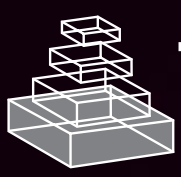
frontiers

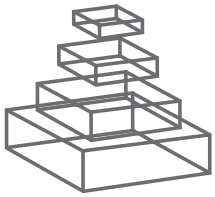
RESEARCH TOPICS

PAIN – NOVEL TARGETS AND NEW TECHNOLOGIES

Topic Editors
Susan Hua and Peter J. Cabot



frontiers in PHARMACOLOGY



frontiers

ISSN 1664-8714
ISBN 978-2-88919-394-3
DOI 10.3389/978-2-88919-394-3

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

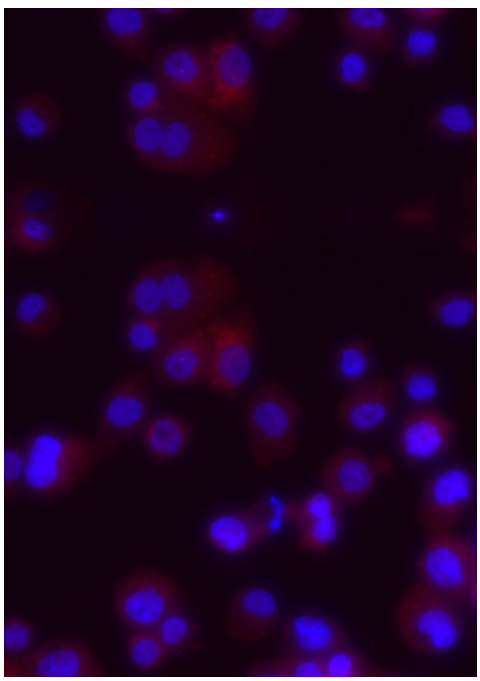
Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

PAIN – NOVEL TARGETS AND NEW TECHNOLOGIES

Topic Editors:
Susan Hua, The University of Newcastle, Australia
Peter J. Cabot, The University of Queensland, Australia



Differentiated THP-1 cells labelled for the mu-opioid receptor (red) and DAPI (blue) provided by Naghmeh Asvadi

The problem of clinical pain management is complex and far-reaching, as it encompasses many different types of pain, such as arthritis, musculoskeletal conditions, neuropathic pain, and visceral pain. It is widely known that many of the well-established analgesic pathways are centrally based, involving spinal and supraspinal sites. However, pain can also be effectively controlled by peripheral pathways. The analgesics market is growing and the driving forces are the aging population and need for better therapeutic benefits. There are various analgesic products that are available that can be administered by various routes, yet research is active in identifying new technologies for better drug targeting and novel targets to gain improved therapeutic efficiency.

This e-Book "PAIN – novel targets and new technologies" has brought together experts in the field of pain at the physiological, pharmacological and pharmaceutical levels to discuss novel pain targets and new pain technologies across the various types of pain. This information is presented as novel research findings, short communications and review articles. The goal of this e-Book is to generate further collaborative discussion on the future and direction of pain therapies.

Table of Contents

frontiers in
PHARMACOLOGY

EDITORIAL
published: 16 September 2014
doi: 10.3389/fphar.2014.00211

Pain—novel targets and new technologies

Susan Hua*[1] *and* ***Peter J. Cabot*[2]**

[1] *The School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia*
[2] *School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia*
**Correspondence: susan.hua@newcastle.edu.au*

Edited and reviewed by:
Nicholas M. Barnes, University of Birmingham, UK

Keywords: pain, analgesics, novel strategies, therapeutic target, targeted drug delivery

Pain is a major health problem that significantly affects the quality of life of patients. It has a significant impact on both the sufferers and the broader community, imparting high health costs, and economic loss to society. The consensus among clinicians and researchers worldwide is that current strategies for the treatment of pain are inadequate. These inadequacies are even greater when chronic pain, which often accompanies chronic illnesses such as arthritis or nerve injury, is involved. Despite major advances in treatment strategies over the last two decades, pain management still remains a major challenge in arthritis; and even with treatment with current therapies, many patients still experience moderate-to-severe pain (Stein and Baerwald, 2013). Patients with rheumatoid arthritis report pain management as their highest priority (Whittle et al., 2013), and osteoarthritis is the leading cause of pain and physical disability in the elderly (Stein and Baerwald, 2013). The burden of disease, especially with osteoarthritis, is growing in relation to the aging population and the increasing levels of obesity in the world population. Similar concerns are growing for other types of pain.

Current analgesics for persistent pain are relatively ineffective, are associated with significant adverse effects or abuse liability, and do not reduce pain in all treated individuals (Woolf, 2010). Opioids (e.g., morphine, codeine, oxycodone) are currently one of the most potent groups of analgesics used clinically (Iwaszkiewicz et al., 2013), with prescriptions increasing by 50% over the past 10 years for chronic, non-cancer pain (Waterman, 2013). However there is clear evidence that as opioid prescription rate rises, there is a corresponding increase in opioid overdose deaths, misuse and addiction, with these adverse effects attributed to their agonist effects on central opioid receptors—causing dependence, tolerance, sedation, and respiratory depression (Hua and Cabot, 2010; Waterman, 2013). Non-steroidal and steroidal anti-inflammatory drugs have serious side effects such as gastric erosions, ulcer formation, bleeding, hypersensitivity reactions, cardiovascular toxicity, renal toxicity, and hepatotoxicity (Warner and Mitchell, 2008; Stein et al., 2009). In addition, they are also not peripherally selective thereby causing a range of central adverse effects (Stein et al., 2009). Over the past 20 years, most analgesic development activity have been limited to reformulation of opioids, production of new cyclooxygenase (COX) inhibitors, amine reuptake inhibitors and anticonvulsants, and introduction of topical local anesthetics—all of these act on well-established targets (Woolf, 2010). Therefore, there is an obvious clinical need to introduce more effective and safe analgesics, suitable for chronic administration.

The problem of clinical pain management is complex and far-reaching, as it encompasses many different types of pain, such as arthritic, musculoskeletal, neuropathic, and visceral pain. Our increasing understanding of the neurobiology of pain further supports that a "one size fits all" policy is not appropriate for the way we treat pain across different pathological pain conditions as well as for individuals with the same underlying condition. Pain is commonly a manifestation of a range of multiple, sometimes irreversible, abnormalities in the functioning of the nervous system. In many cases the problem is the persistent amplification of sensory signals and generation of spontaneous activity in the nervous system, which occurs in conditions such as fibromyalgia, neuropathic pain, irritable bowel syndrome, and headaches (Woolf and Salter, 2000; Latremoliere and Woolf, 2009). The complexity and heterogeneity of pain should be appreciated. Complex interplay of processes operating at multiple peripheral and central sites are involved in initiating or sustaining pain, with each mechanism involving many unique or similar targets (Woolf, 2010).

The driving force for the successful translational development of novel analgesics requires the collaboration of experts in the field of basic pain science, pharmaceutics and clinicians specializing in pain management. Drug delivery and targeting is now recognized as the key to effective development of many novel and existing therapeutics to enable optimal therapeutic use of such molecules, as many drugs are severely compromised by significant obstacles to delivery *in vivo* and by toxic adverse effects (Hua and Wu, 2013). Drug delivery systems have been used in pain therapies to improve toxicity or side effect profiles by targeted delivery to specific sites in the body, increase drug bioavailability, and providing prolonged drug release (Hua and Cabot, 2013; Hua and Wu, 2013). There is also a need for detailed phenotyping of animal models of pain and evaluation of whether the models are appropriate surrogates for human pain syndromes. In cases where there is no good rodent model of the disease, it may be better to model pain mechanisms, such as peripheral sensitization or ectopic excitability in nociceptors using electrophysiology (Woolf, 2010). It may be very likely that a single pain-relieving magic bullet simply does not exist, and instead our focus may need to turn to multiple targeted treatments and/or synergistic therapies that are aimed at the specific mechanisms responsible.

This Research Topic focuses on articles that discuss the mechanisms of various types of pain as well as identifying potential novel targets and new technologies for the development of innovative therapeutic strategies for the treatment of pain.

REFERENCES

Hua, S., and Cabot, P. J. (2010). Mechanisms of peripheral immune-cell-mediated analgesia in inflammation: clinical and therapeutic implications. *Trends Pharmacol. Sci.* 31, 427–433. doi: 10.1016/j.tips.2010.05.008

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16, E199–E216.

Hua, S., and Wu, S. Y. (2013). The use of lipid-based nanocarriers for targeted pain therapies. *Front. Pharmacol.* 4:143. doi: 10.3389/fphar.2013.00143

Iwaszkiewicz, K. S., Schneider, J. J., and Hua, S. (2013). Targeting peripheral opioid receptors to promote analgesic and anti-inflammatory actions. *Front. Pharmacol.* 4:132. doi: 10.3389/fphar.2013.00132

Latremoliere, A., and Woolf, C. J. (2009). Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J. Pain* 10, 895–926. doi: 10.1016/j.jpain.2009.06.012

Stein, C., and Baerwald, C. (2013). Opioids for the treatment of arthritis pain. *Expert Opin. Pharmacother.* 15, 193–202. doi: 10.1517/14656566.2014.861818

Stein, C., Clark, J. D., Oh, U., Vasko, M. R., Wilcox, G. L., Overland, A. C., et al. (2009). Peripheral mechanisms of pain and analgesia. *Brain Res. Rev.* 60, 90–113. doi: 10.1016/j.brainresrev.2008.12.017

Warner, T. D., and Mitchell, J. A. (2008). COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. *Lancet* 371, 270–273. doi: 10.1016/S0140-6736(08)60137-3

Waterman, P. (2013). Prescription addiction - the creeping menace. *Aust. Pharm.* 32, 33–35.

Whittle, S. L., Richards, B. L., and Buchbinder, R. (2013). Opioid analgesics for rheumatoid arthritis pain. *JAMA* 309, 485–486. doi: 10.1001/jama.2012.193412

Woolf, C. J. (2010). Overcoming obstacles to developing new analgesics. *Nat. Med.* 16, 1241–1247. doi: 10.1038/nm.2230

Woolf, C. J., and Salter, M. W. (2000). Neuronal plasticity: increasing the gain in pain. *Science* 288, 1765–1769. doi: 10.1126/science.288.5472.1765

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 July 2014; accepted: 01 September 2014; published online: 16 September 2014.

*Citation: Hua S and Cabot PJ (2014) Pain—novel targets and new technologies. Front. Pharmacol. **5**:211. doi: 10.3389/fphar.2014.00211*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

REVIEW ARTICLE
published: 18 December 2013
doi: 10.3389/fphar.2013.00156

Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN)

Yaqin Han [1,2] ***and Maree T. Smith*** [1,2]*

[1] *Centre for Integrated Preclinical Drug Development, The University of Queensland, Brisbane, QLD, Australia*
[2] *School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Joel S. Greenberger, University of Pittsburgh Medical Center-Shadyside, USA
Andreas Bergdahl, Concordia University, Canada

***Correspondence:**
Maree T. Smith, Centre for Integrated Preclinical Drug Development, The University of Queensland, Level 3, Steele Building, St. Lucia Campus, Brisbane, QLD 4072, Australia
e-mail: maree.smith@uq.edu.au

Chemotherapy induced peripheral neuropathy (CIPN) is a type of neuropathic pain that is a major dose-limiting side-effect of potentially curative cancer chemotherapy treatment regimens that develops in a "stocking and glove" distribution. When pain is severe, a change to less effective chemotherapy agents may be required, or patients may choose to discontinue treatment. Medications used to alleviate CIPN often lack efficacy and/or have unacceptable side-effects. Hence the unmet medical need for novel analgesics for relief of this painful condition has driven establishment of rodent models of CIPN. New insights on the pathobiology of CIPN gained using these models are discussed in this review. These include mitochondrial dysfunction and oxidative stress that are implicated as key mechanisms in the development of CIPN. Associated structural changes in peripheral nerves include neuronopathy, axonopathy and/or myelinopathy, especially intra-epidermal nerve fiber (IENF) degeneration. In patients with CIPN, loss of heat sensitivity is a hallmark symptom due to preferential damage to myelinated primary afferent sensory nerve fibers in the presence or absence of demyelination. The pathobiology of CIPN is complex as cancer chemotherapy treatment regimens frequently involve drug combinations. Adding to this complexity, there are also subtle differences in the pathobiological consequences of commonly used cancer chemotherapy drugs, viz platinum compounds, taxanes, vincristine, bortezomib, thalidomide and ixabepilone, on peripheral nerves.

Keywords: chemotherapy-induced peripheral neuropathy (CIPN), mitochondrial dysfunction, oxidative stress, intraepidermal nerve fiber (IENF) degeneration, loss of heat sensitivity

INTRODUCTION

Chemotherapy-induced peripheral neuropathy (CIPN) is a common and potentially dose-limiting side effect of many cancer chemotherapy drug treatment regimens (Burton et al., 2007). The prevalence of CIPN varies from 10 to 100% depending upon the particular anticancer drug or drug combination administered, the dosing regimen, the methods of pain assessment and the particular patient situation (Balayssac et al., 2011). The development of CIPN may result in dose reduction of the cancer chemotherapy agents or a switch to less efficacious agents or even cessation of treatment in the extreme (Gutiérrez-Gutiérrez et al., 2010).

Typically, CIPN presents in patients with a "stocking and glove" distribution in the feet and hands, respectively, due to the vulnerability of the long nerves (Boland et al., 2010). Sensory symptoms that are commonly reported include paresthesia, dysesthesia, allodynia, hyperalgesia, hypoalgesia or pain that is burning, shooting or electric-shock-like (Boland et al., 2010). Painful symptoms may persist well beyond discontinuation of treatment (so called "coasting") (Quasthoff and Hartung, 2002) resulting in a condition as painful or more painful than the original cancer. Furthermore, although slow recovery of peripheral nerve damage may occur in patients with CIPN, this is not always the case and so pain may persist (Peltier and Russell, 2002).

Anticancer drugs that most commonly induce CIPN are platinum compounds (cisplatin and oxaliplatin), spindle poisons/antitubulins including vincristine and paclitaxel (Wolf et al., 2008; Balayssac et al., 2011), and some newer agents such as the proteasome inhibitor, bortezomib (Hoy, 2013), ixabepilone (Goel et al., 2008) and thalidomide (Kocer et al., 2009). A wide range of solid and hematological malignancies are treated with these compounds and polychemotherapy schedules are used to enhance treatment effectiveness (Cavaletti and Marmiroli, 2010). However, the latter also increase the risk of CIPN (Burton et al., 2007; Argyriou et al., 2013).

The prevalence of cancer is increasing globally with an estimated 17 million new cases projected by 2020 (Kanavos, 2006; Paice, 2011). Cancer survival rates have increased dramatically as new treatments and older therapies are refined to have a greater antitumor effect. This means that the landscape of "cancer pain" has shifted into a form of long term chronic pain in many instances (Burton et al., 2007). In clinical practice, CIPN is poorly diagnosed and under-treated to the detriment of patient quality-of-life and there is no proven method for prevention of CIPN (Balayssac et al., 2011). Although drugs used to provide symptomatic relief of CIPN often lack efficacy and/or have unacceptable side-effects (Balayssac et al., 2005), a recent 5-week randomized, placebo-controlled clinical trial found that oral duloxetine at 60 mg daily produced significant relief of CIPN above placebo (Smith et al., 2013). Despite these promising findings, there is nevertheless a large unmet medical need for novel,

well-tolerated analgesic agents to improve relief of CIPN. In the past decade, new insights on the mechanisms underpinning the pathogenesis of CIPN (Balayssac et al., 2011) have been made possible by the advent of rodent models enabling new targets to be identified for use in pain therapeutics discovery programs. Such studies are discussed in the following sections of this review.

STRUCTURAL CHANGES IN PERIPHERAL NERVES

Cancer chemotherapy agents may differentially affect specific peripheral nervous system (PNS) structures to produce neuronopathy, axonopathy and/or myelinopathy that contribute to the pathogenesis of painful CIPN (Ocean and Vahdat, 2004; Balayssac et al., 2011) (**Table 1** and **Figure 1**).

Cancer chemotherapy-induced peripheral nerve injury appears to be due primarily to axonopathy (McDonald et al., 2005; Persohn et al., 2005; Gilardini et al., 2012) that is seen both in patients with CIPN (Cata et al., 2007; Burakgazi et al., 2011) and in rodent models of CIPN (Cavaletti et al., 2007; Boyette-Davis et al., 2011). Thus, peripheral nerve degeneration or small fiber neuropathy is generally accepted as underpinning the development of CIPN (Liu et al., 2010; Boyette-Davis et al., 2011; Burakgazi et al., 2011; Wang et al., 2012).

THE LONGEST AXONS ARE THE FIRST AFFECTED

Peripheral nerves contain a variety of nerve fibers that differ in their respective morphology, degree of myelination, function and biochemical features (Gutiérrez-Gutiérrez et al., 2010). These various fiber types are differentially sensitive to the neurotoxic effects of cancer chemotherapy agents with the longest nerves having the greatest vulnerability (Wilkes, 2007; Gutiérrez-Gutiérrez et al., 2010). This may be related to their higher metabolic requirements (Chen and Chan, 2006; Mironov, 2007). Clinically, symptoms develop initially in the feet and hands, followed by proximal progression to the ankles and wrists in a "stocking and glove" distribution (Lomonaco et al., 1992; Wolf et al., 2008).

MYELINATED FIBERS ARE DAMAGED WITH/WITHOUT ALTERED MYELIN STRUCTURE WHEREAS UNMYELINATED FIBERS ARE MOSTLY UNAFFECTED

Myelin is a lipid- and protein-rich sheath that insulates axons and facilitates faster conduction of nerve impulses compared with unmyelinated axons (Gilardini et al., 2012). Although myelinated fibers are damaged (Cata et al., 2006), perhaps even by preferential selection (Cavaletti et al., 1995; Dougherty et al., 2004), the extent to which demyelination is a key pathobiological event in CIPN is unclear. For example, using X-ray diffraction capable of detecting even subtle changes in the myelin structure, there were no structural alterations in the myelin sheath of the sciatic and optic nerves in rat models of CIPN induced using cisplatin, paclitaxel or bortezomib (Gilardini et al., 2012). These findings mirror the findings of earlier work that used fixed tissues (spinal cord and DRGs) from rodents administered the same cancer chemotherapy agents (Cavaletti et al., 1995) as well as from humans with paclitaxel-induced CIPN (Postma et al., 1995). In patients with bortezomib-induced CIPN, approximately 50% had pure small fiber neuropathy whereas the remainder had mixed small and large fiber involvement (Richardson et al., 2009).

In rat models of paclitaxel, cisplatin and bortezomib-induced CIPN, there were no clear-cut changes in the structure of internodal myelin (Gilardini et al., 2012). However, higher dosages of bortezomib were associated with an increased risk of peripheral nerve degeneration and possibly demyelination in contrast to lower dosages that nevertheless induced neuropathic pain behaviors (Zheng et al., 2012) (**Table 1**). In earlier work in patients administered paclitaxel, sural nerve biopsy revealed severe nerve fiber loss, axonal atrophy (with absence of axonal regeneration) and secondary demyelination (Sahenk et al., 1994). These peripheral nerve changes argue more for ganglionopathy than axonopathy as the most likely structural change in paclitaxel-induced neurotoxicity (Sahenk et al., 1994).

SLOWING OF SNCV MAY NOT BE DUE TO DEMYELINATION OR DEGENERATION OF PERIPHERAL NERVE AXONS

In CIPN, reduced sensory nerve conduction velocity (SNCV) (Gilardini et al., 2012; Xiao et al., 2012), can only be attributed reliably to myelinopathy if it is associated with preserved nerve compound action potentials (Gilardini et al., 2012). Unfortunately, the technical limitations of current neurophysiological methods do not allow the relative contributions of demyelination and axonal degeneration on reduced SNCV in CIPN to be assessed (Gilardini et al., 2012). In rats with docetaxel-induced CIPN, reduced levels of myelin and mRNA encoding myelin suggest that myelin is targeted in experimental peripheral neuropathies (Roglio et al., 2009). These findings are consistent with observations of taxane-induced axonal damage and secondary demyelination (Sahenk et al., 1994; Quasthoff and Hartung, 2002; Windebank and Grisold, 2008). The extent to which individual anticancer agents or treatment combinations induce differential structural changes in peripheral nerves, is currently unclear. This is a knowledge gap that requires systematic investigation in rodent models for comparison with the changes observed in skin biopsy specimens from patients with CIPN.

IENF LOSS WITHOUT DEGENERATION OF PERIPHERAL NERVE AXONS AND ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Unmyelinated fibers and terminal nerve arbors are major sites of cancer chemotherapy-induced neurotoxicity (Grisold et al., 2012) such that intraepidermal nerve fiber (IENF) loss or terminal arbor degeneration is proposed as a common lesion in various toxic neuropathies (Bennett et al., 2011; Zheng et al., 2012).

In a rodent model of paclitaxel-induced CIPN, significant IENF degeneration was not apparent by approximately 10 days after initiation of the paclitaxel treatment regimen (2 mg/kg on 4 alternate days) with peak effects observed several days later (Xiao et al., 2011). IENF degeneration and the development of pain behavior appear to be linked as both have similar delays to onset and peak effects (Xiao et al., 2011). Using electron microscopy at the time of peak pain severity, there were no signs of axonal degeneration in the saphenous nerve of these animals at a level just below the knee joint (Flatters and Bennett, 2006). Additionally, upregulation of activating transcription factor-3 (ATF-3) expression, a marker of axonal injury (Tsujino et al., 2000), was not observed in the nuclei of afferent neurons (Flatters and Bennett, 2006). Similar findings have been observed in rat

Table 1 | Effects of clinically used cancer chemotherapy agents on peripheral nerve structure in rodent models of CIPN.

Chemotherapy agent	Dosing regime	Rodents	PNS tissue examined	Extent of peripheral nerve damage	References
Bortezomib	ip, 0.2 mg/kg, 5 consecutive days	Male SD rats	Saphenous nerve DRGs and IENFs	IENF decrease but no degenerating axons No DRG neurons with ATF-3 positive nuclei	Zheng et al., 2012
	iv, 0.08, 0.15, 0.2, 0.3 mg/kg, 2 or 3 times a week, 4 weeks	Female Wistar rats	Sciatic nerves	Mild to moderate pathological changes involving predominantly Schwann cells and myelin; primarily characterized by myelin sheath degeneration and axonal degeneration. Unmyelinated fibers were unaffected	Cavaletti et al., 2007
	iv, 0.2 mg/kg ×3/week, 4 weeks	Female Wistar rats	Sciatic nerves Optic nerves	No pathological changes in axons and the surrounding myelin sheath Myelin degeneration in a limited number of fibers, optic nerves normal	Gilardini et al., 2012
	iv, 0.15/0.2 mg/kg × 3/week, 8 weeks	Female Wistar rats	Sciatic nerves DRGs	Nerve fiber degeneration, loss of axonal structures in the most severe cases No morphological alteration in most DRG neurons and satellite cells	Meregalli et al., 2010
	iv, 0.4/0.8 mg/kg × 2/week, 4 weeks	Female BALB/c mice	DRGs Sciatic nerves	No pathological changes in DRGs Axonal degeneration in sciatic nerves at higher dose	Carozzi et al., 2010a
	sc, 0.8, 1 mg/kg × 2/week or × 2/ week, 6 weeks	Swiss OFI female mice	Sciatic and tibial nerves Plantar pads	Lower density of myelinated large fibers and decreased fiber diameter but no signs of degeneration	Bruna et al., 2010
Cisplatin	ip, 1 mg/kg ×3 /week, 2 mg/kg × 2/ week, 3 mg/kg ×1/week, 5 weeks	Male SD rats	Lumbar spinal cord Sciatic nerve and paw skin	Myelin sheath remains normal Unmyelinated fibers were unaffected	Authier et al., 2003a
	ip, 3 mg/kg every 3 days, 4 weeks	Male Wistar rats	Sciatic nerves	Degenerated myelinated axons with altered myelin band and altered unmyelinated axons; axonal damage without demyelination	Arrieta et al., 2011
	ip, 2/4 mg/kg × 2/week, 4 weeks	Female BALB/c mice Wistar rats	DRGs Sciatic nerves	No pathological changes in the DRGs Mild pathological changes at higher dosage regimen in sciatic nerves	Carozzi et al., 2010a; Gilardini et al., 2012
	ip, 2 mg/kg, 2/week in 4.5 weeks	Male Wistar rats	Sciatic nerves	Focal areas of demyelination and degeneration	Al Moundhri et al., 2013
Oxaliplatin	ip, 2 mg/kg, 5 consecutive days	Male SD rats	Saphenous nerves and IENFs	Oxaliplatin evoked SNCV slowing occurred in the absence of demyelination or degeneration of peripheral nerve axons	Xiao et al., 2012

(Continued)

Table 1 | Continued

Chemotherapy agent	Dosing regime	Rodents	PNS tissue examined	Extent of peripheral nerve damage	References
	ip, 2 mg/kg, 4 alternate days	Male SD rats	Nerve fibers	Significantly fewer IENFs	Boyette-Davis and Dougherty, 2011
	ip, 4 mg/kg, 2/week in 4.5 weeks	Male Wistar rats	Sciatic nerves	Focal areas of demyelination and degeneration	Al Moundhri et al., 2013
	ip, 3, 6 or 12 mg/kg, single	Male SD rats	Lumbar spinal cord	No difference in immunoreactivity for CGRP but substance P was significant higher than for vehicle control group (12 vs. 5%)	Ling et al., 2007
Vincristine	iv, 50, 100 and 150 μg/kg, every second day, up to five injections	Male SD rats	Paw skin	Myelin sheaths remained unaffected	Authier et al., 2003b
	ip, 0.2 mg/kg ×1/week, 5 weeks, 0.1 mg/kg and increase by 0.05 mg/kg each week, 5 weeks	Male rats	Sciatic nerve	Reduction in action potential amplitude associated with axonal degeneration with or without minor changes of segmental demyelination	Ja'afer et al., 2006
Paclitaxel	ip, single 32 mg/kg	Male SD rats	Lumbar spinal cord, Sciatic nerve and paw skin	Axonal degenerative changes while Schwann cells and myelin sheaths remained normal	Authier et al., 2000b
	ip, 0.5, 1, 2, 6 or 8 mg/kg, 4 alternate days	Male SD rats	DRGs Sciatic nerves	No degeneration, no DRG neurons with ATF-3 positive nuclei No degeneration of myelinated or unmyelinated axons	Polomano et al., 2001; Flatters and Bennett, 2006; Bennett et al., 2011
	iv, 18 mg/kg, D0 and D3	Male SD rats	DRGs Sciatic nerve	ATF-3 upregulation	Peters et al., 2007
	ip, 8 mg/kg × 2/week, 4 weeks	Male Wistar rats	Sciatic nerves	Axonal damage without demyelination	Arrieta et al., 2011
	ip, 16mg/kg × 1/week, 4 weeks iv, 5, 10, 12.5 mg/kg × 1/week, 4 weeks	Female Wistar rats	Axons (sciatic nerve)	Most myelinated fibers have normal histology, some fibers show axonal degeneration	Persohn et al., 2005
	ip, 12.5 mg/kg × 1/week, 9 weeks	Female Wistar rats	DRGs	Increased immunohistochemical staining for ATF-3	Jamieson et al., 2007
	iv, 10 mg/kg × 1/week, 4 weeks	Female Wistar rats	Sciatic nerves Optic nerves	No pathological changes in axons and surrounding myelin sheath	Gilardini et al., 2012
	iv, 18 mg/kg, twice, every 3 days	Male SD rats	Trigeminal ganglia DRGs	Increased immunohistochemical staining for ATF-3	Jimenez-Andrade et al., 2006
	ip, 4.5 mg/kg, 25 mg/kg, or 60 mg/kg	Female C57BL/6 mice	Sciatic nerves	Macrophage-mediated demyelination, axons completely stripped of their myelin sheaths and surrounded by the cytoplasm of debris-filled phagocytes in some cases	Mo et al., 2012

(Continued)

Table 1 | Continued

Chemotherapy agent	Dosing regime	Rodents	PNS tissue examined	Extent of peripheral nerve damage	References
	ip, 8 or 16 mg/kg × 1/week, 5 weeks	Female Wistar rats	Sciatic/peroneal nerves and DRGs	Decrease in number of large myelinated fibers, but not due to a reduction in myelin thickness, mild axonal loss with minimal demyelination	Cavaletti et al., 1995
	iv, 50.70 mg/kg, × 1/week, 4 weeks	Female BALB/c mice	DRGs Sciatic nerves	No pathological changes	Carozzi et al., 2010a
	ip, 30 mg/kg once or several times at different intervals	BDF1 mice	Dorsal funiculus Dorsal spinal roots Peripheral nerves	Nerve fiber degeneration characterized by axonal and myelin fragmentations and phagocytosis	Mimura et al., 2000

ATF, activating transcription factor; CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; IENFs, intraepidermal nerve fibers; iv, intravenous injection; ip, intraperitoneal injection; sc, subcutaneous; SD, Sprague-Dawley; SNCV, sensory nerve conduction velocity.

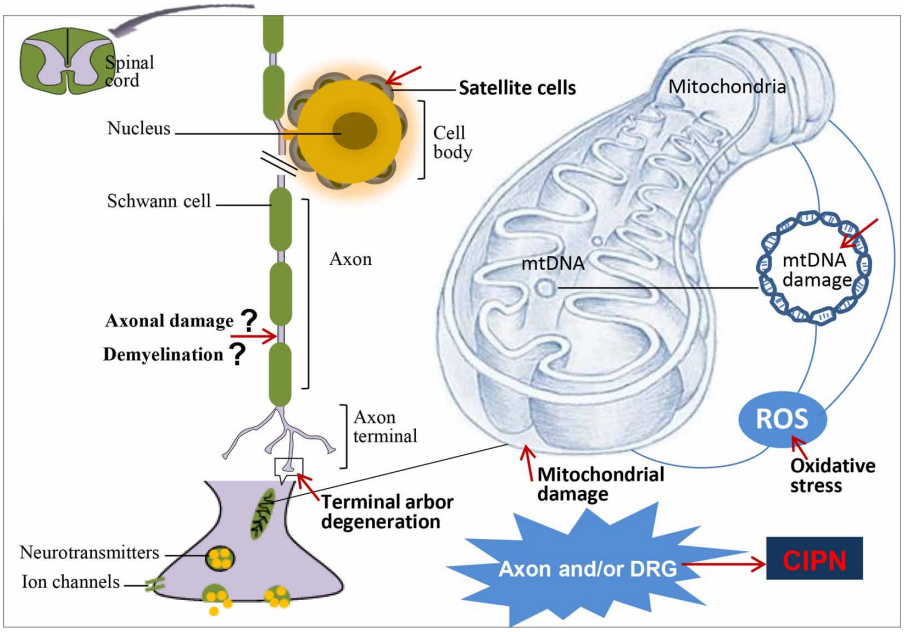


FIGURE 1 | CIPN pathogenesis and associated morphologic changes. The neurotoxic effects of cancer chemotherapy agents adversely affect multiple components of the peripheral nervous system (PNS) including axons and cell bodies of dorsal root ganglion (DRG) neurons to cause axonal damage (IENF loss/terminal arbor degeneration), mitochondrial damage and oxidative stress probably associated with inflammation. DRG neurons and their surrounding satellite cells show pathological changes including alterations in levels of expression of multiple ion channels (Xiao et al., 2007; Anand et al., 2010; Kaur et al., 2010; Descoeur et al., 2011), neurotransmitters (Tatsushima et al., 2011), and their receptors (Carozzi et al., 2010b; Mihara et al., 2011), as well as altered gene expression (Alaedini et al., 2008). Mitochondrial dysfunction and IENF loss appear to be important pathobiological features of CIPN that are correlated directly with pain behaviors in rodent models (Flatters and Bennett, 2006; Zheng et al., 2012). Indeed, direct mitochondrial DNA (mtDNA) damage contributes to cisplatin-induced CIPN (Podratz et al., 2011). Myelinated fibers are damaged (Cata et al., 2006) possibly by preferential selection (Dougherty et al., 2004) but the extent to which demyelination is a key pathobiological event is currently unclear.

models of vincristine, oxaliplatin and bortezomib-induced CIPN such that neuropathic pain behaviors were associated with IENF degeneration in the absence of peripheral nerve axonal degeneration (Aley et al., 1996; Tanner et al., 1998; Topp et al., 2000; Siau and Bennett, 2006; Bennett et al., 2011).

Clinically, there is IENF loss in patients with CIPN (Boyette-Davis et al., 2011; Giannoccaro et al., 2011) despite these individuals having normal peripheral nerve axon counts (Holland et al., 1998; Herrmann et al., 1999) and normal nerve conduction results (Periquet et al., 1999; Devigili et al., 2008; Løseth et al.,

2008). This led Holland et al. (1998) to coin the term "terminal axonopathy" that is akin to the more recently promulgated "terminal arbor degeneration" concept (Bennett et al., 2011). In patients, an increase in the swelling ratio of IENFs appeared to be predictive of a decrease in IENF density and this was correlated with the severity of painful neuropathy induced in the feet by paclitaxel (CIPN), diabetes, AIDS, and idiopathic neuropathy (Schmidt et al., 1997; Lauria et al., 2003). However, administration of much larger doses of cancer chemotherapy agents in rats, such as paclitaxel either as a single bolus (12.5–32 mg/kg) (Authier et al., 2000b; Jamieson et al., 2007) or as cumulative doses (8 and 16 mg/kg once-weekly for 5 weeks) (Cavaletti et al., 1995) or bortezomib at 2.4–4.8 mg/kg (Cavaletti et al., 2007; Meregalli et al., 2010; Gilardini et al., 2012), resulted in degeneration of peripheral nerve axons and DRG neurons, together with ATF-3 up-regulation in DRG neurons (Jamieson et al., 2007; Peters et al., 2007). Thus, the extent to which peripheral nerve axons are damaged by chemotherapy agents appear to be directly related to the dosing regimen (**Table 1**).

Comparatively high concentrations of paclitaxel are found in the DRGs relative to peripheral nerve and spinal cord (Herrmann et al., 1999), that may be underpinned by the fact that the subepidermal axon bundles in peripheral nerves lack a perineurium (a component of the blood-nerve barrier). Additionally, anterograde transport of paclitaxel from sensory neuron cell bodies to the IENFs would take time for toxic levels to be reached in the terminal arbors (Bennett et al., 2011). Such a lag period may potentially explain the coasting effect, i.e., the delay between treatment cessation relative to the loss of IENFs and the appearance of pain hypersensitivity (Bennett et al., 2011).

IENF degeneration and abnormal spontaneous discharge of primary afferent nerve fibers in rat models of CIPN may be underpinned by mitochondrial dysfunction and consequent energy deficiency (Boyette-Davis and Dougherty, 2011; Xiao et al., 2012; Zheng et al., 2012). Mitochondria are concentrated in regions of high metabolic demand (Chen and Chan, 2006; Mironov, 2007) such as sensory terminal boutons that are packed with mitochondria (Breathnach, 1977; Ribeiro-Da-Silva et al., 1991; Bennett et al., 2011). The high energy requirement of the intraepidermal terminal arbor is thought to be due, at least in part, to the constant degeneration and regeneration (remodeling) of the arbor in its ever changing microenvironment (Bennett et al., 2011). This is because the epidermis is in a continuous state of renewal with a total epidermal turnover time of approximately 45 days in humans (Bergstresser and Taylor, 1977).

MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS

Mitochondria are the energy-generating structures in cells with their dysfunction implicated in the pathogenesis of cancer and a range of neurodegenerative diseases (Florea and Büsselberg, 2011). Abnormalities in mitochondrial structure and function in peripheral sensory nerve fibers are postulated as key CIPN mechanisms and appear to be correlated directly with pain behavior (Flatters and Bennett, 2006; Zheng et al., 2012). In multiple myeloma patients administered cycles of bortezomib in combination with dexamethasone, bortezomib toxicity on mitochondria resulted in impairment of the electrogenic Na^+-K^+-ATPase-dependent pump resulting in axonal membrane depolarization that preceded axonal degeneration (Nasu et al., 2013). In patients with vincristine and bortezomib-induced CIPN, there were significant changes in the expression of genes involved in the control of mitochondrial function in myeloma plasma cells and peripheral blood (Broyl et al., 2010). Interestingly, exposure of cultured DRG neurons to cisplatin and paclitaxel *in vitro* induced mitochondrial damage that was reversed by pretreatment with the antioxidant, α-lipoic acid (Melli et al., 2008). Additionally, the development of CIPN in rodent models (**Table 2**) and patients (**Table 3**) can be prevented by treatment with drugs that enhance mitochondrial function. Conversely, as mitochondrial poisons exacerbate neuropathic pain behaviors in rodent models of CIPN (Xiao and Bennett, 2012), CIPN appears to be linked to mitotoxicity (**Figure 1**).

MITOTOXICITY

Direct mitochondrial DNA (mtDNA) damage

Cisplatin forms adducts with mitochondrial DNA resulting in direct mitochondrial DNA (mtDNA) damage that is a novel mechanism for cisplatin-induced CIPN and is distinct from the established nuclear DNA (nDNA) damage pathway (Podratz et al., 2011). DRG neurons accumulate high levels of cisplatin-DNA adducts both *in vitro* and *in vivo* (McDonald et al., 2005; Ta et al., 2006) such that the cisplatin concentration in the PNS is comparable with that in tumor tissue (Gregg et al., 1992; Screnci and McKeage, 1999; Melli et al., 2008).

Cisplatin-DNA adducts can be removed and DNA repaired by the nucleotide excision repair (NER) system that is present in nDNA (McDonald et al., 2005; Podratz et al., 2011), in contrast to mtDNA where the NER system is absent (Croteau et al., 1999). Hence, cisplatin-mtDNA adducts inhibit mtDNA replication and mtRNA transcription to cause mitochondrial degradation (Podratz et al., 2011) in DRG neurons.

Increased mitochondrial swelling and vacuolation in peripheral nerve axons

In rat models of paclitaxel, oxaliplatin and bortezomib-induced CIPN, the number of swollen and vacuolated mitochondria in the axons of A- and C-primary afferent sensory nerve fibers was significantly higher (37.3 and 152%, respectively) than for vehicle-treated control rats (Xiao et al., 2011, 2012; Zheng et al., 2012). These changes resulted in mitochondrial dysfunction characterized by significant deficits in mitochondrial respiration and ATP production that were rescued by prophylactic treatment with acetyl-L-carnitine. The latter is an acetylated derivative of the natural amino acid, L-carnitine, that has an essential role in the transport of long-chain free fatty acids into mitochondria (Zheng et al., 2011, 2012). Interestingly, there was a relative sparing of mitochondria in the corresponding peripheral nerve Schwann cells (Flatters and Bennett, 2006; Zheng et al., 2011, 2012; Xiao and Bennett, 2012; Xiao et al., 2012).

In DRG satellite cells, bortezomib induced intracytoplasmic vacuolation characterized by damage to mitochondria and the endoplasmic reticulum (Cavaletti et al., 2007). These changes

Table 2 | Summary of pharmacological agents that enhance mitochondrial function as well as prevent and/or alleviate CIPN in rodent models.

Pharmacological agent	Rodent model	Efficacy outcome	Dose and route	References
Acetyl-L-carnitine (antioxidant)	Paclitaxel	+ (intervention)	100 mg/kg, p.o. Daily ×10	Flatters et al., 2006
	Paclitaxel	+(prophylactic)	50 and 100 mg/kg, p.o. Daily ×21	Flatters et al., 2006
	Paclitaxel	+ (prophylactic and intervention)	100 mg/kg, s.c. Daily	Ghirardi et al., 2005
	Vincristine	+ (prophylactic and intervention)	100 mg/kg, s.c. Daily	Ghirardi et al., 2005
	Cisplatin	+ (prophylactic and intervention)	100 mg/kg, s.c. Daily	Ghirardi et al., 2005
	Oxaliplatin	+ (prophylactic and intervention)	100 mg/kg, s.c. Daily	Orlando et al., 2005
	Oxaliplatin	+ (prophylactic)	100 mg/ml/kg, p.o. Daily	Xiao et al., 2012
Olesoxime	Paclitaxel	+ (prophylactic)	3 or 30 mg/kg, p.o. Daily	Xiao et al., 2009
	Oxaliplatin	+ (prophylactic)	30 mg/ml/kg, p.o. Daily	Xiao et al., 2012
Silibinin(antioxidant)	Oxaliplatin	+ (prophylactic)	100 mg/kg, p.o. Daily	Di Cesare Mannelli et al., 2012
Allopregnanolone	Oxaliplatin	+ (prophylactic and intervention)	2 or 4 mg/kg, Every 2 or 4 days	Meyer et al., 2011

p.o., per os; s.c., subcutaneous.

Table 3 | Clinical trial evidence for the role antioxidants in the relief of CIPN.

Medications	Patients involved	Chemotherapy agent	Trial	Efficacy	Weather interfere with anticancer efficacy	References
α-Lipoic acid (Treatment)	14	Docetaxel and isplatin	Randomised	Yes	–	Gedlicka et al., 2003
	15	Oxaliplatin	–	Yes	–	Gedlicka et al., 2002
Acetyl-L-carnitine (Treatment)	25	Cisplatin and/or Paclitaxel	–	Yes	–	Bianchi et al., 2005
	27	Cisplatin and/or Paclitaxel	–	Yes	–	Maestri et al., 2005
	409	Taxane-based	RCT	No; pain worsened	–	Hershman et al., 2013
Glutathione (Prevention)	31	Cisplatin	Randomized	Yes	No	Colombo et al., 1995
	151	Cisplatin	–	Yes	–	Smyth et al., 1997
	27	Oxaliplatin/5-fluorouracil/ leucovorin (FOLFOX)	Randomized	Yes	No	Milla et al., 2009
	52	Oxaliplatin-based	RCT	Yes	–	Cascinu et al., 2002
Amifostine (Prevention)	92	Oxaliplatin (FOLFOX4)	Randomized	Yes	No	Lu et al., 2008
	187	Paclitaxel and Carboplatin	Randomized	yes	–	Lorusso et al., 2003
	27	Cisplatin and Paclitaxel	–	Not really	–	Moore et al., 2003
	38	Paclitaxel and Carboplatin	Randomized	Yes	–	Kanat et al., 2003
	72	Paclitaxel and Carboplatin-based	RCT	Yes	±	Hilpert et al., 2005
Org 2766 (Prevention)	196	Cisplatin and cyclophosphamide	–	No	–	Roberts et al., 1997
	55	Cisplatin and cyclophosphamide	RCT	Yes	No	van et al., 1990
N-acetylcysteine (Prevention)	14	Cisplatin-based	Randomized placebo controlled	Yes	–	Lin et al., 2006

RCT, Randomized, Double-Blind, Placebo-Controlled Trial.

appear to be underpinned by activation of the mitochondrial-based apoptotic pathway including caspase activation (Broyl et al., 2010; Lee et al., 2012) as well as dysregulation of calcium homeostasis (Landowski et al., 2005). Paclitaxel-induced mitochondrial damage was confined to the axons of primary afferent sensory with sparing of motor neurons (Xiao et al., 2011). The high and persistent exposure of primary sensory neuron cell bodies in the DRGs to paclitaxel may contribute to this selective effect (Xiao et al., 2011).

Opening of the mPTP and dysregulation of calcium homoeostasis

Paclitaxel opens the mitochondrial permeability transition pore (mPTP), a multi-molecular complex containing a voltage-dependent anion channel that induces mitochondrial calcium release (Kidd et al., 2002; Flatters and Bennett, 2006). Acetyl-L-carnitine can prevent mPTP opening (Pastorino et al., 1993) and is associated with a reduction in paclitaxel, oxaliplatin and bortezomib-induced CIPN when administered prophylactically in rodents (Jin et al., 2008; Bujalska and Makulska-Nowak, 2009; Carozzi et al., 2010b; Xiao et al., 2012; Zheng et al., 2012).

Mitochondria have a large calcium buffering capacity and so impaired calcium uptake or increased calcium leakage from mitochondrial stores may have a pathological role in CIPN (Jaggi and Singh, 2012). This notion is supported by the fact that vincristine-induced neurotoxicity in rats was reversed by drugs that reduce elevated intra-neuronal calcium concentrations (Muthuraman et al., 2008; Kaur et al., 2010). In other work, increased expression levels of the $\alpha_2\delta$ subunit of voltage-gated Ca^{2+} channels in the DRGs were correlated with the development of mechanical allodynia (Luo et al., 2001). Conversely, drugs that bind to the $\alpha_2\delta$ subunit such as gabapentin (Flatters and Bennett, 2004; Xiao et al., 2007) and pregabalin (Saif et al., 2010; Nakashima et al., 2012; Peng et al., 2012), as well as the L-type calcium channel blocker, lercanidipine (Saha et al., 2012), showed efficacy for prevention of CIPN in rodent models and patients (Nguyen and Lawrence, 2004; Saif et al., 2010; Nakashima et al., 2012).

A retrospective review of 69 patients administered oxaliplatin concluded that calcium channel blockers reduce CIPN (Tatsushima et al., 2013). Although intravenous Ca^{2+}/Mg^{2+}infusions reportedly attenuate the development of oxaliplatin-induced CIPN without compromising cancer treatment efficacy (Wolf et al., 2008; Kurniali et al., 2010; Wen et al., 2013), there are lingering concerns regarding a negative effect on cancer chemotherapy treatment efficacy. Hence, this needs to be evaluated for each class of cancer chemotherapy agent (Kurniali et al., 2010).

OXIDATIVE STRESS

In a rat model of oxaliplatin-induced neuropathy, markers of oxidative stress including lipid peroxidation, carbonylated proteins, and DNA oxidation increased in the systemic circulation, the sciatic nerve and the lumbar spinal cord (Di Cesare Mannelli et al., 2012), with these changes prevented by antioxidant treatment (Di Cesare Mannelli et al., 2012; Nasu et al., 2013). Similarly, production of reactive oxygen species (ROS) was increased by cisplatin (Florea and Büsselberg, 2011), and bortezomib (Wang et al., 2011). In patients receiving docetaxel for the treatment of cancer, the occurrence of grade ≥2 CIPN was more frequent in individuals homozygous for *GSTP1* ^{105}Ile allele, that encodes glutathione S-transferase pi 1 (GSTP1), an enzyme involved in the regulation of oxidative stress (Mir et al., 2009).

A role for oxidative stress in the pathobiology of CIPN is supported by multiple *in vitro* and *in vivo* studies showing that antioxidants have neuroprotective effects in CIPN (**Table 2**). In particular, the non-specific ROS scavenger, phenyl N-tert-butylnitrone (PBN), administered according to an intervention protocol in rats administered paclitaxel, attenuated development of mechanical (Kim et al., 2010) and cold hypersensitivity in the hindpaws (Fidanboylu et al., 2011). Conversely, for rats administered auranofin, a compound that increased oxidative stress, oxaliplatin and paclitaxel-induced neuropathic pain behaviors were exacerbated (Xiao and Bennett, 2012). Furthermore, as the superoxide-specific scavenger, TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) neither alleviated established paclitaxel-induced CIPN nor prevented its development in rodents, ROS but not superoxide radicals alone, are implicated in CIPN pathogenesis (Fidanboylu et al., 2011).

Although a benefit of antioxidants for the treatment and/or prevention of CIPN has been shown in multiple clinical studies (**Table 3**), most did not report on their impact on anticancer efficacy, and so this is a knowledge gap.

Increased spinal dorsal horn levels of peroxynitrite in rats with paclitaxel-induced CIPN (Doyle et al., 2012) implicate a role for reactive nitrogen species (RNS) in CIPN pathogenesis (Kamei et al., 2005; Mihara et al., 2011). Augmented peroxynitrite production may occur via two mechanisms with the first involving activation of nitric oxide synthase and NADPH oxidase to induce formation of the peroxynitrite precursors, NO and SO (Doyle et al., 2012). The second involves inactivation of the enzyme (manganese superoxide dismutase) that catalyzes peroxynitrite degradation (Doyle et al., 2012). This latter mechanism is supported by observations that peroxynitrite decomposition catalysts ($FeTMPyP^{5+}$ and $MnTE\text{-}2\text{-}PyP^{5+}$) prevented development of neuropathic pain behaviors in rat models of paclitaxel, oxaliplatin and bortezomib-induced CIPN (Doyle et al., 2012; Janes et al., 2013).

CIPN-induced nitro-oxidative stress results in increased production of proinflammatory cytokines (TNF-α and IL-1β), reduced production of anti-inflammatory cytokines (IL-10 and IL-4), as well as post-translational nitration of glutamate transporters and glutamine synthetase in astrocytes, the net result of which is enhanced pro-nociceptive glutamatergic signaling (Doyle et al., 2012). Treatment strategies that shift the balance in favor of anti-inflammatory cytokines have potential for slowing the development and progression of peripheral neuropathy in patients receiving cancer chemotherapy drugs (Wang et al., 2012).

LOSS OF HEAT SENSITIVITY IN CIPN

DIVERSE RESULTS OF HEAT SENSITIVITY IN CIPN

Primary afferent nerve fibers affected by cancer chemotherapy drug treatment regimens often exhibit both positive and negative sensory phenomena resulting in altered nociceptive thresholds (Nahman-Averbuch et al., 2011). Increased nociceptive thresholds may develop due to nerve fiber loss whereas reduced

nociceptive thresholds may develop as a result of peripheral and central sensitization (Nahman-Averbuch et al., 2011).

In general, there is heat hypoalgesia or a loss of heat sensitivity in patients with CIPN (Dougherty et al., 2004; Cata et al., 2006; Attal et al., 2009; Nahman-Averbuch et al., 2011) as well as in most rodent models of this condition (Authier et al., 2000a, 2003a; Fischer et al., 2001; Cata et al., 2006, 2008; Garcia et al., 2008; Hori et al., 2010; Xiao et al., 2012; Zheng et al., 2012). Additionally, cold allodynia is a characteristic symptom of painful CIPN in patients (Cata et al., 2006) as well as in rodent models (Authier et al., 2003a,b; Cata et al., 2006; Xiao et al., 2012).

LOSS OF HEAT SENSITIVITY MAY RESULT FROM SENSITIZATION/DESENSITIZATION OF TRPV1

Loss of heat sensibility may be due to myelinated A-fiber damage and loss of transient receptor potential vanilloid 1 (TRPV1)-expression (Woodbury et al., 2004) C-fibers (Dougherty et al., 2004).

A small increase in ROS production activates transcriptional machinery to enhance TRPV1 expression levels in C-fibers (Suzukawa et al., 2000; Kishi et al., 2002; Schmeichel et al., 2003). Additionally, nerve growth factor (NGF) facilitates increased TRPV1 expression by nociceptive C-fibers and directly increases the number of neurons that respond to noxious heat (Stucky and Lewin, 1999; Amaya et al., 2004). Enhanced thermal sensitivity results from sensitization (phosphorylation) of TRPV1, transduced by protein kinase C (PKC) (Kamei et al., 2001; Di Marzo et al., 2002; Hong and Wiley, 2005) and/or mitogen-activated protein kinases (MAPK) (Ji et al., 2002; Clapham, 2003). In the DRGs and hindpaw skin of hyperalgesic and hypoalgesic mice, TRPV1 expression levels are increased and decreased, respectively (Pabbidi et al., 2008). Thermal hypoalgesia may be underpinned by reduced TRPV1 expression and function, that in turn may lead to more serious complications (Pabbidi et al., 2008).

Other TRP channels implicated in the pathogenesis of CIPN include TRPA1 that is expressed by nociceptors and is activated by oxidative stress. The transient benefit of the TRPA1 antagonist HC-030031 in mice with bortezomib or oxaliplatin-induced CIPN, suggests a role for early activation/sensitization of TRPA1 by oxidative stress by-products in establishment of CIPN (Trevisan et al., 2013). Additionally, TRPV4 may contribute to paclitaxel-induced mechanical hypersensitivity in CIPN (Alessandri-Haber et al., 2004), whereas TRPA1 and TRPM8 over-expression were induced in the DRGs by oxaliplatin (Anand et al., 2010; Descoeur et al., 2011). Cisplatin and oxaliplatin-induced neurotoxicity of DRG neurons in rats results in p38 MAPK and ERK1/2 activation as well as a reduction in JNK/Sapk phosphorylation (Scuteri et al., 2009, 2010). Apart from the foregoing, a broad array of other molecular mechanisms have been implicated in the pathobiology of CIPN and these have been reviewed elsewhere (Jaggi and Singh, 2012; Wang et al., 2012) and are summarized in **Table 4**.

BETWEEN CANCER CHEMOTHERAPY AGENT DIFFERENCES IN THE PATHOBIOLOGY OF CIPN

CIPN affects sensory nerves predominantly; while motor, autonomic or CNS (Schlegel, 2011) involvement is rare (Grisold et al., 2012). Sensory nerves allow the perception of touch, pain, temperature (small fiber); position, and vibration (large fiber) (Wilkes, 2007). The persistent cumulative injury caused by cancer chemotherapy agents most often affects sensory nerve cell bodies in the DRGs (e.g., cisplatin) and/or the afferent and efferent axons lying outside the spinal cord (e.g., paclitaxel, oxaliplatin) (Quasthoff and Hartung, 2002).

It is generally assumed that platinum compounds irreversibly bind to DNA thereby inducing apoptosis of primary sensory neurons (Velasco and Bruna, 2010). Antitubulins (paclitaxel, docetaxel and vincristine) bind to microtubules, interrupt axonal transport, target the soma of sensory neurons as well as nerve axons, to induce neuronal death (Bennett, 2010; Cavaletti and Marmiroli, 2010; Velasco and Bruna, 2010). In cultured rat DRG neurons, paclitaxel increased the release of the pro-nociceptive neuropeptide, substance P, whereas oxaliplatin did not; the extent to which this difference contributes to differences in paclitaxel and oxaliplatin-induced peripheral nerve neurotoxicity, remains to be determined (Tatsushima et al., 2011). In patients with CIPN, sensory testing shows that peripheral nerve abnormalities appear to have distinct features depending upon the cancer chemotherapeutic agent involved (Cata et al., 2006), but the mechanistic basis remains unclear (Gilchrist, 2012).

Conversely, it is also likely that one or more pathobiologic mechanisms are shared among anticancer agents (Dougherty et al., 2004; Grisold et al., 2012; Zheng et al., 2012). For example, nerve biopsies from rodents and patients administered cisplatin (Dougherty et al., 2004), paclitaxel, oxaliplatin, vincristine, and bortezomib show similar morphological changes (loss of IENFs) even though these compounds have different neurotoxic targets (Flatters and Bennett, 2006; Bennett et al., 2011; Boyette-Davis et al., 2011; Burakgazi et al., 2011; Pachman et al., 2011; Xiao et al., 2012; Zheng et al., 2012). Additionally, mitotoxicity appears to be a factor in common in the pathobiology of CIPN induced by the taxane, paclitaxel, the platinum-complex agent, oxaliplatin, and the proteasome-inhibitor, bortezomib, in rodent models (Zheng et al., 2011, 2012; Xiao et al., 2012).

Although CIPN may share mediators in common with other types of neuropathic pain, the disparity in efficacy of anti-neuropathic agents suggests underlying mechanistic differences (Farquhar-Smith, 2011). For example, NGF deficiency in peripheral nerves is a phenomenon in common between cisplatin-induced CIPN (Cavaletti et al., 2002) and early diabetic neuropathy (Anand, 2004). Hypersensitivity to heat is common in the CCI-rat model of neuropathic pain, but it is very minor or absent in rat models of CIPN (Bennett, 2010) and in patients with either CIPN (Dougherty et al., 2004; Hershman et al., 2011) or diabetic neuropathy (Sorensen et al., 2006; Nahman-Averbuch et al., 2011). Such dissociations indicate that the pathophysiological mechanisms responsible for peripheral nerve injury and neuropathic pain are at least in part dependent upon the cause of the nerve injury (Bennett, 2010).

CONCLUSION

CIPN is characterized by multiple sensory changes including the development of (i) mechanical allodynia, whereby light pressure or touch that would normally be perceived as innocuous,

Table 4 | Molecular mechanisms implicated in the pathogenesis of CIPN.

Chemotherapy agents	Rodent CIPN models and human studies	Mechanism	References
Cisplatin Oxaliplatin	Male C57BL6 mice Female Wistar rats-cultured DRGs	Up-regulation of TRPV1, TRPA1 and TRPM8 TRPM8 and/or TRPA1 over-expression; respond to cold allodynia	Anand et al., 2010; Ta et al., 2010; Descoeur et al., 2011; Goswami, 2012
Cisplatin Oxaliplatin	Male SD rats	Activation of p38 MAPK and ERK1/2, along with downregulation of SAPK/JNK in cultured DRGs	Scuteri et al., 2010
Vincristine Paclitaxel	Male SD rats	Calcium increase either by influx of extracellular Ca^{2+}or release from mitochondrial intracellular stores, binding to $\alpha_2\delta$ subunit of Ca^{2+} channel; decreased calcium flux	Xiao et al., 2007; Kaur et al., 2010
Paclitaxel	Human neuroblastoma cell line, SHSY-5Y	Activation of calpain, degradation of neuronal calcium sensor (NCS-1), and loss of intracellular calcium signaling	Benbow et al., 2012
Paclitaxel Vincristine Cisplatin Oxaliplatin Bortezomib	Female/male Wistar rats Male SD rats	NMDA receptor antagonists antagonize CIPN in prevention but not intervention protocol or only at high doses	Pascual et al., 2010; Mihara et al., 2011
Oxaliplatin Cisplatin Vincristine	Male mice- C57BL6J Male SD rats	DNA damage	Brederson et al., 2012; Ta et al., 2013
Oxaliplatin	Male SD rats	Increase in PKC activity in supra-spinal regions	Norcini et al., 2009
Paclitaxel but Not Oxaliplatin	Male SD rats- cultured DRG	Increased release of substance P and altered CGRP and somatostatin release	Tatsushima et al., 2011
Cisplatin Paclitaxel	Female patients Female Wistar rats	Decrease in NGF levels by Total Neuropathy Score (TNS) in patient and in rat plasma samples	Cavaletti et al., 2002, 2004
Oxaliplatin	Patients Rats	Dysfunction of axonal Na^+ channels Dysfunction of axonal K^+channels	Park et al., 2011; Kagiava et al., 2013
Vincristine	Female Inbred C57BL mice	Increase in 5-HT_{2A} receptors in dorsal horn and DRGs	Hansen et al., 2011
Paclitaxel	Male C57BL/6 mice	Antagonists of Kinin B1 and B2 receptors attenuate CIPN	Costa et al., 2011
Cisplatin Paclitaxel	Male SD rats	Activation of cannabinoid CB2 receptors	Deng et al., 2012
Paclitaxel	Female WT and σ_1-KO CD-1mice	Antagonists of the sigma-1 receptor attenuate CIPN	Nieto et al., 2012

(Continued)

Table 4 | Continued

Chemotherapy agents	Rodent CIPN models and human studies	Mechanism	References
Oxaliplatin	Patients	Integrin beta-3 L33P is related to CIPN severity but not the development of CIPN	Antonacopoulou et al., 2010
Paclitaxel Cisplatin	Male SD rats	Inflammation	Alaedini et al., 2008; Wang et al., 2012
Taxol Oxaliplatin	Balb/c mice	Increased glial fibrillary acidic protein expression in satellite glial cells, and gap junction-mediated coupling between satellite glial cells	Warwick and Hanani, 2013
Oxaliplatin	Male SD rats	Activation of spinal astrocytes accompanied by increased expression of astrocyte-astrocyte gap junction connections via Cx43	Yoon et al., 2013
		Activation of drug transporters (nervous system transporters including glutamate, copper transporters, etc.)	Ceresa and Cavaletti, 2011
		Patient's genetic background	Windebank and Grisold, 2008; Broyl et al., 2010; Grisold et al., 2012

CGRP, Calcitonin gene related peptide; IENFs, intraepidermal nerve fibers; MAPK, mitogen activated protein kinase; NMDA (N-methyl-D-aspartate) receptors; TRPV, transient receptor potential vanilloid.

evokes pain, (ii) cold allodynia whereby cold temperature evokes a painful sensation, (iii) slowing of SNCV, and (iv) loss of heat sensitivity.

Although the precise pathobiology of CIPN remains to be fully elucidated, recent research implicates "terminal arbor degeneration" (Bennett et al., 2011) and the associated mitochondrial dysfunction and mitotoxicity (Podratz et al., 2011; Zheng et al., 2012) as well as oxidative stress (Nasu et al., 2013). Additional investigation is required to better define subtle between-chemotherapy agent differences in the pathogenesis of CIPN as a means for enhancing rational discovery of novel treatments with potential to prevent and/or attenuate the development of CIPN.

REFERENCES

Alaedini, A., Xiang, Z., Kim, H., Sung, Y. J., and Latov, N. (2008). Up-regulation of apoptosis and regeneration genes in the dorsal root ganglia during cisplatin treatment. *Exp. Neurol.* 210, 368–374. doi: 10.1016/j.expneurol.2007.11.018

Alessandri-Haber, N., Dina, O. A., Yeh, J. J., Parada, C. A., Reichling, D. B., and Levine, J. D. (2004). Transient receptor potential vanilloid 4 is essential in chemotherapy-induced neuropathic pain in the rat. *J. Neurosci.* 24, 4444–4452. doi: 10.1523/JNEUROSCI.0242-04.2004

Aley, K. O., Reichling, D. B., and Levine, J. D. (1996). Vincristine hyperalgesia in the rat: a model of painful vincristine neuropathy in humans. *Neuroscience* 73, 259–265. doi: 10.1016/0306-4522(96)00020-6

Al Moundhri, M. S., Al-Salam, S., Al Mahrouqee, A., Beegam, S., and Ali, B. H. (2013). The effect of curcumin on oxaliplatin and cisplatin neurotoxicity in rats: some behavioral, biochemical, and histopathological studies. *J. Med. Toxicol.* 9, 25–33. doi: 10.1007/s13181-012-0239-x

Amaya, F., Shimosato, G., Nagano, M., Ueda, M., Hashimoto, S., Tanaka, Y., et al. (2004). NGF and GDNF differentially regulate TRPV1 expression that contributes to development of inflammatory thermal hyperalgesia. *Eur. J. Neurosci.* 20, 2303–2310. doi: 10.1111/j.1460-9568.2004.03701.x

Anand, P. (2004). "Neurotrophic factors and their receptors in human sensory neuropathies," in *Ngf and Related Molecules in Health and Disease*, ed L. C. L. Aloe (Italy: Elsevier), 477–492.

Anand, U., Otto, W. R., and Anand, P. (2010). Sensitization of capsaicin and icilin responses in oxaliplatin treated adult rat DRG neurons. *Mol. Pain* 6. doi: 10.1186/1744-8069-1186-1182

Antonacopoulou, A. G., Argyriou, A. A., Scopa, C. D., Kottorou, A., Kominea, A., Peroukides, S., et al. (2010). Integrin beta-3 L33P: a new insight into the pathogenesis of chronic oxaliplatin-induced peripheral neuropathy? *Eur. J. Neurol.* 17, 963–968. doi: 10.1111/j.1468-1331.2010.02966.x

Argyriou, A. A., Cavaletti, G., Briani, C., Velasco, R., Bruna, J., Campagnolo, M., et al. (2013). Clinical pattern and associations of oxaliplatin acute neurotoxicity: a prospective study in 170 patients with colorectal cancer. *Cancer* 119, 438–444. doi: 10.1002/cncr.27732

Arrieta, O., Hernandez-Pedro, N., Fernandez-Gonzalez-Aragon, M. C., Saavedra-Perez, D., Campos-Parra, A. D., Rios-Trejo, M. A., et al. (2011). Retinoic acid reduces chemotherapy-induced neuropathy in an animal model and patients with lung cancer. *Neurology* 77, 987–995. doi: 10.1212/WNL.0b013e31822e045c

Attal, N., Bouhassira, D., Gautron, M., Vaillant, J. N., Mitry, E., Lepere, C., et al. (2009). Thermal hyperalgesia as a marker of oxaliplatin neurotoxicity: a prospective quantified sensory assessment study. *Pain* 144, 245–252. doi: 10.1016/j.pain.2009.03.024

Authier, N., Fialip, J., Eschalier, A., and Coudore, F. (2000a). Assessment of allodynia and hyperalgesia after cisplatin administration to rats. *Neurosci. Lett.* 291, 73–76. doi: 10.1016/S0304-3940(00)01373-2

Authier, N., Gillet, J.-P., Fialip, J., Eschalier, A., and Coudore, F. (2000b). Description of a short-term Taxol ő-induced nociceptive neuropathy in rats. *Brain Res.* 887, 239–249. doi: 10.1016/S0006-8993(00)02910-3

Authier, N., Gillet, J.P., Fialip, J., Eschalier, A., and Coudore, F. (2003a). An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. *Exp. Neurol.* 182, 12–20. doi: 10.1016/S0014-4886(03)00003-7

Authier, N., Gillet, J. P., Fialip, J., Eschalier, A., and Coudore, F. (2003b). A new animal model of vincristine-induced nociceptive peripheral neuropathy. *Neurotoxicology* 24, 797–805. doi: 10.1016/S0161-813X(03)00043-3

Balayssac, D., Cayre, A., Authier, N., Bourdu, S., Penault-Llorca, F., Gillet, J. P., et al. (2005). Patterns of P-glycoprotein activity in the nervous system during vincristine-induced neuropathy in rats. *J. Peripher. Nerv. Syst.* 10, 301–310. doi: 10.1111/j.1085-9489.2005.10308.x

Balayssac, D., Ferrier, J., Descoeur, J., Ling, B., Pezet, D., Eschalier, A., et al. (2011). Chemotherapy-induced peripheral neuropathies: from clinical relevance to preclinical evidence. *Expert Opin. Drug Saf.* 10, 407–417. doi: 10.1517/14740338.2011.543417

Benbow, J. H., Mann, T., Keeler, C., Fan, C. P., Hodsdon, M. E., Lolis, E., et al. (2012). Inhibition of paclitaxel-induced decreases in calcium signaling. *J. Biol. Chem.* 287, 37907–37916. doi: 10.1074/jbc.M112.385070

Bennett, G. J. (2010). Pathophysiology and animal models of cancer-related painful peripheral neuropathy. *Oncologist* 15, 9–12. doi: 10.1634/theoncologist.2009-S503

Bennett, G. J., Liu, G. K., Xiao, W. H., Jin, H. W., and Siau, C. (2011). Terminal arbor degeneration – a novel lesion produced by the antineoplastic agent paclitaxel. *Eur. J. Neurosci.* 33, 1667–1676. doi: 10.1111/j.1460-9568.2011.07652.x

Bergstresser, P. R., and Taylor, J. R. (1977). Epidermal 'turnover time'—a new examination. *Br. J. Dermatol.* 96, 503–506. doi: 10.1111/j.1365-2133.1977.tb07152.x

Bianchi, G., Vitali, G., Caraceni, A., Ravaglia, S., Capri, G., Cundari, S., et al. (2005). Symptomatic and neurophysiological responses of paclitaxel- or cisplatin-induced neuropathy to oral acetyl-L-carnitine. *Eur. J. Cancer* 41, 1746–1750. doi: 10.1016/j.ejca.2005.04.028

Boland, B. A., Sherry, V., and Polomano, R. C. (2010). Chemotherapy-induced peripheral neuropathy in cancer survivors. *Oncol. Nurse Edn.* 24, 33–38, 42–43.

Boyette-Davis, J., and Dougherty, P. M. (2011). Protection against oxaliplatin-induced mechanical hyperalgesia and intraepidermal nerve fiber loss by minocycline. *Exp. Neurol.* 229, 353–357. doi: 10.1016/j.expneurol.2011.02.019

Boyette-Davis, J., Xin, W., Zhang, H., and Dougherty, P. M. (2011). Intraepidermal nerve fiber loss corresponds to the development of Taxol-induced hyperalgesia and can be prevented by treatment with minocycline. *Pain* 152, 308–313. doi: 10.1016/j.pain.2010.10.030

Breathnach, A. S. (1977). Electron microscopy of cutaneous nerves and receptors. *J. Invest. Dermatol.* 69, 8–26. doi: 10.1111/1523-1747.ep12497857

Brederson, J. D., Joshi, S. K., Browman, K. E., Mikusa, J., Zhong, C., Gauvin, D., et al. (2012). PARP inhibitors attenuate chemotherapy-induced painful neuropathy. *J. Peripher. Nerv. Syst.* 17, 324–330. doi: 10.1111/j.1529-8027.2012.00413.x

Broyl, A., Corthals, S. L., Jongen, J. L. M., van der Holt, B., Kuiper, R., de Knegt, Y., et al. (2010). Mechanisms of peripheral neuropathy associated with bortezomib and vincristine in patients with newly diagnosed multiple myeloma: a prospective analysis of data from the HOVON-65/GMMG-HD4 trial. *Lancet Oncol.* 11, 1057–1065. doi: 10.1016/S1470-2045(10)70206-0

Bruna, J., Udina, E., Alé, A., Vilches, J. J., Vynckier, A., Monbaliu, J., et al. (2010). Neurophysiological, histological and immunohistochemical characterization of bortezomib-induced neuropathy in mice. *Exp. Neurol.* 223, 599–608. doi: 10.1016/j.expneurol.2010.02.006

Bujalska, M., and Makulska-Nowak, H. (2009). Bradykinin receptors antagonists and nitric oxide synthase inhibitors in vincristine and streptozotocin induced hyperalgesia in chemotherapy and diabetic neuropathy rat model. *Neuro Endocrinol. Lett.* 30, 144–152.

Burakgazi, A. Z., Messersmith, W., Vaidya, D., Hauer, P., Hoke, A., and Polydefkis, M. (2011). Longitudinal assessment of oxaliplatin-induced neuropathy. *Neurology* 77, 980–986. doi: 10.1212/WNL.0b013e31822cfc59

Burton, A. W., Fanciullo, G. J., Beasley, R. D., and Fisch, M. J. (2007). Chronic pain in the cancer survivor: a new frontier. *Pain Med.* 8, 189–198. doi: 10.1111/j.1526-4637.2006.00220.x

Carozzi, V. A., Canta, A., Oggioni, N., Sala, B., Chiorazzi, A., Meregalli, C., et al. (2010a). Neurophysiological and neuropathological characterization of new murine models of chemotherapy-induced chronic peripheral neuropathies. *Exp. Neurol.* 226, 301–309. doi: 10.1016/j.expneurol.2010.09.004

Carozzi, V. A., Chiorazzi, A., Canta, A., Lapidus, R. G., Slusher, B. S., Wozniak, K. M., et al. (2010b). Glutamate carboxypeptidase inhibition reduces the severity of chemotherapy-induced peripheral neurotoxicity in rat. *Neurotox. Res.* 17, 380–391. doi: 10.1007/s12640-009-9114-1

Cascinu, S., Catalano, V., Cordella, L., Labianca, R., Giordani, P., Baldelli, A. M., et al. (2002). Neuroprotective effect of reduced glutathione on oxaliplatin-based chemotherapy in advanced colorectal cancer: a randomized, double-blind, placebo-controlled trial. *J. Clin. Oncol.* 20, 3478–3483. doi: 10.1200/JCO.2002.07.061

Cata, J. P., Weng, H. R., Burton, A. W., Villareal, H., Giralt, S., and Dougherty, P. M. (2007). Quantitative sensory findings in patients with bortezomib-induced pain. *J. Pain* 8, 296–306. doi: 10.1016/j.jpain.2006.09.014

Cata, J. P., Weng, H. R., and Dougherty, P. M. (2008). Behavioral and electrophysiological studies in rats with cisplatin-induced chemoneuropathy. *Brain Res.* 1230, 91–98. doi: 10.1016/j.brainres.2008.07.022

Cata, J. P., Weng, H. R., Lee, B. N., Reuben, J. M., and Dougherty, P. M. (2006). Clinical and experimental findings in humans and animals with chemotherapy-induced peripheral neuropathy. *Minerva Anestesiol.* 72, 151–169.

