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Highlights in musculoskeletal pain 2021/22

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Highlights in musculoskeletal pain 2021/22

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Editorial: Highlights in musculoskeletal pain 2021/22

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Editorial on the Research Topic
Highlights in musculoskeletal pain 2021/22

Musculoskeletal pain refers to acute or chronic pain that affects musculoskeletal structures such as bones, muscles, ligaments, tendons, and nerves, which has become the main cause of disability around the world (1). According to the World Health Organization (WHO), there are 1.75 billion people globally with some form of chronic musculoskeletal pain (2). This condition greatly impacts people's life quality and well-being, and creates enormous socio-economic burdens (3). Musculoskeletal pain comprises numerous types, and the prevalence varies. The most common one is low back pain, which affects 30%–40% of adult patients; whilst fibromyalgia only 2% (4). The prevalence of knee pain is 10%–15%, and 15%–20% for neck and shoulder pain (5). Some risk factors have been identified to be associated with musculoskeletal pain, such as smoking, diet, depression, and sedentary lifestyle (6). Though progresses have been made in terms of neural mechanism and management strategy of musculoskeletal pain, challenges still exist especially for the chronic musculoskeletal pain characterized by sustained emotional distress and functional disability. In this special Research Topic *Highlights in Musculoskeletal Pain 2021/22*, we collated a series of articles that provide new knowledge about the epidemiology, mechanism or treatment of musculoskeletal pain.

Overview of the articles included in this Research Topic

Total twelve articles were collected in this Research Topic: four pieces of original clinical research, three review articles, two original basic studies, one survey report, one hypothesis and one perspective article.

Yalew et al. conducted a cross-sectional investigation among restaurant service staff in Ethiopia to assess the prevalence of work-related low back pain and the associated factors. More than two-fifth of those surveyed reported discomfort in low back area. Several predisposing factors associated were identified such as female, long standing duration while working and carrying out repetitive actions. Recommendations to prevent low back pain for restaurant service staff were provided, including regular exercise and delivering safety training.

In another cross-sectional investigation in India, Sankaran et al. focused on school-going children aged 10–16 from an urban and rural location, exploring the prevalence of musculoskeletal pain among them and its relationship with backpack weight. They reported a high prevalence of

musculoskeletal pain in these children, and demonstrated a significant association between backpack weight and musculoskeletal pain.

To study how muscle-muscle interactions act, Dunn et al. tested the modulating effect on hypertonic saline (HS)-induced forearm muscle pain by concurrent infusion of normal saline (NS) into adjacent, contralateral, and remote muscles, that is, the ipsilateral hand, contralateral forearm, and contralateral leg. They showed that subperceptual simultaneous infusion of NS into all these three areas raised the HS-induced overall muscle pain in the forearm. These results implicated the involvement of central nerval system underlying the muscle-muscle interactions.

Administration of non-steroidal anti-inflammatory drugs (NSAIDs) has been noticed to increase the risk of renal complication. Hayashi et al. observed the renal function change of chronic musculoskeletal pain patients with long-term administration of NSAID followed by tramadol hydrochloride/acetaminophen combination tablets (TA). They found that the estimated glomerular filtration rate (eGFR) of patients with NSAIDs administration for 12 months was reduced on cessation of this drug, but there was no reduction of eGFR after TA administration for the following 12 months. This study provides further evidence to highlight the strategy of multimodal analgesic medication against musculoskeletal pain in terms of the potential safety benefit.

Three review articles focused on the role of glia underlying mechanism of nociception. Boakye et al. comprehensively reviewed the process of microglia activation by secondary mediators released from primary afferent neurons, and further the microglia-neuron interaction in the spinal dorsal horn by tertiary mediators released from activated microglia, following peripheral nerve injury in neuropathic pain conditions. They presented an interesting paradox that since many different mediators shared similar effect in the peripheral and central nervous system, how inactivating one mediator can cause the overall pain to be relieved. They also highlighted the different roles of mediators between females and males.

The mini review completed by Gazerani discussed the involvement of peripheral satellite glial cells (SGCs) in pain signaling. The potential future directions in pain research were pointed out by summarizing the promising avenues and the meaningful topics regarding SGCs. Understanding the potential role of SGCs will aid the development of new therapeutics to target pain in the future.

Cedeño et al. reviewed the role of glial cells underlying mechanisms of pain alleviation by spinal cord stimulation using neuropathic pain model in animals. They showed that the approach of differential target multiplexed programming (DTMP) of spinal cord stimulation significantly modulates the transcriptomic profile of neuron and glia cells toward normal levels, indicating a shift in the neuron-glial environment involves in the analgesic effect of spinal cord stimulation.

In their original research article, Ahmed et al. explored whether a gap junction protein (connexin 43) expressed in the trigeminal ganglion is involved in persistent inflammatory hyperalgesia in the temporomandibular joint (TMJ) of both male and female animals. They reported that there was an increased connexin 43 expression following inflammation in TMJ in female rats rather than males. Interestingly, inhibiting connexin 43 in trigeminal ganglion reversed TMJ inflammation-induced masseter muscle overactivity in a sex-independent way, indicating that connexin 43 was involved in the enhancement of jaw muscle activity in both males and females under TMJ inflammation.

In another original research article, Wang et al. explored the effect of c-Jun N-terminal kinase (JNK) on modulating glutamine synthetase (GS) in astrocytes. They observed that GS was activated and phosphorylation of JNK was increased in astrocytes after exposure to lipopolysaccharide (LPS). The changes in GS were reversed following endocannabinoid 2-arachidonoylglycerol (2-AG) administration, but the activation of JNK was not affected, suggesting the phosphorylation of JNK has no effect on modulating of GS in astrocytes by 2-AG.

Clingan et al. presented a brief survey of currently available spinal cord stimulator hardware sold in the United States for the treatment of chronic pain. They introduced the features, indications, and limitations which make each product unique. Understanding each product's nuances will aid the selection of most appropriate device for patients with chronic pain.

In their hypothesis and theory article, Tuckey et al. proposed a novel mechanism of interstitial inflammatory stasis and lymphatic drainage impairment underlying chronic musculoskeletal pain. They hypothesize that inflammatory substance may be entrapped in interstitial space and lymphatic pathways following immune activity or trauma, leading to the interstitial stasis of inflammation. Then the sympathetic mechanism was activated which further decrease blood perfusion and disable the local lymphatic pumping, leading to additional interstitial stasis. This feed-forward loop may play a vital role in the development and maintenance of chronic musculoskeletal pain.

In their perspective article, Schmid et al. come up with a novel cross-disciplinary approach to fill important knowledge gaps in low back pain research, by connecting methods from neuroscience and biomechanics research including functional magnetic resonance imaging, psychological analysis, optical capturing of motion and digital modeling of musculoskeletal system. This novel approach may aid the clarify of motor-control strategy with different phenotypes and the development of better treatment options.

Author contributions

The author contributed to manuscript preparation and approved it for publication.

Conflict of interest

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.

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BRIEF RESEARCH REPORT
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Survey of Spinal Cord Stimulation Hardware Currently Available for the Treatment of Chronic Pain in the United States

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Background: The number of spinal cord stimulator (SCS) units sold in the United States (US) for the treatment of chronic pain has increased with a corresponding expansion in the number of different SCS platforms available. Each marketed stimulator has several unique features, indications, and limitations, which distinguish one from the other and makes the selection of appropriate hardware possible for optimal patient care. There are an even greater number of similar and overlapping features between SCS.

Measures: We used market analysis techniques to survey the currently available SCS technology. We then reviewed published device specifications and manuals for comparison of features.

Outcomes: As of 2020, there are nine commonly used SCS platforms made by four manufacturers including four SCS units from Abbott, three from Boston Scientific, and one each from Medtronic and Nevro.

Conclusions: A working understanding of each SCS product's nuances is needed for selecting the most appropriate device with which to manage chronic pain patients. Here we present a brief survey of currently available SCS hardware in the US and the features that make each product unique.

Keywords: spinal cord stimulation, neuromodulation, chronic pain, practice management, pain treatment, medical devices

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INTRODUCTION

The point prevalence of chronic low back pain (cLBP) among all adults in the United States (US) is 13.1% (1). Several factors have been identified to confer a more than doubling of the adjusted odds ratio (aOR) of cLBP including being between 50 and 69 years old (aOR 2.03–2.07), having less than a high school education (aOR 2.27), having an annual household income <$20,000 (aOR 2.29), income derived primarily from disability (aOR 2.62), depression (aOR 3.30–10.62 depending on severity), sleep disturbances (aOR 3.90), and other medical comorbidities (aOR 2.49–6.09) (1). The lifetime prevalence of acute LBP is nearly 80% in the United States (2). There is concern that as the US population ages and attains increasing risk factors for the development of cLBP, there will be a need for increased treatments (3).

The treatment of cLBP pain represents a major financial burden on the US health-care system. The 12 month health-care expenditures of adult patients with LBP in the US was found to be $25,613 (95% confidence interval $25,569–$25,657) among patients who underwent spine surgery compared to $795 ($790–800) among patients who chose non-surgical treatments (4). The two major considerations when choosing a spinal cord stimulation (SCS) system are efficacy, which is often equivalent to spine surgery, and cost, which is substantially less than spine surgery. SCS represents a continuously evolving technology with evidence for cost-effective management of cLBP. The use of older, non-rechargeable implanted pulse generators (IPGs) was associated with similar incremental cost utilization ratio (ICUR) compared to surgical reoperation for the treatment of LBP (0.59 vs. 0.83) (5). The use of SCS for the treatment of neuropathic leg and LBP was associated with higher upfront costs compared to conventional medical therapy ($19,486 vs. $3,994) but increases in health-care-related quality of life and EuroQol-5D (EQ-5D) scores at 6 months (6).

The utilization of SCS therapy for the treatment of chronic painful conditions continues in the US due to well-documented efficacy. The rapid development of SCS systems over time necessitate continuously updated reviews of available hardware (7). There exists a number of different products available in the US, each with its own unique features, indications, and limitations. The purpose of this review is to succinctly present the unique and differentiating aspects of commonly available SCS systems currently available on the US market. The intention of the review is that it will be a periodically updated resource that will reflect changes in available SCS products.

METHODS

This study only gathered data that was publicly available. As such, the study did not require Internal Review Board approval. Internet search tools including MEDLINE, EMBASE, Google scholar, and Google were used to identify SCS products. Searches included terms such as "spinal cord stimulation" "dorsal column stimulation." Title and abstracts were iteratively reviewed for relevance with particular emphasis placed on high-quality health-care market assessments and product details provided by either device manufactures or independent, non-biased sources (e.g., FDA and other government agencies). Data was excluded if it described products that were not approved and available for patients to use in the United States in 2020 for the intended implantation in spine. This resulted in the exclusion of SCS devices available in other countries as well as for other indications, such as vagal nerve stimulators, which were not relevant to our analysis. Additionally, reviews focusing on mechanism of action or clinical effectiveness were not included as this was not the primary goal of the manuscript. All authors were involved in gathering and interpreting information. Unique features of products were then confirmed using from several sources, including product manuals, medical conference proceedings, published investor and business development reports, publicly available company due diligence reports, peer-reviewed medical literature, and device manufacturer-produced literature. When necessary, clarification was made through requesting additional documentation from device manufacturer sales teams and engineering support personnel. Endnote X9 was used to manage references and data sources (Clarivate Analytics, Philadelphia PA).

RESULTS

There are currently nine different SCS units commonly-available for the treatment of pain in the United States. The features of each device are presented in **Table 1**. Data was derived from a number of sources (8–16). Eight of the product's leads are intended to be placed over the dorsal columns of the spinal cord, and one product's leads are intended to be placed over the dorsal root ganglion. While the dorsal root ganglion is not technically a part of the spinal cord, the provided mechanism of action and treatment indications of this device makes it more appropriately discussed with spinal cord stimulators rather than peripheral nerve stimulators. Different batteries have unique warranty of between 2 and 10 years, while most are expected to last longer than this prior to the need to be replaced. Four of the systems do not use rechargeable batteries and five of the systems do use rechargeable batteries. Recharging times range from 15 to 120 min. The frequency and rate of recharging is generally a function of the stimulation settings. With regards to MRI compatibility, five of the spinal cord stimulator systems are full-body conditional, and two are compatible with only head and extremity imaging, one is not MRI compatible, one is compatible only with cranial imaging. Each device has a unique definition of conditionality with MRI that should be carefully considered prior to imaging. Two of the devices do not need to be deactivated while driving, and seven do need to be deactivated while driving when used to treat LBP and/or lower extremity pain. Seven are capable of burst frequency programing. The exact definition of "burst frequency programming" varies between devices and is provided in the footnotes of **Table 1**. The sizes of the IPG for each system are presented in **Table 2**. The Medtronic Intellis system currently has the thinnest IPG. Older units that still appear on company websites but are not highly marketed are listed in **Table 3**.

CONCLUSIONS

The ongoing development of SCS technology has led to the commercialization of several products on the US market, each with unique properties. This ever-expanding armamentarium allows physicians to individualize pain treatment and overcome previously existing treatment barriers. The current selection of SCS technologies has improved over previous generations through the refinement of SCS technologies including the miniaturization of IPGs, extended battery life, unique/novel waveforms and programming options, improved designs to ease trials and implantation, and a reduction in limitations of use,

TABLE 1 | Features of currently available spinal cord stimulation systems.

Manufacturer	Device	Date of FDA approval	Upgradeable software	Battery life*	Rechargeable battery	Recharging frequency	MRI compatibility	Turn off while driving	Turn off while sleeping	Burst capable	Unique factors	Other
Boston Scientific	WaveWriter	January 2018	No	Five year warranty, usually lasts 12 years	Yes	15–30 min daily	Head only	Yes	At patient's discretion	Yes	1. Paresthesia-free stimulation at 1.2 kHz 2. Paresthesia-free "micro-burst" programing 3. Can run both burst and tonic stimulation simultaneously	Currently involved in litigation with Nevro over patent laws concerning frequency
	Precision Montage	May 2016	No	Five year warranty	Yes	120 min every 2–3 days	Full body conditional	Yes	At patient's discretion	Yes		
	Precision Novi	June 2015	No	Two year warranty, usually lasts 5 years	No		No	Yes	At patient's discretion	Yes	1. Capable of burst or 1.2 KHz stimulation but not recommended as it will decrease battery life 2. Cannot do burst and 1.2 KHz simultaneously	
Medtronic	Intellis	July 2017	Yes	Nine year warranty	Yes	60 min every 1–5 days	Full body conditional	Yes	At patient's discretion	No	1. Can use "low dose" 40 Hz" or "high dose" 1000 Hz stimulation	1. Purchased Stimgenics in January 2020 for undisclosed amount. Conducting RCT for incorporation of proprietary waveform that targets glial cells 2. Smallest battery
Nevro	Senza Omnia	November 2019	Yes	Minimum 10 year	Yes	45 min daily	Full body conditional	No	No	Yes	1. Does not require mapping 2. can simultaneously run burst with high frequency (10 kHz) or lower frequency	
Abbott	Proclaim XR Recharge-Free	September 2019	Yes	Five year warranty	No	NA	Full body conditional	Yes	At patient's discretion	Yes	1. No need to recharge 2. Can be controlled through Apple device, such as iphone, with Bluetooth connection 3. Postural changes affect stimulation intensity	
	Proclaim Elite with burst	October 2016	yes	Up to 10 years	No	NA	Full body conditional	Yes	At patient's discretion	Yes	1. No need to recharge 2. Can be controlled through Apple device, such as iphone, with Bluetooth connection 3. Postural changes affect stimulation intensity	

(Continued)

TABLE 1 | Continued

Manufacturer	Device	Date of FDA approval	Upgradeable software	Battery life*	Rechargeable battery	Recharging frequency	MRI compatibility	Turn off while driving	Turn off while sleeping	Burst capable	Unique factors	Other
	Prodigy MRI IPG	October 2016	Yes	Ten year warranty	Yes	45 min 1–3 times per week	Head and extremity only	Yes	At patient's discretion	Yes	1. Can be controlled through Apple device, such as iphone, with Bluetooth connection 2. Postural changes affect stimulation intensity	
	Proclaim DRG Neurostim	November 2016	Yes	5 to 6 years on average	No	NA	Head and extremity conditional	No	At patient's discretion	No	1. Only DRG stimulation product currently available 2. Can be controlled through Apple device, such as iphone, with Bluetooth connection	

DRG, dorsal root ganglion; FDA, Food and Drug Administration; Hz, hertz; IPG, implantable pulse generator; KHz, kilohertz; MRI, magnetic resonance imaging; RCT, randomized controlled trial.

**Battery life and frequency of charging is technically a byproduct of individual patient usage. Higher usage will result in more frequent charging sessions and decreased battery life. A patient's recharge routine may vary depending on your stimulation parameters. High power users will require more frequent charging.*

Boston Scientific Definition of Burst: 2–6 bursts at 450 Hz frequency.

Boston Scientific Definition of Microbursts: between 0 and 1 s range. Amplitude is set at 50% of the patient perception threshold. Pulse width is 210 ms, rate is 450 Hz intraburst with 40 Hz interburst. Microburst is 6 pulses on 12. Ms, followed by 12.5 ms off.

Nevro Definition of Burst: 2–4 bursts, at 500 Hz.

Abbott Definition of Burst: 40, 500 Hz of 5 s spikes; with cycles of (1) 30 s on, 30 s off, (2) 30 s on, 3 min off, (3) 30 s on, 6 min off.

TABLE 2 | Size comparison of implantable pulse generators.

Manufacturer	Device	Size (depth × height × length) mm
Boston Scientific	Precision Plus	10 × 54 × 45
	Precision Novi	11.3 × 70.9 × 49.5
Medtronic	Intellis	6 × 57 × 47
Nevro	Senza II	10 × 56 × 46
	Omnia	10 × 56 × 46
Abbott/ St. Jude	Eon Mini	9 × 50 × 57
	Prodigy MRI	9 × 48 × 53
	Proclaim Elite	13 × 56 × 50
	Proclaim XR	13 × 56 × 50
	Proclaim DRG	13 × 61 × 50

mm, millimeter.

TABLE 3 | Older products not discussed but still appear on company product websites.

Name	FDA approval date
Boston Scientific Precision	2004
Boston Scientific Precision Plus	2005
Boston Scientific Precision Spectra	2013
Medtronic Restore Advanced	7/2006
Medtronic Restore Ultra	2/2008
Medtronic Restore Sensor	11/2011
Medtronic Prima Advanced Surescan MRI	2013
Nevro Senza	2015
Nevro Senza II	2018

such as the expansion of MRI compatibility. We anticipate that this market will continue to be develop.

DISCUSSION

The technology for SCS is continuously improved with the goals of refining current treatment applications and expanding therapeutic indications. In 2019, there was a decrease in the US SCS market overall. However, by 2025 the US SCS market is expected to increase by 5–10% compounded annual growth (17).

