to its abundance in cardiac tissue (Icho et al., 1994). Later renamed "mitofilin" based on its structure and localization, subsequent studies demonstrated that human Mic60 is a nuclear-expressed mitochondrial protein that is targeted selectively to the inner mitochondrial membrane (Odgren et al., 1996; Gieffers et al., 1997). Human Mic60, which exists in both 88 kDa and 90 kDa isoforms, contains a cleavable mitochondrial targeting sequence, a transmembrane domain near the N-terminus that spans the inner mitochondrial membrane with the bulk of the protein jutting into the intermembrane space (Gieffers et al., 1997), and three coiled-coil domains characteristic of involvement in protein-protein interactions (Odgren et al., 1996; John et al., 2005).

John et al. (2005) first described Mic60/mitofilin as a critical protein for maintaining mitochondrial cristae structure and mitochondrial respiration. Perhaps the most remarkable characteristic that was noted in association with Mic60 was that loss of the protein resulted in the reorganization of the mitochondrial cristae structure. Mitochondria in Mic60/mitofilin-deficient cells exhibited concentric ring-like structures or whorls in place of the normal inner membrane cristae structure (John et al., 2005), an effect since noted by others in various cell and animal models with aberrant Mic60 expression (Rabl et al., 2009; Mun et al., 2010; von der Malsburg et al., 2011; Tsai et al., 2017; Tsai et al., 2018). John et al. also found that Mic60/mitofilin not only formed a homo-oligomeric structure with itself but also was present in a large multimeric protein complex (John et al., 2005). Shortly thereafter, Xie et al. demonstrated that Mic60/mitofilin associated with a protein complex including Sam50, coiled-coil-helix coiled-coil-helix domain-containing (CHCHD) proteins 3 and 6, and metaxins 1 and 2, proteins known to be involved in mitochondrial protein import and assembly (Xie et al., 2007), thus linking Mic60 to both structural and protein maintenance of the mitochondrion.

Subsequent studies confirmed that Mic60/mitofilin is indeed a core component of a larger functional multi-protein complex of the inner membrane, now known as the MICOS complex (Pfanner et al., 2014; Kozjak-Pavlovic, 2017). As previously noted, the MICOS complex is responsible for structural organization of the mitochondria. MICOS subcomplexes interact with mitochondrial membrane lipids to form cristae junctions and organize respiratory complexes; and interact with outer-membrane transport machinery to regulate mitochondrial protein import and biogenesis (von der Malsburg et al., 2011; Bohnert et al., 2012; Zerbes et al., 2012a; Harner et al., 2014; Pfanner et al., 2014; Ding et al., 2015; Friedman et al., 2015; Horvath et al., 2015; Eydt et al., 2017; Hessenberger et al., 2017; Rampelt et al., 2017; Tarasenko et al., 2017). A uniform nomenclature was established for the MICOS complex and its subunits Mic10 through Mic60, the name given to mitofilin (Pfanner et al., 2014). In metazoa, the MICOS complex also interacts with the sorting and assembly machinery (SAM) protein import complex to form the larger mitochondrial intermembrane space bridging complex (MIB) at inner-outer membrane contact sites (Ott et al., 2012, 2015; Guarani et al., 2015; Huynen et al., 2016; Kozjak-Pavlovic, 2017). The organization and function of the MICOS and MIB complexes has been thoroughly reviewed elsewhere (Zerbes et al., 2012b; Pfanner et al., 2014; Kozjak-Pavlovic, 2017; Rampelt et al., 2017). We will therefore focus on Mic60 and its potential role in neurodegenerative disease and PD.