Cavaletti, G., Bogliun, G., Marzorati, L., Zincone, A., Piatti, M., Colombo, N., et al. (2004). Early predictors of peripheral neurotoxicity in cisplatin and paclitaxel combination chemotherapy. *Ann. Oncol.* 15, 1439–1442. doi: 10.1093/annonc/mdh348

Cavaletti, G., Gilardini, A., Canta, A., Rigamonti, L., Rodriguez-Menendez, V., Ceresa, C., et al. (2007). Bortezomib-induced peripheral neurotoxicity: a neurophysiological and pathological study in the rat. *Exp. Neurol.* 204, 317–325. doi: 10.1016/j.expneurol.2006.11.010

Cavaletti, G., and Marmiroli, P. (2010). Chemotherapy-induced peripheral neurotoxicity. *Nat. Rev. Neurol.* 6, 657–666. doi: 10.1038/nrneurol.2010.160

Cavaletti, G., Pezzoni, G., Pisano, C., Oggioni, N., Sala, F., Zoia, C., et al. (2002). Cisplatin-induced peripheral neurotoxicity in rats reduces the circulating levels of nerve growth factor. *Neurosci. Lett.* 322, 103–106. doi: 10.1016/S0304-3940(02)00091-5

Cavaletti, G., Tredici, G., Braga, M., and Tazzari, S. (1995). Experimental peripheral neuropathy induced in adult-rats by repeated intraperitoneal administration of taxol. *Exp. Neurol.* 133, 64–72. doi: 10.1006/exnr.1995.1008

Ceresa, C., and Cavaletti, G. (2011). Drug transporters in chemotherapy induced peripheral neurotoxicity: current knowledge and clinical implications. *Curr. Med. Chem.* 18, 329–341. doi: 10.2174/092986711794839160

Chen, H., and Chan, D. C. (2006). Critical dependence of neurons on mitochondrial dynamics. *Curr. Opin. Cell Biol.* 18, 453–459. doi: 10.1016/j.ceb.2006.06.004

Clapham, D. E. (2003). TRP channels as cellular sensors. *Nature* 426, 517–524. doi: 10.1038/nature02196

Colombo, N., Bini, S., Miceli, D., Bogliun, G., Marzorati, L., Cavaletti, G., et al. (1995). Weekly cisplatin+/-glutathione in relapsed ovarian-carcinoma. *Int. J. Gynecol. Cancer* 5, 81–86. doi: 10.1046/j.1525-1438.1995.05020081.x

Costa, R., Motta, E. M., Dutra, R. C., Manjavachi, M. N., Bento, A. F., Malinsky, F. R., et al. (2011). Anti-nociceptive effect of kinin B-1 and B-2 receptor antagonists on peripheral neuropathy induced by paclitaxel in mice. *Br. J. Pharmacol.* 164, 681–693. doi: 10.1111/j.1476-5381.2011.01408.x

Croteau, D. L., Stierum, R. H., and Bohr, V. A. (1999). Mitochondrial DNA repair pathways. *Mutat. Res. DNA Repair* 434, 137–148. doi: 10.1016/S0921-8777(99)00025-7

Deng, L. T., Guindon, J., Vemuri, V. K., Thakur, G. A., White, F. A., Makriyannis, A., et al. (2012). The maintenance of cisplatin- and paclitaxel-induced mechanical and cold allodynia is suppressed by cannabinoid CB2 receptor activation and independent of CXCR4 signaling in models of chemotherapy-induced peripheral neuropathy. *Mol. Pain* 8, 1–12. doi: 10.1186/1744-8069-1188-1171

Descoeur, J., Pereira, V., Pizzoccaro, A., Francois, A., Ling, B., Maffre, V., et al. (2011). Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors. *Embo. Mol. Med.* 3, 266–278. doi: 10.1002/emmm.201100134

Devigili, G., Tugnoli, V., Penza, P., Camozzi, F., Lombardi, R., Melli, G., et al. (2008). The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. *Brain* 131, 1912–1925. doi: 10.1093/brain/awn093

Di Cesare Mannelli, L., Zanardelli, M., Failli, P., and Ghelardini, C. (2012). Oxaliplatin-induced neuropathy: oxidative stress as pathological mechanism. protective effect of silibinin. *J. Pain* 13, 276–284. doi: 10.1016/j.jpain.2011.11.009

Di Marzo, V., Blumberg, P. M., and Szallasi, A. (2002). Endovanilloid signaling in pain. *Curr. Opin. Neurobiol.* 12, 372–379. doi: 10.1016/S0959-4388(02)00340-9

Dougherty, P. M., Cata, J. P., Cordella, J. V., Burton, A., and Weng, H.-R. (2004). Taxol-induced sensory disturbance is characterized by preferential impairment of myelinated fiber function in cancer patients. *Pain* 109, 132–142. doi: 10.1016/j.pain.2004.01.021

Doyle, T., Chen, Z., Muscoli, C., Bryant, L., Esposito, E., Cuzzocrea, S., et al. (2012). Targeting the overproduction of peroxynitrite for the prevention and reversal of paclitaxel-induced neuropathic pain. *J. Neurosci.* 32, 6149–6160. doi: 10.1523/JNEUROSCI.6343-11.2012

Farquhar-Smith, P. (2011). Chemotherapy-induced neuropathic pain. *Curr. Opin. Support Palliat. Care* 5, 1–7. doi: 10.1097/SPC.0b013e328342f9cc

Fidanboylu, M., Griffiths, L. A., and Flatters, S. J. L. (2011). Global inhibition of reactive oxygen species (ROS) inhibits paclitaxel-induced painful peripheral neuropathy. *PLoS ONE* 6:e25212. doi: 10.1371/journal.pone.0025212

Fischer, S. J., McDonald, E. S., Gross, L., and Windebank, A. J. (2001). Alterations in cell cycle regulation underlie cisplatin induced apoptosis of dorsal root ganglion neurons *in vivo*. *Neurobiol. Dis.* 8, 1027–1035. doi: 10.1006/nbdi.2001.0426

Flatters, S. J. L., and Bennett, G. J. (2004). Ethosuximide reverses paclitaxel- and vincristine-induced painful peripheral neuropathy. *Pain* 109, 150–161. doi: 10.1016/j.pain.2004.01.029

Flatters, S. J. L., and Bennett, G. J. (2006). Studies of peripheral sensory nerves in paclitaxel-induced painful peripheral neuropathy: evidence for mitochondrial dysfunction. *Pain* 122, 245–257. doi: 10.1016/j.pain.2006.01.037

Flatters, S. J. L., Xiao, W. H., and Bennett, G. J. (2006). Acetyl-L-carnitine prevents and reduces paclitaxel-induced painful peripheral neuropathy. *Neurosci. Lett.* 397, 219–223. doi: 10.1016/j.neulet.2005.12.013

Florea, A.-M., and Büsselberg, D. (2011). Cisplatin as an anti-tumor drug: cellular mechanisms of activity, drug resistance and induced side effects. *Cancers* 3, 1351–1371. doi: 10.3390/cancers3011351

Garcia, J. M., Cata, J. P., Dougherty, P. M., and Smith, R. G. (2008). Ghrelin prevents cisplatin-induced mechanical hyperalgesia and cachexia. *Endocrinology* 149, 455–460. doi: 10.1210/en.2007-0828

Gedlicka, C., Kornek, G. V., Schmid, K., and Scheithauer, W. (2003). Amelioration of docetaxel/cisplatin induced polyneuropathy by alpha-lipoic acid. *Ann. Oncol.* 14, 339–340. doi: 10.1093/annonc/mdg051

Gedlicka, C., Scheithauer, W., Schull, B., and Kornek, G. V. (2002). Effective treatment of oxaliplatin-induced cumulative polyneuropathy with alpha-lipoic acid. *J. Clin. Oncol.* 20, 3359–3361. doi: 10.1200/JCO.2002.99.502

Ghirardi, O., Vertechy, M., Vesci, L., Canta, A., Nicolini, G., Galbiati, S., et al. (2005). Chemotherapy-induced allodinia: neuroprotective effect of acetyl-L-carnitine. *In Vivo* 19, 631–637.

Giannoccaro, M. P., Donadio, V., Gomis Perez, C., Borsini, W., Di Stasi, V., and Liguori, R. (2011). Somatic and autonomic small fiber neuropathy induced by bortezomib therapy: an immunofluorescence study. *Neurol. Sci.* 32, 361–363. doi: 10.1007/s10072-010-0475-2

Gilardini, A., Avila, R. L., Oggioni, N., Rodriguez-Menendez, V., Bossi, M., Canta, A., et al. (2012). Myelin structure is unaltered in chemotherapy-induced peripheral neuropathy. *Neurotoxicology* 33, 1–7. doi: 10.1016/j.neuro.2011.10.010

Gilchrist, L. (2012). Chemotherapy-induced peripheral neuropathy in pediatric cancer patients. *Semin. Pediatr. Neurol.* 19, 9–17. doi: 10.1016/j.spen.2012.02.011

Goel, S., Goldberg, G. L., Kuo, D. Y., Muggia, F., Arezzo, J., and Mani, S. (2008). Novel neurosensory testing in cancer patients treated with the epothilone B analog, ixabepilone. *Ann. Oncol.* 19, 2048–2052. doi: 10.1093/annonc/mdn420

Goswami, C. (2012). TRPV1-tubulin complex: involvement of membrane tubulin in the regulation of chemotherapy-induced peripheral neuropathy. *J. Neurochem.* 123, 1–13. doi: 10.1111/j.1471-4159.2012.07892.x

Gregg, R. W., Molepo, J. M., Monpetit, V. J. A., Mikael, N. Z., Redmond, D., Gadia, M., et al. (1992). Cisplatin neurotoxicity—the relationship between dosage, time, and platinum concentration in neurologic tissues, and morphological evidence of toxicity. *J. Clin. Oncol.* 10, 795–803.

Grisold, W., Oberndorfer, S., and Windebank, A. J. (2012). Chemotherapy and Polyneuropathies. *Eur. Assoc. NeuroOncol. Mag.* 2, 25–36.

Gutiérrez-Gutiérrez, G., Sereno, M., Miralles, A., Casado-Sáenz, E., and Gutiérrez-Rivas, E. (2010). Chemotherapy-induced peripheral neuropathy: clinical features, diagnosis, prevention and treatment strategies. *Clin. Transl. Oncol.* 12, 81–91. doi: 10.1007/S12094-010-0474-z

Hansen, N., Uceyler, N., Palm, F., Zelenka, M., Biko, L., Lesch, K. P., et al. (2011). Serotonin transporter deficiency protects mice from mechanical allodynia and heat hyperalgesia in vincristine neuropathy. *Neurosci. Lett.* 495, 93–97. doi: 10.1016/j.neulet.2011.03.035

Herrmann, D. N., Griffin, J. W., Hauer, P., Cornblath, D. R., and McArthur, J. C. (1999). Epidermal nerve fiber density and sural nerve morphometry in peripheral neuropathies. *Neurology* 53, 1634–1640. doi: 10.1212/WNL.53.8.1634

Hershman, D. L., Fehrenbacher, L., Wade, R. J. L., Wong, S.-F., Hortobagyi, G. N., Meyskens, F. L., et al. (2013). Randomized double-blind placebo-controlled trial of acetyl-L-carnitine for the prevention of taxane-induced neuropathy in women undergoing adjuvant breast cancer therapy. *J. Clin. Oncol.* 31, 2627. doi: 10.1200/JCO.2012.44.8738

Hershman, D. L., Weimer, L. H., Wang, A., Kranwinkel, G., Brafman, L., Fuentes, D., et al. (2011). Association between patient reported outcomes and quantitative sensory tests for measuring long-term neurotoxicity in breast cancer survivors treated with adjuvant paclitaxel chemotherapy. *Breast Cancer Res. Treat.* 125, 767–774. doi: 10.1007/s10549-010-1278-0

Hilpert, F., Stahle, A., Tome, O., Burges, A., Rossner, D., Spathe, K., et al. (2005). Neuroprotection with amifostine in the first-line treatment of advanced ovarian cancer with carboplatin/paclitaxel-based chemotherapy—a double-blind, placebo-controlled, randomized phase II study from the Arbeitsgemeinschaft Gynakologische Onkologoie (AGO) Ovarian Cancer Study Group. *Support. Care Cancer* 13, 797–805. doi: 10.1007/s00520-005-0782-y

Holland, N. R., Crawford, T. O., Hauer, P., Cornblath, D. R., Griffin, J. W., and McArthur, J. C. (1998). Small-fiber sensory neuropathies: clinical course and neuropathology of idiopathic cases. *Ann. Neurol.* 44, 47–59. doi: 10.1002/ana.410440111

Hong, S. S., and Wiley, J. W. (2005). Early painful diabetic neuropathy is associated with differential changes in the expression and function of vanilloid receptor 1. *J. Biol. Chem.* 280, 618–627.

Hori, K., Ozaki, N., Suzuki, S., and Sugiura, Y. (2010). Upregulations of P2X(3) and ASIC3 involve in hyperalgesia induced by cisplatin administration in rats. *Pain* 149, 393–405. doi: 10.1016/j.pain.2010.03.005

Hoy, S. M. (2013). Subcutaneous bortezomib: in multiple myeloma. *Drugs* 73, 45–54. doi: 10.1007/s40265-013-0006-6

Ja'afer, F. M. H., Hamdan, F. B., and Mohammed, F. H. (2006). Vincristine-induced neuropathy in rat: electrophysiological and histological study. *Exp. Brain Res.* 173, 334–345.doi: 10.1007/s00221-006-0499-2

Jaggi, A. S., and Singh, N. (2012). Mechanisms in cancer-chemotherapeutic drugs-induced peripheral neuropathy. *Toxicology* 291, 1–9. doi: 10.1016/j.tox.2011.10.019

Jamieson, S. M. F., Liu, J. J., Connor, B., Dragunow, M., and McKeage, M. J. (2007). Nucleolar enlargement, nuclear eccentricity and altered cell body immunostaining characteristics of large-sized sensory neurons following treatment of rats with paclitaxel. *Neurotoxicology* 28, 1092–1098. doi: 10.1016/j.neuro.2007.04.009

Janes, K., Doyle, T., Bryant, L., Esposito, E., Cuzzocrea, S., Ryerse, J., et al. (2013). Bioenergetic deficits in peripheral nerve sensory axons during chemotherapy-induced neuropathic pain resulting from peroxynitrite-mediated post-translational nitration of mitochondrial superoxide dismutase. *Pain* 154, 2432–2440. doi: 10.1016/j.pain.2013.07.032

Ji, R. R., Samad, T. A., Jin, S. X., Schmoll, R., and Woolf, C. J. (2002). p38 MAPK activation by NGF in primary sensory neurons after inflammation increases TRPV1 levels and maintains heat hyperalgesia. *Neuron* 36, 57–68. doi: 10.1016/S0896-6273(02)00908-X

Jimenez-Andrade, J. M., Peters, C. M., Mejia, N. A., Ghilardi, J. R., Kuskowski, M. A., and Mantyh, P. W. (2006). Sensory neurons and their supporting cells located in the trigeminal, thoracic and lumbar ganglia differentially express markers of injury following intravenous administration of paclitaxel in the rat. *Neurosci. Lett.* 405, 62–67. doi: 10.1016/j.neulet.2006.06.043

Jin, H. W., Flatters, S. J. L., Xiao, W. H., Mulhem, H. L., and Bennett, G. J. (2008). Prevention of paclitaxel-evoked painful peripheral neuropathy by acetyl-L-carnitine: effects on axonal mitochondria, sensory nerve fiber terminal arbors, and cutaneous Langerhans cells. *Exp. Neurol.* 210, 229–237. doi: 10.1016/j.expneurol.2007.11.001

Kagiava, A., Kosmidis, E. K., and Theophilidis, G. (2013). Oxaliplatin-induced hyperexcitation of rat sciatic nerve fibers: an intra-axonal study. *Anticancer Agent. Med. Chem.* 13, 373–379. doi: 10.2174/1871520611313020023

Kamei, J., Tamura, N., and Saitoh, A. (2005). Possible involvement of the spinal nitric oxide/cGMP pathway in vincristine-induced painful neuropathy in mice. *Pain* 117, 112–120. doi: 10.1016/j.pain.2005.05.026

Kamei, J., Zushida, K., Morita, K., Sasaki, M., and Tanaka, S. (2001). Role of vanilloid VR1 receptor in thermal allodynia and hyperalgesia in diabetic mice. *Eur. J. Pharmacol.* 422, 83–86. doi: 10.1016/S0014-2999(01)01059-7

Kanat, O., Evrensel, T., Baran, I., Coskun, H., Zarifoglu, M., Turan, O. F., et al. (2003). Protective effect of amifostine against toxicity of paclitaxel and carboplatin in non-small cell lung cancer: a single center randomized study. *Med. Oncol.* 20, 237–245. doi: 10.1385/MO:20:3:237

Kanavos, P. (2006). The rising burden of cancer in the developing world. *Ann. Oncol.* 17, 15–23. doi: 10.1093/annonc/mdl983

Kaur, G., Jaggi, A. S., and Singh, N. (2010). Exploring the potential effect of Ocimum sanctum in vincristine-induced neuropathic pain in rats. *J. Brachial. Plex. Peripher. Nerve Inj.* 5:3. doi: 10.1186/1749-7221-5-3

Kidd, J. F., Pilkington, M. F., Schell, M. J., Fogarty, K. E., Skepper, J. N., Taylor, C. W., et al. (2002). Paclitaxel affects cytosolic calcium signals by opening the mitochondrial permeability transition pore. *J. Biol. Chem.* 277, 6504–6510. doi: 10.1074/jbc.M106802200

Kim, H. K., Zhang, Y. P., Gwak, Y. S., and Abdi, S. (2010). Phenyl N-tert-butylnitrone, a free radical scavenger, reduces mechanical allodynia in chemotherapy-induced neuropathic pain in rats. *Anesthesiology* 112, 432–439. doi: 10.1097/ALN.0b013e3181ca31bd

Kishi, M., Tanabe, J., Schmelzer, J. D., and Low, P. A. (2002). Morphometry of dorsal root ganglion in chronic experimental diabetic neuropathy. *Diabetes* 51, 819–824. doi: 10.2337/diabetes.51.3.819

Kocer, B., Sucak, G., Kuruoglu, R., Aki, Z., Haznedar, R., and Erdogmus, N. I. (2009). Clinical and electrophysiological evaluation of patients with thalidomide-induced neuropathy. *Acta Neurol. Belg.* 109, 120–126.

Kurniali, P. C., Luo, L. G., and Weitberg, A. B. (2010). Role of calcium/magnesium infusion in oxaliplatin-based chemotherapy for colorectal cancer patients. *Oncology* 24, 289–292.

Landowski, T. H., Megli, C. J., Nullmeyer, K. D., Lynch, R. M., and Dorr, R. T. (2005). Mitochondrial-mediated disregulation of Ca2+ is a critical determinant of Velcade (PS-341/Bortezomib) cytotoxicity in myeloma cell lines. *Cancer Res.* 65, 3828–3836. doi: 10.1158/0008-5472.CAN-04-3684

Lauria, G., Morbin, M., Lombardi, R., Borgna, M., Mazzoleni, G., Sghirlanzoni, A., et al. (2003). Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. *Neurology* 61, 631–636. doi: 10.1212/01.WNL.0000070781.92512.A4

Lee, J., Giordano, S., and Zhang, J. (2012). Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. *Biochem. J.* 441, 523–540. doi: 10.1042/BJ20111451

Lin, P. C., Lee, M. Y., Wang, W. S., Yen, C. C., Chao, T. C., Hsiao, L. T., et al. (2006). N-acetylcysteine has neuroprotective effects against oxaliplatin-based adjuvant chemotherapy in colon cancer patients: preliminary data. *Support. Care Cancer* 14, 484–487. doi: 10.1007/s00520-006-0018-9

Ling, B., Coudoré-Civiale, M., Balayssac, D., Eschalier, A., Coudoré, F., and Authier, N. (2007). Behavioral and immunohistological assessment of painful neuropathy induced by a single oxaliplatin injection in the rat. *Toxicology* 234, 176–184. doi: 10.1016/j.tox.2007.02.013

Liu, C. C., Lu, N., Cui, Y., Yang, T., Zhao, Z. Q., Xin, W. J., et al. (2010). Prevention of paclitaxel-induced allodynia by minocycline: effect on loss of peripheral nerve fibers and infiltration of macrophages in rats. *Mol. Pain* 6:76. doi: 10.1186/1744-8069-6-76

Lomonaco, M., Milone, M., Batocchi, A. P., Padua, L., Restuccia, D., and Tonali, P. (1992). Cisplatin neuropathy: clinical course and neurophysiological findings. *J. Neurol.* 239, 199–204. doi: 10.1007/BF00839140

Lorusso, D., Ferrandina, G., Greggi, S., Gadducci, A., Pignata, S., Tateo, S., et al. (2003). Phase III multicenter randomized trial of amifostine as cytoprotectant in first-line chemotherapy in ovarian cancer patients. *Ann. Oncol.* 14, 1086–1093. doi: 10.1093/annonc/mdg301

Løseth, S., Stålberg, E., Jorde, R., Mellgren, S. I., Institutionen För, N., Uppsala, U., et al. (2008). Early diabetic neuropathy: thermal thresholds and intraepidermal nerve fibre density in patients with normal nerve conduction studies. *J. Neurol.* 255, 1197–1202. doi: 10.1007/s00415-008-0872-0

Lu, P., Fan, Q. X., Wang, L. X., Wang, X., Zong, H., and Wang, R. L. (2008). Prophylactic effect of amifostine on oxaliplatin-related neurotoxicity in patients with digestive tract tumors. *Ai Zheng* 27, 1117–1120.

Luo, Z. D., Chaplan, S. R., Higuera, E. S., Sorkin, L. S., Stauderman, K. A., Williams, M. E., et al. (2001). Upregulation of dorsal root ganglion alpha(2)delta calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J. Neurosci.* 21, 1868–1875.

Maestri, A., Ceratti, A. D. P., Cundari, S., Zanna, C., Cortesi, E., and Crino, L. (2005). A pilot study on the effect of acetyl-L-carnitine in paclitaxel-and cisplatin-induced peripheral neuropathy. *Tumori* 91, 135–138.

McDonald, E. S., Randon, K. R., Knight, A., and Windebank, A. J. (2005). Cisplatin preferentially binds to DNA in dorsal root ganglion neurons *in vitro* and *in vivo*: a potential mechanism for neurotoxicity. *Neurobiol. Dis.* 18, 305–313. doi: 10.1016/j.nbd.2004.09.013

Melli, G., Taiana, M., Camozzi, F., Triolo, D., Podini, P., Quattrini, A., et al. (2008). Alpha-lipoic acid prevents mitochondrial damage and neurotoxicity in experimental chemotherapy neuropathy. *Exp. Neurol.* 214, 276–284. doi: 10.1016/j.expneurol.2008.08.013

Meregalli, C., Canta, A., Carozzi, V. A., Chiorazzi, A., Oggioni, N., Gilardini, A., et al. (2010). Bortezomib-induced painful neuropathy in rats: a behavioral, neurophysiological and pathological study in rats. *Eur. J. Pain* 14, 343–350. doi: 10.1016/j.ejpain.2009.07.001

Meyer, L., Patte-Mensah, C., Taleb, O., and Mensah-Nyagan, A.G. (2011). Allopregnanolone prevents and suppresses oxaliplatin-evoked painful neuropathy: multi-parametric assessment and direct evidence. *Pain* 152, 170–181. doi: 10.1016/j.pain.2010.10.015

Mihara, Y., Egashira, N., Sada, H., Kawashiri, T., Ushio, S., Yano, T., et al. (2011). Involvement of spinal NR2B-containing NMDA receptors in oxaliplatin-induced mechanical allodynia in rats. *Mol. Pain* 7, 1–7. doi: 10.1186/1744-8069-7-8

Milla, P., Airoldi, M., Weber, G., Drescher, A., Jaehde, U., and Cattel, L. (2009). Administration of reduced glutathione in FOLFOX4 adjuvant treatment for colorectal cancer: effect on oxaliplatin pharmacokinetics, Pt-DNA adduct formation, and neurotoxicity. *Anti-Cancer Drugs* 20, 396–402. doi: 10.1097/CAD.0b013e32832a2dc1

Mimura, Y., Kato, H., Eguchi, K., and Ogawa, T. (2000). Schedule dependency of paclitaxel-induced neuropathy in mice: a morphological study. *Neurotoxicology* 21, 513–520.

Mir, O., Alexandre, J., Tran, A., Durand, J. P., Pons, G., Treluyer, J. M., et al. (2009). Relationship between GSTP1 Ile105Val polymorphism and docetaxel-induced peripheral neuropathy: clinical evidence of a role of oxidative stress in taxane toxicity. *Ann. Oncol.* 20, 736–740. doi: 10.1093/annonc/mdn698

Mironov, S. L. (2007). ADP regulates movements of mitochondria in neurons. *Biophysical J.* 92, 2944–2952. doi: 10.1529/biophysj.106.092981

Mo, M., Erdelyi, I., Szigeti-Buck, K., Benbow, J. H., and Ehrlich, B. E. (2012). Prevention of paclitaxel-induced peripheral neuropathy by lithium pretreatment. *FASEB J.* 26, 4696–4709. doi: 10.1096/fj.12-214643

Moore, D. H., Donnelly, J., McGuire, W. P., Almadrones, L., Cella, D. F., Herzog, T. J., et al. (2003). Limited access trial using amifostine for protection against cisplatin- and three-hour paclitaxel-induced neurotoxicity: a phase II study of the Gynecologic Oncology Group. *J. Clinical Oncol.* 21, 4207–4213. doi: 10.1200/JCO.2003.02.086

Muthuraman, A., Jaggi, A. S., Singh, N., and Singh, D. (2008). Ameliorative effects of amiloride and pralidoxime in chronic constriction injury and vincristine induced painful neuropathy in rats. *Eur. J. Pharmacol.* 587, 104–111. doi: 10.1016/j.ejphar.2008.03.042

Nahman-Averbuch, H., Yarnitsky, D., Granovsky, Y., Sprecher, E., Steiner, M., Tzuk-Shina, T., et al. (2011). Pronociceptive pain modulation in patients with painful chemotherapy-induced polyneuropathy. *J. Pain Symptom Manage.* 42, 229–238. doi: 10.1016/j.jpainsymman.2010.10.268

Nakashima, T., Kiba, T., Ogawa, Y., Hosokawa, A., Shintani, H., Okada, Y., et al. (2012). A case of paclitaxel-induced peripheral neuropathy successfully treated with pregabalin. *Gan To Kagaku Ryoho* 39, 1443–1445.

Nasu, S., Misawa, S., Nakaseko, C., Shibuya, K., Isose, S., Sekiguchi, Y., et al. (2013). Bortezomib-induced neuropathy: axonal membrane depolarization precedes development of neuropathy. *Clin. Neurophysiol.* doi: 10.1016/j.clinph.2013.07.014. [Epub ahead of print].

Nguyen, V. H., and Lawrence, H. J. (2004). Use of gabapentin in the prevention of taxane-induced arthralgias and myalgias. *J. Clin. Oncol.* 22, 1767–1769. doi: 10.1200/JCO.2004.99.298

Nieto, F. R., Cendan, C. M., Sanchez-Fernandez, C., Jose Cobos, E., Entrena, J. M., Tejada, M. A., et al. (2012). Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. *J. Pain* 13, 1107–1121. doi: 10.1016/j.jpain.2012.08.006

Norcini, M., Vivoli, E., Galeotti, N., Bianchi, E., Bartolini, A., and Ghelardini, C. (2009). Supraspinal role of protein kinase C in oxaliplatin-induced neuropathy in rat. *Pain* 146, 141–147. doi: 10.1016/j.pain.2009.07.017

Ocean, A. J., and Vahdat, L. T. (2004). Chemotherapy-induced peripheral neuropathy: pathogenesis and emerging therapies. *Support. Care Cancer* 12, 619–625. doi: 10.1007/s00520-004-0657-7

Orlando, G., Pietro Lo, G., Claudio, P., Mario, V., Augusta, B., Loredana, V., et al. (2005). Acetyl-L-carnitine prevents and reverts experimental chronic neurotoxicity induced by oxaliplatin, without altering its antitumor properties. *Anticancer Res.* 25, 2681–2687.

Pabbidi, R. M., Yu, S. Q., Peng, S., Khardori, R., Pauza, M. E., and Premkumar, L. S. (2008). Influence of TRPV1 on diabetes-induced alterations in thermal pain sensitivity. *Mol. Pain* 4:9. doi: 10.1186/1744-8069-4-9

Pachman, D. R., Barton, D. L., Watson, J. C., and Loprinzi, C. L. (2011). Chemotherapy-induced peripheral neuropathy: prevention and treatment. *Clin. Pharmacol. Ther.* 90, 377–387. doi: 10.1038/clpt.2011.115

Paice, J. A. (2011). Chronic treatment-related pain in cancer survivors. *Pain* 152, S84–S89. doi: 10.1016/j.pain.2010.10.010

Park, S. B., Lin, C. S. Y., Krishnan, A. V., Goldstein, D., Friedlander, M. L., and Kiernan, M. C. (2011). Utilizing natural activity to dissect the pathophysiology of acute oxaliplatin-induced neuropathy. *Exp. Neurol.* 227, 120–127. doi: 10.1016/j.expneurol.2010.10.002

Pascual, D., Goicoechea, C., Burgos, E., and Martin, M. I. (2010). Antinociceptive effect of three common analgesic drugs on peripheral neuropathy induced by paclitaxel in rats. *Pharmacol. Biochem. Behav.* 95, 331–337. doi: 10.1016/j.pbb.2010.02.009

Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B., and Farber, J.L. (1993). Cyclosporine and carnitine prevent the anoxic death of cultured-hepatocytes by inhibiting the mitochondrial permeability transition. *J. Biol. Chem.* 268, 13791–13798.

Peltier, A. C., and Russell, J. W. (2002). Recent advances in drug-induced neuropathies. *Curr. Opin. Neurol.* 15, 633–638. doi: 10.1097/00019052-200210000-00015

Peng, P., Xi, Q. S., Xia, S., Zhuang, L., Gui, Q., Chen, Y., et al. (2012). Pregabalin attenuates docetaxel-induced neuropathy in rats. *J. Huazhong Uni. Sci. Technolog. Med. Sci.* 32, 586–590. doi: 10.1007/s11596-012-1001-y

Periquet, M. I., Mendell, J. R., Novak, V., Collins, M. P., Nagaraja, H. N., Erdem, S., et al. (1999). Painful sensory neuropathy—prospective evaluation using skin biopsy. *Neurology* 53, 1641–1647. doi: 10.1212/WNL.53.8.1641

Persohn, E., Canta, A., Schoepfer, S., Traebert, M., Mueller, L., Gilardini, A., et al. (2005). Morphological and morphometric analysis of paclitaxel and docetaxel-induced peripheral neuropathy in rats. *Eur. J. Cancer* 41, 1460–1466. doi: 10.1016/j.ejca.2005.04.006

Peters, C. M., Jimenez-Andrade, J. M., Kuskowski, M. A., Ghilardi, J. R., and Mantyh, P. W. (2007). An evolving cellular pathology occurs in dorsal root ganglia, peripheral nerve and spinal cord following intravenous administration of paclitaxel in the rat. *Brain Res.* 1168, 46–59. doi: 10.1016/j.brainres.2007.06.066

Podratz, J. L., Knight, A. M., Ta, L. E., Staff, N. P., Gass, J. M., Genelin, K., et al. (2011). Cisplatin induced Mitochondrial DNA damage in dorsal root ganglion neurons. *Neurobiol. Dis.* 41, 661–668. doi: 10.1016/j.nbd.2010.11.017

Polomano, R. C., Mannes, A. J., Clark, U. S., and Bennett, G. J. (2001). A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain* 94, 293–304. doi: 10.1016/S0304-3959(01)00363-3

Postma, T. J., Vermorken, J. B., Liefting, A. J. M., Pinedo, H. M., and Heimans, J. J. (1995). Paclitaxel-induced neuropathy. *Ann. Oncol.* 6, 489–494.

Quasthoff, S., and Hartung, H. P. (2002). Chemotherapy-induced peripheral neuropathy. *J. Neurol.* 249, 9–17. doi: 10.1007/PL00007853

Ribeiro-Da-Silva, A., Kenigsberg, R. L., and Cuello, A. C. (1991). Light and electron microscopic distribution of nerve growth factor receptor-like immunoreactivity in the skin of the rat lower lip. *Neuroscience* 43, 631–646. doi: 10.1016/0306-4522(91)90322-F

Richardson, P. G., Xie, W., Mitsiades, C., Chanan-Khan, A. A., Lonial, S., Hassoun, H., et al. (2009). Single-agent bortezomib in previously untreated multiple myeloma: efficacy, characterization of peripheral neuropathy, and molecular correlations with response and neuropathy. *J. Clinical Oncol.* 27, 3518–3525. doi: 10.1200/JCO.2008.18.3087

Roberts, J. A., Jenison, E. L., Kim, K., Clarke-Pearson, D., and Langleben, A. (1997). A Randomized, multicenter, double-blind, placebo-controlled, dose-finding study of ORG 2766 in the prevention or delay of cisplatin-induced neuropathies in women with ovarian cancer. *Gynecol. Oncol.* 67, 172–177. doi: 10.1006/gyno.1997.4832

Roglio, I., Bianchi, R., Camozzi, F., Carozzi, V., Cervellini, I., Crippa, D., et al. (2009). Docetaxel-induced peripheral neuropathy: protective effects of dihydroprogesterone and progesterone in an experimental model. *JPNS* 14, 36–44. doi: 10.1111/j.1529-8027.2009.00204.x

Saha, L., Hota, D., and Chakrabarti, A. (2012). Evaluation of lercanidipine in Paclitaxel-induced neuropathic pain model in rat: a preliminary study. *Pain res. Treat.* 2012:143579. doi: 10.1155/2012/143579

Sahenk, Z., Barohn, R., New, P., and Mendell, J. R. (1994). Taxol neuropathy. Electrodiagnostic and sural nerve biopsy findings. *Arch. Neurol.* 51, 726–729. doi: 10.1001/archneur.1994.00540190110024

Saif, M. W., Syrigos, K., Kaley, K., and Isufi, I. (2010). Role of pregabalin in treatment of oxaliplatin-induced sensory neuropathy. *Anticancer Res.* 30, 2927–2933.

Schlegel, U. (2011). Central nervous system toxicity of chemotherapy. *Eur. Assoc. NeuroOncol. Mag.* 1, 25–29.

Schmeichel, A. M., Schmelzer, J. D., and Low, P. A. (2003). Oxidative injury and apoptosis of dorsal root ganglion neurons in chronic experimental diabetic neuropathy. *Diabetes* 52, 165–171. doi: 10.2337/diabetes.52.1.165

Schmidt, R. E., Dorsey, D., Parvin, C. A., Beaudet, L. N., Plurad, S. B., and Roth, K. A. (1997). Dystrophic axonal swellings develop as a function of age and diabetes in human dorsal root ganglia. *J. Neuropathol. Exp. Neurol.* 56, 1028–1043. doi: 10.1097/00005072-199709000-00008

Screnci, D., and McKeage, M. J. (1999). Platinum neurotoxicity: clinical profiles, experimental models and neuroprotective approaches. *J. Inorg. Biochem.* 77, 105–110. doi: 10.1016/S0162-0134(99)00135-X

Scuteri, A., Galimberti, A., Maggioni, D., Ravasi, M., Pasini, S., Nicolini, G., et al. (2009). Role of MAPKs in platinum-induced neuronal apoptosis. *Neurotoxicology* 30, 312–319. doi: 10.1016/j.neuro.2009.01.003

Scuteri, A., Galimberti, A., Ravasi, M., Pasini, S., Donzelli, E., Cavaletti, G., et al. (2010). NGF protects dorsal root ganglion neurons from oxaliplatin by modulating JNK/Sapk and ERK1/2. *Neurosci. Lett.* 486, 141–145. doi: 10.1016/j.neulet.2010.09.028

Siau, C., and Bennett, G. J. (2006). Dysregulation of cellular calcium homeostasis in chemotherapy-evoked painful peripheral neuropathy. *Anesth. Analg.* 102, 1485–1490. doi: 10.1213/01.ane.0000204318.35194.ed

Smith, E. M. L., Pang, H., Cirrincione, C., Fleishman, S., Paskett, E. D., Ahles, T., et al. (2013). Effect of duloxetine on pain, function, and quality of life among patients with chemotherapy-induced painful peripheral neuropathy: a randomized clinical trial. *JAMA* 309, 1359–1367. doi: 10.1001/jama.2013.2813

Smyth, J. F., Bowman, A., Perren, T., Wilkinson, P., Prescott, R. J., Quinn, K. J., et al. (1997). Glutathione reduces the toxicity and improves quality of life of women diagnosed with ovarian cancer treated with cisplatin: results of a double-blind, randomised trial. *Ann. Oncol.* 8, 569–573. doi: 10.1023/A:1008211226339

Sorensen, L., Molyneaux, L., and Yue, D. K. (2006). The relationship among pain, sensory loss, and small nerve fibers in diabetes. *Diabetes Care* 29, 883–887. doi: 10.2337/diacare.29.04.06.dc05-2180

Stucky, C. L., and Lewin, G. R. (1999). Isolectin B4-positive and -negative nociceptors are functionally distinct. *J. Neurosci.* 19, 6497–6505.

Suzukawa, K., Miura, K., Mitsushita, J., Resau, J., Hirose, K., Crystal, R., et al. (2000). Nerve growth factor-induced neuronal differentiation requires generation of Rac1-regulated reactive oxygen species. *J. Biol. Chem.* 275, 13175–13178. doi: 10.1074/jbc.275.18.13175

Ta, L. E., Bieber, A. J., Carlton, S. M., Loprinzi, C. L., Low, P. A., and Windebank, A. J. (2010). Transient Receptor Potential Vanilloid 1 is essential for cisplatin-induced heat hyperalgesia in mice. *Mol. Pain* 6:15. doi: 10.1186/1744-8069-6-15

Ta, L. E., Espeset, L., Podratz, J., and Windebank, A. J. (2006). Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurons correlates with platinum-DNA binding. *Neurotoxicology* 27, 992–1002. doi: 10.1016/j.neuro.2006.04.010

Ta, L. E., Schmelzer, J. D., Bieber, A. J., Loprinzi, C. L., Sieck, G. C., Brederson, J. D., et al. (2013). A novel and selective poly (ADP-ribose) polymerase inhibitor ameliorates chemotherapy-induced painful neuropathy. *PLoS ONE* 8:e54261. doi: 10.1371/journal.pone.0054161

Tanner, K. D., Levine, J. D., and Topp, K. S. (1998). Microtubule disorientation and axonal swelling in unmyelinated sensory axons during vincristine-induced painful neuropathy in rat. *J. Comp. Neurol.* 395, 481–492. doi: 10.1002/(SICI)1096-9861(19980615)395:4<481::AID-CNE5>3.3.CO;2-T

Tatsushima, Y., Egashira, N., Kawashiri, T., Mihara, Y., Yano, T., Mishima, K., et al. (2011). Involvement of substance P in peripheral neuropathy induced by paclitaxel but not oxaliplatin. *J. Pharmacol. Exp. Ther.* 337, 226–235. doi: 10.1124/jpet.110.175976

Tatsushima, Y., Egashira, N., Narishige, Y., Fukui, S., Kawashiri, T., Yamauchi, Y., et al. (2013). Calcium channel blockers reduce oxaliplatin-induced acute neuropathy: a retrospective study of 69 male patients receiving modified FOLFOX6 therapy. *Biomed. Pharmacother.* 67, 39–42. doi: 10.1016/j.biopha.2012.10.006

Topp, K. S., Tanner, K. D., and Levine, J. D. (2000). Damage to the cytoskeleton of large diameter sensory neurons and myelinated axons in vincristine-induced painful peripheral neuropathy in the rat. *J. Comp. Neurol.* 424, 563–576. doi: 10.1002/1096-9861(20000904)424:4<563::AID-CNE1>3.3.CO;2-L

Trevisan, G., Materazzi, S., Fusi, C., Altomare, A., Aldini, G., Lodovici, M., et al. (2013). Novel therapeutic strategy to prevent chemotherapy-induced persistent sensory neuropathy by TRPA1 blockade. *Cancer Res.*73, 3120. doi: 10.1158/0008-5472.CAN-12-4370

Tsujino, H., Kondo, E., Fukuoka, T., Dai, Y., Tokunaga, A., Miki, K., et al. (2000). Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: a novel neuronal marker of nerve injury. *Mol. Cell Neurosci.* 15, 170–182. doi: 10.1006/mcne.1999.0814

van, D. H. R. G., Vecht, C. J., van, D. B. M. E., Elderson, A., Boogerd, W., Heimans, J. J., et al. (1990). Prevention of cisplatin neurotoxicity with an ACTH(4-9) analogue in patients with ovarian cancer. *N. Engl. J. Med.* 322, 89–94. doi: 10.1056/NEJM199001113220204

Velasco, R., and Bruna, J. (2010). Chemotherapy-induced peripheral neuropathy: an unresolved issue. *Neurologia* 25, 116–131. doi: 10.1016/S0213-4853(10)70036-0

Wang, H.-T., Liu, Z.-G., Yang, W., Liao, A.-J., Zhang, R., Wu, B., et al. (2011). Study on mechanism of bortezomib inducing peripheral neuropathy and the reversing effect of reduced glutathione. *Zhonghua xue ye xue za zhi* 32, 107–111.

Wang, X.-M., Lehky, T. J., Brell, J. M., and Dorsey, S. G. (2012). Discovering cytokines as targets for chemotherapy-induced painful peripheral neuropathy. *Cytokine* 59, 3–9. doi: 10.1016/j.cyto.2012.03.027

Warwick, R. A., and Hanani, M. (2013). The contribution of satellite glial cells to chemotherapy-induced neuropathic pain. *Eur. J. Pain* 17, 571–580. doi: 10.1002/j.1532-2149.2012.00219.x

Wen, F., Zhou, Y., Wang, W., Hu, Q. C., Liu, Y. T., Zhang, P. F., et al. (2013). Ca/Mg infusions for the prevention of oxaliplatin-related neurotoxicity in patients with colorectal cancer: a meta-analysis. *Ann. Oncol.* 24, 171–178. doi: 10.1093/annonc/mds211

Wilkes, G. (2007). Peripheral neuropathy related to chemotherapy. *Semin. Oncol. Nurs.* 23, 162–173. doi: 10.1016/j.soncn.2007.05.001

Windebank, A. J., and Grisold, W. (2008). Chemotherapy-induced neuropathy. *J. Peripheral Nervous Sys.* 13, 27–46. doi: 10.1111/j.1529-8027.2008.00156.x

Wolf, S., Barton, D., Kottschade, L., Grothey, A., and Loprinzi, C. (2008). Chemotherapy-induced peripheral neuropathy: prevention and treatment strategies. *Eur. J. Cancer* 44, 1507–1515. doi: 10.1016/j.ejca.2008.04.018

Woodbury, C. J., Zwick, M., Wang, S. Y., Lawson, J. J., Caterina, M. J., Koltzenburg, M., et al. (2004). Nociceptors lacking TRPV1 and TRPV2 have normal heat responses. *J. Neurosci.* 24, 6410–6415. doi: 10.1523/JNEUROSCI.1421-04.2004

Xiao, W., Boroujerdi, A., Bennett, G. J., and Luo, Z. D. (2007). Chemotherapy-evoked painful peripheral neuropathy: analgesic effects of gabapentin and effects on expression of the alpha-2-delta type-1 calcium channel subunit. *Neuroscience* 144, 714–720. doi: 10.1016/j.neuroscience.2006.09.044

Xiao, W. H., and Bennett, G. J. (2012). Effects of mitochondrial poisons on the neuropathic pain produced by the chemotherapeutic agents, paclitaxel and oxaliplatin. *Pain* 153, 704–709. doi: 10.1016/j.pain.2011.12.011

Xiao, W. H., Zheng, F. Y., Bennett, G. J., Bordet, T., and Pruss, R. M. (2009). Olesoxime (cholest-4-en-3-one, oxime): analgesic and neuroprotective effects in a rat model of painful peripheral neuropathy produced by the chemotherapeutic agent, paclitaxel. *Pain* 147, 202–209. doi: 10.1016/j.pain.2009.09.006

Xiao, W. H., Zheng, H., and Bennett, G. J. (2012). Characterization of oxaliplatin-induced chronic painful peripheral neuropathy in the rat and comparison with the neuropathy induced by paclitaxel. *Neuroscience* 203, 194–206. doi: 10.1016/j.neuroscience.2011.12.023

Xiao, W. H., Zheng, H., Zheng, F. Y., Nuydens, R., Meert, T. F., and Bennett, G. J. (2011). Mitochondrial abnormality in sensory, but not motor, axons in paclitaxel-evoked painful peripheral neuropathy in the rat. *Neuroscience* 199, 461–469. doi: 10.1016/j.neuroscience.2011.10.010

Yoon, S. Y., Robinson, C. R., Zhang, H., and Dougherty, P. M. (2013). Spinal astrocyte gap junctions contribute to oxaliplatin-induced mechanical hypersensitivity. *J. Pain* 14, 205–214. doi: 10.1016/j.jpain.2012.11.002

Zheng, H., Xiao, W. H., and Bennett, G. J. (2011). Functional deficits in peripheral nerve mitochondria in rats with paclitaxel- and oxaliplatin-evoked painful peripheral neuropathy. *Exp. Neurol.* 232, 154–161. doi: 10.1016/j.expneurol.2011.08.016

Zheng, H., Xiao, W. H., and Bennett, G. J. (2012). Mitotoxicity and bortezomib-induced chronic painful peripheral neuropathy. *Exp. Neur.* 238, 225–234. doi: 10.1016/j.expneurol.2012.08.023

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 03 October 2013; accepted: 28 November 2013; published online: 18 December 2013.

*Citation: Han Y and Smith MT (2013) Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). Front. Pharmacol. **4**:156. doi: 10.3389/fphar.2013.00156*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

REVIEW ARTICLE
published: 17 July 2014
doi: 10.3389/fphar.2014.00169

Development of novel treatment strategies for inflammatory diseases—similarities and divergence between glucocorticoids and GILZ

Qiang Cheng, Eric Morand and Yuan Hang Yang*

Centre for Inflammatory Diseases, Department of Medicine, Southern Clinical School, Monash University Faculty of Medicine, Nursing and Health Sciences, Monash Medical Centre, Clayton, VIC, Australia

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Emanuela Ricciotti, University of Pennsylvania, USA
Heiko Mühl, University Hospital Goethe University Frankfurt, Germany

***Correspondence:**
Yuan Hang Yang, Centre for Inflammatory Diseases, Monash University Faculty of Medicine, Nursing and Health Sciences, Monash Medical Centre, 246 Clayton Road, Clayton, VIC 3168, Australia
e-mail: yuan.yang@monash.edu

Glucocorticoids (GC) are the most commonly prescribed medications for patients with inflammatory diseases, despite their well-known adverse metabolic effects. Previously, it was understood that the anti-inflammatory effects of the GC/GC receptor (GR) complex were mediated via transrepression, whilst the adverse metabolic effects were mediated via transactivation. It has recently become clear that this "divergent actions" paradigm of GC actions is likely insufficient. It has been reported that the GC/GR-mediated transactivation also contributes to the anti-inflammatory actions of GC, via up-regulation of key anti-inflammatory proteins. One of these is glucocorticoid-induced leucine zipper (GILZ), which inhibits inflammatory responses in a number of important immune cell lineages *in vitro*, as well as in animal models of inflammatory diseases *in vivo*. This review aims to compare the GILZ and GC effects on specific cell lineages and animal models of inflammatory diseases. The fact that the actions of GILZ permit a GILZ-based gene therapy to lack GC-like adverse effects presents the potential for development of new strategies to treat patients with inflammatory diseases.

Keywords: glucocorticoids, GILZ, inflammation, anti-inflammation, immune response, cell biology

INTRODUCTION

Glucocorticoids (GC) remain the most common prescription to treat patients with immune-mediated inflammatory diseases, despite their well-known side effects. The efficacy of GCs is underpinned by GC effects on intracellular pathways mediated via forming a complex with the glucocorticoid receptor (GR). The effects of the GC/GR complex depend on a combination of several effects. These include (i) transrepression, whereby the GC-GR complex tethers to pro-inflammatory transcription factors such as NF-κB and AP-1, constraining their activity; (ii) cis-repression, whereby the GC-GR complex binds directly to DNA and exerts inhibitory effects on gene transcription; and (iii) transactivation, whereby a GC-GR dimer acts as a *bona fides* transcription factor and activates gene transcription (McKay and Cidlowski, 1999; De Bosscher et al., 2003; Barnes, 2006; Beaulieu and Morand, 2011). Despite their beneficial effects, adverse effects of GC treatment have been noted since the beginning of their usage, because the amount of GCs required therapeutically to inhibit the immune system is in excess of metabolic homeostatic requirements. Until lately, it was believed that GC/GR mediated transrepression was predominantly responsible for the immune suppressive function of GCs, while GC/GR induced transactivation was linked to GC adverse metabolic effects (Reichardt et al., 2001; Schacke et al., 2005, 2007). Recently, however, a new role of GC/GR transactivation in immune suppressive actions has emerged, due to the functional characterization of several GC induced anti-inflammatory proteins. Many studies have shown that one such protein, glucocorticoid-induced leucine zipper (GILZ), inhibits activation of a wide range of immune cells under inflamed conditions (Beaulieu and Morand, 2011; Esposito et al., 2012; Cheng et al., 2013; Ngo et al., 2013a). Importantly, published data to date have not indicated GC-like adverse effects associated with GILZ, suggesting that GILZ exerts immunosuppressive effects that mimic those of GCs but occur via distinct pathways. In this review, we aim to summarize current knowledge of GILZ biological functions and compare them with the known effects of GC on immune system. The similarities and divergence between the effects of GILZ and GC suggest the potential use of GILZ-based therapies in improving GC efficacy while reducing GC metabolic toxicity, and thus the development of new treatment strategies to replace supplement or even replace GC.

GLUCOCORTICOIDS AND GLUCOCORTICOID RECEPTORS

Inflammation is a self-protective process that forms part of the host organism responses to harmful insults, such as cell damage, foreign pathogens or irritants. To avoid excessive damage to the healthy tissues, the activated immune system needs to be rapidly "switched off" once the noxious stimuli are eliminated. Therefore, inflammatory responses are tightly regulated via competition between stimulative and suppressive signals. GC, produced by the adrenal gland, represents one of the most powerful endogenous pathways to temper the intensity of immune responses. Under certain conditions, however, the ability of endogenous GC to suppress immune response is overwhelmed,

resulting in the hyper-activation of the immune system that leads to chronic auto-immune and inflammatory disease. In the majority of these diseases, treatment with synthetic GCs is used to control inflammation, exploiting the natural pathways that evolved to permit endogenous GC to regulate immune responses. The profound effectiveness of GCs underpins their use in a wide range of diseases. Despite their effectiveness, however, the use of GCs is accompanied by a litany of serious adverse effects, particularly in patients under long term or high dose treatment. These unwanted effects include diabetes, immunosuppression, osteoporosis and increased risk of cardiovascular events, all of which are closely associated with the physiological metabolic functions of GC (Rhen and Cidlowski, 2005). The continued use of glucocorticoids reflects a failure to discover a reliable GC replacement. To develop such glucocorticoid mimics, a large body of research has been conducted to understand the molecular actions of GC.

MOLECULAR MECHANISMS OF GLUCOCORTICOID ACTIONS

GCs impact on intracellular pathways through binding to the GR (Rhen and Cidlowski, 2005). The GC/GR complex exerts its transrepression effects by tethering to and interfering with the function of proinflammatory transcription factors, such as NF-κB and AP-1, which consequently results in repression of a large number of proinflammatory mediators, cytokines, chemokines and adhesion molecules (Rhen and Cidlowski, 2005; Nixon et al., 2013). The GC/GR complex also suppresses gene expression via binding to negative GC response elements (nGRE) located within the promoters of genes, a phenomena called cis-repression (Newton, 2000; Clark and Belvisi, 2012; Nixon et al., 2013; Vandevyver et al., 2013). These GC/GR-induced transrepression and cis-repression effects are important in the therapeutic activity of GCs. On the other hand, the dimerized GC/GR complex is also capable of binding to positive GREs and thereby up-regulating the expression of multiple genes, many of which are related to the metabolic adverse effects of GCs (Rhen and Cidlowski, 2005; Clark and Belvisi, 2012; Nixon et al., 2013; Vandevyver et al., 2013). It has previously been believed that anti-inflammatory effects of GCs are mediated via transrepression, whereas the metabolic effects are mediated via transactivation (**Figure 1A**). Thus, a significant effort has been invested by pharmaceutical companies worldwide toward the discovery of "selective glucocorticoid receptor modulators" (SGRM), based on the idea that GR ligands that favor transrepression over transactivation would be equally effective but safer (**Figure 1B**) (Weinstein et al., 2011). As we have recently reviewed, however, it has become clear that the "divergent actions" paradigm of GC actions does not fully explain the parallel therapeutic and metabolic effects of GCs (Fan and Morand, 2012). For examples, nGREs were recently identified within the promoters of a large number of genes, including insulin, osteoprotegerin and anti-apoptotic proteins (Surjit et al., 2011). GC/GR binding to these nGREs results in down-regulation of gene expression, directly contributing to GC adverse metabolic effects. Moreover, GC/GR-dimer induced transactivation effects include the induction of the expression of several important anti-inflammatory proteins, including GILZ, annexin A1 (AnxA1) and MAPK phosphatase-1 (MKP-1) (Clark, 2003; Perretti and D'Acquisto, 2009; Beaulieu and Morand, 2011; Clark and Belvisi, 2012; Ratman et al., 2013; Yang et al., 2013). Indeed, two recent studies indicate that GC-GR-dimer-dependent effects, likely due to transactivation of anti-inflammatory proteins, are essential for the full anti-inflammatory effects of GCs (Baschant et al., 2011; Kleiman et al., 2012). These findings demonstrate a previously under-recognized role of GC-induced proteins in the anti-inflammatory effects of GC, suggesting that the paradigm separating GC anti-inflammatory and metabolic effects on the basis of transrepression and transactivation is inadequate to explain the effects of GC.

To develop a new strategy to mimic GC immune-suppressive functions but limit the metabolic effects, an alternate approach would be to discover GC-mediated inhibitory effects on the immune system that bypass the mechanism of GC metabolic effects, i.e., molecules that do not utilize the GR for their

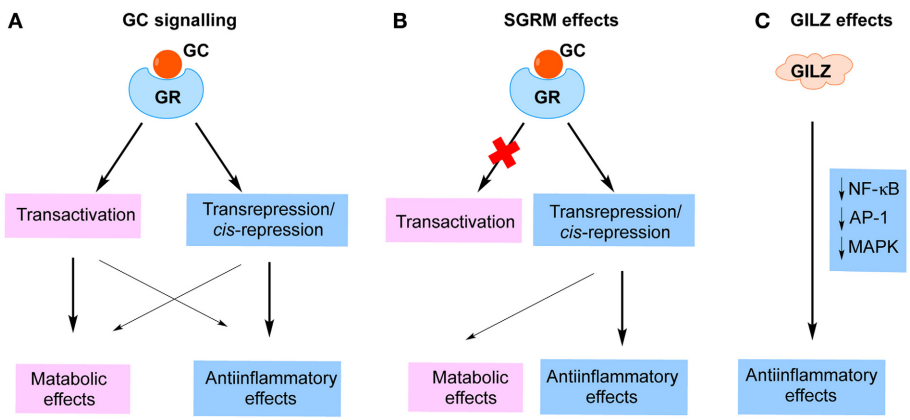


FIGURE 1 | Strategies to improve glucocorticoid therapy. (A) Cellular effects of glucocorticoids (GC). GCs bind to glucocorticoid receptor (GR) and activate intracellular signaling pathways. Previously, it was believed that transactivation predominantly accounts for the adverse metabolic effects, whereas transrepression/cis-repression mainly mediates anti-inflammatory functions of GCs. **(B)** The strategy to develop "selective glucocorticoid receptor modulators (SGRM)" based on the paradigm that metabolic effects but not immune-suppressive effects depended on transactivation. **(C)** The anti-inflammatory effects of GILZ are independent of GR function and may conceivably avoid metabolic effects entirely.

actions on inflammation. Accumulating evidence indicates that one of the GC-induced proteins, GILZ, represents the basis for such an approach (**Figure 1C**). GILZ has been detected and functionally characterized in a wide range of immune cells, including T lymphocytes, B lymphocytes, dendritic cells (DCs), monocyte/macrophages, mast cells and endothelial cells (ECs) (D'Adamio et al., 1997; Glynne et al., 2000; Berrebi et al., 2003; Cannarile et al., 2006; Cohen et al., 2006; Godot et al., 2006; Cheng et al., 2013). Within the most of cell types investigated, the main function of GILZ is immune suppressive. The molecular actions of GILZ were recently reviewed by us and others (Ayroldi and Riccardi, 2009; Beaulieu and Morand, 2011). Although GILZ expression is up-regulated by GCs, via GC/GR binding to GREs within the *Gilz* promoter (Asselin-Labat et al., 2005), divergent effects of GILZ and GCs on mesenchymal stem cell (MSC) differentiation have been reported, suggesting that GILZ lacks an ability to induce GC-related adverse effects (Shi et al., 2000; Zhang et al., 2008). This notion is further supported by the observation that GC immune suppressive functions do not always require the presence of GILZ, suggesting that GILZ and GCs work in parallel and impact on immune system through divergent signaling pathways (Cheng et al., 2013; Ngo et al., 2013a). In the following sections we shall review the known effects of GC and GILZ in various cells and compartments relevant to inflammatory disease and illustrate evidence that their effects are divergent.

COMPARISON OF THE EFFECTS OF GC AND GILZ

To better understand the similarities and differences between GILZ and GCs, their effects on specific cell lineages and animal models of inflammatory diseases are directly compared in the following sections, as summarized in **Tables 1**, **2**.

THYMOCYTES

GCs induce thymocyte apoptosis and this effect requires GR-dependent protein synthesis (Thomas et al., 1983; Cohen and Duke, 1984; Ashwell et al., 2000; Bruscoli et al., 2006). Although the mechanisms remain only partially understood, mitochondrial apoptotic signaling pathways mediated by Bcl-2 family members play a dominant role (Sentman et al., 1991; Grillot et al., 1995). In addition, GCs suppress NF-κB signals in thymocytes, leading to increased thymocyte cell death (Wang et al., 1999). GILZ was initially discovered in murine thymocytes as its expression is exquisitely sensitive to GCs (D'Adamio et al., 1997). To elucidate the role of GILZ in regulation of thymocyte survival, a transgenic (TG) mouse line, in which GILZ expression is driven by a CD2 promoter, was generated (Delfino et al., 2004). Consistent with GC pro-apoptotic effects, overexpression of GILZ led to a reduction of Bcl-xL expression and accelerated anti-CD3 Ab induced thymocyte apoptosis, which was accompanied by inhibition of NF-κB p65 nuclear translocation and DNA binding ability (Delfino et al., 2004, 2006). As a result, adult TG mice show a decrease in CD4 and CD8 double positive thymocytes. Moreover, a mouse strain ($GR^{lck-Cre}$), in which GR expression is conditionally knocked out in thymocytes prior to selections, was recently generated (Mittelstadt et al., 2012). Deletion of GR led to an absence of GC signaling and GC-induced GILZ expression. As a result, $GR^{lck-Cre}$ thymoctyes are completely resistance to GC-induced apoptosis. Thus, these studies suggest that GCs and GILZ both play a similar pro-apoptotic role in regulation of thymocyte apoptosis.

T LYMPHOCYTES

ACTIVATION-INDUCED APOPTOSIS

In contrast to their pro-apoptotic effects on thymocytes, GC and GILZ effects on activated T lymphocytes are anti-apoptotic (D'Adamio et al., 1997). GCs directly interfere with AP-1 and suppress activation-induced FasL expression, to inhibit T lymphocyte apoptosis (Paliogianni et al., 1993; Yang et al., 1995). The GC dexamethasone (DEX) also blocks NF-κB activation through inhibiting IκB degradation, to reduce anti-CD3-induced FasL expression and T cell death (Auphan et al., 1995; Liberman et al., 2012). Similarly, GILZ inhibits anti-CD3 Ab-induced cell apoptosis in a T cell line via directly interfering with the AP-1 transcription factor, leading to inhibition of FasL expression and pro-apoptotic signaling (D'Adamio et al., 1997; Mittelstadt and Ashwell, 2001). GILZ overexpression also protects IL-2 withdrawal-induced T lymphocyte cell death, which is correspondingly accelerated in GILZ-deficient cells (Asselin-Labat et al., 2004). IL-2 withdrawal rapidly induces binding of the FOXO3 transcription factor to the GILZ promoter, leading to increased GILZ expression (Asselin-Labat et al., 2004, 2005). On the other hand, GILZ inhibits FOXO3 transcriptional activity via CRM1 (a nuclear transport receptor)-dependent nuclear exclusion of FOXO3 (Latre de Late et al., 2010), leading to decreased expression of itself and BIM, a pro-apoptotic member of the Bcl-2 family. These findings suggest a bidirectional regulation between FOXO3 and GILZ proteins to regulate cell apoptosis in T lymphocytes.

IMMUNE RESPONSE

GCs suppress Th1 development and promote Th2 differentiation in CD4 T lymphocytes, through mediating the production of a wide range of cytokines at the transcriptional level (Kunicka et al., 1993; Almawi et al., 1996; Miyaura and Iwata, 2002). This notion is supported by the observation that GCs inhibit the activation of several important T cell pro-inflammatory transcription factors, including AP-1, NFAT and NF-κB (Tsitoura and Rothman, 2004). Upon TCR activation of murine CD4 T cells, GCs reduce production of Th1 cytokines (e.g., IL-2) and promote the expression of Th2 cytokines (e.g., IL-4) (Daynes and Araneo, 1989). It is now clear that GCs suppress IL-2 expression via direct inhibition of AP-1 and NF-κB DNA binding activity (Vacca et al., 1992; Adcock et al., 1995). A similar skew toward Th2 phenotype was observed in T lymphocytes from GILZ TG mice (Cannarile et al., 2006). Anti-CD3/anti-CD28 antibody-induced activation of the Th2 specific transcription factors GATA3 and STAT6 is increased in GILZ TG T lymphocytes, whereas activity of T-bet, a Th1 transcription factor, was reduced (Cannarile et al., 2006). As a result, GILZ induces T lymphocytes to produce more Th2 cytokines (e.g., IL-4, IL-5, and IL-10) and less Th1 cytokines (e.g., INF-γ). The concept that GILZ suppresses Th1 responses is supported by the observation that increased IFN-γ production is detected in GILZ knockout (KO) T cells (Ngo et al., 2013a). Moreover, GILZ inhibits the transcriptional activity of AP-1, NF-κB and NFAT, transcription factors known to regulate T cell activation

Table 1 | Comparison of GILZ and GC *in vitro* effects on specific cell lineages.

	GC effects	GILZ effects
Thymocytes	↑ Apoptosis *via* ↓ Bcl-xL, NF-κB (Wang et al., 1999; Bianchini et al., 2006; Bruscoli et al., 2006)	↑ Apoptosis *via* ↓ Bcl-xL, NF-κB (Delfino et al., 2004, 2006)
T lymphocytes	↓ Apoptosis *via* ↓ AP-1, FasL (Zacharchuk et al., 1990; Paliogianni et al., 1993; Yang et al., 1995)	↓ Apoptosis *via* ↓ AP-1, FasL, FOXO3, BIM (Mittelstadt and Ashwell, 2001; Asselin-Labat et al., 2004; Latre de Late et al., 2010)
	↓ Th1 *via* ↓ IL-2, IFNγ (Daynes and Araneo, 1989; Miyaura and Iwata, 2002)	↓ Th1 *via* ↓ IL-2, IFNγ (Ayroldi et al., 2001; Mittelstadt and Ashwell, 2001; Cannarile et al., 2006; Ngo et al., 2013a)
	↑ Th2 *via* ↑ IL-4, IL-10 (Daynes and Araneo, 1989; Miyaura and Iwata, 2002)	↑ Th2 *via* ↑ GATA-3, STAT6, IL-4, IL-10 (Cannarile et al., 2006)
	↓ Activation *via* ↓ AP-1, NF-κB, No effect on ERK (Vacca et al., 1992; Adcock et al., 1995; Tsitoura and Rothman, 2004)	↓ Activation *via* ↓ AP-1, NF-κB, NFAT, ↓ ERK (Ayroldi et al., 2001, 2002; Cannarile et al., 2006)
	No effect on Th17 (McKinley et al., 2008; Nanzer et al., 2013)	↓ Th17 *via* ↓ IL17A (Ngo et al., 2013a)
Dendritic cells	↓ Maturation *via* ↓ CD80, CD86, IL-12 (Kitajima et al., 1996; Rea et al., 2000)	↓ Maturation *via* ↓ CD80, CD86 (Cohen et al., 2006)
	↑ Tolerance *via* ↑ IL-10, B7-H1 (Rea et al., 2000; Cohen et al., 2006)	↑ Tolerance *via* ↑ IL-10, B7-H1 (Cohen et al., 2006)
	↑ DC induced Treg *via* ↑ FOXP3+ Treg (Hamdi et al., 2007b; Unger et al., 2009)	↑ DC induced Treg *via* ↑ FOXP3+ Treg (Hamdi et al., 2007b)
Endothelial cells	↓ Activation *via* ↓ NF-κB, p38, ↑ MKP-1 (Ray et al., 1997; Furst et al., 2007; Cheng et al., 2013)	↓ Activation *via* ↓ NF-κB, p38, ERK, JNK, ↑ MKP-1 (Cheng et al., 2013)
Monocyte/Macrophages	↓ Activation *via* ↓ NF-κB, IL-1β, TNFα (Jeon et al., 2000; Steer et al., 2000; Joyce et al., 2001)	↓ Activation *via* ↓ NF-κB, IL-1β, TNFα (Berrebi et al., 2003; Hamdi et al., 2007a)
MSC	↑ AP, ↓ OB *via* ↑ C/EBPδ, PPARγ2 (Shi et al., 2000; Yao et al., 2008)	↓ AP, ↓ OB *via* ↓ PPARγ2 (Shi et al., 2003; Zhang et al., 2008)

The effects of GCs and GILZ on each cell lineage and related mechanisms are shown. Underlined text indicates the GILZ and GC effects are divergent. Abbreviation: AP, adipocytes; OB, osteoblasts; MSC, mesenchymal stem cells.

and differentiation (Ayroldi et al., 2001, 2002; Mittelstadt and Ashwell, 2001), thus mimicking GC effects. GILZ directly interacts with NF-κB p65 subunit through its C-terminal domain (Di Marco et al., 2007), and inhibits p65 nuclear translocation and DNA binding, resulting in reduced expression of IL-2 and IL-2R (Ayroldi et al., 2001). On the other hand, the N-terminal of GILZ interacts with AP-1 and subsequently inhibits AP-1 DNA binding, resulting in decreased IL-2 expression (Mittelstadt and Ashwell, 2001).

Despite many similarities, several divergent effects of GCs and GILZ on T lymphocytes activation and differentiation have also been reported. GC effects on Th17 phenotype are not fully understood. For example, GCs have no effect on Th17 cytokine production from polarized Th17 T cells *in vitro* (McKinley et al., 2008). As a result, neutrophil infiltration induced by adoptive transfer of Th17 cells cannot be attenuated by GCs *in vivo*. Consequently, the increased production of IL-17A and IL-22 by asthmatic patient peripheral blood monocytes (PBMC) is insensitive to GCs *in vitro* (Nanzer et al., 2013). In contrast, early evidence suggests that GILZ inhibits IL-17A production as GILZ KO T cells produce significantly more IL-17A than WT cells (Ngo et al., 2013a). Moreover, GCs have no effect on phosphorylation of ERK MAP kinase in T lymphocytes activated by anti-CD3 Ab (Tsitoura and Rothman, 2004), whereas GILZ markedly inhibits ERK activation (Ayroldi et al., 2002). GILZ directly interacts with and inhibits Ras/Raf, upstream kinases in the ERK pathway (Ayroldi et al., 2002, 2007; Soundararajan et al., 2007). Together, these observations indicate that while GILZ mimics most GC

Table 2 | Comparison of GILZ and GC *in vivo* effects on animal models of diseases.

Diseases	GC effects	GILZ effects	Techniques
Th1 Colitis (DNBS)	↓ *via* ↓ TNFα, IL-6 (Antonioli et al., 2007)	↓ *via* ↓ NF-κB, TNFα, IFNγ, FasL (Cannarile et al., 2009)	GILZ TG
Th2 Colitis (Oxazolone)	Unknown	↑ *via* ↑ MPO (Cannarile et al., 2009)	
SCI	↓ *via* ↓ ROS (Hall, 2011)	↓ *via* ↓ NF-κB, TNFα, FasL, ↓ Bcl-2 (Esposito et al., 2012)	
EAE	↓ (Chen et al., 2006)	↓ *via* ↓ NF-κB, IFNγ, IL-17, ↓ GATA-3 (Srinivasan and Janardhanam, 2011)	rGILZ
Experimental arthritis	↓ CIA, AIA, K/BxN (Yang et al., 2004; Beaulieu et al., 2010; Patel et al., 2012; Ngo et al., 2013a)	↓ CIA *via* ↓ IL-1, TNFα (Beaulieu et al., 2010; Ngo et al., 2013a)	GILZ-AAV
		No effect (Ngo et al., 2013a)	Endogenous GILZ via KO
Endotoxemia	↓ (Yang et al., 2009)	↓ *via* ↓ IL-6 (Pinheiro et al., 2013)	SPRET/Ei mice
		No effect(Ngo et al., 2013a)	Endogenous GILZ via KO
DTH	↓ (Taube and Carlsten, 2000)	↓ *via* ↓ IFNγ, IL-17, proliferation (Ngo et al., 2013a)	Endogenous GILZ via KO
Male infertility	↑ SSC apoptosis *via* ↑ FasL (Khorsandi et al., 2008; Orazizadeh et al., 2010)	↓ SSC apoptosis *via* ↓ ERK, AKT, FOXO1, BIM (Bruscoli et al., 2012; Ngo et al., 2013b)	

The effects of GCs and GILZ on each disease model and related mechanisms are shown. Underlined text indicates the GILZ and GC effects are divergent. The technique by which GILZ function was examined is also listed. MPO, myeloperoxidase; SCI, spinal cord injury; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis; AIA, antigen-induced arthritis; SPRET/Ei, a wild-derived inbred murine strain; SSC, spermatogonia stem cells; DTH, delayed-type hypersensitivity.

effects on T cell activation, effects on IL-17A production and ERK activation are divergent.

DENDRITIC CELLS

DCs play a crucial role in priming naive T lymphocytes via their ability to recognize and present antigens to T cells (Hackstein and Thomson, 2004). GCs and GILZ have very similar suppressive effects on DC activation, and many GC effects have been suggested to be GILZ-dependent (Cohen et al., 2006; Hamdi et al., 2007b). To mature, immature DCs need to be activated by pro-inflammatory stimuli (e.g., TNFα), bacterial components (e.g., LPS) or T lymphocytes expressing CD40 ligand (CD40L) (Hackstein and Thomson, 2004). GCs inhibit DC maturation via suppressing expression of DC maturation markers (e.g., CD80, CD86, CD83 and IL-12), and promoting tolerance markers (e.g., IL-10 and B7-H1) (Kitajima et al., 1996; Rea et al., 2000; Cohen et al., 2006). GCs also inhibit chemokine production (e.g., CCL-3, CCL-5 and CXCL-8) by human DCs and, therefore, impair DC ability to activate T cells (Cohen et al., 2006). Moreover, GC-treated DCs promote the generation of regulatory T (Treg) cells derived from pathogen-specific human CD4 T lymphocytes (Hamdi et al., 2007b). Hamdi et al. reported that GC treatment increases DC ability to induce the $CD25^{hi}FOXP3$ phenotype among human $CD4^{+}$ T cells. The function of these inducible Tregs is confirmed by their capacity to inhibit human PBMC proliferation (Hamdi et al., 2007b). GILZ expression is highly sensitive to GC treatment in human DCs and GILZ overexpression mimics the known GC inhibitory effects on DC maturation and activation (Cohen et al., 2006; Hamdi et al., 2007b). Consistent with this, deficiency of endogenous GILZ induced by silencing via siRNA reverses these GC effects, suggesting that GILZ expression plays a critical role in mediating GC actions in DCs (Cohen et al., 2006).