The most common new trend is the development of multiple waveform-capable product lines and individual products, such as the non-rechargeable Abbott Proclaim (burst and traditional) and the Nevro Omnia (burst, traditional, and high frequency). The optimal waveform and programming for the treatment of different painful phenotypes is currently being investigated in several ongoing clinical trials with results expected in 2022 or later (NCT03681262, NCT03957395, and NCT03014583). Currently there is a paucity of evidence from direct comparison of different waveforms in pragmatic clinical trial settings to adequately inform healthcare decisions.

The development of future SCS technology, including novel platforms and programming, will continue to occur in order to satisfy ongoing and unmet patient needs. Predictions of any new technology remains would be vague for two reasons.

First, any new SCS technology would need to be formally evaluated in clinical trials for both safety and effectiveness prior to commercialization. Second, the need for protections of novel intellectual property makes very little information available to the public. Future iterations of this or similar manuscripts will strive to provide details of such new and emerging technology. SCS leads are a crucial component of an implantable SCS system. The use of different numbers and types of leads (paddles vs. percutaneous, one lead or two) can result in significant changes in the clinical profile of many SCS systems and is an additional important consideration for implanting physicians to consider.

The evidence on SCS for the treatment of pain is expanding. While the focus of this manuscript was to survey the characteristics of the hardware, unique clinical outcomes and head-to-head comparisons are extremely important considerations. The currently published reviews of SCS clinical utility do not allow for several practical questions to be answered such as the ability to decrease opioid use or increase in functional capacity. There is also a dearth of large-scale and long-term data regarding the utilization of high-cost health-care resources after implantation of a spinal cord stimulator, such as the avoidance of spine surgery. With the increased utilization of SCS to treat LBP in non-previously operated spines, additional data will be needed to delineate the most effective SCS treatment algorithms in these patients. Physicians who use SCS to treat pain are now faced with several options in the US market with both unique and overlapping features.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors contributed equally and meet the criteria for authorship based on International Committee of Medical Journal Editors.

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BRIEF RESEARCH REPORT
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Modulation of Muscle Pain Is Not Somatotopically Restricted: An Experimental Model Using Concurrent Hypertonic-Normal Saline Infusions in Humans

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We have previously shown that during muscle pain induced by infusion of hypertonic saline (HS), concurrent application of vibration and gentle brushing to overlying and adjacent skin regions increases the overall pain. In the current study, we focused on muscle-muscle interactions and tested whether HS-induced muscle pain can be modulated by innocuous/sub-perceptual stimulation of adjacent, contralateral, and remote muscles. Psychophysical observations were made in 23 healthy participants. HS (5%) was infused into a forearm muscle (flexor carpi ulnaris) to produce a stable baseline pain. In separate experiments, in each of the three test locations (n = 10 per site)—ipsilateral hand (abductor digiti minimi), contralateral forearm (flexor carpi ulnaris), and contralateral leg (tibialis anterior)—50 μl of 0.9% normal saline (NS) was infused (in triplicate) before, during, and upon cessation of HS-induced muscle pain in the forearm. In the absence of background pain, the infusion of NS was imperceptible to all participants. In the presence of HS-induced pain in the forearm, the concurrent infusion of NS into the ipsilateral hand, contralateral forearm, and contralateral leg increased the overall pain by 16, 12, and 15%, respectively. These effects were significant, reproducible, and time-locked to NS infusions. Further, the NS-evoked increase in pain was almost always ascribed to the forearm where HS was infused with no discernible percept attributed to the sites of NS infusion. Based on these observations, we conclude that intramuscular infusion of HS results in muscle hyperalgesia to sub-perceptual stimulation of muscle afferents in a somatotopically unrestricted manner, indicating the involvement of a central (likely supra-spinal) mechanism.

Keywords: normal saline, muscle afferent, somatotopy, muscle pain, hypertonic saline, hyperalgesia, central sensitization

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INTRODUCTION

For most individuals, it is relatively easy to distinguish between innocuous and noxious stimuli. However, in a subset of individuals afflicted with chronic pain, there is a disturbance of normal somatosensory function, such that a normally innocuous stimulus can evoke pain, for example, the emergence of tactile allodynia in patients with sciatica (1). This can have a debilitating impact on both the individual and society (2, 3).

Studies using hypertonic saline (HS) infusions have shown a touch-evoked pain (allodynia) that extends to overlying (4) and adjacent (5, 6) skin regions. Intramuscular HS administration produces a deep musculoskeletal pain that often extends or refers to distal regions (7–9). Repeated intramuscular injections of HS reveal plastic processes with a decrease in the area and intensity of local pain and an increase in the expression of referred pain (10) in addition to the emergence of pain hypersensitivity that extends bilaterally (11). These complex interactions cannot readily be explained by changes in peripheral circuitry and appear to mimic characteristics of chronic pain conditions such as fibromyalgia. Within such chronic pain conditions, current arguments favor an explanation based on a central change in, or sensitization of, the neural function that results in the observed widespread and diffuse musculoskeletal pain, pressure-pain hypersensitivity, cutaneous allodynia, and tactile dysesthesia (12–14).

In the current study, a HS infusion model was used to examine whether the interaction previously observed between muscle and skin (4, 6, 11) can be replicated between adjacent and remote muscles. We hypothesized that the presence of background nociceptive activity using HS infusion would produce a state of central sensitization resulting in an exacerbation of the overall pain (hyperalgesia) to the application of a normally innocuous stimulus (normal saline, NS). We also hypothesized that this effect would occur regardless of whether the NS was infused into an adjacent or a remote muscle.

METHODS

Twenty-three healthy naïve participants aged 18–28 years (six females), with no reported history of musculoskeletal or neurological disorders, were recruited for this study. Participants were asked to abstain from intensive bouts of exercise for 48 h preceding the experiment so as not to sensitize the target muscles (15). Six participants took part in multiple arms of the study across different experimental sittings (30 experiments total), the inclusion of these participants in multiple study arms was random. One participant took part in all arms of the study, whilst a further five participated in both the contralateral and remote testing procedures. To minimize the risk of a placebo effect or familiarization with the protocol, repeat participants did not undertake experiments in any prescribed order with control recordings in the absence of HS-infusion (i.e., no-pain) obtained at the commencement of each separate experiment session across each of the test locations.

Informed written consent was obtained from each participant prior to the experiment. This study was approved by the Human Research Ethics Committee (approval numbers: H9190 and H13204) of Western Sydney University in accordance with the revised Declaration of Helsinki.

Participants were comfortably seated in a chair throughout the experiment. HS and NS were infused using a Syringe Infusion Pump (Harvard Apparatus, South Natick, Massachusetts, USA) and a 25G winged infusion set. Importantly, the Syringe Infusion Pump used for NS-infusion was obscured from sight and did not include any audible cues. Pain ratings were continuously recorded using the ADInstruments Response Meter connected to the ADInstruments PowerLab (ADInstruments, Dunedin, New Zealand). The Response Meter had a slide control, and the pain scale was divided into ten equal segments within a range of 0 (no pain) to 10 (worst pain). In addition, participants were asked to verbally report the location of pain during the course of the experiment.

Infusion of Hypertonic Saline

Across all parts of the study, 5% HS was infused into the belly of the flexor carpi ulnaris (FCU) muscle of the forearm for ∼10 min to establish a stable baseline pain. The muscle belly was palpated whilst the participant performed light flexion and adduction of the wrist to identify the boundaries of the FCU muscle. The needle was inserted ∼0.8–1 cm into the center of the muscle belly at an angle perpendicular to the skin at the site of insertion. The infusion rate of HS in the FCU varied between subjects (30–175 μl/min) to establish a moderate pain intensity preferably between 4 and 6 (out of 10) on the pain scale. Once a stable baseline pain was achieved, no further changes were made to the infusion rate.

Infusion of Normal Saline

After a stable baseline pain was maintained for at least a minute, NS (0.9%) at room temperature was concurrently infused at the rate of 50 μl/min for 1 min per trial (tested in triplicate). This duration was chosen based on the data collected in a pilot study which indicated a delay of several seconds before the onset of an increase in pain levels. The delayed response has been reported in previous studies (6, 16). The triplicate NS trials were performed at 1-min intervals.

Participants were asked to continuously rate the overall pain intensity, and any changes thereof, on the pain scale. Care was taken to avoid the use of suggestive language with participants informed that the HS-induced pain could remain the same, increase or decrease during the co-infusion with NS.

In addition to concurrent HS-NS infusions, NS alone was infused in triplicate trials prior to the commencement and upon cessation of HS-evoked pain in all experiments. Typically, the HS-evoked pain disappeared over a time course of under 10 min. After a 3- to 5-min wait following cessation of pain, NS infusion was repeated at each site. Collectively, ∼450 μl of NS was infused per muscle.

Part 1: Interactions With Adjacent Muscles

NS was infused into the ipsilateral abductor digiti minimi (ADM) muscle of the hand to examine potential interactions between adjacent muscles in response to HS-induced acute muscular pain. The muscle belly of the ADM was identified by palpation whilst the participant abducted the fifth digit of their hand. The infusion needle was inserted to a depth of ∼0.5 cm into the center of the ADM muscle belly. The ADM muscle was chosen as it shares the same peripheral innervation (ulnar nerve) as the HS-infused FCU.

Part 2: Contralateral Interactions

NS was infused into the belly of the contralateral FCU muscle of the forearm to test whether the HS-NS interactions were limited to muscles within the same nerve territory or spread to contralateral muscles as well. The needle location and insertion for the contralateral FCU were identical to the HS-infusion site described prior.

Part 3: Remote Interactions

NS was delivered to the belly of the tibialis anterior (TA) muscle of the contralateral leg to determine the spatial extent of inter-muscle interactions in an acute pain state. The muscle belly of the TA was identified by palpation during dorsiflexion of the ankle. The needle was inserted into the middle of the belly of the TA muscle perpendicular to the skin to a depth of ∼1 cm.

Statistical Analysis

Repeated measures two-way analysis of variance (RM 2-way ANOVA) was used to compare pain ratings at baseline (HS infusion alone) with evoked responses (co-infusion of NS and HS) at each location (adjacent, contralateral, and remote). Where a significant change ($P < 0.05$) was found, individual comparisons were made using Tukey's multiple comparison test. The normal distribution of data was confirmed in all groups using D'Agostino and Pearson omnibus normality test. Pain scores for the baseline (HS) and co-infusion (HS and NS) conditions are presented as mean ± standard error of the mean (SEM) for all parts of the study. Statistical analysis was performed using GraphPad Prism (version 7.04, La Jolla, California, USA).

RESULTS

Prior to the induction and following the cessation of HS-evoked muscle pain, all participants reported NS infusion (50 μl/min) to be innocuous (i.e., rated as 0 out of 10 on the pain scale) and imperceptible regardless of the NS infusion site (**Figure 1A**). The infusion of 5% HS into the FCU always resulted in a diffuse, deep pain in the muscle that extended down the medial aspect of the forearm. This baseline pain remained stable in the absence of NS co-infusions (**Figure 1A**) and did not significantly differ between the different parts of the study ($P = 0.66$).

At all three test locations (adjacent, contralateral, and remote), the co-infusion of NS significantly increased the overall pain in all trials (T1-3, $P < 0.0001$, **Figures 1B–D** left-hand panel). All observed increases in pain scores during co-infusion were transient and time-locked to the NS-infusion, with the pain returning to baseline (HS) within 1 min of the cessation of NS co-infusion (example shown in **Figure 1A**). Further, the increases in pain scores did not vary in amplitude based on the location (adjacent, contralateral, and remote) of the NS co-infusion ($P = 0.30$).

The pooled mean response of all participants in each part of the study, with respective HS and HS + NS data points linked, are shown in the right-hand panel of **Figures 1B–D** and described further in the following sections.

Part 1: Interactions With Adjacent Muscles

The infusion of HS into the FCU resulted in a pooled mean score of 4.3 ± 0.5 ($n = 10$). When NS was co-infused into the adjacent ADM in the presence of this background pain, the pooled mean score increased to 5.0 ± 0.4 (**Figure 1B**). This constitutes a pain increase of ∼16% and when comparing baseline and co-infusion pain scores the increase in pain ratings was significant [$P < 0.0001$, $F_{(1,27)} = 318.5$]. This indicates that muscle pain can be modulated by low-threshold/sub-perceptual stimulation of an adjacent muscle.

Part 2: Contralateral Interactions

The infusion of HS into the FCU resulted in a pooled mean score of 4.3 ± 0.1 ($n = 10$). The co-infusion of NS into the contralateral FCU increased this pooled mean score to 4.8 ± 0.2 (**Figure 1C**). This represents a ∼12% increase in the pain scores during co-infusion, an effect found to be significant [$P < 0.0001$, $F_{(1,27)} = 156.7$]. This demonstrates that muscle pain can be modulated by normally sub-perceptual stimulation across contralateral muscles, thereby suggesting a central (spinal/supra-spinal) phenomenon.

Part 3: Remote Interactions

Within this aspect of the study, participants reported a pooled mean score of 4.0 ± 0.1 in response to infusions of HS into the FCU ($n = 10$). During concomitant infusion of NS into the contralateral TA, participants reported a pain increase of 15% with the pooled mean score increasing to 4.6 ± 0.1 (**Figure 1D**). A comparison of the baseline and co-infusion pain scores revealed a significant difference [$P < 0.0001$, $F_{(1,27)} = 97.84$]. The observed interaction between the site of noxious muscle stimulation and remote innocuous muscle stimulation alludes to the involvement of a supra-spinal mechanism.

In **Figure 2**, triplicate responses for each individual ($n = 10$ per test location) at all three test sites ($n = 90$) to transient NS infusion during HS infusion (i.e., HS + NS) have been plotted as a function of the baseline pain evoked by HS alone. When plotted in this manner, all data points fell to the left of the line of equivalence (x = y or HS = HS + NS) indicating that the NS infusion evoked a reproducible pain increase across a broad range (pain scale 1.4–6.7) of baseline pain levels.

When participants were asked about the location of pain, all participants—except 2 in part 1 and one each in parts 2–3—ascribed it to the forearm where hypertonic saline was infused with no discernible percept attributed to the sites of NS infusion. This was true not only for HS-evoked pain but also for pain increases during HS-NS co-infusions. The four subjects who did not ascribe the pain increase to the HS-infusion site instead ascribed it to the NS-infusion site. Importantly, these subjects always reported NS-infusion as imperceptible at the local site under

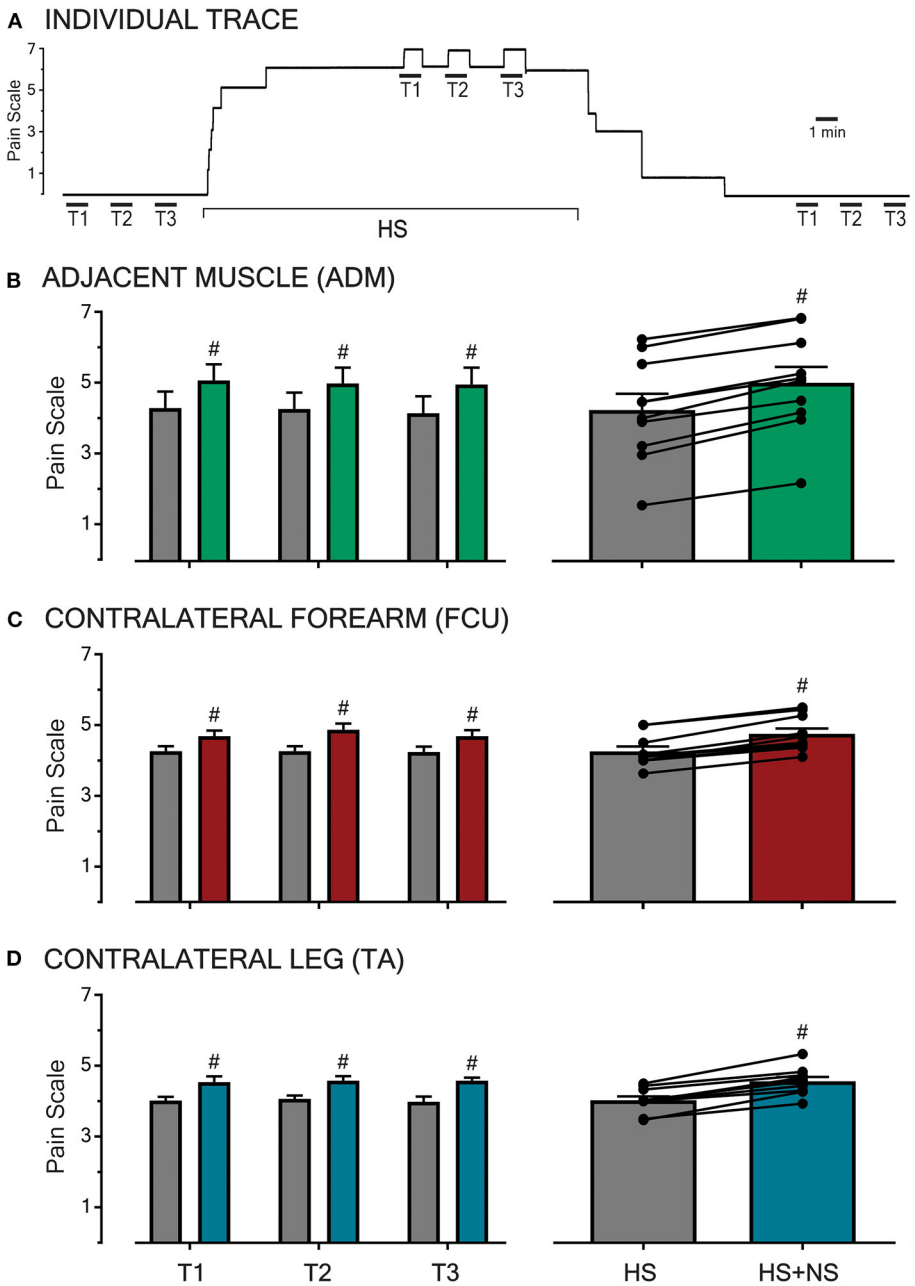

FIGURE 1 | Pain intensities in response to HS-infusion and subsequent to transient NS-infusions at various sites across the body. An example raw trace of a participant's pain ratings throughout an experimental sitting is shown **(A)**. In the absence of background pain, infusions of NS for 1 min (T1, T2, T3 with infusion time-course shown by the overlying bar) were imperceptible. During baseline HS-induced muscle pain in the FCU, co-infusion of NS (triplicate, left **B–D)** produced a reproducible increase in overall pain. Following the cessation of HS infusion and the associated background pain (0 out of 10 on the pain scale), NS trials were once again imperceptible. In all three sessions, HS pain was generated in the FCU, and the test location for NS infusion was the adjacent ADM muscle **(B)**, the contralateral FCU **(C)**, or the contralateral TA muscle (**D**, remote). At each test location, NS co-infusion during HS background pain resulted in a reproducible and significant increase in overall pain ($P < 0.0001$, right **B–D**). The transient pain increase was reproducible across trials at all sites. Significant changes ($P < 0.0001$, #) were confirmed between baseline (HS) and co-infusion (HS + NS) using RM 2-way ANOVA.

control and recovery conditions (no HS-pain). This suggests that NS infusion was almost always nonpainful regardless of whether there was HS pain or not, but in the presence of HS pain, the NS co-infusion resulted in hyperalgesia at the HS site, and this modulation of HS pain was not somatotopically restricted.