Mic60 is a key component of both the MICOS and MIB complexes, interacting either directly or indirectly with the other known components of these complexes (Xie et al., 2007; Harner et al., 2011; von der Malsburg et al., 2011; Ott et al., 2012), and is possibly the oldest evolutionarily conserved component of this structural system (Huynen et al., 2016). Loss of Mic60 leads to destabilization and even loss of MICOS and MIB components (Ott et al., 2015). Further, Mic60 analogs appear to be highly conserved and expressed in all cells containing mitochondria—including plant, yeast, and animal cells—as would be predicted for a protein critical for mitochondrial functions (Odgren et al., 1996; Gieffers et al., 1997; Munoz-Gomez et al., 2015a,b, 2017; Michaud et al., 2016; Wideman and Munoz-Gomez, 2016; Tarasenko et al., 2017).

Multiple studies have now shown that Mic60 is essential for maintaining mitochondrial structure and respiration (John et al., 2005; Rabl et al., 2009; Mun et al., 2010; von der Malsburg et al., 2011; Bohnert et al., 2012; Yang et al., 2012, 2015; Ott et al., 2015; Li et al., 2016; Van Laar et al., 2016; Tsai et al., 2017, 2018). Mic60 loss detrimentally affects cellular viability,

especially in response to stress. Viability may be affected by the rearrangement of mitochondrial cristae, impaired mitochondrial respiration, impaired homeostasis, impaired fission and fusion, and disrupted protein import associated with Mic60 deficiency (John et al., 2005; Rabl et al., 2009; von der Malsburg et al., 2011; Bohnert et al., 2012; Zerbes et al., 2012a; Yang et al., 2015; Li et al., 2016; Van Laar et al., 2016). Many of these effects appear to be associated with the reorganization of the mitochondrial membrane structures and protein complexes (Friedman et al., 2015; Eydt et al., 2017; Kozjak-Pavlovic, 2017). In addition to respiratory deficiency and dynamics dysfunction, loss of Mic60 is also linked with mitochondrially associated apoptosis. Yang et al. (2012) demonstrated that reduction of Mic60 expression in HeLa cells resulted in a remodeling of mitochondrial cristae, correlating with increased release of cytochrome c and decreased cell viability in response to apoptosis inducers (Yang et al., 2012). Mic60 knockdown has also been shown to trigger increased calpain activity and apoptosis-inducing factor (AIF) – poly(ADP-ribose) polymerase (PARP) dependent apoptosis in H9c2 myoblasts and HEK 293 cells (Madungwe et al., 2018). Of note, Rossi et al. (2009) found that mitochondrial localization of PARP-1, which they found also regulates mitochondrial DNA (mtDNA) integrity, is dependent on an interaction with Mic60. Multiple studies have now found that suppressed Mic60 affects mtDNA stability, leading to aberrant nucleoid formation, accumulated mtDNA damage, and attenuated mtDNA transcription (Rossi et al., 2009; Yang et al., 2015; Li et al., 2016), and potentially further impairing mitochondrial function. These functions of Mic60 become particularly relevant to PD, where Complex I dysfunction, ROS production, lipid membrane integrity, and hindered mitochondrial quality control are major drivers of pathogenesis. Thus, the stability of Mic60 becomes a key issue in maintaining mitochondrial and cellular health, particularly in cells such as neurons that highly utilize their mitochondria.

Mic60 IS A TARGET FOR ALTERED EXPRESSION AND OXIDATIVE MODIFICATION DURING CELLULAR STRESS

Mic60 abundance is highly susceptible to oxidative stress (Magi et al., 2004; Van Laar et al., 2008), which is of particular relevance given that the mitochondrial environment produces high levels of ROS. Exposure to ROS-generating photodynamic therapy, a cancer-treatment method, demonstrated a marked decrease in Mic60 protein levels in cultured HL60 and MCF-7 cells (Magi et al., 2004; Kratassiouk et al., 2006). HL60 cells exposed to the apoptosis-inducing compound homoharringtonine (HTT) showed an initial decrease in Mic60 mRNA expression, followed by a rapid increase (6-fold) in mRNA expression within 6 hrs of treatment, one of only a few genes detected to behave in this manner (Jin et al., 2004). Such a response may suggest that the cells are attempting to recover following a toxic insult. Along this line, Navet et al. (2007) found that expression of Mic60 is significantly increased, along with altered expression of other mitochondrial proteins, in rat brown adipocyte cells during acclimation to colder temperatures, which requires high-energy usage. Of relevance to PD pathogenesis, we demonstrated that Mic60 abundance was significantly decreased in isolated rat brain mitochondria following exposure to DA quinone, as well as in mitochondria isolated from PC12 cells exposed to exogenous DA (Van Laar et al., 2008).