ENDOTHELIAL CELLS

ECs play a critical role in mediating the recruitment of fast-traveling leukocytes from the blood stream to inflamed tissues. This occurs via upregulation of the pro-inflammatory adhesion

molecules (E-selectin, VCAM-1 and ICAM-1), and cytokines and chemokines such as IL-6, CXCL8 and CCL2 (Ley et al., 2007). The expression of endothelial pro-inflammatory molecules is predominantly controlled by NF-κB and MAP kinase signaling pathways (Chen and Manning, 1995; Kuldo et al., 2005; Cheng et al., 2010). GC effects on the activation of human umbilical cord vein endothelial cells (HUVECs) require GR, as GR-deficient HUVECs show prolonged NF-κB activation (Goodwin et al., 2013). GCs inhibit E-selectin, IL-6, VCAM-1 and ICAM-1 expression in HUVECs via interference with NF-κB (Ray et al., 1997; Pan et al., 2011; Goodwin et al., 2013). Additionally, GCs regulate endothelial activation via NF-κB-independent mechanisms (Furst et al., 2007). In HUVEC, GCs induce the expression of MKP-1, a MAPK inhibitory phosphatase, leading to inhibition of p38 MAPK-dependent E-selectin expression without affecting NF-κB DNA binding activity (Furst et al., 2007). Consistent with these observations, GC-treated HUVECs have impaired ability to support leukocyte rolling and transmigration under flow conditions (Cheng et al., 2013). GILZ is highly expressed in ECs in synovial tissue from patients with rheumatoid arthritis (RA), and plays an important role in regulation of endothelial adhesive functions (Beaulieu et al., 2010; Cheng et al., 2013). Similar to the known GC effects, GILZ expression in HUVECs inhibits their capacity to support leukocyte rolling, adhesion and transmigration, accompanied by reduced expression of E-selecting, ICAM-1, IL-6, CXCL8, and CCL2 (Cheng et al., 2013). Differing from observations reported in T lymphocytes, however, in a human microvascular endothelial cell line (HMEC), GILZ inhibits NF-κB transcription activity via suppressing NF-κB p65 DNA binding ability, without affecting its nuclear translocation, suggesting a novel mechanism by which GILZ regulates NF-κB signaling in human ECs. GILZ also upregulates MKP-1 expression and, thereby, inhibits TNFα-induced p38, ERK, and JNK MAP kinase activation, mimicking GC effects. On the other hand, GILZ silencing via siRNA does not alter the inhibitory effects of exogenous GC on HUVEC adhesive function, suggesting redundancy of GILZ in GC effects on ECs despite the GC-mimicking effects of GILZ (Cheng et al., 2013). Together, these observations suggest that GCs and GILZ both play an anti-inflammatory role in regulation of human EC activation, although GILZ is not required for GC actions.

MONOCYTES/MACROPHAGES

GCs inhibit monocyte/macrophage activation via suppressing AP-1 and NF-κB signaling, leading to reduced expression of a wide range of pro-inflammatory genes (Mukaida et al., 1991; Berkman et al., 1995; Marfaing-Koka et al., 1996; Jeon et al., 2000; Steer et al., 2000; Cao et al., 2003). GILZ is constitutively expressed in monocytes/macrophages and its expression is also sensitive to GC treatment (Berrebi et al., 2003; Hamdi et al., 2007a). GILZ inhibits LPS-induced production of cytokines and chemokines such as TNFα, RANTES, IL-6, and IL-1β by human monocytes and macrophages *in vitro*, mimicking the effects of GC (Hamdi et al., 2007a; Wang et al., 2012). Mechanistic studies in THP-1 cells, a human monocyte cell line, show that GILZ directly interacts with the NF-κB p65 subunit and suppresses transcriptional activity, resulting in reduced expression of macrophage activation markers CD80, CD86, and TLR2, and chemoknies CCL5 and CCL3 (Berrebi et al., 2003). Moreover, another GC induced anti-inflammatory protein, AnxA1, requires GILZ to mediate inhibitory effects of GC in murine macrophage (Yang et al., 2009). In keeping with these findings, GILZ expression is inhibited in macrophages from patients with Crohn's disease, tuberculosis and alcoholic hepatitis (AH), typical inflammatory diseases associated with macrophage activation (Berrebi et al., 2003; Hamdi et al., 2007a). This impairment of GILZ expression has been suggested to be implicated in prolonged macrophage responses in these patients. GILZ mRNA stability was found to be reduced in human macrophages upon Toll-like receptor (TLR) activation (Hoppstadter et al., 2012), suggesting a mechanism for permissive reductions in GILZ expression during inflammation. LPS triggers TLR signaling and caused GILZ mRNA degradation in a tristetraprolin (TTP) and MyD88 dependent manner (Hoppstadter et al., 2012). Together, these observations support the notion that GILZ and GCs have similar inhibitory effects on monocyte/macrophages activation via interference with NF-κB signaling.

MESENCHYMAL STEM CELLS (MSCs)/OSTEOBLASTS

One of the main functions of multipotent MSCs is to differentiate into important cell lineages, including adipocytes and osteoblasts (Pittenger et al., 1999). The differentiation of MSCs into adipocytes and osteoblasts is reciprocally modulated, mainly via regulation of the activity of peroxisome proliferator-activated receptor gamma (PPARγ2), a master transcription factor that promotes adipogenic differentiation (Tontonoz et al., 1994; Weinstein et al., 1998; Ahdjoudj et al., 2001; Akune et al., 2004). GCs induce MSC differentiation toward adipocytes and suppress osteogenic differentiation, via promoting CCAAT/enhancer-binding protein δ (C/EBPδ) expression and its binding to the C/EBP binding site within the PPARγ2 promoter, resulting in increased PPARγ2 mRNA expression and transcriptional activity (Shi et al., 2000; Yao et al., 2008). This GC inhibitory effect on osteoblast formation accounts in part for the rapid bone loss observed in patients during GC treatment. Despite the fact that GCs rapidly induces GILZ expression in MSCs, GILZ exerts a completely opposite effect in the regulation of MSC differentiation (Eddleston et al., 2007). GILZ expression in MSCs increases osteogenic differentiation, and inhibits adipocyte formation (Shi et al., 2003; Zhang et al., 2008). GILZ was reported in these studies to bind to the C/EBP site in competition with C/EBPδ, leading to a decreased PPARγ2 expression. Together, these observations suggest that GC and GILZ play opposite roles in regulation of adipocyte and osteoblast formation.

ANIMAL MODELS OF INFLAMMATORY DISEASES

To better understand the role of GILZ in the regulation of inflammation, GILZ effects on animal models of inflammatory diseases have been studied. Consistent with GILZ effects on Th1/Th2 balance *in vitro*, the GILZ TG mice showed increased sensitivity to bleomycin-induced neutrophil infiltration and edema, a typical Th2 dependent model of inflammation (Cannarile et al., 2006). Similarly, GILZ TG mice were more susceptible to oxazolone induced Th2 colitis (Cannarile et al., 2009), consistent with the

pro-Th2 and anti-Th1 effects of GILZ discussed above. On the other hand, a beneficial effect of GILZ has been described in several animal models of Th1-related inflammatory diseases. T cell infiltration is reduced in GILZ TG mice during dinitrobenzene sulfonic acid (DNBS)-induced colitis, accompanied by a decrease in tissue damage, epithelial cell apoptosis, cytokine production, as well as NF-κB activation (Cannarile et al., 2009). Using the same GILZ TG mice, similar protective effects of GILZ were also detected in a spinal cord injury (SCI) model, mainly due to the GILZ inhibition of T cell activation and recruitment (Esposito et al., 2012). Consistently, an ability of GILZ to suppress inflammation was demonstrated in a rat model of experimental autoimmune encephalomyelitis (EAE) using an amphipathic chariot peptide (Pep-1) for systemic administration of GILZ proteins or peptides (Srinivasan and Janardhanam, 2011). Recently, the role of GILZ in the murine collagen-induced arthritis (CIA) model of RA was investigated, via local induction of GILZ expression in response to local injection of GILZ-adeno-associated virus (GILZ-AAV) into the joints (Ngo et al., 2013a). GILZ expression inhibited disease development in the CIA model, mimicking the effects of therapeutic dosing with the GC dexamethasone. Consistent with this observation, GILZ depletion by systemic administration of GILZ siRNA was previously found to increase disease severity in CIA (Beaulieu et al., 2010). Moreover, the anti-inflammatory function of GILZ has been demonstrated in a wild-derived inbred SPRET/Ei mouse strain (Pinheiro et al., 2013). These mice are resistant to LPS-induced endotoxemia, as a result of increased GILZ expression due to genetic variations (Pinheiro et al., 2013). LPS-induced IL-6 and IL-12 production were also reduced in SPRET/Ei macrophages, and silencing GILZ by siRNA, in contrast, completely abolished this effect (Pinheiro et al., 2013). These studies provide direct evidence that GILZ is a key mediator of inflammatory responses in both innate and adaptive immune systems.

To further study biological functions of endogenous GILZ, GILZ knockout (KO) mouse strains were recently generated independently by four laboratories (Bruscoli et al., 2012; Romero et al., 2012; Suarez et al., 2012; Ngo et al., 2013a). Surprisingly, male KO animals are infertile and lack the ability to produce sperm. Further research demonstrated that GILZ plays critical role in regulation of spermatogonia stem cell (SSC) survival and differentiation, likely via mediating ERK, AKT, and FOXO1 activity (Bruscoli et al., 2012; Ngo et al., 2013b). On the other hand, no major alteration in immune responses was detected in GILZ KO mice, despite a moderate increase in T cell proliferation and DTH response (Ngo et al., 2013a). Surprisingly, given the effects of GILZ therapy, GILZ deficiency failed to alter disease phenotypes in antigen-induced arthritis (AIA), K/BxN serum-transfer arthritis, CIA, and LPS-induced cytokinemia (Ngo et al., 2013a). GILZ deficiency also had no effect on GC sensitivity in these models (Ngo et al., 2013a). Consistent with this, bone marrow macrophages of GILZ KO mice showed neither impaired inflammatory response to LPS, nor reduced level of sensitivity to GCs, suggesting that GILZ is redundant for GC immune suppressive functions in these phenomena (Suarez et al., 2012). This result is in line with the findings described in ECs, in which GC actions were not GILZ dependent despite inhibitory actions of GILZ in these cells (Cheng et al., 2013). In contrast, GC effects on DCs were abrogated on GILZ silencing, suggesting variation in GILZ and GC impacts in different cell types. Further research is required to elucidate the divergent molecular pathways involved in GILZ and GC actions in these cells.

CONCLUSIONS

The studies reviewed here suggest that GILZ and GCs share many anti-inflammatory effects on immune cells. However, differences between GILZ and GC impacts on immune cell function and animal models of disease are evident, indicating that the mechanisms involved in the actions of GILZ and GC in specific cell types may be different. Moreover, the requirement for GILZ in GCs actions varies between different cell types, suggesting that GILZ mediation of GCs functions is cell-type-specific. The opposite effects of GILZ and GCs on MSC differentiation raise the possibility that development of a GILZ based gene therapy to treat inflammatory disease could result in effects opposite to those of GC in terms of adiposity and osteoporosis. Further investigation is required to determine whether GILZ induces GC-related adverse metabolic effects. If confirmed, this will support the hypothesis that GILZ therapeutic effects mimic those of GCs but lack GC-like metabolic effects. As a result, GILZ-based gene therapy has great potential in the therapy of human diseases.

KEY CONCEPTS

1. GILZ inhibits inflammatory responses in a number of important immune cell lineages *in vitro*;
2. GILZ reduces disease severity in animal models of inflammatory diseases *in vivo*, mimicking glucocorticoid effects;
3. GILZ exerts inhibitory effects on immune system independent of glucocorticoid receptor and, thus, avoids glucocorticoid-induced adverse metabolic effects;
4. Further investigation is required to confirm the lack of ability of GILZ to induce GC-related side effects;
5. GILZ-based gene therapy has great potential in development of new treatment strategies for human diseases.

REFERENCES

Adcock, I. M., Brown, C. R., Gelder, C. M., Shirasaki, H., Peters, M. J., and Barnes, P. J. (1995). Effects of glucocorticoids on transcription factor activation in human peripheral blood mononuclear cells. *Am. J. Physiol.* 268(2 Pt 1), C331–C338.

Ahdjoudj, S., Lasmoles, F., Oyajobi, B. O., Lomri, A., Delannoy, P., and Marie, P. J. (2001). Reciprocal control of osteoblast/chondroblast and osteoblast/adipocyte differentiation of multipotential clonal human marrow stromal F/STRO-1(+) cells. *J. Cell. Biochem.* 81, 23–38. doi: 10.1002/1097-4644(20010401)

Akune, T., Ohba, S., Kamekura, S., Yamaguchi, M., Chung, U. I., Kubota, N., et al. (2004). PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. *J. Clin. Invest.* 113, 846–855. doi: 10.1172/JCI19900

Almawi, W. Y., Beyhum, H. N., Rahme, A. A., and Rieder, M. J. (1996). Regulation of cytokine and cytokine receptor expression by glucocorticoids. *J. Leukoc. Biol.* 60, 563–572.

Antonioli, L., Fornai, M., Colucci, R., Ghisu, N., Da Settimo, F., Natale, G., et al. (2007). Inhibition of adenosine deaminase attenuates inflammation in experimental colitis. *J. Pharmacol. Exp. Ther.* 322, 435–442. doi: 10.1124/jpet.107.122762

Ashwell, J. D., Lu, F. W., and Vacchio, M. S. (2000). Glucocorticoids in T cell development and function. *Annu. Rev. Immunol.* 18, 309–345. doi: 10.1146/annurev.immunol.18.1.309

Asselin-Labat, M. L., Biola-Vidamment, A., Kerbrat, S., Lombes, M., Bertoglio, J., and Pallardy, M. (2005). FoxO3 mediates antagonistic effects of glucocorticoids and interleukin-2 on glucocorticoid-induced leucine zipper expression. *Mol. Endocrinol.* 19, 1752–1764. doi: 10.1210/me.2004-0206

Asselin-Labat, M. L., David, M., Biola-Vidamment, A., Lecoeuche, D., Zennaro, M. C., Bertoglio, J., et al. (2004). GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. *Blood* 104, 215–223. doi: 10.1182/blood-2003-12-4295

Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., and Karin, M. (1995). Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science* 270, 286–290.

Ayroldi, E., Migliorati, G., Bruscoli, S., Marchetti, C., Zollo, O., Cannarile, L., et al. (2001). Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. *Blood* 98, 743–753. doi: 10.1182/blood.V98.3.743

Ayroldi, E., and Riccardi, C. (2009). Glucocorticoid-induced leucine zipper (GILZ): a new important mediator of glucocorticoid action. *FASEB J.* 23, 3649–3658. doi: 10.1096/fj.09-134684

Ayroldi, E., Zollo, O., Bastianelli, A., Marchetti, C., Agostini, M., Di Virgilio, R., et al. (2007). GILZ mediates the antiproliferative activity of glucocorticoids by negative regulation of Ras signaling. *J. Clin. Invest.* 117, 1605–1615. doi: 10.1172/JCI30724

Ayroldi, E., Zollo, O., Macchiarulo, A., Di Marco, B., Marchetti, C., and Riccardi, C. (2002). Glucocorticoid-induced leucine zipper inhibits the Raf-extracellular signal-regulated kinase pathway by binding to Raf-1. *Mol. Cell. Biol.* 22, 7929–7941. doi: 10.1128/MCB.22.22.7929-7941.2002

Barnes, P. J. (2006). How corticosteroids control inflammation: Quintiles Prize Lecture 2005. *Br. J. Pharmacol.* 148, 245–254. doi: 10.1038/sj.bjp.0706736

Baschant, U., Frappart, L., Rauchhaus, U., Bruns, L., Reichardt, H. M., Kamradt, T., et al. (2011). Glucocorticoid therapy of antigen-induced arthritis depends on the dimerized glucocorticoid receptor in T cells. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19317–19322. doi: 10.1073/pnas.1105857108

Beaulieu, E., and Morand, E. F. (2011). Role of GILZ in immune regulation, glucocorticoid actions and rheumatoid arthritis. *Nat. Rev. Rheumatol.* 7, 340–348. doi: 10.1038/nrrheum.2011.59

Beaulieu, E., Ngo, D., Santos, L., Yang, Y. H., Smith, M., Jorgensen, C., et al. (2010). Glucocorticoid-induced leucine zipper is an endogenous antiinflammatory mediator in arthritis. *Arthritis Rheum.* 62, 2651–2661. doi: 10.1002/art.27566

Berkman, N., Jose, P. J., Williams, T. J., Schall, T. J., Barnes, P. J., and Chung, K. F. (1995). Corticosteroid inhibition of macrophage inflammatory protein-1 alpha in human monocytes and alveolar macrophages. *Am. J. Physiol.* 269(4 Pt 1), L443–L452.

Berrebi, D., Bruscoli, S., Cohen, N., Foussat, A., Migliorati, G., Bouchet-Delbos, L., et al. (2003). Synthesis of glucocorticoid-induced leucine zipper (GILZ) by macrophages: an anti-inflammatory and immunosuppressive mechanism shared by glucocorticoids and IL-10. *Blood* 101, 729–738. doi: 10.1182/blood-2002-02-0538

Bianchini, R., Nocentini, G., Krausz, L. T., Fettucciari, K., Coaccioli, S., Ronchetti, S., et al. (2006). Modulation of pro- and antiapoptotic molecules in double-positive (CD4+CD8+) thymocytes following dexamethasone treatment. *J. Pharmacol. Exp. Ther.* 319, 887–897. doi: 10.1124/jpet.106.108480

Bruscoli, S., Di Virgilio, R., Donato, V., Velardi, E., Baldoni, M., Marchetti, C., et al. (2006). Genomic and non-genomic effects of different glucocorticoids on mouse thymocyte apoptosis. *Eur. J. Pharmacol.* 529, 63–70. doi: 10.1016/j.ejphar.2005.10.053

Bruscoli, S., Velardi, E., Di Sante, M., Bereshchenko, O., Venanzi, A., Coppo, M., et al. (2012). Long glucocorticoid-induced leucine zipper (L-GILZ) protein interacts with ras protein pathway and contributes to spermatogenesis control. *J. Biol. Chem.* 287, 1242–1251. doi: 10.1074/jbc.M111.316372

Cannarile, L., Cuzzocrea, S., Santucci, L., Agostini, M., Mazzon, E., Esposito, E., et al. (2009). Glucocorticoid-induced leucine zipper is protective in Th1-mediated models of colitis. *Gastroenterology* 136, 530–541. doi: 10.1053/j.gastro.2008.09.024

Cannarile, L., Fallarino, F., Agostini, M., Cuzzocrea, S., Mazzon, E., Vacca, C., et al. (2006). Increased GILZ expression in transgenic mice up-regulates Th-2 lymphokines. *Blood* 107, 1039–1047. doi: 10.1182/blood-2005-05-2183

Cao, L., Qian, L. L., Zhu, Y. R., Guo, C. B., Gong, X. H., and Sun, B. (2003). Regulation of activity of nuclear factor-kappaB and activator protein-1 by nitric oxide, surfactant and glucocorticoids in alveolar macrophages from piglets with acute lung injury. *Acta Pharmacol. Sin.* 24, 1316–1323.

Chen, C. C., and Manning, A. M. (1995). Transcriptional regulation of endothelial cell adhesion molecules: a dominant role for NF-kappa B. *Agents Actions Suppl.* 47, 135–141.

Chen, X., Oppenheim, J. J., Winkler-Pickett, R. T., Ortaldo, J. R., and Howard, O. M. (2006). Glucocorticoid amplifies IL-2-dependent expansion of functional FoxP3(+)CD4(+)CD25(+) T regulatory cells in vivo and enhances their capacity to suppress EAE. *Eur. J. Immunol.* 36, 2139–2149. doi: 10.1002/eji.200635873

Cheng, Q., Fan, H., Ngo, D., Beaulieu, E., Leung, P., Lo, C. Y., et al. (2013). GILZ overexpression inhibits endothelial cell adhesive function through regulation of NF-κB and MAPK activity. *J. Immunol.* 191, 424–433. doi: 10.4049/jimmunol.1202662

Cheng, Q., McKeown, S. J., Santos, L., Santiago, F. S., Khachigian, L. M., Morand, E. F., et al. (2010). Macrophage migration inhibitory factor increases leukocyte-endothelial interactions in human endothelial cells via promotion of expression of adhesion molecules. *J. Immunol.* 185, 1238–1247. doi: 10.4049/jimmunol.0904104

Clark, A. R. (2003). MAP kinase phosphatase 1: a novel mediator of biological effects of glucocorticoids? *J. Endocrinol.* 178, 5–12. doi: 10.1677/joe.0.1780005

Clark, A. R., and Belvisi, M. G. (2012). Maps and legends: the quest for dissociated ligands of the glucocorticoid receptor. *Pharmacol. Ther.* 134, 54–67. doi: 10.1016/j.pharmthera.2011.12.004

Cohen, J. J., and Duke, R. C. (1984). Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* 132, 38–42.

Cohen, N., Mouly, E., Hamdi, H., Maillot, M. C., Pallardy, M., Godot, V., et al. (2006). GILZ expression in human dendritic cells redirects their maturation and prevents antigen-specific T lymphocyte response. *Blood* 107, 2037–2044. doi: 10.1182/blood-2005-07-2760

D'Adamio, F., Zollo, O., Moraca, R., Ayroldi, E., Bruscoli, S., Bartoli, A., et al. (1997). A new dexamethasone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* 7, 803–812.

Daynes, R. A., and Araneo, B. A. (1989). Contrasting effects of glucocorticoids on the capacity of T cells to produce the growth factors interleukin 2 and interleukin 4. *Eur. J. Immunol.* 19, 2319–2325. doi: 10.1002/eji.1830191221

De Bosscher, K., Vanden Berghe, W., and Haegeman, G. (2003). The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. *Endocr. Rev.* 24, 488–522. doi: 10.1210/er.2002-0006

Delfino, D. V., Agostini, M., Spinicelli, S., Vacca, C., and Riccardi, C. (2006). Inhibited cell death, NF-kappaB activity and increased IL-10 in TCR-triggered thymocytes of transgenic mice overexpressing the glucocorticoid-induced protein GILZ. *Int. Immunopharmacol.* 6, 1126–1134. doi: 10.1016/j.intimp.2006.02.001

Delfino, D. V., Agostini, M., Spinicelli, S., Vito, P., and Riccardi, C. (2004). Decrease of Bcl-xL and augmentation of thymocyte apoptosis in GILZ overexpressing transgenic mice. *Blood* 104, 4134–4141. doi: 10.1182/blood-2004-03-0920

Di Marco, B., Massetti, M., Bruscoli, S., Macchiarulo, A., Di Virgilio, R., Velardi, E., et al. (2007). Glucocorticoid-induced leucine zipper (GILZ)/NF-kappaB interaction: role of GILZ homo-dimerization and C-terminal domain. *Nucleic Acids Res.* 35, 517–528. doi: 10.1093/nar/gkl1080

Eddleston, J., Herschbach, J., Wagelie-Steffen, A. L., Christiansen, S. C., and Zuraw, B. L. (2007). The anti-inflammatory effect of glucocorticoids is mediated by glucocorticoid-induced leucine zipper in epithelial cells. *J. Allergy Clin. Immunol.* 119, 115–122. doi: 10.1016/j.jaci.2006.08.027

Esposito, E., Bruscoli, S., Mazzon, E., Paterniti, I., Coppo, M., Velardi, E., et al. (2012). Glucocorticoid-induced leucine zipper (GILZ) over-expression in T lymphocytes inhibits inflammation and tissue damage in spinal cord injury. *Neurotherapeutics* 9, 210–225. doi: 10.1007/s13311-011-0084-7

Fan, H., and Morand, E. F. (2012). Targeting the side effects of steroid therapy in autoimmune diseases: the role of GILZ. *Discov. Med.* 13, 123–133.

Furst, R., Schroeder, T., Eilken, H. M., Bubik, M. F., Kiemer, A. K., Zahler, S., et al. (2007). MAPK phosphatase-1 represents a novel anti-inflammatory target of glucocorticoids in the human endothelium. *FASEB J.* 21, 74–80. doi: 10.1096/fj.06-6752com

Glynne, R., Ghandour, G., Rayner, J., Mack, D. H., and Goodnow, C. C. (2000). B-lymphocyte quiescence, tolerance and activation as viewed by global gene

expression profiling on microarrays. *Immunol. Rev.* 176, 216–246. doi: 10.1034/j.1600-065X.2000.00614.x

Godot, V., Garcia, G., Capel, F., Arock, M., Durand-Gasselin, I., Asselin-Labat, M. L., et al. (2006). Dexamethasone and IL-10 stimulate glucocorticoid-induced leucine zipper synthesis by human mast cells. *Allergy* 61, 886–890. doi: 10.1111/j.1398-9995.2006.01065.x

Goodwin, J. E., Feng, Y., Velazquez, H., and Sessa, W. C. (2013). Endothelial glucocorticoid receptor is required for protection against sepsis. *Proc. Natl. Acad. Sci. U.S.A.* 110, 306–311. doi: 10.1073/pnas.1210200110

Grillot, D. A., Merino, R., and Nunez, G. (1995). Bcl-XL displays restricted distribution during T cell development and inhibits multiple forms of apoptosis but not clonal deletion in transgenic mice. *J. Exp. Med.* 182, 1973–1983.

Hackstein, H., and Thomson, A. W. (2004). Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. *Nat. Rev. Immunol.* 4, 24–34. doi: 10.1038/nri1256

Hall, E. D. (2011). Antioxidant therapies for acute spinal cord injury. *Neurotherapeutics* 8, 152–167. doi: 10.1007/s13311-011-0026-4

Hamdi, H., Bigorgne, A., Naveau, S., Balian, A., Bouchet-Delbos, L., Cassard-Doulcier, A. M., et al. (2007a). Glucocorticoid-induced leucine zipper: A key protein in the sensitization of monocytes to lipopolysaccharide in alcoholic hepatitis. *Hepatology* 46, 1986–1992. doi: 10.1002/hep.21880

Hamdi, H., Godot, V., Maillot, M. C., Prejean, M. V., Cohen, N., Krzysiek, R., et al. (2007b). Induction of antigen-specific regulatory T lymphocytes by human dendritic cells expressing the glucocorticoid-induced leucine zipper. *Blood* 110, 211–219. doi: 10.1182/blood-2006-10-052506

Hoppstadter, J., Diesel, B., Eifler, L. K., Schmid, T., Brune, B., and Kiemer, A. K. (2012). Glucocorticoid-induced leucine zipper is downregulated in human alveolar macrophages upon Toll-like receptor activation. *Eur. J. Immunol.* 42, 1282–1293. doi: 10.1002/eji.201142081

Jeon, Y. J., Han, S. H., Lee, Y. W., Lee, M., Yang, K. H., and Kim, H. M. (2000). Dexamethasone inhibits IL-1 beta gene expression in LPS-stimulated RAW 264.7 cells by blocking NF-kappa B/Rel and AP-1 activation. *Immunopharmacology* 48, 173–183. doi: 10.1016/S0162-3109(00)00199-5

Joyce, D. A., Gimblett, G., and Steer, J. H. (2001). Targets of glucocorticoid action on TNF-alpha release by macrophages. *Inflamm. Res.* 50, 337–340.

Khorsandi, L. S., Hashemitabar, M., Orazizadeh, M., and Albughobeish, N. (2008). Dexamethasone effects on fas ligand expression in mouse testicular germ cells. *Pak. J. Biol. Sci.* 11, 2231–2236. doi: 10.3923/pjbs.2008.2231.2236

Kitajima, T., Ariizumi, K., Bergstresser, P. R., and Takashima, A. (1996). A novel mechanism of glucocorticoid-induced immune suppression: the inhibiton of T cell-mediated terminal maturation of a murine dendritic cell line. *J. Clin. Invest.* 98, 142–147. doi: 10.1172/JCI118759

Kleiman, A., Hubner, S., Rodriguez Parkitna, J. M., Neumann, A., Hofer, S., Weigand, M. A., et al. (2012). Glucocorticoid receptor dimerization is required for survival in septic shock via suppression of interleukin-1 in macrophages. *FASEB J.* 26, 722–729. doi: 10.1096/fj.11-192112

Kuldo, J. M., Westra, J., Asgeirsdottir, S. A., Kok, R. J., Oosterhuis, K., Rots, M. G., et al. (2005). Differential effects of NF-κB and p38 MAPK inhibitors and combinations thereof on TNF-α- and IL-1β-induced proinflammatory status of endothelial cells in vitro. *Am. J. Physiol. Cell Physiol.* 289, C1229–C1239. doi: 10.1152/ajpcell.00620.2004

Kunicka, J. E., Talle, M. A., Denhardt, G. H., Brown, M., Prince, L. A., and Goldstein, G. (1993). Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. *Cell. Immunol.* 149, 39–49. doi: 10.1006/cimm.1993.1134

Latre de Late, P., Pepin, A., Assaf-Vandecasteele, H., Espinasse, C., Nicolas, V., Asselin-Labat, M. L., et al. (2010). Glucocorticoid-induced leucine zipper (GILZ) promotes the nuclear exclusion of FOXO3 in a Crm1-dependent manner. *J. Biol. Chem.* 285, 5594–5605. doi: 10.1074/jbc.M109.068346

Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007). Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* 7, 678–689. doi: 10.1038/nri2156

Liberman, A. C., Refojo, D., Antunica-Noguerol, M., Holsboer, F., and Arzt, E. (2012). Underlying mechanisms of cAMP- and glucocorticoid-mediated inhibition of FasL expression in activation-induced cell death. *Mol. Immunol.* 50, 220–235. doi: 10.1016/j.molimm.2012.01.008

Marfaing-Koka, A., Maravic, M., Humbert, M., Galanaud, P., and Emilie, D. (1996). Contrasting effects of IL-4, IL-10 and corticosteroids on RANTES production by human monocytes. *Int. Immunol.* 8, 1587–1594.

McKay, L. I., and Cidlowski, J. A. (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr. Rev.* 20, 435–459.

McKinley, L., Alcorn, J. F., Peterson, A., Dupont, R. B., Kapadia, S., Logar, A., et al. (2008). TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. *J. Immunol.* 181, 4089–4097. doi: 10.4049/jimmunol.181.6.4089

Mittelstadt, P. R., and Ashwell, J. D. (2001). Inhibition of AP-1 by the glucocorticoid-inducible protein GILZ. *J. Biol. Chem.* 276, 29603–29610. doi: 10.1074/jbc.M101522200

Mittelstadt, P. R., Monteiro, J. P., and Ashwell, J. D. (2012). Thymocyte responsiveness to endogenous glucocorticoids is required for immunological fitness. *J. Clin. Invest.* 122, 2384–2394. doi: 10.1172/JCI63067

Miyaura, H., and Iwata, M. (2002). Direct and indirect inhibition of Th1 development by progesterone and glucocorticoids. *J. Immunol.* 168, 1087–1094. doi: 10.4049/jimmunol.168.3.1087

Mukaida, N., Zachariae, C. C., Gusella, G. L., and Matsushima, K. (1991). Dexamethasone inhibits the induction of monocyte chemotactic-activating factor production by IL-1 or tumor necrosis factor. *J. Immunol.* 146, 1212–1215.

Nanzer, A. M., Chambers, E. S., Ryanna, K., Richards, D. F., Black, C., Timms, P. M., et al. (2013). Enhanced production of IL-17A in patients with severe asthma is inhibited by 1alpha,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion. *J. Allergy Clin. Immunol.* 132, 297–304.e3. doi: 10.1016/j.jaci.2013.03.037

Newton, R. (2000). Molecular mechanisms of glucocorticoid action: what is important? *Thorax* 55, 603–613. doi: 10.1136/thorax.55.7.603

Ngo, D., Beaulieu, E., Gu, R., Leaney, A., Santos, L., Fan, H., et al. (2013a). Divergent effects of endogenous and exogenous glucocorticoid-induced leucine zipper in animal models of inflammation and arthritis. *Arthritis Rheum.* 65, 1203–1212. doi: 10.1002/art.37858

Ngo, D., Cheng, Q., AE, O. C., Deboer, K. D., Lo, C. Y., Beaulieu, E., et al. (2013b). Glucocorticoid-induced leucine zipper (GILZ) regulates testicular FOXO1 activity and spermatogonial stem cell (SSC) function. *PLoS ONE* 8:e59149. doi: 10.1371/journal.pone.0059149

Nixon, M., Andrew, R., and Chapman, K. E. (2013). It takes two to tango: dimerisation of glucocorticoid receptor and its anti-inflammatory functions. *Steroids* 78, 59–68. doi: 10.1016/j.steroids.2012.09.013

Orazizadeh, M., Khorsandi, L. S., and Hashemitabar, M. (2010). Toxic effects of dexamethasone on mouse testicular germ cells. *Andrologia* 42, 247–253. doi: 10.1111/j.1439-0272.2009.00985.x

Paliogianni, F., Raptis, A., Ahuja, S. S., Najjar, S. M., and Boumpas, D. T. (1993). Negative transcriptional regulation of human interleukin 2 (IL-2) gene by glucocorticoids through interference with nuclear transcription factors AP-1 and NF-AT. *J. Clin. Invest.* 91, 1481–1489. doi: 10.1172/JCI116353

Pan, L. L., Liu, X. H., Gong, Q. H., Wu, D., and Zhu, Y. Z. (2011). Hydrogen sulfide attenuated tumor necrosis factor-alpha-induced inflammatory signaling and dysfunction in vascular endothelial cells. *PLoS ONE* 6:e19766. doi: 10.1371/journal.pone.0019766

Patel, H. B., Kornerup, K. N., Sampaio, A. L., D'Acquisto, F., Seed, M. P., Girol, A. P., et al. (2012). The impact of endogenous annexin A1 on glucocorticoid control of inflammatory arthritis. *Ann. Rheum. Dis.* 71, 1872–1880. doi: 10.1136/annrheumdis-2011-201180

Perretti, M., and D'Acquisto, F. (2009). Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat. Rev. Immunol.* 9, 62–70. doi: 10.1038/nri2470

Pinheiro, I., Dejager, L., Petta, I., Vandevyver, S., Puimege, L., Mahieu, T., et al. (2013). LPS resistance of SPRET/Ei mice is mediated by Gilz, encoded by the Tsc22d3 gene on the X chromosome. *EMBO Mol. Med.* 5, 456–470. doi: 10.1002/emmm.201201683

Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., et al. (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143–147.

Ratman, D., Vanden Berghe, W., Dejager, L., Libert, C., Tavernier, J., Beck, I. M., et al. (2013). How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. *Mol. Cell. Endocrinol.* 380, 41–54. doi: 10.1016/j.mce.2012.12.014

Ray, K. P., Farrow, S., Daly, M., Talabot, F., and Searle, N. (1997). Induction of the E-selectin promoter by interleukin 1 and tumour necrosis factor alpha, and inhibition by glucocorticoids. *Biochem. J.* 328(Pt 2), 707–715.

Rea, D., van Kooten, C., van Meijgaarden, K. E., Ottenhoff, T. H., Melief, C. J., and Offringa, R. (2000). Glucocorticoids transform CD40-triggering of dendritic cells into an alternative activation pathway resulting in antigen-presenting cells that secrete IL-10. *Blood* 95, 3162–3167.

Reichardt, H. M., Tuckermann, J. P., Gottlicher, M., Vujic, M., Weih, F., Angel, P., et al. (2001). Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J.* 20, 7168–7173. doi: 10.1093/emboj/20.24.7168

Rhen, T., and Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. *N. Engl. J. Med.* 353, 1711–1723. doi: 10.1056/NEJMra050541

Romero, Y., Vuandaba, M., Suarez, P., Grey, C., Calvel, P., Conne, B., et al. (2012). The Glucocorticoid-induced leucine zipper (GILZ) Is essential for spermatogonial survival and spermatogenesis. *Sex Dev.* 6, 169–177. doi: 10.1159/000338415

Schacke, H., Berger, M., Rehwinkel, H., and Asadullah, K. (2007). Selective glucocorticoid receptor agonists (SEGRAs): novel ligands with an improved therapeutic index. *Mol. Cell. Endocrinol.* 275, 109–117. doi: 10.1016/j.mce.2007.05.014

Schacke, H., Rehwinkel, H., and Asadullah, K. (2005). Dissociated glucocorticoid receptor ligands: compounds with an improved therapeutic index. *Curr. Opin. Investig. Drugs* 6, 503–507.

Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., and Korsmeyer, S. J. (1991). bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* 67, 879–888.

Shi, X. M., Blair, H. C., Yang, X., McDonald, J. M., and Cao, X. (2000). Tandem repeat of C/EBP binding sites mediates PPARgamma2 gene transcription in glucocorticoid-induced adipocyte differentiation. *J. Cell. Biochem.* 76, 518–527. doi: 10.1002/(SICI)1097-4644(20000301)76:3<518::AID-JCB18>3.0.CO;2-M

Shi, X., Shi, W., Li, Q., Song, B., Wan, M., Bai, S., et al. (2003). A glucocorticoid-induced leucine-zipper protein, GILZ, inhibits adipogenesis of mesenchymal cells. *EMBO Rep.* 4, 374–380. doi: 10.1038/sj.embor.embor805

Soundararajan, R., Wang, J., Melters, D., and Pearce, D. (2007). Differential activities of glucocorticoid-induced leucine zipper protein isoforms. *J. Biol. Chem.* 282, 36303–36313. doi: 10.1074/jbc.M707287200

Srinivasan, M., and Janardhanam, S. (2011). Novel p65 binding glucocorticoid-induced leucine zipper peptide suppresses experimental autoimmune encephalomyelitis. *J. Biol. Chem.* 286, 44799–44810. doi: 10.1074/jbc.M111.279257

Steer, J. H., Kroeger, K. M., Abraham, L. J., and Joyce, D. A. (2000). Glucocorticoids suppress tumor necrosis factor-alpha expression by human monocytic THP-1 cells by suppressing transactivation through adjacent NF-kappa B and c-Jun-activating transcription factor-2 binding sites in the promoter. *J. Biol. Chem.* 275, 18432–18440. doi: 10.1074/jbc.M906304199

Suarez, P. E., Rodriguez, E. G., Soundararajan, R., Merillat, A. M., Stehle, J. C., Rotman, S., et al. (2012). The glucocorticoid-induced leucine zipper (gilz/Tsc22d3-2) gene locus plays a crucial role in male fertility. *Mol. Endocrinol.* 26, 1000–1013. doi: 10.1210/me.2011-1249

Surjit, M., Ganti, K. P., Mukherji, A., Ye, T., Hua, G., Metzger, D., et al. (2011). Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 145, 224–241. doi: 10.1016/j.cell.2011.03.027

Taube, M., and Carlsten, H. (2000). Action of dexamethasone in the suppression of delayed-type hypersensitivity in reconstituted SCID mice. *Inflamm. Res.* 49, 548–552. doi: 10.1007/s000110050630

Thomas, N., Edwards, J. L., and Bell, P. A. (1983). Studies of the mechanism of glucocorticoid-induced pyknosis in isolated rat thymocytes. *J. Steroid Biochem.* 18, 519–524.

Tontonoz, P., Hu, E., and Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 79, 1147–1156.

Tsitoura, D. C., and Rothman, P. B. (2004). Enhancement of MEK/ERK signaling promotes glucocorticoid resistance in CD4+ T cells. *J. Clin. Invest.* 113, 619–627. doi: 10.1172/JCI18975

Unger, W. W., Laban, S., Kleijwegt, F. S., van der Slik, A. R., and Roep, B. O. (2009). Induction of Treg by monocyte-derived DC modulated by vitamin D3 or dexamethasone: differential role for PD-L1. *Eur. J. Immunol.* 39, 3147–3159. doi: 10.1002/eji.200839103

Vacca, A., Felli, M. P., Farina, A. R., Martinotti, S., Maroder, M., Screpanti, I., et al. (1992). Glucocorticoid receptor-mediated suppression of the interleukin 2 gene expression through impairment of the cooperativity between nuclear factor of activated T cells and AP-1 enhancer elements. *J. Exp. Med.* 175, 637–646.

Vandevyver, S., Dejager, L., Tuckermann, J., and Libert, C. (2013). New insights into the anti-inflammatory mechanisms of glucocorticoids: an emerging role for glucocorticoid-receptor-mediated transactivation. *Endocrinology* 154, 993–1007. doi: 10.1210/en.2012-2045

Wang, W., Wykrzykowska, J., Johnson, T., Sen, R., and Sen, J. (1999). A NF-kappa B/c-myc-dependent survival pathway is targeted by corticosteroids in immature thymocytes. *J. Immunol.* 162, 314–322.

Wang, Y., Ma, Y. Y., Song, X. L., Cai, H. Y., Chen, J. C., Song, L. N., et al. (2012). Upregulations of glucocorticoid-induced leucine zipper by hypoxia and glucocorticoid inhibit proinflammatory cytokines under hypoxic conditions in macrophages. *J. Immunol.* 188, 222–229. doi: 10.4049/jimmunol.1002958

Weinstein, D. S., Gong, H., Doweyko, A. M., Cunningham, M., Habte, S., Wang, J. H., et al. (2011). Azaxanthene based selective glucocorticoid receptor modulators: design, synthesis, and pharmacological evaluation of (S)-4-(5-(1-((1,3,4-thiadiazol-2-yl)amino)-2-methyl-1-oxopropan-2-yl)-5H-chromeno [2,3-b]pyridin-2-yl)-2-fluoro-N,N-dimethylbenzamide (BMS-776532) and its methylene homologue (BMS-791826). *J. Med. Chem.* 54, 7318–7333. doi: 10.1021/jm200879j

Weinstein, R. S., Jilka, R. L., Parfitt, A. M., and Manolagas, S. C. (1998). Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. *J. Clin. Invest.* 102, 274–282. doi: 10.1172/JCI2799

Yang, Y. H., Aeberli, D., Dacumos, A., Xue, J. R., and Morand, E. F. (2009). Annexin-1 regulates macrophage IL-6 and TNF via glucocorticoid-induced leucine zipper. *J. Immunol.* 183, 1435–1445. doi: 10.4049/jimmunol.0804000

Yang, Y. H., Morand, E. F., Getting, S. J., Paul-Clark, M., Liu, D. L., Yona, S., et al. (2004). Modulation of inflammation and response to dexamethasone by Annexin 1 in antigen-induced arthritis. *Arthritis Rheum.* 50, 976–984. doi: 10.1002/art.20201

Yang, Y. H., Morand, E., and Leech, M. (2013). Annexin A1: potential for glucocorticoid sparing in RA. *Nat. Rev. Rheumatol.* 9, 595–603. doi: 10.1038/nrrheum.2013.126

Yang, Y., Mercep, M., Ware, C. F., and Ashwell, J. D. (1995). Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. *J. Exp. Med.* 181, 1673–1682.

Yao, W., Cheng, Z., Busse, C., Pham, A., Nakamura, M. C., and Lane, N. E. (2008). Glucocorticoid excess in mice results in early activation of osteoclastogenesis and adipogenesis and prolonged suppression of osteogenesis: a longitudinal study of gene expression in bone tissue from glucocorticoid-treated mice. *Arthritis Rheum.* 58, 1674–1686. doi: 10.1002/art.23454

Zacharchuk, C. M., Mercep, M., Chakraborti, P. K., Simons, S. S. Jr., and Ashwell, J. D. (1990). Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. *J. Immunol.* 145, 4037–4045.

Zhang, W., Yang, N., and Shi, X. M. (2008). Regulation of mesenchymal stem cell osteogenic differentiation by glucocorticoid-induced leucine zipper (GILZ). *J. Biol. Chem.* 283, 4723–4729. doi: 10.1074/jbc.M704147200

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 May 2014; paper pending published: 12 June 2014; accepted: 25 June 2014; published online: 17 July 2014.

*Citation: Cheng Q, Morand E and Yang YH (2014) Development of novel treatment strategies for inflammatory diseases—similarities and divergence between glucocorticoids and GILZ. Front. Pharmacol. **5**:169. doi: 10.3389/fphar.2014.00169*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

REVIEW ARTICLE
published: 13 December 2013
doi: 10.3389/fphar.2013.00155

Lipid- and sugar-modified endomorphins: novel targets for the treatment of neuropathic pain

Pegah Varamini*[1] *and Istvan Toth*[1,2]

[1] *Medicinal Chemistry Department, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia*
[2] *Medicinal Chemistry Department, School of Pharmacy, The University of Queensland, Brisbane, QLD, Australia*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Karen K. Szumlinski, University of California, Santa Barbara, USA
George Kokotos, University of Athens, Greece
Robin Polt, The University of Arizona, USA

****Correspondence:***
Istvan Toth, School of Chemistry and Molecular Biosciences, The University of Queensland, Chemistry Bld. 68, Cooper Road, St. Lucia, Brisbane, QLD 4072, Australia
e-mail: i.toth@uq.edu.au

Endomorphins are endogenous opioid peptides that cause potent antinociception in rodent models of acute and neuropathic pain with less undesirable side effects than opioid alkaloids. However, endomorphins are poorly suited to clinical applications because of low membrane permeability and a susceptibility to enzymatic degradation. Glycosylation and lipidation have proven to be two of the most robust approaches for the generation of new therapeutic endomorphin derivatives. Conjugation with lipoamino acids (LAA) confers an amphipathic character to the peptide, which improved interaction between the peptide and the lipid bilayer of the cell membranes, increasing permeability. Glycosylation can also improve peptide stability and blood brain barrier (BBB) transport. It is believed that an endocytotic mechanism (transcytosis) is responsible for the systemic delivery of water-soluble glycopeptides. This review discusses the application of glycosylation and lipidation strategies to improve the drug-like properties of endomorphins. Pharmacologically active endomorphin analogs with less adverse effects are also discussed.

Keywords: endomorphin, peptide delivery, neuropathic pain, lipoamino acid, glycosylation, lipopeptide, glycopeptide, blood brain barrier

INTRODUCTION

Opioid analgesics such as morphine are among the most commonly used for the treatment of severe pain. Although opioid analgesics are useful for the relief of nociceptive pain, their efficacy against neuropathic pain is limited. Furthermore, they are associated with a range of undesirable side effects such as constipation, respiratory depression, tolerance, and physical dependence, particularly with long-term use. Investigations into new effective treatments for neuropathic pain that could replace opioid alkaloids have predominantly focused on the development of peptide analogs with selectivity for μ-opioid (MOP) receptors (Vaccarino and Kastin, 2000). In order to be effective as an analgesic for clinical application, the peptide analog must confer high bioavailability, which is achieved through good blood brain barrier (BBB) permeability and resistance to enzymatic degradation. Delivery of pharmaceutical agents to the brain is highly challenging (Banks et al., 1992). Various properties combine to make the BBB a formidable barrier, including tight junctions, minimized surface area, electrostatic interactions and increased metabolism, as well as an active efflux system. Different approaches have been used to improve the brain penetration of pharmaceutical agents (Banks and Kastin, 1985; Begley, 1996). Endomorphin-1 and -2 are naturally occurring peptides with excellent therapeutic potential as replacements for morphine-like opioids. They are potent, highly selective MOP receptor agonists with remarkable anti-neuropathic properties in different rodent models of neuropathic pain (Przewlocki and Przewlocka, 2001) and cause less adverse effects than opioid alkaloids (Vaccarino et al., 1999; Czapla et al., 2000). Nevertheless, like most peptide neurotransmitters and neuromodulators in the CNS, modifications are required to transport endomorphins to the brain.

HISTORICAL PERSPECTIVE

All endogenous opioid peptides that contain Tyr-Gly-Gly-Phe (including endorphins, enkephakins, and dynorphins), possess affinity for the three opioid receptors: μ (MOP), δ (DOP), and κ (KOP), with low to moderate specificity. β-endorphins have comparable affinity for both MOP and DOP receptors. Met- and leu-enkephalins are DOP receptor endogenous ligands and dynorphins are ligands of KOP receptors. Mammalian peptides with high selectivity and affinity for MOP receptors were not known until the discovery of endomorphins. In 1997, Zadina et al.. replaced the Gly in the endogenous peptide sequence (Tyr-Pro-Trp-Gly-NH_2) which had high MOP receptor selectivity, but low affinity (Hughes et al., 1975),with all possible natural amino acids (Zadina et al., 1997). Among all derivatives, the Phe-substituted sequence showed the highest affinity and selectivity for MOP receptors and was called endomorphin-1 (Tyr-Pro-Trp-Phe-NH2). Endomorphin-1 and -2, (Tyr-Pro-Phe-Phe-NH2) (**Figure 1**) were first isolated from bovine brain (Zadina et al., 1997) and then from human cortex (Hackler et al., 1997). Although the precursor(s) of endomorphins remain unidentified, their extraordinarily high affinity and selectivity for MOP receptors in the brain supports the proposal that they are endogenous MOP receptor ligands.

STRATEGIES TO IMPROVE BIOAVAILABILITY

Different strategies have been investigated for the effective delivery of endomorphin.

Use of peptidase inhibitors such as tripeptides diprotin A and B, Tyr-Pro-Ala-NH2 (EMDB-2), and Tyr-Pro-Ala-OH (EMDB-3), enhanced and prolonged the antinociceptive effects of endomorphins (Sakurada et al., 2003; Cravezic et al., 2011). However, due to the need for central administration of both peptides and peptidase inhibitors (Fichna et al., 2010; Cravezic et al., 2011), this approach achieved a low degree of success.

Several strategies have been developed to manipulate the structure of endomorphins. The current data indicate that is conformational adaptation of the neuropeptides to the different MOP, DOP, and KOP receptor topographies. Thus, the design of conformationally restricted analogs is of great importance for the selective targeting of a single distinct receptor type (Schiller and DiMaio, 1982). The greater rigidity of the bioactive peptide epitope affects the receptor binding affinity in a receptor-specific way (Bock et al., 2013). Therefore, selectivity of the peptide can be tuned based on the level of conformational restriction imposed by the constraints (Clark et al., 2010).

Glycosylation and lipidation are two successful strategies where peptide conformation is locally restricted. The incorporation of lipids and sugars as conformational constraints (Hruby et al., 1990; Kawai et al., 1990; Hruby and Balse, 2000) significantly improved the pharmacological properties of various peptides (Polt et al., 1994; Egleton et al., 2000; Falconer and Toth, 2007; Cros et al., 2011).

GLYCOSYLATION

Conjugation of carbohydrates to peptides increased the biodistribution of opioid peptides by enhancing permeation across intestinal epithelium and BBB via natural transporters (Poduslo and Curran, 1994; Egleton and Davis, 2005). Membrane proteins such as glucose transporters, GLUT and SGLUT, allowed for the uptake of specific carbohydrates through active, or facilitated transport pathways to deliver peptides across biological barriers (Wood and Trayhurn, 2003). In spite of difficulties associated with their synthesis (Buskas et al., 2006), carbohydrates improved the water solubility, stability and bioavailability of the peptide analogs (Albert et al., 1993; Polt et al., 1994; Negri et al., 1999; Bilsky et al., 2000; Egleton et al., 2000). Although glycosylation reduces passive diffusion of the peptides by reducing their lipophilicity, it makes the peptide a favorable substrate for glucose transporters. Thus, this modification often results in increased penetration through the BBB and GI membranes (Horvat, 2001; Gentilucci, 2004). However, the exact mechanism through which glycosylation improves BBB or GI transport is yet to be elucidated. Octanol/saline distribution studies indicated that transport does not occur via passive diffusion, due to significantly lower lipophilicity (Egleton et al., 2000). Alternatively, the amphipathic nature of glycopeptides was suggested to be responsible for their enhanced permeability through barriers particularly BBB (Palian et al., 2003). For the glycopeptides to be effectively delivered to the brain, it is necessary to produce "biousian" activity (Polt et al., 2005a). *Ousia* means "essence" in Greek. It is important for the glycopeptides to have two essences, an amphipathic state that promotes adsorption to biological membranes and a random coil state that is water-soluble. Biousian effect enabled the compound to undergo endocytosis or permits "membrane hopping" (Egleton et al., 2005). Through extensive studies on a library of glycopeptides, negative membrane curvature on the surface of endothelial cells was shown to be promoted by permeable glycopeptides (Dhanasekaran et al., 2005). This in turn led to an increase in BBB transport (**Figure 2**) (Broadwell et al., 1988; Egleton et al., 2001; Polt et al., 2005b).

Distribution and pharmacodynamic of the peptides are immensely affected by glycosylation. This allows glycosidic moieties to be used as vectors for targeting specific carbohydrate-recognition receptors (Eduardo, 1994).

LIPIDATION

Lipidation is a post-translational peptide modification that significantly influences the properties of peptides and is used in the design of peptide drugs. The presence of polar groups reduced the peptides' partition coefficients and subsequently decreased their membrane permeability (Chikhale et al., 1994). Lipidation provided a simple way to modulate peptide lipophilicity, and facilitates their interaction with cell membranes and penetration across biological barriers by passive diffusion (Balaz, 2000; Griffin and O'Driscoll, 2011). Through increasing the membrane-like properties of the peptides, lipidation improved their interaction with the lipid bilayer within the cell membrane (Pignatello et al., 2005). Both lipoamino acids (LAA) and fatty acid chains have been attached to the peptides to enhance their permeability across biological membranes (Desino et al., 2009). LAAs are α-amino acids with varying length (usually C8–20) alkyl side chains (**Figure 3**). Having both the hydrophobic properties of lipids and the hydrophilic characteristics of α-amino acids, LAAs are appropriate conjugates to incorporate into the structure of peptides (Toth, 1994; Kokotos et al., 1996). Although the conjugation of fatty acids to the peptides will ultimately result in an increase

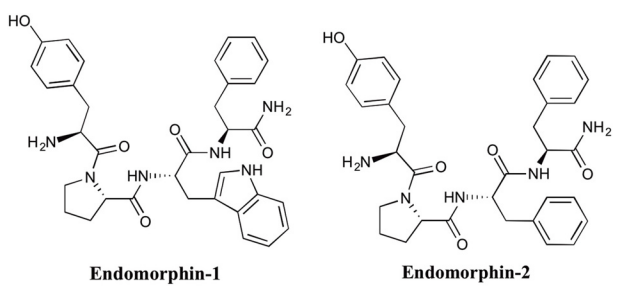


FIGURE 1 | The structure of endomorphin-1 and -2.

FIGURE 2 | Endocytosis of glucopeptides (Polt, 2008).

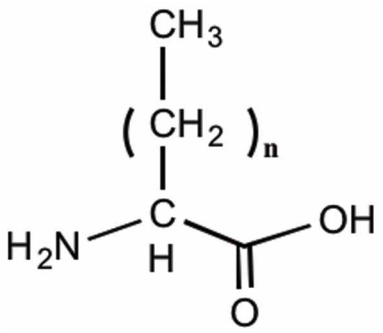


FIGURE 3 | Structure of lipoamino acids.

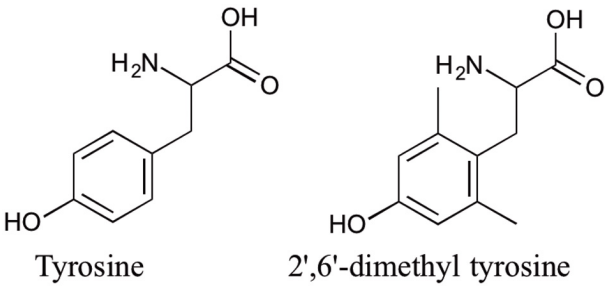


FIGURE 4 | Structure of Tyrosine and 2′,6′-dimethyltyrosine.

in their lipophilicity, the addition of LAAs is more advantageous due to their amphipathic character (Toth, 1994). In addition it plays an important role in enhancing peptide's stability against enzymatic degradation (Wang et al., 2006). This in turn affects the absorption, distribution, metabolism and excretion (ADME) and bioavailability of drugs and makes it an attractive strategy to convert peptides into drug leads (Silvius, 2002).

PHYSIOLOGICAL AND PHARMACOLOGICAL PROPERTIES OF LIPO- AND GLYCO-ENDOMORPHINS

METABOLIC STABILITY AND MEMBRANE PERMEABILITY

Lipoamino acid modification

The endogenous opioid peptide leu-enkephalin was chemically modified by a lipophilic dimethylmaleic anhydride analog. This analog showed a 12- and 32-fold increase in mouse small intestinal mucosal homogenate and liver homogenate (Wang et al., 2006).

A series of glycosylated endomorphin-1 peptides were synthesized by modifying either the N- or C-terminus of endomorphin-1 with glucose succinate or glucose, respectively. The half-life of the analog conjugated with glucose at the N-terminus increased from 5 min for endomorphin-1 to 38 min in the Caco-2 cell homogenates. However, the C8LAA-modified glycosylated analog produced even higher stability in the Caco-2 cell homogenate assay with a half-life of 75 min (Koda et al., 2008). Although there was a 3-fold increase in the apparent permeability (P_{app}) of glucose-C8LAA derivative, this was not as pronounced as the P_{app} of the compounds modified only with C8LAA compared to endomorphin-1. Due to a significant reduction in the receptor binding affinity of the C8LAA analog, a further modification was performed on the backbone structure of the parent peptide. The unnatural amino acid 2′,6′-dimethyltyrosine (Dmt) the more hydrophobic and conformationally restricted residue compared to Tyr (**Figure 4**). Substitution of Tyr with Dmt was shown to enhance the MOP receptor binding affinity and potency of several opioid peptides (Li et al., 2005) including endomorphin-1 (Jinsmaa et al., 2006). Therefore, a Dmt analog, C8LAA[Dmt^1]endomorphin-1 was synthesized which preserved MOP receptor binding affinity (0.08 nM) relative to the parent peptide. This analog exhibited enhanced stability and permeability (Koda et al., 2008). In another trial, the 10-carbon LAA modified peptides with/without substitution of Tyr^1 with Dmt^1 were examined for their biological activity. These endomorphin-1 analogs produced 2–3.5 times higher stability and 2–2.6 times higher permeability for Tyr^1 and DMT^1 analogs, respectively, in comparison with their corresponding C8 derivatives (Varamini et al., 2012b).

Substance P is a neuropeptide, which was first reported to elicit analgesic activity in 1976 by Stewart et al. (Stewart et al., 1976). Previously, a hybrid alkaloid/peptide compound, comprised of morphine sulpfate bound to SP_{3-11}, produced a strong antinociceptive response with little or no development of opioid tolerance or dependence (Kream, 2003; Kream et al., 2007). C10LAA hybrid endomorphin-1 peptides were designed and synthesized by conjugation of the last 4 or 5 C-terminal amino acids of substance P (SP_{8-11} and SP_{7-11}) with all compounds bearing an overlapping Phe in the sequence. It was proposed that the addition of substance P fragments to lipo-endomorphin-1 may decrease the development of tolerance and physical dependence. Two C10-modified endomorphin-1/SP hybrid peptides showed significantly improved half-life and membrane permeability. However, of the two promising LAA-modified derivatives with high stability and permeability, only the one that contained the SP_{7-11} fragment produced potent activity at MOP receptors with significant binding affinity and retained selectivity. The docking scores obtained from conformational studies were also in agreement with the $K_{i\mu}$ values obtained in the receptor binding affinity experiments (**Figure 5**) (Varamini et al., 2012a).

Glycosylation

A sugar-modified derivative of endomorphin was synthesized by attachment of lactose succinamic acid to the peptide (**Figure 6**). This glycosylated analog produced an unprecedented 700-fold increase in membrane permeability and 21-fold increase in plasma stability relative to the native peptide (Varamini et al., 2012c). In this study a receptor-mediated or lactose selective transporter-mediated absorption was suggested for as the mechanism of transport across Caco-2 cell monolayer. According to the Biopharmaceutics Classification System, there is a high correlation between Caco-2 cell permeability coefficients (P_{app} values) and fractional absorption values (Fa) in humans. This makes the results from Caco-2 cell studies a reliable predictor of the oral absorption of compounds in human (Smetanova et al.,

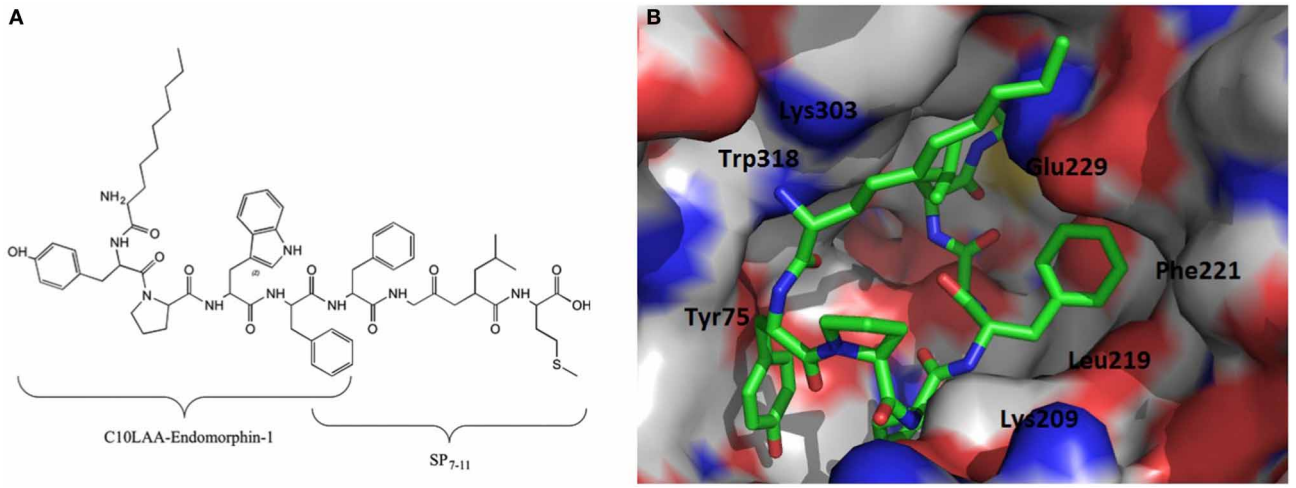


FIGURE 5 | *In vitro* biological analyses revealed that the C10LAA-modified analog conjugated with SP_{7-11} fragment was the most promising derivative. (A) Structure of the compounds. **(B)** Surface view of the active site of the MOP receptor for the highest docking score conformation of MOP receptor with the compound docked into the active site. For clarity, color of the atoms are as follows: blue—nitrogen, red—oxygen, white—carbon (mu-opioid receptor), and green—carbon (on ligand) (Varamini et al., 2012a).

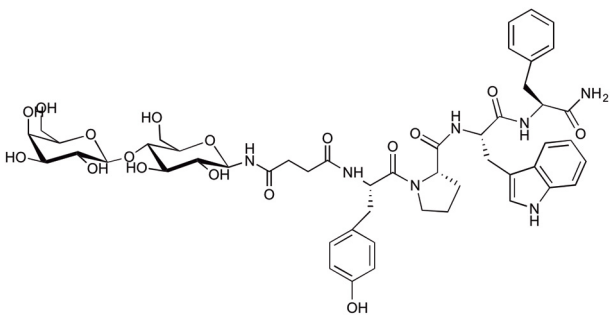


FIGURE 6 | Structure of lactose succinamic acid-conjugated endomorphin-1.

2011). Based on this classification, lactose-endomorphin-1 was considered an ideal candidate for oral delivery.

Opioid peptides are predominantly cleared from the body via fecal-oral routes (Weber et al., 1992; Witt et al., 2001). It has previously been shown that shifting from hepatic to renal clearance can increase plasma stability and improve the antinociceptive effects of opioid peptides (Witt et al., 2001). Since hydrophilicity of the glycopeptides is increased compared with the parent peptides, it is plausible that glycosylation shifts elimination to the renal pathway, thus contributing to the higher stability of these conjugates.

In vivo ANTINOCICEPTIVE ACTIVITY

Lipoamino acid modification

Targeting of neuropeptide drugs to the CNS through systemic and oral delivery routes is a formidable obstacle. The delivery of peptide drugs is limited by their poor bioavailability to the brain due to low metabolic stability, high clearance by the liver, and the obstacle posed by the BBB (Egleton et al., 2001).

The 8- and 10-carbon LAA derivatives of endomorphin-1, with either Tyr or Dmt at position 1, have been shown to produce significant dose dependent analgesia in a chronic constriction injury (CCI) model of pain in rats. This analgesia was opioid receptor-mediated and was produced following intravenous (i.v.) administration. The two C10LAA-modified endomorphin-1 peptides produced higher potency than C8LAA analogs and even morphine in this model of rats with ED_{50} values of about 1 μmol/kg. However, no significant difference was observed between ED_{50} values obtained for lipo-endomorphin-1 analogs with Tyr^1 as in the native form of the peptide, or Dmt^1 in the modified form. Although C8-Endo-1 and C8-Dmt-Endo-1 (Koda et al., 2008) were reported to have higher MOP receptor binding affinity and agonist activity than their corresponding C10-peptides, their potency in relieving neuropathic pain in CCI rats was less than C10-analogs. The higher antinociceptive potency of C10-modified peptides compared to C8-modified analogs was explained by the increase in their permeability and metabolic stability. Longer alkyl chain length possibly increased analgesic activity by permitting improved transport across lipophilic membranes including the BBB (Varamini et al., 2012b). Furthermore, these lipidated analogs did not cause significant respiratory depression (Varamini et al., 2013), or constipation, and resulted in less tolerance than morphine at analgesic doses (Varamini et al., 2012b). The effect of attaching LAA residues containing different length of alkyl side chain to drug molecules with *in vitro* monoamine oxidase inhibitory activity was also investigated. Consistently, analogs with different LAAs produced significantly different potencies (Pignatello et al., 2005). It is plausible that the divergence in the antinociceptive and side effect profiles of morphine and the lipidic endomorphin-1 derivatives may be due to their different interactions with pharmacologically defined MOP receptor subtypes (Dworkin et al., 2007). Opioid receptor hetero-oligomerization was suggested to be responsible for the

diverse pharmacology displayed by these compounds (Jordan and Devi, 1999; Levac et al., 2002).

Glycosylation

To date, many glycosylated analogs of various neuropeptides such as deltorphin (Negri et al., 1999), Met-enkephalin (Polt et al., 1994; Egleton et al., 2000), and Leu-enkephalin (Bilsky et al., 2000) have shown improved analgesic activity after peripheral administration. Structure-activity studies of enkephalin-based opioid glycopeptides revealed that disaccharide derivatives were significantly more potent than any of the monosaccharides after peripheral administration (Elmagbari et al., 2004). However, there are only limited reports of the characterization of endomorphin glycopeptides because only few potent derivatives have been developed so far. Biondi *et al.* synthesized glycosylated endomorphin-2 analogs by conjugating glucose or 2,3,4,6-tetra-O-acetyl glucose with the hydroxyproline (Hyp) residue. The MOP receptor binding affinity and agonist activity was abolished in all of these analogs therefore the compounds were not further investigated for their *in vivo* pain relieving activity (Biondi et al., 2006).

N-terminal conjugation of endomorphin-1 with lactose succinamic acid resulted in a significant antinociception following oral administration to CCI rats. This effect was comparable with that of morphine, for which various oral dosage forms are currently in clinical use (Varamini et al., 2012c). In contrast to morphine, the pain-relieving effect of the lactose-endomorphin-1 analog was selective to the injured hindpaw with insignificant effects produced in the contralateral hindpaw. This effect was antagonized by naloxone, which indicated the key role of opioid receptors (Varamini et al., 2012c).

CONCLUSION

Endomorphins have been shown to elicit a potent pain relieving effect in different acute and neuropathic pain models in animals after central administration. More importantly this effect is concomitant with little to no undesirable side effects associated with the application of opioids like morphine. These promising effects have been strong motivation for investigators to design and synthesize a substantial number of endomorphin derivatives. Numerous studies report developments in our understanding of the structure activity relationship properties, bioactive conformation, physiological characterizations, *in vivo* and *in vitro* biological activity of endomorphin analogs. Over fifteen years since the discovery of endomorphins, investigations have led to the production of derivatives with acceptable selectivity and MOP receptor-binding affinity. However, only limited progress has been made in the production of compounds with outstanding metabolic stability and membrane permeability, while retaining their pharmacodynamic properties. These are critical criteria in the field of peptide drug delivery to overcome the obstacles and succeed in the development of peripherally active and BBB-permeable analgesics suitable for clinical applications. Thus far, two of the most successful strategies have been shown to be glycosylation and lipid modification. Two examples are the development of an orally active lactose-modified and systemically effective LAA-conjugated derivative of endomorphin-1 with high potential for the treatment of neuropathic pain.

This review highlights the two modifications that have made the most improvements to the therapeutic and side effect profile of endomorphins. Presently potent and promising lead compounds have been developed which are prospective to proceed from research to the pharmaceutical industry. These achievements are the outcome of extensive research having been made to develop opioid peptide-based analgesics like endomorphins for the effective management of neuropathic pain.

ACKNOWLEDGMENTS

We thank Miss Thalia Guerin for her assistance in editing this manuscript.

REFERENCES

Albert, R., Marbach, P., Bauer, W., Briner, U., Fricker, G., Bruns, C., et al. (1993). SDZ CO 611: a highly potent glycated analog of somatostatin with improved oral activity. *Life Sci.* 53, 517–525. doi: 10.1016/0024-3205(93)90703-6

Balaz, S. (2000). Lipophilicity in trans-bilayer transport and subcellular pharmacokinetics. *Perspect. Drug Discov.* 19, 157–177. doi: 10.1023/A:1008775707749

Banks, W. A., Audus, K. L., and Davis, T. P. (1992). Permeability of the blood-brain barrier to peptides: an approach to the development of therapeutically useful analogs. *Peptides* 13, 1289–1294. doi: 10.1016/0196-9781(92)90037-4

Banks, W. A., and Kastin, A. J. (1985). Peptides and the blood-brain barrier: lipophilicity as a predictor of permeability. *Brain Res. Bull.* 15, 287–292. doi: 10.1016/0361-9230(85)90153-4

Begley, D. J. (1996). The blood-brain barrier: principles for targeting peptides and drugs to the central nervous system. *J. Pharm. Pharmacol.* 48, 136–146. doi: 10.1111/j.2042-7158.1996.tb07112.x

Bilsky, E. J., Egleton, R. D., Mitchell, S. A., Palian, M. M., Davis, P., Huber, J. D., et al. (2000). Enkephalin glycopeptide analogues produce analgesia with reduced dependence liability. *J. Med. Chem.* 43, 2586–2590. doi: 10.1021/jm000077y

Biondi, B., Giannini, E., Negri, L., Melchiorri, P., Lattanzi, R., Rosso, F., et al. (2006). Opioid peptides: synthesis and biological activity of new endomorphin analogues. *Int. J. Pept. Res. Ther.* 12, 145–151. doi: 10.1007/s10989-006-9015-6

Bock, J. E., Gavenonis, J., and Kritzer, J. A. (2013). Getting in shape: controlling peptide bioactivity and bioavailability using conformational constraints. *ACS Chem. Biol.* 8, 488–499. doi: 10.1021/cb300515u

Broadwell, R. D., Balin, B. J., and Salcman, M. (1988). Transcytotic pathway for blood-borne protein through the blood-brain barrier. *Proc. Natl. Acad. Sci. U.S.A.* 85, 632–636. doi: 10.1073/pnas.85.2.632

Buskas, T., Ingale, S., and Boons, G. J. (2006). Glycopeptides as versatile tools for glycobiology. *Glycobiology* 16, 113R–136R doi: 10.1093/glycob/cwj125

Chikhale, E. G., Ng, K.-Y., Burton, P. S., and Borchardt, R. T. (1994). Hydrogen bonding potential as a determinant of the *in vitro* and *in situ* blood-brain barrier permeability of peptides. *Pharm Res* 11, 412–419. doi: 10.1023/A:1018969222130

Clark, R. J., Jensen, J., Nevin, S. T., Callaghan, B. P., Adams, D. J., and Craik, D. J. (2010). The engineering of an orally active conotoxin for the treatment of neuropathic pain. *Angew. Chem. Int. Ed. Engl.* 49, 6545–6548. doi: 10.1002/anie.201000620

Cravezic, A., Fichna, J., Gach, K., Wyrebska, A., Perlikowska, R., Costentin, J., et al. (2011). Effect of potent endomorphin degradation blockers on analgesic and antidepressant-like responses in mice. *Neuropharmacology* 61, 1229–1238. doi: 10.1016/j.neuropharm.2011.07.021

Cros, C. D., Toth, I., and Blanchfield, J. T. (2011). Lipophilic derivatives of leu-enkephalinamide: *in vitro* permeability, stability and *in vivo* nasal delivery. *Bioorg. Med. Chem.* 19, 1528–1534. doi: 10.1016/j.bmc.2010.12.042

Czapla, M. A., Gozal, D., Alea, O. A., Beckerman, R. C., and Zadina, J. E. (2000). Differential cardiorespiratory effects of endomorphin 1, endomorphin 2, DAMGO, and morphine. *Am. J. Respir. Crit. Care. Med.* 162, 994–999. doi: 10.1164/ajrccm.162.3.9911102

Desino, K. E., Pignatello, R., Guccione, S., Basile, L., Ansar, S., Michaelis, M. L., et al. (2009). TCP-FA4: a derivative of tranylcypromine showing

improved blood-brain permeability. *Biochem. Pharmacol.* 78, 1412–1417. doi: 10.1016/j.bcp.2009.07.027

Dhanasekaran, M., Palian, M. M., Alves, I., Yeomans, L., Keyari, C. M., Davis, P., et al. (2005). Glycopeptides related to β-endorphin adopt helical amphipathic conformations in the presence of lipid bilayers. *J. Am. Chem. Soc.* 127, 5435–5448. doi: 10.1021/ja0432158

Dworkin, R. H., O'Connor, A. B., Backonja, M., Farrar, J. T., Finnerup, N. B., Jensen, T. S., et al. (2007). Pharmacologic management of neuropathic pain: evidence-based recommendations. *Pain* 132, 237–251. doi: 10.1016/j.pain.2007.08.033

Eduardo, P. (1994). 'Carbohydrate handles' as natural resources in drug delivery. *Adv. Drug Delivery Rev.* 13, 311–323. doi: 10.1016/0169-409X(94)90017-5

Egleton, D. R., Bilsky, J. E., Tollin, G., Dhanasekaran, M., Lowery, J., Alves, I., et al. (2005). Biousian glycopeptides penetrate the blood-brain barrier. *Tetrahedron Asymmetry* 16, 65–75. doi: 10.1016/j.tetasy.2004.11.038

Egleton, R., and Davis, T. (2005). Development of neuropeptide drugs that cross the blood-brain barrier. *Neurotherapeutics* 2, 44–53. doi: 10.1602/neurorx.2.1.44

Egleton, R. D., Mitchell, S. A., Huber, J. D., Janders, J., Stropova, D., Polt, R., et al. (2000). Improved bioavailability to the brain of glycosylated Met-enkephalin analogs. *Brain Res.* 881, 37–46. doi: 10.1016/S0006-8993(00)02794-3

Egleton, R. D., Mitchell, S. A., Huber, J. D., Palian, M. M., Polt, R., and Davis, T. P. (2001). Improved blood-brain barrier penetration and enhanced analgesia of an opioid peptide by glycosylation. *J. Pharmacol. Exp. Ther.* 299, 967–972. doi: 10.1602/neurorx.2.1.44

Elmagbari, N. O., Egleton, R. D., Palian, M. M., Lowery, J. J., Schmid, W. R., Davis, P., et al. (2004). Antinociceptive structure-activity studies with enkephalin-based opioid glycopeptides. *J. Pharmacol. Exp. Ther.* 311, 290–297. doi: 10.1124/jpet.104.069393

Falconer, R. A., and Toth, I. (2007). Design, synthesis and biological evaluation of novel lipoamino acid-based glycolipids for oral drug delivery. *Bioorg. Med. Chem.* 15, 7012–7020. doi: 10.1016/j.bmc.2007.07.048

Fichna, J., Perlikowska, R., Gach, K., do-Rego, J. C., Cravezic, A., Janecka, A., et al. (2010). The novel endomorphin degradation blockers Tyr-Pro-DClPhe-Phe-NH2 (EMDB-1) and Tyr-Pro-Ala-NH2 (EMDB-2) prolong endomorphin-2 action in rat ileum *in vitro*. *Chem. Biol. Drug Des.* 76, 77–81. doi: 10.1111/j.1747-0285.2010.00977.x

Gentilucci, L. (2004). New trends in the development of opioid peptide analogues as advanced remedies for pain relief. *Curr. Top. Med. Chem.* 4, 19–38. doi: 10.2174/1568026043451663

Griffin, B. D., and O'Driscoll, C. M. (2011). Opportunities and challenges for oral delivery of hydrophobic versus hydrophilic peptide and protein-like drugs using lipid-based technologies. *Ther. Deliv.* 2, 1633–1653.