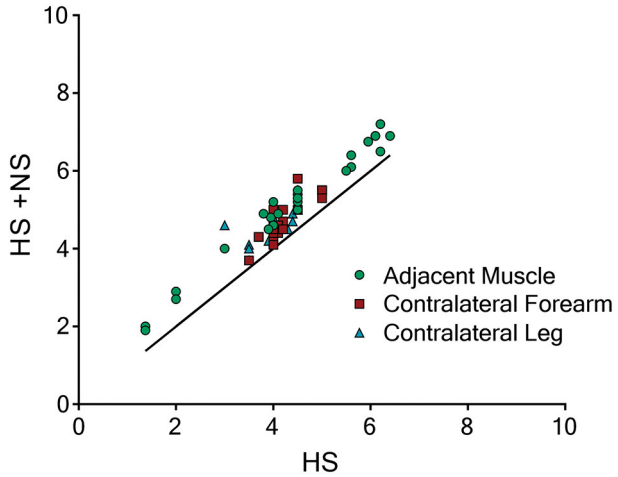

FIGURE 2 | Triplicate data points for each participant during HS-NS co-infusion plotted as a function of baseline pain. When triplicate responses from each participant at each location ($n = 10$ per location, total $n = 90$) to NS infusion during HS infusion (HS + NS) are plotted as a function of baseline pain (i.e., HS alone) all data points fall to the right of the line of equivalence (x = y or HS = HS + NS). This indicates that the NS infusion evoked a reproducible effect between trials and across all test sites and over a broad range of baseline pain levels.

DISCUSSION

The current study has provided evidence that muscle pain can be modulated (hyperalgesia) by sub-perceptual stimulation of muscle afferents in a somatotopically unrestricted manner. This finding not only builds upon the previous observation that an intramuscular HS infusion can result in allodynia in the overlying and adjacent skin regions (4, 6, 17) but the spatial extent of this modulation, spanning several spinal segments, suggests the involvement of a central, likely supra-spinal, mechanism.

The sub-perceptual nature of repeated intermittent NS infusions (50 μl over 1 min) under control (no HS-pain) condition suggests that localized muscle distension does not activate the nociceptors (18) but may activate low-threshold stretch-sensitive receptors within the muscle. In this respect, these weak mechanical stimuli resemble the inability of weak (micro) intraneural electrical stimulation to produce a discernible pain sensation at recording sites dominated by muscle spindles (19, 20). We have also previously shown that intradermal infusions of NS (50 μl/min for 2 min) do not produce a percept (5).

The conversion of the sub-perceptual NS stimulus to one that enhances pain, during HS infusion in the FCU muscle, is unlikely to be due to peripheral sensitization given the anatomical separation (forearm vs. hand, >15 cm) and the small volume of intermittently infused NS. Likewise, the increase in pain evoked by NS-infusion into the contralateral forearm is more consistent with a central involvement. Furthermore, the interaction between the FCU and the contralateral TA suggests that the central involvement likely extends to supra-spinal structures. Assertions as to the exact location of this central involvement cannot be resolved by this study, but the acute/short-lasting and reversible nature of these interactions do suggest that the requisite circuitry may already be present, and thus an elaborate anatomical reorganization need not be necessary for these to occur.

The broad-ranging muscle-muscle interactions observed here appear to be in marked contrast to the somatotopically constrained interactions observed in the skin; for example, the confinement of secondary hyperalgesia to the region immediately surrounding intradermal capsaicin injection (21–23) or the inability of microstimulation of large-diameter mechanoreceptors innervating a skin region beyond the site of secondary hyperalgesia to produce a painful percept (16).

The effects observed in the current study are most likely driven by a transient and reversible episode of central sensitization (increased excitability and synaptic efficacy of central nociceptive pathways) (24) in response to the HS-induced muscle pain. The HS infusion alone was run for ∼10 min prior to the commencement of NS co-infusion, and this may have resulted in a state of central sensitization. Indeed, the clinical correlates of central sensitization (25, 26) are apparent in a HS-infusion model with hyperalgesia and allodynia reported in this and previous work (4, 6, 11, 17).

The generalized modulation of the exacerbated pain response at the HS-induced muscle site during NS-infusion in the adjacent, contralateral, and remote muscles is noteworthy and warrants further study using more quantitative measures of pain localization than verbal reporting. Further, the quality and temporal characteristics of this hyperalgesia need further investigation. In addition to the prerequisite of ongoing nociceptive input (HS infusion), we observed that the onset of NS-evoked increase in pain tended to be delayed by several seconds, which suggests a possible need for temporal summation.

Previous findings in humans have shown that repeated intramuscular HS injections in the TA result in a pressure-pain hypersensitivity developing across both the ipsilateral and contralateral TA muscles (11). Further, it has been shown in animals that a unilateral forelimb injury can produce sensory perturbations in the contralateral limb (27). In the case of intramuscular HS, the evidence for centralized effects necessitates the need for control data collection prior to any HS administration and warrants an investigation into other commonly used pain models.

In the current study, the co-infusion of sub-perceptual NS resulted in increased HS-pain (i.e., hyperalgesia). In HS and other experimental models as well as chronic pain conditions, tactile and thermal stimuli can produce allodynia (pain to a normally nonpainful stimulus) and hyperalgesia (increased pain from a painful stimulus) (1, 4, 6), but paradoxically, these modulatory stimuli—both painful and nonpainful—can also reduce pain with slow gentle brushing of the skin shown to reduce cutaneous heat pain (28). Conditioned pain modulation is a well-recognized phenomenon in which a painful stimulus can

be inhibited by a second painful stimulus applied to a different body site (i.e., pain inhibits pain) (29–31). The underlying mechanisms are not fully understood but likely involve a complex interplay between excitatory and inhibitory circuits in the central nervous system.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of Western Sydney University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SN and DM contributed to the conception and design of the study. JD performed the experiments and organized the database and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Satellite Glial Cells in Pain Research: A Targeted Viewpoint of Potential and Future Directions

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Chronic pain is known to be caused by sensitization within the pain circuits. An imbalance occurs between excitatory and inhibitory transmission that enables this sensitization to form. In addition to neurons, the contribution of central glia, especially astrocytes and microglia, to the pathogenesis of pain induction and maintenance has been identified. This has led to the targeting of astrogliosis and microgliosis to restore the normal functions of astrocytes and microglia to help reverse chronic pain. Gliosis is broadly defined as a reactive response of glial cells in response to insults to the central nervous system (CNS). The role of glia in the peripheral nervous system (PNS) has been less investigated. Accumulating evidence, however, points to the contribution of satellite glial cells (SGCs) to chronic pain. Hence, understanding the potential role of these cells and their interaction with sensory neurons has become important for identifying the mechanisms underlying pain signaling. This would, in turn, provide future therapeutic options to target pain. Here, a viewpoint will be presented regarding potential future directions in pain research, with a focus on SGCs to trigger further research. Promising avenues and new directions include the potential use of cell lines, cell live imaging, computational analysis, 3D tissue prints and new markers, investigation of glia–glia and macrophage–glia interactions, the time course of glial activation under acute and chronic pathological pain compared with spontaneous pain, pharmacological and non-pharmacological responses of glia, and potential restoration of normal function of glia considering sex-related differences.

Keywords: satellite glial cells (SGCs), pain, sensory ganglia, trigeminal ganglion (TG), dorsal root ganglion (DRG), nociception, peripheral nervous system

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INTRODUCTION

Chronic pain is a debilitating and common condition (1), and it has a substantial impact on affected individuals, society, and the health-care system (2). It is generally accepted that pathological chronic pain is caused by a maladaptive process that occurs when an imbalance is present between excitation and inhibition signaling pathways underlying pain (3). Both functional and structural alterations have been identified. Altered neuronal activity, manifested as sensitization of peripheral primary sensory neurons in the sensory ganglia [e.g., dorsal root ganglia (DRG) and trigeminal ganglia (TG)] and central sensitization of nociceptive neurons within the central nervous system (CNS), including the spinal cord, trigeminal nucleus, brain stem, and cortex, has been reported

(4). Treatment of chronic pain is complicated and often results in inadequate response or side effects. Attempts are ongoing for a better understanding of pain processes, mechanism-based treatment and targeting, implications of multidisciplinary pain management, and patient-centered strategies (5).

Generally, there has been increasing interest in the role of the non-neuronal components of the nervous system (glial cells) in the health and diseases of the nervous system (6–8). These cells have been markedly recognized to contribute to the development or maintenance of abnormal neuronal excitability (9). In this line, accumulating evidence supports the contribution of glial cells in the initiation or maintenance of chronic pain (10). Major glial residents in the CNS, namely, astrocytes and microglia, have been the subject of extensive research, and their important role in the pathogenesis of persistent pain is becoming definitive (11–13). Cross talk between astrocytes, microglia, and neurons has been suggested to promote pathological chronic pain or pain chronification, i.e., transition from acute to chronic pain (14). Interestingly, in the context of pain, gliopathy (e.g., astrogliosis and microgliosis) seems to play distinct roles (10, 15). Gliosis is non-specifically defined as a reactive response of glial cells in response to insults to the CNS. Differences in the response of microglia and astrocytes depend on the type of pain (16), the time course of insult (17), and sex (18). Excellent reviews are available to comprehend the role of astrocytes and microglia in chronic pain (10, 19–24). Recently, the potential role of other central glia, oligodendrocytes, has also been investigated, and current findings collectively support their participation in the central pain process and contribution to persistent pain (25). Targeting central glia to reverse chronic pain or to prevent its development has also emerged (26, 27).

Glial cells of the peripheral nervous system (PNS) have also been investigated in the context of chronic pain pathology and targeting (28–30). These cells include satellite glial cells (SGCs), Schwann cells (SCs), and enteric glial cells (EGCs). The latter two cell types are less investigated than SGCs. Within ganglia, SGCs surround the cell bodies of neurons very closely and create a unique structure, a unit of neuron–SGC, which is not found in other parts of the nervous system (31). In different pain models with a neuropathic or inflammatory nature, SGCs have been shown to undergo alterations in structure and function (31, 32). Consequently, the neuronal activity of sensory ganglia neurons is affected, which is reflected in hyperactivity of neurons, neuron–SGC coupling, elevated responses to adenosine triphosphate (ATP), release of cytokines, and downregulation of potassium channels (32). It is proposed that this increase in neuronal activity is linked to the development of chronic pain. A distinct pattern is seen in SGCs following insult to the PNS. A recent review summarized common changes that occurred in SGCs in four major pain models: systemic inflammation, postoperative pain, diabetic neuropathy, and postherpetic pain (32). SGC alterations have been documented in response to both injury and inflammation. These cells, therefore, have become another potential target for therapeutic purposes, i.e., for the prevention or treatment of chronic pain. An argument has been formed around preference in targeting these cells, as SGCs are located outside the blood–brain barrier (BBB), which might offer a better potential for blocking pain transmission at the periphery. Considering that these cells seem first to respond to injury or inflammation prior to central glial cells, they may also offer potential for minimizing the risk of chronification and transition from peripheral to central sensitization (15, 33). Elegant reviews are available to deepen the knowledge of what has been investigated and found in exploring the roles of SGCs in pain (32, 34–37) or its targeting (27). The purpose is therefore not to provide a comprehensive systematic review of the current literature on the role of glial cells in pain, since several excellent reviews are already available, where the readers are referred to (10, 19–24, 32, 34–37). Instead, this paper aims to provide a viewpoint on potential future directions and avenues to stimulate further interest and to form scientific hypotheses with a focus on peripheral glia, mainly SGCs. Further investigation of glia in relation to pain and its targeting is not only a truly fascinating field of science but also highly valuable in understanding pain mechanisms and mechanistic-based optimized targeting.

FUTURE DIRECTIONS FOR SATELLITE GLIAL CELLS IN PAIN RESEARCH

Satellite Glial Cells' Characterizations by Aid of Novel Tools

Historically, SGCs were considered cells that share some common features with astrocytes; hence, the expression of some proteins that were known for astrocytes was expected in these cells, such as glial fibrillary acidic protein (GFAP), glutamine synthetase, glutamate aspartate transporter, and connexin 43 gap junction (31, 38). However, it was determined that these cells have their own morphology and characteristics that are unique, and differences might exist between SGCs that are located in the DRG and those located in the TG. Heterogeneity was also observed in terms of the morphology and distribution of these cells within sensory ganglia around different neuronal populations, e.g., with different sizes (39). These observations highlighted the fact that the characterization of these cells in the TG and DRG under physiological and pathological pain with different natures (e.g., neuropathic and inflammatory) is valuable. This is crucial when pain conditions in humans are modeled in laboratory animals and to test potential targets for pain. It has gradually become evident that accurate information on SGC and macrophage morphology and function will facilitate research on the roles of these cells in pain and as potential therapeutic targets (15, 28). Perhaps one of the limitations that have slowed down the process has been the lack of proper methods or tools to facilitate dynamic visualization of these cells.

A recent study (40) focused on the characterization of SGCs and macrophages in the DRG. The authors applied the method of specific gene expression or deletion and examined Ca^{2+} dynamics in these cells. Both immunohistochemistry and 2-photon Ca^{2+} imaging have been used to characterize SGCs in the DRG in the available and most commonly used genetically modified mouse lines that are used to study astrocytes or microglia. Interestingly, findings from this study pointed out that the majority of lines used in studying astrocyte

functions were not efficient in studying SGCs in the DRG, with the exception of two mouse lines. The authors used mouse lines of S100β-eGFP, ALDH1L1-eGFP, GFAP-Cre::GCaMP6f, GLAST-CreERT2::GCaMP6f, Cx30-CreERT2::GCaMP6f, and Cx43-CreERT2::GCaMP6f for SGCs in the DRG and similar lines for astrocytes in the visual cortex (40). The double transgenic line Cx43-CreERT2::GCaMP6f permitted inducible GCaMP6f expression in more than 90% of DRG SGCs (92.6%), where the expression of GCaMP6f in neurons was only 4%. It remains to be determined whether GCaMP6f is expressed in other cells within the DRG, such as endothelial cells, fibroblasts, or SCs (40). Interestingly, not only was the expression of Cx43 found to be very stable, but also it was upregulated after injury insult in the PNS (40). Hence, it seems that this mouse line can be a useful tool in pain research focused on PNS and pain. The results from this study (40) also demonstrated that the knock-in CX3CR1-eGFP mouse line presents specific eGFP expression in the majority of microglial cells and macrophages in both the DRG and the visual cortex. Therefore, this line can be an option when studying specific targeting of SGCs in the DRG. These two validated mouse lines (Cx43-CreERT2::GCaMP6f and CX3CR1-eGFP) could be used as proper tools for further investigation of SGCs in the DRG under healthy and painful conditions (40). This direction presents new avenues toward the development and application of research tools to enable progress in research on SGCs in relation to pain. For example, it has been proposed that genetically encoded animals can allow studying sensory neuron–SGC interactions (41, 42). This would provide potential for studying the specific roles of target genes that are expressed in SGCs following pathological pain. This approach has similarly been proposed for investigating the roles of SCs in neuropathic pain (30).

Another attempt is to properly isolate SGCs to characterize them and study their function (43). This approach has been used to examine whether isolation would dramatically change the natural milieu that SGCs normally experience *in vivo*. A contradiction exists in the literature, but cell-based platforms have been used for the characterization of SGCs (44–47) and their function (48, 49). Recent studies have shown that the transcriptomes of SGCs can be determined under normal and pain conditions (50). Next-generation RNA sequencing by Jager et al. (50) provided the first evidence on the state of SGCs under normal conditions and following peripheral nerve injury. Findings from this study show similarities between naïve SGCs and astrocytes, being enriched in genes associated with the immune system and cell-to-cell communication. Data from this study (50) show that 3 days following injury, several genes linked to cholesterol biosynthesis are downregulated in SGCs, and this pattern was also present 14 days postinjury. SGC transcriptional analysis, however, shows a signature that 14 days postinjury, a higher expression of genes associated with MHCII and migration of leukocytes is present. Access to the full transcriptome has been offered by the authors (on the gene omnibus database) (50) and can serve as an important and valuable tool to understand cell function and regulation of different gene products. This study is also the first to provide evidence that postinjury perineuronal proliferating cells are not SGCs but macrophages.

Transcriptomics, focused on the characterization of individual cells, is increasingly used (51). This approach is valuable because single-cell RNA sequencing allows analysis of subtypes of SGCs and comparison of these cells in different sensory ganglia in one species or comparison between species, for example, between rodents and humans. This line of research will be particularly important when researchers are focused on pain conditions that are specific to one type of sensory ganglia, for example, dental pain, headache, and other types of orofacial pain that need a focus on SGCs in the TG (52, 53).

In addition, due to the nature of translational gaps between human glia and glia in rodents (54), the identification of human sensory ganglion cells would reveal similarities and differences and hence provide a more accurate understanding based on transcriptome profiling. Some attempts have already been initiated (55, 56). Having access to human sensory ganglia (healthy and pain patients) for research would close the gaps in the findings obtained from rodent models (32). A study in 2018 (55) examined the transcriptomic analyses of DRGs obtained from human donors and mouse tissues, including DRGs. This study has also created an online, searchable repository to provide access to data on cross-species analysis of DRGs. This would be highly valuable to speed up the screening of valuable targets for therapeutic purposes (55). This indeed also emphasizes an urgent need to access databases for researchers working in SGC-related pain research.

Extracellular vesicles (EVs) are released by cells into the extracellular space. EVs are secreted by a range of cell types, can be isolated, and can be characterized. Their roles in the nervous system (e.g., in cell–cell communication) under health and disease have been reviewed recently (57). Proteomic profiling of EVs shed from SGCs has been reported (45), and the findings have revealed differentially regulated proteins when SGCs are stimulated by lipopolysaccharides (LPSs; mimicking inflammation). These proteins include junction plakoglobin and myosin 9, which can be considered markers of SGC responses under inflammatory conditions.

An elegant recent review (58) summarized the ncRNAs in neuropathic pain within the PNS and the CNS. The findings cover both neuronal and non-neuronal cell sources of these molecules and, interestingly, those related to SGCs in the TG and DRG. For example, NONRATT021972 and uc.48+ upregulate the ionotropic purinoreceptor P2X7 in SGCs (59, 60). Interestingly, inhibition of uc.48+ has been shown to reduce mechanical hypersensitivity in a rat model of trigeminal neuralgia by inhibiting the expression of the P2X7 receptor in trigeminal SGCs (61).

ncRNAs' roles in pain are not limited to neuropathic pain. A recent study (62) provided information on the role of lncRNA X inactivate-specific transcript (XIST) in inflammatory pain. In this study, a complete Freund's adjuvant (CFA) model of inflammatory pain was established in rats, where high expression of XIST and voltage-gated sodium channel (VGSC) 1.7 (Nav1.7) was observed in the DRG. When the authors applied XIST inhibition, pain behavior (reflected on mechanical withdrawal threshold) and SGC expression of GFAP, inflammatory cytokine levels of interleukin-6, and tumor necrosis factor-α were

diminished (62). In contrast, downregulation of XIST increased the mechanical pain threshold and decreased the expression of miR-146a. To identify the role of XIST, the authors ran an *in vitro* test and identified that XIST acted as a sponge of miR-146a, which targeted Nav1.7 and concluded that based on these observations, XIST can regulate SGCs in the DRG under inflammatory pain condition and hence can be a future therapeutic target (62).