In addition to regulation of Mic60 expression and abundance, the protein itself is also highly susceptible to oxidative modification under stress. Suh et al. (2004) found that exposure of human hepatoma cells to alcohol led to oxidation of Mic60 cysteine residues (Suh et al., 2004). Taylor et al. (2003) examined normal human cardiac tissue mitochondria for oxidative modification of tryptophan residues and found oxidation of selective Mic60 tryptophan residues, suggesting "hot spots" of oxidative susceptibility (Taylor et al., 2003). Mic60 was also found to be carbonylated in kainic acid excitotoxicity-induced neuronal injury in hippocampal cells (Furukawa et al., 2011). Recently, a study found that Mic60 in the brains of aged rats exhibited an age-related increase in oxidative sulfonation of cysteines, with implications for declining neuronal mitochondrial function with age (Yang X. et al., 2018). As discussed in greater detail below, we demonstrated that DA quinone covalently modifies Mic60 in isolated rat brain mitochondria (Van Laar et al., 2009). These studies demonstrate that Mic60 protein and protein levels are highly susceptible to oxidative stress, including PD-relevant oxidative stress. While the consequences of Mic60 protein loss are well documented, the functional consequences of Mic60 oxidative modifications are not known. Modifications that interrupt critical protein-protein interactions could significantly impair Mic60 and MICOS function.

Mic60 has also been found to exhibit other post-translational modifications that potentially regulate its function. In a rat model of traumatic brain injury, Mic60 was found to undergo poly ADP-ribosylation, though the significance of this modification is undetermined (Lai et al., 2008). Studies have now shown that Mic60 function is regulated under cellular and mitochondrial stress via direct phosphorylation by protein kinase A (PKA) and PD-associated mitochondrial kinase PINK1, altering interaction between Mic60 and other proteins and its inner-membrane structural shaping function (Akabane et al., 2016; Tsai et al., 2018). The specific effects of this phospho-regulation and their relevance to PD are further discussed below.

A ROLE FOR Mic60 IN MITOCHONDRIAL DYNAMICS AND IMPLICATIONS FOR NEURODEGENERATION

In addition to respiratory regulation and apoptosis signaling, Mic60 also appears to be a key player in regulating the mitochondrial dynamics of fission, fusion, transport, degradation, and biogenesis (Weihofen et al., 2009; Ding et al., 2015; Li et al., 2016; Van Laar et al., 2016; Akabane et al., 2016; Cho et al., 2017; Tsai et al., 2017, 2018). Balance of these dynamic properties is critical for mitochondrial health and cellular viability, particularly in mitochondria-dependent

neurons (Van Laar and Berman, 2013). We were the first to show a functional relationship between mitochondrial fission-fusion dynamics and Mic60 abundance in neurons, demonstrating that increased Mic60 suppressed mitochondrial fission in neurites, leading to longer neuritic mitochondria (Van Laar et al., 2016). Loss of Mic60 in mammalian cell lines was associated with decreased levels of multiple fission and fusion proteins, and corresponding lower fission and fusion rates (Ding et al., 2015; Li et al., 2016). Mic60 loss also impaired mtDNA nucleoid formation and mtDNA transcription (Li et al., 2016), key steps in mitochondrial division and biogenesis. Recent evidence shows that Mic60 also regulates transport. Mic60 associates with a complex containing Miro, a mitochondrial outer membrane protein that regulates kinesin-based mitochondrial anterograde axonal transport (Wang and Schwarz, 2009; Weihofen et al., 2009). Recently, Tsai et al. (2017) demonstrated that Mic60 loss in *Drosophila* was associated with a loss of Miro, leading to an arrest of neuronal mitochondrial movement. This was also associated with functional and structural disruption of neuromuscular junction synapses, suggesting that Mic60 loss has a detrimental impact on axons and axonal mitochondrial health (Tsai et al., 2017).