Hackler, L., Zadina, J. E., Ge, L. J., and Kastin, A. J. (1997). Isolation of relatively large amounts of endomorphin-1 and endomorphin-2 from human brain cortex. *Peptides* 18, 1635–1639. doi: 10.1016/S0196-9781(97)00259-3

Horvat, S. (2001). Opioid peptides and their glycoconjugates: structure-activity relationships. *Curr. Med. Chem.* 1, 133–154. Available online at: http://dx.doi.org/10.2174/1568015013358581

Hruby, V. J., al-Obeidi, F., and Kazmierski, W. (1990). Emerging approaches in the molecular design of receptor-selective peptide ligands: conformational, topographical and dynamic considerations. *Biochem. J.* 268, 249–262.

Hruby, V. J., and Balse, P. M. (2000). Conformational and topographical considerations in designing agonist peptidomimetics from peptide leads. *Curr. Med. Chem.* 7, 945–970. doi: 10.2174/0929867003374499

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975). Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* 258, 577–580. doi: 10.1038/258577a0

Jinsmaa, Y., Marczak, E., Fujita, Y., Shiotani, K., Miyazaki, A., Li, T., et al. (2006). Potent *in vivo* antinociception and opioid receptor preference of the novel analogue [Dmt1]endomorphin-1. *Pharmacol. Biochem. Behav.* 84, 252–258. doi: 10.1016/j.pbb.2006.05.005

Jordan, B. A., and Devi, L. A. (1999). G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399, 697–700. doi: 10.1038/21441

Kawai, M., Fukuta, N., Ito, N., Kagami, T., Butsugan, Y., Maruyama, M., et al. (1990). Preparation and opioid activities of N-methylated analogs of [D-Ala2,Leu5]enkephalin. *Int. J. Pept. Protein Res.* 35, 452–459. doi: 10.1111/j.1399-3011.1990.tb00072.x

Koda, Y., Del Borgo, M., Wessling, S. T., Lazarus, L. H., Okada, Y., Toth, I., et al. (2008). Synthesis and *in vitro* evaluation of a library of modified endomorphin 1 peptides. *Bioorg. Med. Chem.* 16, 6286–6296. doi: 10.1016/j.bmc.2008.04.020

Kokotos, G., Martin, V., Constantinou-Kokotou, V., and Gibbons, W. A. (1996). Synthesis of medicinally useful lipidicalpha-amino acids, 2-amino alcohols and diamines. *Amino Acids* 11, 329–343. doi: 10.1007/BF00807940

Kream, R. M. (Inventor). (2003). *Chimeric Hybrid Analgesics*. Patent Number: WO2003090697 US.

Kream, R. M., Liu, N. L., Zhuang, M., Esposito, P. L., Esposito, T. R., Stefano, G. B., et al. (2007). Synthesis and pharmacological analysis of a morphine/substance P chimeric molecule with full analgesic potency in morphine-tolerant rats. *Med. Sci. Monit.* 13, BR25–BR31.

Levac, B. A., O'Dowd, B. F., and George, S. R. (2002). Oligomerization of opioid receptors: generation of novel signaling units. *Curr. Opin. Pharmacol.* 2, 76–81. doi: 10.1016/S1471-4892(02)00124-8

Li, T. Y., Fujita, Y., Tsuda, Y., Miyazaki, A., Ambo, A., Sasaki, Y., et al. (2005). Development of potent mu-opioid receptor ligands using unique tyrosine analogues of endomorphin-2. *J. Med. Chem.* 48, 586–592. doi: 10.1021/jm049384k

Negri, L., Lattanzi, R., Tabacco, F., Orru, L., Severini, C., Scolaro, B., et al. (1999). Dermorphin and deltorphin glycosylated analogues:? synthesis and antinociceptive activity after systemic administration. *J. Med. Chem.* 42, 400–404. doi: 10.1021/jm9810699

Palian, M. M., Boguslavsky, V. I., O'Brie, D. F., and Polt, R. (2003). Glycopeptide-membrane interactions: glycosyl enkephalin analogues adopt turn conformations by NMR and CD in amphipathic media. *J. Am. Chem. Soc.* 125, 5823–5831. doi: 10.1021/ja0268635

Pignatello, R., Puleo, A., Guccione, S., Raciti, G., Acquaviva, R., Campisi, A., et al. (2005). Enhancement of drug affinity for cell membranes by conjugation with lipoamino acids. I. Synthesis and biological evaluation of lipophilic conjugates of tranylcypromine. *Eur. J. Med. Chem.* 40, 1074–1079. doi: 10.1016/j.ejmech.2005.05.009

Poduslo, J. F., and Curran, G. L. (1994). Glycation increases the permeability of proteins across the blood-nerve and blood-brain barriers. *Brain. Res. Mol. Brain Res.* 23, 157–162. doi: 10.1016/0169-328X(94)90222-4

Polt, D. R., Dhanasekaran, M., Egleton, R. D., Bilsky, E. J., Yamamura, H. I., Porreca, F., et al. (2005a). Endorphins: biousian glycopeptides cross the BBB due to surfactant properties. *Abstr. Pap. Am. Chem. Soc.* 229, U116.

Polt, R., Dhanasekaran, M., and Keyari, C. M. (2005b). Glycosylated neuropeptides: a new vista for neuropsychopharmacology? *Med. Res. Rev.* 25, 557–585. doi: 10.1002/med.20039

Polt, R. (2008). "Biomedicine of enkephalin-derived glycopeptide analgesics," in *Glycoscience* eds B. Fraser-Reid, K. Tatsuta, J. Thiem (Berlin; Heidelberg: Springer), 2525–2543. doi: 10.1007/978-3-540-30429-6_65

Polt, R., Porreca, F., Szabo, L. Z., Bilsky, E. J., Davis, P., Abbruscato, T. J., et al. (1994). Glycopeptide enkephalin analogues produce analgesia in mice: evidence for penetration of the blood-brain barrier. *Proc. Natl. Acad. Sci. U.S.A.* 91, 7114–7118. doi: 10.1073/pnas.91.15.7114

Przewlocki, R., and Przewlocka, B. (2001). Opioids in chronic pain. *Eur. J. Pharmacol.* 429, 79–91. doi: 10.1016/S0014-2999(01)01308-5

Sakurada, C., Sakurada, S., Hayashi, T., Katsuyama, S., Tan-No, K., and Sakurada, T. (2003). Degradation of endomorphin-2 at the supraspinal level in mice is initiated by dipeptidyl peptidase IV: an *in vitro* and *in vivo* study. *Biochem. Pharmacol.* 66, 653–661. doi: 10.1016/S0006-2952(03)00391-5

Schiller, P. W., and DiMaio, J. (1982). Opiate receptor subclasses differ in their conformational requirements. *Nature* 297, 74–76. doi: 10.1038/297074a0

Silvius, J. R. (2002). Lipidated peptides as tools for understanding the membrane interactions of lipid-modified proteins. *Curr. Top. Membr.* 52, 371–95. doi: 10.1016/S1063-5823(02)52015-9

Smetanova, L., Stetinova, V., Svoboda, Z., and Kvetina, J. (2011). Caco-2 cells, biopharmaceutics classification system (BCS) and biowaiver. *Acta Medica (Hradec Kralove)* 54, 3–8.

Stewart, J. M., Getto, C. J., Neldner, K., Reeve, E. B., Krivoy, W. A., and Zimmermann, E. (1976). Substance P and analgesia. *Nature* 262, 784–785. doi: 10.1038/262784a0

Toth, I. (1994). A novel chemical approach to drug delivery: lipidic amino acid conjugates. *J. Drug Target.* 2, 217–239. doi: 10.3109/10611869408996805

Vaccarino, A. L., and Kastin, A. J. (2000). Endogenous opiates: 1999. *Peptides* 21, 1975–2034. doi: 10.1016/S0196-9781(00)00345-4

Vaccarino, A. L., Olson, G. A., Olson, R. D., and Kastin, A. J. (1999). Endogenous opiates: 1998. *Peptides* 20, 1527–1574. doi: 10.1016/S0196-9781(99)00166-7

Varamini, P., Goh, W. H., Mansfeld, F. M., Blanchfield, J. T., Wyse, B. D., Smith, M. T., et al. (2013). Peripherally acting novel lipo-endomorphin-1 peptides in neuropathic pain without producing constipation. *Bioorg. Med. Chem.* 21, 1898–1904. doi: 10.1016/j.bmc.2013.01.044

Varamini, P., Hussein, W. M., Mansfeld, F. M., and Toth, I. (2012a). Synthesis, biological activity and structure-activity relationship of endomorphin-1/substance P derivatives. *Bioorg. Med. Chem.* 20, 6335–6343. doi: 10.1016/j.bmc.2012.09.003

Varamini, P., Mansfeld, F. M., Blanchfield, J. T., Wyse, B. D., Smith, M. T., and Toth, I. (2012b). Lipo-endomorphin-1 derivatives with systemic activity against neuropathic pain without producing constipation. *PLoS ONE* 7:e41909. doi: 10.1371/journal.pone.0041909

Varamini, P., Mansfeld, F. M., Blanchfield, J. T., Wyse, B. D., Smith, M. T., and Toth, I. (2012c). Synthesis and biological evaluation of an orally active glycosylated endomorphin-1. *J. Med. Chem.* 55, 5859–5867. doi: 10.1021/jm300418d

Wang, J., Hogenkamp, D. J., Tran, M., Li, W. Y., Yoshimura, R. F., Johnstone, T. B. C., et al. (2006). Reversible lipidization for the oral delivery of leu-enkephalin. *J. Drug. Target.* 14, 127–136. doi: 10.1080/10611860600648221

Weber, S. J., Greene, D. L., Hruby, V. J., Yamamura, H. I., Porreca, F., and Davis, T. P. (1992). Whole body and brain distribution of [^{3}H]cyclic [D-Pen^2,D-Pen^5] enkephalin after intraperitoneal, intravenous, oral and subcutaneous administration. *J. Pharmacol. Exp. Ther.* 263, 1308–1316.

Witt, K. A., Huber, J. D., Egleton, R. D., Roberts, M. J., Bentley, M. D., Guo, L., et al. (2001). Pharmacodynamic and pharmacokinetic characterization of poly(ethylene glycol) conjugation to met-enkephalin analog [D-Pen^2, D-Pen^5]-enkephalin (DPDPE). *J. Pharmacol. Exp. Ther.* 298, 848–856.

Wood, I. S., and Trayhurn, P. (2003). Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *The British journal of nutrition* 89, 3–9. doi: 10.1079/BJN2002763

Zadina, J. E., Hackler, L., Ge, L.-J., and Kastin, A. J. (1997). A potent and selective endogenous agonist for the μ-opiate receptor. *Nature* 386, 499–502. doi: 10.1038/386499a0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 17 October 2013; accepted: 27 November 2013; published online: 13 December 2013.

*Citation: Varamini P and Toth I (2013) Lipid- and sugar-modified endomorphins: novel targets for the treatment of neuropathic pain. Front. Pharmacol. **4**:155. doi: 10.3389/fphar.2013.00155*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

MINI REVIEW ARTICLE
published: 04 October 2013
doi: 10.3389/fphar.2013.00127

Targeting sites of inflammation: intercellular adhesion molecule-1 as a target for novel inflammatory therapies

Susan Hua*

The School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, New South Wales, Australia

Edited by:
Peter John Cabot, The University of Queensland, Australia

Reviewed by:
Muzamil Ahmad, Indian Institute of Integrative Medicine, India
Heather Hain, Psychogenics, Inc., USA

****Correspondence:***
Susan Hua, The School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, New South Wales 2038, Australia
e-mail: susan.hua@newcastle.edu.au

Targeted drug delivery to sites of inflammation will provide effective, precise, and safe therapeutic interventions for treatment of diverse disease conditions, by limiting toxic side effects and/or increasing drug action. Disease-site targeting is believed to play a major role in the enhanced efficacy observed for a variety of drugs when formulated inside lipid vesicles. This article will focus on the factors and mechanisms involved in drug targeting to sites of inflammation and the importance of cell adhesion molecules, in particular intercellular adhesion molecule-1, in this process.

Keywords: inflammation, targeted drug delivery, ICAM-1, cell adhesion molecules, liposomes

INTRODUCTION

Targeted delivery of therapeutics to sites of inflammation is an important goal. The endothelium represents a key target for pharmacological interventions in many disease conditions, including rheumatological, cardiovascular, hematological, pulmonary, and oncological (Koning et al., 2002; Metselaar and Storm, 2005; Muro and Muzykantov, 2005; Ding et al., 2006). The goal of endothelial targeting is to achieve specific and safe delivery of a drug to, into, or across endothelial cells, in order to localize effects in the lumen, desired intracellular endothelial cell compartments, or extravascular space, thereby improving pharmacological interventions. However, due to their lack of affinity to the endothelium, only a small fraction of injected therapeutics binds to endothelial cells (Ding et al., 2006). Progress in understanding disease mechanisms provides better selection of drugs for endothelial interventions and a deeper insight into designing drug delivery carriers to target inflammatory specific destinations.

DRUG TARGETING TO SITES OF INFLAMMATION

In inflamed tissues, the permeability of the vasculature is often increased to the extent that particulate carriers, which are normally excluded from these tissues, can extravasate and localize in the tissue interstitial space. Endothelial cells also start to express several types of adhesion molecules: the selectins, the integrins, and the immunoglobulins, which mediate recruitment of leukocytes into the inflamed tissue (Koning et al., 2002; Metselaar and Storm, 2005; Ding et al., 2006). Furthermore, the process of angiogenesis (formation of new blood vessels from pre-existing vasculature) may occur in several chronic inflammatory disorders, such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease (Koning et al., 2002; Metselaar and Storm, 2005). In this complex cascade of events, numerous cell-surface receptors, adhesion molecules, and growth factors are involved, which may serve as potential targets for therapeutic intervention.

Targeted intervention in inflammatory disease at the vascular endothelial cell (VEC) level has great potential. Rational design of such drug delivery systems includes: (1) selection of proper target determinants on endothelial surfaces, such as cell adhesion molecules (CAMs); (2) production of affinity ligands useful for targeting, such as affinity peptides, antibodies, or their fragments; (3) selection and adopting of suitable delivery vehicles, such as liposomes; and (4) formulation of drug delivery system with optimal targeting and therapeutic features (Ding et al., 2006). Specific drug delivery should concentrate the drug at the targeted site, increasing efficacy, and also decreasing side effects in other tissues (Willis and Forssen, 1998; Maruyama, 2002). This concept is particularly attractive in cancer therapy, where the dose a patient can tolerate is limited due to high toxicity to non-target cells. Targeted delivery to tumor tissue may allow the use of lower drug-concentrations. Moreover, targeting therapeutic agents to the vasculature of tumors offer additional advantages; in particular blood vessels are more readily accessible to intravenously administered therapy than tumor cells (Bendas, 2001). In the same respects, targeted drug delivery of opioid analgesics to peripheral opioid receptors upregulated at sites of inflammation will significantly alleviate nociception without the central opioid mediated side effects (Hua and Cabot, 2013).

TARGETING ADHESION MOLECULES

Adhesion molecules are glycoproteins expressed on cell surfaces, where they mediate the contact between two cells (both homotypic and heterotypic interactions) or between cells and the extracellular matrix. They are essential for the regulation of immune cell responses and migration of inflammatory cells from the blood vessels into inflamed tissues (Bloemen et al., 1995; Mastrobattista et al., 1999). In fact, the expression of particular CAMs [e.g., intercellular adhesion molecule-1 (ICAM-1), E-selectin, P-selectin, vascular cell adhesion molecule-1

(VCAM-1)] are locally induced or enhanced at areas of inflammation (Bloemen et al., 1995; Spragg et al., 1997; Mastrobattista et al., 1999; Koning et al., 2002; Sakhalkar et al., 2003; Muro and Muzykantov, 2005; Voinea et al., 2005). Upregulated and/or overexpressed CAMs can be found in a multitude of clinical diseases where inflammation and immune cells are involved (e.g., ischemia-reperfusion injury, transplant rejection, and inflammatory diseases of the cardiovascular system, skin, kidneys, gastrointestinal tract, brain, and liver; Bloemen et al., 1995; Spragg et al., 1997; Mastrobattista et al., 1999; Koning et al., 2002; Muro and Muzykantov, 2005). Additionally, tumor cells use adhesion molecules to grow and spread throughout the body (Janssen et al., 2003).

Of particular note is the implication of CAMs in the pathogenesis of several rheumatic diseases. For example, rheumatoid arthritis is a chronic inflammatory disease in which adhesion molecules play an important role in the invasion of leukocytes into synovial tissues, leading to tissue damage (Mojcik and Shevach, 1997; Metselaar et al., 2003). Not only have increased expression of E-selectin, VCAM-1, and ICAM-1 been found on the vascular endothelium of synovial tissues, but immunohistochemical studies have shown elevated levels of adhesion molecule expression in ongoing inflammatory lesions (Mojcik and Shevach, 1997; Koning et al., 2002). Although reduction or blockade of the expression or function of a specific CAM is a possible therapeutic way to diminish infiltration and/or activation of inflammatory immune cells in order to reduce inflammation, this approach is complicated by the fact that most types of adhesion molecules are expressed on more than one cell type, that most cells express more than one adhesion molecule on their surface, and that several molecules can function as a ligand for a single adhesion molecule (Mojcik and Shevach, 1997; Koning et al., 2002). Importantly, blockade of CAMs can interfere with functions of immune cells essential for host defense (Mojcik and Shevach, 1997; Koning et al., 2002). Induction and/or increased expression of certain CAMs at inflammatory loci associated with various diseases offers opportunities for the development of new therapeutic strategies aimed toward selective drug-targeting. Adhesion molecules represent an easily accessible target molecule for therapeutics circulating in the blood compartment (Bloemen et al., 1995; Koning et al., 2002; Metselaar and Storm, 2005; Muro and Muzykantov, 2005; Ding et al., 2006).

INTERCELLULAR ADHESION MOLECULE-1

Intercellular adhesion molecules (ICAMs) are structurally related transmembrane glycoproteins of the immunoglobulin supergene family and are ligands for the β2 integrin molecules present on leukocytes (Almenar-Queralt et al., 1995; Hubbard and Rothlein, 2000). Of the five ICAMs identified, ICAM-1 is the most extensively studied (Koning et al., 2002; Muro and Muzykantov, 2005). ICAM-1 specifically participates in trafficking of inflammatory cells, in leukocyte effector functions, in adhesion of antigen-presenting cells to T lymphocytes, in microbial pathogenesis, and in signal transduction pathways through outside-in signaling events (Almenar-Queralt et al., 1995; Hubbard and Rothlein, 2000; Muro and Muzykantov, 2005). This adhesion molecule is localized to both the apical and basolateral surface of endothelial cells, making it ideally positioned to facilitate transendothelial migration of leukocytes (Almenar-Queralt et al., 1995). In fact, ICAM-1 (along with VCAM-1) is considered to represent the most important adhesion molecule for leukocyte recruitment to inflamed sites (Koning et al., 2002). Additionally, ICAM-1 has been shown to exist in a soluble form in circulation, which results from proteolytic cleavage mediated by neutrophil proteases (leukocyte elastase and cathepsin G) in a process independent of ICAM-1 surface density (Muro and Muzykantov, 2005).

Intercellular adhesion molecule-1 is widely distributed and expressed constitutively at low levels on leukocytes, VECs, fibroblasts, and epithelial cells. Although ICAM-1 is present in several cell types, the level of expression is orders of magnitude lower than that of VECs (Almenar-Queralt et al., 1995; Scholz et al., 1996; Mojcik and Shevach, 1997; Hubbard and Rothlein, 2000; Koning et al., 2002; Muro et al., 2003b, 2005; Muro and Muzykantov, 2005). Stimulation of a variety of cells with inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) has been documented to increase ICAM-1 expression on multiple cell types (Almenar-Queralt et al., 1995; Scholz et al., 1996; Hubbard and Rothlein, 2000; Muro et al., 2003b, 2005; Muro and Muzykantov, 2005). Strong upregulation of ICAM-1 is observed under inflammatory conditions within 24 h (Scholz et al., 1996). In contrast to the selectins, which are rapidly down regulated after induction, ICAM-1 and VCAM-1, once upregulated, remain on the cell surface for more than 48 h (Koning et al., 2002). Information on the internalization capacity of ICAM-1 is, however, rather contradictory (Koning et al., 2002). Some authors report the total absence of internalization on TNF-activated human umbilical vein endothelial cells (HUVEC) or a rather slow process of internalization by HUVECs, whereas others report on (rapid) internalization of ICAM-1-binding peptides by lymphocytes, antibody-targeted liposomes or poly lactic-co-glycolic acid (PLGA) nanoparticles by epithelial cells (Koning et al., 2002; Chittasupho et al., 2009). In fact, it has been reported that ICAM-1 internalization levels are practically indistinguishable from background (<10% of surface expressed ICAM-1; Muro and Muzykantov, 2005). These contradictory findings may not only be attributed to the difference in cell types and targeting ligands used, but also to the timeframe of the study (Koning et al., 2002). It is therefore of great importance to test the internalization capacity of the developed drug delivery system. Targeting to non- or slow-internalizing epitopes may be of specific interest for drugs that work at the luminal site of the VECs, whereas fast-internalizing epitopes are interesting for drugs with an intracellular address (Koning et al., 2002; Muro and Muzykantov, 2005).

Several adhesion molecules involved in the leukocyte adhesion cascade, in principle, comply with the requirements for achieving targeted delivery of drugs into VECs. However of these, ICAM-1 represents an attractive target since it is a high-density determinant stably exposed from the endothelial surface, which is upregulated and functionally involved in inflammation (Almenar-Queralt et al., 1995; Scholz et al., 1996; Mojcik and Shevach, 1997; Hubbard and Rothlein, 2000; Koning et al., 2002; Muro et al., 2003b, 2005; Muro and Muzykantov, 2005).

In particular, ICAM-1 seems to be well suited for drug targeting to the luminal surface, due to ineffective internalization of either monomolecular or large anti-ICAM conjugates (Muro and Muzykantov, 2005). Potentially this will allow extravasation of the delivery carrier across the endothelial cells and release of the therapeutic drug specifically into the inflammatory site of action (Metselaar and Storm, 2005; Ding et al., 2006). This feature distinguishes ICAM from other similarly prevalent endothelial determinants all of which are rapidly internalized, therefore leading to early release of the drug within the endothelial cells themselves (Koning et al., 2002; Muro and Muzykantov, 2005).

Antibodies to CAMs are being explored as therapeutics and delivery carriers in cell cultures, animal models, and early clinical studies (Muro et al., 2005). A small number of studies have demonstrated the validity of such an approach, in particular showing specific binding and drug delivery to VECs *in vitro* and *in vivo* (Bloemen et al., 1995; Spragg et al., 1997; Bendas et al., 1998; Mastrobattista et al., 1999; Kessner et al., 2001; Jaafari and Foldvari, 2002b; Koning et al., 2002; Asgeirsdottir et al., 2003; Everts et al., 2003; Murciano et al., 2003; Muro et al., 2003a, 2005; Muro and Muzykantov, 2005; Voinea et al., 2005; Ding et al., 2006). Presumably, the specific and strong upregulation of these CAMs at sites of inflammation still allows specific targeting to be observed. Therefore, ICAM-1 targeting seems attractive, as this CAM shows basal levels of expression on VECs in general, but is strongly upregulated on VECs at inflamed sites (Almenar-Queralt et al., 1995; Scholz et al., 1996; Mojcik and Shevach, 1997; Hubbard and Rothlein, 2000; Koning et al., 2002; Muro et al., 2003b, 2005; Muro and Muzykantov, 2005). These developments in drug targeting to VECs will result in increasing knowledge on the role of the endothelium in inflammatory disorders and will further improve clinical therapy.

SELECTIVE INTERACTION WITH ICAM-1 AND UPTAKE BY TARGET CELLS

There are a number of potential modes of delivery of encapsulated therapeutics from ICAM-1 targeted carriers, which will affect its therapeutic availability and action. Contradicting results have been reported of the extent of internalization of ICAM-1-directed carriers by endothelial cells (Koning et al., 2002). The capacity of endothelial cells to uptake anti-CAM multimeric conjugates may depend on the size of the particles, with conjugates having diameters from 100 to 300 nm readily entering endothelial cells, whereas conjugates of larger size (500 nm to 1 μm) remained attached to the cell surface at 37°C (Murciano et al., 2003; Muro et al., 2003a; Muro and Muzykantov, 2005). The notion that small multimeric ligands can undergo internalization within endothelial cells by CAM-mediated endocytosis is of pharmacological and physiological relevance (Murciano et al., 2003; Muro et al., 2003a; Muro and Muzykantov, 2005). The signaling and cytoskeletal events involved in endothelial internalization of anti-CAM conjugates are similar to those triggered by CAM-clustering in course of leukocyte adhesion and transmigration (Muro and Muzykantov, 2005). This parallelism supports the notion that intracellular drug delivery mediated by anti-CAM conjugates may be further enhanced in inflammation and pathological conditions that activate such transduction pathways in endothelial cells (Muro and Muzykantov, 2005).

In addition to delivering therapeutic cargoes intracellularly or to the luminal surface to have an anti-inflammatory effect on the endothelial cells involved in inflammation (Przewlocki and Przewlocka, 2001; Stein et al., 2001), it is plausible for liposomes under pathological conditions to extravasate through the endothelial barrier directed by ICAM-1 on the surface of endothelial cells at sites of inflammation to release drugs within the extravascular tissue space (Oku and Namba, 1994; Vingerhoeds et al., 1994; Willis and Forssen, 1998; Koning et al., 2002; Antohe et al., 2004; Metselaar and Storm, 2005).

FACTORS INFLUENCING TARGET ACCUMULATION IN INFLAMMATION

Drug targeting using liposomes as carriers holds much promise, especially in reducing toxicity and targeting delivery to pathological sites of inflammation (e.g., musculoskeletal conditions, infection, burns, tumors) that are characterized by increased vascular permeability (Oku and Namba, 1994; Vingerhoeds et al., 1994; Yuan et al., 1994; Thurston et al., 1998; Willis and Forssen, 1998; Klimuk et al., 1999; Laverman et al., 1999; Bendas, 2001; Koning et al., 2002; Maruyama, 2002; Antohe et al., 2004; Metselaar and Storm, 2005). Long-circulating liposomes are currently used in targeted drug delivery to tumors and inflammatory regions, and have shown impressive improvement of the therapeutic index of encapsulated drugs (Oku and Namba, 1994; Torchilin, 1994, 1996; Laverman et al., 1999; Bendas, 2001; Koning et al., 2002; Metselaar and Storm, 2005; Ding et al., 2006). For example, rats and mice with arthritis treated with a single intravenous (IV) injection of sterically stabilized liposomes (SL) containing prednisolone phosphate resulted in complete remission of paw inflammation for 1 week in comparison to free drug (Metselaar and Storm, 2005). Mechanistic studies showed that the increased therapeutic benefit was a result of selective joint targeting (Metselaar and Storm, 2005).

Within inflamed tissues the permeability of the vasculature is often increased to the extent that particulate carriers, which are normally excluded from these tissues, can extravasate and localize in the tissue interstitial space (Antohe et al., 2004; Metselaar and Storm, 2005). This selective accumulation and increase in drug concentration at inflamed target sites is due to the so-called enhanced permeability and retention (EPR) effect (Maruyama, 2002; Metselaar and Storm, 2005). Inflammation results in a dramatic change in blood vessel permeability as the capillary vasculature undergoes structural remodeling to allow leukocyte diapedesis into the peripheral tissue (Klimuk et al., 1999). The width of the tight junctional regions between endothelial cells *in vivo* has been reported to be from 12 to 20 nm (Antohe et al., 2004), however exposure of endothelial cells to inflammatory mediators increases permeability of the microvasculature, with the formation of gaps of up to 1 μm (Antohe et al., 2004). In fact, pore sizes ranging from 0.2 to 1.2 μm have been observed, though the size and number of pores are dependent upon the microenvironment of the pathological site (Klimuk et al., 1999; Antohe et al., 2004). Observations using fluorescence and electron microscopy have shown that SL can indeed extravasate beyond the endothelial barrier, mainly in postcapillary venules, with

SL ranging from 100 to 200 nm in diameter having a higher probability of encountering the leaky vessels of the inflamed tissue (Willis and Forssen, 1998; Antohe et al., 2004; Metselaar and Storm, 2005). Leukocytes are able to open intercellular junctions of the endothelium monolayer by stimulating contraction of the endothelial cells or by causing a gap by passing between the cells (Antohe et al., 2004). It is therefore plausible that liposomal carriers may cross the monolayer in association with leukocytes or migrate independently across gaps formed in the monolayer by leukocyte migration (Klimuk et al., 1999; Sipkins et al., 2000; Antohe et al., 2004; Metselaar and Storm, 2005).

Currently, systemic liposome targeting strategies investigated are able to deliver no more than a few percent of the administered dose to their desired sites *in vivo* (Willis and Forssen, 1998). Although these formulations represent significant improvements over corresponding conventional drug therapies, much of the administered dose is still delivered to non-targeted tissues (Willis and Forssen, 1998). For example, a biodisposition study of polyethylene glycol (PEG)-coated lipid microspheres of indomethacin in arthritic rats reported an overall drug targeting efficiency of 7.5-fold higher than the conventional lipid microspheres (Palakurthi et al., 2005). The enhanced accumulation of the drug in the inflammatory tissue may be attributed to extravasation through the leaky vasculature and their possible uptake by circulating monocytes, which would subsequently be concentrated in the rheumatic joints (Palakurthi et al., 2005). Importantly, formulation as lipid microspheres drastically reduced the concentration of the drug in the brain (C_{max}) from 1.73 to 0.69 μg/g of the tissue, thereby reducing central nervous system (CNS) adverse effects (Palakurthi et al., 2005). PEG-coated lipid microspheres further reduced the concentration to 0.58 μg/g (Palakurthi et al., 2005). The lower accumulation in sensitive non-target tissues (e.g., brain, kidneys) may be due to the reduced availability of the free drug in the blood (Palakurthi et al., 2005). It should be noted that the blood–brain barrier is often the rate-limiting factor in determining permeation of therapeutic drugs into the brain due to both physical (tight junctions) and metabolic (enzymes) barriers (Rousseau et al., 1999; Schmidt et al., 2003). Thus liposomal carriers are only able to localize in the brain more efficiently when this barrier has been altered (Rousseau et al., 1999; Schmidt et al., 2003; Palakurthi et al., 2005).

Attachment of target-specific ligands to the liposome surface (active targeting) has been shown to further enhance targeting to specific cells or tissues (Senior, 1987; Torchilin, 1994, 1996; Vingerhoeds et al., 1994; Willis and Forssen, 1998; Bendas, 2001; Maruyama, 2002; Ulrich, 2002). Targeting endothelial cells by exploiting cell-specific surface markers has been widely investigated *in vitro* (Bloemen et al., 1995; Willis and Forssen, 1998; Koning et al., 2002; Muro and Muzykantov, 2005; Ding et al., 2006). Liposomes have been modified with ligands that can selectively interact with E-selectin (Bendas et al., 1998; Kessner et al., 2001; Everts et al., 2003), ICAM-1 (Bloemen et al., 1995; Willis and Forssen, 1998; Mastrobattista et al., 1999; Sipkins et al., 2000; Jaafari and Foldvari, 2002a,b; Muro and Muzykantov, 2005; Ding et al., 2006) and VCAM-1 (Voinea et al., 2005) molecules that are upregulated on the surface of endothelial cells following activation by inflammatory signals. For example, P_0-peptide-1 linked to liposome surfaces is capable of mediating the specific binding of liposomes to melanoma cells expressing high levels of ICAM-1, thus making it possible to target cancerous melanocytes in lymph nodes and skin melanomas (Jaafari and Foldvari, 2002b). Most of the work with liposomes in inflammatory disorders are based on imaging agents, with only few *in vivo* studies having been conducted using ligand-targeted liposomes incorporating therapeutic agents (Metselaar and Storm, 2005). For example, the biodistribution and target localization of E-selectin-targeted dexamethasone-containing liposomes was examined in a murine delayed-type hypersensitivity model, which reported enhanced uptake by activated endothelium at inflamed sites as compared with control tissue (Everts et al., 2003). Similarly, selective interaction with target cells following extravasation of targeted liposomes into the inflamed tissue has hardly been addressed *in vivo* (Metselaar and Storm, 2005). Boot et al. (2005) reported that the surface receptor CD134, specifically expressed by auto-aggressive T cells at sites of inflammation, could efficiently be targeted by liposomes modified with anti-CD134 antibody. It was observed that encapsulation of 5′-fluorodeoxyuridine dipalmitate in these liposomes could lead to inactivation of auto-aggressive T cells and amelioration of experimental arthritis (Boot et al., 2005). In addition, loperamide-encapsulated ICAM-1 targeted immunoliposomes have been shown to induce significant peripheral antinociceptive and anti-inflammatory activity in rats with complete Freund's adjuvant-induced inflammation of the paw via an opioid receptor dependent mechanism (Hua and Cabot, 2013).

This phenomenon of disease-site targeting is believed to play a major role in the enhanced efficacy observed for a variety of drugs when formulated inside lipid vesicles (Oku and Namba, 1994; Vingerhoeds et al., 1994; Torchilin, 1996; Willis and Forssen, 1998; Bendas, 2001; Maruyama, 2002; Ulrich, 2002). Formulation of ICAM-1-directed sterically stabilized immunoliposomes (SIL) will not only allow prolonged circulation but also active targeting to sites of inflammation (Bloemen et al., 1995; Willis and Forssen, 1998; Koning et al., 2002; Muro and Muzykantov, 2005; Ding et al., 2006). Such drug carriers may escape from the gaps between adjacent endothelial cells and openings at the vessel termini during inflammation by passive convective transport and/or ligand-directed targeting (Antohe et al., 2004; Metselaar and Storm, 2005). It is also plausible that some liposomes can attach onto activated leukocytes undergoing diapedesis into inflammatory sites (Sipkins et al., 2000), as CAMs such as ICAM-1 are expressed not only on the surface of vascular endothelium and neurones, but also by activated T lymphocytes (Sipkins et al., 2000; Koning et al., 2002; Hua et al., 2006). This field of research of targeting therapeutics to sites of inflammation specific to pathological disease states will improve the efficacy of therapeutic agents and reduce the toxicity to other parts of the body.

ACKNOWLEDGMENT

This work was supported by The Pharmacy Research Trust of New South Wales.

REFERENCES

Almenar-Queralt, A., Duperray, A., Miles, L. A., Felez, J., and Altieri, D. C. (1995). Apical topography and modulation of ICAM-1 expression on activated endothelium. *Am. J. Pathol.* 147, 1278–1288.

Antohe, F., Lin, L., Kao, G. Y., Poznansky, M. J., and Allen, T. M. (2004). Transendothelial movement of liposomes in vitro mediated by cancer cells, neutrophils or histamine. *J. Liposome Res.* 14, 1–25. doi: 10.1081/LPR-120039660

Asgeirsdottir, S. A., Kok, R. J., Everts, M., Meijer, D. K., and Molema, G. (2003). Delivery of pharmacologically active dexamethasone into activated endothelial cells by dexamethasone-anti-E-selectin immunoconjugate. *Biochem. Pharmacol.* 65, 1729–1739. doi: 10.1016/S0006-2952(03)00122-9

Bendas, G. (2001). Immunoliposomes: a promising approach to targeting cancer therapy. *BioDrugs* 15, 215–224. doi: 10.2165/00063030-200115040-00002

Bendas, G., Krause, A., Schmidt, R., Vogel, J., and Rothe, U. (1998). Selectins as new targets for immunoliposome-mediated drug delivery. A potential way of anti-inflammatory therapy. *Pharm. Acta Helv.* 73, 19–26. doi: 10.1016/S0031-6865(97)00043-5

Bloemen, P. G., Henricks, P. A., van Bloois, L., van den Tweel, M. C., Bloem, A. C., Nijkamp, F. P., et al. (1995). Adhesion molecules: a new target for immunoliposome-mediated drug delivery. *FEBS Lett.* 357, 140–144. doi: 10.1016/0014-5793(94)01350-A

Boot, E. P., Koning, G. A., Storm, G., Wagenaar-Hilbers, J. P., van Eden, W., Everse, L. A., et al. (2005). CD134 as target for specific drug delivery to auto-aggressive $CD4^+$ T cells in adjuvant arthritis. *Arthritis Res. Ther.* 7, R604–R615. doi: 10.1186/ar1722

Chittasupho, C., Xie, S., Baoum, A., Yakovleva, T., Siahaan, T. J., and Berkland, C. J. (2009). ICAM-1 targeting of doxorubicin-loaded PLGA nanoparticles to lung epithelial cells. *Eur. J. Pharm. Sci.* 2009, 141–150. doi: 10.1016/j.ejps.2009.02.008

Ding, B. S., Dziubla, T., Shuvaev, V. V., Muro, S., and Muzykantov, V. R. (2006). Advanced drug delivery systems that target the vascular endothelium. *Mol. Interv.* 6, 98–112. doi: 10.1124/mi.6.2.7

Everts, M., Koning, G. A., Kok, R. J., Asgeirsdottir, S. A., Vestweber, D., Meijer, D. K., et al. (2003). In vitro cellular handling and in vivo targeting of E-selectin-directed immunoconjugates and immunoliposomes used for drug delivery to inflamed endothelium. *Pharm. Res.* 20, 64–72. doi: 10.1023/A:1022298725165

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16, E199–E216.

Hua, S., Hermanussen, S., Tang, L., Monteith, G. R., and Cabot, P. J. (2006). The neural cell adhesion molecule antibody blocks cold water swim stress-induced analgesia and cell adhesion between lymphocytes and cultured dorsal root ganglion neurons. *Anesth. Analg.* 103, 1558–1564. doi: 10.1213/01.ane.0000243410.61451.c1

Hubbard, A. K., and Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. *Free Radic. Biol. Med.* 28, 1379–1386. doi: 10.1016/S0891-5849(00)00223-9

Jaafari, M. R., and Foldvari, M. (2002a). Targeting of liposomes to human keratinocytes through adhesive peptides from immunoglobulin domains in the presence of IFN-gamma. *Drug Deliv.* 9, 1–9. doi: 10.1080/107175402753413127

Jaafari, M. R., and Foldvari, M. (2002b). Targeting of liposomes to melanoma cells with high levels of ICAM-1 expression through adhesive peptides from immunoglobulin domains. *J. Pharm. Sci.* 91, 396–404. doi: 10.1002/jps.10062

Janssen, A. P., Schiffelers, R. M., ten Hagen, T. L., Koning, G. A., Schraa, A. J., Kok, R. J., et al. (2003). Peptide-targeted PEG-liposomes in anti-angiogenic therapy. *Int. J. Pharm.* 254, 55–58. doi: 10.1016/S0378-5173(02)00682-8

Kessner, S., Krause, A., Rothe, U., and Bendas, G. (2001). Investigation of the cellular uptake of E-Selectin-targeted immunoliposomes by activated human endothelial cells. *Biochim. Biophys. Acta* 1514, 177–190. doi: 10.1016/S0005-2736(01)00368-6

Klimuk, S. K., Semple, S. C., Scherrer, P., and Hope, M. J. (1999). Contact hypersensitivity: a simple model for the characterization of disease-site targeting by liposomes. *Biochim. Biophys. Acta* 1417, 191–201. doi: 10.1016/S0005-2736(98)00261-2

Koning, G. A., Schiffelers, R. M., and Storm, G. (2002). Endothelial cells at inflammatory sites as target for therapeutic intervention. *Endothelium* 9, 161–171. doi: 10.1080/10623320213631

Laverman, P., Boerman, O. C., Oyen, W. J., Dams, E. T., Storm, G., and Corstens, F. H. (1999). Liposomes for scintigraphic detection of infection and inflammation. *Adv. Drug Deliv. Rev.* 37, 225–235. doi: 10.1016/S0169-409X(98)00095-7

Maruyama, K. (2002). PEG-immunoliposome. *Biosci. Rep.* 22, 251–266. doi: 10.1023/A:1020138622686

Mastrobattista, E., Storm, G., van Bloois, L., Reszka, R., Bloemen, P. G., Crommelin, D. J., et al. (1999). Cellular uptake of liposomes targeted to intercellular adhesion molecule-1 (ICAM-1) on bronchial epithelial cells. *Biochim. Biophys. Acta* 1419, 353–363. doi: 10.1016/S0005-2736(99)00074-7

Metselaar, J. M., and Storm, G. (2005). Liposomes in the treatment of inflammatory disorders. *Expert Opin. Drug Deliv.* 2, 465–476. doi: 10.1517/17425247.2.3.465

Metselaar, J. M., Wauben, M. H., Wagenaar-Hilbers, J. P., Boerman, O. C., and Storm, G. (2003). Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. *Arthritis Rheum.* 48, 2059–2066. doi: 10.1002/art.11140

Mojcik, C. F., and Shevach, E. M. (1997). Adhesion molecules: a rheumatologic perspective. *Arthritis Rheum.* 40, 991–1004. doi: 10.1002/art.1780400602

Murciano, J. C., Muro, S., Koniaris, L., Christofidou-Solomidou, M., Harshaw, D. W., Albelda, S. M., et al. (2003). ICAM-directed vascular immunotargeting of antithrombotic agents to the endothelial luminal surface. *Blood* 101, 3977–3984. doi: 10.1182/blood-2002-09-2853

Muro, S., Cui, X., Gajewski, C., Murciano, J. C., Muzykantov, V. R., and Koval, M. (2003a). Slow intracellular trafficking of catalase nanoparticles targeted to ICAM-1 protects endothelial cells from oxidative stress. *Am. J. Physiol. Cell Physiol.* 285, C1339–C1347. doi: 10.1152/ajpcell.00099.2003

Muro, S., Wiewrodt, R., Thomas, A., Koniaris, L., Albelda, S. M., Muzykantov, V. R., et al. (2003b). A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. *J. Cell Sci.* 116(Pt 8), 1599–1609. doi: 10.1242/jcs.00367

Muro, S., Gajewski, C., Koval, M., and Muzykantov, V. R. (2005). ICAM-1 recycling in endothelial cells: a novel pathway for sustained intracellular delivery and prolonged effects of drugs. *Blood* 105, 650–658. doi: 10.1182/blood-2004-05-1714

Muro, S., and Muzykantov, V. R. (2005). Targeting of antioxidant and anti-thrombotic drugs to endothelial cell adhesion molecules. *Curr. Pharm. Des.* 11, 2383–2401. doi: 10.2174/1381612054367274

Oku, N., and Namba, Y. (1994). Long-circulating liposomes. *Crit. Rev. Ther. Drug Carrier Syst.* 11, 231–270.

Palakurthi, S., Vyas, S. P., and Diwan, P. V. (2005). Biodisposition of PEG-coated lipid microspheres of indomethacin in arthritic rats. *Int. J. Pharm.* 290, 55–62. doi: 10.1016/j.ijpharm.2004.11.017

Przewlocki, R., and Przewlocka, B. (2001). Opioids in chronic pain. *Eur. J. Pharmacol.* 429, 79–91. doi: 10.1016/S0014-2999(01)01308-5

Rousseau, V., Denizot, B., Le Jeune, J. J., and Jallet, P. (1999). Early detection of liposome brain localization in rat experimental allergic encephalomyelitis. *Exp. Brain Res.* 125, 255–264. doi: 10.1007/s002210050681

Sakhalkar, H. S., Dalal, M. K., Salem, A. K., Ansari, R., Fu, J., Kiani, M. F., et al. (2003). Leukocyte-inspired biodegradable particles that selectively and avidly adhere to inflamed endothelium in vitro and in vivo. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15895–15900. doi: 10.1073/pnas.2631433100

Schmidt, J., Metselaar, J. M., Wauben, M. H., Toyka, K. V., Storm, G., and Gold, R. (2003). Drug targeting by long-circulating liposomal glucocorticosteroids increases therapeutic efficacy in a model of multiple sclerosis. *Brain* 126(Pt 8), 1895–1904. doi: 10.1093/brain/awg176

Scholz, D., Devaux, B., Hirche, A., Potzsch, B., Kropp, B., Schaper, W., et al. (1996). Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. *Cell Tissue Res.* 284, 415–423. doi: 10.1007/s004410050602

Senior, J. H. (1987). Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 123–193.

Sipkins, D. A., Gijbels, K., Tropper, F. D., Bednarski, M., Li, K. C., and Steinman, L. (2000). ICAM-1 expression in autoimmune encephalitis visualized using magnetic resonance

imaging. *J. Neuroimmunol.* 104, 1–9. doi: 10.1016/S0165-5728(99)00248-9

Spragg, D. D., Alford, D. R., Greferath, R., Larsen, C. E., Lee, K. D., Gurtner, G. C., et al. (1997). Immunotargeting of liposomes to activated vascular endothelial cells: a strategy for site-selective delivery in the cardiovascular system. *Proc. Natl. Acad. Sci. U.S.A.* 94, 8795–8800. doi: 10.1073/pnas.94.16.8795

Stein, C., Machelska, H., and Schafer, M. (2001). Peripheral analgesic and antiinflammatory effects of opioids. *Z. Rheumatol.* 60, 416–424. doi: 10.1007/s003930170004

Thurston, G., McLean, J. W., Rizen, M., Baluk, P., Haskell, A., Murphy, T. J., et al. (1998). Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J. Clin. Invest.* 101, 1401–1413. doi: 10.1172/JCI965

Torchilin, V. P. (1994). Immunoliposomes and PEGylated immunoliposomes: possible use for targeted delivery of imaging agents. *Immunomethods* 4, 244–258. doi: 10.1006/immu.1994.1027

Torchilin, V. P. (1996). Affinity liposomes in vivo: factors influencing target accumulation. *J. Mol. Recognit.* 9, 335–346. doi: 10.1002/(SICI)1099-1352(199634/12)9:5/6<335::AID-JMR309>3.0.CO;2-0

Ulrich, A. S. (2002). Biophysical aspects of using liposomes as delivery vehicles. *Biosci. Rep.* 22, 129–150. doi: 10.1023/A:1020178304031

Vingerhoeds, M. H., Storm, G., and Crommelin, D. J. (1994). Immunoliposomes in vivo. *Immunomethods* 4, 259–272. doi: 10.1006/immu.1994.1028

Voinea, M., Manduteanu, I., Dragomir, E., Capraru, M., and Simionescu, M. (2005). Immunoliposomes directed toward VCAM-1 interact specifically with activated endothelial cells – a potential tool for specific drug delivery. *Pharm. Res.* 22, 1906–1917. doi: 10.1007/s11095-005-7247-3

Willis, M., and Forssen, E. (1998). Ligand-targeted liposomes. *Adv. Drug Deliv. Rev.* 29, 249–271. doi: 10.1016/S0169-409X(97)00083-5

Yuan, F., Leunig, M., Huang, S. K., Berk, D. A., Papahadjopoulos, D., and Jain, R. K. (1994). Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft. *Cancer Res.* 54, 3352–3356.

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 August 2013; paper pending published: 31 August 2013; accepted: 14 September 2013; published online: 04 October 2013.

Citation: Hua S (2013) Targeting sites of inflammation: intercellular adhesion molecule-1 as a target for novel inflammatory therapies. Front. Pharmacol. ***4****:127. doi: 10.3389/fphar.2013.00127*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

MINI REVIEW ARTICLE
published: 21 November 2013
doi: 10.3389/fphar.2013.00143

The use of lipid-based nanocarriers for targeted pain therapies

***Susan Hua*[1]* and *Sherry Y. Wu*[2]**

[1] School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia
[2] Department of Gynecologic Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

Edited by:
Peter John Cabot, The University of Queensland, Australia

Reviewed by:
Gopalkumar Rakesh, National Institute of Mental Health and Neurosciences, India
Kruthika Sampathgiri, Stony Brook University, USA

***Correspondence:**
Susan Hua, School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW 2038, Australia
e-mail: susan.hua@newcastle.edu.au

Sustained delivery of analgesic agents at target sites remains a critical issue for effective pain management. The use of nanocarriers has been reported to facilitate effective delivery of these agents to target sites while minimizing systemic toxicity. These include the use of biodegradable liposomal or polymeric carriers. Of these, liposomes present as an attractive delivery system due to their flexible physicochemical properties which allow easy manipulation in order to address different delivery considerations. Their favorable toxicity profiles and ease of large scale production also make their clinical use feasible. In this review, we will discuss the concept of using liposomes as a drug delivery carrier, their *in vitro* characteristics as well as *in vivo* behavior. Current advances in the targeted liposomal delivery of analgesic agents and their impacts on the field of pain management will be presented.

Keywords: pain, inflammation, liposomes, nanocarriers, targeted drug delivery

INTRODUCTION

Targeted drug delivery provides effective, precise, and safe therapeutic interventions for treatment of diverse disease conditions, by limiting toxic side effects and/or increasing drug action. Effective drug targeting depends on several factors that are either carrier or target related. The drug carrier must be stable, protect the drug from degradation, protect the body from harmful side effects, and allow delivery to the target cell population *in vivo* (Koning et al., 2002). The target must be well accessible for the drug-targeting system and must display specific cell-surface molecules that allow selective targeting and efficient drug delivery (Vingerhoeds et al., 1994; Willis and Forssen, 1998; Ding et al., 2006). The field of site-specific drug delivery has been continuously explored to develop formulations with a therapeutically acceptable degree of target specificity. Many different approaches using various physical and biochemical principles have been proposed and examined, with targeted liposomes as a carrier for both hydrophobic and hydrophilic drugs having attracted much attention.

LIPOSOMES AS DRUG DELIVERY CARRIERS

Liposomes have long been considered good candidates for efficient drug carrier and delivery systems. They have been used as delivery vehicles for stabilizing drugs, overcoming barriers to cellular and tissue uptake, and for directing their contents toward specific sites *in vivo* (Senior, 1987; Oku and Namba, 1994; Vingerhoeds et al., 1994; Woodle et al., 1994; Torchilin, 1996; Willis and Forssen, 1998; Bendas, 2001; Maruyama, 2002; Moghimi and Szebeni, 2003; Metselaar and Storm, 2005; Ding et al., 2006). The unique ability of liposomes to entrap drugs both in an aqueous and a lipid phase make such delivery systems attractive for hydrophilic and hydrophobic drugs. Hydrophobic molecules are intercalated within the bilayer membrane, and hydrophilic molecules can be entrapped in the internal aqueous region. Additionally, by virtue of their large aqueous interior and biocompatible lipid exterior, they offer a possible means of local delivery of a large variety of drug structures, from small molecules to macromolecules such as proteins and DNA, to the site of interest while reducing systemic toxicity (Senior, 1987; Oku and Namba, 1994; Torchilin, 1996; Ulrich, 2002; Sahoo and Labhasetwar, 2003; Ding et al., 2006).

Liposomes offer several advantages over other delivery systems. Liposomes are generally considered non-toxic, biodegradable, and non-immunogenic, as they are typically composed of naturally occurring lipids. Association of a drug with liposomes generally prolongs circulation half-life, reduces volume of distribution, and lowers systemic toxicity. Moreover, the drug is protected from early degradation, inactivation, and dilution in circulation (Oku and Namba, 1994; Torchilin, 1996; Laverman et al., 1999; Ulrich, 2002; Sahoo and Labhasetwar, 2003). *In vivo* behavior of liposomes can be easily modified by changing their characteristics, such as size, lipid composition, and charge (Senior, 1987; Oku and Namba, 1994; Willis and Forssen, 1998; Laverman et al., 1999; Ulrich, 2002). In addition, the liposome surface can be modified with polymer structures such as poly(ethylene glycol) (PEG), which inhibits macrophage uptake and thereby increases liposome circulation time, and with targeting moieties such as antibodies or peptides (Senior, 1987; Oku and Namba, 1994; Torchilin, 1994; Woodle et al., 1994; Maruyama, 2002; Moghimi and Szebeni, 2003). Site-directing ligands incorporated into the liposome membrane surface therefore have been investigated intensely in an effort to further enhance the selectivity of liposomal drug delivery (Sawant and Torchilin, 2012; Allen and Cullis, 2013; Koshkaryev et al., 2013). Unlike solid polymeric carrier systems, liposome membranes are dynamic structures, allowing surface-coupled ligands a greater degree of freedom with the ability to move about

within the bilayer plane, positioning themselves for optimal substrate interactions (Willis and Forssen, 1998). Critical factors for successful *in vivo* delivery of ligand-targeted liposomes will involve selection of accessible and appropriate targets, use of ligands with adequate selectivity and affinity for these targets, and suitable liposome surface coupling methods for correct presentation of ligands to their binding sites (Vingerhoeds et al., 1994; Torchilin, 1996; Willis and Forssen, 1998; Metselaar and Storm, 2005; Ding et al., 2006). The benefit of liposomes as therapeutic carriers stimulates the accumulation of novel experiences in the practical aspects of liposomes, as well as new developments in basic research.

IN VIVO STABILITY, BIODISTRIBUTION, AND BIOAVAILABILITY OF LIPOSOMES

Several major hurdles must be overcome in order to prolong liposome circulation times. These include stabilizing the vesicles against leakage of entrapped contents, avoiding opsonization, and minimizing removal by the reticuloendothelial system (RES; Willis and Forssen, 1998). The rate at which liposomes are cleared depends on their size, surface charge, and stability (Oku and Namba, 1994; Laverman et al., 1999; Ishida et al., 2001; Ulrich, 2002). The presence of a high electrostatic surface charge promotes the interaction of liposomes with biomolecules that could serve as opsonins and with cells (Laverman et al., 1999; Ishida et al., 2001). In general, unmodified large liposomes are cleared more rapidly than small, neutral, or positively charged liposomes (Oku and Namba, 1994; Laverman et al., 1999; Ishida et al., 2001; Ulrich, 2002). Previous studies have demonstrated that the liver removes large, charged liposomes rapidly, with spleen clearance half-life of less than 1 h (Chrai et al., 2002). The presence of cholesterol is another important factor both for enhancing stability against leakage and in minimizing phospholipid exchange (Willis and Forssen, 1998; Laverman et al., 1999). This minimizes lipid exchange with other structures in the circulation (red blood cells, lipoproteins), which can lead to depletion of the high phase transition temperature lipids and their replacement with less physiologically stable components (Willis and Forssen, 1998; Laverman et al., 1999; Ulrich, 2002).

A major concern in using liposomes for therapeutic purposes is their fast removal from blood circulation by components of the RES. The RES is the major site of liposome accumulation after systemic administration. Primary organs associated with the RES are the liver, spleen, kidneys, lungs, bone marrow, and lymph nodes (Senior, 1987; Oku and Namba, 1994; Vingerhoeds et al., 1994; Ishida et al., 2001; Chrai et al., 2002). The liver exhibits the largest capacity for uptake, whereas the spleen can accumulate liposomes so that its tissue concentration is 10-fold higher than those of other organs (Chrai et al., 2002). Removal of liposomes from the blood is attributed to phagocytic cells that reside in the RES and is mediated through direct interactions between those cells and the liposomes (Senior, 1987; Oku and Namba, 1994; Vingerhoeds et al., 1994; Ishida et al., 2001; Chrai et al., 2002). Although clearance of liposomes by the RES occurs predominantly after opsonization of the vesicles, that is the adsorption of plasma proteins (e.g. immunoglobulins, fibronectin, complement components, C-reactive protein) onto their surface, *in vitro* studies have shown that liposomal uptake into macrophages can also occur in the absence of serum proteins (Ishida et al., 2001; Chrai et al., 2002). The extent of opsonization decreases with a decrease in liposome size from 800 to 200 nm in diameter (Chrai et al., 2002). Small liposomes could not support opsonic activity, whereas the larger ones did so substantially. The profound effect of size on complement recognition affects liver uptake, depending on the extent of liposome opsonization (Laverman et al., 1999; Ishida et al., 2001; Chrai et al., 2002). One of the major steps in improving circulation time and preventing removal by RES was sterically stabilizing the liposomes through the introduction of PEG modification (Oku and Namba, 1994; Torchilin, 1994, 1996; Vingerhoeds et al., 1994; Woodle et al., 1994; Willis and Forssen, 1998; Maruyama, 2002; Ulrich, 2002; Moghimi and Szebeni, 2003). More specifically, stabilization of liposomes with PEG creates a local surface concentration of highly hydrated groups which sterically inhibits both electrostatic and hydrophobic interactions with a variety of serum proteins or cells, thus resulting in a reduced uptake by cells of the RES (Ishida et al., 2001). Many targeting systems with promising outlook based on *in vitro* results have faced the above problems when tested *in vivo* (Sahoo and Labhasetwar, 2003). Therefore, having an understanding of the events that take place *in vivo* is essential for the design of particles with optimal circulation profiles.

The accumulation of liposomes at the target site is a prerequisite but does not necessarily guarantee a therapeutic effect of the encapsulated drug. Therefore, the crucial role of the liposome-cell interaction has to be taken into account (Vingerhoeds et al., 1994; Willis and Forssen, 1998; Ulrich, 2002). Multiple factors such as activation state of the target cell or size, charge, sterical stabilization, and pH-dependence of the liposomes have an important impact on this interaction (Vingerhoeds et al., 1994; Willis and Forssen, 1998; Laverman et al., 1999; Ulrich, 2002; Muro and Muzykantov, 2005). The cellular incorporation of liposomal content can occur in different ways: (i) extracellular release of the soluble content and uptake via diffusion or pore formation; (ii) liposomal fusion within the cell membrane followed by an intracellular release of the liposomal content; and (iii) active uptake of the liposomes via an endocytotic or phagocytotic pathway (Vingerhoeds et al., 1994; Willis and Forssen, 1998; Bendas, 2001; Ulrich, 2002). In receptor-mediated endocytosis, small particles (<150 nm diameter) bind to cell surface receptors and are taken up by clathrin-coated pits to form coated vesicles. After internalization, the clathrin coat is removed and the vesicle fuses with lysosomes, which induces the breakdown of the lipids and release of their contents. Large particles (>150 nm), on the other hand, are taken up principally by phagocytosis, which is usually limited to specific cells such as macrophages but can be induced in many other cell types with appropriate ligands. In both cases, liposomes could either be degraded in the low pH environment, or they could fuse directly with the endosomal or lysosomal membrane (Willis and Forssen, 1998; Ulrich, 2002). In addition, macromolecules can cross the endothelial barrier in three ways: (Koning et al., 2002) between the cells, through cell junctions (paracellular); (Ding et al., 2006) through the endothelial cell, via pores; and (Vingerhoeds et al., 1994) transcellularly, via shuttling vesicles and specific receptors (van Hinsbergh, 1997; Antohe et al., 2004).

It is generally believed that the charge and compactness of the endothelial matrix contribute additionally to the selectivity of the endothelial barrier toward molecules of different size and charge (van Hinsbergh, 1997).

LIPOSOMES – THERAPEUTIC OPPORTUNITIES

The use of liposomes as drug sustained release systems or as drug delivery systems for passive targeting is well established, with several drug formulations in the clinic or in late clinical trials (Sawant and Torchilin, 2012; Allen and Cullis, 2013; Koshkaryev et al., 2013). Several laboratories have reported the use of liposomes as drug carriers in the treatment of cancer, fungal diseases, and inflammatory or immune diseases (Oku and Namba, 1994; Vingerhoeds et al., 1994; Woodle et al., 1994; Willis and Forssen, 1998; Sahoo and Labhasetwar, 2003; Metselaar and Storm, 2005). Innovative research in liposomal drugs has led to commercialization of several liposomal formulations, including anticancer therapeutics (Doxil® and Myocet®) and an antifungal drug formulation (AmBisome®). These products have demonstrated improved therapeutic indices over their corresponding conventional drugs by avoiding sensitive tissues and/or increasing delivery to specific targets *in vivo* (Oku and Namba, 1994; Vingerhoeds et al., 1994; Willis and Forssen, 1998). Liposomes offer several advantages over other delivery systems including biocompatibility, capacity for self-assembly, ability to carry large payloads of active agent, and a wide range of physical properties that can be modified to control their biological properties (Senior, 1987; Woodle et al., 1994; Torchilin, 1996; Willis and Forssen, 1998; Bendas, 2001; Moghimi and Szebeni, 2003). Additionally, the delivery system itself is pharmacologically inactive with minimal toxicity, and is readily metabolized and cleared from the circulation once its carrier function has been completed (Willis and Forssen, 1998). An advantage that liposomes possess over solid particulate delivery systems is their ability to transport and deliver biologically active molecules without the need for covalent coupling (Willis and Forssen, 1998). To improve upon these therapies, clinically active liposomal delivery systems may need to include site-directed surface ligands to further enhance their selective delivery. The concept of drug targeting and controlled drug delivery is used in attempts to improve the therapeutic index of drugs by increasing their localization to specific organs, tissues, or cells and by decreasing their activity and potential toxic side effects in normal organs (e.g. heart, liver, or kidneys). This concept is especially important for drugs with a narrow therapeutic window which has the potential of having detrimental effects (Vingerhoeds et al., 1994; Willis and Forssen, 1998; Bendas, 2001; Maruyama, 2002).

USE OF NANOCARRIERS FOR PAIN THERAPIES

Drug delivery systems have been used in pain therapies to improve toxicity or side effect profiles by targeted delivery to specific sites in the body, increase drug upload or bioavailability, and to provide prolonged drug release. For example, an area of interest has been the delivery of opioid-based compounds to target peripheral opioid receptors within injured tissue to promote analgesic and anti-inflammatory activity (Hua and Cabot, 2010). It is well-established that many conventional opioid agonists have been shown to produce potent opioid receptor mediated analgesia when administered locally into injured tissue of rodents, non-human primates, and humans (Stein et al., 2001, 2003; Rittner and Stein, 2005; Rittner et al., 2005). However, with increased blood flow secondary to inflammation, drugs may still be absorbed into the systemic circulation, leading to side effects mediated by activation of central or peripheral opioid receptor activity (e.g., sedation, respiratory depression, dependence, tolerance, nausea, or constipation) (Stein et al., 2001, 2003; Menendez et al., 2005; Rittner et al., 2005; Rittner and Stein, 2005; Sevostianova et al., 2005). This area of research of applying targeted drug delivery and the use of nanocarriers in the management of pain is a novel and exciting area of research, with much potential for growth and clinical benefits. The remainder of the review will focus on the progress made in this area of research in experimental and clinical studies (**Figure 1**).

EXPERIMENTAL USE OF NANOCARRIERS FOR PAIN THERAPIES

Nanosystems used for delivering compounds intended for pain therapies, such as local anesthetics (de Paula et al., 2012) or non-steroidal anti-inflammatory drugs (NSAIDS), have been reviewed previously (Puglia et al., 2013). The encapsulation of local anesthetics into liposomes, for instance, presents advantages such as slow release, prolonged duration of action, reduced plasma concentrations, and low toxicity to the central nervous and cardiovascular systems. A number of pre-clinical studies have been conducted encapsulating local anesthetics, such as bupivacaine or lidocaine, into multilamellar or unilamellar liposomes using different phospholipid and pH combinations (de Paula et al., 2012). These studies report increased duration of anesthesia and sensory blockade following parenteral administration of these formulations.

Targeted delivery of glucocorticosteroids has been widely studied for the treatment of rheumatoid arthritis and other inflammatory joint conditions (Metselaar et al., 2003, 2004; Metselaar and Storm, 2005). Although corticosteroids are not classified as an analgesic, the pain relieving effects are secondary to their anti-inflammatory activity. Long-circulating PEGylated liposomes containing methylprednisolone or betamethasone have been used to treat Lewis rats with adjuvant-induced arthritis (AIA) both at early (before clinical signs appear) and late (at the peak of the disease) stages of the disease (Avnir et al., 2008). In addition, Ulmansky et al. (2012) showed that intravenous treatment with sterically stabilized nano-liposomes (NSSL) encapsulated with methylprednisolone or betamethasone significantly decreased the severity of adjuvant arthritis in Lewis rats throughout all disease stages. They reported that both subcutaneous and intravenous administration of glucocorticoid-encapsulated NSSL was able to suppress arthritis significantly compared to higher doses of the free drugs or to TNF-α antagonists (Ulmansky et al., 2012).

Non-steroidal anti-inflammatory drugs have long been used as an analgesic and anti-inflammatory agent. However, they are associated with numerous interactions with other medications and have serious side effects to the gastrointestinal tract, kidneys, and cardiovascular system (Rittner et al., 2005; Warner and Mitchell, 2008). Nanocarriers have been used to enhance the efficacy and reduce the toxicity of NSAIDs by targeted delivery to

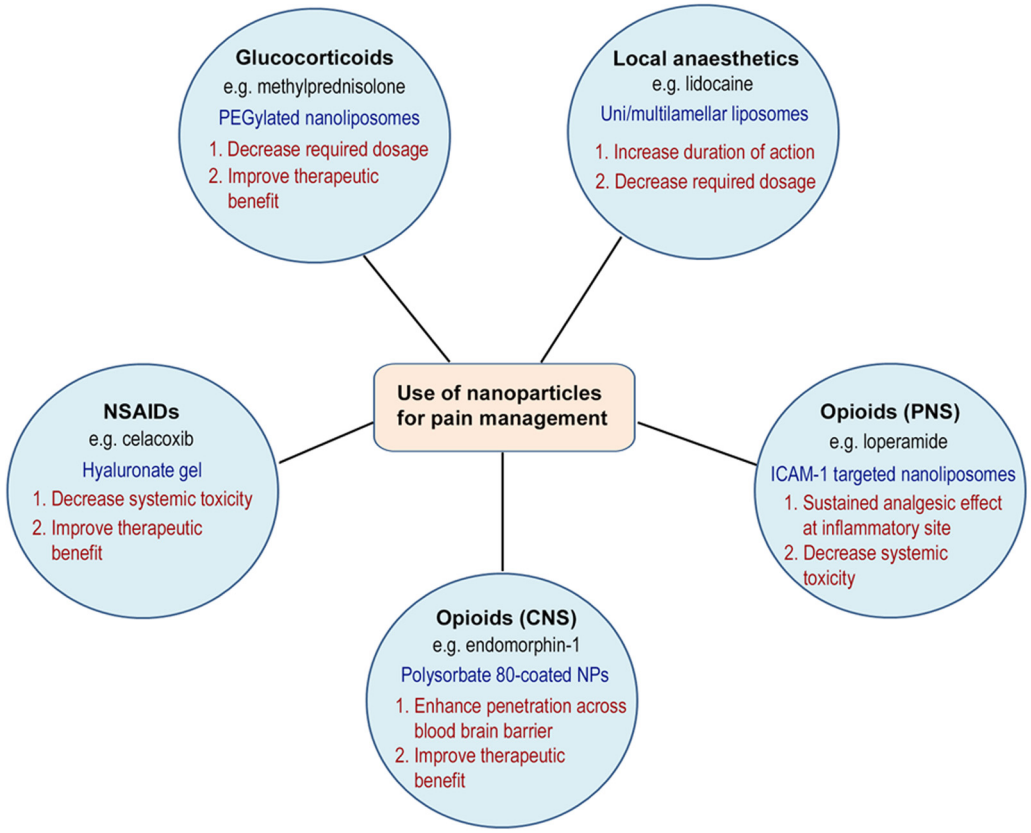


FIGURE 1 | Use of nanocarriers for pain management.

the site of inflammatory pain. A number of topical and parenteral nano-formulations have been utilized and have shown success in preclinical studies (Bansal et al., 2007; Raffin et al., 2012; Tarţău et al., 2012; Dong et al., 2013; Puglia et al., 2013). Dong et al. (2013) recently demonstrated that celecoxib-loaded liposomes embedded into hyaluronate gel was more effective than either single agent in pain control and cartilage protection in a rabbit knee osteoarthritis model following intra-articular injection.

Targeted nanoparticles have recently been engineered to deliver opioids, in particular loperamide HCl, specifically to peripheral opioid receptors to induce analgesic and anti-inflammatory actions for use in painful inflammatory conditions (Hua and Cabot, 2013). Loperamide is a peripherally-selective mu-opioid receptor agonist that does not have analgesic effects following intravenous or oral application due to its physicochemical properties. These nanoparticles are conjugated with antibodies targeted against intercellular adhesion molecule-1 (anti-ICAM-1) which mimics the properties of opioid-containing immune cells. These targeted nanoparticles produced highly significant analgesic and anti-inflammatory effects over the 48-h time course studied following intravenous administration in rats with Complete Freund's Adjuvant-induced inflammation of the paw. Biodistribution data demonstrated specific localization of the targeted nanoparticles to peripheral inflammatory tissue with no significant uptake into the brain (Hua and Cabot, 2013). Other sustained release systems have also been engineered to prolong the duration of action of opioid analgesics (Ward et al., 2013).