Therefore, the identification of signatures or biomarkers in SGCs can offer a further characterization of these cells under health and pathological pain conditions. The literature presents some data available for both the DRG (50) and TG (45, 63). lncRNAs and circRNAs and the computational construction of interaction networks between lncRNAs/circRNAs–miRNAs–mRNAs can provide new directions and potential therapeutic targets.

Computational Modeling of Satellite Glial Cells' Behavior Within the Sensory Ganglia

Another path that researchers started exploring is the potential of computational modeling. For example, a group of researchers (64) have tried to investigate and determine the characteristics of intercellular communication between sensory neurons in the DRG and SGCs by applying ATP. Researchers of this study have proposed that the neural engineering approach provides a physiologically constrained computational model that can be used for several purposes, in addition to physiological communication of neurons and SGCs (64), for example, understanding of various factors that control this communication, such as changes in receptor expression or activity, e.g., Kir 4.1 current density that occurs in SGCs under pain. Perhaps by expansion in the use of artificial intelligence in neural engineering, this field can also benefit from further advancement to deepen the knowledge on predictive parameters affecting SGC–neuron interactions in relation to pain. Such an attempt has been presented for neuron–astrocyte interactions (65). Biocomputational modeling can potentially provide a platform to test hypotheses about SGC–neuron interactions or SGC–SGC interactions and parameters influencing those interactions within the sensory ganglia.

Satellite Glial Cells' Role in Nerve Repair

Research on nerve repair has long focused on sensory neurons and their signaling alterations after injury in addition to SCs that insulate axons (66–68). Only recently have sparks been raised about the contribution of SGCs that envelop the neuronal soma. Evidence started to accumulate supporting their roles in nerve repair. A recent study (69) provided results indicating that the synthesis of fatty acids in SGCs promotes sensory neuron repair after injury and results in regeneration. In this study (69), first, the researchers identified a new marker in SGCs via transcriptional profiling, which is called Fabp7/BLBP (fatty acid binding protein 7/brain lipid-binding protein). Upon nerve injury, alterations in gene expression were observed in SGCs that were mainly related to fatty acid synthesis and peroxisome proliferator-activated receptor alpha (PPARα) signaling. Based on this observation, researchers (69) modeled the injury condition, where deletion of fatty acid synthase (Fasn) resulted in the absence of axon regeneration. To reverse this condition, they applied fenofibrate, which is a PPARα agonist, and axon regeneration returned in mice lacking Fasn in SGC. These findings (69) demonstrated that fatty acid synthesis in SGC is a crucial step in nerve repair in adults after peripheral nerve injury. In the context of pain, this can offer a new direction in regenerative responses after nerve injury promoted by SGCs. Interestingly, astrocytes have been identified as essential for the development and function of axons *in vivo*, and lipid metabolism in these cells has been found to be a critical step in this process (70). Therefore, the authors of this study (69) have suggested further investigations to identify how lipid metabolism in SGCs influences axon regeneration, for example, via a paracrine effect or other mechanisms. In addition, they left open questions for further investigation of the potential effects of fenofibrate on centrally projecting sensory axon growth (69). The clinical implication of fenofibrate to yield beneficial neuroprotective effects has already been discussed for diabetic retinopathy (71) and brain trauma (69, 72). Considering the complexity of the changes that occur after peripheral nerve injury and the involvement of several cell types, it has been suggested to investigate interactions between SCs, fibroblasts, and macrophages in addition to sensory nerves and SGCs (73). This would facilitate a better understanding of cell-specific roles in repair phenomena following peripheral nerve injury. In addition, much remains to be investigated in relation to myelinating and non-myelinating forms of SCs (74). Identification of cell–cell interactions might be achievable by new high-resolution live imaging techniques (75) to characterize dynamic changes in neuropathies over time, e.g., changes in SGCs of damaged nerves or development of new SGCs, and identification of acute vs. chronic responses for event time-course analyses. In addition, it has been demonstrated that macrophages interact with SGCs within sensory ganglia (76). Therefore, the interaction of SGCs with other cells is valuable to consider in future studies and how the interaction may influence the overall neuronal response.

It has been shown that transplantation of SCs might be a promising method to promote neural repair. SCs from rats were cultured and microencapsulated in a research study (77) and then administered to rats that underwent chronic constriction injury (CCI). Data showed that microencapsulated SC transplantation could block the expression of the purinergic receptor P2X3 in the DRG and diminish the behavioral components of a neuropathic pain model (77). It is not yet known whether such a method can be applicable for SGCs considering that fatty acid synthesis in SGCs has been identified as a crucial step in nerve repair in adults after peripheral nerve injury (69).

An increased number of studies are becoming available to present the responses of SCs, in particular to nerve injury and contributions to neuropathic pain (30, 78). An emerging line of investigation related to SCs in pain is the identification and characterization of different roles of myelinating and non-myelinating SCs in neuropathic pain. There is also interest in drugs that can target SCs in addition to the possibility of SC transplantation as potential future options in the treatment of neuropathic pain (30).

Recently, a specialized type of peripheral glial cell was discovered in the skin (79), where it produces a mesh-like network that plays an essential role in sensing noxious stimuli to thermal and mechanical stimuli. These glial cells are closely associated with unmyelinated nociceptors and convey nociceptive information to the nerve; hence, they are called nociceptive SCs. Further investigation is expected to emerge on these cutaneous SCs and their role, now that they have been found to be able to initiate pain-like behavior (79).

Functional Roles of Satellite Glial Cells

A general view is that activation of glial cells contributes to the development of pain due to the release of proinflammatory cytokines and chemokines and other substances and factors that drive pain signaling, such as glutamate, calcitonin gene-related peptide, and substance P (80). However, since glial cells also release anti-inflammatory substances, one can consider that beneficial effects might also be present, for instance, to reverse neurotoxicity and pain (80). Considering this side of the coin, we might be able to promote the protective function. This is particularly interesting, as glial inhibitors *per se* have not been successful in alleviating pain, mainly because the normal activity of glia must remain reserved, as they have critical roles with the PNS and CNS. This is not an easy path in the production of glia-associated drugs because the way that glial cells behave is complex and depends on numerous factors, such as the type of stimuli, location, and length of stimuli. Information on SGCs is very limited in this area, but some literature exists for microglia and astrocytes. The challenge is still to determine whether and how the proinflammatory nature of SGC activation can be prevented while its anti-inflammatory nature can be promoted. It has been shown that activation of central glia by LPS leads to the release of proinflammatory cytokines, but when growth factors or anti-inflammatory cytokines are applied, glial cells release factors that can promote neuronal survival (80).

This field needs further *in vitro* research (e.g., rodent and human cell cultures), *in vivo* research (e.g., transplantation of human glia in rodents), and translational research in humans [e.g., by application of positron emission tomography (PET) technique and tracers (81, 82), to follow glial activation at different time points and in response to different stimuli or in pain patients with acute or chronic pain conditions]. Eventually, by better understanding the molecular mechanisms behind the role of glia in pain, proper, and safer therapeutic agents might be developed. Focusing on central glia in this line does not necessarily close the field for more research in peripheral glia, including SGCs. In addition, considering different pain conditions, one can reserve possibilities for the activation of SGCs in the TG that contributes to orofacial and craniofacial pain conditions vs. their activation in the DRG that relates to pain in other body regions, even though overlap occurs, for example, in diabetic neuropathy manifested in the feet and eye (83) or musculoskeletal pain (84).

Sex-Dependent Characteristics and Function of Satellite Glial Cells

Considering that pain is a sexually dimorphic phenomenon (85) and that some painful conditions are predominant in one sex (e.g., migraine in females) or only exist in one sex (e.g., pelvic pain due to endometriosis in females), it is important to include this aspect in further glial-associated pain research (86). A number of reports propose that pathological pain in males is regulated by microglial signaling (21, 87); however, astrocyte signaling seems not to show a sex-dependent nature in inflammatory and neuropathic pain models (18).

The literature shows that following peripheral nerve injury, proliferation, and morphological changes occur in microglia in males and females (85). However, only in male animals has the functional role of microglia been observed, which is proposed to drive neuropathic pain (88, 89).

We still do not know whether any sex-related characteristics or functional responses exist in the activation of SGCs following PNS insult. Further research can present the value and importance and whether any natural protective mechanism or susceptibility might exist in either sex related to SGCs and whether this can be manipulated or targeted for pain control.

Satellite Glial Cells in Sympathetic and Parasympathetic Ganglia

The behavior of SGCs in the sympathetic ganglia has rarely been investigated (32, 90); hence, the role of these cells is not yet clear. A study from 2004 (91) reported that sciatic nerve transection resulted in changes in both the DRG and lumbar sympathetic ganglia, where neuroinflammatory responses were evident in both ganglia, and interestingly, some markers were more affected in the sympathetic ganglia than in the DRG, such as GFAP reactivity, macrophage reactivity, and T cell responses (91).

Another study examined the recruitment of T-lymphocytes and macrophages into lumbar sympathetic ganglia and DRG in a rat model of spinal nerve ligation (SNL), where different patterns of response were found. The authors suggested that this pattern difference between these ganglia may provide information about contribution of macrophages in neuronal insult and post injury hyperexcitability (92). Another study (93) demonstrated that when the sympathetic nerves in the superior cervical ganglia were damaged, SGCs became activated and underwent alterations consisting of coupling, higher sensitivity to ATP, and less responsiveness to acetylcholine. Interestingly, in this study, SGCs of the TG were not affected (93).

Glial coupling is not limited to autonomic ganglia and has also been studied in sensory ganglia (41, 94). Coupling is defined as the formation of connectivity between SGCs that can be observed as an elevated number of gap junctions between these cells, which was reported in response to peripheral nerve injury (37). Next, electrophysiological methods and dye injection were applied to confirm the initial observations that higher coupling occurred postinjury. This finding has been reported consistently in the literature in several pain studies in which both inflammatory and neuropathic models were applied (95, 96). Consequently, it was reported that gap junction blockers

such as carbenoxolone, meclofenamic acid, and palmitoleic acid could diminish coupling between SGCs and reduce pain in experimental animal models (97). Collectively, evidence supports the notion that enhancement of SGC coupling through gap junctions is associated with the development and maintenance of neuropathic pain (37).

Among connexins, connexin 43 (Cx43), a gap-junction protein that is expressed in activated SGCs (98), has been investigated rather extensively. This has gained attention because connexins could be targeted to block pain. Connexin proteins are presented with 20 subtypes and, among other roles, function as gap junctions between cells. Recent studies highlight the role of connexins in the induction and maintenance of chronic pain, where their modulation has resulted in pain amelioration in several chronic pain models (99). Interestingly, chemotherapy-induced neuropathic pain, for example, following the administration of oxaliplatin and taxol, has been linked to SGC activation, with a proposed mechanism involving coupling by gap junctions (100). This has also been shown *in vitro* (101). Blocking gap junctions, for example, by administration of carbenoxolone, which blocks connexin 43, has been shown to reduce chemotherapeutic-induced hypersensitivity in animal models of pain (100).

Collectively, and based on limited available data (102), research on SGC activation, coupling, and their influence on neurons within autonomic ganglia (sympathetic and parasympathetic) and sensory ganglia—in relation to pain—will continue to emerge (32, 103). Further investigation would also help identify the exact underlying mechanisms of gap junctions and inhibitors in pain control (90). Drug-induced peripheral neuropathy, as has been seen with chemotherapeutic agents, can also be studied further, and research should examine the role of SGCs in limiting the side effects of these agents.

Miscellaneous

In addition to pharmacological approaches as powerful tools to study SGCs in pain research, it is also of great interest to test non-pharmacological approaches, for example, whether neuromodulation techniques that are used to alleviate pain of different types could exert any effect on non-neuronal cells in chronic pain.

Investigation of the effects of environmental factors on SGCs, such as dietary control, microbiome, and other environmental factors, such as stress, under health and pathological pain conditions remains open.

The role of the SGC in the overall immune responses in ganglia, for instance, pathogen defense against viral infection, is becoming more evident. This avenue might not be directly linked to pain research but will allow for further characterization of the non-neuronal response of SGCs within PNS ganglia.

EGCs reside within the enteric nervous system (ENS) (104). These cells have some common features with astrocytes from the functional and structural points of view. EGCs regulate ENS homeostasis, and violation of their normal function leads to gastrointestinal disorders, such as functional gastrointestinal disorders and inflammatory bowel diseases (105, 106). In addition, EGCs have been identified to modify visceral pain signaling via cross talk with neurons and immune cells. This observation has potential in understanding the mechanisms underlying chronic abdominal pain and its targeting (107, 108). This direction of research is also proposing to expand further, in particular in relation to an increasing amount of research on gut microbiota and its interaction with the brain. In addition, since enteric glia are affected by stress, they are considered to play a substantial role in visceral hypersensitivity and the immune response to stress (107).

CONCLUSION

In the past few years, several breakthroughs have been achieved with a focus on glia and cross talk of glia with neurons and other cells that have revolutionized pain research and inspired implications for pain management in the future and further research.

The development and availability of sophisticated technology and tools (109) to study the molecular, genetic, morphological, physiological, and pathological aspects of glia *in vitro* and *in vivo* have definitely advanced the field. Translational research has moved the field from experimental rodent models toward human studies, although limited, but substantial achievements have been made. Some clinical trials have attempted to use available compounds with glial modulatory effects in humans with various outcomes, such as vitamin D (110) and ibudilast (111) in migraine patients, minocycline for lumbar radicular neuropathic pain (112), and palmitoylethanolamide for the treatment of different types of pain (113).

Investigation of human glia moves the field one step closer to testing and potential application of strategies for prevention and therapy of chronic pain, with fewer risks due to interspecies gaps from preclinical to clinical stage. Realization of parameters that can influence the complex behavior of glia has also advanced formulation of testable hypotheses, for example, interactions of SGCs with other SGCs, neurons, and macrophages that collectively determine pain responses to nerve injury and inflammation. Considering age and sex is gaining further attention in studying glia in pain research.

Bioinformatics, neuronal engineering, complex modeling, and dynamic and live assessment techniques together with profiling these cells via quantitative methods such as mapping the transcriptome and evaluating the responses of SGCs to indirect environmental changes in the host that can influence pain response and sensitivity have become more familiar to researchers and have inspired drug designer and pharmaceutical and non-pharmaceutical strategies to maintain the protective and positive functions of SGCs while shifting their negative influence on pain toward pain relief.

In addition, finding the crucial role of SGCs in nerve repair deserves further investigation. A focus on peripheral nerve regeneration via the contribution of both SGCs and SCs sounds logical. At a system level, one can also consider how much knowledge can be obtained if information can be collected from different types of glia within different systems, e.g., from both PNS and CNS glia, considering the time course of acute and

chronic pain and response to manipulating factors. This would enhance the visibility of the big picture in the entire body system to unmask some missing pieces.

These and several more dimensions, such as 3D tissue prints, potential use of induced pluripotent stem cells (iPS cells), and cell transplantation techniques, with a wide range of research applications in this field, have become available and should be further researched to not only advance the fascinating science of glia but also to pave the way for potential targeted therapy that can offer safer and efficient options for pain patients.

AUTHOR CONTRIBUTIONS

PG conceptualized and carried out the literature search and wrote this mini review.

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Impact of Non-steroidal Anti-inflammatory Drug Administration for 12 Months on Renal Function

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Background: The use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with an increased risk of renal complications. Resolution of renal adverse effects after NSAID administration has been observed after short-term use. Thus, the present study aimed to investigate a series of patients with chronic musculoskeletal pain who underwent long-term NSAID administration followed by switching to tramadol hydrochloride/acetaminophen (TA) combination tablets to study the impact of NSAID-induced renal adverse effects.

Methods: This was a longitudinal retrospective study of 99 patients with chronic musculoskeletal pain. The patients were administrated with NSAIDs daily during the first 12 months, followed by daily TA combination tablets for 12 months. Estimated glomerular filtration rate (eGFR) and serum levels of aspartate aminotransferase and alanine transaminase were measured at baseline, after NSAID administration and after TA administration.

Results: eGFR was significantly reduced after 12-month NSAID administration (median, from 84.0 to 72.8 ml/min/1.73 m^2), and the reduction was not shown after the subsequent 12-month TA administration (median, 71.5 ml/min/1.73 m^2). Reduction in eGFR was less in patients who received celecoxib (median, −1.8 ml/min/1.73 m^2) during the first 12 months. There was no significant difference in aspartate aminotransferase and alanine transaminase in each period.

Conclusions: Thus, patients receiving NSAIDs for 12 months displayed both reversible and irreversible reduction of eGFR upon cessation of NSAIDs and switching to TA. Our data highlight the potential safety benefit of utilizing multimodal analgesic therapies to minimize the chronic administration of NSAIDs.

Keywords: anti-inflammatory agents, analgesics, drug-related side effects and adverse reactions, longitudinal studies, kidney, musculoskeletal pain

INTRODUCTION

The administration of non-steroidal anti-inflammatory drugs (NSAIDs) to treat chronic musculoskeletal pain has become widely used in the clinic due to its ability to provide effective levels of pain relief (1–6). However, regular administration of NSAIDs has an increased risk of gastrointestinal, cardiovascular, and renal complications (1–6). There is a linear relationship between NSAID cumulative dose and change in renal function over a 2-year period (7). Despite the high incidence of dose/duration-dependent renal adverse effects (estimated at 1–5%) (7, 8), there is a paucity of data regarding the long-term safety of NSAID therapy, and the risk of renal damage has prompted an increasing appreciation in the value of multimodal analgesia in the management of moderate-to-severe pain. For example, tramadol hydrochloride/acetaminophen (TA) combination tablets have emerged as a particularly useful option for chronic pain management (5, 6).

Previous studies have demonstrated that the renal adverse effects of NSAIDs are usually reversible (8–10), but such studies have several limitations. For example, Chou et al. showed the risk of kidney injury is higher in current NSAID users than in past NSAID users *vs.* control (9), which suggests the renal risks from NSAIDs could be reversible. However, they defined past NSAID users as having a termination date of 31–180 days before the index date, regardless of the administration period. Moreover, Shukla et al. reported that rises in kidney injury biomarkers resulting from regular NSAID therapy for spondyloarthritis are seen as early as 1 week and continue to rise up to 6 weeks (10). Notably, the same study also showed reversibility in the rise of kidney injury biomarkers at 12 weeks upon stopping the drug (10). Taken together, these studies show that regular administration of NSAIDs results in chronic renal failure (8), but patients taking NSAIDs for 6 weeks or less may have a chance of recovery (10). Based on the potentially intolerable adverse effects or suboptimal pain relief, substantial proportions of musculoskeletal pain patients are often switched to a different treatment within 12 months of initiating NSAID treatment (11–13). However, no study to date has evaluated the potential safety benefit of this common practice: reversing renal adverse effects after cessation of long-term NSAID therapy.