Mic60 also interacts with proteins involved directly in the general regulation of mitochondrial dynamic processes, as well as ones linked to neurodegenerative diseases. This places it in a unique position to regulate the response to PD-relevant stress. Mic60 interacts with the optic atrophy-linked protein OPA1 (Darshi et al., 2011; Banerjee and Chinthapalli, 2014; Barrera et al., 2016; Glytsou et al., 2016; Hering et al., 2017). OPA1 regulates fusion of the inner mitochondrial membrane between two mitochondria and has been implicated in cristae remodeling (Frezza et al., 2006). Evidence suggests that the relationship between Mic60 / MICOS complex and OPA1 is key in regulating mitochondrial fusion (Cho et al., 2017). However, there are conflicting results as to whether OPA1 plays an integral role in the function of Mic60 and the MICOS complex to organize cristae junctions (Barrera et al., 2016; Glytsou et al., 2016). Mic60 has also been associated with PINK1, a protein involved in regulating mitochondrial homeostasis and linked to a familial form of PD (Weihofen et al., 2009; Akabane et al., 2016; Tsai et al., 2018). This association is discussed in further detail below. The effects of Mic60 specifically on neuronal mitochondrial dynamics, along with the interactions of Mic60 with regulators of mitochondrial dynamics, support that Mic60 may play an important role in maintenance of neuronal health, and potentially in neurodegenerative pathogenesis.

EVIDENCE ASSOCIATES Mic60 WITH PD PATHOGENESIS

With such an important role in mitochondrial function, Mic60 is likely to be a key player in the health of post-mitotic mitochondria-dependent neurons, especially in times of stress. Indeed, Mic60 has previously been linked to neurological disorders, including fetal Down syndrome (Bernert et al., 2002; Myung et al., 2003), seizure (Omori et al., 2002; Furukawa et al., 2011), schizophrenia (Millar et al., 2005; Park et al., 2010; Atkin et al., 2011), Amyotrophic Lateral Sclerosis (ALS) (Fukada et al., 2004), optic atrophy (Abrams et al., 2015; Abrams et al., 2018), and neurodegeneration in animal models (Wang et al., 2008). While the evidence for Mic60 and these neurological disorders may represent a general effect of aberrant Mic60 expression, protein modification, or protein-protein interactions on neuronal health, little evidence has directly implicated Mic60 itself as a major causative factor in these diseases. However, emerging evidence from multiple studies has begun to demonstrate a strong association between Mic60 and the pathogenic processes in PD.

Mic60 as a Target of Covalent Modification by DA Quinone, and Loss in DA and MPTP Toxicity

Studies have demonstrated that Mic60 protein abundance is affected by PD-relevant toxicants *in vitro*. We identified Mic60 in a proteomic screen for mitochondrial proteins sensitive to oxidative stress in the DA oxidation model of PD. Following exposure of isolated rat brain mitochondria to DA quinone, Mic60 was found to be covalently modified by DAQ and its abundance was decreased by more than half, amongst the most decreased of all proteins identified in our study (Van Laar et al., 2008, 2009). Similarly, in we found that Mic60 abundance was decreased and the protein covalently modified by DA in neuronally differentiated dopaminergic cells exposed to exogenous DA (Van Laar et al., 2008, 2009). Consistent with our findings, Burte et al. (2011), found that exposing neuronally differentiated dopaminergic mouse N2a cells to MPTP also lead to decreased levels of Mic60 expression (Burte et al., 2011).