A number of non-lipid-based nanocarrier formulations have also been studied to improve the oral (Martín-Banderas et al., 2012; Tang et al., 2012), intranasal (Kumar et al., 2013; Patel et al., 2013), and CNS delivery of analgesic agents (Liu et al., 2006; Tosi et al., 2007; Chen et al., 2013). Local or systemic administration of endogenous opioid peptides (e.g. β-endorphin) is not viable due to its short half-life in the blood and within inflamed tissue. Liu et al. (2006) demonstrated that opioid peptides, in particular endomorphin-1, adsorbed onto the surface of butylcyanoacrylate nanoparticles and coated with polysorbate 80 could penetrate the blood-brain barrier following intravenous administration to cause analgesia. Tosi et al. (2007) investigated the *in vivo* antinociceptive efficacy of peptide-derivatised nanoparticles loaded with loperamide HCl for delivery to central opioid receptors, and reported a peak percentage of maximum possible effect (% MPE) of 60% at 4 h and a significant sustained release effect for 6 h after tail vein injection of a dose equivalent of 0.7 mg of loperamide HCl in Wistar rats. In addition, Chen et al. (2013) showed that nanoparticles consisting of loperamide and PLGA-PEG-PLGA triblock copolymer coated with poloxamer 188 or polysorbate 80 had improved penetration

across the blood-brain barrier in comparison to PLGA-PEG-PLGA nanoparticles and PLGA nanoparticles. These studies demonstrate that the use of surface modification for nanoparticles is an efficient strategy to deliver opioid analgesics to specific sites in the body.

CLINICAL USE OF NANOCARRIERS FOR PAIN THERAPIES

Although liposomes and nanoparticles present an exciting opportunity to improve the management of a variety of painful conditions, current clinical use is limited and few products appear to be in use for human clinical trials. Liposome encapsulation of local anesthetics, NSAIDs, and opioids has been studied in humans with promising results. For example, liposomal formulations of local anesthetics have been demonstrated to provide significantly prolonged pain relief after surgical procedures and in chronic cancer (de Paula et al., 2012). Gorfine et al. (2011) compared the magnitude and duration of postoperative analgesia from a single dose of bupivacaine extended-release injection with placebo administered intraoperatively via wound infiltration in 184 patients undergoing hemorrhoidectomy in a multicenter, randomized, double-blind, placebo-controlled trial. The results showed that the liposomal formulation significantly reduced pain over 72 h and decreased opioid requirements, compared to placebo (Gorfine et al., 2011). Similarly, Lafont et al. (1996) reported prolonged pain relief in a patient with chronic cancer that lasted for 11 h after injection of a liposomal bupivacaine formulation, compared to 4 h for plain bupivacaine.

The efficacy of topical liposomal NSAID-based formulations has also been demonstrated in clinical studies (Puglia et al., 2013). For example, indomethacin-loaded liposomes incorporated into hydrogels were studied in UVB-induced erythema on healthy human volunteers. The results provided a more prolonged anti-inflammatory effect in comparison to a gel formulation containing free drug, allowing a sustained release of the drug to deeper skin layers (Puglia et al., 2004).

Strategies to restrict the access of opioid agonists to the CNS have also been of major interest in pain research (Menendez et al., 2005; Sevostianova et al., 2005). With regards to incorporation of hydrophilic opioids into liposomal formulations, an extended-release morphine preparation based on a multivesicular lipid suspension foam technology is available in the United States (Rose et al., 2005; Viscusi et al., 2005). This preparation is indicated for pain relief after major surgery (e.g., orthopedic surgery involving lower extremities, lower abdominal surgery, or cesarean delivery) as a single lumbar epidural injection. Studies have demonstrated effective, dose-related analgesia for up to 48 h after a single dose (Rose et al., 2005; Viscusi et al., 2005). Although the safety profile was largely consistent with those for other epidurally administered opioid analgesics, systemic adverse effects were still reported. In fact, the rate of respiratory depression was higher in the liposomal morphine group compared with the intravenous patient controlled analgesia (PCA) fentanyl group, which suggest that patient characteristics are important in choosing an appropriate dose of liposomal morphine (Viscusi et al., 2005). While benefits were seen with its use following cesarean section (Carvalho et al., 2007), another study showed no benefit over traditional opioids following abdominal surgery with breakthrough pain relief still required and a similar side effect profile to traditional opioids (Gambling et al., 2005). To date, the clinical studies for pain therapies have only investigated the use of conventional liposomes which permits passive targeting. It is anticipated that the use of ligand-targeted nanocarriers (active targeting) for pain therapies will further improve the efficacy and side effect profile of the conventional liposome formulations.

CONCLUSION

This phenomenon of disease-site targeting is believed to play a major role in the enhanced efficacy observed for a variety of drugs when formulated inside lipid vesicles. Despite the clinical need, the use of nano-based therapeutics to target and treat inflammation and pain is only beginning to be exploited. The use of drug-loaded liposomes for this application would be promising for a multitude of acute and chronic pain conditions (e.g., post-operative pain, visceral cancer pain, rheumatoid arthritis, or neuropathic pain). Their use will ultimately lead to improved efficacy, increased duration of action, and improved side effect profile of analgesic and anti-inflammatory therapeutics.

ACKNOWLEDGMENT

This work was supported by The Pharmacy Research Trust of New South Wales.

REFERENCES

Allen, T. M., and Cullis, P. R. (2013). Liposomal drug delivery systems: from concept to clinical applications. *Adv. Drug Del. Rev.* 65, 36–48. doi: 10.1016/j.addr.2012.09.037

Antohe, F., Lin, L., Kao, G. Y., Poznansky, M. J., and Allen, T. M. (2004). Transendothelial movement of liposomes in vitro mediated by cancer cells, neutrophils or histamine. *J. Liposome Res.* 14, 1–25. doi: 10.1081/LPR-120039660

Avnir, Y., Ulmansky, R., Wasserman, V., Even-Chen, S., Broyer, M., Barenholz, Y., et al., (2008). Amphipathic weak acid glucocorticoid prodrugs remote-loaded into sterically stabilized nanoliposomes evaluated in arthritic rats and in a Beagle dog: a novel approach to treating autoimmune arthritis. *Arthritis Rheum.* 58, 119–129. doi: 10.1002/art.23230

Bansal, S. S., Joshi, A., and Bansal, A. K. (2007). New dosage formulations for targeted delivery of cyclo-oxygenase-2 inhibitors: focus on use in the elderly. *Drugs Aging* 24, 441–451. doi: 10.2165/00002512-200724060-00001

Bendas, G. (2001). Immunoliposomes: a promising approach to targeting cancer therapy. *BioDrugs* 15, 215–224. doi: 10.2165/00063030-200115040-00002

Carvalho, B., Roland, L. M., Chu, L. F., Campitelli, V. A., and Riley, E. T. (2007). Single-dose, extended-release epidural morphine (DepoDur) compared to conventional epidural morphine for post-cesarean pain. *Anesth. Analg.* 105, 176–183. doi: 10.1213/01.ane.0000265533.13477.26

Chen, Y. C., Hsieh, W. Y., Lee, W. F., and Zeng, D. T. (2013). Effects of surface modification of PLGA-PEG-PLGA nanoparticles on loperamide delivery efficiency across the blood-brain barrier. *J. Biomater. Appl.* 27, 909–922. doi: 10.1177/0885328211429495

Chrai, S. S., Murari, R., and Ahmad, I. (2002). Liposomes (a review) part two: drug delivery systems. *BioPharm* 17, 40–43.

de Paula, E., Cereda, C. M., Fraceto, L. F., de Araújo, D. R., Franz-Montan, M., Tofoli, G. R., et al., (2012). Micro and nanosystems for delivering local anesthetics. *Expert Opin. Drug Deliv.* 9, 1505–1524. doi: 10.1517/17425247.2012.738664

Ding, B. S., Dziubla, T., Shuvaev, V. V., Muro, S., and Muzykantov, V. R. (2006). Advanced drug delivery systems that target the vascular endothelium. *Mol. Interv.* 6, 98–112. doi: 10.1124/mi.6.2.7

Dong, J., Jiang, D., Wang, Z., Wu, G., Miao, L., and Huang, L. (2013). Intra-articular delivery of liposomal celecoxib-hyaluronate combination for the treatment of osteoarthritis in rabbit model. *Int. J. Pharm.* 441, 285–290. doi: 10.1016/j.ijpharm.2012.11.031

Gambling, D., Hughes, T., Martin, G., Horton, W., and Manvelian, G. (2005). A comparison of Depodur, a novel, single-dose extended-release epidural morphine, with standard epidural morphine for pain relief after lower abdominal surgery. *Anesth. Analg.* 100, 1065–1074. doi: 10.1213/01.ANE.0000145009.03574.78

Gorfine, S. R., Onel, E., Patou, G., and Krivokapic, Z. V. (2011). Bupivacaine extended-release liposome injection for prolonged postsurgical analgesia in patients undergoing hemorrhoidectomy: a multicenter, randomized, double-blind, placebo-controlled trial. *Dis. Colon Rectum* 54, 1552–1559. doi: 10.1097/DCR.0b013e318232d4c1

Hua, S., and Cabot, P. J. (2010). Mechanisms of peripheral immune-cell-mediated analgesia in inflammation: clinical and therapeutic implications. *Trends Pharmacol. Sci.* 31, 427–433. doi: 10.1016/j.tips.2010.05.008

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician.* 16, E199–E216.

Ishida, T., Harashima, H., and Kiwada, H. (2001). Interactions of liposomes with cells in vitro and in vivo: opsonins and receptors. *Curr. Drug Metab.* 2, 397–409. doi: 10.2174/1389200013338306

Koning, G. A., Schiffelers, R. M., and Storm, G. (2002). Endothelial cells at inflammatory sites as target for therapeutic intervention. *Endothelium* 9, 161–171. doi: 10.1080/10623320213631

Koshkaryev, A., Sawant, R., Deshpande, M., and Torchilin, V. (2013). Immunoconjugates and long circulating systems: origins, current state of the art and future directions. *Adv. Drug Del. Rev.* 65, 24–35. doi: 10.1016/j.addr.2012.08.009

Kumar, M., Pandey, R. S., Patra, K. C., Jain, S. K., Soni, M. L., Dangi, J. S., et al., (2013). Evaluation of neuropeptide loaded trimethyl chitosan nanoparticles for nose to brain delivery. *Int. J. Biol. Macromol.* 61, 189–195. doi: 10.1016/j.ijbiomac.2013.06.041

Lafont, N. D., Legros, F. J., and Boogaerts, J. G. (1996). Use of liposome-associated bupivacaine in a cancer pain syndrome. *Anaesthesia* 51, 578–579. doi: 10.1111/j.1365-2044.1996.tb12569.x

Laverman, P., Boerman, O. C., Oyen, W. J., Dams, E. T., Storm, G., and Corstens, F. H. (1999). Liposomes for scintigraphic detection of infection and inflammation. *Adv. Drug Deliv. Rev.* 37, 225–235. doi: 10.1016/S0169-409X(98)00095-7

Liu, H., Ni, J., and Wang, R. (2006). In vitro release performance and analgesic activity of endomorphin-1 loaded nanoparticles. *Pharmazie* 61, 450–452.

Martín-Banderas, L., Alvarez-Fuentes, J., Durán-Lobato, M., Prados, J., Melguizo, C., Fernández-Arévalo, M., et al., (2012). Cannabinoid derivate-loaded PLGA nanocarriers for oral administration: formulation, characterization, and cytotoxicity studies. *Int. J. Nanomed.* 7, 5793–5806.

Maruyama, K. (2002). PEG-immunoliposome. *Biosci. Rep.* 22, 251–266. doi: 10.1023/A:1020138622686

Menendez, L., Lastra, A., Meana, A., Hidalgo, A., and Baamonde, A. (2005). Analgesic effects of loperamide in bone cancer pain in mice. *Pharmacol. Biochem. Behav.* 81, 114–121. doi: 10.1016/j.pbb.2005.02.007

Metselaar, J. M., and Storm, G. (2005). Liposomes in the treatment of inflammatory disorders. *Expert. Opin. Drug Deliv.* 2, 465–476. doi: 10.1517/17425247.2.3.465

Metselaar, J. M., Van den Berg, W. B., Holthuysen, A. E., Wauben, M. H., Storm, G., and Van Lent, P. L. (2004). Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. *Ann. Rheum. Dis.* 63, 348–353. doi: 10.1136/ard.2003.009944

Metselaar, J. M., Wauben, M. H., Wagenaar-Hilbers, J. P., Boerman, O. C., and Storm, G. (2003). Complete remission of experimental arthritis by joint targeting of glucocorticoids with long-circulating liposomes. *Arthritis Rheum.* 48, 2059–2066. doi: 10.1002/art.11140

Moghimi, S. M., and Szebeni, J. (2003). Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog. Lipid Res.* 42, 463–478. doi: 10.1016/S0163-7827(03)00033-X

Muro, S., and Muzykantov, V. R. (2005). Targeting of antioxidant and antithrombotic drugs to endothelial cell adhesion molecules. *Curr. Pharm. Des.* 112383–112401. doi: 10.2174/1381612054367274

Oku, N., and Namba, Y. (1994). Long-circulating liposomes. *Crit. Rev. Ther. Drug Carrier Syst.* 11, 231–270.

Patel, D., Naik, S., Chuttani, K., Mathur, R., and Mishra, A. K. (2013). Intranasal delivery of cyclobenzaprine hydrochloride-loaded thiolated chitosan nanoparticles for pain relief. *J. Drug Target.* 21, 759–769. doi: 10.3109/1061186X.2013.818676

Puglia, C., Tirendi, G. G., and Bonina, F. (2013). Emerging role of colloidal drug delivery systems (CDDS) in NSAID topical administration. *Curr. Med. Chem.* 20, 1847–1857. doi: 10.2174/11847

Puglia, C., Trombetta, D., Venuti, V., Saija, A., and Bonina, F. (2004). Evaluation of in-vivo topical anti-inflammatory activity of indometacin from liposomal vesicles. *J. Pharm. Pharmacol.* 56, 1225–1232. doi: 10.1211/0022357044445

Raffin, R. P., Lima, A., Lorenzoni, R., Antonow, M. B., Turra, C., Alves, M. P., et al. (2012). Natural lipid nanoparticles containing nimesulide: synthesis, characterization and in vivo antiedematogenic and antinociceptive activities. *J. Biomed. Nanotechnol.* 8, 309–315. doi: 10.1166/jbn.2012.1377

Rittner, H. L., and Stein, C. (2005). Involvement of cytokines, chemokines and adhesion molecules in opioid analgesia. *Eur. J. Pain.* 9, 109–112. doi: 10.1016/j.ejpain.2004.05.009

Rittner, H. L., Machelska, H., and Stein, C. (2005). Leukocytes in the regulation of pain and analgesia. *J. Leukoc. Biol.* 78, 1215–1222. doi: 10.1189/jlb.0405223

Rose, J. S., Neal, J. M., and Kopacz, D. J. (2005). Extended-duration analgesia: update on microspheres and liposomes. *Reg. Anesth. Pain Med.* 30, 275–285.

Sahoo, S. K., and Labhasetwar, V. (2003). Nanotech approaches to drug delivery and imaging. *Drug Discov. Today* 8, 1112–1120. doi: 10.1016/S1359-6446(03)02903-9

Sawant, R. R., and Torchilin, V. P. (2012). Challenges in development of targeted liposomal therapeutics. *AAPS J.* 14, 303–315. doi: 10.1208/s12248-012-9330-0

Senior, J. H. (1987). Fate and behavior of liposomes in vivo: a review of controlling factors. *Crit. Rev. Ther. Drug Carrier Syst.* 3, 123–193.

Sevostianova, N., Danysz, W., and Bespalov, A. Y. (2005). Analgesic effects of morphine and loperamide in the rat formalin test: interactions with NMDA receptor antagonists. *Eur. J. Pharmacol.* 525, 83–90. doi: 10.1016/j.ejphar.2005.10.010

Stein, C., Machelska, H., and Schafer, M. (2001). Peripheral analgesic and antiinflammatory effects of opioids. *Z. Rheumatol.* 60, 416–424. doi: 10.1007/s003930170004

Stein, C., Schafer, M., and Machelska, H. (2003). Attacking pain at its source: new perspectives on opioids. *Nat. Med.* 9, 1003–1008. doi: 10.1038/nm908

Tang, S. Y., Sivakumar, M., Ng, A. M., and Shridharan, P. (2012). Anti-inflammatory and analgesic activity of novel oral aspirin-loaded nanoemulsion and nano multiple emulsion formulations generated using ultrasound cavitation. *Int. J. Pharm.* 430, 299–306. doi: 10.1016/j.ijpharm.2012.03.055

Tarţău, L., Cazacu, A., and Melnig, V. (2012). Ketoprofen-liposomes formulation for clinical therapy. *J. Mater. Sci. Mater. Med.* 23, 2499–2507. doi: 10.1007/s10856-012-4712-5

Torchilin, V. P. (1994). Immunoliposomes and PEGylated immunoliposomes: possible use for targeted delivery of imaging agents. *Immunomethods* 4, 244–258. doi: 10.1006/immu.1994.1027

Torchilin, V. P. (1996). Affinity liposomes in vivo: factors influencing target accumulation. *J. Mol. Recognit.* 9, 335–346. doi: 10.1002/(SICI)1099-1352(199634/12)9:5/6<335::AID-JMR309>3.0.CO;2-0

Tosi, G., Costantino, L., Rivasi, F., Ruozi, B., Leo, E., Vergoni, A. V., et al., (2007). Targeting the central nervous system: in vivo experiments with peptide-derivatized nanoparticles loaded with Loperamide and Rhodamine-123. *J. Control Release* 122, 1–9. doi: 10.1016/j.jconrel.2007.05.022

Ulmansky, R., Turjeman, K., Baru, M., Katzavian, G., Harel, M., Sigal, A., et al., (2012). Glucocorticoids in nano-liposomes administered intravenously and subcutaneously to adjuvant arthritis rats are superior to the free drugs in suppressing arthritis and inflammatory cytokines. *J. Control Release* 160, 299–305. doi: 10.1016/j.jconrel.2011.12.024

Ulrich, A. S. (2002). Biophysical aspects of using liposomes as delivery vehicles. *Biosci. Rep.* 22, 129–150. doi: 10.1023/A:1020178304031

van Hinsbergh, W. M. (1997). Endothelial permeability for macromolecules. Mechanistic aspects of pathophysiological modulation. *Arterioscler. Thromb. Vasc. Biol.* 17, 1018–1023. doi: 10.1161/01.ATV.17.6.1018

Vingerhoeds, M. H., Storm, G., and Crommelin, D. J. (1994). Immunoliposomes in vivo. *Immunomethods* 4, 259–272. doi: 10.1006/immu.1994.1028

Viscusi, E. R., Martin, G., Hartrick, C. T., Singla, N., and Manvelian, G. (2005). Forty-eight hours of postoperative pain relief after total hip arthroplasty with a novel, extended-release epidural morphine formulation. *Anesthesiology* 102, 1014–1022. doi: 10.1097/00000542-200505000-00022

Ward, B. B., Huang, B., Desai, A., Cheng, X. M., Vartanian, M., Zong, H., et al., (2013). Sustained analgesia achieved through esterase-activated morphine prodrugs complexed with PAMAM dendrimer. *Pharm. Res.* 30, 247–256. doi: 10.1007/s11095-012-0869-3

Warner, T. D., and Mitchell, J. A. (2008). COX-2 selectivity alone does not define the cardiovascular risks associated with non-steroidal anti-inflammatory drugs. *Lancet* 371, 270–273. doi: 10.1016/S0140-6736(08)60137-3

Willis, M., and Forssen, E. (1998). Ligand-targeted liposomes. *Adv. Drug Deliv. Rev.* 29, 249–271. doi: 10.1016/S0169-409X(97)00083-5

Woodle, M. C., Newman, M. S., and Cohen, J. A. (1994). Sterically stabilized liposomes: physical and biological properties. *J. Drug Target.* 2, 397–403. doi: 10.3109/10611869408996815

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 September 2013; paper pending published: 21 October 2013; accepted: 04 November 2013; published online: 21 November 2013.

*Citation: Hua S and Wu SY (2013) The use of lipid-based nanocarriers for targeted pain therapies. Front. Pharmacol. **4**:143. doi: 10.3389/fphar.2013.00143*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

MINI REVIEW ARTICLE
published: 13 February 2014
doi: 10.3389/fphar.2014.00017

Female reproductive tract pain: targets, challenges, and outcomes

Phillip Jobling*, Kate O'Hara and Susan Hua

School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia

Edited by:
Peter John Cabot, The University of Queensland, Australia

Reviewed by:
Ciaran J. Faherty, Cadence Pharmaceuticals, USA
Leighann Litcher-Kelly, Stony Brook University, USA

****Correspondence:***
Phillip Jobling, School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW 2038, Australia
e-mail: phillip.jobling@newcastle.edu.au

Pain from the female reproductive tract (FRT) is a significant clinical problem for which there are few effective therapies. The complex neuroanatomy of pelvic organs not only makes diagnosis of pelvic pain disorders difficult but represents a challenge to development of targeted therapies. A number of potential therapeutic targets have been identified on sensory neurons supplying the FRT but our knowledge on the basic neurophysiology of these neurons is limited compared with other viscera. Until this is addressed we can only guess if the new experimental therapies proposed for somatic, gastrointestinal, or bladder pain will translate to the FRT. Once suitable therapeutic targets become clear, the next challenge is drug delivery. The FRT represents a promising system for topical drug delivery that could be tailored to act locally or systemically depending on formulation. Development of these therapies and their delivery systems will need to be done in concert with more robust *in vivo* and *in vitro* models of FRT pain.

Keywords: pelvic pain, vagina, cervix, uterus, drug delivery

INTRODUCTION

Pain syndromes represent one of the major challenges of neurology. Pain has many definitions but essentially it is a concept generated across the brain in response to internal or external stimuli that the individual associates with real or perceived tissue damage or imminent threat (Merskey and Bogduk, 1994). Pain is difficult enough to treat when it arises from a relatively straightforward injury to a defined region like a small piece of skin or a single joint. Pain from pelvic organs, particularly the reproductive tract, is notoriously difficult to treat. In this review we will examine the complex and unique innervation of the female reproductive tract (FRT), current treatments and the potential for topical therapies.

The prevalence of transient pelvic pain (usually dysmenorrhea) has been placed as high as 70–80% of women surveyed while chronic pelvic pain was reported at >20% (Hillen et al., 1999; Pitts et al., 2008). Ten percent of outpatient gynecological visits are for intractable pelvic pain (Ryder, 1996), and pelvic pain is the primary reason for 12–18% of hysterectomies (Kramer and Reiter, 1997). United kingdom estimates from 2000 placed direct healthcare costs at £158 million (Stones et al., 2000) whereas 1996 data from the USA placed patients' out of pocket expenses at $1.9 billion dollars and indirect costs due to time off work at over $500 million (Mathias et al., 1996). Importantly many women do not seek treatment for their pain (Mathias et al., 1996).

Chronic pelvic pain is further divided into "specific disease-associated pelvic pain" and "chronic pelvic pain syndrome" where the underlying pathology remains obscure (International Association for the Study of Pain, 2011). Pelvic pain may arise from a number of structures, both somatic (e.g., striated pelvic floor muscles), and visceral (reproductive tract, bladder, and lower bowel). Focusing on reproductive structures, clinical observations have identified numerous predictors of chronic pelvic pain including endometriosis, pelvic inflammatory disease, childbirth, and urinogenital atrophy following menopause (Giamberardino, 2008; Lara et al., 2009; Paterson et al., 2009).

THE COMPLEX NATURE OF PAIN FROM THE FRT

Sexual behavior and reproduction rely on the integration of nervous and hormonal signals to a widely distributed collection of structures. The external genitalia are essentially somatic structures and the distribution of sensory axons and their neurochemical coding are similar to cutaneous tissues (Martin-Alguacil et al., 2008; Moszkowicz et al., 2011; Vilimas et al., 2011). Sensory neurons innervating the hollow organs show different patterns of neurochemical expression compared with those that supply somatic structures (skin, muscle, and joints; Cervero and Laird, 2004; Song et al., 2009) and marked differences in central axons termination in the spinal cord (Sugiura et al., 1989, 1993).

Pain arising from the vagina, cervix, and uterus is an example of visceral nociception – or pain that comes from distension, injury, or inflammation of hollow organs (Cervero and Laird, 1999). Visceral pain is diffuse, poorly localized, often referred to other body regions, and can be accompanied by disrupted motor and autonomic reflexes (Janig and Morrison, 1986; McMahon, 1997; Westlund, 2000).

EXTERNAL GENITALIA

The most widely reported pain syndromes associated with external genitalia are the vulvodynias (Petersen et al., 2008; Fugl-Meyer et al., 2012). These have a prevalence of around 10% in U.S studies (Harlow and Stewart, 2003; Petersen et al., 2008). Most sensations from the external genitalia are transmitted via axons in the pudendal nerve (Martin-Alguacil et al., 2008; Moszkowicz et al., 2011;

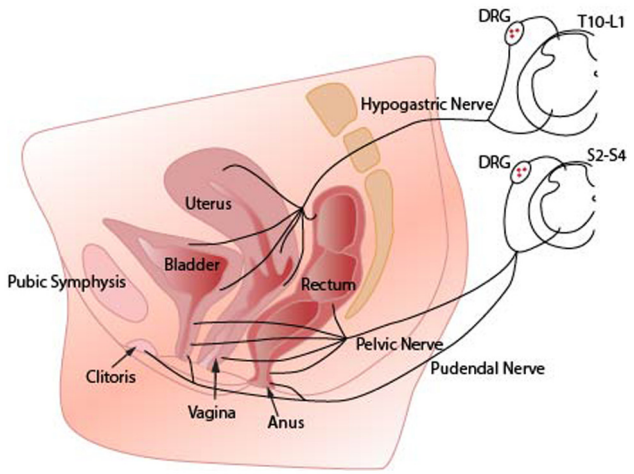


FIGURE 1 | Innervation of pelvic organs. Sensory axons innervating the vagina reach the spinal cord via pelvic nerves and terminate in sacral spinal cord segments (S2–S4). Axons innervating the uterus travel in the hypogastric nerves and terminate in the thoracolumbar spinal cord segments (T10–L2). The region surrounding the cervix represents a transitional zone and is innervated by fibers that travel in both nerves. Sensory axons from the clitoris and vulva follow the pudendal nerves to sacral spinal cord. Note that sensory information from all pelvic organs may converge onto the same spinal cord neural circuits. DRG (dorsal root ganglia).

Vilimas et al., 2011; **Figure 1**). Limited data indicate that often pain from these structures is similar to generalized somatic pain as opposed to visceral pain (Bachmann et al., 2006; Goldstein and Burrows, 2008).

SENSATIONS FROM THE VAGINA, CERVIX AND UTERUS ENTER THE CNS AT MULTIPLE LEVELS

The walls of the FRT are innervated with sensory afferent terminals that respond to both distension and inflammatory mediators (Berkley et al., 1993b; Papka and Traurig, 1993). The FRT is innervated via two main spinal nerve trunks; the hypogastric and pelvic nerves that send sensory information to a number of spinal cord segments (**Figure 1**; Berkley et al., 1993a,b; Wesselmann and Lai, 1997; Wesselmann, 2001; Jobling et al., 2003, 2010).

Electrical recordings from axons in rodents indicate that there is a heterogeneous distribution of receptors. Most axons respond to distension, whilst others respond to both distension and chemical stimuli (e.g., bradykinin or serotonin; Berkley et al., 1993a,b). Compared with the FRT, sensory axons supplying the gastrointestinal tract (GIT) and bladder have been better studied. Five functional classes of sensory axons have been described in the GIT (Page et al., 2002; Brierley et al., 2004; Song et al., 2009) and four functional classes of bladder sensory axons have been identified (Zagorodnyuk et al., 2007). There is an extensive interaction between vagina, cervix, uterus, and somatic structures (Hotta et al., 1999) where there is considerable convergence of these pathways in the spinal cord. The most widely and long-recognized consequence of this convergence is referred pain (Head, 1893).

CENTRAL SENSITIZATION "PELVIC PAIN WITHOUT PELVIC ORGANS"

A significant barrier to treatment is the observation that pelvic pain can exist in the absence of any obvious pathology. In fact pelvic pain is often resistant to the removal of the allegedly offending organs (Baskin and Tanagho, 1992). This observation is thought to be caused by the phenomenon of central sensitization. The mechanisms underlying central sensitization for somatic afferents have been examined in detail (Millan, 1999; Jones and Sorkin, 2003; Lu et al., 2009), however, central sensitization from FRT afferents remain poorly understood. Inflammation of the rat uterus increased receptive field size, and decreased thresholds for cervix afferents (Berkley et al., 1993a). Another study has shown that FRT inflammation recruits large numbers of neurons in the dorsal horn (Wesselmann et al., 2000).

PELVIC ORGAN CROSSTALK – LINKING VAGINA, CERVIX, UTERUS BLADDER, AND BOWEL

Epidemiological data suggest strong comorbidity between inflammatory bowel disorders, interstitial cystitis, and pelvic pain (Whorwell et al., 1986). Some of this comorbidity might be explained by the sensory and motor pathways that link FRT, bladder, and lower bowel (Winnard et al., 2006; Klumpp and Rudick, 2008). Sensations from these organs share synaptic circuits in the spinal cord (Wyndaele et al., 2013) and may even share individual sensory neurons (**Figure 1**; Christianson et al., 2007). These functional and anatomical interactions have implications for the effectiveness of local topical therapies designed to act on one organ only.

OVARIAN HORMONES ALTER SENSORY INNERVATION AND PAIN THRESHOLDS

Fluctuations in levels of ovarian hormones, particularly estrogen, are associated with changes in sensation, including pain, in a variety of tissues (Martin, 2009). This effect of estrogen on pain responses is no doubt due to the widespread expression of estrogen receptors which are located, not only in the FRT, but also on primary sensory neurons, spinal cord neurons, and higher brain centers (Papka et al., 2001; Papka and Mowa, 2003; Vanderhorst et al., 2009; Takanami et al., 2010). Notably, estrogen receptors are particularly concentrated in sacral spinal cord segments that are crucial to the control of pelvic organs (Vanderhorst et al., 2009). The role of estrogen in modulation of the nervous system is unclear (Balthazart and Ball, 2006). Estrogen can influence several receptors and ion channels in peripheral, spinal and supraspinal pathways. For example transient receptor potential (TRP) channels on primary afferent neurons are inhibited by activation of the beta subtype estrogen receptor (ERβ; Xu et al., 2008).

OVARIAN HORMONE WITHDRAWAL ALTERS FRT AND CUTANEOUS SENSITIVITY

In humans menopause is associated with a drastic decrease in levels of ovarian hormones (Martin, 2009). With this altered hormonal status many women have increased pain from the FRT, especially the vagina, and some somatic tissues (Fillingim and Edwards, 2001; Samsioe, 2007; Martin, 2009). This post-menopausal

vaginal hyperalgesia, typically presents as pain during intercourse (dyspareunia; Davis et al., 2005; Mac Bride et al., 2010). Various explanations have been proposed including vaginal atrophy (Forsberg, 1995). However the severity of pain is only loosely correlated with vaginal wall thickness (Kao et al., 2008), suggesting other factors are critical in this condition. Many of the painful urinogenital symptoms can be reversed by conventional systemic estrogen replacement although an increasing alternative is the use of local intravaginal estrogen replacement (Mac Bride et al., 2010).

PERIPHERAL THERAPEUTIC TARGETS

Precise information about the nature of primary sensory afferent endings in uterus, cervix, and vagina is scant compared with somatic (Woolf and Ma, 2007) or other visceral targets (Blackshaw et al., 2007). Anatomical data from animals (Papka et al., 1985, 1995, 1999; Shew et al., 1991; Collins et al., 2002) and, rarely, humans (Fried et al., 1990; Bokor et al., 2009; Malvasi et al., 2010) suggest they express the same neurochemical markers and receptors as nearby viscera, e.g., bladder and bowel.

OPIOIDS

Enkephalin immunoreactive axons in uterus and vagina have been reported in some mammals (Lakomy et al., 1994; Skobowiat et al., 2009), while mu and delta opioid receptors are present in human and mouse myometrium (Zhu and Pintar, 1998; Fanning et al., 2013). Functional measures of peripheral opioid receptor activation are not well documented for FRT afferents. However in GIT (Armstrong et al., 2005; Page et al., 2008) and bladder (Su et al., 1997) mechanosensitive sensory axons are modulated by opioid receptor agonists.

TRP CHANNELS

The TRP family of channels have been a focus of somatic pain research for some time. TRPV1 channels are present on presumed nociceptive axons in rat vagina (Liao and Smith, 2011) and human cervix (Tingåker et al., 2008). Interestingly these have been proposed to underlie some of the adverse side effects of clotrimazole, an anti-mycotic agent (Meseguer et al., 2008). Furthermore estrogen amplifies pain evoked by uterine distension, via a TRPV1 receptor dependent mechanism (Yan et al., 2007). Information on other TRP channels (e.g., TRPM8 and TRPA1) is limited in the FRT although they are expressed on sensory nerves supplying the GIT (Blackshaw et al., 2010).

TROPHIC FACTORS

Various growth factors particularly nerve growth factor (NGF) and members of the glial cell line-derived neurotrophic factor family of ligands are implicated not only in survival of some sensory neurons but their receptors have been suggested as targets for alleviating neuropathic pain (Koltzenburg et al., 1999; Boucher et al., 2000; Malin et al., 2006; Malin and Davis, 2008). Within the FRT NGF has been reported in the uterus (Lobos et al., 2005) and cervix (Chalar et al., 2003) and neurturin mRNA has been found in uterus (Widenfalk et al., 2000). Sensory neurons innervating the uterus were shown to express tyrosine receptor kinase A receptors (Chalar et al., 2003). Whether growth factors or their receptors modulate sensory afferent neurons from the FRT is unknown although they are implicated in bladder signaling (Klinger and Vizzard, 2008; Schnegelsberg et al., 2010).

P2X RECEPTORS

ATP was implicated in pain signaling nearly four decades ago (Bleehen and Keele, 1977). The subsequent discovery of P2X receptors on sensory nerves (Cook et al., 1997) had led to much work on identifying subtypes of P2X receptors as therapeutic targets (North and Jarvis, 2013). Detailed studies of the FRT are lacking however P2X receptors have been identified on both uterine and cervical sensory axons (Papka et al., 2005).

METABOTROPIC GLUTAMATE RECEPTORS (mGluRs)

mGluR have been implicated in uterine and cervix sensory signaling (Ghosh et al., 2007) where they may modulate sensory discharge during parturition. Within the GIT peripheral mGluR on sensory endings modulate excitability (Page et al., 2005) where they have been proposed as therapeutic targets (Blackshaw et al., 2011).

ACID SENSING ION CHANNELS (ASICs)

No studies to date have tested whether ASIC are expressed on sensory neurons innervating the FRT. However, they are expressed in vagal (Page et al., 2007) and colonic (Jones et al., 2005) sensory neurons where they represent a potential therapeutic target.

NITRIC OXIDE

Nitric oxide generated by neuronal, inducible or endothelial nitric oxide synthase (NOS) plays many roles in the FRT. It is most notably released by autonomic vasodilator neurons to dramatically increase blood flow (Morris et al., 2005). However nNOS is also expressed in a subpopulation of sensory nerves (Papka et al., 1995). The role of nNOS in sensory signaling in the reproductive tract is unknown. However a role for pain modulation has been proposed in somatic pain models (Boettger et al., 2007; Keilhoff et al., 2013) and therapeutic agents that target nNOS have been proposed (Mladenova et al., 2012).

CANNABINOIDS

The cannabinoid signaling pathways have long been proposed as therapeutic targets (Roques et al., 2012). The FRT has some of the highest levels of endogenous cannabinoids (Schmid et al., 1997) and cannabinoid receptors are expressed in human and rodent myometrium (Das et al., 1995; Dennedy et al., 2004) where they act on smooth muscle. Cannabinoid receptors on sensory axons associated with the FRT have not been reported. However activation of cannabinoid type 1 receptors modulates sensory afferent signaling from the urinary bladder (Walczak et al., 2009) and jejunum (Yuce et al., 2010).

CURRENT THERAPIES FOR FRT PAIN

Currently evidence based treatment for FRT pain is limited. Standard pain therapies such as paracetamol, non-steroidal anti-inflammatory drugs (NSAIDs), opioids, or neuropathic pain therapies have been used, as the type of pain is not well defined and directing treatment is difficult. Topical therapies are increasing as options for treating vulvar and vaginal pain and are recommended

as first line treatment (Nunns et al., 2010). Topical therapy presents an attractive alternative to systemic therapy as they are generally well tolerated and are associated with less systemic adverse effects. However, some topical therapies may cause irritation that can worsen symptoms (Nunns et al., 2010). Currently, due to a lack of evidence of effectiveness, all topical therapies used in this condition would be considered experimental (Andrews, 2011). Topical treatments have also been associated with a high placebo response (Nunns et al., 2010).

Vulvodynia and vestibulodynia treatment has been the subject of several recent reviews, which concluded there was insufficient evidence of effectiveness and safety for a range of therapies. It was determined there was evidence of a lack of efficacy for botulinum toxin injection, topical 5% xylocaine, and topical nifedipine. There was insufficient evidence to evaluate the effectiveness of steroid, local anesthetic injections, nerve blocks, intramuscular or intralesional interferon or topical capsaicin, montelukast, steroids, gabapentin, and ketoconazole (Andrews, 2011). Oral treatments that include tricyclic antidepressants, serotonin–norepinephrine uptake inhibitors and anticonvulsants (Cox and Neville, 2012) lack good quality evidence of effectiveness and have systemic adverse effects. Physical and alternative therapies are also used but there are only anecdotal reports of effectiveness (Andrews, 2011; Cox and Neville, 2012). Surgery has also been used effectively to treat vulvar vestibular pain (Nunns et al., 2010; Andrews, 2011; Cox and Neville, 2012). Estrogen therapies are effective in patients where the pain is linked to low estrogen levels following menopause or breast cancer treatment (Goetsch, 2012). Less common therapeutic options that have shown success in small clinical trials include cutaneous fibroblast lysate cream (Donders and Bellen, 2012), nitroglycerin cream (Walsh et al., 2002), and amitriptyline-baclofen cream (Nyirjesy et al., 2009).

Treatment of uterine pain is limited to systemic options with little evidence. Dysmenorrhea is the best-studied uterine pain syndrome. Primary dysmenorrhea is treated with simple analgesics, usually naproxen, while secondary dysmenorrhea treatment relies on removal of the underlying cause of the pain (Kohle and Deb, 2011). As other pelvic organs can cause pelvic pain a thorough investigation is important. Non-pharmacological therapies including nutrition and lifestyle changes, along with surgery may play a role. Further investigation is needed to determine the most appropriate treatments for uterine pain.

Further investigation is needed to determine specific targets for pharmacological management of the various FRT pain sub-types. The use of drug delivery systems may be required to effectively deliver existing or experimental compounds to the target site for improved efficacy and/or to reduce systemic adverse effects.

DELIVERING THERAPIES TO THE FRT

The intravaginal route of drug administration has been studied as a suitable site for local and systemic delivery of therapeutic agents. The degree to which therapies act locally or systemically is formulation dependent. Presently intravaginal therapies are typically prescribed for vaginal infections and vaginal dryness. Systemic drug delivery includes uterine targeting or treatment of migraines (Bassi and Kaur, 2012). In relation to pain stemming from the FRT, the intravaginal route shows promise for the local or systemic delivery of analgesic and anti-inflammatory agents.

The vagina has unique features that can be exploited for optimal therapeutic responses, such as the presence of a dense network of blood vessels, large surface area, and permeability (Srikrishna and Cardozo, 2013). In addition, unlike conventional oral therapy, the vaginal route avoids hepatic first-pass metabolism, significant enzymatic degradation of active ingredients and drug interactions (Bassi and Kaur, 2012). Absorption from vaginal delivery systems occurs by dissolution, followed by penetration of drug through the vaginal membrane to reach the systemic circulation (Hussain and Ahsan, 2005). Physiological factors can affect the drug release from intravaginal delivery systems and/or vaginal absorption of drugs, such as cyclic changes in thickness of the vaginal epithelium, fluid volume and composition, pH and sexual arousal (Hussain and Ahsan, 2005; das Neves et al., 2011). Although physiological factors are difficult to alter, the physicochemical properties of a drug compound (e.g., molecular weight, lipophilicity, ionization, surface charge, chemical nature; Hussain and Ahsan, 2005) as well as the formulation can be selected to regulate local versus systemic activity.

Despite its therapeutic potential, vaginal preparations show low patient acceptability due to factors including multiple daily dosing; leakage and messiness following application; and the need for night-time dosing. The effectiveness of commonly available vaginal dosage forms (creams, gels, solutions, foams, pessaries) is often limited by their low retention to the vaginal epithelium (Pavelić et al., 2004). In order to overcome these limitations, novel vaginal delivery systems are being developed that possess desirable distribution, bioadhesion, and release properties – such as vaginal rings, bioadhesive delivery systems, and nanosystems.

VAGINAL RINGS

Intravaginal rings (IVRs) are circular drug delivery devices that are designed to provide both sustained and controlled drug release, lasting for several weeks to several months following insertion into the vagina. IVR have been shown to be effective in delivering a multitude of compounds, such as contraceptive steroids and steroids for the treatment of post-menopausal atrophy. This delivery device has been previously reviewed (Baloglu et al., 2009; Thurman et al., 2013; Srikrishna and Cardozo, 2013).

BIOADHESIVE DRUG DELIVERY (BDD) SYSTEMS

BDD systems were developed to circumvent the issues associated with conventional vaginal formulations, by adhering to the vaginal mucosal tissue and prolonging the residence time of the formulation. Vaginal BDD systems have been exploited for both local as well as systemic delivery of drugs (Merabet et al., 2005; Bassi and Kaur, 2012). Several studies have focused on BDD systems in the form of tablets, films, patches, and gels for the vaginal mucosal route that are composed of bioadhesive polymers that are biocompatible, biodegradable and stable. Common mucoadhesive polymers include tragacanth (acacia), carbopol resins, sodium alginate, carboxymethylcellulose, and chitosan. Vaginal BDD systems have been previously reviewed (Baloglu et al., 2009; Bassi and Kaur, 2012).

NANOSYSTEMS

Nanocarriers [e.g., dendrimers, liposomes, Poly(lactic-co-glycolic acid) nanoparticles, silver and gold nanoparticles] have been utilized in topical drug delivery to enhance the penetration of drug compounds. For example, encapsulation of drugs within liposomes can provide characteristics such as enhanced skin or mucosal permeability, sustained release as well as controlled release (Pavelić et al., 2004). Such nanosystems are usually incorporated within a bioadhesive base (e.g., Carbopol resin) to enhance the viscosity of the formulation for retention on the mucosal surface (Pavelić et al., 2004; das Neves et al., 2011). The use of nanosystems is promising for intravaginal drug delivery, however, similar to BDD systems, the interaction of these formulations with mucosal fluids present in the vagina at different stages of the menstrual cycle and age is not yet well defined (Bassi and Kaur, 2012).

CONCLUSION

Pain attributed to the FRT is complex and involves several classes of nociceptive and non-nociceptive sensory neurons. The unique neural anatomy of pelvic organs provides challenges in the delivery of selective therapies. There is little evidence that current treatments are effective and new strategies need to be developed. Relative to somatic pain, or pain from the GIT, there is a lack of information on the basic neurophysiology of FRT sensory neurons. Well defined animal models of neuropathic or inflammatory pain exist for somatic structures (e.g., chronic constriction injury models) and to some extent colitis (trinitrobenzene sulfonic acid models). At present there is no consistent approach to FRT pain. This will need to be addressed if we are to explore the many potential therapeutic targets present on FRT sensory neurons. Exciting opportunities exist for development of intravaginal drug delivery systems for either local or systemic drug delivery. Similar targeted delivery systems can be developed for the vulvodynias. Finally larger clinical trials of the few currently available promising therapies could provide useful insights in directing preclinical studies.

ACKNOWLEDGMENTS

We would like to thank Kelly Smith for her artwork and Jessica Madden for editing the manuscript.

REFERENCES

Andrews, J. C. (2011). Vulvodynia interventions–systematic review and evidence grading. *Obstet. Gynecol. Surv.* 66, 299–315. doi: 10.1097/OGX.0b013e3182277fb7

Armstrong, D. S., Arvieux, J., Asaturyan, R., Averett, T., Bailey, S. L., Batigne, G., et al. (2005). Strange-quark contributions to parity-violating asymmetries in the forward g0 electron-proton scattering experiment. *Phys. Rev. Lett.* 95, 092001. doi: 10.1103/PhysRevLett.95.092001

Bachmann, G. A., Rosen, R., Pinn, V. W., Utian, W. H., Ayers, C., Basson, R. (2006). Vulvodynia: a state-of-the-art consensus on definitions, diagnosis and management. *J. Reprod. Med.* 51, 447–456.

Baloglu, E., Senyigit, Z. A., Karavana, S. Y., and Bernkop-Schnürch, A. (2009). Strategies to prolong the intravaginal residence time of drug delivery systems. *J. Pharm. Pharmaceut. Sci.* 12, 312–336.

Balthazart, J., and Ball, G. F. (2006). Is brain estradiol a hormone or a neurotransmitter? *Trends Neurosci.* 29, 241–249. doi: 10.1016/j.tins.2006.03.004

Baskin, L. S., and Tanagho, E. A. (1992). Pelvic pain without pelvic organs. *J. Urol.* 147, 683–686.

Bassi, P., and Kaur, G. (2012). Innovations in bioadhesive vaginal drug delivery system. *Expert. Opin. Ther. Pat.* 22, 1019–1032. doi: 10.1517/13543776.2012.714369

Berkley, K. J., Hubscher, C. H., and Wall, P. D. (1993a). Neuronal responses to stimulation of the cervix, uterus, colon, and skin in the rat spinal cord. *J. Neurophysiol.* 69, 545–556.

Berkley, K. J., Robbins, A., and Sato, Y. (1993b). Functional differences between afferent fibers in the hypogastric and pelvic nerves innervating female reproductive organs in the rat. *J. Neurophysiol.* 69, 533–544.

Blackshaw, L. A., Brierley, S. M., and Hughes, P. A. (2010). TRP channels: new targets for visceral pain. *Gut* 59, 126–135. doi: 10.1136/gut.2009.179523

Blackshaw, L. A., Brookes, S. J., Grundy, D., and Schemann, M. (2007). Sensory transmission in the gastrointestinal tract. *Neurogastroenterol. Motil.* 19, 1–19. doi: 10.1111/j.1365-2982.2006.00871.x

Blackshaw, L. A., Page, A. J., and Young, R. L. (2011). Metabotropic glutamate receptors as novel therapeutic targets on visceral sensory pathways. *Front. Neurosci.* 5:40. doi: 10.3389/fnins.2011.00040

Bleehen, T., and Keele, C. A. (1977). Observations on the algogenic actions of adenosine compounds on the human blister base preparation. *Pain* 3, 367–377. doi: 10.1016/0304-3959(77)90066-5

Boettger, M. K., Uceyler, N., Zelenka, M., Schmitt, A., Reif, A., Chen, Y., et al. (2007). Differences in inflammatory pain in nNOS-, iNOS- and eNOS-deficient mice. *Eur. J. Pain* 11, 810–818. doi: 10.1016/j.ejpain.2006.12.008

Bokor, A., Kyama, C. M., Vercruysse, L., Fassbender, A., Gevaert, O., Vodolazkaia, A., et al. (2009). Density of small diameter sensory nerve fibres in endometrium: a semi-invasive diagnostic test for minimal to mild endometriosis. *Hum. Reprod.* 24, 3025–3032. doi: 10.1093/humrep/dep283

Boucher, T. J., Okuse, K., Bennett, D. L., Munson, J. B., Wood, J. N., and McMahon, S. B. (2000). Potent analgesic effects of GDNF in neuropathic pain states. *Science* 290, 124–127. doi: 10.1126/science.290.5489.124

Brierley, S. M., Jones, R. C. III, Gebhart, G. F., and Blackshaw, L. A. (2004). Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* 127, 166–178. doi: 10.1053/j.gastro.2004.04.008

Cervero, F., and Laird, J. M. (1999). Visceral pain. *Lancet* 353, 2145–2148. doi: 10.1016/S0140-6736(99)01306-9

Cervero, F., and Laird, J. M. (2004). Understanding the signaling and transmission of visceral nociceptive events. *J. Neurobiol.* 61, 45–54. doi: 10.1002/neu.20084

Chalar, C., Richeri, A., Viettro, L., Chavez-Genaro, R., Bianchimano, P., Marmol, N. M., et al. (2003). Plasticity in developing rat uterine sensory nerves: the role of NGF and TrkA. *Cell Tissue Res.* 314, 191–205. doi: 10.1007/s00441-003-0799-9

Christianson, J. A., Liang, R., Ustinova, E. E., Davis, B. M., Fraser, M. O., and Pezzone, M. A. (2007). Convergence of bladder and colon sensory innervation occurs at the primary afferent level. *Pain* 128, 235–243. doi: 10.1016/j.pain.2006.09.023

Collins, J. J., Usip, S., McCarson, K. E., and Papka, R. E. (2002). Sensory nerves and neuropeptides in uterine cervical ripening. *Peptides* 23, 167–183. doi: 10.1016/S0196-9781(01)00593-9

Cook, S. P., Vulchanova, L., Hargreaves, K. M., Elde, R., and McCleskey, E. W. (1997). Distinct ATP receptors on pain-sensing and stretch-sensing neurons. *Nature* 387, 505–508. doi: 10.1038/387505a0

Cox, K. J., and Neville, C. E. (2012). Assessment and management options for women with vulvodynia. *J. Midwifery Womens Health* 57, 231–240. doi: 10.1111/j.1542-2011.2012.00162.x

das Neves, J., Amiji, M., and Sarmento, B. (2011). Mucoadhesive nanosystems for vaginal microbicide development: friend or foe? *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* 3, 389–399. doi: 10.1002/wnan.144

Das, S. K., Paria, B. C., Chakraborty, I., and Dey, S. K. (1995). Cannabinoid ligand-receptor signaling in the mouse uterus. *Proc. Natl. Acad. Sci. U.S.A.* 92, 4332–4336. doi: 10.1073/pnas.92.10.4332

Davis, S. R., Dinatale, I., Rivera-Woll, L., and Davison, S. (2005). Postmenopausal hormone therapy: from monkey glands to transdermal patches. *J. Endocrinol.* 185, 207–222. doi: 10.1677/joe.1.05847

Dennedy, M. C., Friel, A. M., Houlihan, D. D., Broderick, V. M., Smith, T., and Morrison, J. J. (2004). Cannabinoids and the human uterus during pregnancy. *Am. J. Obstet. Gynecol.* 190, 2–9; discussion 3A.

Donders, G. G., and Bellen, G. (2012). Cream with cutaneous fibroblast lysate for the treatment of provoked vestibulodynia: a double-blind randomized placebo-controlled crossover study. *J. Low. Genit. Tract Dis.* 16, 427–436. doi: 10.1097/LGT.0b013e31825a2274

Fanning, R. A., McMorrow, J. P., Campion, D. P., Carey, M. F., and O'Connor, J. J. (2013). Opioid mediated activity and expression of mu and delta opioid receptors in isolated human term non-labouring myometrium. *Eur. J. Pharmacol.* 698, 170–177. doi: 10.1016/j.ejphar.2012.09.045

Fillingim, R. B., and Edwards, R. R. (2001). The association of hormone replacement therapy with experimental pain responses in postmenopausal women. *Pain* 92, 229–234. doi: 10.1016/S0304-3959(01)00256-1

Forsberg, J. G. (1995). A morphologist's approach to the vagina–age-related changes and estrogen sensitivity. *Maturitas* 22(Suppl.), S7–S15. doi: 10.1016/0378-5122(95)00957-4

Fried, G., Meister, B., and Radestad, A. (1990). Peptide-containing nerves in the human pregnant uterine cervix: an immunohistochemical study exploring the effect of RU 486 (mifepristone). *Hum. Reprod.* 5, 870–876.

Fugl-Meyer, K. S., Bohm-Starke, N., Damsted Petersen, C., Fugl-Meyer, A., Parish, S., and Giraldi, A. (2012). Standard operating procedures for female genital sexual pain. *J. Sex. Med.* 10, 83–93. doi: 10.1111/j.1743-6109.2012.02867.x

Ghosh, C., Storey-Workley, M., Usip, S., Hafemeister, J., Miller, K. E., and Papka, R. E. (2007). Glutamate and metabotropic glutamate receptors associated with innervation of the uterine cervix during pregnancy: receptor antagonism inhibits c-Fos expression in rat lumbosacral spinal cord at parturition. *J. Neurosci. Res.* 85, 1318–1335. doi: 10.1002/jnr.21225

Giamberardino, M. A. (2008). Women and visceral pain: are the reproductive organs the main protagonists? Mini-review at the occasion of the "European Week Against Pain in Women 2007". *Eur. J. Pain* 12, 257–260. doi: 10.1016/j.ejpain.2007.11.007

Goetsch, M. F. (2012). Unprovoked vestibular burning in late estrogen-deprived menopause: a case series. *J. Low. Genit. Tract Dis.* 16, 442–446. doi: 10.1097/LGT.0b013e31825c2d28

Goldstein, A. T., and Burrows, L. (2008). Vulvodynia. *J. Sex. Med.* 5, 5–14; quiz 15. doi: 10.1111/j.1743-6109.2007.00679.x

Harlow, B. L., and Stewart, E. G. (2003). A population-based assessment of chronic unexplained vulvar pain: have we underestimated the prevalence of vulvodynia? *J. Am. Med. Womens Assoc.* 58, 82–88.

Head, H. (1893). On disturbances of sensation with especial reference to the pain of visceral disease. *Brain* 16, 1–133. doi: 10.1093/brain/16.1-2.1

Hillen, T. I., Grbavac, S. L., Johnston, P. J., Straton, J. A., and Keogh, J. M. (1999). Primary dysmenorrhea in young Western Australian women: prevalence, impact, and knowledge of treatment. *J. Adolesc. Health* 25, 40–45. doi: 10.1016/S1054-139X(98)00147-5

Hotta, H., Uchida, S., Shimura, M., and Suzuki, H. (1999). Uterine contractility and blood flow are reflexively regulated by cutaneous afferent stimulation in anesthetized rats. *J. Auton. Nerv. Syst.* 75, 23–31. doi: 10.1016/S0165-1838(98)00170-2

Hussain, A., and Ahsan, F. (2005). The vagina as a route for systemic drug delivery. *J. Control. Release* 103, 301–313. doi: 10.1016/j.jconrel.2004.11.034

International Association for the Study of Pain. (2011). Available at: http://www.iasp-pain.org/AM/Template.cfm?Section=Pain_Definitions

Janig, W., and Morrison, J. F. (1986). Functional properties of spinal visceral afferents supplying abdominal and pelvic organs, with special emphasis on visceral nociception. *Prog. Brain Res.* 67, 87–114. doi: 10.1016/S0079-6123(08)62758-2

Jobling, P., Gibbins, I. L., and Morris, J. L. (2003). Functional organization of vasodilator neurons in pelvic ganglia of female guinea pigs: comparison with uterine motor neurons. *J. Comp. Neurol.* 459, 223–241. doi: 10.1002/cne.10584

Jobling, P., Graham, B. A., Brichta, A. M., and Callister, R. J. (2010). Cervix stimulation evokes predominantly subthreshold synaptic responses in mouse thoracolumbar and lumbosacral superficial dorsal horn neurons. *J. Sex. Med.* 7, 2068–2076. doi: 10.1111/j.1743-6109.2010.01768.x

Jones, R. C. III, Xu, L., and Gebhart, G. F. (2005). The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J. Neurosci.* 25, 10981–10989. doi: 10.1523/JNEUROSCI.0703-05.2005

Jones, T. L., and Sorkin, L. S. (2003). Basic neurochemistry of central sensitization. *Semin. Pain Med.* 1, 184–194. doi: 10.1016/S1537-5897(03)00043-0

Kao, A., Binik, Y. M., Kapuscinski, A., and Khalife, S. (2008). Dyspareunia in postmenopausal women: a critical review. *Pain Res. Manag.* 13, 243–254.

Keilhoff, G., Schroder, H., Peters, B., and Becker, A. (2013). Time-course of neuropathic pain in mice deficient in neuronal or inducible nitric oxide synthase. *Neurosci. Res.* 77, 215–221. doi: 10.1016/j.neures.2013.08.008

Klinger, M. B., and Vizzard, M. A. (2008). Role of p75NTR in female rat urinary bladder with cyclophosphamide-induced cystitis. *Am. J. Physiol. Renal Physiol.* 295, F1778–F1789. doi: 10.1152/ajprenal.90501.2008

Klumpp, D. J., and Rudick, C. N. (2008). Summation model of pelvic pain in interstitial cystitis. *Nat. Clin. Pract.* 5, 494–500. doi: 10.1038/ncpuro1203

Kohle, S., and Deb, S. (2011). Dysmenorrhoea. *Obstet. Gynaecol. Reprod. Med.* 21, 311–316. doi: 10.1016/j.ogrm.2011.09.006

Koltzenburg, M., Bennett, D. L., Shelton, D. L., and McMahon, S. B. (1999). Neutralization of endogenous NGF prevents the sensitization of nociceptors supplying inflamed skin. *Eur. J. Neurosci.* 11, 1698–1704. doi: 10.1046/j.1460-9568.1999.00590.x

Kramer, M. G., and Reiter, R. C. (1997). Hysterectomy: indications, alternatives and predictors. *Am. Fam. Physician.* 55, 827–834.

Lakomy, M., Happola, O., Majewski, M., and Kaleczyc, J., (1994). Immunohistochemical localization of neuropeptides in nerve fibers of the porcine vagina and uterine cervix. *Folia Histochem. Cytobiol.* 32, 167–175.

Lara, L. A., Useche, B., Ferriani, R. A., Reis, R. M., de Sa, M. F., de Freitas, M. M. (2009). The effects of hypoestrogenism on the vaginal wall: interference with the normal sexual response. *J. Sex. Med.* 6, 30–39. doi: 10.1111/j.1743-6109.2008.01052.x

Liao, Z., and Smith, P. G. (2011). Adaptive plasticity of vaginal innervation in term pregnant rats. *Reprod. Sci.* 18, 1237–1245. doi: 10.1177/1933719111410706

Lobos, E., Gebhardt, C., Kluge, A., and Spanel-Borowski, K. (2005). Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. *Endocrinology* 146, 1922–1929. doi: 10.1210/en.2004-0925

Lu, V. B., Biggs, J. E., Stebbing, M. J., Balasubramanyan, S., Todd, K. G., Lai, A. Y. et al. (2009). Brain-derived neurotrophic factor drives the changes in excitatory synaptic transmission in the rat superficial dorsal horn that follow sciatic nerve injury. *J. Physiol.* 587, 1013–1032. doi: 10.1113/jphysiol.2008.166306

Mac Bride, M. B., Rhodes, D. J., and Shuster, L. T. (2010). Vulvovaginal atrophy. *Mayo Clin. Proc.* 85, 87–94. doi: 10.4065/mcp.2009.0413

Malin, S. A., and Davis, B. M. (2008). Postnatal roles of glial cell line-derived neurotrophic factor family members in nociceptors plasticity. *Sheng Li Xue Bao* 60, 571–578.

Malin, S. A., Molliver, D. C., Koerber, H. R., Cornuet, P., Frye, R., Albers, K. M., et al. (2006). Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. *J. Neurosci.* 26, 8588–8599. doi: 10.1523/JNEUROSCI.1726-06.2006

Malvasi, A., Tinelli, A., Cavallotti, C., Bettocchi, S., Di Renzo, G. C., and Stark, M. (2010). Substance P (SP) and vasoactive intestinal polypeptide (VIP) in the lower uterine segment in first and repeated cesarean sections. *Peptides* 31, 2052–2059. doi: 10.1016/j.peptides.2010.07.025

Martin, V. T. (2009). Ovarian hormones and pain response: a review of clinical and basic science studies. *Gend. Med.* 6(Suppl.2), 168–192. doi: 10.1016/j.genm.2009.03.006

Martin-Alguacil, N., Schober, J. M., Sengelaub, D. R., Pfaff, D. W., and Shelley, D. N. (2008). Clitoral sexual arousal: neuronal tracing study from the clitoris through the spinal tracts. *J. Urol.* 180, 1241–1248. doi: 10.1016/j.juro.2008.06.009

Mathias, S. D., Kuppermann, M., Liberman, R. F., Lipschutz, R. C., and Steege, J. F. (1996). Chronic pelvic pain: prevalence, health-related quality of life, and economic correlates. *Obstet. Gynecol.* 87, 321–327. doi: 10.1016/0029-7844(95)00458-0

McMahon, S. B. (1997). Are there fundamental differences in the peripheral mechanisms of visceral and somatic pain? *Behav. Brain Sci.* 20, 381–391; discussion 435–513. doi: 10.1017/S0140525X97231481

Merabet, J., Thompson, D., and Saul Levinson, R. (2005). Advancing vaginal drug delivery. *Expert Opin. Drug Deliv.* 2, 769–777. doi: 10.1517/17425247.2.4.769

Merskey, H., and Bogduk, N. (1994). *Classification of Chronic Pain. Descriptions of Chronic Pain Syndromes and Definitions of Pain Terms.* Seattle, WA: IASP Press.

Meseguer, V., Karashima, Y., Talavera, K., D'Hoedt, D., Donovan-Rodriguez, T., Viana, F., et al. (2008). Transient receptor potential channels in sensory neurons are targets of the antimycotic agent clotrimazole. *J. Neurosci.* 28, 576–586. doi: 10.1523/JNEUROSCI.4772-07.2008

Millan, M. J. (1999). The induction of pain: an integrative review. *Prog. Neurobiol.* 57, 1–164. doi: 10.1016/S0301-0082(98)00048-3

Mladenova, G., Annedi, S. C., Ramnauth, J., Maddaford, S. P., Rakhit, S., Andrews, J. S., et al. (2012). First-in-class, dual-action, 3,5-disubstituted indole derivatives having human nitric oxide synthase (nNOS) and norepinephrine reuptake inhibitory (NERI) activity for the treatment of neuropathic pain. *J. Med. Chem.* 55, 3488–3501. doi: 10.1021/jm300138g

Morris, J. L., Gibbins, I. L., and Jobling, P. (2005). Post-stimulus potentiation of transmission in pelvic ganglia enhances sympathetic dilatation of guinea-pig

uterine artery in vitro. *J. Physiol.* 566, 189–203. doi: 10.1113/jphysiol.2005.083493

Moszkowicz, D., Alsaid, B., Bessede, T., Zaitouna, M., Penna, C., Benoît, G. (2011). Neural supply to the clitoris: immunohistochemical study with three-dimensional reconstruction of cavernous nerve, spongious nerve, and dorsal clitoris nerve in human fetus. *J. Sex. Med.* 8, 1112–1122. doi: 10.1111/j.1743-6109.2010.02182.x

North, R. A., and Jarvis, M. F. (2013). P2X receptors as drug targets. *Mol. Pharmacol.* 83, 759–769. doi: 10.1124/mol.112.083758

Nunns, D., Mandal, D., Byrne, M., McLelland, J., Rani, R., Cullimore, J., et al. (2010). Guidelines for the management of vulvodynia. *Br. J. Dermatol.* 162, 1180–1185. doi: 10.1111/j.1365-2133.2010.09684.x

Nyirjesy, P., Lev-Sagie, A., Mathew, L., and Culhane, J. F. (2009). Topical amitriptyline-baclofen cream for the treatment of provoked vestibulodynia. *J. Low. Genit. Tract Dis.* 13, 230–236. doi: 10.1097/LGT.0b013e31819663ee

Page, A. J., Brierley, S. M., Martin, C. M., Hughes, P. A., and Blackshaw, L. A. (2007). Acid sensing ion channels 2 and 3 are required for inhibition of visceral nociceptors by benzamil. *Pain* 133, 150–160. doi: 10.1016/j.pain.2007.03.019

Page, A. J., Martin, C. M., and Blackshaw, L. A. (2002). Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. *J. Neurophysiol.* 87, 2095–2103.

Page, A. J., O'Donnell, T. A., and Blackshaw, L. A. (2008). Opioid modulation of ferret vagal afferent mechanosensitivity. *Am. J. Physiol. Gastrointest. Liver Physiol.* 294, G963–G970. doi: 10.1152/ajpgi.00562.2007

Page, A. J., Young, R. L., Martin, C. M., Umaerus, M., O'Donnell, T. A., et al. (2005). Metabotropic glutamate receptors inhibit mechanosensitivity in vagal sensory neurons. *Gastroenterology* 128, 402–410. doi: 10.1053/j.gastro.2004.11.062

Papka, R. E., and Mowa, C. N. (2003). Estrogen receptors in the spinal cord, sensory ganglia, and pelvic autonomic ganglia. *Int. Rev. Cytol.* 231, 91–127. doi: 10.1016/S0074-7696(03)31003-4

Papka, R. E., and Traurig, H. H. (1993). "Autonomic efferent and visceral sensory innervation of the female reproductive system: special reference to neurochemical markers in nerves and ganglionic connections," in *Nervous Control of the Urogenital System,* ed. C. Maggi (Chur: Harwood, Luxembourg), 423–466.

Papka, R. E., Collins, J., Copelin, T., and Wilson, K. (1999). Calretinin-immunoreactive nerves in the uterus, pelvic autonomic ganglia, lumbosacral dorsal root ganglia and lumbosacral spinal cord. *Cell Tissue Res.* 298, 63–74. doi: 10.1007/s004419900071

Papka, R. E., Cotton, J. P., and Traurig, H. H. (1985). Comparative distribution of neuropeptide tyrosine-, vasoactive intestinal polypeptide-, substance P-immunoreactive, acetylcholinesterase-positive and noradrenergic nerves in the reproductive tract of the female rat. *Cell Tissue Res.* 242, 475–490. doi: 10.1007/BF00225412

Papka, R. E., Hafemeister, J., and Storey-Workley, M. (2005). P2X receptors in the rat uterine cervix, lumbosacral dorsal root ganglia, and spinal cord during pregnancy. *Cell Tissue Res.* 321, 35–44. doi: 10.1007/s00441-005-1114-8

Papka, R. E., McNeill, D. L., Thompson, D., and Schmidt, H. H. (1995). Nitric oxide nerves in the uterus are parasympathetic, sensory, and contain neuropeptides. *Cell Tissue Res.* 279, 339–349. doi: 10.1007/BF00318490

Papka, R. E., Storey-Workley, M., Shughrue, P. J., Merchenthaler, I., Collins, J. J., Usip, S., et al. (2001). Estrogen receptor-alpha and beta- immunoreactivity and mRNA in neurons of sensory and autonomic ganglia and spinal cord. *Cell Tissue Res.* 304, 193–214. doi: 10.1007/s004410100363

Paterson, L. Q., Davis, S. N., Khalife, S., Amsel, R., and Binik, Y. M. (2009). Persistent genital and pelvic pain after childbirth. *J. Sex. Med.* 6, 215–221. doi: 10.1111/j.1743-6109.2008.01063.x

Pavelić, Z., Skalko-Basnet, N., Schubert, R., and Jalsenjak, I. (2004). Liposomal gels for vaginal drug delivery. *Methods Enzymol.* 387, 287–299. doi: 10.1016/S0076-6879(04)87018-6

Petersen, C. D., Lundvall, L., Kristensen, E., and Giraldi, A. (2008). Vulvodynia. Definition, diagnosis and treatment. *Acta obstet. Gynecol. Scand.* 87, 893–901. doi: 10.1080/00016340802323321

Pitts, M. K., Ferris, J. A., Smith, A. M., Shelley, J. M., and Richters, J. (2008). Prevalence and correlates of three types of pelvic pain in a nationally representative sample of Australian women. *Med. J. Aust.* 189, 138–143.

Roques, B. P., Fournie-Zaluski, M. C., and Wurm, M. (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nat. Rev. Drug Discov.* 11, 292–310. doi: 10.1038/nrd3673

Ryder, R. M. (1996). Chronic pelvic pain. *Am. Fam. Physician.* 54, 2225–2232, 2237.

Samsioe, G. (2007). "Urogenital symptoms around the menopause and beyond," in *Treatment of the Postmenopausal Woman*, ed. R. A. Lobo (Burlington: Elsevier).

Schmid, P. C., Paria, B. C., Krebsbach, R. J., Schmid, H. H., and Dey, S. K. (1997). Changes in anandamide levels in mouse uterus are associated with uterine receptivity for embryo implantation. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4188–4192.

Schnegelsberg, B., Sun, T. T., Cain, G., Bhattacharya, A., Nunn, P. A., Ford, A. P., et al. (2010). Overexpression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 298, R534–R547. doi: 10.1152/ajpregu.00367.2009

Shew, R. L., Papka, R. E., and McNeill, D. L. (1991). Substance P and calcitonin gene-related peptide immunoreactivity in nerves of the rat uterus: localization, colocalization and effects on uterine contractility. *Peptides* 12, 593–600. doi: 10.1016/0196-9781(91)90107-Z

Skobowiat, C., Gonkowski, S., Janiuk, I., Calka, J., and Majewski, M., (2009). The relationship and co-localization of vasoactive intestinal peptide (VIP)- and Leu5-enkephalin (LENK)-immunoreactivity in the female genital tract of the pig. *Pol. J. Vet. Sci.* 12, 347–353.

Song, X., Chen, B. N., Zagorodnyuk, V. P., Lynn, P. A., Blackshaw, L. A., Grundy, D. et al. (2009). Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract. *Gastroenterology* 137, 274–284, 284.e1. doi: 10.1053/j.gastro.2009.02.061

Srikrishna, S., and Cardozo, L. (2013). The vagina as a route for drug delivery: a review. *Int. Urogynecol. J.* 24, 537–543. doi: 10.1007/s00192-012-2009-3

Stones, R. W., Selfe, S. A., Fransman, S., and Horn, S. A. (2000). Psychosocial and economic impact of chronic pelvic pain. *Baillieres Best Pract. Res. Clin. Obstet. Gynaecol.* 14, 415–431. doi: 10.1053/beog.1999.0084

Su, X., Sengupta, J. N., and Gebhart, G. F. (1997). Effects of opioids on mechanosensitive pelvic nerve afferent fibers innervating the urinary bladder of the rat. *J. Neurophysiol.* 77, 1566–1580.

Sugiura, Y., Terui, N., and Hosoya, Y. (1989). Difference in distribution of central terminals between visceral and somatic unmyelinated (C) primary afferent fibers. *J. Neurophysiol.* 62, 834–840.

Sugiura, Y., Terui, N., Hosoya, Y., Tonosaki, Y., Nishiyama, K., and Honda, T. (1993). Quantitative analysis of central terminal projections of visceral and somatic unmyelinated (C) primary afferent fibers in the guinea pig. *J. Comp. Neurol.* 332, 315–325. doi: 10.1002/cne.903320305

Takanami, K., Sakamoto, H., Matsuda, K.-I., Hosokawa, K., Nishi, M., Prossnitz, E. R., et al. (2010). Expression of G protein-coupled receptor 30 in the spinal somatosensory system. *Brain Res.* 1310, 17–28. doi: 10.1016/j.brainres.2009.11.004

Thurman, A. R., Clark, M. R., Hurlburt, J. A., and Doncel, G. F. (2013). Intravaginal rings as delivery systems for microbicides and multipurpose prevention technologies. *Int. J. Womens Health* 5, 695–708. doi: 10.2147/IJWH.S34030

Tingåker, B. K., Ekman-Ordeberg, G., Facer, P., Irestedt, L., and Anand, P. (2008). Influence of pregnancy and labor on the occurrence of nerve fibers expressing the capsaicin receptor TRPV1 in human corpus and cervix uteri. *Reprod. Biol. Endocrinol.* 6:8. doi: 10.1186/1477-7827-6-8

Vanderhorst, V. G. J. M., Terasawa, E., and Ralston, H. J. (2009). Estrogen receptor-alpha immunoreactive neurons in the brainstem and spinal cord of the female rhesus monkey: species-specific characteristics. *Neuroscience* 158, 798–810. doi: 10.1016/j.neuroscience.2008.10.017

Vilimas, P. I., Yuan, S. Y., Haberberger, R. V., and Gibbins, I. L. (2011). Sensory innervation of the external genital tract of female guinea pigs and mice. *J. Sex. Med.* 8, 1985–1995. doi: 10.1111/j.1743-6109.2011.02258.x

Walczak, J. S., Price, T. J., and Cervero, F. (2009). Cannabinoid CB1 receptors are expressed in the mouse urinary bladder and their activation modulates afferent bladder activity. *Neuroscience* 159, 1154–1163. doi: 10.1016/j.neuroscience.2009.01.050

Walsh, K. E., Berman, J. R., Berman, L. A., and Vierregger, K. (2002). Safety and efficacy of topical nitroglycerin for treatment of vulvar pain in women with vulvodynia: a pilot study. *J. Gend. Specif. Med.* 5, 21–27.