Thus, the present study aimed to investigate a series of patients with chronic musculoskeletal pain who underwent long-term NSAID administration followed by switching to TA combination tablets to study the impact of NSAID-induced renal adverse effects.

MATERIALS AND METHODS

Subjects

The Research Ethics Committee of Amagasaki Central Hospital approved this study (no. H23022501). Data were retrospectively collected from medical records of 602 consecutive outpatients with chronic musculoskeletal pain from July 2011 to February 2012 at a primary care clinic. Inclusion criteria included age ≥ 20 years old, the existence of chronic musculoskeletal pain over the follow-up period of 2 years, and receiving daily NSAIDs during the first 12 months followed by receiving daily TA combination tablets for 12 months. Chronic musculoskeletal pain was defined as persisting, continuous, or intermittent pain for longer than 3 months (14). Exclusion criteria were cancer-related pain, presence of neurological signs, evidence of bone fractures, recent surgery within the past 6 months, positive pregnancy test, American Society of Anesthesiologists' physical status ≥ 3, allergy or contraindication to the tested substances, severe kidney [estimated glomerular filtration rate (eGFR) < 30] or liver function disorders (Child–Pugh classes A, B, and C), acute duodenal or ventricular ulcer, or laboratory data outside of normal ranges. Finally, 99 patients receiving daily NSAIDs during the first 12 months followed by receiving daily TA combination tablets for 12 months were analyzed in this study (**Figure 1**). The patients were included regardless of administration dose. Concomitant medications were not permitted.

The number of subjects was determined by a sample size estimation using G*Power software (v 3.0.10; Franz Faul, Kiel University, Kiel, Germany). On the basis of the effect size of 0.3, the minimum number of subjects was estimated to be 90 for an α-level of 0.05 and a power (1–β) of 0.80.

Treatment Characteristics

NSAIDs used in the study included meloxicam, loxoprofen, diclofenac, celecoxib, and others. During the latter 12 month period, all study participants were administrated daily TA combination tablets (Ultracet®). Change of administration dose was permitted. The initial dosage and administration of TA was one tablet (tramadol hydrochloride 37.5 mg and acetaminophen 325 mg) given orally four times per day (15). The dose could be increased or decreased depending on patients' symptoms, but no more than two tablets per administration were permitted (up to a maximum of eight tablets daily). No other supplementary analgesic medications were given during the study. Discontinuation of medication for the treatment of internal comorbidities was not required.

Outcomes

Patient characteristics included age, sex, major diagnosis, comorbidities, number of medications for comorbidities, and administration dose. Laboratory values were routinely collected at baseline, after 12-month NSAID administration and after 12-month TA administration. Comparisons of laboratory results during the 12 months with daily NSAIDs and during the following 12 months with daily administration of TA combination tablets were made in the same patient.

The primary outcome measure was serum levels of eGFR. eGFR was calculated as follows (16): 194 × age-0.287 × serum creatinine-1.094 (if female, ×0.739). The eGFR values (ml/min/1.73 m^2) in a given range were stratified into one of the following published chronic kidney disease (CKD) categories (17): grade 1, normal or high, ≥90; grade 2, mildly decreased, 60–89; grade 3a, mildly to moderately decreased, 45–59; grade 3b, moderately to severely decreased, 30–44; grade 4, severely decreased, 15–29; grade 5, kidney failure, <15; or dialysis. Patients categorized with an increase in severity of at least

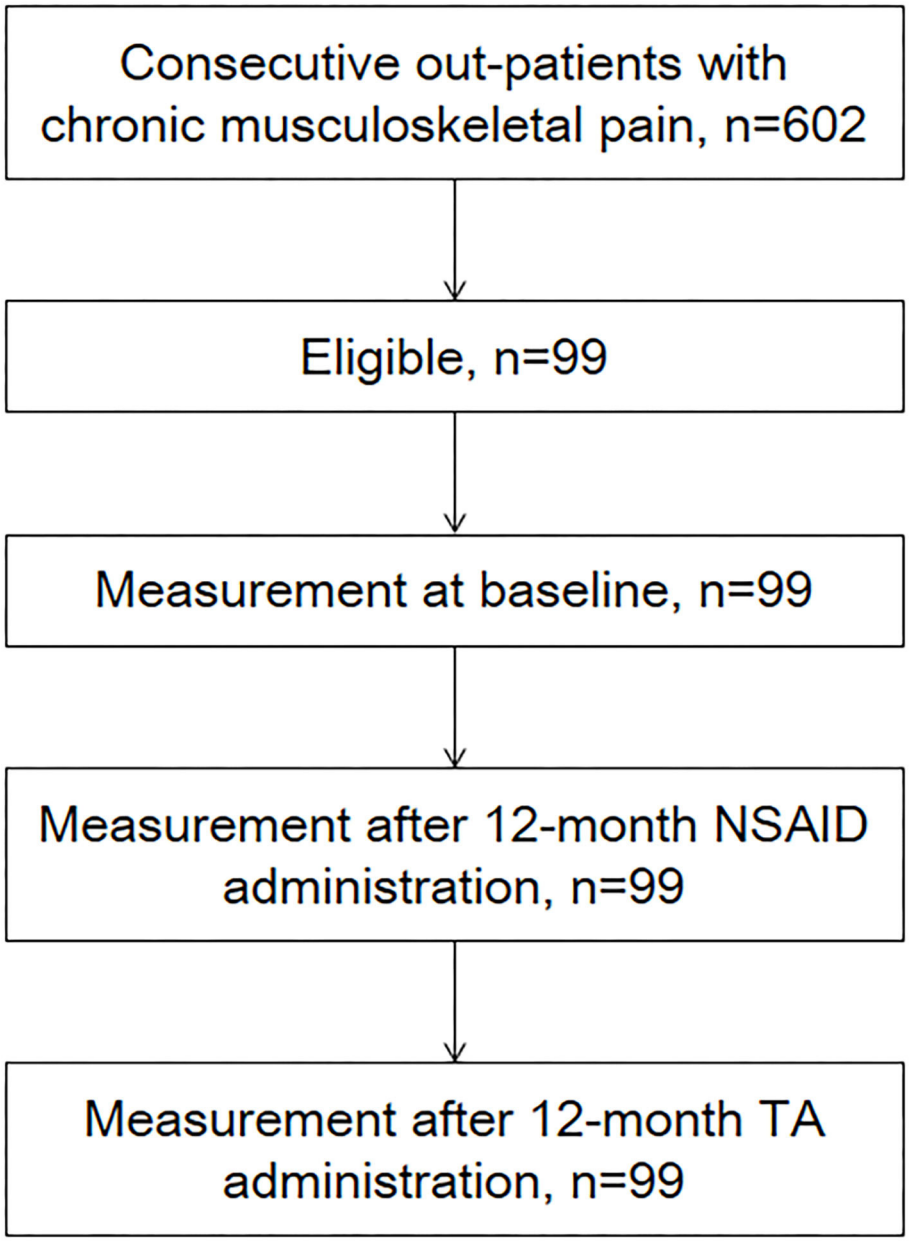

FIGURE 1 | Flowchart of participants through the study. Ninety-nine patients were analyzed in this study.

one grade in the CKD category were enrolled for NSAID and TA administration.

Secondary outcome measures were serum levels of aspartate transaminase (AST) and alanine transaminase (ALT). Other information regarding adverse events during treatment was also collected. Treatment outcome measures were assessed at baseline and after treatment, in each treatment, by using a pain-Numeric Rating Scale (NRS) (18). The pain-NRS was used to measure pain severity at each assessment, where 0 = no pain and 10 = worst pain imaginable (18).

Statistical Analysis

Relative change in eGFR, AST, and ALT from baseline (xb) and measurements was calculated using the equation (x–xb)/xb, where x is the measured value. The normality of distribution for each measurement was evaluated using the Shapiro–Wilk test for continuous variables. The outcome variables were not normally distributed; thus, continuous data are expressed as medians and interquartile ranges (IQRs). Categorical variables were analyzed using the chi-square test. Continuous variables were analyzed using the Mann–Whitney *U*-test, the Kruskal–Wallis test, the

Friedman test, the Steel–Dwass test, and the Spearman's rank correlation coefficient test.

All data were statistically analyzed using the SPSS 25.0J program, and $P < 0.05$ were considered significant.

RESULTS

Of the 99 patients, 70 (71%) were female (**Table 1**). The median age was 73 years (IQR, 47–81). Major diagnoses (multiple allowed) of the patients included lumbago (n = 45), osteoarthritis (n = 28), and rheumatoid arthritis (n = 3). NSAIDs taken during the first 12 months included meloxicam (n = 31), loxoprofen (n = 25), diclofenac (n = 13), and celecoxib (n = 20). No significant difference in patient characteristics, pain conditions, comorbidities, number of medications for comorbidities, and pain-NRS were observed based on the particular NSAIDs used (**Tables 1**, **2**). No other serious and minor complications occurred during the 2-year research period.

The median baseline for eGFR was 84.0 ml/min/1.73 m^2 (IQR, 67.6–102.0), the median baseline for AST was 20.0 U/L (IQR, 17.0–24.0), and the median baseline for ALT was 16.0 U/L (IQR, 11.0–22.0) (**Table 3**). eGFR level was significantly correlated with age at baseline ($r = -0.606$), after NSAID administration for 12 months ($r = -0.682$) and after TA administration for 12 months ($r = -0.645$).

As shown in **Table 3** and **Figure 2**, eGFR levels after NSAID administration for 12 months followed by TA for 12

TABLE 1 | Patient characteristics.

	Overall (n = 99)	Meloxicam (n = 31)	Loxoprofen (n = 25)	Diclofenac (n = 13)	Celecoxib (n = 20)	Other (n = 10)
Demographics						
Age [year]	73 [47–81]	68 [45–81]	80 [59–83]	73 [45–82]	71 [47–80]	77 [68–83]
Female, n (%)	70 (71%)	23 (74%)	17 (68%)	8 (62%)	16 (80%)	6 (60%)
Major diagnoses (multiple allowed)						
Lumbago, n (%)	45 (45%)	11 (35%)	17 (68%)	5 (38%)	8 (40%)	4 (40%)
Osteoarthritis, n (%)	28 (28%)	7 (23%)	4 (16%)	4 (31%)	9 (45%)	4 (40%)
Rheumatoid arthritis, n (%)	3 (3%)	3 (10%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Comorbidities						
Diabetes, n (%)	4 (4%)	0 (0%)	2 (8%)	1 (8%)	0 (0%)	1 (10%)
Hypertension, n (%)	22 (22%)	4 (13%)	9 (36%)	4 (31%)	3 (15%)	2 (20%)
Chronic heart failure, n (%)	3 (3%)	0 (0%)	2 (8%)	0 (0%)	1 (5%)	0 (0%)
Dyslipidemia, n (%)	1 (1%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	0 (0%)
Hypothyroidism, n (%)	2 (2%)	2 (6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Osteoporosis, n (%)	5 (5%)	2 (6%)	1 (4%)	0 (0%)	2 (10%)	0 (0%)
Migraine, n (%)	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)
Depression, n (%)	2 (2%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	1 (10%)
Number of medications for comorbidities, n (%)						
0	61 (62%)	21 (68%)	11 (44%)	9 (69%)	14 (70%)	6 (60%)
1	24 (24%)	9 (29%)	9 (36%)	1 (8%)	4 (20%)	1 (10%)
2	9 (9%)	1 (3%)	4 (16%)	1 (8%)	2 (10%)	1 (10%)
3	3 (3%)	0 (0%)	1 (4%)	1 (8%)	0 (0%)	1 (10%)
4	2 (2%)	0 (0%)	0 (0%)	1 (8%)	0 (0%)	1 (10%)
Administration dose per day						
NSAIDs [mg]	75 [10–180]	10 [10–10]	180 [75–180]	62.5 [75–110]	200 [200–200]	62.5 [12–450]
TA [tablets]	2 [1–4]	3 [2–4]	2 [1–3]	2 [2–4]	2 [1–4]	2 [1–3]
Pain-NRS [points]						
Baseline	6 [5–7]	7 [5–7]	5 [5–7]	6 [5–7]	5 [4–6]	6 [4–6]
After NSAIDs for 12 months	5 [4–6]	5 [4–7]	4 [4–6]	5 [3–7]	4 [3–5]	6 [4–6]
After TA for 12 months	4 [3–5]	5 [3–7]	4 [3–5]	4 [3–5]	3 [2–5]	4 [2–6]

NRS, Numeric Rating Scale; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen.
Data of sex, major diagnoses, and comorbidities are number and (%) of patients. Data of age, administration dose, and pain-NRS are medians and interquartile ranges [IQR].

months were significantly reduced compared with baseline. eGFR was significantly reduced during the first 12 months with NSAID administration (median, from 84.0 to 72.8 ml/min/1.73 m^2), whereas the reduction was not shown during the following 12 months with TA administration (median, 71.5 ml/min/1.73 m^2). Some patients showed an increase of eGFR after cessation of NSAIDs and switching to TA. There was no significant difference in eGFR between after the 12-month NSAIDs period and after the 12-month TA period. With respect to the four specific NSAIDs, reduction of eGFR was significantly less in patients taking celecoxib (median, −1.8 ml/min/1.73 m^2) than those on meloxicam or diclofenac (**Figure 3**). As shown in **Table 3** and **Figures 4**, **5**, there was no significant difference in AST or ALT in each period.

Table 4 shows the number of patients for each grade of the CKD category. Of the 99 patients, 37 patients (37%) experienced an increase in severity of at least one grade in the CKD

TABLE 2 | Course of pain-NRS.

	Lumbago	Osteoarthritis	Rheumatoid arthritis
Baseline	5 [5–6]	5 [6–7]	7 [7–7]
After NSAIDs for 12 months	4 [4–6]	3 [4–6]	5 [5–5]
After TA for 12 months	3 [4–5]	2 [3–4]	3 [4–5]

NRS, Numeric Rating Scale; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen.
Data of pain-NRS are medians and interquartile ranges [IQR].

TABLE 3 | Course of laboratory levels.

	Overall ($n = 99$)	Meloxicam ($n = 31$)	Loxoprofen ($n = 25$)	Diclofenac ($n = 13$)	Celecoxib ($n = 20$)	Other ($n = 10$)
eGFR [ml/min/1.73 m^2]						
Baseline	84.0 [67.6–102.0]	86.0 [75.7–104.0]	84.0 [65.1–93.8]	92.1 [65.0–116.5]	83.1 [57.1–98.1]	74.6 [64.3–88.3]
After NSAIDs for 12 months	72.8 [57.5–89.6]*	73.8 [60.9–89.6]*	72.1 [49.3–92.2]	72.6 [48.2–85.7]	76.2 [61.4–90.5]	66.7 [50.8–75.5]
After TA for 12 months	71.5 [57.7–88.7]*	72.9 [64.1–92.7]*	71.7 [53.7–90.4]	71.3 [56.2–97.5]	75.3 [58.0–84.5]	57.5 [43.8–77.3]
Changes during NSAIDs use	−13.8 [−25.0–0.0]	−18.8 [−28.7 to −5.9]†	−2.7 [−19.3–0.0]	−21.5 [−31.2 to −12.5]†	−1.8 [−14.1–0.0]	−14.8 [−27.6 to −5.8]
Changes during TA use	0.4 [−7.5–11.8]	4.0 [−7.5–14.0]	1.9 [−6.6–13.5]	1.5 [−1.1–15.0]	−2.8 [−9.9–11.0]	−8.5 [18.6–8.7]
AST [U/L]						
Baseline	20.0 [17.0–24.0]	20.0 [17.0–22.0]	22.0 [18.5–26.5]	21.0 [15.5–30.0]	19.0 [16.0–28.8]	22.5 [15.0–25.3]
After NSAIDs for 12 months	21.0 [16.0–25.0]	21.0 [16.0–24.0]	22.0 [16.5–26.0]	18.0 [15.5–21.0]	23.0 [15.0–31.0]	20.0 [16.5–22.5]
After TA for 12 months	19.0 [16.0–24.0]	19.0 [16.0–22.0]	22.0 [19.0–27.0]	18.0 [15.0–21.5]	19.5 [17.0–28.0]	17.0 [15.8–21.8]
Changes during NSAIDs use	0.0 [−12.5–17.6]	5.0 [−6.3–17.6]	−4.3 [−15.0–16.3]	−4.5 [−22.8–6.7]	1.6 [−16.9–18.5]	0.0 [−18.2–19.1]
Changes during TA use	−5.6 [−17.1–5.9]	−6.7 [−20.0–4.8]	0.0 [−7.7–14.4]	−5.3 [−14.8–0.0]	−10.2 [−16.6–6.5]	−16.7 [−20.0–3.3]
ALT [U/L]						
Baseline	16.0 [11.0–22.0]	15.0 [11.0–20.0]	17.0 [11.0–27.5]	15.0 [10.5–29.5]	17.5 [10.3–23.5]	16.5 [10.0–25.0]
After NSAIDs for 12 months	15.0 [10.0–21.0]	14.0 [10.0–19.0]	16.0 [9.0–28.5]	12.0 [9.0–24.5]	15.0 [11.0–25.0]	15.0 [10.5–20.3]
After TA for 12 months	14.0 [10.0–21.0]	12.0 [9.0–16.0]	17.0 [12.0–23.0]	12.0 [8.0–19.5]	14.5 [11.0–25.8]	13.5 [10.0–21.0]
Changes during NSAIDs use	0.0 [−25.0–23.5]	8.3 [−20.0–23.5]	−10.0 [−38.7–17.7]	−10.0 [−33.9–1.8]	6.5 [−29.6–37.2]	−9.2 [−25.3–19.8]
Changes during TA use	−9.1 [−27.6–8.3]	−11.1 [−40.0–7.7]	0.0 [−26.1–20.8]	−12.5 [−28.1–17.8]	−8.1 [−27.3–8.4]	−10.1 [−34.2–5.0]

eGFR, estimated glomerular filtration rate; AST, aspartate transaminase; ALT, alanine transaminase; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen.
*Data are medians and interquartile ranges [IQR]. These data were analyzed using Kruskal–Wallis test and Steel–Dwass test. Significance level was set at <5%. *Significant difference vs. baseline. †Significant difference vs. celecoxib. eGFR after NSAIDs for 12 months and after TA for 12 months were significantly decreased than baseline, in overall and meloxicam.*

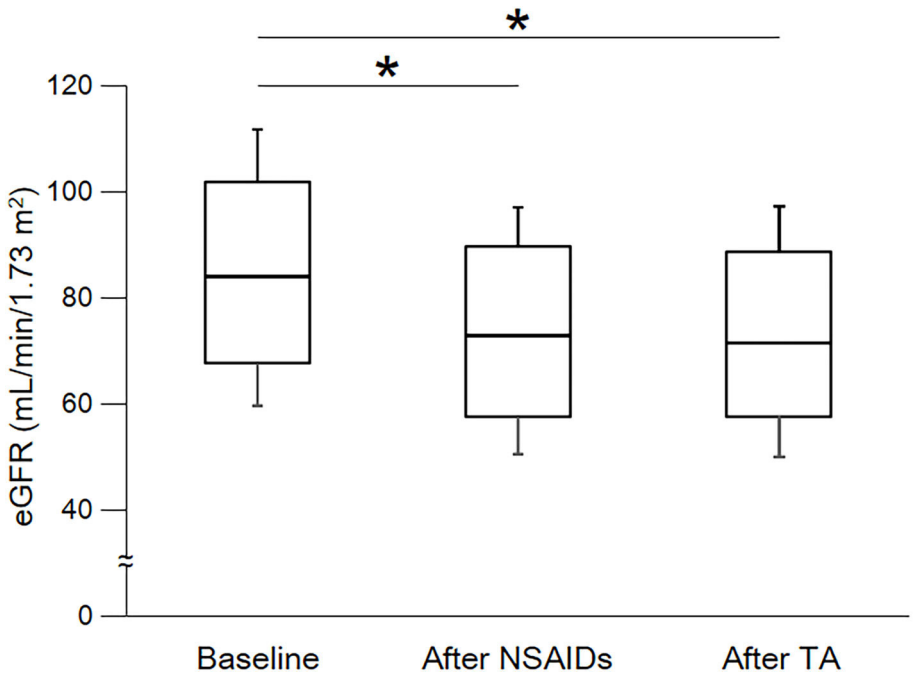

FIGURE 2 | Course of eGFR. eGFR, estimated glomerular filtration rate; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen. Each box plot represents the 75 percentile, median, and 25 percentile. Error bar shows standard deviation. eGFR after NSAIDs for 12 months and after TA for 12 months were significantly decreased than baseline. There was no significant difference between after NSAIDs for 12 months and after TA for 12 months. These data were analyzed using Friedman test and Steel–Dwass test. Significance level was set at <5%. *Significant difference vs. baseline.

category during the first 12 months with NSAID administration. Interestingly, the extent of severity varied by NSAID type, where 15% of patients on celecoxib ($n = 3$) were affected, compared with 77% of patients on diclofenac ($n = 10$) ($p = 0.003$). On the other hand, during the 24 months with NSAID and TA administration, 35 patients (35%) increased severity by at least one grade of the CKD category. There were 30 patients in more than three categories after NSAIDs for 12 months, whereas 28 patients in more than three categories after TA for 12 months. The number of patients increasing severity by at least one grade of the CKD category over 24 months showed no significant difference among the four specific NSAIDs used. The variables other than NSAID type were not significantly different between patients who fell into at least one grade worse of the CKD category or not (**Table 5**).