While the effects of loss of Mic60 on mitochondrial function have been well demonstrated, the specific effects of covalent DA modifications to Mic60 on cellular health are not clear. Notably, we observed larger molecular weight bands immunopositive for Mic60 in Western analysis of DA-treated cells, suggesting DA oxidation-induced SDS-insoluble interaction of Mic60 proteins. As cysteines are typically utilized in protein-protein interactions, it is likely that such modifications disrupt the ability of Mic60 to properly interact with and regulate the MICOS complex.

Mic60 Loss Exacerbates Vulnerability to PD Toxicants, and Overexpression Protects Against Models of PD

We recently demonstrated that a modest loss of Mic60 (−30%) did not affect the basal cellular viability of dopaminergic neuronal cells, but significantly exacerbated cellular vulnerability to the PD-relevant toxicant exogenous DA. (Van Laar et al., 2016). This suggests even a slight loss can greatly impact cellular response to mitochondrial stress. Conversely, Mic60 overexpression in dopaminergic cells *in vitro* increased mitochondrial respiratory capacity and promoted cellular survival in response to both toxicants rotenone and exogenous DA (Van Laar et al., 2016). This was the first demonstration that Mic60 loss increased the vulnerability of dopaminergic neuronal cells and the first demonstration that increased Mic60 expression in dopaminergic

cells was protective in a toxicant model of PD. In a recent study, Mic60 also appeared to be protective in a genetic model of PD (Tsai et al., 2018).

As noted previously, perhaps the most dramatic characteristic associated with Mic60 loss is the severe reorganization of the mitochondrial cristae structure, resulting in concentric ring-like "onion" structures, or whorls (John et al., 2005; von der Malsburg et al., 2011), and these have recently been seen *in vivo* in Mic60 mutant flies (Tsai et al., 2017, 2018). Similar effects of mitochondrial structural dysregulation have been noted in other models of PD. PINK1 and Parkin knockout flies exhibit mitochondria with abnormal morphology and disorganized internal structures, including whorls (Park et al., 2006; Deng et al., 2008; Poole et al., 2008; Tsai et al., 2018).

Excitingly, a recent study demonstrated that Mic60 overexpression was protective in a genetic PD model, the PINK1 knockout model in flies (Tsai et al., 2018). Mic60 overexpression rescued the mitochondrial cristae disorganization that is exhibited in this PD model, in addition to protecting against multiple other parkinsonian phenotypes, including mitochondrial complex 1 activity deficits, ATP levels, DA neuron degeneration, and behavioral defects. In fact, Mic60 overexpression rescued the PINK1 PD phenotype to a greater extent than overexpression of Parkin, another familial PD-associated protein that functions downstream of PINK1 (Tsai et al., 2018). As Mic60 is a known interactor of PINK1 (Weihofen et al., 2009; Akabane et al., 2016; Tsai et al., 2018), this finding strengthens the relationship between multiple genetic forms of PD and a shared, convergent pathway in regulating mitochondrial function.

In human studies, Tsai et al. identified rare mutations in the mitochondrial targeting sequence of Mic60, some of which were associated with PD patients (Tsai et al., 2018). The mutations were shown to impair the mitochondrial targeting of Mic60 and resulted in disrupted mitochondrial structure when expressed in *Drosophila* (Tsai et al., 2018). These studies suggest a possible genetic link between Mic60 deficiency and PD risk.

Mic60 Interacts With PINK1 and Is Regulated via Phosphorylation by PINK1 and PKA: Implications for PD Pathogenesis

Previous studies have found Mic60 interacts with PINK1, a protein whose recessive mutations cause familial PD (Weihofen et al., 2009; Akabane et al., 2016; Tsai et al., 2018). New evidence suggests that the Mic60 interaction is regulated via phosphorylation of Mic60, affecting its interaction with PINK1 and MICOS proteins. Both protein kinase A (PKA) and PINK1 itself have now been demonstrated to phosphorylate Mic60 directly and influence function (Akabane et al., 2016; Tsai et al., 2018), which carries implications for Mic60 having a key role in PD pathogenesis.