Wesselmann, U. (2001). Neurogenic inflammation and chronic pelvic pain. *World J. Urol.* 19, 180–185. doi: 10.1007/s003450100201

Wesselmann, U., and Lai, J. (1997). Mechanisms of referred visceral pain: uterine inflammation in the adult virgin rat results in neurogenic plasma extravasation in the skin. *Pain* 73, 309–317. doi: 10.1016/S0304-3959(97)00112-7

Wesselmann, U., Czakanski, P. P., and Sanders, C. (2000). Altered CNS processing of nociceptive messages from the vagina in rats, that have recovered from uterine inflammation. *Prog. Pain Res. Manag.* 16, 581–588.

Westlund, K. N. (2000). Visceral nociception. *Curr. Rev. Pain* 4, 478–487. doi: 10.1007/s11916-000-0072-9

Whorwell, P. J., Lupton, E. W., Erduran, D., and Wilson, K. (1986). Bladder smooth muscle dysfunction in patients with irritable bowel syndrome. *Gut* 27, 1014–1017. doi: 10.1136/gut.27.9.1014

Widenfalk, J., Parvinen, M., Lindqvist, E., and Olson, L. (2000). Neurturin, RET, GFRalpha-1 and GFRalpha-2, but not GFRalpha-3, mRNA are expressed in mice gonads. *Cell Tissue Res.* 299, 409–415.

Winnard, K. P., Dmitrieva, N., and Berkley, K. J. (2006). Cross-organ interactions between reproductive, gastrointestinal, and urinary tracts: modulation by estrous stage and involvement of the hypogastric nerve. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 291, R1592–R1601. doi: 10.1152/ajpregu.00455.2006

Woolf, C. J., and Ma, Q. (2007). Nociceptors–noxious stimulus detectors. *Neuron* 55, 353–364. doi: 10.1016/j.neuron.2007.07.016

Wyndaele, M., De Wachter, S., De Man, J., Minagawa, T., Wyndaele, J. J., Pelckmans, P. A. et al. (2013). Mechanisms of pelvic organ crosstalk: 1. Peripheral modulation of bladder inhibition by colorectal distention in rats. *J. Urol.* 190, 765–771. doi: 10.1016/j.juro.2013.03.045

Xu, S., Cheng, Y., Keast, J. R., and Osborne, P. B. (2008). 17beta-estradiol activates estrogen receptor beta-signalling and inhibits transient receptor potential vanilloid receptor 1 activation by capsaicin in adult rat nociceptor neurons. *Endocrinology* 149, 5540–5548. doi: 10.1210/en.2008-0278

Yan, T., Liu, B., Du, D., Eisenach, J. C., and Tong, C. (2007). Estrogen amplifies pain responses to uterine cervical distension in rats by altering transient receptor potential-1 function. *Anesth. Analg.* 104, 1246–1250, tables of contents. doi: 10.1213/01.ane.0000263270.39480.a2

Yuce, B., Kemmer, M., Qian, G., Muller, M., Sibaev, A., Li, Y., et al. (2010). Cannabinoid 1 receptors modulate intestinal sensory and motor function in rat. *Neurogastroenterol. Motil.* 22, 672–e205. doi: 10.1111/j.1365-2982.2010.01473.x

Zagorodnyuk, V. P., Gibbins, I. L., Costa, M., Brookes, S. J., and Gregory, S. J. (2007). Properties of the major classes of mechanoreceptors in the guinea pig bladder. *J. Physiol.* 585, 147–163. doi: 10.1113/jphysiol.2007.140244

Zhu, Y., and Pintar, J. E. (1998). Expression of opioid receptors and ligands in pregnant mouse uterus and placenta. *Biol. Reprod.* 59, 925–932. doi: 10.1095/biolreprod59.4.925

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 December 2013; paper pending published: 16 January 2014; accepted: 27 January 2014; published online: 13 February 2014.

*Citation: Jobling P, O'Hara K and Hua S (2014) Female reproductive tract pain: targets, challenges, and outcomes. Front. Pharmacol. **5**:17. doi: 10.3389/fphar.2014.00017*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 03 March 2014
doi: 10.3389/fphar.2014.00028

Targeting the endogenous cannabinoid system to treat neuropathic pain

Benjamin K. Lau *† and Christopher W. Vaughan †

Pain Management Research Institute, Kolling Institute of Medical Research, Northern Clinical School, University of Sydney, Sydney, NSW, Australia
**Correspondence: benjamin.lau@sydney.edu.au*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Matthias Klugmann, University of New South Wales, Australia
Lih-Chu Chiou, National Taiwan University, Taiwan
† These authors contributed equally to this work.

Keywords: neuropathic pain, endocannabinoid, 2-arachidnoylglycerol, anandamide, FAAH, MAGL

Chronic neuropathic pain is a debilitating condition that remains poorly treated by current medications. Preclinical studies have indicated that cannabinoid receptor agonists have analgesic efficacy in neuropathic pain models, but this is accompanied by undesirable side effects. In recent years, novel strategies targeting the endogenous cannabinoid system have emerged, which are being mooted as safer alternatives. A recent clinical trial, however, has demonstrated that a new endocannabinoid modulator is ineffective against osteoarthritic pain, despite exhibiting efficacy during the preclinical stage. Further basic and clinical work is needed to resolve this disparity.

CANNABINOIDS AND CHRONIC PAIN

Chronic pain is a prevalent and costly health care problem (Torrance et al., 2006). A particularly persistent, severe and debilitating form of chronic pain is neuropathic pain. This syndrome manifests following damage or dysfunction of the peripheral nerves, spinal cord, or brain; or can be caused by stroke, multiple sclerosis or diabetes. Unfortunately, the currently recommended pharmacological treatments for neuropathic pain display poor efficacy and illicit undesirable side effects (Dworkin et al., 2010).

There is growing clinical evidence indicating that the cannabis constituent, Δ^9-tetrahydrocannabinol (THC), and synthetic cannabinoid agonists have efficacy in chronic pain states (Lynch and Campbell, 2011). These human studies are based upon substantial preclinical evidence. A wide range of animal models of neuropathic pain have demonstrated that cannabinoid agonists reverse the common symptoms of neuropathic pain, including allodynia (to cool and innocuous mechanical stimuli) and hyperalgesia (to noxious thermal and mechanical stimuli) (Fox et al., 2001; Scott et al., 2004). Unfortunately, this therapeutic intervention is also associated with a number of adverse effects, including sedation and motor/cognitive impairment. Having said this, the therapeutic window between the desired and adverse effects of cannabinoids has not been systematically examined. Thus, it remains to be determined whether cannabinoids can produce pain relief at doses below the side effect threshold.

THE ENDOGENOUS CANNABINOID SYSTEM—A TARGET FOR PAIN RELIEF

Since exogenous cannabinoid agonists act globally throughout the central nervous system to produce their effects, isolation of the desired therapeutic action from the unwanted side effects has remained a difficult challenge. To overcome this problem, alternative approaches at targeting cannabinoid signaling have been explored (Petrosino and Di Marzo, 2010; Piscitelli and Di Marzo, 2012). Unlike exogenous cannabinoids, endogenous ligands of the cannabinoid system are synthesized in an "on demand" fashion. This suggests specific, localized release of these transmitters only in regions where their actions are pertinent. Thus, targeting endocannabinoids may provide a more effective strategy in relieving pain devoid of side effects.

Endocannabinoids are present in multiple pain-modulating regions throughout the CNS, including the periaqueductal gray (PAG), rostral ventral medulla (RVM) and spinal cord dorsal horn, where their levels are enhanced by acute nociceptive stimuli and stress (e.g., Walker et al., 1999; Hohmann et al., 2005). Interestingly, endocannabinoid levels within these regions are also enhanced in chronic pain models (Jhaveri et al., 2007; Petrosino et al., 2007; Guindon et al., 2013). Exogenous administration of AEA or 2-AG produces THC-like effects, but each elicits a distinct subset of the total effects observed with exogenous cannabinoid administration. Specifically, in animal models, AEA administration has been reported to produce antinociception alone (Cravatt et al., 2001), or evoke the full tetrad of cannabinoid agonist effects—antinociception, hypothermia, hypolocomotion and catalepsy (Smith et al., 1994). By contrast, 2-AG administration evokes only a subset of these effects (Lichtman et al., 2002). While major endocannabinoids, such as N-arachidonoyl ethanolamide (anandamide or AEA) and 2-arachidonoyl glycerol (2-AG), act via cannabinoid CB1 and CB2 receptors in a manner similar to THC and synthetic cannabinoid agonists, they may also modulate nociception via non-cannabinoid receptor targets. This is particularly the case for anandamide, which has actions on transient receptor potential vanilloid 1 (TRPV1) and peroxisome proliferator-activated receptor-α (PPAR-α), both of which are cellular targets implicated in nociception (Jhaveri et al., 2007; Di Marzo and De Petrocellis, 2012).

The actions of endocannabinoids are tightly regulated by enzymatic degradation. In particular, AEA is degraded via fatty acid amide hydrolase (FAAH), and 2-AG via monoacylglycerol lipase (MAGL) (Cravatt et al., 1996; Dinh et al., 2002). In addition, two serine hydrolases, ABHD6 and ABHD12, have recently been implicated in the hydrolysis of 2-AG (Blankman et al., 2007; Savinainen et al., 2012); however, their presence in pain pathways has yet to be confirmed. In recent years, a number of pharmacological tools have been developed which selectively inhibit FAAH and MAGL (Kathuria et al., 2003; Long et al., 2009a; Ahn et al., 2011). Inhibition of these degradative enzymes is thought to specifically enhance endocannabinoids where they are produced on demand, resulting in more localized receptor activation compared to globally acting exogenous agonists. Considerable attention has been focused on developing degradation inhibitors to indirectly target the endocannabinoid system.

NOVEL ENDOCANNABINOID MODULATORS—FAAH AND MAGL INHIBITORS

A number of groups have demonstrated that selective FAAH inhibitors, such as URB597, OL-135, PF-3845, PF- 04457845 and ST-4070 alleviate the mechanical and cold allodynia induced in a range of animal models of chronic pain (Russo et al., 2007; Kinsey et al., 2009, 2010; Ahn et al., 2011; Caprioli et al., 2012; Guindon et al., 2013). Although it has not been examined systematically, this analgesic effect occurs at doses that do not elicit the tetrad of cannabinoid-induced side effects. The evidence in support of FAAH inhibitors, however, is not unanimous. For example, our group originally found that systemic administration of URB597 had no effect on mechanical allodynia in the partial nerve ligation (PNL) model of neuropathic pain (Jayamanne et al., 2006), despite the synthetic analog of THC, HU-210 abolishing allodynia in the same study.

The contrasting findings between these studies might be due to a number of factors. Firstly, the above studies examined a range of different neuropathic pain models. Indeed, we have observed that, unlike the PNL model, URB597 reduces allodynia (albeit at higher doses) in the sciatic nerve chronic constriction injury (CCI) model (unpublished data). Secondly, the wide range of different FAAH inhibitor compounds administered at varying doses may account for the contrasting efficacy on allodynia, particularly since some of compounds in question did not display clear dose dependence in pain assays (e.g., Ahn et al., 2011). Thirdly, depending on the acute or chronic pain assay examined, it is possible that endocannabinoid activation of CB1 and TRPV1 receptors may have opposing effects on nociception, thus confounding the observed results (Maione et al., 2006). Together, these studies suggest that FAAH inhibitors may be efficacious for neuropathic pain, but more systematic studies need to be conducted to confirm their therapeutic potential.

Compared to FAAH inhibitors, there have been relatively fewer studies examining MAGL inhibitors in pain models. This is mainly due to their later identification and development. Nevertheless, systemic administration of the MAGL inhibitors, JZL-184 and KML-29, have been shown to alleviate allodynia in certain neuropathic pain models, without eliciting the full tetrad of cannabinoid-induced effects (Kinsey et al., 2009, 2010, 2013; Schlosburg et al., 2010; Guindon et al., 2013; Ignatowska-Jankowska et al., 2013). Interestingly, while analgesic efficacy is maintained during chronic administration of low doses of JZL184, tolerance has been shown to develop at higher doses (Kinsey et al., 2013). This latter observation is clinically relevant, since conditions like chronic pain often require long-term drug treatment.

THERAPEUTIC POTENTIAL OF ENDOCANNABINOID MODULATORS

In a recent clinical trial, the FAAH inhibitor, PF-04457845 was found ineffective in patients with osteoarthritic pain (Huggins et al., 2012). This was despite a clear abolition of FAAH activity and elevation in plasma AEA levels in almost all subjects tested. This finding directly contrasts those observed by the same group in an animal model of inflammatory and non-inflammatory pain, in which PF-04457845 produced a potent antinociceptive effect (Ahn et al., 2011). While the reason for this contrasting efficacy between humans and animals is not clear, it should be noted that the former study was likely confounded by a number of human factors (Di Marzo, 2012). Furthermore, the study examined only an osteoarthritic model of pain. Thus, the utility of FAAH inhibitors in the treatment of neuropathic pain remains to be explored.

SYNERGISTIC/ANTAGONISTIC INTERACTIONS OF ENDOCANNABINOIDS

In chronic pain states, patients often receive drugs in combination. The aim of this approach is to produce greater or potentially synergistic analgesia at lower drug doses. Indeed, there is some clinical evidence in favour of the combined use of cannabinoid and opioid agonists in chronic pain patients, although synergy has yet to be established (Abrams et al., 2011).

This combinatorial approach might also be applied to the endocannabinoid modulators described above. One promising idea would be to inhibit both FAAH and MAGL (Pertwee, 2013). In this regard, dual inhibitors of both FAAH and MAGL have recently been developed (Long et al., 2009b; Ramesh et al., 2013), although they remain to be systematically tested in chronic pain models. Because FAAH and MAGL act via distinct pathways to degrade AEA and 2-AG, dual inhibition of these degradative enzymes should boost levels of both endocannabinoids. It is unknown whether this will produce an additive or synergistic effect. Therefore, it would be interesting to observe the effect of dual FAAH/MAGL inhibition in neuropathic pain models, particularly in terms of their efficacy and therapeutic window relative to FAAH or MAGL inhibition.

It should be noted that whilst the antinociceptive effect of MAGL inhibitors is specifically abolished by CB1 receptor antagonism or knockout (Kinsey et al., 2009, 2010), the effect by FAAH inhibitors is not only abolished/reduced by CB1 and CB2 receptor blockade (Russo et al., 2007; Kinsey et al., 2009, 2010), but also antagonism of TRPV1 receptors and PPAR-α (Caprioli et al., 2012). Since TRPV1 and PPAR-α are predominantly pro-nociceptive targets, this suggests FAAH inhibition may produce competing, opposing actions on antinociception. Thus, it would be interesting to examine

the effect of a FAAH inhibitor in combination with a TRPV1 antagonist. In this regard, the endocannabinoid-related agent, N-arachidonoyl-serotonin is a dual FAAH/TRPV1 inhibitor found to be highly effective in neuropathic pain models (De Novellis et al., 2011). In addition to pro-nociceptive targets, FAAH inhibition has been demonstrated to produce pro-nociceptive metabolites by diverting AEA breakdown through other metabolic pathways, such as cyclooxygenase-2 (COX-2) (Gatta et al., 2012). Hence, another potential interaction worth examining is dual inhibition of FAAH and COX-2. Such an approach may also lead to enhanced analgesic efficacy (Fowler et al., 2009).

Together, the above studies importantly highlight that while some endocannabinoid pathways act synergistically, others may act antagonistically to reduce antinociception. Therefore, prizing apart these subcellular pathways may provide a means to further improve the safety and efficacy of endocannabinoid modulators.

FUTURE DIRECTIONS

While the lack of efficacy of a FAAH inhibitor in a recent clinical trial is disappointing, there are alternative approaches targeting the endocannabinoid system which may still be of promise. These need to be first addressed in animal studies before proceeding with further clinical trials. In particular, the efficacy of the highly specific, individual MAGL inhibitors and the recently developed, dual FAAH/MAGL inhibitors need to be explored in neuropathic pain models. Furthermore, the use of these degradation inhibitors in combination with agents that act on related targets should be investigated (Di Marzo, 2012). Both drug efficacy and side-effect profiles need to be examined systematically. This may eventually lead to an effective pharmacotherapy to treat the problematic condition of neuropathic pain.

REFERENCES

Abrams, D. I., Couey, P., Shade, S. B., Kelly, M. E., and Benowitz, N. L. (2011). Cannabinoid-opioid interaction in chronic pain. *Clin. Pharmacol. Ther.* 90, 844–851. doi: 10.1038/clpt.2011.188

Ahn, K., Smith, S. E., Liimatta, M. B., Beidler, D., Sadagopan, N., Dudley, D. T., et al. (2011). Mechanistic and pharmacological characterization of PF-04457845: a highly potent and selective fatty acid amide hydrolase inhibitor that reduces inflammatory and noninflammatory pain. *J. Pharmacol. Exp. Ther.* 338, 114–124. doi: 10.1124/jpet.111.180257

Blankman, J. L., Simon, G. M., and Cravatt, B. F. (2007). A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chem. Biol.* 14, 1347–1356. doi: 10.1016/j.chembiol.2007.11.006

Caprioli, A., Coccurello, R., Rapino, C., Di Serio, S., Di Tommaso, M., Vertechy, M., et al. (2012). The novel reversible fatty acid amide hydrolase inhibitor ST4070 increases endocannabinoid brain levels and counteracts neuropathic pain in different animal models. *J. Pharmacol. Exp. Ther.* 342, 188–195. doi: 10.1124/jpet.111.191403

Cravatt, B. F., Demarest, K., Patricelli, M. P., Bracey, M. H., Giang, D. K., Martin, B. R., et al. (2001). Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proc. Natl. Acad. Sci. U.S.A.* 98, 9371–9376. doi: 10.1073/pnas.161191698

Cravatt, B. F., Giang, D. K., Mayfield, S. P., Boger, D. L., Lerner, R. A., and Gilula, N. B. (1996). Molecular characterization of an enzyme that degrades neuromodulatory fatty-acid amides. *Nature* 384, 83–87. doi: 10.1038/384083a0

De Novellis, V., Vita, D., Gatta, L., Luongo, L., Bellini, G., De Chiaro, M., et al. (2011). The blockade of the transient receptor potential vanilloid type 1 and fatty acid amide hydrolase decreases symptoms and central sequelae in the medial prefrontal cortex of neuropathic rats. *Mol. Pain* 7, 7. doi: 10.1186/1744-8069-7-7

Di Marzo, V. (2012). Inhibitors of endocannabinoid breakdown for pain: not so FA(AH)cile, after all. *Pain* 153, 1785–1786. doi: 10.1016/j.pain.2012.06.016

Di Marzo, V., and De Petrocellis, L. (2012). Why do cannabinoid receptors have more than one endogenous ligand? *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367, 3216–3228. doi: 10.1098/rstb.2011.0382

Dinh, T. P., Carpenter, D., Leslie, F. M., Freund, T. F., Katona, I., Sensi, S. L., et al. (2002). Brain monoglyceride lipase participating in endocannabinoid inactivation. *Proc. Natl. Acad. Sci. U.S.A.* 99, 10819–10824. doi: 10.1073/pnas.152334899

Dworkin, R. H., O'Connor, A. B., Audette, J., Baron, R., Gourlay, G. K., Haanpaa, M. L., et al. (2010). Recommendations for the pharmacological management of neuropathic pain: an overview and literature update. *Mayo Clin. Proc.* 85, S3–14. doi: 10.4065/mcp.2009.0649

Fowler, C. J., Naidu, P. S., Lichtman, A., and Onnis, V. (2009). The case for the development of novel analgesic agents targeting both fatty acid amide hydrolase and either cyclooxygenase or TRPV1. *Br. J. Pharmacol.* 156, 412–419. doi: 10.1111/j.1476-5381.2008.00029.x

Fox, A., Kesingland, A., Gentry, C., McNair, K., Patel, S., Urban, L., et al. (2001). The role of central and peripheral Cannabinoid(1) receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain* 92, 91–100. doi: 10.1016/S0304-3959(00)00474-7

Gatta, L., Piscitelli, F., Giordano, C., Boccella, S., Lichtman, A., Maione, S., et al. (2012). Discovery of prostamide F2alpha and its role in inflammatory pain and dorsal horn nociceptive neuron hyperexcitability. *PLoS ONE* 7:e31111. doi: 10.1371/journal.pone.0031111

Guindon, J., Lai, Y., Takacs, S. M., Bradshaw, H. B., and Hohmann, A. G. (2013). Alterations in endocannabinoid tone following chemotherapy-induced peripheral neuropathy: effects of endocannabinoid deactivation inhibitors targeting fatty-acid amide hydrolase and monoacylglycerol lipase in comparison to reference analgesics following cisplatin treatment. *Pharmacol. Res.* 67, 94–109. doi: 10.1016/j.phrs.2012.10.013

Hohmann, A. G., Suplita, R. L., Bolton, N. M., Neely, M. H., Fegley, D., Mangieri, R., et al. (2005). An endocannabinoid mechanism for stress-induced analgesia. *Nature* 435, 1108–1112. doi: 10.1038/nature03658

Huggins, J. P., Smart, T. S., Langman, S., Taylor, L., and Young, T. (2012). An efficient randomised, placebo-controlled clinical trial with the irreversible fatty acid amide hydrolase-1 inhibitor PF-04457845, which modulates endocannabinoids but fails to induce effective analgesia in patients with pain due to osteoarthritis of the knee. *Pain* 153, 1837–1846. doi: 10.1016/j.pain.2012.04.020

Ignatowska-Jankowska, B. M., Ghosh, S., Crowe, M. S., Kinsey, S. G., Niphakis, M. J., Abdullah, R. A., et al. (2013). *In vivo* characterization of the highly selective monoacylglycerol lipase inhibitor KML29: antinociceptive activity without cannabimimetic side effects. *Br. J. Pharmacol.* 345, 492–501. doi: 10.1111/bph.12298. [Epub ahead of print].

Jayamanne, A., Greenwood, R., Mitchell, V. A., Aslan, S., Piomelli, D., and Vaughan, C. W. (2006). Actions of the FAAH inhibitor URB597 in neuropathic and inflammatory chronic pain models. *Br. J. Pharmacol.* 147, 281–288. doi: 10.1038/sj.bjp.0706510

Jhaveri, M. D., Richardson, D., and Chapman, V. (2007). Endocannabinoid metabolism and uptake: novel targets for neuropathic and inflammatory pain. *Br. J. Pharmacol.* 152, 624–632. doi: 10.1038/sj.bjp.0707433

Kathuria, S., Gaetani, S., Fegley, D., Valino, F., Duranti, A., Tontini, A., et al. (2003). Modulation of anxiety through blockade of anandamide hydrolysis. *Nat. Med.* 9, 76–81. doi: 10.1038/nm803

Kinsey, S. G., Long, J. Z., Cravatt, B. F., and Lichtman, A. H. (2010). Fatty acid amide hydrolase and monoacylglycerol lipase inhibitors produce anti-allodynic effects in mice through distinct cannabinoid receptor mechanisms. *J. Pain* 11, 1420–1428. doi: 10.1016/j.jpain.2010.04.001

Kinsey, S. G., Long, J. Z., O'Neal, S. T., Abdullah, R. A., Poklis, J. L., Boger, D. L., et al. (2009). Blockade of endocannabinoid-degrading enzymes attenuates neuropathic pain. *J. Pharmacol. Exp. Ther.* 330, 902–910. doi: 10.1124/jpet.109.155465

Kinsey, S. G., Wise, L. E., Ramesh, D., Abdullah, R., Selley, D. E., Cravatt, B. F., et al. (2013). Repeated low dose administration of the monoacylglycerol lipase inhibitor JZL184 retains CB1 receptor mediated antinociceptive and gastroprotective effects. *J. Pharmacol. Exp. Ther.* 345, 492–501. doi: 10.1124/jpet.112.201426

Lichtman, A. H., Hawkins, E. G., Griffin, G., and Cravatt, B. F. (2002). Pharmacological activity of fatty acid amides is regulated, but not mediated,

by fatty acid amide hydrolase *in vivo*. *J. Pharmacol. Exp. Ther.* 302, 73–79. doi: 10.1124/jpet.302.1.73

Long, J. Z., Li, W., Booker, L., Burston, J. J., Kinsey, S. G., Schlosburg, J. E., et al. (2009a). Selective blockade of 2-arachidonoylglycerol hydrolysis produces cannabinoid behavioral effects. *Nat. Chem. Biol.* 5, 37–44. doi: 10.1038/nchembio.129

Long, J. Z., Nomura, D. K., Vann, R. E., Walentiny, D. M., Booker, L., Jin, X., et al. (2009b). Dual blockade of FAAH and MAGL identifies behavioral processes regulated by endocannabinoid crosstalk *in vivo*. *Proc. Natl. Acad. Sci. U.S.A.* 106, 20270–20275. doi: 10.1073/pnas.0909411106

Lynch, M. E., and Campbell, F. (2011). Cannabinoids for treatment of chronic non-cancer pain; a systematic review of randomized trials. *Br. J. Clin. Pharmacol.* 72, 735–744. doi: 10.1111/j.1365-2125.2011.03970.x

Maione, S., Bisogno, T., De Novellis, V., Palazzo, E., Cristino, L., Valenti, M., et al. (2006). Elevation of endocannabinoid levels in the ventrolateral periaqueductal grey through inhibition of fatty acid amide hydrolase affects descending nociceptive pathways via both cannabinoid receptor type 1 and transient receptor potential vanilloid type-1 receptors. *J. Pharmacol. Exp. Ther.* 316, 969–982. doi: 10.1124/jpet.105.093286

Pertwee, R. G. (2013). Elevating endocannabinoid levels: pharmacological strategies and potential therapeutic applications. *Proc. Nutr. Soc.* 73, 96–105. doi: 10.1017/S0029665113003649

Petrosino, S., and Di Marzo, V. (2010). FAAH and MAGL inhibitors: therapeutic opportunities from regulating endocannabinoid levels. *Curr. Opin. Investig. Drugs* 11, 51–62.

Petrosino, S., Palazzo, E., De Novellis, V., Bisogno, T., Rossi, F., Maione, S., et al. (2007). Changes in spinal and supraspinal endocannabinoid levels in neuropathic rats. *Neuropharmacology* 52, 415–422. doi: 10.1016/j.neuropharm.2006.08.011

Piscitelli, F., and Di Marzo, V. (2012). "Redundancy" of endocannabinoid inactivation: new challenges and opportunities for pain control. *ACS Chem. Neurosci.* 3, 356–363. doi: 10.1021/cn300015x

Ramesh, D., Gamage, T. F., Vanuytsel, T., Owens, R. A., Abdullah, R. A., Niphakis, M. J., et al. (2013). Dual inhibition of endocannabinoid catabolic enzymes produces enhanced antiwithdrawal effects in morphine-dependent mice. *Neuropsychopharmacology* 38, 1039–1049. doi: 10.1038/npp.2012.269

Russo, R., Loverme, J., La Rana, G., Compton, T. R., Parrott, J., Duranti, A., et al. (2007). The fatty acid amide hydrolase inhibitor URB597 (cyclohexylcarbamic acid 3'-carbamoylbiphenyl-3-yl ester) reduces neuropathic pain after oral administration in mice. *J. Pharmacol. Exp. Ther.* 322, 236–242. doi: 10.1124/jpet.107.119941

Savinainen, J. R., Saario, S. M., and Laitinen, J. T. (2012). The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signalling through cannabinoid receptors. *Acta Physiol. (Oxf).* 204, 267–276. doi: 10.1111/j.1748-1716.2011.02280.x

Schlosburg, J. E., Blankman, J. L., Long, J. Z., Nomura, D. K., Pan, B., Kinsey, S. G., et al. (2010). Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat. Neurosci.* 13, 1113–1119. doi: 10.1038/nn.2616

Scott, D. A., Wright, C. E., and Angus, J. A. (2004). Evidence that CB-1 and CB-2 cannabinoid receptors mediate antinociception in neuropathic pain in the rat. *Pain* 109, 124–131. doi: 10.1016/j.pain.2004.01.020

Smith, P. B., Compton, D. R., Welch, S. P., Razdan, R. K., Mechoulam, R., and Martin, B. R. (1994). The pharmacological activity of anandamide, a putative endogenous cannabinoid, in mice. *J. Pharmacol. Exp. Ther.* 270, 219–227.

Torrance, N., Smith, B. H., Bennett, M. I., and Lee, A. J. (2006). The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. *J. Pain* 7, 281–289. doi: 10.1016/j.jpain.2005.11.008

Walker, J. M., Huang, S. M., Strangman, N. M., Tsou, K., and Sanudo-Pena, M. C. (1999). Pain modulation by release of the endogenous cannabinoid anandamide. *Proc. Natl. Acad. Sci. U.S.A.* 96, 12198–12203. doi: 10.1073/pnas.96.21.12198

Received: 17 December 2013; accepted: 19 January 2014; published online: 03 March 2014.

Citation: Lau BK and Vaughan CW (2014) Targeting the endogenous cannabinoid system to treat neuropathic pain. Front. Pharmacol. ***5****:28. doi: 10.3389/fphar.2014.00028*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 22 November 2013
doi: 10.3389/fphar.2013.00142

Immunotherapy targeting cytokines in neuropathic pain

Justin G. Lees, Samuel S. Duffy and Gila Moalem-Taylor*

Department of Anatomy, School of Medical Sciences, University of New South Wales, Sydney, NSW, Australia
**Correspondence: gila@unsw.edu.au*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Zubair Ahmed, University of Birmingham, UK
Orion Furmanski, Johns Hopkins University School of Medicine, USA

Keywords: neuropathic pain, cytokines, peripheral nerve injury, neuroimmunology, proinflammatory cytokines

Pain is a complex warning system activated in response to potential or apparent danger and the absence of pain is detrimental. Nociceptive pain is high-threshold pain activated in the presence of intense stimuli, such as contact with a burning object, and is a protective system essential for detection of noxious stimuli. Inflammatory pain, caused by immune system activation in response to tissue injury or infection, is also protective, as it discourages physical contact with the damaged area and assists healing (Woolf, 2010). In contrast, neuropathic pain emanating from disease or damage to the somatosensory system, is somewhat unique in that it is not protective, but rather pathological. Neuropathic pain encompasses a series of heterogeneous conditions with some similar clinical manifestations. Peripheral examples include traumatic nerve injury, diabetic peripheral neuropathy and chemotherapy-induced peripheral neuropathy, whilst multiple sclerosis is an example of a disease which can result in centrally derived neuropathic pain. These conditions are characterized by a low-threshold chronic pain emanating from aberrant peripheral and central neuronal sensitization. Symptoms include paraesthesia, spontaneous ongoing pain, and evoked pain (e.g., hyperalgesia and allodynia). Recent studies investigating neuropathic pain have demonstrated significant associated immune system activation and a fundamental role for cytokine signaling (Austin and Moalem-Taylor, 2010).

In this Opinion Article, we briefly summarize the progress made on research of cytokine involvement in neuropathic pain states and suggest that targeting key cytokines may prove useful in the development of new immune-therapeutics. However, further studies are required to determine which cytokine is the appropriate target for specific neuropathic pain conditions.

CYTOKINE EXPRESSION AFTER NERVE INJURY

Dysregulation of cytokines has been implicated in a variety of neuropathic pain conditions in both humans and animals. For example, differential expression of blood and cerebrospinal fluid cytokines has been demonstrated in patients with painful neuropathies. Compared to healthy controls, these patients show higher levels of proinflammatory cytokines [e.g., interleukin (IL)-1β and tumor necrosis factor-α (TNFα)], and lower levels of anti-inflammatory cytokines (e.g., IL-10) (Uceyler et al., 2007; Backonja et al., 2008). Animal models of peripheral nerve injury, such as chronic constriction injury (CCI) of the sciatic nerve, demonstrate extensive infiltration into the peripheral nervous system by cytokine producing immune cells (Moalem et al., 2004; Hu et al., 2007). Nerve injury increases expression and secretion of proinflammatory cytokines, including TNFα, IL-1β, IL-6, and interferon-γ, all of which are required for the development of pain hypersensitivity (Murphy et al., 1995; Costigan et al., 2009). Injury to neurons of the central nervous system (CNS) also initiates an immune response involving cytokine signaling (Guptarak et al., 2013).

In cases of peripheral nerve injury, the local inflammatory response is followed by a proximal response in both the dorsal root ganglion (DRG) and the spinal cord. Activated glial cells within the dorsal horn are critically involved in transmission of pain and are pivotal to the maintenance of chronic neuropathic pain (Austin and Moalem-Taylor, 2010). Both the major types of glia within the CNS (astrocytes and microglia) are particularly sensitive to activation following nerve injury (Mika et al., 2013). Furthermore, glia secrete cytokines and are likely to be the major source of central proinflammatory cytokines TNFα, IL-1β, and IL-6 (Whitehead et al., 2010). Classically characterized as messengers of the immune system, cytokines can act upon many different cell types, including neurons. For example IL-1β (Binshtok et al., 2008), TNFα (Jin and Gereau, 2006), and IL-17A (Richter et al., 2012) all can directly activate nociceptors and induce pain hypersensitivity.

Shortly after CCI, mRNA coding for the cytokine TNFα is rapidly elevated in the sciatic nerve (within hours of injury) and subsequently in the DRG (1–3 days following injury) (Sacerdote et al., 2008). TNFα alters the excitability of neurons and promotes continued inflammation (Sorkin et al., 1997). For at least 2 weeks, TNFα and its receptor TNFR1, display elevated expression in the ipsilateral and contralateral DRG of nerve-injured animals (Dubovy et al., 2006). Within the DRG and spinal cord, signaling via TNFR1, results in activation of NF-κB pathway followed by up-regulation of IL-6 (Lee et al., 2009). Bilateral elevated levels of IL-6 are observed in the DRG following unilateral CCI and furthermore in distant cervical DRG, suggesting a wider neuroinflammatory response (Dubovy et al., 2013). IL-1β precipitates pain hypersensitivity when administered to the sciatic nerve (Zelenka et al., 2005) or intrathecally (Sung et al., 2004). *In vitro* exposure to IL-1β leads to increased excitability in medium and small DRG neurons (Stemkowski and Smith, 2012). IL-1β also acts in higher CNS regions whereby supraspinal changes in

IL-1β expression alter over time in the brainstem, thalamus and prefrontal cortex following sciatic nerve injury (Apkarian et al., 2006). Furthermore, IL-1β is elevated in the contralateral hippocampus of rats with CCI and spinal nerve ligation (SNL) (del Rey et al., 2011).

THERAPEUTICS TARGETING PROINFLAMMATORY CYTOKINES

Numerous studies have demonstrated that antagonism of proinflammatory cytokine signaling attenuates neuronal hypersensitivity and inflammation associated with nerve injury. For example, intrathecal injection of both IL-1β and TNFα antagonists alleviated pain induced by gp120 activated spinal injury (Milligan et al., 2001). Similarly, intrathecal administration of an IL-6 neutralizing antibody significantly reduced gp120-induced mechanical allodynia and down-regulated the expression of IL-1β and TNFα within the CNS (Schoeniger-Skinner et al., 2007). Biologics which target the activity of specific proinflammatory cytokines have been and continue to be developed. A common theme associated with their clinical use is that they are generally well-tolerated (Dinarello et al., 2012).

TNFα INHIBITORS

TNFα inhibitors are demonstrated to significantly reduce mechanical and thermal pain hypersensitivity associated with peripheral nerve injury (Iwatsuki et al., 2013). There are several TNFα inhibitors which have been developed, including the humanized monoclonal antibody infliximab and the receptor fusion protein etanercept. These and other TNFα inhibitors are currently approved for clinical use to treat a range of immune disorders including rheumatoid arthritis, chrohn's disease, and psoriasis. Infliximab was tested in a human clinical trial for the treatment of pain associated with disc herniation induced sciatica. The drug failed to show a significant reduction in pain across the entire treatment group, but patients with L3–L4 and L4–L5 disc herniation appeared to benefit (Korhonen et al., 2006). Etanercept was tested in humans suffering from sciatica and shown to have significant benefits in a pilot study (Genevay et al., 2004). Similarly in another small trial, epidural administration of etanercept was effective in reducing pain associated with spinal stenosis (Ohtori et al., 2012a). However, results from a randomized, double blind, placebo controlled trial for the treatment of radicular or discogenic back pain indicated that etanercept did not demonstrate any significant benefit (Cohen et al., 2007). TNFα activates the pain mediator p38 following nerve injury, a signaling pathway which is thought to be critical for the mediation of pain transmission. Pre-treatment with inhibitors of p38 (SB203580) or etanercept significantly reduced mechanical allodynia in rats with SNL injury (Schafers et al., 2003). In a human clinical trial of patients with nerve trauma, radiculopathy, or carpal tunnel syndrome, the selective p38 inhibitor dilmapimod was shown to have a statistically significant effect in reducing patients pain scores (Anand et al., 2011). Conversely, a similar p38 selective inhibitor losmapimod did not show any significant effect in reducing pain in a human clinical trial of patients with peripheral focal neuropathic pain related to nerve injury caused by trauma or surgery (Ostenfeld et al., 2013). Despite some variable clinical trial outcomes, there is evidence to suggest that TNFα inhibitors and next generation p38 inhibitors may prove effective in the treatment of different forms of neuropathic pain.

IL-1β INHIBITORS

Both knockout of the IL-1 receptor and transgenic over expression of the endogenous receptor antagonist IL-1RA reduced mechanical and thermal pain hypersensitivity in mice with spinal nerve injury (Wolf et al., 2006). Similarly, a neutralizing antibody targeting the IL-1β receptor (IL-1R1) alleviated allodynia in mice with CCI (Sommer et al., 1999). Therapeutics targeting the IL-1 receptor such as anakinra, a recombinant form of IL-1RA, the soluble decoy receptor rilonacept and anti IL-1β neutralizing antibody cannikumab, are all currently approved for use in a number of inflammatory diseases, including rheumatoid arthritis, gout, and stills diseases. A current clinical trial is testing whether preoperative administration of anakinra reduces incisional pain in patients undergoing vascular or orthopedic surgical procedures by lowering the concentration of inflammatory mediators in surgical wounds (NCT01466764). Given the key role of IL-1β in the development and propagation of neuropathic pain in animal models, it is noteworthy that very few trials have been conducted testing the effects of IL-1β inhibitors against neuropathic pain conditions in humans.

IL-6 INHIBITORS

IL-6 is widely expressed following nerve injury and intrathecal administration of IL-6 results in mechanical allodynia. An IL-6 receptor neutralizing antibody abolished mechanical allodynia associated with spinal cord injury pain 14 days after a single intraperitoneal injection (Guptarak et al., 2013). The humanized IL-6 receptor neutralizing antibody tocilizimab has been approved for use in rheumatoid arthritis and juvenile idiopathic arthritis. There have also been case reports where this antibody has been effective in reducing pain associated with neuromyelitis optica (Araki et al., 2013) and sciatica (Ohtori et al., 2012b). Alternative antibodies that bind directly to IL-6 (BMS945439) are being tested in clinical trials for the treatment of rheumatoid arthritis. Due to the substantial evidence indicating a role for IL-6 in the propagation of neuropathic pain, therapeutic antibodies targeting both the IL-6 and its receptor may be beneficial in treating certain neuropathic pain conditions.

IL-17

Although less conspicuous than other proinflammatory cytokines, IL-17 is a key orchestrator of cytokine signaling in neuropathic pain. IL-17 expression is significantly elevated following CCI in mice, peaking at day 7 after injury (Kleinschnitz et al., 2006). Knockout of IL-17 reduces pain hypersensitivity and the activation of CNS glia when compared to wild type mice following partial sciatic nerve ligation (PSNL), conversely injection of IL-17 results in increased mechanical and thermal hypersensitivity and neutrophil migration to the site of injection (Kim and Moalem-Taylor, 2011). IL-17 mediated hyperalgesia has been shown to be dependent on TNFα/TNFRA1 signaling (McNamee et al., 2011). Intraperitoneal injection of a monoclonal antibody against

IL-17 into rats with antigen induced arthritis reduced paw guarding and hyperalgesia (Richter et al., 2012). Humanized monoclonal antibodies against IL-17, such as secukinumab, are now being assessed for efficacy in treating a range of inflammatory conditions including psoriasis and rheumatoid arthritis, and based on results with animal models these biologics could be tested in human clinical trials for treatment of neuropathic pain.

THERAPEUTICS AIMED AT RESOLVING INFLAMMATION

As an alternative to targeting proinflammatory cytokines, another treatment option is to promote resolution of the inflammation by stimulating the expression of anti-inflammatory cytokines. A perceived advantage of this strategy is that it does not directly inhibit the activity of proinflammatory cytokines, which may be required for processes such as Wallerian degeneration and peripheral axonal regeneration.

ANTI-INFLAMMATORY CYTOKINES

A single dose of IL-10 had long-lasting behavioral effects in rats with excitotoxic spinal cord injury (Plunkett et al., 2001). Intrathecal gene therapy targeting the expression of IL-10 has been shown to be efficacious; delivery of adeno-associated viral IL-10 transiently reversed allodynia (Milligan et al., 2005), whilst repeated delivery of naked DNA encoding IL-10 reversed allodynia for up to 2 months after CCI (Milligan et al., 2006). Administration of IL-4 inhibited the production of TNFα and IL-1β in hyperalgesia models (Cunha et al., 1999) and intraneural injection of TGFβ caused a delayed and reduced pain hypersensitivity in rats with PSNL. This attenuated the homing of cytokine producing MAC+ macrophages and reduced the infiltration of T cells into the injured nerve (Echeverry et al., 2013). Data on the effectiveness of anti-inflammatory therapies in treating neuropathic pain in humans is limited, and has to date focused largely on IL-10. Recombinant human IL-10 (such as Ilodecakin/Tenovil) has been tested with variable success in treating chronic inflammatory conditions such as psoriasis, Crohn's disease, and rheumatoid arthritis. To the best of our knowledge, human trials investigating the efficacy of similar therapies in treating neuropathic pain are yet to be conducted.

SUMMARY AND PERSPECTIVE

The remarkable success of targeted inhibition of several cytokines, such as TNFα, in patients with rheumatoid arthritis, psoriasis and many other diseases has fundamentally revised the treatment of chronic inflammatory diseases. This suggests that different conditions may share common pathophysiology and may benefit from disruption of the cytokine network. Indeed, several neuropathic pain conditions have been shown to have dysregulation of cytokines, and the use of biologics targeting cytokines is an exciting and promising strategy in the quest to find more effective treatments for neuropathic pain. There are two basic sub-strategies that can be followed: (1) to target certain proinflammatory cytokines or their receptors to inhibit their activity, (2) to enhance the resolution of inflammation by promoting the activity of anti-inflammatory cytokines. The few clinical trials that have tested the efficacy of cytokine inhibitors in chronic neuropathic pain so far have demonstrated mixed results, suggesting that human validation studies will be necessary to identify the appropriate cytokines for a given neuropathic pain syndrome. It is anticipated that new effective cytokine targets will be discovered and will allow future novel treatment strategies for neuropathic pain.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council of Australia and the NSW Office for Science and Medical Research to Gila Moalem-Taylor.

REFERENCES

Anand, P., Shenoy, R., Palmer, J. E., Baines, A. J., Lai, R. Y., Robertson, J., et al. (2011). Clinical trial of the p38 MAP kinase inhibitor dilmapimod in neuropathic pain following nerve injury. *Eur. J. Pain* 15, 1040–1048. doi: 10.1016/j.ejpain.2011.04.005

Apkarian, A. V., Lavarello, S., Randolf, A., Berra, H. H., Chialvo, D. R., Besedovsky, H. O., et al. (2006). Expression of IL-1beta in supraspinal brain regions in rats with neuropathic pain. *Neurosci. Lett.* 407, 176–181. doi: 10.1016/j.neulet.2006.08.034

Araki, M., Aranami, T., Matsuoka, T., Nakamura, M., Miyake, S., and Yamamura, T. (2013). Clinical improvement in a patient with neuromyelitis optica following therapy with the anti-IL-6 receptor monoclonal antibody tocilizumab. *Mod. Rheumatol.* 23, 827–831. doi: 10.1007/s10165-012-0715-9

Austin, P. J., and Moalem-Taylor, G. (2010). The neuro-immune balance in neuropathic pain: Involvement of inflammatory immune cells, immune-like glial cells and cytokines. *J. Neuroimmunol.* 229, 26–50. doi: 10.1016/j.jneuroim.2010.08.013

Backonja, M. M., Coe, C. L., Muller, D. A., and Schell, K. (2008). Altered cytokine levels in the blood and cerebrospinal fluid of chronic pain patients. *J. Neuroimmunol.* 195, 157–163. doi: 10.1016/j.jneuroim.2008.01.005

Binshtok, A. M., Wang, H., Zimmermann, K., Amaya, F., Vardeh, D., Shi, L., et al. (2008). Nociceptors are interleukin-1beta sensors. *J. Neurosci.* 28, 14062–14073. doi: 10.1523/JNEUROSCI.3795-08.2008

Cohen, S. P., Wenzell, D., Hurley, R. W., Kurihara, C., Buckenmaier, C. C. 3rd., Griffith, S., et al. (2007). A double-blind, placebo-controlled, dose-response pilot study evaluating intradiscal etanercept in patients with chronic discogenic low back pain or lumbosacral radiculopathy. *Anesthesiology* 107, 99–105. doi: 10.1097/01.anes.0000267518.20363.0d

Costigan, M., Moss, A., Latremoliere, A., Johnston, C., Verma-Gandhu, M., Herbert, T. A., et al. (2009). T-cell infiltration and signaling in the adult dorsal spinal cord is a major contributor to neuropathic pain-like hypersensitivity. *J. Neurosci.* 29, 14415–14422. doi: 10.1523/JNEUROSCI.4569-09.2009

Cunha, F. Q., Poole, S., Lorenzetti, B. B., Veiga, F. H., and Ferreira, S. H. (1999). Cytokine-mediated inflammatory hyperalgesia limited by interleukin-4. *Br. J. Pharmacol.* 126, 45–50. doi: 10.1038/sj.bjp.0702266

del Rey, A., Yau, H. J., Randolf, A., Centeno, M. V., Wildmann, J., Martina, M., et al. (2011). Chronic neuropathic pain-like behavior correlates with IL-1beta expression and disrupts cytokine interactions in the hippocampus. *Pain* 152, 2827–2835. doi: 10.1016/j.pain.2011.09.013

Dinarello, C. A., Simon, A., and van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* 11, 633–652. doi: 10.1038/nrd3800

Dubovy, P., Brazda, V., Klusakova, I., and Hradilova-Svizenska, I. (2013). Bilateral elevation of interleukin-6 protein and mRNA in both lumbar and cervical dorsal root ganglia following unilateral chronic compression injury of the sciatic nerve. *J. Neuroinflammation* 10, 55. doi: 10.1186/1742-2094-10-55

Dubovy, P., Jancalek, R., Klusakova, I., Svizenska, I., and Pejchalova, K. (2006). Intra- and extraneuronal changes of immunofluorescence staining for TNF-alpha and TNFR1 in the dorsal root ganglia of rat peripheral neuropathic pain models. *Cell. Mol. Neurobiol.* 26, 1205–1217. doi: 10.1007/s10571-006-9006-3

Echeverry, S., Wu, Y., and Zhang, J. (2013). Selectively reducing cytokine/chemokine expressing macrophages in injured nerves impairs the development of neuropathic pain. *Exp. Neurol.*

240, 205–218. doi: 10.1016/j.expneurol.2012.11.013

Genevay, S., Stingelin, S., and Gabay, C. (2004). Efficacy of etanercept in the treatment of acute, severe sciatica: a pilot study. *Ann. Rheum. Dis.* 63, 1120–1123. doi: 10.1136/ard.2003.016451

Guptarak, J., Wanchoo, S., Durham-Lee, J., Wu, Y., Zivadinovic, D., Paulucci-Holthauzen, A., et al. (2013). Inhibition of IL-6 signaling: a novel therapeutic approach to treating spinal cord injury pain. *Pain* 154, 1115–1128. doi: 10.1016/j.pain.2013.03.026

Hu, P., Bembrick, A. L., Keay, K. A., and McLachlan, E. M. (2007). Immune cell involvement in dorsal root ganglia and spinal cord after chronic constriction or transection of the rat sciatic nerve. *Brain Behav. Immun.* 21, 599–616. doi: 10.1016/j.bbi.2006.10.013

Iwatsuki, K., Arai, T., Ota, H., Kato, S., Natsume, T., Kurimoto, S., et al. (2013). Targeting anti-inflammatory treatment can ameliorate injury-induced neuropathic pain. *PLoS ONE* 8:e57721. doi: 10.1371/journal.pone.0057721

Jin, X., and Gereau, R. W. (2006). Acute p38-mediated modulation of tetrodotoxin-resistant sodium channels in mouse sensory neurons by tumor necrosis factor-alpha. *J. Neurosci.* 26, 246–255. doi: 10.1523/JNEUROSCI.3858-05.2006

Kim, C. F., and Moalem-Taylor, G. (2011). Interleukin-17 contributes to neuroinflammation and neuropathic pain following peripheral nerve injury in mice. *J. Pain* 12, 370–383. doi: 10.1016/j.jpain.2010.08.003

Kleinschnitz, C., Hofstetter, H. H., Meuth, S. G., Braeuninger, S., Sommer, C., and Stoll, G. (2006). T cell infiltration after chronic constriction injury of mouse sciatic nerve is associated with interleukin-17 expression. *Exp. Neurol.* 200, 480–485. doi: 10.1016/j.expneurol.2006.03.014

Korhonen, T., Karppinen, J., Paimela, L., Malmivaara, A., Lindgren, K. A., Bowman, C., et al. (2006). The treatment of disc-herniation-induced sciatica with infliximab: one-year follow-up results of FIRST II, a randomized controlled trial. *Spine (Phila Pa 1976)* 31, 2759–2766. doi: 10.1097/01.brs.0000245873.23876.1e

Lee, K. M., Jeon, S. M., and Cho, H. J. (2009). Tumor necrosis factor receptor 1 induces interleukin-6 upregulation through NF-kappaB in a rat neuropathic pain model. *Eur. J. Pain* 13, 794–806. doi: 10.1016/j.ejpain.2008.09.009

McNamee, K. E., Alzabin, S., Hughes, J. P., Anand, P., Feldmann, M., Williams, R. O., et al. (2011). IL-17 induces hyperalgesia via TNF-dependent neutrophil infiltration. *Pain* 152, 1838–1845. doi: 10.1016/j.pain.2011.03.035

Mika, J., Zychowska, M., Popiolek-Barczyk, K., Rojewska, E., and Przewlocka, B. (2013). Importance of glial activation in neuropathic pain. *Eur. J. Pharmacol.* 716, 106–119. doi: 10.1016/j.ejphar.2013.01.072

Milligan, E. D., O'Connor, K. A., Nguyen, K. T., Armstrong, C. B., Twining, C., Gaykema, R. P., et al. (2001). Intrathecal HIV-1 envelope glycoprotein gp120 induces enhanced pain states mediated by spinal cord proinflammatory cytokines. *J. Neurosci.* 21, 2808–2819.

Milligan, E. D., Sloane, E. M., Langer, S. J., Cruz, P. E., Chacur, M., Spataro, L., et al. (2005). Controlling neuropathic pain by adeno-associated virus driven production of the anti-inflammatory cytokine, interleukin-10. *Mol. Pain* 1, 9. doi: 10.1186/1744-8069-1-9

Milligan, E. D., Sloane, E. M., Langer, S. J., Hughes, T. S., Jekich, B. M., Frank, M. G., et al. (2006). Repeated intrathecal injections of plasmid DNA encoding interleukin-10 produce prolonged reversal of neuropathic pain. *Pain* 126, 294–308. doi: 10.1016/j.pain.2006.07.009

Moalem, G., Xu, K., and Yu, L. (2004). T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. *Neuroscience* 129, 767–777. doi: 10.1016/j.neuroscience.2004.08.035

Murphy, P. G., Grondin, J., Altares, M., and Richardson, P. M. (1995). Induction of interleukin-6 in axotomized sensory neurons. *J. Neurosci.* 15(7 Pt 2), 5130–5138.

Ohtori, S., Miyagi, M., Eguchi, Y., Inoue, G., Orita, S., Ochiai, N., et al. (2012a). Epidural administration of spinal nerves with the tumor necrosis factor-alpha inhibitor, etanercept, compared with dexamethasone for treatment of sciatica in patients with lumbar spinal stenosis: a prospective randomized study. *Spine (Phila Pa 1976)* 37, 439–444. doi: 10.1097/BRS.0b013e318238af83

Ohtori, S., Miyagi, M., Eguchi, Y., Inoue, G., Orita, S., Ochiai, N., et al. (2012b). Efficacy of epidural administration of anti-interleukin-6 receptor antibody onto spinal nerve for treatment of sciatica. *Eur. Spine J.* 21, 2079–2084. doi: 10.1007/s00586-012-2183-5

Ostenfeld, T., Krishen, A., Lai, R. Y., Bullman, J., Baines, A. J., Green, J., et al. (2013). Analgesic efficacy and safety of the novel p38 MAP kinase inhibitor, losmapimod, in patients with neuropathic pain following peripheral nerve injury: a double-blind, placebo-controlled study. *Eur. J. Pain* 17, 844–857. doi: 10.1002/j.1532-2149.2012.00256.x

Plunkett, J. A., Yu, C. G., Easton, J. M., Bethea, J. R., and Yezierski, R. P. (2001). Effects of interleukin-10 (IL-10) on pain behavior and gene expression following excitotoxic spinal cord injury in the rat. *Exp. Neurol.* 168, 144–154. doi: 10.1006/exnr.2000.7604

Richter, F., Natura, G., Ebbinghaus, M., von Banchet, G. S., Hensellek, S., Konig, C., et al. (2012). Interleukin-17 sensitizes joint nociceptors to mechanical stimuli and contributes to arthritic pain through neuronal interleukin-17 receptors in rodents. *Arthritis Rheum.* 64, 4125–4134. doi: 10.1002/art.37695

Sacerdote, P., Franchi, S., Trovato, A. E., Valsecchi, A. E., Panerai, A. E., and Colleoni, M. (2008). Transient early expression of TNF-alpha in sciatic nerve and dorsal root ganglia in a mouse model of painful peripheral neuropathy. *Neurosci. Lett.* 436, 210–213. doi: 10.1016/j.neulet.2008.03.023

Schafers, M., Svensson, C. I., Sommer, C., and Sorkin, L. S. (2003). Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J. Neurosci.* 23, 2517–2521.

Schoeniger-Skinner, D. K., Ledeboer, A., Frank, M. G., Milligan, E. D., Poole, S., Martin, D., et al. (2007). Interleukin-6 mediates low-threshold mechanical allodynia induced by intrathecal HIV-1 envelope glycoprotein gp120. *Brain Behav. Immun.* 21, 660–667. doi: 10.1016/j.bbi.2006.10.010

Sommer, C., Petrausch, S., Lindenlaub, T., and Toyka, K. V. (1999). Neutralizing antibodies to interleukin 1-receptor reduce pain associated behavior in mice with experimental neuropathy. *Neurosci. Lett.* 270, 25–28. doi: 10.1016/S0304-3940(99)00450-4

Sorkin, L. S., Xiao, W. H., Wagner, R., and Myers, R. R. (1997). Tumour necrosis factor-alpha induces ectopic activity in nociceptive primary afferent fibres. *Neuroscience* 81, 255–262. doi: 10.1016/S0306-4522(97)00147-4

Stemkowski, P. L., and Smith, P. A. (2012). Long-term IL-1beta exposure causes subpopulation-dependent alterations in rat dorsal root ganglion neuron excitability. *J. Neurophysiol.* 107, 1586–1597. doi: 10.1152/jn.00587.2011

Sung, C. S., Wen, Z. H., Chang, W. K., Ho, S. T., Tsai, S. K., Chang, Y. C., et al. (2004). Intrathecal interleukin-1beta administration induces thermal hyperalgesia by activating inducible nitric oxide synthase expression in the rat spinal cord. *Brain Res.* 1015, 145–153. doi: 10.1016/j.brainres.2004.04.068

Uceyler, N., Rogausch, J. P., Toyka, K. V., and Sommer, C. (2007). Differential expression of cytokines in painful and painless neuropathies. *Neurology* 69, 42–49. doi: 10.1212/01.wnl.0000265062.92340.a5

Whitehead, K. J., Smith, C. G., Delaney, S. A., Curnow, S. J., Salmon, M., Hughes, J. P., et al. (2010). Dynamic regulation of spinal pro-inflammatory cytokine release in the rat in vivo following peripheral nerve injury. *Brain Behav. Immun.* 24, 569–576. doi: 10.1016/j.bbi.2009.12.007

Wolf, G., Gabay, E., Tal, M., Yirmiya, R., and Shavit, Y. (2006). Genetic impairment of interleukin-1 signaling attenuates neuropathic pain, autotomy, and spontaneous ectopic neuronal activity, following nerve injury in mice. *Pain* 120, 315–324. doi: 10.1016/j.pain.2005.11.011

Woolf, C. J. (2010). What is this thing called pain? *J. Clin. Invest.* 120, 3742–3744. doi: 10.1172/JCI45178

Zelenka, M., Schafers, M., and Sommer, C. (2005). Intraneural injection of interleukin-1beta and tumor necrosis factor-alpha into rat sciatic nerve at physiological doses induces signs of neuropathic pain. *Pain* 116, 257–263. doi: 10.1016/j.pain.2005.04.018

Received: 13 September 2013; accepted: 03 November 2013; published online: 22 November 2013.

*Citation: Lees JG, Duffy SS and Moalem-Taylor G (2013) Immunotherapy targeting cytokines in neuropathic pain. Front. Pharmacol. **4**:142. doi: 10.3389/fphar.2013.00142*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 23 September 2013
doi: 10.3389/fphar.2013.00123

Targeting pain and inflammation by peripherally acting opioids

Christoph Stein*

Department of Anesthesiology and Critical Care Medicine, Charité Campus Benjamin Franklin Freie Universitaet Berlin, Berlin, Germany
**Correspondence: christoph.stein@charite.de*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Peter J. Cabot, The University of Queensland, Australia

Keywords: opioid receptor, opioid peptide, inflammation, pain, analgesia

INTRODUCTION

Opioids can produce potent analgesia by activating opioid receptors outside the central nervous system, thus avoiding centrally mediated unwanted effects. Peripheral opioid receptors are expressed in peripheral sensory (dorsal root ganglion) neurons and can interact with exogenous or endogenous opioid ligands both in animals and in humans. Inflammation of peripheral tissue leads to upregulation of such opioid receptors and to local production of endogenous opioid peptides in immune cells. This article will summarize recent mechanistic, preclinical, and clinical findings.

OPIOID RECEPTOR SIGNALING IN PERIPHERAL SENSORY NEURONS

Co-localization and electrophysiological studies have confirmed the presence of opioid receptors on C- and A-fibers, on dorsal root ganglion neurons expressing transient receptor potential vanilloid subtype-1 (TRPV-1) and G-protein-coupled inwardly rectifying K^+ (GIRK) channels, and on fibers expressing isolectin B4, substance P, and/or calcitonin-gene-related peptide, consistent with the phenotype of nociceptors. The activation of such opioid receptors results in inhibition of high-voltage activated Ca^{++}- and enhancement of GIRK-currents. These effects are mediated by G-proteins (G_i and/or G_o). In addition, opioids—via inhibition of adenylyl cyclase—suppress tetrodotoxin-resistant Na^+-, TRPV1- and other non-selective cation currents stimulated by inflammatory agents, which may account for the notable efficacy of peripheral opioids in inflammatory and neuropathic pain. Consistent with their effects on ion channels, opioids attenuate the excitability of peripheral nociceptor terminals, the propagation of action potentials, the release of excitatory proinflammatory neuropeptides (substance P, calcitonin gene-related peptide) from peripheral sensory nerve endings, and vasodilatation evoked by stimulation of C-fibers. These mechanisms result in analgesia and/or anti-inflammatory actions (Endres-Becker et al., 2007; Vetter et al., 2008; Stein and Machelska, 2011; Moshourab and Stein, 2012; Nockemann et al., 2013; Spahn et al., 2013; Stein and Küchler, 2013).

PERIPHERAL OPIOID RECEPTORS AND TISSUE INJURY

Peripheral opioid analgesic effects are particularly prominent in inflamed tissue (Kalso et al., 2002; Stein et al., 2003; Vadivelu et al., 2011). Under such conditions the synthesis and expression of opioid receptors in dorsal root ganglia is elevated. Subsequently, the axonal transport and membrane-directed trafficking of opioid receptors increases, leading to their upregulation on peripheral neuron terminals (Patwardhan et al., 2005; Cayla et al., 2012; Pettinger et al., 2013). These events are dependent on neuronal electrical activity, cytokines, and nerve growth factor from the damaged tissue. In mechanical nerve injury leading to neuropathic pain, opioid receptors accumulate proximal and distal to the lesion, indicating anterograde and retrograde transport (Labuz et al., 2009). Inflammatory milieu (low pH, prostanoids, bradykinin) can augment opioid receptor function e.g., by more efficient G-protein coupling and inhibition of elevated neuronal cyclic adenosine monophosphate production (Stein and Machelska, 2011; Stein, 2013). Inflammation also leads to sprouting of sensory nerve terminals and disruption of the perineurial barrier, thus facilitating the access of opioid agonists to their receptors (Rittner et al., 2012). Endogenous opioid ligands derived from inflammatory cells stimulate recycling of opioid receptors to the membrane of sensory neurons, which can prevent the development of tolerance to peripherally active opioid agonists (Zöllner et al., 2008). Consistently, clinical studies have indicated a lack of cross-tolerance between peripheral exogenous and endogenous opioids in synovial inflammation. All of these mechanisms can contribute to enhanced antinociceptive efficacy of opioid agonists in injured tissue (Stein and Machelska, 2011).

ENDOGENOUS LIGANDS OF PERIPHERAL OPIOID RECEPTORS

Concurrent with the development of inflammation, opioid peptide-producing immune cells are recruited to the site of injury. The most thoroughly characterized peptides are β-endorphin and enkephalins deriving from the respective precursors proopiomelanocortin (POMC) and proenkephalin. Transcripts and peptides derived from POMC and proenkephalin, as well as the prohormone convertases PC1/3 and PC2, necessary for their posttranslational processing, were detected in such cells. The expression of immune-derived opioids is stimulated by viruses, endotoxins, cytokines, corticotropin releasing hormone (CRH) and adrenergic agonists. In painful tissue inflammation and neuropathy, POMC mRNA, β-endorphin, met-enkephalin, and dynorphin are

detectable in circulating cells and lymph nodes, and are upregulated in resident lymphocytes, monocytes/macrophages, and granulocytes. Circulating opioid-containing leukocytes migrate to injured tissue attracted by adhesion molecules, chemokines, and neurokinins. In inflamed tissue, opioid-containing leukocytes, vascular P-selectin, ICAM-1, and PECAM-1 are simultaneously upregulated. Blocking chemokines, selectins, or ICAM-1 reduces the extravasation of opioid-containing cells and increases inflammatory and neuropathic pain. Consistently, immunosuppression can exacerbate pain (Labuz et al., 2009; Stein and Machelska, 2011; Busch-Dienstfertig et al., 2012).

Stimuli such as environmental stress, noradrenaline, CRH, interleukin-1β, chemokines, or mycobacteria can elicit opioid peptide release from immune cells via specific receptors and the regulated secretory pathway. Depending on the cell type and agent, intracellular Ca^{++} release from endoplasmic reticulum or extracellular Ca^{++} is required. *In vivo*, the secreted opioid peptides bind to opioid receptors on sensory neurons and elicit analgesia in injured tissue and neuropathy (Labuz et al., 2009; Rittner et al., 2009). Not only stimulated but also tonic release of opioids from immune cells decreases pain in animals (Rittner et al., 2009) and in humans (Stein et al., 1993). Thus, the development of inflammatory and neuropathic pain is counteracted by immune cells producing and secreting opioid peptides. Gene therapeutic approaches are aiming to increase the production of opioid peptides and receptors in inflammatory cells and peripheral sensory neurons, respectively (Stein and Machelska, 2011; Raja, 2012). Preventing the extracellular degradation of endogenous opioid peptides by peptidase inhibitors as well as nanocarrier-directed transport of opioids have been shown to diminish inflammatory pain (Roques et al., 2012; Schreiter et al., 2012; Hua and Cabot, 2013).

PRECLINICAL STUDIES ON PERIPHERAL OPIOID ANALGESICS

This basic research has stimulated the development of novel opioid ligands acting exclusively in the periphery without central side-effects. A common approach is the use of hydrophilic compounds with minimal capability to cross the blood-brain-barrier. Among the first compounds were the mu-agonist loperamide (known as an antidiarrheal drug) and the kappa-agonist asimadoline. Peripheral restriction was also achieved with glucuronidation, arylacetamide (ADL 10-0101), morphinan-based (TRK-820, HS-731), triazaspiro (DiPOA) and peptidic compounds (DALDA, FE200665, CR845). While earlier attempts to demonstrate peripheral opioid analgesia in normal tissue failed, they were much more successful in models of pathological pain (Stein, 1993). For example, in subcutaneous inflammation the local injection of low, systemically inactive doses of mu-, delta-, and kappa-agonists produces dose-dependent and opioid receptor-specific antinociception. Such effects were also shown in models of nerve damage, visceral, thermal, cancer and bone pain (Stein and Machelska, 2011).

EFFECTS ON INFLAMMATION

Inflammation contributes to many diverse disorders such as trauma, arthritis, neuropathy, fibromyalgia, endometriosis, diabetes, cancer, and chronic pain. Therapeutic inhibition of inflammation is indicated when it becomes dysregulated, chronic, recurrent or inappropriate. However, standard treatments such as steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and disease-modifying drugs have severe side effects (ulcers, bleeding, myocardial infarction, stroke, infections) (Trelle et al., 2011) and biological anti-inflammatory treatments such as inhibitors of tumor necrosis factor-α or of Janus kinases can only be used in a limited number of patients due to their prohibitive cost, parenteral formulation and risk for infection and tumor induction. A large number of *in vitro* and animal investigations have produced evidence that peripherally active opioids can reduce release of proinflammatory neuropeptides, cytokines, plasma extravasation, vasodilation, immune mediators, expression of adhesion molecules and tissue destruction (Stein and Küchler, 2012). In contrast to currently available anti-inflammatory agents, opioids have no demonstrated organ toxicity, making them interesting candidates for drug development. However, there is a lack of clinical studies in this area at present.

CLINICAL STUDIES ON PERIPHERAL OPIOID ANALGESICS

The most extensively examined clinical application is the intraarticular injection of morphine. Both in human and veterinary medicine, numerous controlled clinical studies have demonstrated dose-dependent and peripherally mediated reduction of pain and/or supplemental analgesic consumption without significant side effects (Kalso et al., 2002; Stein, 2013). Intraarticular morphine is effective in acute (postoperative) and chronic (arthritic) pain, its effect is similar to intraarticular local anesthetics and steroids, and it is long lasting, possibly due to anti-inflammatory activity. Locally applied opioids were also effective in dental pain, skin ulcers, corneal abrasions and visceral pain (Sawynok, 2003; Farley, 2011; Vadivelu et al., 2011). Some studies found no peripheral effects of opioids, e.g., after injection into the non-inflamed environment along nerve trunks (Picard et al., 1997). The latter observation suggests that intraaxonal opioid receptors are "in transit," and not available as functional receptors at the membrane. Peripherally restricted opioids are under investigation for human use (morphine-6-glucuronide, CR845), and were shown to reduce postoperative and visceral pain with similar efficacy as morphine but limited central side-effects (Dahan et al., 2008; Binning et al., 2011; Stein and Machelska, 2011).

SUMMARY

Opioids can reduce pain and inflammation by activating opioid receptors outside the central nervous system. Inflammation of peripheral tissue leads to upregulation of opioid receptors on peripheral sensory neurons and to local production of endogenous opioid peptides in immune cells. Future aims in drug development include the design of peripherally restricted opioid agonists, selective targeting of opioids to sites of painful injury and the augmentation of peripheral ligand and receptor synthesis, e.g., by gene therapy. The ultimate goal is to avoid detrimental side effects of currently available opioid and nonopioid drugs such as apnoea, cognitive impairment,

addiction, gastrointestinal bleeding, and thromboembolic complications.

ACKNOWLEDGMENTS

Supported by Bundesministerium für Bildung und Forschung (VIP0272/AZ 03V0364; 0316177B/C1).

REFERENCES

Binning, A. R., Przesmycki, K., Sowinski, P., Morrison, L. M., Smith, T. W., Marcus, P., et al. (2011). A randomised controlled trial on the efficacy and side-effect profile (nausea/vomiting/sedation) of morphine-6-glucuronide versus morphine for post-operative pain relief after major abdominal surgery. *Eur. J. Pain* 15, 402–408. doi: 10.1016/j.ejpain.2010.09.007

Busch-Dienstfertig, M., Labuz, D., Wolfram, T., Vogel, N. N., and Stein, C. (2012). JAK-STAT1/3-induced expression of signal sequence-encoding proopiomelanocortin mRNA in lymphocytes reduces inflammatory pain in rats. *Mol. Pain* 8, 83. doi: 10.1186/1744-8069-8-83

Cayla, C., Labuz, D., Machelska, H., Bader, M., Schäfer, M., and Stein, C. (2012). Impaired nociception and peripheral opioid antinociception in mice lacking both kinin B1 and B2 receptors. *Anesthesiology* 116, 448–457. doi: 10.1097/ALN.0b013e318242b2ea

Dahan, A., van Dorp, E., Smith, T., and Yassen, A. (2008). Morphine-6-glucuronide (M6G) for post-operative pain relief. *Eur. J. Pain* 12, 403–411. doi: 10.1016/j.ejpain.2007.07.009

Endres-Becker, J., Heppenstall, P. A., Mousa, S. A., Labuz, D., Oksche, A., Schäfer, M., et al. (2007). Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol. Pharmacol.* 71, 12–18. doi: 10.1124/mol.106.026740

Farley, P. (2011). Should topical opioid analgesics be regarded as effective and safe when applied to chronic cutaneous lesions. *J. Pharm. Pharmacol.* 63, 747–756. doi: 10.1111/j.2042-7158.2011.01252.x

Hua, S., and Cabot, P. J. (2013). Targeted nanoparticles that mimic immune cells in pain control inducing analgesic and anti-inflammatory actions: a potential novel treatment of acute and chronic pain condition. *Pain Physician* 16, E199–E216.