DISCUSSION

The present study showed that NSAID administration for 12 months significantly reduced serum levels of eGFR. However, the reduction was not shown after 12 months of TA administration. Several patients showed an increase of eGFR upon cessation of NSAIDs followed by switching to TA.

Most forms of acute renal failure from NSAID administration are short-term and reversible upon NSAID discontinuation (8). The adverse effects of NSAIDs are the consequences of inhibiting prostaglandin synthesis and can result in acute renal failure. Moreover, there is the possibility that chronic administration of any NSAIDs can cause chronic renal failure in some patients despite previous data suggesting it is safe (8, 19). The underlying pathology of chronicity is considered chronic papillary necrosis or chronic interstitial nephritis (20). NSAID administration for the short term for up to 6 weeks may preserve the chance for recovery (10); however, there has previously been no study to test the reversibility of renal adverse effects after long-term NSAID use. The present study suggested that the eGFR was not reduced after the cessation of NSAIDs and switching to TA, but the reversibility as the change was not significant.

NSAIDs inhibit the peripheral production of prostaglandins and inflammatory processes (21). NSAIDs could have a role in central neurons across the blood–brain barrier (22). In osteoarthritis, NSAIDs could have favorable effects on articular cartilage and osteoarthritis progression, although there are no convincing data (23). The present study showed a decrease in pain with a reduction of eGFR. The favorable and unfavorable effects of NSAIDs should be considered. Drug-induced renal failure is mostly induced by an antirheumatic drug, calcineurin inhibitors, an antitumor drug, and NSAIDs (24). The nephrotoxic potential of dual or triple combinations of NSAIDs with renin–angiotensin system inhibitors and/or

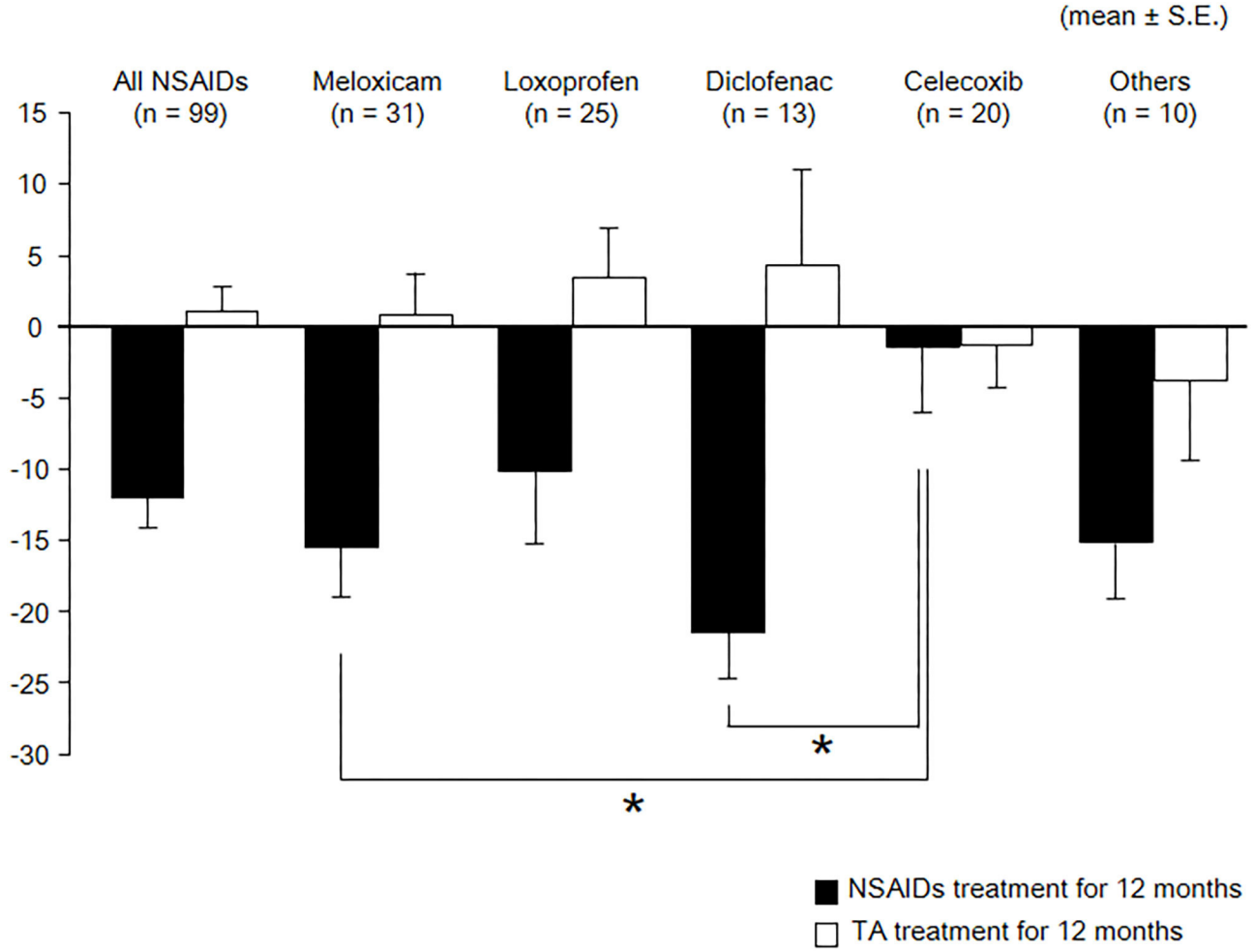

FIGURE 3 | Course of eGFR among specific NSAIDs. eGFR, estimated glomerular filtration rate; TA, tramadol hydrochloride/acetaminophen. Values are means of change of eGFR, and the error bar shows standard error. Reduction of eGFR was significantly lesser in patients with celecoxib than those with meloxicam and diclofenac. These data were analyzed using Kruskal–Wallis test and Steel–Dwass test. Significance level was set at <5%. *Significant difference among specific NSAIDs.

diuretics yields a high incidence of acute kidney injury (25, 26). More than half of patients have no medications for comorbidities in the present study. NSAIDs also have serious adverse effects of heart attack and stroke. Other adverse effects include stomach pain, constipation, diarrhea, gas, heartburn, nausea, vomiting, and dizziness (27). The patients in the present study showed no adverse effects. When the patients have any of the adverse effects, a physician should reconsider the subscription of NSAIDs.

The risk profiles of adverse effects are different for every NSAID (28–30). A randomized control trial for patients with osteoarthritis or rheumatoid arthritis shows celecoxib treatment results in lower rates of renal adverse events than did ibuprofen (28). In a meta-analysis of 114 clinical trials, Zhang et al. showed that rofecoxib intensified the risk for renal adverse effects. By contrast, among NSAIDs, celecoxib had a low risk for renal adverse effects (29). Other NSAIDs were not significantly associated with the risk, although some trends were evident. Similarly, Winkelmayer et al. showed rofecoxib, ibuprofen, and indomethacin were associated with a higher risk of acute kidney injury than celecoxib (30). In the present study, the reduction of renal function after administering NSAIDs for 12 months tended to be less in patients receiving celecoxib compared with patients receiving other NSAIDs.

TA combination tablets, which combine tramadol hydrochloride and acetaminophen, are a widely used analgesic (15). Tramadol is a synthetic opioid receptor agonist with analgesic properties that also has a unique monoaminergic action through serotonin-noradrenaline reuptake inhibition (31). Acetaminophen is one of the more traditional and better-tolerated among fast-acting analgesics that block pain through different pathways than opioids (32). The effectiveness of TA in the treatment of chronic non-cancer pain is clinically acceptable,

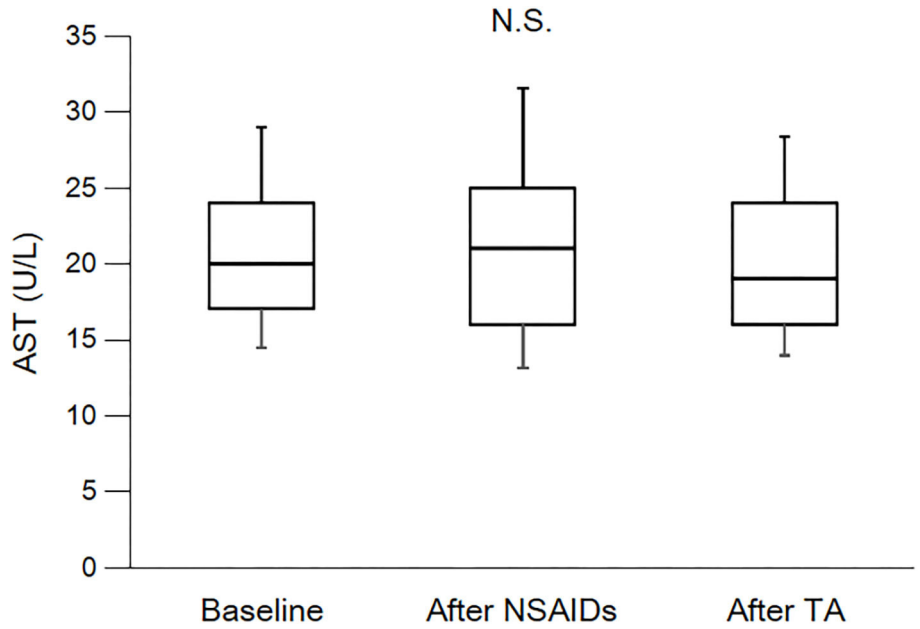

FIGURE 4 | Course of AST. AST, aspartate transaminase; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen. Each box plot represents the 75 percentile, median, and 25 percentile. Error bar shows standard deviation. There was no significant difference in each period. These data were analyzed using Friedman test and Steel–Dwass test. Significance level was set at <5%.

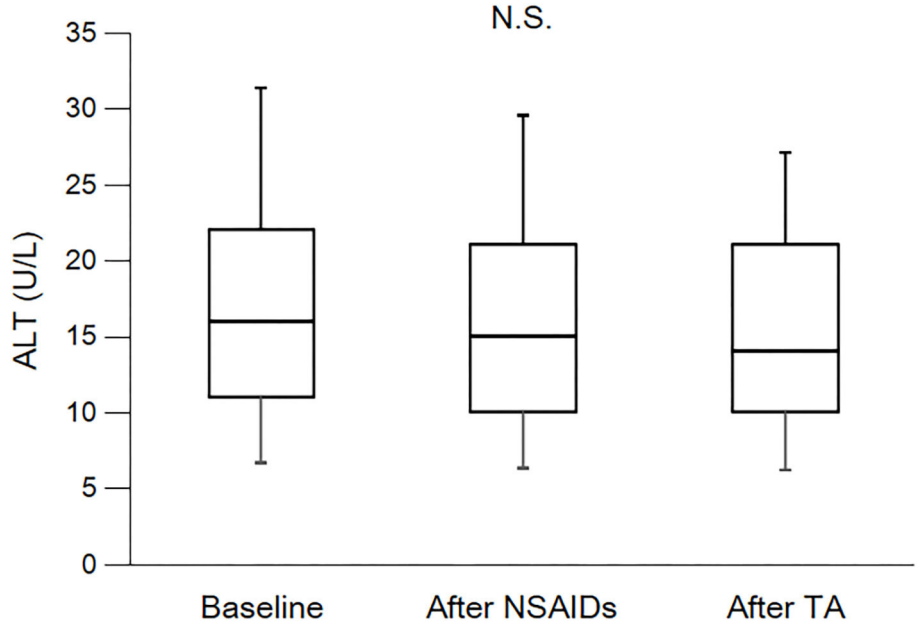

FIGURE 5 | Course of ALT. ALT, alanine transaminase; NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen. Each box plot represents the 75 percentile, median, and 25 percentile. Error bar shows standard deviation. There was no significant difference in each period. These data were analyzed using Friedman test and Steel–Dwass test. Significance level was set at <5%.

and improvements in pain contribute to improvements in quality of life in practice (15). Most of the adverse effects of TA are non-serious (15, 33–35); it is suggested that liver enzymes are elevated in the presence of acetaminophen at doses higher than normal therapeutic levels (36). In addition, previous work showed that concomitant treatment with opioids does

TABLE 4 | Number of patients each grade of CKD category.

	Overall ($n = 99$)	Meloxicam ($n = 31$)	Loxoprofen ($n = 25$)	Diclofenac ($n = 13$)	Celecoxib ($n = 20$)	Other ($n = 10$)
Baseline, n (%)						
1	38 (38%)	13 (42%)	8 (32%)	8 (62%)	7 (35%)	2 (20%)
2	43 (43%)	16 (52%)	12 (48%)	2 (15%)	7 (35%)	6 (60%)
3a	13 (13%)	1 (3%)	3 (12%)	3 (23%)	5 (25%)	1 (10%)
3b	5 (5%)	1 (3%)	2 (8%)	0 (0%)	1 (5%)	1 (10%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
After NSAIDs for 12 months, n (%)						
1	23 (23%)	7 (23%)	8 (32%)	2 (15%)	5 (25%)	1 (10%)
2	46 (46%)	17 (55%)	7 (28%)	6 (46%)	11 (55%)	5 (50%)
3a	19 (19%)	5 (16%)	5 (20%)	3 (23%)	3 (15%)	3 (30%)
3b	9 (9%)	2 (6%)	4 (16%)	2 (15%)	1 (5%)	0 (0%)
4	2 (2%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	1 (10%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
After TA for 12 months, n (%)						
1	22 (22%)	8 (26%)	6 (24%)	3 (23%)	4 (20%)	1 (10%)
2	49 (49%)	18 (58%)	12 (48%)	5 (38%)	11 (55%)	3 (30%)
3a	16 (16%)	3 (10%)	2 (8%)	4 (31%)	4 (20%)	3 (30%)
3b	9 (9%)	1 (3%)	4 (16%)	0 (0%)	1 (5%)	3 (30%)
4	3 (3%)	1 (3%)	1 (4%)	1 (3%)	0 (0%)	0 (0%)
5	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Fell into at least worse one grade of CKD category, n (%)						
during NSAIDs use (12 months)	37 (37%)	13 (42%)	7 (28%)	10 (77%)†	3 (15%)*	4 (40%)
during NSAIDs and TA use (24 months)	35 (35%)	11 (35%)	7 (28%)	8 (62%)	4 (20%)	5 (50%)

NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen; CKD, chronic kidney disease.
*Data are number and (%) of patients. Of 99 patients, 37 patients (37%) experienced an increase in severity of at least one grade in CKD category during first 12 months with NSAID administration. On the other hand, during 24 months with NSAIDs and TA administration, 35 patients (35%) increased severity by at least one grade of CKD category. These data were analyzed using chi-square test. Significance level was set at < 5%. *Significantly fewer number of patients. †Significantly more number of patients.*

not lead to an elevation of liver enzyme levels (36). Similarly, in our study, we did not observe any significant elevations in liver enzymes.

There are several limitations in the present study. First, the present study is a retrospective study limited only to patients receiving daily NSAIDs during the first 12 months followed by 12 months of administration of TA combination tablets daily. There is no group receiving only daily NSAIDs or TA combination tablets during the 24-month periods. The renal function might already have reached a stable but lower plateau in the present study. In addition, many patients had concomitant medications. Thus, our observations must be interpreted with caution. Second, the administration protocol was variable, and the overall impact of administration dose on serum levels was not determined. Third, patients were mostly of advanced age in the present study. The reduction of eGFR could be overestimated. Finally, we included only a small number of participants with different pain conditions at a single medical center. Further studies that investigate larger patient cohorts and additional treatment regimens are required to clarify the effects of long-term use of NSAIDs on serum levels.

CONCLUSIONS

The present study suggests that patients who have undergone long-term NSAID therapy for 12 months can experience reversible or irreversible renal damage after the cessation of NSAIDs and switching to TA, as determined by measuring eGFR. Given this risk identified in our current series of patients, our data highlight the potential safety of utilizing multimodal analgesic

TABLE 5 | Comparison between patients with fell into at least worse one grade of CKD category or not.