Protein kinase A is a tetrameric holoenzyme (Corbin et al., 1978), and is sub-cellularly targeted, where binding of cyclic-AMP (cAMP), a major activator of PKA, releases catalytic subunits to act in several downstream pathways (Herberg et al., 2000; Paulucci-Holthauzen et al., 2009; Christian et al., 2011), some of which are directly relevant to PD pathophysiology. Mitochondrially localized PKA (PKAmt) has been shown to phosphorylate several targets involved in mitochondrial homeostasis and function, regulating their function, including subunits of Complex I (Papa et al., 2001; Valsecchi et al., 2013), the pro-apoptotic protein BAD (Martin et al., 2005), and the mitochondrial fission protein DRP1 (Chang and Blackstone, 2007), and promote mitochondrial function under stress and even blunting mitophagy (Dagda et al., 2011). But recently, direct links to PD neurodegeneration were suggested by finding that PKAmt affects the stability of the PINK1-Parkin complex at the mitochondria via its phosphorylation of MICOS proteins Mic60 and Mic19, thus potentially regulating Parkin-mediated mitophagy of damaged mitochondria. Ackbane et al. found that the phosphorylation status of Mic60 regulates the stability of PINK1 upstream of the PINK1-Parkin mitophagy pathway (Akabane et al., 2016). Specifically, PKA-mediated phosphorylation of Mic60 at serine 528 (S*528) negatively affects MICOS complex assembly and inhibits Mic60 interaction with PINK1. This prevents the stabilization of PINK1 on the surface of damaged mitochondria, thereby inhibiting Parkin recruitment (Akabane et al., 2016). They also found that Mic19, another important MICOS component, was similarly regulated by PKA phosphorylation. These results reinforce the role of PKA signaling in regulating mitochondrial function and homeostasis, clearly defining it as an essential component and regulator of cellular metabolism.

Evidence suggests that in addition to PKA, PINK1 itself may directly phosphorylate Mic60 and thus regulate function of the MICOS complex. PINK1 is a nuclear-expressed mitochondrially targeted kinase first identified as an autosomal recessive form of juvenile-onset PD (Valente et al., 2004). Individuals possessing homozygous or compound-heterozygous mutations for PINK1 exhibit mood and cognitive dysfunction similar to sporadic and PDD/LBD (Gandhi et al., 2006; Steinlechner et al., 2007; Feligioni et al., 2016) and are at increased risk for early onset Parkinson's disease. Under basal conditions, PINK1 is imported into the mitochondria and processed by matrix processing peptidase (MPP) and presenilin-associated rhomboid-like (PARL) (Jin et al., 2010; Deas et al., 2011; Greene et al., 2012), then released post-processing into the cytosol for further downstream signaling (Dagda et al., 2014; Steer et al., 2015) and degradation (Yamano and Youle, 2013). *In vivo* and *in vitro* studies have shown PINK1 is neuroprotective (Haque et al., 2008; Dagda et al., 2014; Khalil et al., 2015; Steer et al., 2015). Until 2007 PINK1 had only been associated with mitochondrial oxidative stress and dysfunction (Exner et al., 2007; Gautier et al., 2008; Weihofen et al., 2008). Then in 2008, Poole et al. performed a series of experiments in *Drosophila* that identified PINK1 and Parkin as major players in mitochondrial fission, fusion, and morphology (Poole et al., 2008). Shortly thereafter in 2009, Weihofen et al. (2009) demonstrated that PINK1 interacts with MIRO and Milton placing PINK1 in a position to regulate mitochondrial trafficking. In this study, Mic60 was also found to associate in a complex with PINK1. This seminal work was the first to demonstrate that Mic60 may play a role in the observed

changes in PINK1-deficient cells. However, how PINK1 and Mic60 interact would remain elusive for nearly 10 years.