Kalso, E., Smith, L., McQuay, H. J., Moore, R. A. (2002). No pain, no gain: clinical excellence and scientific rigour - lessons learned from IA morphine. *Pain* 98, 269–275. doi: 10.1016/S0304-3959(02)00019-2

Labuz, D., Schmidt, Y., Schreiter, A., Rittner, H. L., Mousa, S. A., and Machelska, H. (2009). Immune cell-derived opioids protect against neuropathic pain in mice. *J. Clin. Invest.* 119, 278–286. doi: 10.1172/JCI36246

Moshourab, R., and Stein, C. (2012). Fentanyl decreases discharges of C and A nociceptors to suprathreshold mechanical stimulation in chronic inflammation. *J. Neurophysiol.* 108, 2827–2836. doi: 10.1152/jn.00082.2012

Nockemann, D., Rouault, M., Labuz, D., Hublitz, P., McKnelly, K., Reis, F. C., et al. (2013). The K channel GIRK2 is both necessary and sufficient for peripheral opioid-mediated analgesia. *EMBO Mol. Med.* 5, 1263–1277. doi: 10.1002/emmm.201201980

Patwardhan, A. M., Berg, K. A., Akopain, A. N., Jeske, N. A., Gamper, N., Clarke, W. P., et al. (2005). Bradykinin-induced functional competence and trafficking of the delta-opioid receptor in trigeminal nociceptors. *J. Neurosci.* 25, 8825–8832. doi: 10.1523/JNEUROSCI.0160-05.2005

Pettinger, L., Gigout, S., Linley, J. E., and Gamper, N. (2013). Bradykinin controls pool size of sensory neurons expressing functional delta-opioid receptors. *J. Neurosci.* 33, 10762–10771. doi: 10.1523/JNEUROSCI.0123-13.2013

Picard, P. R., Tramer, M. R., McQuay, H. J., and Moore, R. A. (1997). Analgesic efficacy of peripheral opioids (all except intra-articular): a qualitative systematic review of randomised controlled trials. *Pain* 72, 309–318. doi: 10.1016/S0304-3959(97)00040-7

Raja, S. N. (2012). Modulating pain in the periphery: gene-based therapies to enhance peripheral opioid analgesia: bonica lecture, ASRA (2010). *Reg. Anesth. Pain Med.* 37, 210–214. doi: 10.1097/AAP.0b013e31823b145f

Rittner, H. L., Amasheh, S., Moshourab, R., Hackel, D., Yamdeu, R. S., Mousa, S. A., et al. (2012). Modulation of tight junction proteins in the perineurium to facilitate peripheral opioid analgesia. *Anesthesiology* 116, 1323–1334. doi: 10.1097/ALN.0b013e318256eeeb

Rittner, H. L., Hackel, D., Voigt, P., Mousa, S., Stolz, A., Labuz, D., et al. (2009). Mycobacteria attenuate nociceptive responses by formyl peptide receptor triggered opioid peptide release from neutrophils. *PLoS Pathog.* 5:e1000362. doi: 10.1371/journal.ppat.1000362

Roques, B. P., Fournie-Zaluski, M. C., and Wurm, M. (2012). Inhibiting the breakdown of endogenous opioids and cannabinoids to alleviate pain. *Nat. Rev. Drug Discov.* 11, 292–310. doi: 10.1038/nrd3673

Sawynok, J. (2003). Topical and peripherally acting analgesics. *Pharmacol. Rev.* 55, 1–20. doi: 10.1124/pr.55.1.1

Schreiter, A., Gore, C., Labuz, D., Fournie-Zaluski, M. C., Roques, B. P., Stein, C., et al. (2012). Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue. *FASEB J.* 26, 5161–5171. doi: 10.1096/fj.12-208678

Spahn, V., Fischer, O., Endres-Becker, J., Schäfer, M., Stein, C., and Zöllner, C. (2013). Opioid withdrawal increases transient receptor potential vanilloid 1 activity in a protein kinase A-dependent manner. *Pain* 154, 598–608. doi: 10.1016/j.pain.2012.12.026

Stein, C. (1993). Peripheral mechanisms of opioid analgesia. *Anesth. Analg.* 76, 182–191. doi: 10.1213/00000539-199301000-00031

Stein, C. (2013). Towards safer and more effective analgesia. *Vet. J.* 196, 6–7. doi: 10.1016/j.tvjl.2012.09.013

Stein, C., Hassan, A. H. S., Lehrberger, K., Giefing, J., and Yassouridis, A. (1993). Local analgesic effect of endogenous opioid peptides. *Lancet* 342, 321–324. doi: 10.1016/0140-6736(93)91471-W

Stein, C., and Küchler, S. (2012). Non-analgesic effects of opioids: peripheral opioid effects on inflammation and wound healing. *Curr. Pharm. Des.* 18, 6053–6069. doi: 10.2174/13816121280 3582513

Stein, C., and Küchler, S. (2013). Targeting inflammation and wound healing by opioids. *Trends Pharmacol. Sci.* 34, 303–312. doi: 10.1016/j.tips.2013.03.006

Stein, C., and Machelska, H. (2011). Modulation of peripheral sensory neurons by the immune system: implications for pain therapy. *Pharmacol. Rev.* 63, 860–881. doi: 10.1124/pr.110.003145

Stein, C., Schäfer, M., and Machelska, H. (2003). Attacking pain at its source: new perspectives on opioids. *Nat. Med.* 9, 1003–1008. doi: 10.1038/nm908

Trelle, S., Reichenbach, S., Wandel, S., Hildebrand, P., Tschannen, B., Villiger, P. M., et al. (2011). Cardiovascular safety of non-steroidal anti-inflammatory drugs: network meta-analysis. *BMJ* 342:c7086. doi: 10.1136/bmj.c7086

Vadivelu, N., Mitra, S., and Hines, R. L. (2011). Peripheral opioid receptor agonists for analgesia: a comprehensive review. *J. Opioid Manag.* 7, 55–68. doi: 10.5055/jom.2011.0049

Vetter, I., Cheng, W., Peiris, M., Wyse, B. D., Roberts-Thomson, S. J., Zheng, J., et al. (2008). Rapid, opioid-sensitive mechanisms involved in transient receptor potential vanilloid 1 sensitization. *J. Biol. Chem.* 283, 19540–19550. doi: 10.1074/jbc.M707865200

Zöllner, C., Mousa, S. A., Fischer, O., Rittner, H. L., Shaqura, M., Brack, A., et al. (2008). Chronic morphine use does not induce peripheral tolerance in a rat model of inflammatory pain. *J. Clin. Invest.* 118, 1065–1073. doi: 10.1172/JCI25911

Received: 05 August 2013; accepted: 05 September 2013; published online: 23 September 2013.
*Citation: Stein C (2013) Targeting pain and inflammation by peripherally acting opioids. Front. Pharmacol. **4**:123. doi: 10.3389/fphar.2013.00123*
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 19 February 2014
doi: 10.3389/fphar.2014.00018

Biotransformation of beta-endorphin and possible therapeutic implications

Naghmeh H. Asvadi, Michael Morgan, Amitha K. Hewavitharana, P. Nicholas Shaw and Peter J. Cabot*

School of Pharmacy, The University of Queensland, Brisbane, Queensland, Australia
**Correspondence: p.cabot@uq.edu.au*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
James W. Grau, Texas A&M University, USA
Claudia Bregonzio, IFEC-CONICET-UNC, Argentina

Keywords: peptide, biotransformation, inflammation, opioid, proteomics, pain

Endogenous opioid peptides have been aligned with a diverse array of effects. Their activity is not only attributable to action the three main opioid receptors, mu (MOR), delta (DOR), and kappa (KOR) opioid receptors but their impacts appear to extend to activities at sodium channels, cytokine receptors (Finley et al., 2008), calcium channels and non-specific and partially undefined pharmacological effects inconsistent with G-protein coupled opioid receptor activity.

Of the family of opioid peptides beta-endorphin (BE 1-31) is one of the most prominent and is the prototypical endogenous peptide for the MOR class of opioid receptors and is found within the CNS and the immune system (Cabot et al., 1997). BE 1-31 is derived from pro-opiomelanocortin (POMC) in the cytosol of cell bodies. BE has been shown to possess peripheral and central analgesic activity (Van Den Burg et al., 2001), producing a morphine-like effect by inhibiting the signals of C- and Aδ-fiber activation (Duggan and Fleetwood-Walker, 1993). In addition, BE 1-31 is a non-selective endogenous peptide with the highest affinities for MOR and DOR (Binder et al., 2004), suggesting that the endogenous system is not modulated by specific and selective opioid agonists in isolation.

This concept touches on a new theme evolving in novel therapeutic strategies in the pain field, i.e. the targeting of multiple channels with either one non-selective ligand or a combination of selective ligands to produce effects that are either synergistic or, at a minimum, differential in terms of side effects. This could seemingly point to a multitude of combinations of drugs of both G-protein receptor targeting ligands or extend to those targeting other receptor classes including sodium channels (Su et al., 2002), potassium channels (Welch and Dunlow, 1993) and calcium channels (Smart et al., 1995). The scope of the possible therapeutic targets is immense and, potentially of even greater complexity, is the dose determination for such combinations. Perhaps the answer in part lies in the endogenous opioid system, which is, in essence, the system designed to mediate noxious stimuli as well as interact with the immune system in disease (**Figure 1**).

ENZYMATIC PROCESSING OF BETA-ENDORPHIN

It is well known that peptides including opioid peptides are susceptible to rapid enzymatic degradation (McKnight et al., 1983). The major peptidases involved in the degradation of opioid peptides are aminopeptidases (Montiel et al., 1997), angiotensin-converting enzyme (ACE), insulin degrading enzyme (Reed et al., 2008), serine peptidases (Sandin et al., 1998), dipeptidyl peptidase III and IV (DPP III, DPP IV) (Sakurada et al., 2003). DPP IV is a serine protease (Shane et al., 1999) and has demonstrated a structural preference for the cleavage of opioid peptides at proline (Augustyns et al., 2005) and is a likely candidate responsible for the cleavage of BE 1–31 producing BE 1–13. Insulin degrading enzyme similarly has been shown to have selective cleaving properties, producing BE 1–17 and BE 1–18 from BE 1–31. In addition, BE 1–19 and BE 20–31 are the likely products of the enzymatic activity of metallo sensitive serine proteases (Sandin et al., 1998). ACE is however has broad peptide cleavage properties and is found widely distributed in many cells (Brownson et al., 1994). Undoubtedly, a major degradative pathway to non-opioid metabolites will be via aminopeptidases, yet to be demonstrated for BE 1-31 but has been shown to be responsible for cleavage of dynorphin A 1–13 to 2–13 (Müller and Hochhaus, 1995), a conserved region within BE 1–31 and dynorphin A 1–13.

INHIBITING BETA-ENDORPHIN BIOTRANSFORMATION AS A THERAPEUTIC STRATEGY

Peptidase inhibition has been investigated as a therapeutic strategy with some success. This is not necessarily a novel approach but it has received a recent resurgence with the development of more selective inhibitors (Schreiter et al., 2012). Certainly, peptidases have been blocked selectively and non-selectively by a number of strategies, e.g., di-isopropyl fluorophosphate and metal ions (Pb^{2+}, Hg^{2+}, Zn^{2+}) are effective inhibitors for DPP IV, albeit di-isopropyl fluorophosphate has been shown to induce compensatory anticholinesterase activity producing tremors due to its irreversible nature. Spinorphin (Tien et al., 2004), which is endogenous factor derived from bovine spinal cord,

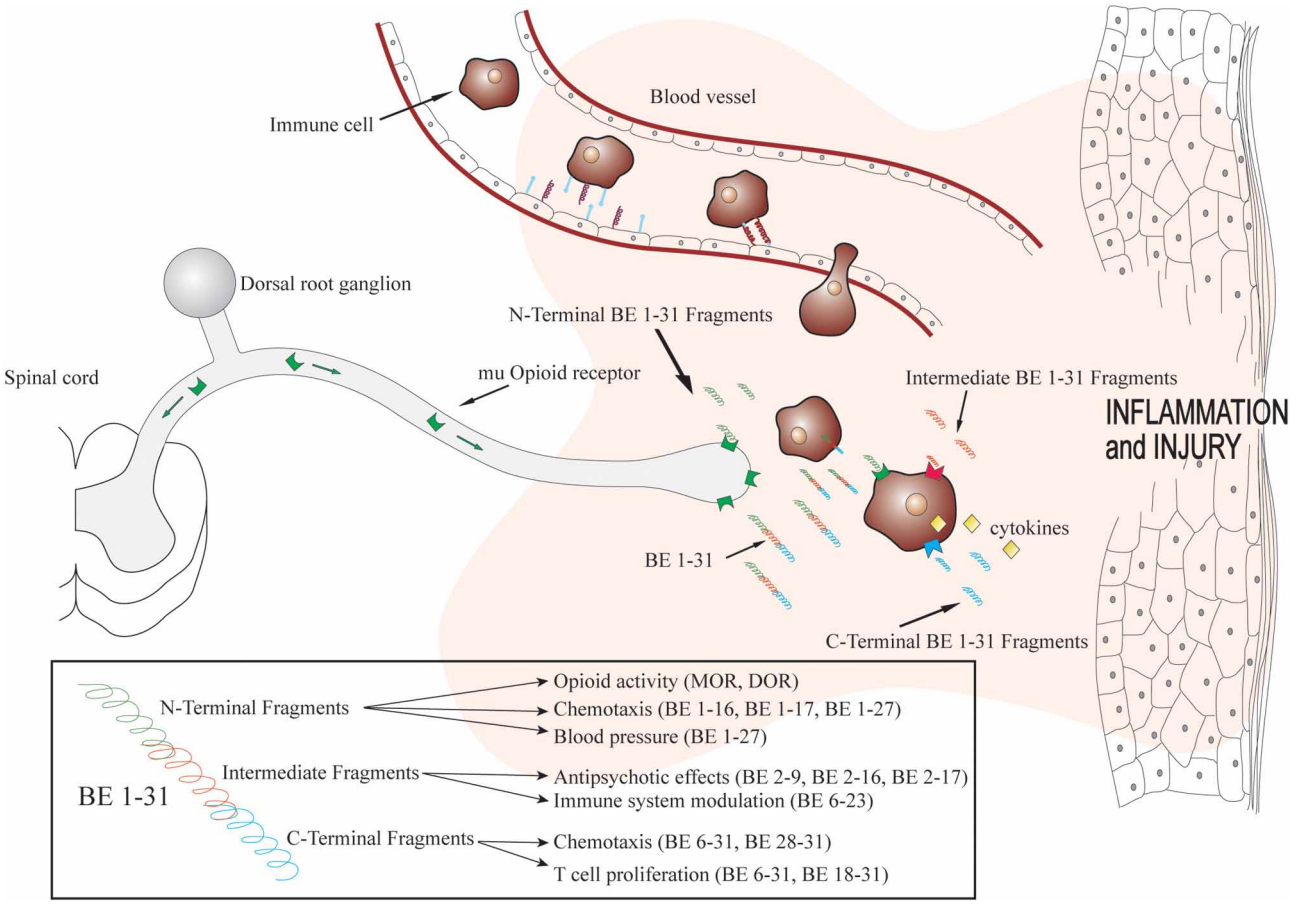


FIGURE 1 | Biotransformation of beta-endorphin 1–31 within inflamed tissue and fragment actions. Immune cells containing beta-endorphin migrate to inflamed tissue in a site directed manner. Beta-endorphin is released within the inflammatory mileu and biotransformed rapidly producing fragments with various pharmacological actions. Adapted with permission from Kapitzke et al. (2005).

and its truncated fragment, tynorphin, are both inhibitors of DPP III (Yamamoto et al., 2000). Leupeptin has been shown to block cysteine-containing enzymes and EDTA and phenanthroline inhibit metalloproteases generally (Mentlein, 1999), whilst aminopeptidases are inhibited non-selectively by bestatin without affecting DPP IV (Scornik and Botbol, 2001). A different approach undertaken is to modify the structure of endogenous peptides at specific labile or susceptible bonds. These include the routine modification at Gly^2 with substitution by D or L Ala^2 or N-methylation of the $Tyrosine^1$, both increasing stability of the peptide by reducing the N-terminal degradation (Hiramatsu et al., 2001). These approaches have been shown to produce long lasting analgesia, thereby suggesting that peptide processing is simply a means to facilitate the degradation of bioactive peptides to their non-pharmacologically active forms. It is likely that this, however, may not be the complete story, opioid peptide fragment and *in-situ* biotransformation may be an integral part of the bodies efforts in addressing disease and pain.

BIOTRANSFORMATION ALTERATION IN DISEASE

A recent study in our laboratory has identified biotransformation fragments of BE 1–31 in rat inflamed tissue (Herath et al., 2012). This study demonstrated that the hydrolytic metabolism of BE 1–31 in homogenized inflamed tissue was faster than in serum and trypsin incubation; similar results have been noted for the processing of dynorphin (the endogenous ligand for KOR) within inflamed tissue homogenates (Morgan et al., 2012). The rate of metabolism of BE 1–31 at pH 5.5 was also higher than the rate of metabolism of BE 1–31 at pH 7.4. These acidic pH values have been shown to be concordant with those found within inflamed tissue (Dray, 1995). In addition, the nature of the biotransformation hydrolysis was altered, BE 1–31 was shown in inflamed tissue homogenates to be most susceptible for hydrolytic degradation at specific amino acid bonds: (Tyr1-Gly2), (Lys9-Ser10), (Leu17-Phe18-Lys19-Asn20), (Lys24-Asn25), (Lys28-Lys29-Gly30-Gln31) (Herath et al., 2012). This is likely to be a consequence of the inflammatory conditions that affect the enzymes independently and specifically (Lin et al., 2001). These results highlight the presence of a unique panel of peptides which would be produced

dependent upon the disease state, possessing potentially unique pharmacological properties.

BIOTRANSFORMATION AND OPIOID ACTIVITY

Many studies have investigated the pharmacological changes observed following opioid peptide modification and truncation. Deakin et al. showed that the removal of one, two, or four amino acids from the C-terminal of BE 1–31 reduced the analgesic effect of fragments and that the removal of eight amino acids from the N-terminal of BE 1–31 resulted in an absence of analgesic activity (Deakin et al., 1980). Many other studies have provided evidence for the structural necessity of a tyrosine residue at position 1 in BE 1–31 for the retention of analgesic activity. In agreement with this notion, N-acetyl derivatives of BE 1–31 naturally found in the pituitary do not produce opioid activity (Deakin et al., 1980). In addition, a number of studies have demonstrated the C-terminal sequence of BE 1–31determines the potency of opioid peptide in analgesia. Naturally occurring forms of BE 1–31, truncated at the C-terminal, BE 1–28, BE 1–27, and BE 1–26 are found in the pituitary (Zakarian and Smyth, 1982). These compounds are not only ineffective as analgesics but BE 1–27 intra-cerebroventricularly injected into mice has been shown to block the analgesia produced by BE 1–31, with a potency four times greater than that of naloxone - the non-selective opioid antagonist (Hammonds et al., 1984). However, further truncation to BE 1-26 decreased the antagonist effect whilst further reduction of the peptide chain resulted in the complete loss of inhibition of analgesic activity (Nicolas and Choh Hao, 1985). The analgesic potency of further abbreviated forms remains from peptide sequences of BE 1–31 right down to BE 1–4, the overwhelming consequence of the truncation to smaller N-terminal conserved sequences is decreased affinity for MOR, but increased activity at DOR and KOR (Jaba et al., 2007).

BIOTRANSFORMATION AND NON-OPIOID ACTIVITY

The presence of BE 1–31 in both the neuronal and immune systems indicates that the pharmacological effects of these peptides may extend past those of the management of nociceptive signals. A number of studies have examined potential immune-related mechanisms for BE 1–31 and a variety of truncated forms. Interestingly, effects on human monocyte chemotaxis showed both a lack of requirement for opioid receptor action and the presence of the N-terminal Tyrosine. These effects occurred for a range of truncated forms of BE 1–31 (namely: BE 1–16, BE 1–17, BE 1–27, BE 6–31, BE 28–31) (Sacerdote and Panerai, 1989). Similarly, T cell proliferation was modulated at non-opioid receptors by BE 1–31, BE 6–31, and BE 18–31 (Van Den Bergh et al., 1993). Separate to their immune system effects but aligned with the systemic availability of these peptides, the effects on blood pressure and heart rate in anesthetized rats have also been examined for BE 1–31 and truncated peptides. BE 1–27, shown in previous studies to possess opioid antagonist activity against BE 1–31, and reduced blood pressure to an extent which was similar to that of the effects of the parent molecule, BE 1–31 (Giersbergen et al., 1991). In neurological experiments BE 1–16 and BE 1–17 modulated avoidance behavior and this was not inhibited by naltrexone, an opioid receptor antagonist. The non-opioid peptide fragment BE 2–17 also displayed strong anti-psychotic effects in schizophrenic patients (De Wied, 1979). This non-opioid effect of truncated BE 1–31 was supported in a separate study that showed similar effects with BE 2–16 and BE 2–9 (Van Ree and De Wied, 1982). Furthermore, BE 1–31, when cultured with rat splenocytes, showed suppression of plaque-forming cells (PFC) in response to coculture with sheep red blood cells, not reversed by naloxone (Hemmick and Bidlack, 1989). BE 1–31 has also been shown to interact with protein S in a C-terminal specific manner, implicating BE 1–31 in anticoagulation through antithrombin III (Hildebrand et al., 1989).

NON-OPIOID SITE OF ACTION

The search for the sites of action for the non-opioid effects of endogenous opioids has been largely focused on the immune system (Rittner et al., 2008). There is evidence of receptor binding sites for BE 1–31 on a number of immune cells that are not modulated by common analgesics or opioid selective antagonists. There is also a substantial body of evidence for opioids interacting with Toll-like receptors within the immune system (Franchi et al., 2012), with stereo selectivity for the plus isomers of common opioids such as morphine-3-glucuronide (Lewis et al., 2010), naloxone and naltrexone (Hutchinson et al., 2008). These effects have been correlated with modulation of cytokine expression or release, and result in changes that may effect cell proliferation and chemotaxis. Consistent with immune system modulation a non-opioid binding site for BE 1–31 has been demonstrated in immune cells, which would appear to exist in combination with classical opioid receptors and naloxone dependent effects. These sites have been proposed to be activated by restricted sequences of BE 1–31 to BE 6–23 and not modulated by naloxone or alkaloid agonists such as morphine (Kovalitskaya and Navolotskaya, 2011).

CONCLUDING REMARKS

Increasing our understanding of the role of beta-endorphin and its biotransformation fragments provides an insight into the complexity of the endogenous opioid system. The current analgesics are targeted at the modulation of analgesia by directly binding to one or more of the opioid receptors, with the analgesic being predominantly designed as a MOP agonist. The above observations would suggest that this is solely one aspect of opioid pharmacology, albeit one that has been explored widely and utilized in therapy. Biotransformation is a process that produces an array of compounds having a plethora of specific actions which contribute to the body's and its biological systems response to disease or injury. Future therapeutic strategies should consider such actions in designing better treatments or disease modulators.

ACKNOWLEDGMENTS

Naghmeh Asvadi was funded off a University of Queensland International Postgraduate Research Scholarship.

REFERENCES

Augustyns, K., Veken, P. V. D., Senten, K., and Haemers, A. (2005). The therapeutic potential of inhibitors of dipeptidyl peptidase IV (DPP IV) and related proline-specific dipeptidyl aminopeptidases. *Curr. Med. Chem.* 12, 971–998. doi: 10.2174/0929867053507298

Binder, W., Mousa, S. A., Sitte, N., Kaiser, M., Stein, C., and Schäfer, M. (2004). Sympathetic activation triggers endogenous opioid release and analgesia within peripheral inflamed tissue. *Eur. J. Neurosci.* 20, 92–100. doi: 10.1111/j.1460-9568.2004.03459.x

Brownson, E. A., Abbruscato, T. J., Gillespie, T. J., Hruby, V. J., and Davis, T. P. (1994). Effect of peptidases at the blood brain barrier on the permeability of enkephalin. *J. Pharmacol. Exp. Ther.* 270, 675–680.

Cabot, P. J., Carter, L., Gaiddon, C., Zhang, Q., Schäfer, M., Loeffler, J. P., et al. (1997). Immune cell-derived beta-endorphin. Production, release, and control of inflammatory pain in rats. *J. Clin. Invest.* 100, 142–148.

Deakin, J. F., Doströvsky, J. O., and Smyth, D. G. (1980). Influence of N-terminal acetylation and C-terminal proteolysis on the analgesic activity of beta-endorphin. *Biochem. J.* 189, 501–506.

De Wied, D. (1979). Schizophrenia as an inborn error in the degradation of β-endorphin a hypothesis. *Trends Neurosci.* 2, 79–82.

Dray, A. (1995). Inflammatory mediators of pain. *Br. J. Anaesth.* 75, 125–131. doi: 10.1093/bja/75.2.125

Duggan, A. W., and Fleetwood-Walker, S. M. (1993). "Opioids and sensory processing in the central nervous system," in *Opioids,* eds A. Herz, H. Akil and E. Simon (Berlin; Heidelberg: Springer), 731–771.

Finley, M. J., Happel, C. M., Kaminsky, D. E., and Rogers, T. J. (2008). Opioid and nociceptin receptors regulate cytokine and cytokine receptor expression. *Cell. Immunol.* 252, 146–154. doi: 10.1016/j.cellimm.2007.09.008

Franchi, S., Moretti, S., Castelli, M., Lattuada, D., Scavullo, C., Panerai, A. E., et al. (2012). Mu opioid receptor activation modulates Toll like receptor 4 in murine macrophages. *Brain Behav. Immun.* 26, 480–488. doi: 10.1016/j.bbi.2011.12.010

Giersbergen, P. L. M. V., Lang, H. D., and Jong, W. D. (1991). Effects of dynorphin A(1 – 13) and of fragments of β-endorphin on blood pressure and heart rate of anesthetized rats. *Can. J. Physiol. Pharmacol.* 69, 327–333. doi: 10.1139/y91-050

Hammonds, R. G., Nicolas, P., and Li, C. H. (1984). beta-endorphin-(1-27) is an antagonist of beta-endorphin analgesia. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1389–1390.

Hemmick, L. M., and Bidlack, J. M. (1989). Beta-endorphin suppresses rat plaque-forming cell response by a non-opioid mechanism. *J. Neuroimmunol.* 23, 67–71.

Herath, H., Cabot, P., Shaw, P., and Hewavitharana, A. (2012). Study of beta endorphin metabolism in inflamed tissue, serum and trypsin solution by liquid chromatography–tandem mass spectrometric analysis. *Anal. Bioanal. Chem.* 402, 2089–2100. doi: 10.1007/s00216-011-5686-8

Hildebrand, A., Preissner, K. T., Müller-Berghaus, G., and Teschemacher, H. (1989). A novel beta-endorphin binding protein. Complement S protein (= vitronectin) exhibits specific non-opioid binding sites for beta-endorphin upon interaction with heparin or surfaces. *J. Biol. Chem.* 264, 15429–15434.

Hiramatsu, M., Inoue, K., Ambo, A., Sasaki, Y., and Kameyama, T. (2001). Long-lasting antinociceptive effects of a novel dynorphin analogue, Tyr-D-Ala-Phe-Leu-Arg ψ (CH2NH) Arg-NH2, in mice. *Br. J. Pharmacol.* 132, 1948–1956. doi: 10.1038/sj.bjp.0703982

Hutchinson, M. R., Zhang, Y., Brown, K., Coats, B. D., Shridhar, M., Sholar, P. W., et al. (2008). Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur. J. Neurosci.* 28, 20–29. doi: 10.1111/j.1460-9568.2008.06321.x

Jaba, I., Tamba, B., Manolidis, G., and Mungiu, O. (2007). Variation in the analgesic activity of opioid peptide fragments in correlation with the amino acidic sequence. *Cent. Eur. J. Med.* 2, 417–429. doi: 10.2478/s11536-007-0036-0

Kapitzke, D., Vetter, I., and Cabot, P. J. (2005). Endogenous opioid analgesia in peripheral tissues and the clinical implications for pain control. *Ther Clin. Risk Manag.* 1, 279–297.

Kovalitskaya, Y. A., and Navolotskaya, E. V. (2011). Nonopioid effect of β-endorphin. *Biochemistry (Mosc.)* 76, 379–393. doi: 10.1134/S0006297911040018

Lewis, S. S., Hutchinson, M. R., Rezvani, N., Loram, L. C., Zhang, Y., Maier, S. F., et al. (2010). Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1β. *Neuroscience* 165, 569–583. doi: 10.1016/j.neuroscience.2009.10.011

Lin, L., Sohar, I., Lackland, H., and Lobel, P. (2001). The human CLN2 Protein/Tripeptidyl-peptidase I is a serine protease that autoactivates at acidic pH. *J. Biol. Chem.* 276, 2249–2255. doi: 10.1074/jbc.M008562200

McKnight, A. T., Corbett, A. D., and Kosterlitz, H. W. (1983). Increase in potencies of opioid peptides after peptidase inhibition. *Eur. J. Pharmacol.* 86, 393–402. doi: 10.1016/0014-2999(83)90189-9

Mentlein, R. (1999). Dipeptidyl-peptidase IV (CD26)-role in the inactivation of regulatory peptides. *Regul. Pept.* 85, 9–24. doi: 10.1016/S0167-0115(99)00089-0

Montiel, J.-L., Cornille, F., Roques, B. P., and Noble, F. (1997). Nociceptin/Orphanin FQ metabolism: role of aminopeptidase and endopeptidase 24.15. *J. Neurochem.* 68, 354–361. doi: 10.1046/j.1471-4159.1997.68010354.x

Morgan, M., Herath, H. M. D. R., Cabot, P. J., Shaw, P. N., and Hewavitharana, A. K. (2012). Dynorphin A 1–17 biotransformation in inflamed tissue, serum and trypsin solution analysed by liquid chromatography–tandem mass spectrometry. *Anal. Bioanal. Chem.* 404, 3111–3121. doi: 10.1007/s00216-012-6406-8.

Müller, S., and Hochhaus, G. (1995). Metabolism of dynorphin A 1-13 in human blood and plasma. *Pharm. Res.* 12, 1165–1170. doi: 10.1023/A:1016211910107

Nicolas, P., and Choh Hao, L. (1985). Inhibition of analgesia by C-terminal deletion analogs of human β-endorphin. *Biochem. Biophys. Res. Commun.* 127, 649–655. doi: 10.1016/s0006-291x(85)80210-2

Reed, B., Bidlack, J. M., Chait, B. T., and Kreek, M. J. (2008). Extracellular biotransformation of β-endorphin in rat striatum and cerebrospinal fluid. *J. Neuroendocrinol.* 20, 606–616. doi: 10.1111/j.1365-2826.2008.01705.x

Rittner, H. L., Brack, A., and Stein, C. (2008). Pain and the immune system. *Br. J. Anaesth.* 101, 40–44. doi: 10.1093/bja/aen078

Sacerdote, P., and Panerai, A. E. (1989). Analysis of the beta-endorphin structure-related activity on human monocyte chemotaxis: importance of the N- and C-terminal. *Peptides* 10, 565–569. doi: 10.1016/0196-9781(89)90143-5

Sakurada, C., Sakurada, S., Hayashi, T., Katsuyama, S., Tan-No, K., and Sakurada, T. (2003). Degradation of endomorphin-2 at the supraspinal level in mice is initiated by dipeptidyl peptidase IV: an *in vitro* and *in vivo* study. *Biochem. Pharmacol.* 66, 653–661. doi: 10.1016/S0006-2952(03)00391-5

Sandin, J., Nylander, I., and Silberring, J. (1998). Metabolism of β-endorphin in plasma studied by liquid chromatography–electrospray ionization mass spectrometry. *Regul. Pept.* 73, 67–72. doi: 10.1016/s0167-0115(97)01065-3

Schreiter, A., Gore, C., Labuz, D., Fournie-Zaluski, M.-C., Roques, B. P., Stein, C., et al. (2012). Pain inhibition by blocking leukocytic and neuronal opioid peptidases in peripheral inflamed tissue. *FASEB J.* 26, 5161–5171. doi: 10.1096/fj.12-208678

Scornik, O. A., and Botbol, V. (2001). Bestatin as an experimental tool in mammals. *Curr. Drug Metab.* 2, 67–85. doi: 10.2174/1389200013338748

Shane, R., Wilk, S., and Bodnar, R. J. (1999). Modulation of endomorphin-2-induced analgesia by dipeptidyl peptidase IV. *Brain Res.* 815, 278–286. doi: 10.1016/S0006-8993(98)01121-4

Smart, D., Smith, G., and Lambert, D. G. (1995). Mu-opioids activate phospholipase C in SH-SY5Y human neuroblastoma cells via calcium-channel opening. *Biochem. J.* 305, 577–581.

Su, X., Joshi, S. K., Kardos, S., and Gebhart, G. F. (2002). Sodium channel blocking actions of the κ-opioid receptor agonist U50,488 contribute to its visceral antinociceptive effects. *J. Neurophysiol.* 87, 1271–1279.

Tien, L.-T., Fan, L.-W., Ma, T., Loh, H. H., and Ho, I.-K. (2004). Increased diisopropylfluorophosphate-induced toxicity in μ-opioid receptor knockout mice. *J. Neurosci. Res.* 78, 259–267. doi: 10.1002/jnr.20259

Van Den Bergh, P., Rozing, J., and Nagelkerken, L. (1993). Identification of two moieties of beta-endorphin with opposing effects on rat T-cell proliferation. *Immunology* 79, 18–23.

Van Den Burg, E., Metz, J. R., Arends, R., Devreese, B., Vandenberghe, I., Van Beeumen, J., et al. (2001). Identification of beta-endorphins in the pituitary gland and blood plasma of the common carp (*Cyprinus carpio*). *J. Endocrinol.* 169, 271–280. doi: 10.1677/joe.0.1690271

Van Ree, J. M., and De Wied, D. (1982). Behavioral effects of the beta-endorphin fragment 2-9. *Life Sci.* 31, 2383–2386.

Welch, S. P., and Dunlow, L. D. (1993). Antinociceptive activity of intrathecally administered potassium channel openers and opioid agonists: a common mechanism of action? *J. Pharmacol. Exp. Ther.* 267, 390–399.

Yamamoto, Y., Hashimoto, J.-I., Shimamura, M., Yamaguchi, T., and Hazato, T. (2000). Characterization of tynorphin, a potent endogenous inhibitor of dipeptidyl peptidaseIII. *Peptides* 21, 503–508. doi: 10.1016/S0196-9781(00)00174-1

Zakarian, S., and Smyth, D. G. (1982). [beta]-Endorphin is processed differently in specific regions of rat pituitary and brain. *Nature* 296, 250–252.

Received: 13 December 2013; accepted: 28 January 2014; published online: 19 February 2014.

Citation: Asvadi NH, Morgan M, Hewavitharana AK, Shaw PN and Cabot PJ (2014) Biotransformation of beta-endorphin and possible therapeutic implications. Front. Pharmacol. ***5****:18. doi: 10.3389/fphar.2014.00018*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 14 April 2014
doi: 10.3389/fphar.2014.00072

Inhibition of visceral nociceptors

David E. Reed and L. Ashley Blackshaw*

Neurogastroenterolohy Group, Centre for Digestive Diseases, Blizard Institute, Queen Mary University of London, London, UK
**Correspondence: a.blackshaw@qmul.ac.uk*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Peregrine B. Osborne, University of Melbourne, Australia

Keywords: nociceptors, inhibition, visceral pain, enteroendocrine cells, intestinal mucosa

Abdominal pain is one of the most frequent reasons for consulting a doctor. Despite it being a common clinical presentation, abdominal pain remains a difficult entity to treat. This is a result of multiple factors including time course (i.e., acute vs. chronic), etiology (e.g., inflammatory vs. post-inflammatory), and stimulus (i.e., mechanical vs. chemical). About 10% of the population suffer from chronic visceral pain in the form of irritable bowel syndrome (IBS), many of which go undiagnosed. They are hypersensitive to contraction and distension of the gut (Barbara et al., 2011; Keszthelyi et al., 2012), but the pathophysiology of pain is poorly understood. While the intestinal epithelium is a site of high metabolic activity, including digestion/absorption, secretion, hormone release, and immune interactions, it is generally not viewed as a site of pain modulation. However, there are numerous factors at the level of the epithelium capable of modulating pain. This article will highlight the potential role of these factors in nociceptive signaling to identify new therapeutic targets.

NOCICEPTIVE INNERVATION OF THE GUT

Like in other regions of the body and internal organs, the identification of which gastrointestinal primary afferent neurons transmit signals giving rise to pain relies on classification according to their adequate stimuli. It is well-known that cutting and burning of the gut is not necessarily perceived as painful, but generation of intense force by distension or contraction normally is (Cervero, 1994). In the diseased gut less intense forces are required (Coutinho et al., 1996). Therefore, a gut nociceptor is defined for the purposes of this article as a primary afferent fiber that has a high threshold to mechanical stimuli in the healthy gut. They usually also respond directly to inflammatory mediators (Blackshaw et al., 2007). A number of investigations over the last five decades have shown these to innervate blood vessels either within or outside the gut wall (Bessou and Perl, 1966; Blumberg et al., 1983; Song et al., 2009). They are therefore a form of vascular endings, whereas low-threshold afferents innervate the muscle layers of the gut wall or the villi of the mucosa (Brookes et al., 2013). An exception to this rule may well be esophageal afferents which may transmit pain directly from the squamous epithelium in response to acid (which may reflux from the stomach) (Bhat and Bielefeldt, 2006). In the abdominal viscera, it makes sense to place nociceptive endings on blood vessels, since these are less likely to be exposed to mechanical force than those in smooth muscle, and would serve an alarm function for impending dangerous events like rupture or bleeding of the gut. In general, nociceptive afferents innervate the gut via the splanchnic nerves, or in the esophagus via the thoracic sympathetic nerves. There are also sub-populations that innervate via the pelvic and vagal parasympathetic pathways in the rectum and esophagus, respectively.

Whether or not visceral nociceptors differ from those in the rest of the body is controversial, but there are accounts of differing gene expression between the two systems, e.g., Brierley et al. (2008). Another important issue is how nociceptors change in disease states, which may change the way we define them. It is clear from a number of studies that their mechanical thresholds are substantially reduced during inflammation and after healing, so that they respond within the physiological range of stimuli (Jones et al., 2005). In fact Hughes et al found that only nociceptors became markedly sensitized after recovery from inflammation, in both pelvic and splanchnic pathways, whereas other types of afferent fibers were affected little or not at all (Hughes et al., 2009).

The central endings of visceral afferents are most commonly found in the dorsal horn of the spinal cord, where they synapse on projection neurons that send axons to pain processing areas of the brain (Honoré et al., 2002). The dorsal horn is a site of major modulation of pain signals which can override or exacerbate the peripheral signal. Another major site of modulation that is emerging is at the site of signal generation in peripheral endings by a number of endogenous factors. These may be derived from enterocytes, the most numerous cells in the epithelium, or from more specialized cells such as enteroendocrine or immune cells. The examples given below are the best we can find currently and represent derivatives from these three cell types, respectively.

CYCLIC-GUANOSINE-3′,5′-MONOPHOSPHATE (cGMP)

Recently it has been demonstrated that stimulation of guanylate-cyclase C (GC-C) on epithelial cells causes release of cGMP (Blackshaw and Brierley, 2013). This extracellular cGMP inhibits nociceptors in the colon and rectum (Castro et al., 2013; Feng et al., 2013). This is believed to be a mechanism by which linaclotide, a GC-C agonist, alleviates pain in patients with IBS constipation subtype. Demonstration of this epithelial-afferent anti-nociceptive signaling raises the possibility that other mediators released from epithelial cells can inhibit painful signals arising from the gut.

GLUCAGON-LIKE PEPTIDE-1 (GLP-1)

GLP-1 is an incretin located within enteroendocrine L-cells of the intestine that is released upon food intake (Punjabi et al., 2011). It plays an important role in postprandial glucose homeostasis and can alter gastrointestinal (GI) motility (Näslund et al., 1999; Hellström et al., 2008; Edholm et al., 2009; Punjabi et al., 2011). Interestingly, in a phase II trial of IBS patients the GLP-1 analog ROSE-010 reduced acute exacerbations of abdominal pain compared to placebo (Hellström et al., 2009). Whether this analgesic effect was a primary effect on afferent fibers innervating the gut or secondary to a reduction in dysmotility could not be determined. It has been shown that GLP-1 can directly activate vagal afferents within the upper GI tract (Gaisano et al., 2010). However, the expression of GLP-1 is greatest in the distal GI tract. Taken together, this raises an intriguing possibility that release of GLP-1 from L-cells in the distal gut may inhibit spinal afferents responsible for nociceptive transmission. Many treatments for type 2 diabetes are aimed at augmentation of the action of GLP-1 on the endocrine pancreas to boost insulin release. It would be interesting to determine if these treatments have a parallel effect on visceral pain, although this may be small since the effect of GLP-1 on afferent fibers is probably a paracrine effect whereas GLP-1 mimetics augment endocrine actions.

SOMATOSTATIN

There are other epithelial derived mediators with more prominent roles in the upper GI tract that may be capable of inhibiting visceral pain. Somatostatin is found in a variety of cell types in the gut including D-cells in the gut mucosa (Patel, 1999). Its role is largely inhibitory of a number of physiological functions within the GI tract including secretion and motility (Patel, 1999). There is also evidence that it can inhibit visceral perception. Somatostatin receptor agonists reduced afferent firing in "wide-dynamic range" fibers, which would be expected to transmit noxious stimuli (Booth et al., 2001). Jejunal afferents in knockout mice lacking the somatostatin receptor *sst*2 had augmented responses to both low and high threshold distension as well as chemical stimulation suggesting a tonic inhibitory role of somatostatin in visceral sensitivity (Rong et al., 2007). Conversely, in recordings from pelvic afferents in rats, the somatostatin receptor agonist octreotide appeared to reduce visceral pain via a central mechanism rather than at the peripheral site (Su et al., 2001). Interestingly, octreotide inhibited sensation to rectal balloon distension in both healthy volunteers and IBS patients (Hasler et al., 1993, 1994). The investigators believed this was likely due to a peripheral action of octreotide as previous work strongly suggested that octreotide peripherally inhibited cerebral and spinal electrical potentials after electrical stimulation of the rectum (Chey et al., 1995; Schwetz et al., 2004). These contrasting findings suggest that somatostatin may signal to specific afferent subtypes and/or there are species differences. Therefore, further investigations of an inhibitory role of somatostatin on visceral nociception in the distal gut are needed.

GALANIN

Galanin is an important neuromodulator in the enteric nervous system, which acts on three types of G-protein coupled receptor—GAL1, 2, and 3, which are inhibitory, excitatory, and inhibitory respectively. GAL1 and 2 have corresponding effects on mechanosensitivity of vagal afferents at low concentrations (Page et al., 2007), and may indeed have an effect endogenously, but we have only preliminary unpublished data indicating an inhibitory effect of galanin on spinal afferents. It is clear that galanin may have potent central effects on pain processing, especially in neuropathic and inflammatory pain (Wynick et al., 2001), but this peripheral action in the gut is unexplored.

OPIOIDS

T-lymphocytes contain and release beta-endorphin, which activates the mu opioid receptor. This is considered to be an important mechanism of endogenous pain relief, and part of the action of exogenous opioids given therapeutically. We recently showed this is also the case for the gut, where these cells migrate into the tissue and inhibit the response of nociceptors to mechanical stimuli (Hughes et al., 2013b). However, T-cells also release interleukins and other cytokines that can augment the sensitivity of nociceptors or even activate them directly, so there is a balance of excitatory and inhibitory immune modulation of peripheral nociceptive signaling. It is not known if stimuli to the gut in health or disease can preferentially evoke release of opioids or other mediators from white cells, but this would provide a convenient means of pain suppression during normal digestion.

In addition to mu receptors, visceral afferents also express kappa opioid receptors, for which the natural ligand is dynorphin, although it is unclear if endogenous dynorphin plays a role in peripheral pain modulation. However, it has been known for some time that kappa opioid receptor agonists reduce activation of colorectal afferents by distension, and correspondingly pain behaviors evoked in conscious animals (Gebhart et al., 2000). Recent data indicates that visceral nociceptors increase their expression of kappa receptors in a model of chronic visceral hypersensitivity (Hughes et al., 2013a), and correspondingly their inhibition by kappa agonists. This probably underlies the clinical efficacy of kappa ligands on pain in moderate to severe IBS (Mangel and Hicks, 2012).

CONCLUSIONS

In addition to the scope for many interventions that could reduce visceral pain in the clinic targeted at inhibitory mechanisms, there are also many sources of endogenous inhibitors, only some of which have been explored. It is clear nutrients, microbiota and other immunomodulatory influences may impact on the behavior of nociceptors, for example by releasing enteroendocrine mediators or immune-derived mediators. In the case of microbiota, there is also the possibility of direct actions of microbial products on sensory endings, as has already been shown to occur elsewhere in the nervous system (Hsiao et al., 2013).

ACKNOWLEDGMENTS

David E. Reed is supported by a Canadian Association of Gastroenterology/Canadian Institutes for Health Research/Crohn's and Colitis Foundation of Canada Fellowship. L. Ashley Blackshaw is supported by a Wellcome Trust University Award.

REFERENCES

Barbara, G., Cremon, C., De Giorgio, R., Dothel, G., Zecchi, L., Bellacosa, L., et al. (2011). Mechanisms underlying visceral hypersensitivity in irritable bowel syndrome. *Curr. Gastroenterol. Rep.* 13, 308–315. doi: 10.1007/s11894-011-0195-7

Bessou, P., and Perl, E. R. (1966). Amovement receptor of the small intestine. *J. Physiol.* 182, 404–426.

Bhat, Y. M., and Bielefeldt, K. (2006). Capsaicin receptor (TRPV1) and non-erosive reflux disease. *Eur. J. Gastroenterol. Hepatol.* 18, 263–270. doi: 10.1097/00042737-200603000-00006

Blackshaw, L. A., and Brierley, S. M. (2013). Emerging receptor target in the pharmacotherapy of irritable bowel syndrome with constipation. *Expert Rev. Gastroenterol. Hepatol.* 7, 15–19. doi: 10.1586/17474124.2013.820045

Blackshaw, L. A., Brookes, S. J., Grundy, D., and Schemann, M. (2007). Sensory transmission in the gastrointestinal tract. *Neurogastroenterol. Motil.* 19, 1–19. doi: 10.1111/j.1365-2982.2006.00871.x

Blumberg, H., Haupt, P., Jänig, W., and Kohler, W. (1983). Encoding of visceral noxious stimuli in the discharge patterns of visceral afferent fibres from the colon. *Pflugers Arch.* 398, 33–40. doi: 10.1007/BF00584710

Booth, C. E., Kirkup, A. J., Hicks, G. A., Humphrey, P. P., and Grundy, D. (2001). Somatostatin sst(2) receptor-mediated inhibition of mesenteric afferent nerves of the jejunum in the anesthetized rat. *Gastroenterology* 121, 358–369. doi: 10.1053/gast.2001.26335

Brierley, S. M., Page, A. J., Hughes, P. A., Adam, B., Liebregts, T., Cooper, N. J., et al. (2008). Selective role for TRPV4 ion channels in visceral sensory pathways. *Gastroenterology* 134, 2059–2069. doi: 10.1053/j.gastro.2008.01.074

Brookes, S. J., Spencer, N. J., Costa, M., and Zagorodnyuk, V. P. (2013). Extrinsic primary afferent signalling in the gut. *Nat. Rev. Gastroenterol. Hepatol.* 10, 286–296. doi: 10.1038/nrgastro.2013.29

Castro, J., Harrington, A. M., Hughes, P. A., Martin, C. M., Ge, P., Shea, C. M., et al. (2013). Linaclotide inhibits colonic nociceptors and relieves abdominal pain via guanylate cyclase-C and extracellular cyclic guanosine 3',5'-monophosphate. *Gastroenterology* 145, 1334–1346.e1311. doi: 10.1053/j.gastro.2013.08.017

Cervero, F. (1994). Sensory innervation of the viscera: peripheral basis of visceral pain. *Physiol. Rev.* 74, 95–138.

Chey, W. D., Beydoun, A., Roberts, D. J., Hasler, W. L., and Owyang, C. (1995). Octreotide reduces perception of rectal electrical stimulation by spinal afferent pathway inhibition. *Am. J. Physiol.* 269, G821–G826.

Coutinho, S. V., Meller, S. T., and Gebhart, G. F. (1996). Intracolonic zymosan produces visceral hyperalgesia in the rat that is mediated by spinal NMDA and non-NMDA receptors. *Brain Res.* 736, 7–15. doi: 10.1016/0006-8993(96)00661-0

Edholm, T., Cejvan, K., Abdel-Halim, S. M., Efendic, S., Schmidt, P. T., and Hellström, P. M. (2009). The incretin hormones GIP and GLP-1 in diabetic rats: effects on insulin secretion and small bowel motility. *Neurogastroenterol. Motil.* 21, 313–321. doi: 10.1111/j.1365-2982.2008.01229.x

Feng, B., Kiyatkin, M. E., La, J. H., Ge, P., Solinga, R., Silos-Santiago, I., et al. (2013). Activation of guanylate cyclase-C attenuates stretch responses and sensitization of mouse colorectal afferents. *J. Neurosci.* 33, 9831–9839. doi: 10.1523/JNEUROSCI.5114-12.2013

Gaisano, G. G., Park, S. J., Daly, D. M., and Beyak, M. J. (2010). Glucagon-like peptide-1 inhibits voltage-gated potassium currents in mouse nodose ganglion neurons. *Neurogastroenterol. Motil.* 22, 470–479, e111. doi: 10.1111/j.1365-2982.2009.01430.x

Gebhart, G. F., Su, X., Joshi, S., Ozaki, N., and Sengupta, J. N. (2000). Peripheral opioid modulation of visceral pain. *Ann. N.Y. Acad. Sci.* 909, 41–50. doi: 10.1111/j.1749-6632.2000.tb06675.x

Hasler, W. L., Soudah, H. C., and Owyang, C. (1993). A somatostatin analogue inhibits afferent pathways mediating perception of rectal distention. *Gastroenterology* 104, 1390–1397.

Hasler, W. L., Soudah, H. C., and Owyang, C. (1994). Somatostatin analog inhibits afferent response to rectal distention in diarrhea-predominant irritable bowel patients. *J. Pharmacol. Exp. Ther.* 268, 1206–1211.

Hellström, P. M., Hein, J., Bytzer, P., Björnssön, E., Kristensen, J., and Schambye, H. (2009). Clinical trial: the glucagon-like peptide-1 analogue ROSE-010 for management of acute pain in patients with irritable bowel syndrome: a randomized, placebo-controlled, double-blind study. *Aliment. Pharmacol. Ther.* 29, 198–206. doi: 10.1111/j.1365-2036.2008.03870.x

Hellström, P. M., Näslund, E., Edholm, T., Schmidt, P. T., Kristensen, J., Theodorsson, E., et al. (2008). GLP-1 suppresses gastrointestinal motility and inhibits the migrating motor complex in healthy subjects and patients with irritable bowel syndrome. *Neurogastroenterol. Motil.* 20, 649–659. doi: 10.1111/j.1365-2982.2007.01079.x

Honoré, P., Kamp, E. H., Rogers, S. D., Gebhart, G. F., and Mantyh, P. W. (2002). Activation of lamina I spinal cord neurons that express the substance P receptor in visceral nociception and hyperalgesia. *J. Pain* 3, 3–11. doi: 10.1054/jpai.2002.27001

Hsiao, E. Y., McBride, S. W., Hsien, S., Sharon, G., Hyde, E. R., McCue, T., et al. (2013). Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155, 1451–1463. doi: 10.1016/j.cell.2013.11.024

Hughes, P. A., Brierley, S. M., Martin, C. M., Brookes, S. J., Linden, D. R., and Blackshaw, L. A. (2009). Post-inflammatory colonic afferent sensitisation: different subtypes, different pathways and different time courses. *Gut* 58, 1333–1341. doi: 10.1136/gut.2008.170811

Hughes, P. A., Castro, J., Harrington, A. M., Isaacs, N., Moretta, M., Hicks, G. A., et al. (2013a). Increased κ-opioid receptor expression and function during chronic visceral hypersensitivity. *Gut*. doi: 10.1136/gutjnl-2013-306240. [Epub ahead of print].

Hughes, P. A., Harrington, A. M., Castro, J., Liebregts, T., Adam, B., Grasby, D. J., et al. (2013b). Sensory neuro-immune interactions differ between irritable bowel syndrome subtypes. *Gut* 62, 1456–1465. doi: 10.1136/gutjnl-2011-301856

Jones, R. C., Xu, L., and Gebhart, G. F. (2005). The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J. Neurosci.* 25, 10981–10989. doi: 10.1523/JNEUROSCI.0703-05.2005

Keszthelyi, D., Troost, F. J., Simrén, M., Ludidi, S., Kruimel, J. W., Conchillo, J. M., et al. (2012). Revisiting concepts of visceral nociception in irritable bowel syndrome. *Eur. J. Pain* 16, 1444–1454. doi: 10.1002/j.1532-2149.2012.00147.x

Mangel, A. W., and Hicks, G. A. (2012). Asimadoline and its potential for the treatment of diarrhea-predominant irritable bowel syndrome: a review. *Clin. Exp. Gastroenterol.* 5, 1–10. doi: 10.2147/CEG.S23274

Näslund, E., Bogefors, J., Skogar, S., Grybäck, P., Jacobsson, H., Holst, J. J., et al. (1999). GLP-1 slows solid gastric emptying and inhibits insulin, glucagon, and PYY release in humans. *Am. J. Physiol.* 277, R910–R916.

Page, A. J., Slattery, J. A., Brierley, S. M., Jacoby, A. S., and Blackshaw, L. A. (2007). Involvement of galanin receptors 1 and 2 in the modulation of mouse vagal afferent mechanosensitivity. *J. Physiol.* 583, 675–684. doi: 10.1113/jphysiol.2007.135939

Patel, Y. C. (1999). Somatostatin and its receptor family. *Front. Neuroendocrinol.* 20, 157–198. doi: 10.1006/frne.1999.0183

Punjabi, M., Arnold, M., Geary, N., Langhans, W., and Pacheco-López, G. (2011). Peripheral glucagon-like peptide-1 (GLP-1) and satiation. *Physiol. Behav.* 105, 71–76. doi: 10.1016/j.physbeh.2011.02.038

Rong, W., Winchester, W. J., and Grundy, D. (2007). Spontaneous hypersensitivity in mesenteric afferent nerves of mice deficient in the sst2 subtype of somatostatin receptor. *J. Physiol.* 581, 779–786. doi: 10.1113/jphysiol.2006.125187

Schwetz, I., Naliboff, B., Munakata, J., Lembo, T., Chang, L., Matin, K., et al. (2004). Antihyperalgesic effect of octreotide in patients with irritable bowel syndrome. *Aliment. Pharmacol. Ther.* 19, 123–131. doi: 10.1111/j.1365-2036.2004.01774.x

Song, X., Chen, B. N., Zagorodnyuk, V. P., Lynn, P. A., Blackshaw, L. A., Grundy, D., et al. (2009). Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract. *Gastroenterology* 137, 274–284, 284.e271. doi: 10.1053/j.gastro.2009.02.061

Su, X., Burton, M. B., and Gebhart, G. F. (2001). Effects of octreotide on responses to colorectal

distension in the rat. *Gut* 48, 676–682. doi: 10.1136/gut.48.5.676

Wynick, D., Thompson, S. W., and McMahon, S. B. (2001). The role of galanin as a multi-functional neuropeptide in the nervous system. *Curr. Opin. Pharmacol.* 1, 73–77. doi: 10.1016/S1471-4892(01)00006-6

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 06 February 2014; accepted: 27 March 2014; published online: 14 April 2014.

Citation: Reed DE and Blackshaw LA (2014) Inhibition of visceral nociceptors. Front. Pharmacol. ***5****:72. doi: 10.3389/fphar.2014.00072*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

OPINION ARTICLE
published: 05 March 2014
doi: 10.3389/fphar.2014.00027

Understanding and targeting centrally mediated visceral pain in inflammatory bowel disease

Kristen E. Farrell[1], Robert J. Callister[1] and Simon Keely[1,2]*

[1] *School of Biomedical Sciences and Pharmacy, The University of Newcastle, Callaghan, NSW, Australia*
[2] *Gastrointestinal Research Group, Viruses, Infection/Immunity, Vaccines and Asthma Program, Hunter Medical Research Institute, New Lambton Heights, NSW, Australia*
**Correspondence: simon.keely@newcastle.edu.au*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Bruno Bonaz, Grenoble Faculty of Medicine and Hospital, France
Matthew W. Carroll, University of Alberta, Canada
Rajaraman D. Eri, University of Tasmania, Australia

Keywords: inflammatory bowel diseases, colitis, visceral pain, spinal cord, central sensitization

Chronic abdominal pain is a debilitating symptom of inflammatory bowel disease (IBD): a chronic inflammatory condition of the gastrointestinal tract, which includes Crohn's disease (CD) and ulcerative colitis (UC), and is characterized by periods of inflammation and remission. During inflammation, pain is often present due to the activation of afferent nerve endings in the gut by inflammatory mediators (Beyak and Vanner, 2005). Importantly, pain associated with IBD often persists after inflammation has resolved, based on clinical and endoscopic examination, and is often "referred" from the gut to cutaneous or other visceral regions (Minderhoud et al., 2004). Based on what we know about pain processing circuits, this suggests the source of chronic and referred pain lies within the central nervous system (CNS). These clinical observations are supported by work in animal models of visceral inflammation that have provided behavioral, anatomical, molecular, and physiological evidence to implicate spinal circuits in the development of altered pain responses in IBD (Farrell et al., 2014). In spite of this evidence, little is known about the precise mechanisms of chronic pain development and maintenance in IBD. This lack of understanding severely limits current therapeutic approaches for IBD pain management.

PAIN MANAGEMENT IN IBD

Abdominal pain is a common symptom of IBD, with up to 70% of patients presenting with pain during disease onset, or during periods of relapse (Wagtmans et al., 1998). Pain is an indicator of inflammation, and in the case of IBD, occurs in response to the sensitization of intestinal sensory neurons by inflammatory cytokines. Importantly, inflammation does not appear to be the sole cause of pain in IBD, as 30–50% of patients in clinical remission (i.e., no detectable inflammation in the gut) continue to experience severe abdominal pain (Minderhoud et al., 2004; Farrokhyar et al., 2006; Siegel and MacDermott, 2009). The lack of effective pain management for this patient cohort is problematic in its own right, but is also associated with significantly decreased health-related quality of life scores, increased stress, anxiety, and depression (Farrokhyar et al., 2006; Schirbel et al., 2010). As a consequence, a significant number of IBD patients are chronically treated with narcotics (Edwards et al., 2001; Cross et al., 2005; Makharia, 2011). Unfortunately, long-term narcotic use gives rise to a range of side effects in IBD patients, such as nausea, reduced gastrointestinal motility, and narcotic bowel syndrome; i.e., increased abdominal pain despite escalating narcotic use. These detrimental effects worsen with continued drug use (Grunkemeier et al., 2007), yet the use of narcotics persists, especially for IBD patients during hospitalization and surgery associated with relapse (Lian et al., 2010; Long et al., 2012). These issues are compounded by the lack of alternative pain management therapies, as analgesics such as non-steroidal anti-inflammatory drugs (NSAIDS), including selective COX-2 inhibitors, are linked to disease exacerbation (Bielefeldt et al., 2009; Srinath et al., 2012). Given the lack of options and efficacy issues with current pharmaceutics, it is not surprising that narcotic addiction is a major problem in IBD. Rates of narcotic addiction are over 5% in Crohn's patients and 2.7% overall in IBD patients (Edwards et al., 2001), with the risk increased by 8-fold in those patients with concurrent psychiatric disorders, including depression, anxiety, sexual, emotional and physical abuse and substance abuse (Edwards et al., 2001; Hanson et al., 2009). Thus, there is critical need for new approaches and therapies for IBD pain management. However, before new therapeutics can be designed, the mechanisms responsible for the development and maintenance of chronic abdominal pain in IBD must be understood.

SENSORY TRANSMISSION IN THE GASTROINTESTINAL TRACT

The gastrointestinal tract is unique as its function is regulated by both intrinsic and extrinsic components of the autonomic nervous system. Intrinsic innervation is controlled by the enteric nervous system (ENS), which consists of connecting nerve plexuses that run between the muscular layers and the submucosa of the gut wall. The ENS controls gastrointestinal motility, secretion and absorption, which are all essential for gut function, but plays no major role in pain transmission (Blackshaw et al., 2007).

The extrinsic innervation of the gastrointestinal tract includes efferent

parasympathetic and sympathetic autonomic pathways that are involved in the modulation of ENS activity. There are also extrinsic sensory afferents that convey information, including (but not exclusive to) noxious (potentially painful) and innocuous sensations, to the spinal cord and brainstem. Brookes et al. have compiled a comprehensive review of extrinsic sensory afferent types that innervate the gut wall (Brookes et al., 2013). Sensory afferents convey signals (including noxious signals that cause pain) from the lower gastrointestinal tract to the CNS via two major nerve trunks, the splanchnic and pelvic nerves (Gebhart, 2000; Blackshaw et al., 2007). These nerves enter the CNS via the spinal cord dorsal horn, where sensory information is subject to extensive modulation via local interneuron networks and descending influences (Melzack and Wall, 1965). Finally, signals from the gut ascend to the cortex via several pathways, including the spinothalamic, spinoreticular and spinomesencephalic tracts, where perception can occur (Craig, 2002; Jones et al., 2006).

ALTERED NERVOUS SYSTEM SIGNALING IN IBD-RELATED PAIN

There is a growing body of evidence from animal studies, suggesting that a dysfunction of the nervous system plays a role in the development of a chronic pain state in IBD (Willis and Westlund, 1997; Gebhart, 1999; Hughes et al., 2009). Most research on the neural mechanisms responsible for pain in IBD has focused on the peripheral nervous system, where strong evidence of hyperexcitability in afferent nerves has been documented. For example, in animal models of chemically induced colitis, hypersensitivity of mechanosensitive sensory afferents following inflammation has been demonstrated, with reduced thresholds for activation and increased frequencies of action potential discharge reported (Hughes et al., 2009; Feng et al., 2012). Importantly, this hypersensitivity is sustained following recovery from inflammation, which may be a contributing factor in the development of chronic pain (Hughes et al., 2009; Feng et al., 2012).

Other work has shown that colonic inflammation can result in increased behavioral responses to mechanical distension and intraluminal administration of capsaicin into the colon, and that this hypersensitivity can be reversed by antagonism of TRPV1 ion channels, implicating TRPV1 expression in the development of peripheral hyperalgesia (Miranda et al., 2007). Taken together, these data indicate that after colonic inflammation, sensory afferents can become hypersensitive to both mechanical and chemical stimulation, and this can be attributed, at least in part, to changes in the expression of ion channels within sensory afferents.

Although the evidence for peripheral contributions to colonic hypersensitivity is strong, the existence of referred pain in IBD patients and in animal models of colitis suggests the CNS, particularly the spinal cord dorsal horn, is implicated in the development of abnormal pain, as referred pain cannot be explained by hypersensitive colon afferents alone (Bernstein et al., 1996). Referred pain is a common sequelae of visceral pain, in which diffuse, poorly localized pain from the viscera "referred" to other areas: most often to the skin (Cervero and Laird, 1999). For IBD, pain is often referred to the mid-lower back, abdomen, and legs (Ritchie, 1973; Ness et al., 1990; Accarino et al., 1995; Bernstein et al., 1996) and, not surprisingly, is a source of great discomfort. It is thought that referred pain occurs when there is overlap between visceral and somatic pathways within the CNS (Traub, 2000). The region of the CNS where this cross over is most likely to occur is within the spinal cord dorsal horn, as the dorsal horn receives inputs from skin, joints and muscle as well as from the viscera (Almeida et al., 2004).

Evidence that the CNS is involved in the processing of inflammation-induced visceral pain has been demonstrated in animal models at behavioral, anatomical, molecular, and physiological levels of analysis (Farrell et al., 2014). Briefly, it has been shown that animals develop referred hypersensitivity of the hind paw and abdomen following colitis (Lamb et al., 2006), and that this sensitivity remains after the resolution of inflammation (Eijkelkamp et al., 2009). Likewise, colitis results in increased dorsal horn expression of markers of neural activation (cFos and pERK) following distension (Traub and Murphy, 2002; Harrington et al., 2012), as well as neuropeptides commonly linked with pain signaling, such as Substance P and CGRP (Sun and Luo, 2004). Finally, the excitability of spinal dorsal horn neurons has been shown to increase following colitis, with extracellular recordings demonstrating a decrease in action potential threshold and increases in spontaneous neural activity (Al-Chaer et al., 1997). We have previously presented a systematic review outlining the evidence for altered CNS activity following gastrointestinal inflammation (Farrell et al., 2014).

Magnetic resonance imaging studies have also demonstrated changes in the structure and functional activation of cortical and subcortical regions in IBD patients compared with healthy controls. Using fMRI, patients with IBD were shown to have differing patterns of cortical activation and deactivation in response to noxious rectal balloon distension (Bernstein et al., 2002). Likewise, CD patients exhibit altered gray matter volumes in cortical (frontal and anterior midcingulate corticies) and subcortical regions that are associated with emotion, cognition, and nociception (Agostini et al., 2013). Therefore, there is evidence for both spinal and supra-spinal alterations as a consequence of chronic pain in IBD.

FUTURE ADVANCES IN IBD PAIN MANAGEMENT

The presence of chronic pain in IBD patients represents a major health burden, as it can impact significantly on the quality of life and mental health of long-term sufferers. Current pain management strategies are not optimal: often ineffective and associated with a number of detrimental off-target effects (Edwards et al., 2001; Hanson et al., 2009; Siegel and MacDermott, 2009; Srinath et al., 2012). Recent studies have demonstrated that targeting specific CNS pathways could alleviate visceral pain in animal models. For instance, targeting of central N-methyl-D-aspartate (NMDA) receptors is of particular interest, as there is strong evidence for spinal NMDA receptor-dependent neurotransmission underling the development of central sensitization (Haley et al., 1990; Ren et al., 1992). In models of colonic

inflammation, microinjection of the NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) into the rostral ventromedial medulla (RVM) was shown to attenuate exaggerated behavioral responses to colon distension. This attenuation was not observed for injections outside the RVM (Coutinho et al., 1998). Since spinal nociceptive transmission is subject to modulation from supraspinal sites such as the RVM, this demonstrates that visceral hyperalgesia can be influenced by NMDA-dependent descending pain modulation. Likewise, cross-organ sensitization between the inflamed colon and the urethra was reversed by intrathecal injection of APV, and by antagonism NR2B: a subunit of the NMDA receptor, using Co-101244 (Peng et al., 2009). Therefore, direct antagonism of spinal NMDA receptors, specifically the NR2B subunit, can attenuate central sensitization caused by inflammation of the colon.

Another potential candidate recently investigated for visceral pain management is the nociceptive ion channel TPVR1. TRPV1 channels are activated by heat (> 43°) and capsasin (Christoph et al., 2006) and are expressed in up to 80% of visceral afferents (vs. less than one third in somatic afferents) (Robinson and Gebhart, 2008). This makes these channels an appealing pharmacological target. Silencing of TRPV1 in the CNS using intrathecal injection of TRPV1-specific siRNA 4 days prior to intrarectal capsaicin administration was shown to reduce capsasin-induced spontaneous pain behaviors in rats (Christoph et al., 2006). Importantly, in models of neuropathic pain, TRPV1-specific siRNA analgesia could be maintained for 4–5 days (Christoph et al., 2006). Interestingly, the siRNA used, VsiR1, is directed against a segment of TRPV1 mRNA that is conserved in mouse, rat and human, suggesting this approach may be easily translatable into humans.

Although new CNS targets for managing chronic visceral pain are emerging, the blood brain barrier represents a continual obstacle in the development of new therapeutics. The blood brain barrier presents physical, transport and metabolic barriers that can be modulated and regulated under both normal and pathological states (Abbott et al., 2010). Direct intrathecal injection of therapeutics is the most direct way to bypass these barriers, however, it is by far the least practical solution in a clinical setting. Importantly, recent advances in nanoparticle carrier technologies may allow better targeting of CNS pathways involved in visceral pain through site-directed drug delivery and enhanced penetration of the blood brain barrier (Hua and Wu, 2013).

In summary, visceral pain in chronic inflammatory diseases is poorly understood and current therapeutic strategies are limited. Further research is required to improve our knowledge of the events leading to the development chronic visceral pain. In addition, the discovery of improved CNS targets for pain management along with improved methods for drug delivery, are urgently required for the management IBD patients with chronic pain.

ACKNOWLEDGMENTS

This work was supported by grants form from the National Health and Medical Research Council of Australia (APP1021582 to Simon Keely, APP1067146 to Robert J. Callister and Simon Keely), the Hunter Medical Research Institute and the University of Newcastle Priority Research Centre for Translational Neuroscience.

REFERENCES

Abbott, N. J., Patabendige, A. A., Dolman, D. E., Yusof, S. R., and Begley, D. J. (2010). Structure and function of the blood-brain barrier. *Neurobiol. Dis.* 37, 13–25. doi: 10.1016/j.nbd.2009.07.030

Accarino, A. M., Azpiroz, F., and Malagelada, J. R. (1995). Selective dysfunction of mechanosensitive intestinal afferents in irritable bowel syndrome. *Gastroenterology* 108, 636–643. doi: 10.1016/0016-5085(95)90434-4

Agostini, A., Benuzzi, F., Filippini, N., Bertani, A., Scarcelli, A., Farinelli, V., et al. (2013). New insights into the brain involvement in patients with Crohn's disease: a voxel-based morphometry study. *Neurogastroenterol. Motil.* 25, 147–e82. doi: 10.1111/nmo.12017

Al-Chaer, E. D., Westlund, K. N., and Willis, W. D. (1997). Sensitization of postsynaptic dorsal column neuronal responses by colon inflammation. *Neuroreport* 8, 3267–3273. doi: 10.1097/00001756-199710200-00016

Almeida, T. F., Roizenblatt, S., and Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain Res.* 1000, 40–56. doi: 10.1016/j.brainres.2003.10.073

Bernstein, C. N., Frankenstein, U. N., Rawsthorne, P., Pitz, M., Summers, R., and McIntyre, M. C. (2002). Cortical mapping of visceral pain in patients with GI disorders using functional magnetic resonance imaging. *Am. J. Gastroenterol.* 97, 319–327. doi: 10.1111/j.1572-0241.2002.05464.x

Bernstein, C. N., Niazi, N., Robert, M., Mertz, H., Kodner, A., Munakata, J., et al. (1996). Rectal afferent function in patients with inflammatory and functional intestinal disorders. *Pain* 66, 151–161. doi: 10.1016/0304-3959(96)03062-X

Beyak, M. J., and Vanner, S. (2005). Inflammation-induced hyperexcitability of nociceptive gastrointestinal DRG neurones: the role of voltage-gated ion channels. *Neurogastroenterol. Motil.* 17, 175–186. doi: 10.1111/j.1365-2982.2004.00596.x

Bielefeldt, K., Davis, B., and Binion, D. G. (2009). Pain and inflammatory bowel disease. *Inflamm. Bowel Dis.* 15, 778–788. doi: 10.1002/ibd.20848

Blackshaw, L. A., Brookes, S. J., Grundy, D., and Schemann, M. (2007). Sensory transmission in the gastrointestinal tract. *Neurogastroenterol. Motil.* 19, 1–19. doi: 10.1111/j.1365-2982.2006.00871.x

Brookes, S. J., Spencer, N. J., Costa, M., and Zagorodnyuk, V. P. (2013). Extrinsic primary afferent signalling in the gut. *Nat. Rev. Gastroenterol. Hepatol.* 10, 286–296. doi: 10.1038/nrgastro.2013.29

Cervero, F., and Laird, J. M. (1999). Visceral pain. *Lancet* 353, 2145–2148. doi: 10.1016/S0140-6736(99)01306-9

Christoph, T., Grunweller, A., Mika, J., Schafer, M. K., Wade, E. J., Weihe, E., et al. (2006). Silencing of vanilloid receptor TRPV1 by RNAi reduces neuropathic and visceral pain *in vivo*. *Biochem. Biophys. Res. Commun.* 350, 238–243. doi: 10.1016/j.bbrc.2006.09.037

Coutinho, S. V., Urban, M. O., and Gebhart, G. F. (1998). Role of glutamate receptors and nitric oxide in the rostral ventromedial medulla in visceral hyperalgesia. *Pain* 78, 59–69. doi: 10.1016/S0304-3959(98)00137-7

Craig, A. D. (2002). How do you feel? Interoception: the sense of the physiological condition of the body. *Nat. Rev. Neurosci.* 3, 655–666. doi: 10.1038/nrn894

Cross, R. K., Wilson, K. T., and Binion, D. G. (2005). Narcotic use in patients with Crohn's disease. *Am. J. Gastroenterol.* 100, 2225–2229. doi: 10.1111/j.1572-0241.2005.00256.x

Edwards, J. T., Radford-Smith, G. L., and Florin, T. H. (2001). Chronic narcotic use in inflammatory bowel disease patients: prevalence and clinical characteristics. *J. Gastroenterol. Hepatol.* 16, 1235–1238. doi: 10.1046/j.1440-1746.2001.02468.x

Eijkelkamp, N., Heijnen, C. J., Elsenbruch, S., Holtmann, G., Schedlowski, M., and Kavelaars, A. (2009). G protein-coupled receptor kinase 6 controls post-inflammatory visceral hyperalgesia. *Brain Behav. Immun.* 23, 18–26. doi: 10.1016/j.bbi.2008.07.006

Farrell, K. E., Keely, S., Graham, B. A., Callister, R., and Callister, R. J. (2014). A systematic review of the evidence for central nervous system plasticity in animal models of inflammatory-mediated gastrointestinal pain. *Inflamm. Bowel Dis.* 20, 176–195. doi: 10.1097/01.MIB.0000437499.52922.b1

Farrokhyar, F., Marshall, J. K., Easterbrook, B., and Irvine, E. J. (2006). Functional gastrointestinal disorders and mood disorders in patients

with inactive inflammatory bowel disease: prevalence and impact on health. *Inflamm. Bowel Dis.* 12, 38–46. doi: 10.1097/01.MIB.0000195391.49762.89

Feng, B., La, J. H., Schwartz, E. S., Tanaka, T., McMurray, T. P., and Gebhart, G. F. (2012). Long-term sensitization of mechanosensitive and -insensitive afferents in mice with persistent colorectal hypersensitivity. *Am. J. Physiol. Gastrointest. Liver Physiol.* 302, G676–G683. doi: 10.1152/ajpgi.00490.2011

Gebhart, G. F. (1999). Peripheral contributions to visceral hyperalgesia. *Can. J. Gastroenterol.* 13 (Suppl. A), 37A–41A.