	During NSAIDs use (12 months)		During NSAIDs and TA use (24 months)	
	Fell category (*n* = 37)	**Not fell category (*n* = 62)**	**Fell category (*n* = 35)**	**Not fell category (*n* = 64)**
Demographics				
Age [year]	76 [61–84]	72 [47–80]	73 [60–83]	73 [46–80]
Female, n (%)	11 (30%)	18 (29%)	12 (34%)	17 (27%)
Major diagnoses (multiple allowed)				
Lumbago, n (%)	21 (57%)	24 (39%)	18 (51%)	27 (42%)
Osteoarthritis, n (%)	7 (19%)	21 (34%)	9 (26%)	19 (30%)
Rheumatoid arthritis, n (%)	1 (3%)	2 (3%)	1 (3%)	2 (3%)
Comorbidities				
Diabetes, n (%)	3 (8%)	1 (2%)	2 (6%)	2 (3%)
Hypertension, n (%)	11 (30%)	11 (18%)	8 (23%)	14 (22%)
Chronic heart failure, n (%)	2 (5%)	1 (2%)	2 (6%)	1 (2%)
Dyslipidemia, n (%)	1 (3%)	0 (0%)	0 (0%)	1 (2%)
Hypothyroidism, n (%)	0 (0%)	2 (3%)	0 (0%)	2 (3%)
Osteoporosis, n (%)	1 (3%)	4 (6%)	1 (3%)	4 (6%)
Migraine, n (%)	0 (0%)	1 (2%)	0 (0%)	1 (2%)
Depression, n (%)	1 (3%)	1 (2%)	1 (3%)	1 (2%)
Number of medications for comorbidities				
0, n (%)	21 (57%)	40 (65%)	23 (66%)	38 (59%)
1, n (%)	9 (24%)	15 (24%)	6 (17%)	18 (28%)
2, n (%)	4 (11%)	5 (8%)	4 (11%)	5 (8%)
3, n (%)	1 (3%)	2 (3%)	1 (3%)	2 (3%)
4, n (%)	2 (5%)	0 (0%)	1 (3%)	1 (2%)
Type of NSAIDs				
Meloxicam, n (%)	13 (35%)	18 (29%)	11 (31%)	20 (31%)
Loxoprofen, n (%)	7 (19%)	18 (29%)	7 (20%)	18 (28%)
Diclofenac, n (%)	10 (27%)†	3 (5%)*	8 (23%)	5 (8%)
Celecoxib, n (%)	3 (8%)*	17 (27%)†	4 (11%)	16 (25%)
Other, n (%)	4 (11%)	6 (10%)	5 (14%)	5 (8%)
Administration dose per day				
NSAIDs [mg]	75 [10–150]	160 [10–200]	75 [10–180]	100 [10–180]
TA [tablets]	2 [1–4]	2 [1–3]	2 [1–4]	2 [1–4]

NSAIDs, non-steroidal anti-inflammatory drugs; TA, tramadol hydrochloride/acetaminophen; CKD, chronic kidney disease.
*Data of sex, major diagnoses, and comorbidities are number and (%) of patients. Data of age and administration doses are medians and interquartile ranges [IQR]. Of 99 patients, 37 patients (37%) experienced an increase in severity of at least one grade in CKD category during first 12 months with NSAID administration. On the other hand, during 24 months with NSAIDs and TA administration, 35 patients (35%) increased severity by at least one grade of CKD category. These data were analyzed using chi-square test or Mann–Whitney U-test. Significance level was set at <5%. *Significantly fewer number of patients. †Significantly more number of patients.*

therapies to minimize the chronic administration of NSAIDs wherever possible.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The Research Ethics Committee of Amagasaki Central Hospital approved this study.

AUTHOR CONTRIBUTIONS

KM led the design of the study design with KH, HK, TI, and MY. KH led the analysis and the interpretation of data and drafted the manuscript with KM, HK, TI, and MY. All authors were involved in the interpretation of the results, writing of the manuscript, and they all approved the final manuscript.

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Modulation of Astrocytic Glutamine Synthetase by Endocannabinoid 2-Arachidonoylglycerol in JNK-Independent Pathway

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Background and Objective: The glutamine synthetase (GS), an astrocyte-specific enzyme, plays an important role in neuroprotection through the glutamate/glutamine shuttle and can be modulated by endocannabinoid (eCB) 2-arachidonoylglycerol (2-AG) through extracellular signal-regulated protein kinase ½ (ERK1/2) and p38 signaling pathways. However, the role of c-Jun N-terminal kinase (JNK) signaling pathway in the modulation of GS in astrocytes by 2-AG is not clear.

Materials and Methods: The expression of GS and JNK in astrocytes following the exposure to lipopolysaccharide (LPS) was examined with Western blotting and immunochemistry.

Results: The results revealed that short-term exposure to LPS activated GS and increased phosphorylation of JNK in astrocytes in a time-dependent manner. Treatment with 2-AG reversed the changes in GS but had no effect on the activation of JNK.

Conclusions: These findings suggest that the activation of JNK induced by LPS is not involved in the modulation of astrocytic GS by 2-AG.

Keywords: JNK, cannabinoid receptor, 2-AG, astrocyte, glutamine synthetase

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INTRODUCTION

The astrocytic glutamine synthetase (GS) can modulate the extracellular concentration of glutamate by converting glutamate into glutamine and is verified to be involved in a variety of neurological disorders such as neurodegenerative diseases and chronic pain (1). Endocannabinoids (eCBs) are endogenous mediators of lipid signaling with the capabilities to modulate the synaptic function and to provide neuroprotective and anti-inflammatory effects (2). 2-Arachidonoylglycerol (2-AG) is one of the most abundant eCBs and plays a potential role in protecting neurocytes from injuries induced by inflammation and insults of neurodegenerative diseases (3). In addition, previous studies found that 2-AG has the capacity of attenuating neuropathic pain and mechanical hyperalgesia in several preclinical models of chronic pain (4, 5).

The previous study indicates that 2-AG is involved in the modulation of synaptic function, neuroprotection, and stimulation of mitogen-activated protein kinase (MAPK) family by binding to and activating the G-protein-coupled receptors (GPCR), cannabinoid receptor type 1 (CB_1R), and cannabinoid receptor type 2 (CB_2R), which are expressed in astrocytes (6, 7). A variety of

studies also indicate that the activation of CB_1R or CB_2R produced effects of anti-inflammation, antinociception, and neuroprotection (8), and activation of MAPK signaling (9). In addition, activation of CB_1R or CB_2R can inhibit the activation of MAPK cascade induced by stress, and the discrepancy remains to be further studied. Our recent study indicates that astrocytic MAPK subunits, extracellular signal-regulated protein kinase ½ (ERK1/2) and p38, are involved in the modulation of GS by CB_1R and CB_2R (10). Other studies indicate that c-Jun N-terminal kinase (JNK) participates in the CB_1R-mediated inflammation signaling (11) and CB_2R-mediated suppression of leukocyte migration under inflammation (12). However, there is no study about whether JNK participates in the modulation of astrocytic GS by eCBs. Taking into consideration the importance of cannabinoids (CBs) and astrocytic GS in chronic inflammatory pain, it is necessary to eliminate the role of JNK in astrocytic GS in CB-mediated chronic inflammation, which may be helpful for therapeutic strategy.

MATERIALS AND METHODS

This study was approved by the Ethics Committees of Animal Usage of Lanzhou University, Southern Medical University, and Shenzhen University.

Primary Cultures of Astrocyte

The primary culture of astrocytes was performed as described previously (10). In brief, the newborn Sprague-Dawley (SD) rats (postnatal 1–3 days) from the experimental animal center of the Gansu University of Chinese Medicine were decapitated and the cerebral hemispheres were aseptically harvested into Hank's balanced salt solution (HBSS). After the removal of meninges, the cerebral cortices were trimmed into small pieces, followed by digestion with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco Life Technology, CA, USA), mechanical dissociation by gentle pipetting with Pasteur pipette, and then centrifugation at 400 g for 5 min. The cells were resuspended in a culture medium supplemented with 90% Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco Life Technology, CA, USA) and 10% fetal bovine serum (FBS; PAN-Biotech, Germany) and plated at a density of 3–5 $\times$ 10^5 cells/cm^2 in 25 cm^2 flasks. Cells in flasks were cultured at 37°C in a carbon dioxide (CO_2) incubator for 5–7 days to reach the first confluence. To achieve high pure astrocytes (>95%), the confluent cells in flasks were shaken at 200 rpm overnight to diminish contamination from microglia. Afterward, the astrocytes were evenly passaged into 35 mm dishes and treated with 1 μg/ml lipopolysaccharide (LPS), which is one commonly used chemical to induce inflammation and can activate astrocytes via the JNK signaling pathway (13), JNK phosphorylation inhibitor SP600125, or with 0.01 μM 2-AG.

Abbreviations: 2-AG, 2-arachidonylglycerol; CBR, cannabinoid receptor; ERK1/2, extracellular signal-regulated protein kinase 1/2; GS, glutamine synthetase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase.

Protein Isolation and Western Blotting

According to the previous report, the Western blotting was carried out with the manual (10). In brief, astrocytes in 35 mm dishes were lysed in 100 μl radioimmunoprecipitation assay (RIPA) lysis buffer containing 1% phenylmethanesulfonyl fluoride (PMSF) after different treatments. Lysates were centrifuged at 12,000 rpm for 10 min to remove cell debris, and the pellet was diluted with 30 μl sample buffer. The total protein in lysates was measured for concentration by bicinchoninic acid (BCA) and loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels at 5–20 μg/lane and then separated by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Following the non-specific binding sites blockade with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) for 2 h at room temperature (RT), the PVDF membranes were incubated overnight at 4°C with primary antibodies according to the manual of the manufacturer [at a dilution of 1:1,000 for JNK and phospo-JNK (p-JNK) antibodies, #9252 and #9255, Cell Signaling Technology, MA, USA; or 1:10,000 for GS antibody; #ab176562, Abcam, St. Louis, MO, USA] and then washed extensively with TBST three times, 10 min for each time, and incubated with corresponding secondary antibodies (1:10,000; Cell Signaling Technology, MA, USA) at RT for 2 h. The membranes were then washed three times with TBST at 10-min intervals, and the immunolabeled protein bands on membranes were detected by using an enhanced chemiluminescence kit.

Immunocytochemistry

After different treatments, the astrocytes cultured on coverslips were fixed with 4% paraformaldehyde for 30 min and rinsed with phosphate-buffered saline (PBS). The fixed cells were then permeabilized with 0.4% Triton X-100 for 20 min, rinsed again with PBS, incubated with 3% normal goat serum (NGS) for 30 min, and then incubated with different primary antibodies (GS, 1:5,000; JNK and p-JNK, 1:500) overnight at 4°C, respectively. After 24 h, the coverslips were rinsed with PBS and incubated with corresponding secondary antibodies conjugated with Alexa Fluor® 488 (green staining) or 594 (red staining) (Invitrogen, UK) for 2 h at RT. Then, the coverslips were mounted onto the slide with a mounting medium with 4′,6-diamidino-2-phenylindole (DAPI) for the observation of nuclei and sealed with nail gel. The cells were visualized by immunofluorescence microscope (Olympus, Japan).

Statistical Analysis

All experiments were performed in triplicate and repeated at least three times. STATA software version 14.2 (Stata Corp, College Station, TX, USA) was used for statistical analysis, and the data were expressed as the mean ± SEM. One-way ANOVA followed by the Newman-Keuls test was used to assess the significant differences, and $p < 0.05$ was considered as significantly different.

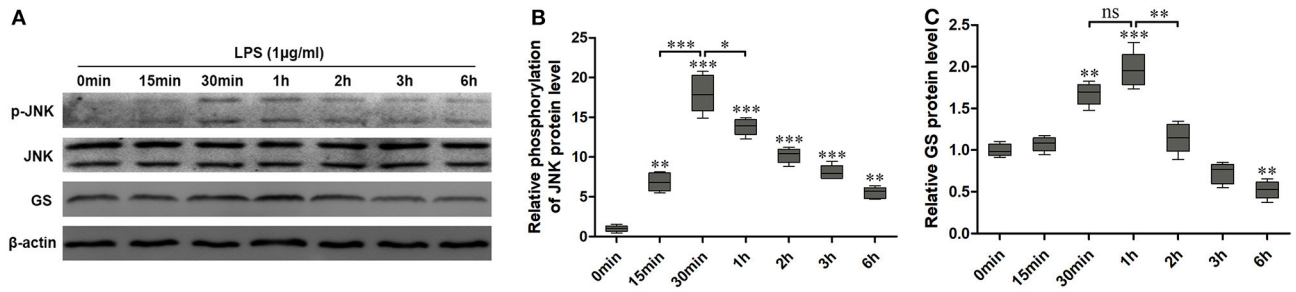

FIGURE 1 | Exposure to lipopolysaccharide (LPS) induced changes in the expression of glutamine synthetase (GS) and activation of c-Jun N-terminal kinase (JNK). Astrocytes were treated with 1 μg/ml LPS for different times, and protein levels of GS **(A,B)** and phospo-JNK (p-JNK) **(A,C)** were analyzed using Western blotting. Error bars were ± SEM. $n = 3$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. control. ns, not significant.

RESULTS

Lipopolysaccharide (LPS) Activated Expression of GS and Phosphorylation of JNK and Translocation in Primary Astrocytes of Rats

Similar to our previous study (10), 1 μg/ml LPS was used to activate the astrocytes. To investigate the effects of LPS on the JNK pathway and expression of GS in astrocytes, the expressions of p-JNK, JNK, and GS in primary astrocyte culture were evaluated using Western blotting after treatment with DMEM/F12 containing 1 μg/ml LPS at 0 min, 15 min, 30 min, 1 h, 2 h, 3 h, and 6 h. The data showed that the exposure to LPS induced time-dependent biphasic changes in the expression of GS in astrocytes, i.e., in contrast to baseline (0 min), expression of GS began to increase at 30 min (1.62 ± 0.08, $p < 0.01$), peaked at 1 h (1.86 ± 0.08, $p < 0.001$), declined to control level at 2–3 h, and then decreased at 6 h (0.57 ± 0.05, $p < 0.01$; **Figures 1A,B**). Regarding the JNK pathway, LPS significantly increased the phosphorylation of JNK in a time-dependent manner while without effect on the total JNK. The protein level of p-JNK increased at 15 min (6.96 ± 0.63, $p < 0.01$), reached a maximal level at 30 min (18.12 ± 0.90, $p < 0.001$), and then gradually declined but was still higher at 6 h (5.60 ± 0.49, $p < 0.01$) than control (**Figures 1A,C**). In addition, previous studies indicated that the JNK pathway exerted its role through translocation from the cytoplasm to nucleus (14). As expected, 1 μg/ml LPS for 1-h exposure induced the translocation of JNK from cytoplasm to nucleus, which was prevented by JNK phosphorylation inhibitor SP600125 (**Figure 2**). In addition, SP600125 also prevented the expression of GS by LPS.

2-Arachidonoylglycerol (2-AG) Reversed Changes in LPS-Induced GS Independently on the p-JNK Pathway

To explore the effects of 2-AG on the activation of astrocytes induced by exposure to LPS, the astrocytes were exposed to 1 μg/ml LPS for 1 h and were chosen on the basis of the acquired data from **Figure 1**. The cells were pretreated with 1 μM 2-AG for 1 h and/or 1 μg/ml LPS for 1 h. Compared with control (0 μg/ml LPS), exposure of astrocytes to 1 μg/ml LPS for 1 h significantly elevated the expressions of p-JNK (2.18 ± 0.18, $p < 0.01$) and GS (3.41 ± 0.29, $p < 0.01$) and treatment with 2-AG could significantly reverse the changes in the expression of GS induced by exposure to LPS (1.13 ± 0.09, $p < 0.01$) when compared with LPS group (**Figures 3A,B**) but without significant effect on the expression of p-JNK (**Figures 3A,C**).

Dephosphorylation of JNK Increased the Expression Level of GS

According to the above results, phosphorylation of JNK is not the pathway of 2-AG modulating the expression of GS in astrocytes. To further address the question of whether the JNK pathway was involved in the process of regulating the expression of GS, a specific inhibitor for the JNK signaling pathway, SP600125, was used to investigate the relationship between the JNK pathway and the expression of GS in astrocytes. SP600125 at the concentration of 50 μM and 100 μM could significantly inhibit LPS-induced activation of JNK in a dose-dependent manner (16.71 ± 0.75, $p < 0.05$; 5.03 ± 0.33, $p < 0.001$) when compared with LPS alone (22.41 ± 1.18) (**Figures 4A,C**). Meanwhile, SP600125 at the concentration of 50 and 100 μM could also significantly suppress the LPS-induced upregulation of expression of GS in a dose-dependent manner (1.15 ± 0.84, $p < 0.01$; 0.79 ± 0.06, $p < 0.01$) when compared with LPS alone (2.14 ± 0.15) (**Figures 4A,B**). In addition, SP600125 also inhibited the translocation of p-JNK (**Figure 2**). Briefly, these data suggested that LPS activated JNK resulting in the upregulation of expression of GS. In the other words, GS was the downstream target of JNK signaling in astrocytes.

DISCUSSION

Previous studies indicate that GS is involved in suppressing the development of glutamate/ammonia neurotoxicity and a variety of neurological diseases, such as neuropathic pain and inflammatory pain (1). Intriguingly, both the increase and the decrease of GS are reported in the same diseases, such as hepatic encephalopathy, traumatic brain injury, and epilepsy, but

contrarily, controlling the expression of GS can diminish these diseases. Consistent with our previous study (10), this study finds that exposure to LPS resulted in the expression of GS in a biphasic form in astrocytes, i.e., the expression of GS is increased with short-term exposure to LPS and decreased with long-term exposure to LPS.

The MAPK family of kinases including p38 and ERK participate in pain and neurodegenerative diseases and exist in activated astrocytes induced by pathological stimulation (6). Our previous study indicates that exposure to LPS could activate p38 and ERK1/2 in astrocytes with different patterns (10). In this study, we find that inhibition of JNK blocks the increase in GS by LPS, of which the mechanism may be through suppression of glucocorticoid receptor transcriptional activity (15). This study further indicates that exposure to LPS produces a uniphasic activation of JNK in astrocytes, which enriches the involvement of MAPK signaling in LPS-induced changes in GS in astrocytes. However, the mechanism remains to be further studied.

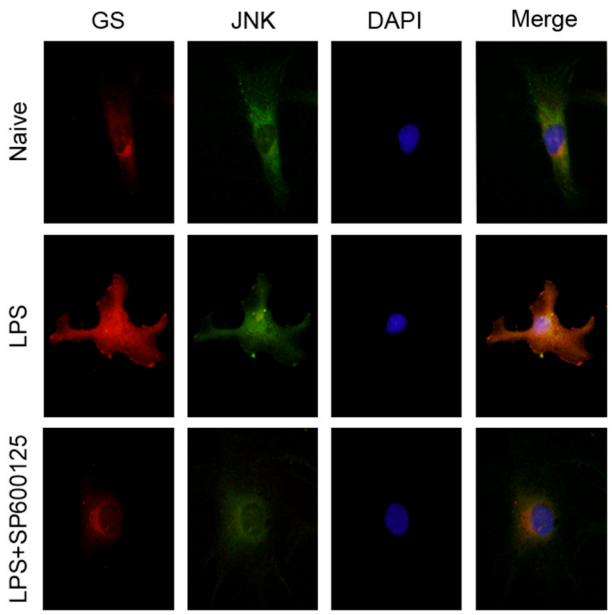

FIGURE 2 | Inhibition of phosphorylation of JNK prevented the translocation of JNK and upregulation of GS induced by exposure to LPS. Astrocytes were pretreated with SP600125 for 1 h and exposed to 1 μg/ml LPS for 1 h. Immunocytochemistry assay was used to analyze the translocation of JNK and changes in the expression of GS. Scar bar = 10 μm.