In 2010, multiple groups established that PINK1 was stabilized on the mitochondrial outer membrane (OMM) following a collapse of the mitochondrial transmembrane potential, leading to accumulation of PINK1 on the OMM (Matsuda et al., 2010; Narendra et al., 2010; Vives-Bauza et al., 2010). Once PINK1 aggregates on the OMM, it interacts with and phosphorylates both Parkin and Ubiquitin to initiate mitophagy (Kondapalli et al., 2012; Kane et al., 2014; Lazarou et al., 2015). PINK1 has since been extensively studied for its role as a sensor of mitochondrial damage and in inducing mitophagy (Nguyen et al., 2016). Although the PINK1-Parkin mitophagy pathway can be activated under stress conditions *in vivo* (Pickrell et al., 2015), more data are emerging that PINK1 is in some cases dispensable for mitophagy and that PINK1 has other distinct and uncharacterized functions (Lee et al., 2018; McLelland et al., 2018; Yang W. et al., 2018).

In this regard, recent work has shown that Mic60 is a substrate of PINK1, providing evidence that PINK1 may directly alter mitochondrial architecture (Tsai et al., 2018). Tsai et al. showed that PINK1 is necessary for mitochondria to maintain cristae junctions in *Drosophila*, and that this function is mediated by PINK1 directly phosphorylating Mic60. Phosphorylation by PINK1 stabilized the oligomerization of Mic60 and increased cristae junctions (Tsai et al., 2018). Further, Mic60 overexpression could rescue multiple phenotypes of PD model PINK1 knockout flies, as mentioned previously, demonstrating that PINK1 modulates the ability of Mic60 to regulate cristae structure and mitochondrial function (Tsai et al., 2018). This regulatory interaction was found to be preserved in human cells, as well, and may be influenced by increased energy demands depending on cell type and/or the location of the mitochondrion within the cell (Tsai et al., 2018).

This phospho-regulation of Mic60 may provide insight into the cellular control of mitochondrial function under various bioenergetic and stress conditions. The evidence suggests that while PKA phosphorylation appears to disrupt the function of Mic60 to interact with and stabilize PINK1 on the OMM, phosphorylation by PINK1 increases Mic60 stability within the MICOS complex, allowing for increased mitochondrial function (**Figures 1B–G**). This suggests a dual regulation based on the stress status of the cell and on which stress pathways are activated. Interestingly, overexpression of PKA has been observed to rescue PINK1 deficiency, so it is possible that PKA is in part regulating Mic60 in a manner similar and parallel to PINK1 (Dagda et al., 2011; Kostic et al., 2015) The significance of these pathways to PD pathology, or their relevance to one another, remains to be elucidated.

A question regarding these findings is how these systems work in balance to regulate mitochondrial structure and degradation. In the studies by Akabane et al. (2016), PKA phosphorylation of Mic60 affected PINK1-Parkin recruitment, but not mitochondrial cristae structure. This observation occurred despite the decrease in PINK1-Mic60 interaction and the disruption of the MICOS complex. This seems to be in opposition to the observations in *Drosophila* by Tsai et al. (2018), where disrupted PINK1-Mic60 interaction dramatically interrupted Mic60 oligomerization and cristae organization. However, Tsai et al. also found that the function of Mic60 in maintaining mitochondrial structure is, at least in part, independent of PINK1, as overexpressing Mic60 compensated for the loss of PINK1 on cristae organization in *PINK1-null* flies (Tsai et al., 2018). Thus, any possible structural regulation effects of Mic60 phosphorylation status at its PKA-phosphorylation sites may be influenced by the level of overexpression of the Mic60 protein in the studies by Akabane et al. More studies should be conducted to definitively address these discrepancies.

Another issue is whether these pathways are relevant to all tissues, or even all species. Tsai et al. noted differences in cristae structure depending on the cell type or subcellular localization of the mitochondria, suggesting the possibility of differential regulation of cristae structure proteins depending on local energy demands (Tsai et al., 2018). While Mic60 itself is shown to be a highly conserved crucial component for mitochondrial structure across species, its phospho-regulation may not be. While the PINK1 phosphorylation sites on Mic60 seem to be preserved across vertebrate and invertebrate species (Tsai et al., 2018), the PKA site appears absent in *Drosophila* and *C. elegans* (Akabane et al., 2016). On the other hand, the PKA site is conserved across all examined species in another MICOS complex protein, Mic19 (Akabane et al., 2016). This variation of phosphorylation-sites carries implications for the evolution of MICOS complex regulation across species and merits further investigation.