Gebhart, G. F. (2000). Visceral pain-peripheral sensitisation. *Gut* 47 (Suppl. 4), iv54–iv55. discussion: iv58. doi: 10.1136/gut.47.suppl_4.iv54

Grunkemeier, D. M., Cassara, J. E., Dalton, C. B., and Drossman, D. A. (2007). The narcotic bowel syndrome: clinical features, pathophysiology, and management. *Clin. Gastroenterol. Hepatol.* 5, 1126–1139; quiz 1121–1122. doi: 10.1016/j.cgh.2007.06.013

Haley, J. E., Sullivan, A. F., and Dickenson, A. H. (1990). Evidence for spinal N-methyl-D-aspartate receptor involvement in prolonged chemical nociception in the rat. *Brain Res.* 518, 218–226. doi: 10.1016/0006-8993(90)90975-H

Hanson, K. A., Loftus, E. V. Jr., Harmsen, W. S., Diehl, N. N., Zinsmeister, A. R., and Sandborn, W. J. (2009). Clinical features and outcome of patients with inflammatory bowel disease who use narcotics: a case-control study. *Inflamm. Bowel Dis.* 15, 772–777. doi: 10.1002/ibd.20847

Harrington, A. M., Brierley, S. M., Isaacs, N., Hughes, P. A., Castro, J., and Blackshaw, L. A. (2012). Sprouting of colonic afferent central terminals and increased spinal mitogen-activated protein kinase expression in a mouse model of chronic visceral hypersensitivity. *J. Comp. Neurol.* 520, 2241–2255. doi: 10.1002/cne.23042

Hua, S., and Wu, S. Y. (2013). The use of lipid-based nanocarriers for targeted pain therapies. *Front. Pharmacol.* 4:143. doi: 10.3389/fphar.2013.00143

Hughes, P. A., Brierley, S. M., Martin, C. M., Brookes, S. J., Linden, D. R., and Blackshaw, L. A. (2009). Post-inflammatory colonic afferent sensitisation: different subtypes, different pathways and different time courses. *Gut* 58, 1333–1341. doi: 10.1136/gut.2008.170811

Jones, M. P., Dilley, J. B., Drossman, D., and Crowell, M. D. (2006). Brain-gut connections in functional GI disorders: anatomic and physiologic relationships. *Neurogastroenterol. Motil.* 18, 91–103. doi: 10.1111/j.1365-2982.2005.00730.x

Lamb, K., Zhong, F., Gebhart, G. F., and Bielefeldt, K. (2006). Experimental colitis in mice and sensitization of converging visceral and somatic afferent pathways. *Am. J. Physiol. Gastrointest. Liver Physiol.* 290, G451–G457. doi: 10.1152/ajpgi.00353.2005

Lian, L., Fazio, V. W., Hammel, J., and Shen, B. (2010). Impact of narcotic use on the requirement for colectomy in inpatients with ulcerative colitis. *Dis. Colon Rectum* 53, 1295–1300. doi: 10.1007/DCR.0b013e3181e7562c

Long, M. D., Barnes, E. L., Herfarth, H. H., and Drossman, D. A. (2012). Narcotic use for inflammatory bowel disease and risk factors during hospitalization. *Inflamm. Bowel Dis.* 18, 869–876. doi: 10.1002/ibd.21806

Makharia, G. K. (2011). Understanding and treating abdominal pain and spasms in organic gastrointestinal diseases: inflammatory bowel disease and biliary diseases. *J. Clin. Gastroenterol.* 45 (Suppl.), S89–S93. doi: 10.1097/MCG.0b013e31821fbd82

Melzack, R., and Wall, P. D. (1965). Pain mechanisms: a new theory. *Science* 150, 971–979. doi: 10.1126/science.150.3699.971

Minderhoud, I. M., Oldenburg, B., Wismeijer, J. A., van Berge Henegouwen, G. P., and Smout, A. J. (2004). IBS-like symptoms in patients with inflammatory bowel disease in remission; relationships with quality of life and coping behavior. *Dig. Dis. Sci.* 49, 469–474. doi: 10.1023/B:DDAS.0000020506.84248.f9

Miranda, A., Nordstrom, E., Mannem, A., Smith, C., Banerjee, B., and Sengupta, J. N. (2007). The role of transient receptor potential vanilloid 1 in mechanical and chemical visceral hyperalgesia following experimental colitis. *Neuroscience* 148, 1021–1032. doi: 10.1016/j.neuroscience.2007.05.034

Ness, T. J., Metcalf, A. M., and Gebhart, G. F. (1990). A psychophysiological study in humans using phasic colonic distension as a noxious visceral stimulus. *Pain*, 43, 377–386. doi: 10.1016/0304-3959(90)90035-C

Peng, H.-Y., Chen, G.-D., Tung, K.-C., Lai, C.-Y., Hsien, M.-C., Chiu, C.-H., et al. (2009). Colon mustard oil instillation induced cross-organ reflex sensitization on the pelvic-urethra reflex activity in rats. *Pain* 142, 75–88. doi: 10.1016/j.pain.2008.11.017

Ren, K., Hylden, J. L., Williams, G. M., Ruda, M. A., and Dubner, R. (1992). The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal horn neuronal activity in rats with unilateral inflammation. *Pain* 50, 331–344. doi: 10.1016/0304-3959(92)90039-E

Ritchie, J. (1973). Pain from distension of the pelvic colon by inflating a balloon in the irritable colon syndrome. *Gut* 14, 125–132. doi: 10.1136/gut.14.2.125

Robinson, D. R., and Gebhart, G. F. (2008). Inside information: the unique features of visceral sensation. *Mol. Interv.* 8, 242–253. doi: 10.1124/mi.8.5.9

Schirbel, A., Reichert, A., Roll, S., Baumgart, D. C., Buning, C., Wittig, B., et al. (2010). Impact of pain on health-related quality of life in patients with inflammatory bowel disease. *World J. Gastroenterol.* 16, 3168–3177. doi: 10.3748/wjg.v16.i25.3168

Siegel, C. A., and MacDermott, R. P. (2009). Is chronic pain an extraintestinal manifestation of IBD? *Inflamm. Bowel Dis.* 15, 769–771. doi: 10.1002/ibd.20844

Srinath, A. I., Walter, C., Newara, M. C., and Szigethy, E. M. (2012). Pain management in patients with inflammatory bowel disease: insights for the clinician. *Therap. Adv. Gastroenterol.* 5, 339–357. doi: 10.1177/1756283X12446158

Sun, Y.-N., and Luo, J.-Y. (2004). Effects of tegaserod on Fos, substance P and calcitonin gene-related peptide expression induced by colon inflammation in lumbarsacral spinal cord. *World J. Gastroenterol.* 10, 1830–1833.

Traub, R. J. (2000). Evidence for thoracolumbar spinal cord processing of inflammatory, but not acute colonic pain. *Neuroreport* 11, 2113–2116. doi: 10.1097/00001756-200007140-00011

Traub, R. J., and Murphy, A. (2002). Colonic inflammation induces fos expression in the thoracolumbar spinal cord increasing activity in the spinoparabrachial pathway. *Pain* 95, 93–102. doi: 10.1016/S0304-3959(01)00381-5

Wagtmans, M. J., Verspaget, H. W., Lamers, C. B., and van Hogezand, R. A. (1998). Crohn's disease in the elderly: a comparison with young adults. *J. Clin. Gastroenterol.* 27, 129–133. doi: 10.1097/00004836-199809000-00005

Willis, W. D., and Westlund, K. N. (1997). Neuroanatomy of the pain system and of the pathways that modulate pain. *J. Clin. Neurophysiol.* 14, 2–31. doi: 10.1097/00004691-199701000-00002

Received: 14 January 2014; accepted: 13 February 2014; published online: 05 March 2014.

Citation: Farrell KE, Callister RJ and Keely S (2014) Understanding and targeting centrally mediated visceral pain in inflammatory bowel disease. Front. Pharmacol. ***5****:27. doi: 10.3389/fphar.2014.00027*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

PERSPECTIVE ARTICLE
published: 25 February 2014
doi: 10.3389/fphar.2014.00022

The search for novel analgesics: re-examining spinal cord circuits with new tools

Kelly M. Smith[1], Jessica F. Madden[1], Robert J. Callister[1], David I. Hughes[2] and Brett A. Graham[1]*

[1] School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, Hunter Medical Research Institute, University of Newcastle, Newcastle, NSW, Australia

[2] Institute of Neuroscience and Psychology, University of Glasgow, Glasgow, UK

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Philippe Seguela, Montreal Neurological Institute, Canada
Ernest Jennings, James Cook University, Australia

***Correspondence:**
Brett A. Graham, School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, Hunter Medical Research Institute, University of Newcastle, Room no. 411 Medical Sciences Building, University Drive, Callaghan, NSW 2308, Australia
e-mail: brett.graham@newcastle.edu.au

In this perspective, we propose the absence of detailed information regarding spinal cord circuits that process sensory information remains a major barrier to advancing analgesia. We highlight recent advances showing that functionally discrete populations of neurons in the spinal cord dorsal horn (DH) play distinct roles in processing sensory information. We then discuss new molecular, electrophysiological, and optogenetic techniques that can be employed to understand how DH circuits process tactile and nociceptive information. We believe this information can drive the development of entirely new classes of pharmacotherapies that target key elements in spinal circuits to selectively modify sensory function and blunt pain.

Keywords: dorsal horn, interneuron, optogenetics, genetic profiling

INTRODUCTION

Pain is an important percept within our somatosensory system. It provides an alert to actual or potential tissue damage and ensures injured tissue is protected during healing (Basbaum et al., 2009). Despite its important biological function, the unpleasant nature of pain and its tendency to often outlast the initial stimulus has driven a search for strategies to relieve it for many millennia. For example, Sumerian clay tablets (∼3400 BC) refer to opium cultivation for pain relief, and to this day opium derivatives remain the gold standard for treating moderate and severe pain (Rosenblum et al., 2008). The fact that an age-old analgesic remains at the frontline of pain treatment emphasizes the slow progress in analgesic research. Together with the problematic side effects of opiates, their addiction and abuse potential, and the "opiate resistance" of many chronic pain conditions (including neuropathic pain) these factors reinforce the urgent need to identify and develop new analgesics (de Leon-Casasola, 2013).

The neuronal pathways that transmit nociceptive signals to the brain contain multiple sites that present opportunities to pharmacologically alter signaling and ultimately influence or block the pain experience. This complex task of sensing, encoding and perceiving stimuli that could generate pain begins with the collection of information in peripheral sensory receptors, termed nociceptors. Nociceptors are located throughout our body in skin, muscle, joints and viscera, forming diverse populations that can encode either exclusively or combinations of high intensity thermal, mechanical and chemical stimuli. The mechanisms that lead to nociceptor activation and sensitisation obviously provide potential analgesic targets [for review see (Stein et al., 2009; Richards and McMahon, 2013)]. However, targeting peripheral components of the nociceptive system has little value in pain conditions where aberrant signaling, in the form of hyperactive neuron populations and circuits, is firmly established in the central nervous system (CNS).

Here we focus on the spinal cord dorsal horn (DH), the first site where nociceptive information enters the CNS, is processed, and subsequently relayed through successively higher levels of the neuroaxis to form a sensory percept. The necessity for nociceptive information to pass through the DH and ascend to higher brain structures before pain is experienced makes it an attractive site for pharmacological targeting. Indeed, this has been well accepted since publication of the gate control theory of pain by Melzack and Wall (1965). Furthermore, we now know that both peripheral and central insults can disrupt normal information flow through the neuroaxis by initiating reorganization of circuits in the spinal cord DH, brainstem, thalamus, limbic, or cortical regions and produce altered sensory perception in conditions such as neuropathic pain or itch (Graham and Callister, 2012). Given this longstanding focus it is not unreasonable to ask, "Why has progress in spinal-based analgesics been so slow"?

We believe an answer to this question may lie in our overly simplified view of the DH. The general view is that this spinal region acts as a single processing unit, even though we know it receives diverse signals from thermal, nociceptive, pruritic (itch), and tactile peripheral receptors. Thus, neurons in the DH must simultaneously fulfil several roles, which are critical for normal sensory experience: i.e., the integration of different types of signals and segregation of others into specific ascending pathways (Todd, 2010). A classic example is the segregation of nociceptive and tactile information in the DH. This ensures peripheral stimuli

such a sharp pin-prick or light touch result in very different and contextually relevant sensory experiences. One of the critical substrates for these processing tasks is the diversity of neuron types, which form DH circuits and have very specific properties. Surprisingly, our knowledge of the discrete neuronal types within DH circuits and their precise role in sensory processing is limited.

These gaps in our knowledge exist because of historical limitations in experimental approaches. Until recently spinal cord researchers have generally been forced to collect data from multiple (unidentified) neuronal classes and then "pool" these results to provide an "averaged view" of sensory processing and function (or dysfunction). This approach clearly overlooks cell-type diversity in the DH as originally observed by Ramon y Cajal (1899) more than a century ago. Since Cajal's work there has been general agreement that if we are to understand nervous system function, we must first understand how neuron types are assembled into processing circuits. Armed with this mechanistic understanding, we may then identify putative drug targets on specific neuron types. We believe this approach offers the promise of targeted analgesics that selectively act on nociceptive circuits.

At the same time, it is important to acknowledge that the difficulty in defining functionally discrete neuron populations within the DH is not due to a lack of effort. In fact, an extensive literature shows that multiple neuron classes do exist as based on any single parameter (e.g., electrophysiology, neurochemistry, morphology). For example, inhibitory interneurons in the superficial DH can be differentiated into four populations based on the non-overlapping expression of neurochemical markers (neuropeptide Y, galanin, parvalbumin, and nitric oxide synthase; Polgár et al., 2013). The same approach reveals up to six populations of excitatory interneurons (calretinin, calbindin, neurotensin, somatostatin, substance P, and neurokinin B; Todd, 2010), though some overlap exists within expression patterns. In contrast, electrophysiological classification distinguishes four to seven types of neurons based on action potential discharge patterns during depolarizing current injection (Grudt and Perl, 2002; Ruscheweyh and Sandkuhler, 2002; Graham, 2004). Finally, using anatomical criteria, four distinct morphologies are commonly differentiated (Grudt and Perl, 2002; Yasaka et al., 2010). The challenge has been to merge this information into a model that defines neuron populations with homogenous properties based on multiple criteria. Only with this information can we begin to understand how DH circuits process both nociceptive and non-nociceptive information and develop tools that allow us to manipulate this region and provide pain relief.

SPINAL SUBPOPULATIONS MATTER FOR PAIN PROCESSING IN THE DH

Some evidence is now accumulating to support a key role for functionally and neurochemically distinct neuronal populations in spinal sensory processing. For example, recent work has examined the role of excitatory DH interneurons in pain and itch. In these experiments knockout of the testicular orphan nuclear receptor 4 (TR4) in the CNS resulted in a substantial loss (∼70%) of excitatory interneurons (Wang et al., 2013). Behavioral analyses then identified an almost complete loss of supraspinally mediated pain and itch responses, elevated mechanical withdrawal thresholds, and nerve injury-induced mechanical hypersensitivity. In contrast, noxious heat evoked reflexes that originate in the spinal cord, nerve injury-induced heat hypersensitivity, and tissue injury-induced heat and mechanical hypersensitivity were unaltered. The authors concluded, "that different subsets of dorsal excitatory interneurons contribute to tissue and nerve injury-induced heat and mechanical pain" (Wang et al., 2013). This study complements a larger body of work, which began with the gate control theory of pain, implicating populations of inhibitory (GABA and glycine containing) DH interneurons in nociceptive processing (Zeilhofer et al., 2011). This work has firmly established that inhibitory dysfunction allows "linking" of tactile and nociceptive circuits to produce allodynia and hyperalgesia. The search for specific neuronal subpopulations directly involved in this process has unfortunately progressed slowly.

Despite the above challenges, work on inhibitory interneurons has succeeded in identifying a functionally distinct neuronal subpopulation in the DH (Ross et al., 2010). This work, which is relevant to itch rather than nociception, assessed the role of a particular transcription factor – Bhlhb5. Mice lacking Bhlhb5 exhibited an itch phenotype and lacked a subset of inhibitory interneurons in the DH. Importantly, the remaining neuronal populations, afferent input, and responses to other sensory modalities were not altered. This confirmed a specific role for the lost inhibitory population in the processing of itch-related stimuli. Together with the data on excitatory populations, this work provides support for the hypothesis that additional unidentified interneuron subpopulations exist in the DH.

TARGETING SPINAL SUBPOPULATIONS

Over the last decade, transgenic techniques have been developed which allow marker proteins such as green fluorescent protein (GFP) to be expressed in specific neurons, thereby enabling us to visualize and target specific neuronal subtypes in the CNS. In simple terms, this is achieved by genetic techniques that couple GFP expression to a promoter protein that only exists in neurons of interest. As long as we have a "genetic signature" for a given neuron type, this approach allows us to address DH interneuron diversity. To date, such studies have been restricted to inhibitory interneurons whereby GFP expression has been linked to proteins involved in neurotransmitter synthesis and membrane transport (Heinke, 2004; Zeilhofer et al., 2004). This work has, however, still reported significant variability in the properties of targeted populations in GFP-positive neurons. Nevertheless, specifically studying GABAergic interneurons labeled by GFP has produced important findings. For example, a small subpopulation of GFP-positive neurons has been identified that receive low-threshold (tactile) input from primary afferents (Daniele and MacDermott, 2009). The ability of these tactile inputs to activate GABAergic interneurons has long been acknowledged as a basic requirement for inhibitory circuits to "segregate" nociceptive and tactile information. While such transgenic approaches provide a powerful tool to study subpopulations of neurons, it seems identifying specific target proteins to drive GFP expression in these populations (i.e., the neuron's genetic "signature") is challenging (Graham et al., 2007). For example if the aim is to

identify discrete classes of neurons with uniform properties and clear roles in sensory processing, labeling neurons based upon the primary neurotransmitter they employ appears too crude an approach.

With these challenges in mind, we have recently characterized a small but significant subpopulation of inhibitory interneurons that express the calcium-binding protein parvalbumin (PV; Hughes et al., 2012). This work places PV-positive interneurons in a putative circuit for mediating feed-forward inhibition in the DH (**Figure 1**). Using GFP to target PV-expressing neurons we showed that the functional and morphological properties of PV-positive interneurons were remarkably homogeneous. PV-positive interneurons exhibited excitable, high frequency AP discharge responses and typically had islet like morphologies. Furthermore, a significant proportion (∼80%) of axons arising from PV-positive DH neurons made selective axoaxonic-synapses onto the central terminals of myelinated afferents. We also showed that PV-positive interneurons received input from the same myelinated afferent population. This connectivity is ideally suited to maintain functional segregation of sensory modalities. Thus, under normal conditions when tactile related information arrives in the DH, PV-neurons are excited and subsequently inhibit tactile inputs. This action prevents further excitation that would otherwise recruit nociceptive circuits (**Figure 1**). By extension, decreased PV-neuron excitability would remove this gating of tactile information, link tactile and nociceptive signaling and produce tactile allodynia. An important caveat to this work is that despite the success of our PV targeting approach, some heterogeneity remained in our sample. Specifically, 20% of the axons arising from PV-positive neurons targeted unidentified structures and some variability remained in our electrophysiological and morphological data. In summary, our findings in the PV-GFP mouse, along with earlier GFP studies, suggest additional analyses are required to uncover more discrete neuronal subpopulations and determine their functional role in spinal sensory processing.

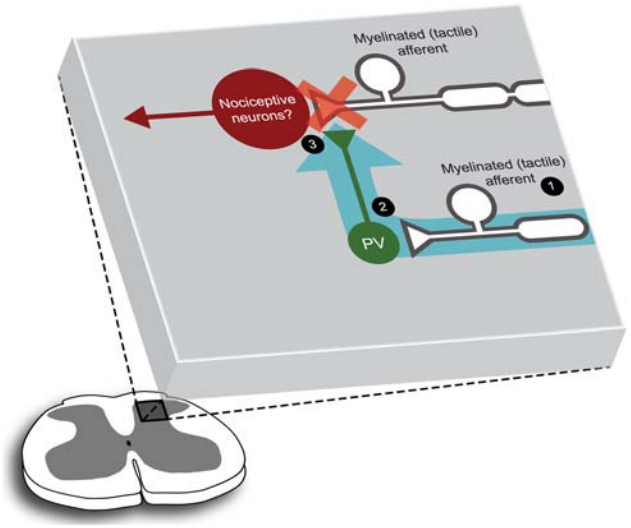


FIGURE 1 | Parvalbumin-positive DH neurons are configured to mediate presynaptic control of myelinated afferent fiber that contribute to DH circuits. Figure summarizes a characterization of PV-positive neurons using a GFP expressing transgenic mouse line (Hughes et al., 2012). PV-positive DH neurons and their axonal arbors (2, green) are concentrated in lamina IIi/III. These cells receive input from myelinated (tactile) primary afferents (1), and their axons appear to target the central terminals of myelinated primary afferents preferentially, forming inhibitory axoaxonic synaptic inputs (3). Thus, when this microcircuit is activated (blue arrow) myelinated afferent input is inhibited, or gated (red cross). The downstream target of this PV-gated input remains to be identified, however, the location of these connections suggests that neurons in nociceptive circuits may be included.

NEW TOOLS TO DEFINE FUNCTIONALLY DISCRETE SUBPOPULATIONS

Fortunately, a number of techniques are becoming available which could expand our analysis of DH neuron subpopulations. For example, several groups have used molecular screening techniques to dissect neuronal heterogeneity. This has only recently been applied to the DH (Wildner et al., 2013). Here two transgenic mice, which lacked key transcriptional regulators that normally define inhibitory DH interneuron lineages, were subjected to molecular screening. Genome-wide expression comparisons identified four genes (pDyn, Kcnip2, Rorb, and Tfap2b) with largely non-overlapping expression patterns that were significantly down regulated in the DH of animals lacking inhibitory interneuron populations (Wildner et al., 2013). The group is now testing how subpopulations that selectively express these four genes contribute to sensory and nociceptive processing in the DH. A variation of this strategy has also been applied to a number of CNS regions whereby gene expression profiling is undertaken in individual, or small numbers of neurons identified via GFP expression. This approach builds on GFP targeting studies by using single-cell quantitative PCR (qPCR) to compare gene expression profiles in subsets of identified neurons. This establishes smaller groupings of neurons within a GFP labeled subpopulation that can be considered distinct according to molecular criteria. Such information can then be used to predict the function of different subpopulations as well as providing novel electrophysiological and anatomical signatures to identify these populations in subsequent studies. The technique also identifies a series of "marker" genes and proteins that can be used to subdivide, label, monitor, and manipulate subpopulations of interest. This procedure has proved valuable in other sensory processing nodes such as the medial vestibular nucleus (MVN; Sugino et al., 2005; Kodama et al., 2012). Gene expression profiling in the MVN identified six distinct neuronal subpopulations. Subsequent combination of this information with electrophysiological and anatomical data allowed several populations to be assigned specific functions in vestibulo-ocular and vestibulo-cerebellar circuits. The DH represents an ideal candidate for this type of analysis as the functional significance of its neuron heterogeneity is yet to be understood and several DH GFP labeled populations are now available. We believe that such studies could identify novel protein targets with a high degree of specificity in terms of the number and identity of DH neurons that could be manipulated and blunt pain.

Once molecular screening has been used to identify specific protein targets in discrete neuronal subpopulations, the next

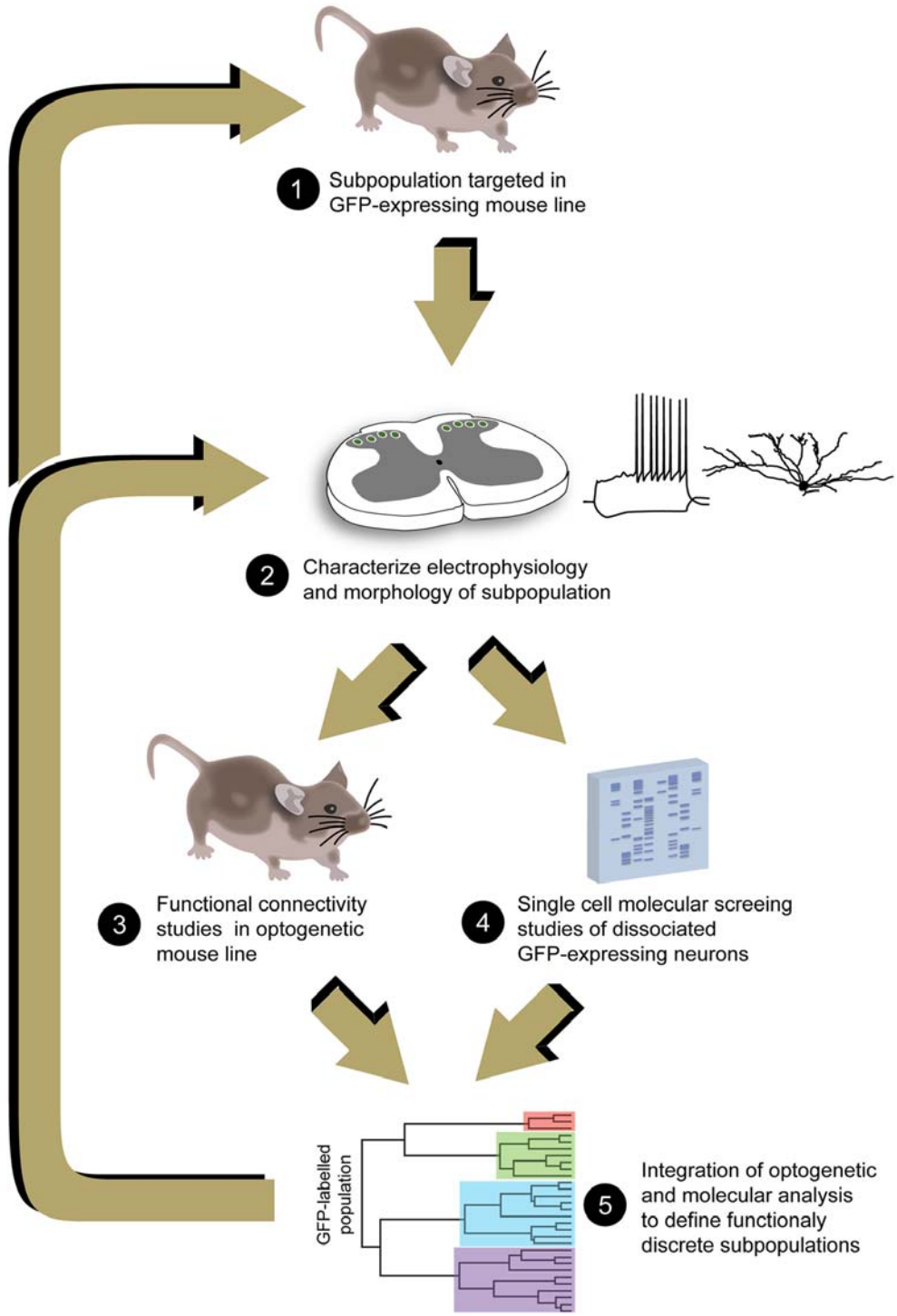


FIGURE 2 | Proposed analysis pathway to resolve DH neuron heterogeneity. Analyses begin with identification of GFP expressing transgenic mouse lines that label DH neuron populations (1). These neurons are first characterized using electrophysiological and anatomical approaches, determining the degree of homogeneity/heterogeneity that exists in the GFP-expressing population (2). Additional transgenic mice can then be employed that express optogenetic probes to selectively activate the subpopulations of interest and study their connectivity in DH circuits (3). GFP-expressing populations can also be subjected to gene expression profiling to further dissect any remaining heterogeneity and identify novel proteins that are selectively expressed in these subpopulations (4). This information can then be fed back into the analysis strategy, further refining the selection of probes to target smaller DH subpopulations, and identifying unique function and neuroanatomical features that can then be used to identify these subpopulations.

critical step is to determine their relevance and role in nociceptive signal processing (versus other modalities) by establishing their connectivity. Until recently it has been virtually impossible to obtain such information for complex neural circuits. The best data for the DH has comes from electrophysiological recordings from pairs of "synaptically connected" neurons in spinal cord slices. These experiments are difficult and typically result in small sample sizes, especially in the DH where connectivity rates are low in slices – [10–15% (Lu and Perl, 2003, 2005)]. More recently a series of elegant publications by the Strassman group have employed laser-scanning microstimulation to investigate connectivity in the DH describing fundamental principles for DH synaptic connectivity patterns (Kato et al., 2009; Kosugi et al., 2013). Notwithstanding the substantial advances this approach has delivered, laser-scanning microstimulation only allows for an identified neuron's inputs to be studied without providing information on the type or types of neurons where these inputs originate. Fortunately, connectivity mapping has recently been revolutionized with the introduction of optogenetic techniques. This approach allows neurons with the "same genetic signature" to be activated by light. Specifically, light-sensitive proteins (channel rhodopsins from algae) are incorporated into a given neuron type. When stimulated by light of an appropriate wavelength, the channel rhodopsins either promote or inhibit neuron activity (Fenno et al., 2011). Optogenetics allows selective activation of neurons within given circuits and vastly increases the speed and accuracy of connectivity studies.

So far optogenetics has not yet been used to study neuronal connectivity within DH circuits. Channel rhodopsins have, however, been expressed in a subpopulation of polymodal unmyelinated (Mrgprd-positive) sensory afferents (Wang and Zylka, 2009). DH neuron responses were recorded in spinal cord slices during selective "light activation" of Mrgprd-positive unmyelinated afferents. This is significant as there are several classes of polymodal afferents and it is not possible to selectively stimulate them electrically. The results of this work showed that Mrgprd-positive afferents provide relatively broad and non-selective input to multiple DH populations, reinforcing a major premise of this perspective – i.e., that functionally discrete DH neuronal populations are critical for appropriate encoding of sensory information. ChR2 has also been expressed in Nav1.8 positive afferent neurons, a manipulation that provides optogenetic control of almost all nociceptors (Daou et al., 2013). In contrast to the Mrgprd experiments, this work was undertaken *in vivo*. Light stimulation of the hindpaw resulted in pain behaviors (i.e., foot withdrawal). These data provide the first proof of concept that optogenetics can be employed under both *in vivo* and *in vitro* conditions and highlights the potential for these approaches to be used in development and testing of novel pain therapeutics.

SUMMARY AND CONCLUSION

The challenge of unraveling cellular heterogeneity in the DH remains a major barrier to understanding how this region encodes our sensory world. The value of this sort of analysis in the CNS has been emphasized in a recent review that reinforced Cajal's original premise: "A complete understanding of nervous system function cannot be achieved without the identification of its component cell types" (Fishell and Heintz, 2013). Our failure to meet this challenge has been largely due to technical limitations, however, a range of leading-edge techniques including molecular phenotyping of individual neurons and optogenetic activation of specific neuronal populations now allows us to proceed (**Figure 2**). In addition, other techniques such as chemogenetic approaches, also referred to as designer receptors exclusively activated by designer drug (or DREADD), are likely to form part of the ever-increasing armory available to manipulate and study the functional role of neurons in the DH (see Rogan and Roth, 2011; Wess et al., 2013). Importantly, the application of these technologies in pain research will require continued advances in molecular probe development, techniques and equipment to delivery light stimulation to the spinal cord. The fundamental information provided by these new techniques will inform future attempts to treat a variety of painful conditions in which sensory processing and perception are disrupted in the DH. For example our dataset on PV-positive DH neurons suggest they play a key role in separating tactile and nociceptive information in the spinal cord. Thus, targetted activation of this functionally discrete DH population may reduce tactile allodynia, a feature of many chronic pain syndromes where tactile sensory input excites nociceptive circuits and produces pain. The information from molecular screening and optogenetic analysis of these DH neurons ultimately aims to identify pharmacological agents that are capable of restoring normal sensory function by abolishing tactile allodynia. Likewise, as additional functionally discrete DH subpopulations are characterized this same strategy could lead to new classes of analgesic drugs with very specific actions.

ACKNOWLEDGMENTS

This research was supported by a National Health and Medical Research Council (NHMRC) of Australia grants 631000 and 1043933 (Brett A. Graham), Hunter Medical Research Institute (HMRI) grant (Brett A. Graham and Robert J. Callister), and BBSRC grant BB/J000620/1 (David I. Hughes).

REFERENCES

Basbaum, A. I., Bautista, D. M., Scherrer, G., and Julius D. (2009). Cellular and molecular mechanisms of pain. *Cell* 139, 267–284. doi: 10.1016/j.cell.2009.09.028

Daniele, C. A., and MacDermott, A. B. (2009). Low-threshold primary afferent drive onto GABAergic interneurons in the superficial dorsal horn of the mouse. *J. Neurosci.* 29, 686–695. doi: 10.1523/JNEUROSCI.5120-08.2009

Daou, I., Tuttle, A. H., Longo, G., Wieskopf, J. S., Bonin, R. P., Ase, A. R., et al. (2013). Remote optogenetic activation and sensitization of pain pathways in freely moving mice. *J. Neurosci.* 33, 18631–18640. doi: 10.1523/JNEUROSCI.2424-13.2013

de Leon-Casasola, O. A. (2013). Opioids for chronic pain: new evidence, new strategies, safe prescribing. *Am. J. Med.* 126, S3–S11. doi: 10.1016/j.amjmed.2012.11.011

Fenno, L., Yizhar, O., and Deisseroth, K. (2011). The development and application of optogenetics. *Annu. Rev. Neurosci.* 34, 389–412. doi: 10.1146/annurev-neuro-061010-113817

Fishell, G., and Heintz, N. (2013). The neuron identity problem: form meets function. *Neuron* 80, 602–612. doi: 10.1016/j.neuron.2013.10.035

Graham, B. A. (2004). In vivo responses of mouse superficial dorsal horn neurones to both current injection and peripheral cutaneous stimulation. *J. Physiol.* 561, 749–763. doi: 10.1113/jphysiol.2004.072645

Graham, B., and Callister, R. (2012). "Pain," in *The Mouse Nervous System*, eds C. Watson, G. Paxinos, and L. Puelles (London: Elsevier), 589–606.

Graham, B. A., Brichta, A. M., and Callister, R. J. (2007). Moving fom an averaged to specific view of spinal cord pain processing circuits. *J. Neurophysiol.* 98, 1057–1063. doi: 10.1152/jn.00581.2007

Grudt, T. J., and Perl, E. R. (2002). Correlations between neuronal morphology and electrophysiological features in the rodent superficial dorsal horn. *J. Physiol.* 540, 189–207. doi: 10.1113/jphysiol.2001.012890

Heinke, B. (2004). Physiological, neurochemical and morphological properties of a subgroup of GABAergic spinal lamina II neurones identified by expression of green fluorescent protein in mice. *J. Physiol.* 560, 249–266. doi: 10.1113/jphysiol.2004.070540

Hughes, D. I., Sikander, S., Kinnon, C. M., Boyle, K. A., Watanabe, M., Callister, R. J., et al. (2012). Morphological, neurochemical and electrophysiological features of parvalbumin-expressing cells: a likely source of axo-axonic inputs in the mouse spinal dorsal horn. *J. Physiol.* 590, 3927–3951. doi: 10.1113/jphysiol.2012.235655

Kato, G., Kawasaki, Y., Koga, K., Uta, D., Kosugi, M., Yasaka, T., et al. (2009). Organization of intralaminar and translaminar neuronal connectivity in the superficial spinal dorsal horn. *J. Neurosci.* 29, 5088–5099. doi: 10.1523/JNEUROSCI.6175-08.2009

Kodama, T., Guerrero, S., Shin, M., Moghadam, S., Faulstich, M., and du Lac, S. (2012). Neuronal classification and marker gene identification via single-cell expression profiling of brainstem vestibular neurons subserving cerebellar learning. *J. Neurosci.* 32, 7819–7831. doi: 10.1523/JNEUROSCI.0543-12.2012

Kosugi, M., Kato, G., Lukashov, S., Pendse, G., Puskar, Z., Kozsurek, M., et al. (2013). Subpopulation-specific patterns of intrinsic connectivity in mouse superficial dorsal horn as revealed by laser scanning photostimulation. *J. Physiol.* 591, 1935–1949. doi: 10.1113/jphysiol.2012.244210

Lu, Y., and Perl, E. R. (2003). A specific inhibitory pathway between substantia gelatinosa neurons receiving direct C-fiber input. *J. Neurosci.* 23, 8752–8758.

Lu, Y., and Perl, E. R. (2005). Modular organization of excitatory circuits between neurons of the spinal superficial dorsal horn (Laminae I and II). *J. Neurosci.* 25, 3900–3907. doi: 10.1523/JNEUROSCI.0102-05.2005

Melzack, R., and Wall, P. D. (1965). Pain mechanisms: a new theory. *Science* 150, 971–979. doi: 10.1126/science.150.3699.971

Polgár, E., Sardella, T. C. P., Tiong, S. Y. X., Locke, S., Watanabe, M., and Todd, A. J. (2013). Functional differences between neurochemically defined populations of inhibitory interneurons in the rat spinal dorsal horn. *Pain* 154, 2606–2615. doi: 10.1016/j.pain.2013.05.001

Ramon y Cajal, S. (1899). *Histology of the Nervous System.* N. Swanson and L. W. Swanson (trans.) (Wien, New York: Springer).

Richards, N., and McMahon, S. B. (2013). Targeting novel peripheral mediators for the treatment of chronic pain. *Br. J. Anaesth.* 111, 46–51. doi: 10.1093/bja/aet216

Rogan, S. C., and Roth, B. L. (2011). Remote control of neuronal signaling. *Pharmacol. Rev.* 63, 291–315. doi: 10.1124/pr.110.003020

Rosenblum, A., Marsch, L. A., Joseph, H., and Portenoy, R. K. (2008). Opioids and the treatment of chronic pain: controversies, current status, and future directions. *Exp. Clin. Psychopharmacol.* 16, 405–416. doi: 10.1037/a0013628

Ross, S. E., Mardinly, A. R., McCord, A. E., Zurawski, J., Cohen, S., Jung, C., et al. (2010). Loss of inhibitory interneurons in the dorsal spinal cord and elevated itch in bhlhb5 mutant mice. *Neuron* 65, 886–898. doi: 10.1016/j.neuron.2010.02.025

Ruscheweyh, R., and Sandkuhler, J. (2002). Lamina-specific membrane and discharge properties of rat spinal dorsal horn neurones in vitro. *J. Physiol.* 541, 231–244. doi: 10.1113/jphysiol.2002.017756

Stein, C., Clark, J. D., Oh, U., Vasko, M. R., Wilcox, G. L., Overland, A. C., et al. (2009). Peripheral mechanisms of pain and analgesia. *Brain Res. Rev.* 60, 90–113. doi: 10.1016/j.brainresrev.2008.12.017

Sugino, K., Hempel, C. M., Miller, M. N., Hattox, A. M., Shapiro, P., Wu, C., et al. (2005). Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nat. Neurosci.* 9, 99–107. doi: 10.1038/nn1618

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nat. Rev. Neurosci.* 11, 823–836. doi: 10.1038/nrn2947

Wang, H., and Zylka, M. J. (2009). Mrgprd-expressing polymodal nociceptive neurons innervate most known classes of substantia gelatinosa neurons. *J. Neurosci.* 29, 13202–13209. doi: 10.1523/JNEUROSCI.3248-09.2009

Wang, X., Zhang, J., Eberhart, D., Urban, R., Meda, K., Solorzano, C., et al. (2013). Excitatory superficial dorsal horn interneurons are functionally heterogeneous and required for the full behavioral expression of pain and itch. *Neuron* 78, 312–324. doi: 10.1016/j.neuron.2013.03.001

Wess, J., Nakajima, K., and Jain, S. (2013). Novel designer receptors to probe GPCR signaling and physiology. *Trends Pharmacol. Sci.* 34, 385–392. doi: 10.1016/j.tips.2013.04.006

Wildner, H., Das Gupta, R., Brohl, D., Heppenstall, P. A., Zeilhofer, H. U., and Birchmeier, C. (2013). Genome-wide expression analysis of ptf1a- and ascl1-deficient mice reveals new markers for distinct dorsal horn interneuron populations contributing to nociceptive reflex plasticity. *J. Neurosci.* 33, 7299–7307. doi: 10.1523/JNEUROSCI.0491-13.2013

Yasaka, T., Tiong, S. Y., and Todd, A. J. (2010). Populations of inhibitory and excitatory interneurons in lamina II of the adult rat spinal dorsal horn revealed by a combined electrophysiological and anatomical approach. *Pain* 151, 475–488. doi: 10.1016/j.pain.2010.08.008

Zeilhofer, H. U., Studler, B., Arabadzisz, D., Schweizer, C., Ahmadi, S., Layh, B., et al. (2004). Glycinergic neurons expressing enhanced green fluorescent protein in bacterial artificial chromosome transgenic mice. *J. Comp. Neurol.* 482, 123–141. doi: 10.1002/cne.20349

Zeilhofer, H. U., Wildner, H., and Yévenes, G. E. (2011). Fast synaptic inhibition in spinal sensory processing and pain control. *Physiol. Rev.* 92, 193–235. doi: 10.1152/physrev.00043.2010

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 08 December 2013; accepted: 03 February 2014; published online: 25 February 2014.

*Citation: Smith KM, Madden JF, Callister RJ, Hughes DI and Graham BA (2014) The search for novel analgesics: re-examining spinal cord circuits with new tools. Front. Pharmacol. **5**:22. doi: 10.3389/fphar.2014.00022*

This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

frontiers in
PHARMACOLOGY

METHODS ARTICLE
published: 30 August 2012
doi: 10.3389/fphar.2012.00158

Telemetric assessment of referred vaginal hyperalgesia and the effect of Indomethacin in a rat model of endometriosis

N. Dmitrieva[1]*, E. K. Faircloth[1], S. Pyatok[1], F. Sacher[2] and V. Patchev[2]

[1] *Program in Neuroscience, Florida State University, Tallahassee, FL, USA*
[2] *Bayer HealthCare Pharmaceuticals, Berlin, Germany*

Edited by:
Susan Hua, The University of Newcastle, Australia

Reviewed by:
Robert L. Barkin, Rush University Medical Center/Northshore University Health System, USA
Jason B. Wu, Cedars-Sinai Medical Center, USA

****Correspondence:***
N. Dmitrieva, Program in Neuroscience/Psychology, Florida State University, 1107 West Call Street, Tallahassee, FL 32306-4301, USA.
e-mail: dmitrieva@psy.fsu.edu

Symptoms of endometriosis (ENDO), among others, include pelvic/abdominal and muscle pain. Non-steroidal anti-inflammatory agents are first-line treatment for this pain. Similar to women, rats with surgically induced ENDO, but not its surgical control, exhibit vaginal hyperalgesia, which in rats is evidenced by a decreased threshold for the visceromotor response (VMR) induced by vaginal distention. Here we assess the VMR in rats with implanted probes that telemetrically transmit EMG activity from the abdominal muscle. The feasibility and sensitivity of this technique for monitoring the VMR threshold across the estrous cycle and the influence of Indomethacin on ENDO-induced vaginal hyperalgesia were evaluated. VMR thresholds in response to vaginal distention with an infusion pump were measured in different estrous stages. Indomethacin (5 or 10 mg/kg i.p. or s.c.) was injected in proestrus rats and 40–60 min later the VMR threshold was measured. The VMR threshold varied across the estrous cycle only in ENDO rats, being lowest in proestrus. Indomethacin increased this threshold in proestrus ENDO rats. These results show that telemetric assessment of the VMR is a sensitive tool, suitable for long-term studies in conscious rats. The results with this technique also suggest that ENDO-associated vaginal hyperalgesia involves COX activity, the feature that also underlies inflammatory pains.

Keywords: analgesia, uterus, prostaglandins, PGE2

INTRODUCTION

Endometriosis (ENDO) is an estrogen-dependent condition defined by the presence of growths of endometrial tissue outside the uterus. ENDO is also considered an inflammatory condition because immune cells and numerous inflammatory mediators including prostaglandins, cytokines, chemokines, and growth factors are involved in its development and maintenance (Bulun et al., 2005; Maybin et al., 2011; Stratton and Berkley, 2011). Among these inflammatory molecules, PGE2 and COX2 appear to play key roles because PGE2 stimulates aromatase activity in endometrial stromal and epithelial cells and thereby promotes local estrogen production that is essential for growth and maintenance of endometrial implants, and COX2 maintains high levels of PGE2 synthesis in ectopic endometrial foci and eutopic endometrium in women with ENDO and itself is subject to regulation by PGE2 and other inflammatory mediators (Noble et al., 1997; Ota et al., 2001; Chishima et al., 2002; Bulun et al., 2005; Banu et al., 2008). In accordance, COX2 inhibitors are effective in reducing ectopic implant growth and improving fertility in murine models when animals are treated in the early stages of ENDO development (Golan et al., 1986; Dogan et al., 2004; Efstathiou et al., 2005; MacHado et al., 2010).

Women with ENDO often experience severe pelvic pain. Non-steroidal anti-inflammatory drugs (NSAIDs) are the first-line treatment for pelvic/abdominal pain but their use is limited because of side effects (Stratton and Berkley, 2011). Surgical removal of lesions often fails to alleviate pain long-term, and hormonal treatments that decrease estrogen levels, although effective for the pain, often have intolerable side effects and are unsafe for long-term use. Therefore, new and long-term treatment options with reduced side effects and efficacy in ENDO are urgently needed.

In an established rat model of ENDO, pieces of uterine tissue are implanted in the abdominal cavity (Vernon and Wilson, 1985). The transplants form cysts that grow rapidly during the first month, continue growing in the following months to reach full size by 8–10 weeks (Vernon and Wilson, 1985). Similar to women, cysts in rats attract their own nerve and blood supply (Zhang et al., 2008; McAllister et al., 2012). Another resemblance to women's signs is significantly elevated levels of inflammatory mediators (Bergqvist et al., 2001; Anaf et al., 2002; Zhang et al., 2008; MacHado et al., 2010). Importantly like women, rats with ENDO exhibit abnormal sensory signaling associated with the pelvic area, such as vaginal hyperalgesia (dyspareunia in women; Cason et al., 2003) and referred abdominal muscle hyperalgesia (Nagabukuro and Berkley, 2007).

In the previous studies, vaginal hyperalgesia in rats has been assessed either with a behavioral psychophysical test (Cason et al., 2003), or with a technique that measures abdominal muscle electrical activity in response to vaginal distention (Nagabukuro and Berkley, 2007). This later measure, which is a referred abdominal muscle response to noxious stimuli, is called visceromotor response (VMR). Although these techniques are sensitive and have been successfully used to reveal estrous differences in

pelvic nociception and effects of drugs and surgical manipulations (Cason et al., 2003; Berkley et al., 2007; Nagabukuro and Berkley, 2007; Dmitrieva et al., 2010), they are labor-intensive and cumbersome to use for drug testing. Here we developed a new technique that combined VMR with vaginal distention and telemetric methodology. We then validated its applicability in two experimental settings. In the first study, we tested changes in ENDO-induced vaginal hyperalgesia across the estrous cycle. In the second study, we tested the hypothesis that COX is involved in pathology of ENDO-associated vaginal hyperalgesia by studying the effect of a non-selective COX inhibitor Indomethacin on the VMR threshold as a proxy of vaginal hyperalgesia.

MATERIALS AND METHODS

This study was approved by the Florida State University's Animal Care and Use Committee, Protocol #0927. The care and use of animals conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals (PHS/NIH) and the Animal Welfare Act (USDA).

SUBJECTS

Sprague-Dawley female rats were used. Estrous cycle was determined daily by cytological examination of vaginal lavage collected approximately 2 h after lights on (Becker et al., 2005).

Endometriosis and sham ENDO surgeries were performed under ketamine/xylazine anesthesia (K/X: 73/8.8 mg/kg i.p.) following aseptic precautions. During surgery and the recovery period, the rat was kept warm on a heating pad. A small incision was made in the middle of the abdomen. A small, approximately 1 cm segment of the middle part of one uterine horn was clamped between two hemostats and excised. The cecum and adjacent intestines were exposed. Four 2 mm × 2 mm biopsies of the excised uterine tissue, or fat in shamENDO rats, were sutured on alternate mesenteric cascade arteries. Muscle and skin were sutured separately. Bupivacaine was given locally and butorphanol, s.c immediately after surgery to alleviate postoperative pain. Rats were monitored on a daily basis for any sign of distress.

TELEMETRIC PROBE IMPLANTATION

Seven weeks after ENDO/shamENDO surgery, under aseptic conditions and K/X anesthesia, a telemetric probe (TA11CTAF40, DSI, St. Paul, MN, USA) was implanted under the skin of the right abdominal flank. Electrodes were tunneled under the skin and implanted in the left inguinal muscle. The skin was sutured. Recovery proceeded as described above. On rare occasions, some rats developed a seroma that was managed by aspiration of clear fluid from the pocket around the probe (under halothane gas anesthesia). This seroma usually subsided in a few days. Rats did not exhibit stress behavior related to the implant. Experiments began at least 7 days after implantation. All rats were habituated to the restraining box and the experimental setting during three to four training sections before VMR recording and did not show any sign of discomfort.

Visceromotor response to vaginal distention in conscious rats was assessed with the Ponemah Telemetry System (DSI, St. Paul, MN, USA), which consisted of a receiver, data exchange matrix, acquisition interface, and a computer with a synchronization board (**Figure 1A**). The rat was placed in a transparent plexiglass box that gently, but not aversively, restrained it. The restraining box was placed on the receiver. A small balloon (∼10 mm diameter when fully inflated) connected to a pressure transducer was inserted into the middle of the vaginal canal. After ∼10 min of resting period, the balloon was inflated by an infusion pump (0.3 ml/min, 1 ml maximum). Electrical activity from the abdominal muscle (electromyography, EMG) was relayed to the DSI system and synchronized with the amplified and digitalized signal from the pressure transducer. EMG activity was recorded during ∼5 min before and during the time when the vaginal balloon was inflated.

The integral of the rectified EMG signal was calculated in 100 ms intervals by the analysis module of the Ponemah System. The volume threshold that corresponded to VMR (**Figure 1B**) 200% or higher than baseline activity was calculated as reported previously (Nagabukuro and Berkley, 2007). VMR thresholds were obtained 1–2 days between the sessions, two to four baselines per each estrous stage (metestrus DI, diestrus DII, proestrus P, and estrus E,) and then averaged for each stage for each rat.

Indomethacin was dissolved in a mixture of DMSO: cremophor: saline (1:1:8). One of two doses (5 mg/kg, i.p. only or 10 mg/kg) or vehicle was injected i.p. or s.c. in the neck area when the rat was in proestrus. The VMR threshold was assessed 40–60 min later.

STATISTICAL ANALYSIS

Differences between VMR thresholds in different estrous stages were analyzed by ANOVA followed by Tukey *post hoc* tests. Differences in VMR thresholds between baseline, vehicle, and Indomethacin groups were analyzed by ANOVA followed by Dunnett or *t*-test as appropriate. Differences with $p < 0.05$ were considered significant.

RESULTS

ESTROUS CYCLE DIFFERENCES

Baseline data were obtained from rats ($n = 20$) in different estrous stages for 3–5 weeks beginning a minimum of 8 weeks after ENDO/sham ENDO surgery when the cyst innervation and vaginal hyperalgesia are fully developed (McAllister et al., 2012). The VMR thresholds were assessed two to four times for each estrous stage for each rat; the individual values remained consistent during the entire study. Average baseline VMR thresholds for each stage are presented for ENDO and shamENDO groups separately in **Figure 2**.

Statistical analysis showed that VMR thresholds in ENDO rats were significantly different from thresholds measured in shamENDO rats ($p < 0.001$). In agreement with our earlier published data obtained using the VMR technique in lightly anesthetized or tethered rats (Nagabukuro and Berkley, 2007; Dmitrieva et al., 2010), the VMR thresholds varied across the estrous cycle in ENDO rats, with the threshold being lowest in proestrus ($p < 0.005$). On average, the VMR threshold in ENDO rats in the present study in proestrus were 15–19% lower than the thresholds observed in other stages. Again, similar to previous observations, the VMR threshold in proestrus ENDO rats was on average 42.4% lower than that in shamENDO rats. Finally,

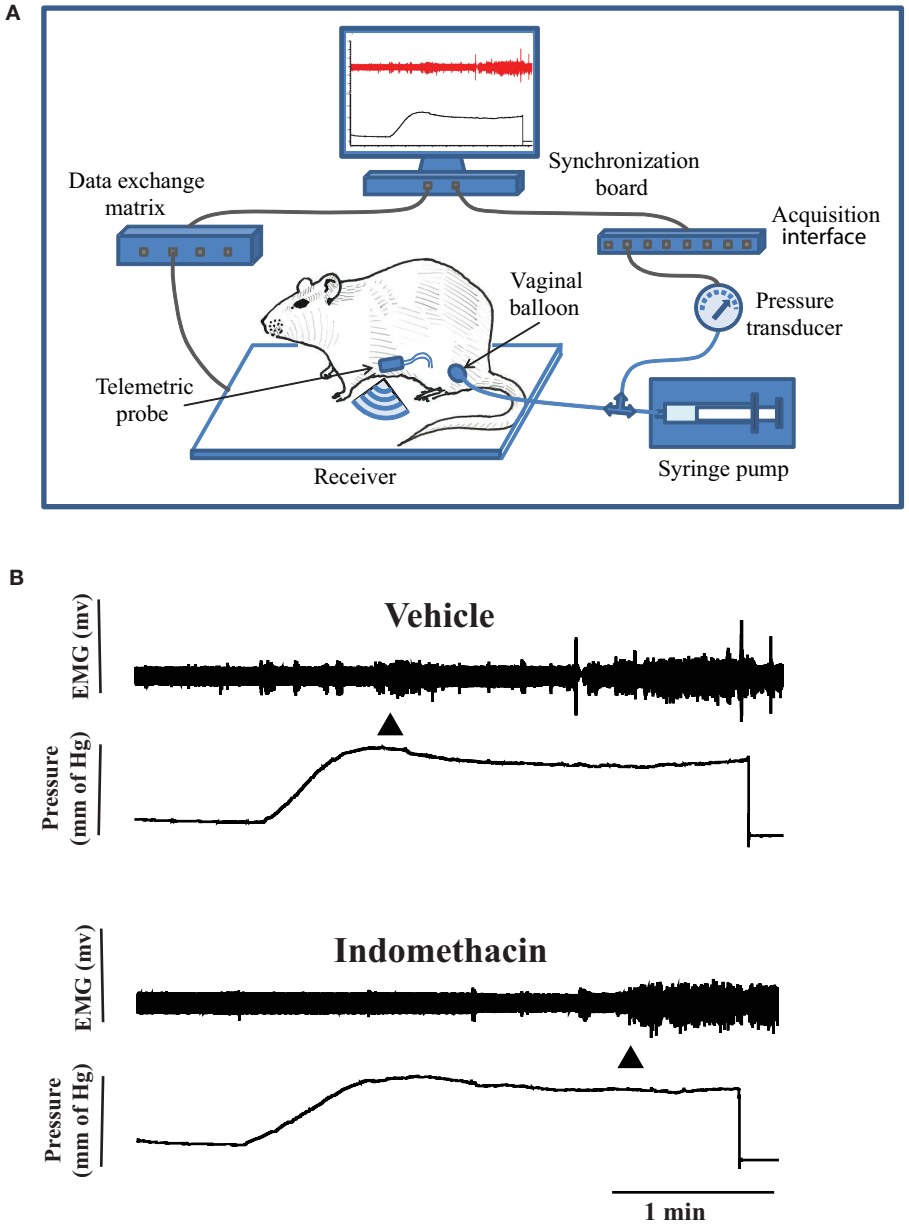


FIGURE 1 | (A) Experimental setting for recording of the visceromotor response (VMR) to vaginal stimulation with the Ponemah Telemetric System (DSI, St. Paul, MN, USA). Pressure in the vaginal balloon was generated by an infusion pump. The EMG signal from the electrode implanted in the abdominal muscle was converted into radio signals by the telemetric probe and transmitted to a receiver connected to the acquisition module of the Ponemah System and synchronized with the incoming signal from the pressure transducer. **(B)** A recording of EMG activity during vaginal distension before and after 10 mg/kg Indomethacin (i.p.) injection in the same rat. The first arrow shows the beginning of vaginal distention, and the second arrow shows the VMR threshold. The VMR threshold increased after Indomethacin.

the threshold in shamENDO rats did not exhibit estrous cycle differences ($p = 0.752$).

Indomethacin was injected first i.p. (5 or 10 mg/kg) in proestrus ENDO and shamENDO rats ($n = 9$). Neither dose of Indomethacin significantly changed the VMR threshold in shamENDO rats. In contrast, the same doses significantly increased the thresholds in ENDO rats in a dose-dependent manner (**Figures 1B** and **3**). The dose 10 mg/kg produced a significant increase in VMR threshold of $81 \pm 14.6\%$ compared with both baseline and the effect produced by vehicle ($p < 0.05$).

This increase produced by Indomethacin was transient: the threshold measured 4 days later, when the rat was in proestrus, was comparable to baseline values before the treatment (data not shown). Although the vehicle injected i.p. produced a small increase (∼28%, **Figure 3**), this effect was not significantly different from baseline ($p = 0.132$). To verify whether the

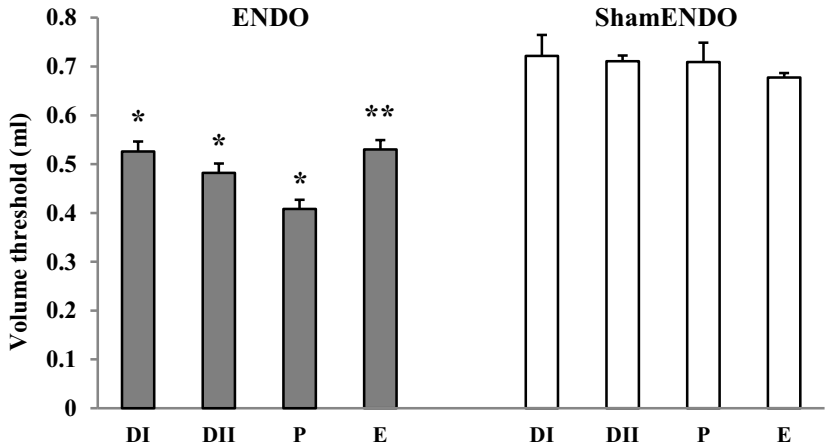


FIGURE 2 | Estrous variations in VMR to vaginal distention (Volume threshold) in rats >8 weeks after ENDO or shamENDO surgery. Baseline thresholds were measured across the estrous cycle over 3–5 weeks and then averaged for each estrous stage (i.e., in metestrus DI, diestrus DII, proestrus P, and estrus E). ANOVA showed that thresholds in ENDO rats were significantly different than thresholds in shamENDO rats ($p < 0.001$). *$p < 0.005$; **$p < 0.001$, compared to P.

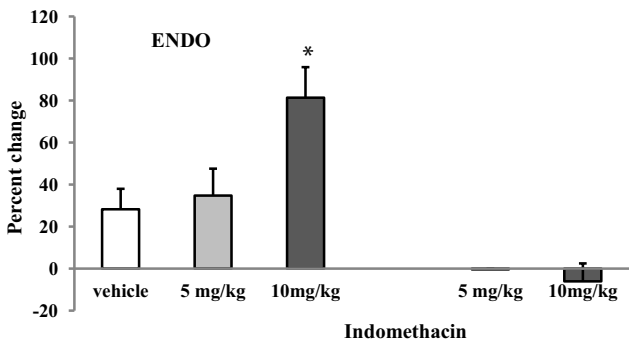


FIGURE 3 | Effects of i.p. injection of two doses of Indomethacin and vehicle on the VMR threshold in rats after ENDO or Sham ENDO surgery. Although vehicle produced a small increase in the VMR threshold, this increase was not significantly different from baseline. The increase produced by 10 mg/kg of Indomethacin was significantly higher than the increase produced by vehicle. *$p < 0.05$ compared to vehicle.

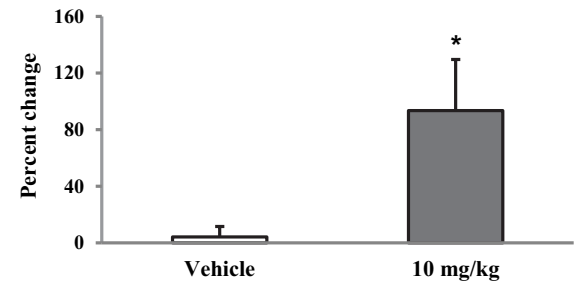


FIGURE 4 | Effect of 10 mg/kg of Indomethacin injected s.c. in the neck on the VMR threshold. *$p < 0.05$ compared to vehicle.

vehicle-induced effect was due to the i.p. injection or the vehicle itself, we injected the vehicle and one dose of Indomethacin (10 mg/kg) s.c. in the neck region in a separate group of ENDO rats ($n = 4$, **Figure 4**). In these rats, Indomethacin but not the vehicle produced a significant increase ($p < 0.05$, $93.5 \pm 36\%$). Therefore, it was the i.p. injection, not the vehicle, that produced the observed increase in the absence of the drug.

DISCUSSION

Using a telemetric data acquisition technique we were able to carry out a long-term study in conscious rats that, first, involved collecting baseline measures of VMR thresholds across the estrous cycle over 3–5 week period, and then, studying the effect of one or two doses of Indomethacin on the VMR threshold.

The first set of findings confirmed our previously published results on estrous cycle differences in the VMR threshold obtained with a non-telemetered system (Nagabukuro and Berkley, 2007). Similar to this earlier study, ENDO, but not its surgical control (shamENDO), significantly decreased the VMR threshold in all stages of the estrous cycle, being lowest in proestrus (when ovarian hormones plasma levels are highest). Therefore, we confirmed here that in cycling rats surgically induced ENDO produces vaginal hyperalgesia, the severity of which parallels estradiol levels.

In the second set of experiments, we further validated the technique by studying the effect of a NSAID Indomethacin on the hyperalgesia: Indomethacin increased the VMR threshold in ENDO rats when injected either i.p. or s.c. but did not affect this threshold in the shamENDO group: i.e., Indomethacin alleviated only ENDO-induced vaginal hyperalgesia. These findings imply that COX plays a role in maintenance of ENDO-associated vaginal hyperalgesia.

Some possible sites of Indomethacin action include (i) peripheral, locally active COX, whose enzymatic products, such as

PGE2, may activate the visceral afferents that innervate the cysts, (ii) COX located in the dorsal root ganglion, and (iii) central sites in the spinal cord and/or brain. Previously, we have shown that afferent fibers, whose branches supply the developed endometrial growths are overactive, which indicates that they are sensitized (Berkley et al., 2004; McAllister et al., 2012). It has been found in other conditions that afferent overactivity can be a result of sensory activation and sensitization by COX2 products (Rendig et al., 1994; Su and Gebhart, 1998; Su et al., 1999).These findings together support a peripheral effect of Indomethacin in the present study. The presence of COX enzymes in dorsal root ganglia and spinal cord, and inhibition of inflammation-induced PGE2 in cerebrospinal fluid and hyperalgesia by intraspinal COX2 inhibitors suggest that Indomethacin could also suppress prostaglandin production centrally (Samad et al., 2001; Yaksh et al., 2001; Dou et al., 2004; Nagabukuro and Berkley, 2007).

The small increase in the VMR threshold that was produced, when the vehicle was injected i.p. was not significant, and was not reproduced after the vehicle was injected s.c. in the neck. This result indicates that the effect was due to the injection itself, not to the vehicle. Thus it is important to recognize that injections may influence the VMR when they are performed close to the recording site (i.e., the abdomen).

Although tethered VMR-based techniques have been proven to be a useful tool in pain-related studies (Nagabukuro and Berkley, 2007; Dmitrieva et al., 2010), frequent repairs of the exposed electrode ends limits its usage in conscious rats to short-term studies. Psychophysics-based technique is an excellent tool for assessing pain-related behavior in rats with ENDO (Cason et al., 2003) but it requires rat pre-screening and training that can limit its applicability when long-term studies are considered. An advantage of telemetry-based VMR technique is that rats require minimal training and are not tethered during the experiment. Together, our findings indicate that the telemetric technique for assessing vaginal hyperalgesia in a rat model of ENDO is feasible and applicable for long-term pharmaceutical studies. Our results also suggest that COX activity plays a role in the vaginal hyperalgesia induced by ENDO. Because products of COX activity can trigger pain in different inflammatory conditions, ENDO-associated pain resembles inflammatory pain. Insofar as findings in the rat relate to ENDO in women, our findings here are in line with other evidence that links COX2 to the development and maintenance of ectopic endometrial growths and together support the conclusion that ENDO-associated pain has inflammatory origins.

ACKNOWLEDGMENTS

We thank Prof. Karen Berkley for her valuable advice overall, and Stacy McAllister for her advice with behavioral procedures. This study was supported by Bayer HealthCare Pharmaceuticals.

REFERENCES

Anaf, V., Simon, P., El, N. I., Fayt, I., Simonart, T., Buxant, F., and Noel, J. C. (2002). Hyperalgesia, nerve infiltration and nerve growth factor expression in deep adenomyotic nodules, peritoneal and ovarian endometriosis. *Hum. Reprod.* 17, 1895–1900.

Banu, S. K., Lee, J., Speights, V. O. Jr., Starzinski-Powitz, A., and Arosh, J. A. (2008). Cyclooxygenase-2 regulates survival, migration, and invasion of human endometriotic cells through multiple mechanisms. *Endocrinology* 149, 1180–1189.

Becker, J. B., Arnold, A. P., Berkley, K. J., Blaustein, J. D., Eckel, L. A., Hampson, E., Herman, J. P., Marts, S., Sadee, W., Steiner, M., Taylor, J., and Young, E. (2005). Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* 146, 1650–1673.

Bergqvist, A., Bruse, C., Carlberg, M., and Carlstrom, K. (2001). Interleukin 1beta, interleukin-6, and tumor necrosis factor-alpha in endometriotic tissue and in endometrium. *Fertil. Steril.* 75, 489–495.

Berkley, K. J., Dmitrieva, N., Curtis, K. S., and Papka, R. E. (2004). Innervation of ectopic endometrium in a rat model of endometriosis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 11094–11098.

Berkley, K. J., McAllister, S. L., Accius, B. E., and Winnard, K. P. (2007). Endometriosis-induced vaginal hyperalgesia in the rat: effect of estropause, ovariectomy, and estradiol replacement. *Pain* 132(Suppl. 1), S150–S159.

Bulun, S. E., Lin, Z., Imir, G., Amin, S., Demura, M., Yilmaz, B., Martin, R., Utsunomiya, H., Thung, S., Gurates, B., Tamura, M., Langoi, D., and Deb, S. (2005). Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. *Pharmacol. Rev.* 57, 359–383.

Cason, A. M., Samuelsen, C. L., and Berkley, K. J. (2003). Estrous changes in vaginal nociception in a rat model of endometriosis. *Horm. Behav.* 44, 123–131.

Chishima, F., Hayakawa, S., Sugita, K., Kinukawa, N., Aleemuzzaman, S., Nemoto, N., Yamamoto, T., and Honda, M. (2002). Increased expression of cyclooxygenase-2 in local lesions of endometriosis patients. *Am. J. Reprod. Immunol.* 48, 50–56.

Dmitrieva, N., Nagabukuro, H., Resuehr, D., Zhang, G., McAllister, S. L., McGinty, K. A., MacKie, K., and Berkley, K. J. (2010). Endocannabinoid involvement in endometriosis. *Pain* 151, 703–710.

Dogan, E., Saygili, U., Posaci, C., Tuna, B., Caliskan, S., Altunyurt, S., and Saatli, B. (2004). Regression of endometrial explants in rats treated with the cyclooxygenase-2 inhibitor rofecoxib. *Fertil. Steril.* 82(Suppl. 3), 1115–1120.

Dou, W., Jiao, Y., Goorha, S., Raghow, R., and Ballou, L. R. (2004). Nociception and the differential expression of cyclooxygenase-1 (COX-1), the COX-1 variant retaining intron-1 (COX-1v), and COX-2 in mouse dorsal root ganglia (DRG). *Prostaglandins Other Lipid Mediat.* 74, 29–43.

Efstathiou, J. A., Sampson, D. A., Levine, Z., Rohan, R. M., Zurakowski, D., Folkman, J., D'Amato, R. J., and Rupnick, M. A. (2005). Nonsteroidal antiinflammatory drugs differentially suppress endometriosis in a murine model. *Fertil. Steril.* 83, 171–181.

Golan, A., Dargenio, R., and Winston, R. M. (1986). The effect of treatment on experimentally produced endometrial peritoneal implants. *Fertil. Steril.* 46, 954–958.

MacHado, D. E., Berardo, P. T., Landgraf, R. G., Fernandes, P. D., Palmero, C., Alves, L. M., Abrao, M. S., and Nasciutti, L. E. (2010). A selective cyclooxygenase-2 inhibitor suppresses the growth of endometriosis with an antiangiogenic effect in a rat model. *Fertil. Steril.* 93, 2674–2679.

Maybin, J. A., Critchley, H. O., and Jabbour, H. N. (2011). Inflammatory pathways in endometrial disorders. *Mol. Cell. Endocrinol.* 335, 42–51.

McAllister, S. L., Dmitrieva, N., and Berkley, K. J. (2012). Sprouted innervation into uterine transplants contributes to the development of hyperalgesia in a rat model of endometriosis. *PLoS ONE* 7, e31758. doi:10.1371/journal.pone.0031758

Nagabukuro, H., and Berkley, K. J. (2007). Influence of endometriosis on visceromotor and cardiovascular responses induced by vaginal distention in the rat. *Pain* 132(Suppl. 1), S96–S103.

Noble, L. S., Takayama, K., Zeitoun, K. M., Putman, J. M., Johns, D. A., Hinshelwood, M. M., Agarwal, V. R., Zhao, Y., Carr, B. R., and Bulun, S. E. (1997). Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. *J. Clin. Endocrinol. Metab.* 82, 600–606.

Ota, H., Igarashi, S., Sasaki, M., and Tanaka, T. (2001). Distribution of cyclooxygenase-2 in eutopic and ectopic endometrium in endometriosis and adenomyosis. *Hum. Reprod.* 16, 561–566.

Rendig, S. V., Pan, H. L., and Longhurst, J. C. (1994). Brief mesenteric ischemia increases PGE2, but not PGI2, in intestinal lymph of cats. *Am. J. Physiol.* 266, R1692–R1696.

Samad, T. A., Moore, K. A., Sapirstein, A., Billet, S., Allchorne, A., Poole, S., Bonventre, J. V., and Woolf, C. J. (2001). Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 410, 471–475.

Stratton, P., and Berkley, K. J. (2011). Chronic pelvic pain and endometriosis: translational evidence of the relationship and implications. *Hum. Reprod. Update* 17, 327–346.

Su, X., and Gebhart, G. F. (1998). Mechanosensitive pelvic nerve afferent fibers innervating the colon of the rat are polymodal in character. *J. Neurophysiol.* 80, 2632–2644.

Su, X., Wachtel, R. E., and Gebhart, G. F. (1999). Capsaicin sensitivity and voltage-gated sodium currents in colon sensory neurons from rat dorsal root ganglia. *Am. J. Physiol.* 277, G1180–G1188.

Vernon, M. W., and Wilson, E. A. (1985). Studies on the surgical induction of endometriosis in the rat. *Fertil. Steril.* 44, 684–694.

Yaksh, T. L., Dirig, D. M., Conway, C. M., Svensson, C., Luo, Z. D., and Isakson, P. C. (2001). The acute antihyperalgesic action of nonsteroidal, anti-inflammatory drugs and release of spinal prostaglandin E2 is mediated by the inhibition of constitutive spinal cyclooxygenase-2 (COX-2) but not COX-1. *J. Neurosci.* 21, 5847–5853.

Zhang, G., Dmitrieva, N., Liu, Y., McGinty, K. A., and Berkley, K. J. (2008). Endometriosis as a neurovascular condition: estrous variations in innervation, vascularization, and growth factor content of ectopic endometrial cysts in the rat. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 294, R162–R171.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 June 2012; paper pending published: 31 July 2012; accepted: 09 August 2012; published online: 30 August 2012.

Citation: Dmitrieva N, Faircloth EK, Pyatok S, Sacher F and Patchev V (2012) Telemetric assessment of referred vaginal hyperalgesia and the effect of Indomethacin in a rat model of endometriosis. Front. Pharmacol. ***3****:158. doi: 10.3389/fphar.2012.00158*

This article was submitted to Frontiers in Neuropharmacology, a specialty of Frontiers in Pharmacology.