Mic60 AS A THERAPEUTIC TARGET FOR PD AND OTHER DISEASES

The known functions of Mic60 and the reported findings on altered Mic60 expression allow us to speculate on a potential role for this protein in neuropathogenesis. The mitochondrial cristae structures are known to undergo reorganization in times of increased energy demands, cellular stress, and apoptosis (Mannella et al., 2001, 2013; Scorrano et al., 2002; Mannella, 2006). It is likely that Mic60 is participating in this reorganization due to oxidation- or phosphorylation-induced alterations affecting its functions within the MICOS complex. Evidence now exists for this process to be regulated by direct phosphorylation. However, excess oxidative stress may either directly modify Mic60, which could alter its structure, affect its ability to be phosphorylated, or target it for degradation, further allowing for detrimental cristae destabilization and reorganization. As the MICOS complex also regulates mitochondrial protein import (Xie et al., 2007; von der Malsburg et al., 2011; Bohnert et al., 2012), a loss of Mic60, and thus mitochondrial protein biogenesis, may further hamper efforts of the mitochondrion to recover from excessive protein damage and loss, setting up a deadly cycle of ROS generation and oxidative protein and lipid damage, ultimately leading to mitochondrial collapse. In dopaminergic neurons, this effect could be amplified by DA oxidation and covalent modifications to Mic60, contributing to the selective vulnerability of these neurons in PD.

The crucial role of Mic60 in regulating so many aspects of mitochondrial homeostasis, combined with a susceptibility to oxidative modification and stress-induced loss of abundance, make it an attractive target for investigating therapeutic strategies for PD and other diseases. Our own evidence suggests that increased Mic60 availability in dopaminergic cells is protective against PD-relevant stressors *in vitro*, and Tsai et al. demonstrated that Mic60 overexpression can rescue PD phenotypes *in vivo* in PINK1-mutant *Drosophila* (Van Laar et al., 2016; Tsai et al., 2018). Given the importance of Mic60 and the reliance of neurons on mitochondrial health, a strategy targeting Mic60 may provide protection in multiple neurological disorders, including PD.

Given the central role of Mic60 in mitochondrial homeostasis and function, it is not surprising that Mic60 upregulation may protective in disorders other than neurologic disorders. Studies in patients and models have also linked Mic60 with obesity, diabetes, osteoporosis, and cardiac dysfunctions (Baseler et al., 2011; Guo et al., 2013; Gutierrez-Salmean et al., 2014; Gorr and Wold, 2015; Lindinger et al., 2015; Wang et al., 2017; Lv et al., 2018), and upregulation of Mic60 has shown to be protective in models of diabetes and osteogenesis (Thapa et al., 2015; Lv et al., 2018). Thus, there is likely a widespread effect of improving mitochondrial stability in general via Mic60 upregulation. That being said, the direct links between Mic60 and multiple specific PD gene products suggest the likelihood of a more specific role in PD neurodegeneration. Further study is needed to expound upon the protective findings of Mic60 overexpression and examine the role and function of increased Mic60 in cellular and neuronal health.

CONCLUSION

The crucial role played by Mic60 at the intersection of mitochondrial structure, function, and homeostasis make it an exciting target to explore for therapeutic intervention in diseases associated with mitochondrial dysfunction, such as PD. Multiple studies have now linked Mic60 deficiency with PD-relevant cellular stress and have clearly placed Mic60 as a player in the PD-associated PINK1-Parkin cellular pathway. Further research into the role of Mic60 in PD may yield exciting new avenues for disease-altering therapeutic interventions.

AUTHOR CONTRIBUTIONS

VV and SB contributed to the initial organization of the review. VV, SB, PO, and TH contributed to the writing of multiple sections and substantive revisions.

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