2-Arachidonoylglycerol (2-AG) is an eCB that binds to CB_1R and CB_2R expressed in astrocytes. The previous study found that astrocytic p38 can be activated by 2-AG, while blocking CB_1R can produce an inhibitory effect on the modulation of 2-AG on p38 (9), implying that 2-AG participates in the modulation of MAPK signaling in astrocytes. Our previous study suggests that, under the condition of short-term exposure to LPS, activation of p38 could increase the expression of GS, and 2-AG could suppress the increased expression of GS by inhibiting the phosphorylation level of p38. While under the condition of long-term exposure to LPS, activation of ERK1/2 results in a decrease of expression of GS and 2-AG reverses the decrease of expression of GS through reducing the activation of ERK1/2 (10). It should be noted, in our previous study, that although ERK1/2 and p38 are activated by short-term exposure to LPS and long-term exposure to LPS, respectively, the activation is relatively weaker compared to long-term and short-term exposure to LPS, respectively. This study indicates that JNK is activated during the short-term exposure to LPS, while 2-AG has no effect on the phosphorylation of JNK. However, other studies indicate that JNKs are involved in the CB_1R-mediated inflammation signaling (11) and CB_2R-mediated suppression of leukocyte migration under inflammation (12). These results imply that activation of JNK may be the upstream pathway of the astrocytic extracellular matrix (ECM) system in modulating inflammation, which remains to be further investigated.

In conclusion, this study indicated that exposure to LPS for the short term and long term can produce different changes in the activities of GS in astrocytes with activation of JNK. ECB 2-AG modulates the expression of GS induced by exposure to LPS, which is not dependent on the activation of JNK. The mechanism of JNK in modulating the astrocytic expression of GS remains to be further studied.

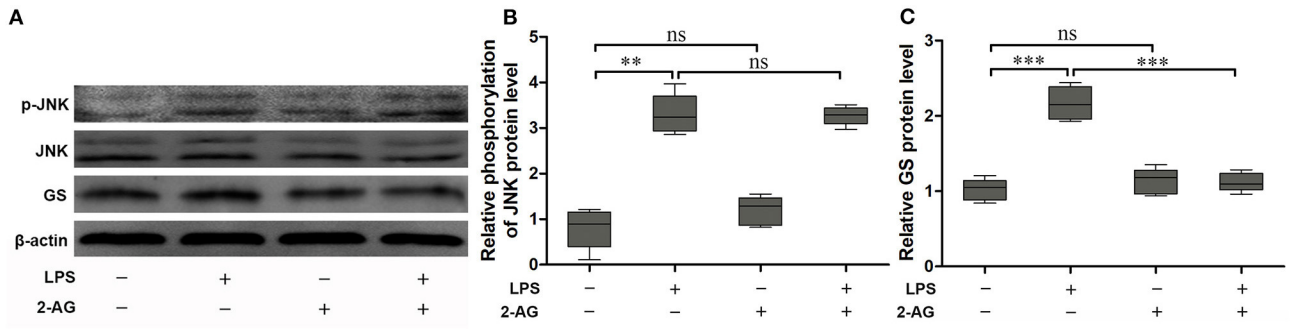

FIGURE 3 | 2-Arachidonoylglycerol (2-AG) suppressed the upregulation of expression of GS but had no effect on phosphorylation of JNK induced by exposure to LPS in astrocytes. Astrocytes were pretreated with 0.01 μM 2-AG for 2 h and exposed to 1 μg/ml LPS for 1 h. The protein levels of GS **(A,B)** and p-JNK **(A,C)** were measured using Western blotting. Error bars were ± SEM. $n = 3$. $^{**}p < 0.01$, and $^{***}p < 0.001$. ns, not significant.

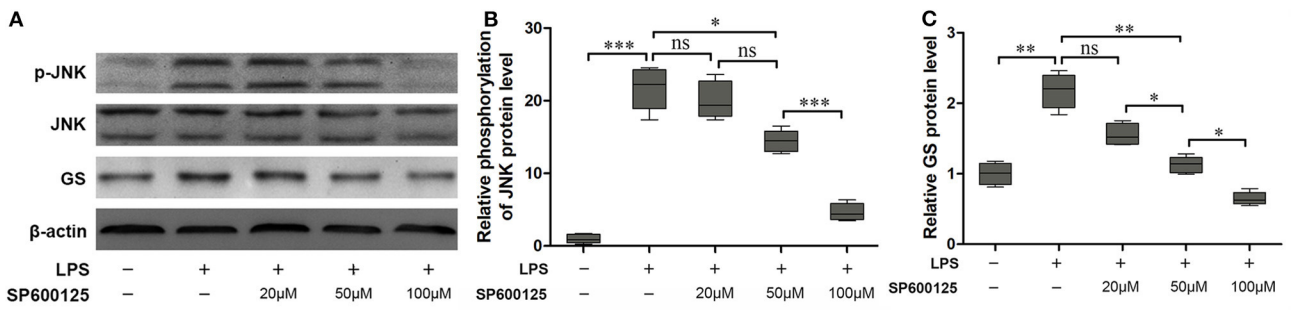

FIGURE 4 | Inhibition of phosphorylation of JNK prevented the effect of LPS-induced upregulation of expression of GS. Astrocytes were pretreated with SP600125 for 1 h and exposed to 1 μg/ml LPS for 1 h. The protein levels of GS **(A,B)** and p-JNK **(A,C)** were measured using Western blotting. Error bars were ± SEM. $n = 3$. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. ns, not significant.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Ethic Committee of Lanzhou University.

CONSENT FOR PUBLICATION

The authors all consented to publication.

AUTHOR CONTRIBUTIONS

SW performed the experiments and analyzed the data. HZ analyzed the data. JW designed the experiments and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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frontiers in Pain Research

REVIEW
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Modulation of Glia-Mediated Processes by Spinal Cord Stimulation in Animal Models of Neuropathic Pain

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Glial cells play an essential role in maintaining the proper functioning of the nervous system. They are more abundant than neurons in most neural tissues and provide metabolic and catabolic regulation, maintaining the homeostatic balance at the synapse. Chronic pain is generated and sustained by the disruption of glia-mediated processes in the central nervous system resulting in unbalanced neuron–glial interactions. Animal models of neuropathic pain have been used to demonstrate that changes in immune and neuroinflammatory processes occur in the course of pain chronification. Spinal cord stimulation (SCS) is an electrical neuromodulation therapy proven safe and effective for treating intractable chronic pain. Traditional SCS therapies were developed based on the gate control theory of pain and rely on stimulating large Aβ neurons to induce paresthesia in the painful dermatome intended to mask nociceptive input carried out by small sensory neurons. A paradigm shift was introduced with SCS treatments that do not require paresthesia to provide effective pain relief. Efforts to understand the mechanism of action of SCS have considered the role of glial cells and the effect of electrical parameters on neuron–glial interactions. Recent work has provided evidence that SCS affects expression levels of glia-related genes and proteins. This inspired the development of a differential target multiplexed programming (DTMP) approach using electrical signals that can rebalance neuroglial interactions by targeting neurons and glial cells differentially. Our group pioneered the utilization of transcriptomic and proteomic analyses to identify the mechanism of action by which SCS works, emphasizing the DTMP approach. This is an account of evidence demonstrating the effect of SCS on glia-mediated processes using neuropathic pain models, emphasizing studies that rely on the evaluation of large sets of genes and proteins. We show that SCS using a DTMP approach strongly affects the expression of neuron and glia-specific transcriptomes while modulating them toward expression levels of healthy animals. The ability of DTMP to modulate key genes and proteins involved in glia-mediated processes affected by pain toward levels found in uninjured animals demonstrates a shift in the neuron–glial environment promoting analgesia.

Keywords: spinal cord stimulation, neuropathic pain, animal models, mechanism of action, neuronal-glial interactions

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INTRODUCTION

Pain is a natural reflex that protects an individual from potentially harmful stimuli. Specialized nerve terminals conduct information from the periphery and internal organs to the brain *via* sensory ganglia and distinct tracts in the spinal cord (SC). When a certain stimulus (mechanical, chemical, thermal, emotional) exceeds a particular threshold, the brain interprets it as pain. Pain is a complex quale encompassing a concerted and balanced interplay of biological processes orchestrated through cellular signaling and interactions throughout the entire nervous system. Acute pain accompanies injuries and is necessary to initiate and sustain a healing and self-protection process to return the damaged tissues to normality. Once the affected part of the body is healed, pain recedes, and the system goes back into balance. However, many individuals continue to experience pain beyond what constitutes the normal healing process from injuries. In this case, persistent or chronic pain sets in due to distorted and unbalanced processing of events. Our understanding of chronic or persistent pain, although limited, has evolved greatly in the last 60 years. Many pain theories have been developed (1, 2), most of them centered on neuronal processing, driven by the fact that neurons are the main carriers of sensory information to the brain. Indeed, the most recent of these, gate control theory (GCT) (3), has served as the foundational development of electrical neuromodulation therapies such as spinal cord stimulation (SCS), dorsal root ganglion stimulation (DRGS), and peripheral nerve stimulation (PNS) for the treatment of intractable chronic neuropathic pain (4–6). Conventional modalities of these treatments consist of applying electric signals to the dorsal columns of the SC, or the DRG, or a peripheral nerve to induce paresthesias that are steered to overlap the affected pain dermatomes. These paresthesias result from the stimulation of neurons in Aβ fibers and are intended to gate out the noxious input transmitted through small, unmyelinated, and slow conductive fibers (7). It is also plausible that conventional SCS exerts an inhibitory effect on wide dynamic range neurons *via* Aβ fibers or directly on these fibers, which are also known to contribute to neuropathic pain (8, 9). Caylor et al. provide a comprehensive review of the various mechanisms of action of SCS (10).

SCS has been proven to be an effective and safe reversible treatment of intractable chronic neuropathic pain of the trunk and limbs (11). Rooted in the foundations of the GCT of pain, it was developed to target neurons to induce paresthesia in the painful area, with electrical signals pulsed at a rate of 40-60 Hz. Technological developments of this paresthesia-based traditional SCS modality have resulted in improvements in clinical outcomes in which ∼50% of treated patients with post-laminectomy pain syndrome obtained ≥50% pain relief (12). Other developments based on the utilization of electrical signals that do not rely on paresthesia have flourished in the last decade (13). Electrical pulses delivered at rates above what was traditionally utilized served as the foundational basis for the development of therapies that use bursts (pulses at 500 Hz delivered every 25 μs) or faster-uninterrupted pulses (>1 kHz). One of such treatments that utilized 10 kHz pulses provided superior outcomes (∼80% of patients obtained ≥50% pain relief) relative to treatment with traditional SCS (14). These results spurred the review of the mechanism of actions that had remained centered on the neuronal doctrine embedded in further developments of the GCT (15).

A largely ignored fact in electrical neuromodulation is the now well-established key role of glial cells in the pathology of chronic pain (16, 17). In a pain state, microglia, the resident immune cells of the central nervous system (CNS), become activated into various phenotypes that promote pro-inflammatory and anti-inflammatory processes *via* the expression and release of cytokines, chemokines, and gliotransmitters (18). Intracellular activation of signaling cascades may cause these changes to become persistent by perpetuating an inflammatory state. Activation of inflammatory processes also triggers the activation of astrocytes, the glial components of the tripartite synapse. These cells modulate calcium signaling, regulate extracellular potassium, and buffer the effect of neurotransmitters (19). Astrocytes monitor the homeostasis of the synaptic clefts and provide neuronal nutrients (glutamine and L-serine) used in the synthesis of neurotransmitters (glutamate, GABA, glycine, and D-serine). Astrocytes communicate *via* calcium waves through gap junctions and regulate calcium-mediated processes central to the signaling of immune and inflammatory processes. They also capture nutrients from the circulatory system and regulate blood supply at the blood–brain barrier while releasing vasoactive molecules. These cells are associated with maintaining a chronic pain state as key players in the long-term potentiation of nociception. In addition to microglia and astrocytes, oligodendrocytes are now recognized to be involved in chronic pain (20, 21). Mature oligodendrocytes myelinate neuronal axons and thus play an important role in maintaining proper signal conduction. Precursor oligodendrocyte cells (OPCs) are mobile and are known to populate and migrate from white and gray matter. These cells can mature to become myelinating as required by the CNS and assist astrocytes via cell-to-cell signaling processes in monitoring the homeostatic balance of the system.

Over a decade ago, Vallejo et al. (22) suggested that electrical stimulation of neural tissue could also target glial cells, acknowledging that they play a fundamental role in the establishment and maintenance of neuropathic pain. This idea has been supported by reports demonstrating that astrocytes and oligodendrocytes respond to electrical stimulation and that such response may be modulated by modifying the characteristics of the electric signal (23–26). Furthermore, Sluka et al. (27) showed that standard low rate (LR) SCS reversed the expression of protein markers associated with glial activation in a rat spared nerve injury (SNI) model of neuropathic pain. Vallejo et al. (28) later showed, using high-throughput transcriptomics, that LR SCS affected the expression of hundreds of genes associated with neuroinflammation, immune response, and ion transport regulation, among others, in the SNI model. Similar results were obtained by Guan et al. (29, 30) using transcriptomic-based analysis of the effects of LR SCS in the rat chronic constricted injury (CCI) model and the rat chemotherapy-induced painful neuropathy (CIPN) model. These studies also validated the involvement of glial cells in immune response and inflammatory

processes associated with the pain models. Vallejo et al. (31–33) have used the knowledge obtained from their molecular biology-based research to develop an SCS approach in which multiple signals are multiplexed to target neurons and glial cells differentially. This approach has been successfully translated to the clinic (34), in which differential target multiplexed SCS programs have provided superior pain relief (∼80% of subjects with ≥50% relief) relative to traditional SCS.

This manuscript provides insight into what has been learned using animal models of SCS on the modulation of glial-based processes, emphasizing evidence obtained using molecular biology methods.

MATERIALS AND METHODS

Animal Models in Spinal Cord Stimulation Used for Glial-Mediated Processes

Animal models for SCS have been recently reviewed (35). Three pain models have mostly been used for studying the molecular effects of SCS on neuropathic pain.

SCS in the Spared Nerve Injury Model

Details of the implementation of the SNI model for SCS are provided by Vallejo et al. (31, 36). The model targets the sciatic nerve at the point of trifurcation into the peroneal, tibial, and sural nerves in the hindlimb of the animal, located under the biceps femoris muscle. Both the tibial and peroneal nerves were ligated with a silk suture, and 2–4 mm of the nerve was sectioned and removed, leaving the sural nerve intact. Nerve injury caused long-lasting mechanical and thermal hypersensitization in the operated limb. For SCS, a cylindrical quadripolar lead was implanted in the dorsal epidural space of the L1-L2 vertebral level *via* a laminectomy at the L4 level. The lead cable was securely anchored to the muscle tissue around the L5 spinal process to reduce migration risk. The lead cable terminals were connected to a block with an ethernet plug attached to a custom-made harness. An ethernet spiral cable connected the block to an assembly that was connected to an external neurostimulator, which delivered the electrical signals to be studied. This setup is capable of providing continuous SCS for many days. In their work, Vallejo et al. (31) have studied various SCS modalities, including traditional low rate (LR, 50 Hz, 20 or 150 μs pulse width, PW), high rate (HR, 1.2 kHz, 50 μs PW), or differential target multiplexed programming (DTMP, 50 Hz and 1.2 kHz, 50 and 150 μs PW). Current intensities were set at 70% of the motor threshold (MT). The effects were studied at an early stage of the pain model, as SCS was started 5 days post-SNI surgery.

The SNI was also similarly implemented by Sluka et al., except for using a quadripolar paddle lead implanted *via* laminectomy at the T13 level (27, 37). Signals were pulsed at 4 or 60 Hz with voltage intensities set at 90% of the MT and delivered 6 h a day for 4 days. PW was likely 250 μs based on another report from this group that utilized the same rate and intensity (37). SCS was started 2 weeks post-nerve injury.

SCS in the Chronic Constriction Injury Model

Details of the implementation of the CCI for SCS are provided by Guan et al. (29, 38). The model targets the sciatic nerve in the hindlimb of the animal located under the biceps femoris muscle. Rather than axotomizing the nerve branches, the sciatic nerve trunk proximal to the trifurcation is loosely ligated with four 4-0 silk sutures about 0.5 mm apart. Nerve injury also developed into a stable and persistent pain model. A quadripolar paddle lead covering the T13-L1 vertebral levels was epidurally implanted *via* a laminectomy at the T13 level in this implementation. Lead cables were tunneled subcutaneously rostrally to exit at the cervical level near the head. These were connected to an external neurostimulator which delivered electrical signals at 50 Hz, 200 μs PW, and current intensities set to 80% of the MT. SCS was delivered twice a day (2 h per session) for 3.5 consecutive days. SCS was started 36 days post-nerve injury.

SCS in the Chemotherapy-Induced Painful Neuropathy Model

Details of the implementation of the CIPN for SCS are provided by Guan et al. (30). This model uses intraperitoneal administration of paclitaxel (1.5 mg/kg) for 4 consecutive days. Animals reached maximum hypersensitivity manifested in the limbs about 2 days after the final dose of paclitaxel. A quadripolar paddle lead was epidurally implanted via a laminectomy at the T13 vertebral level. The lead-covered the dorsal T13-L1 levels. Lead cables were tunneled subcutaneously rostrally to exit near the head. These were connected to an external neurostimulator which delivered electrical signals at 50 Hz, 200 μs PW, and current intensities set to 80% of the MT. SCS was delivered preemptively for 14 days (6–8 h per day), starting 1 day before starting paclitaxel injection.

Molecular Biology Methods

Transcriptomics Using Microarrays

High-throughput quantification of gene expression using microarray technology was used by Vallejo et al. (28) to study the effects of traditional LR SCS (see section SCS in the Spared Nerve Injury Model above) on the stimulated SC section (dorsal ipsilateral quadrant) and the L5 DRG of SNI animals and uninjured animals. This was the first time the transcriptome of SCS was reported. RNA from 48 samples (2-5 from 6 experimental groups) was extracted and quantitated from frozen tissue, preserved in RNAlater solution, using standard procedures (39). The RNA was hybridized to Agilent rat gene expression 4 × 44 microarray kits. Half of the samples were labeled with Cy5 and the other half with Cy3 fluorescent dyes. When a particular hybridized RNA of the sample is complementary to the cDNA probe in the microarray, the fluorescent dye is activated and detected using optical methods. The arrays used in this work interrogated 26,930 genes using 30,367 probes in the microarrays.

Transcriptomics Using RNA Sequencing

Although microarray analysis is a convenient way of quantifying a large amount of protein-coding messenger RNAs (mRNAs), it is limited to those genes that have been characterized and

built into the microarrays. In contrast, RNA sequencing allows sampling of the total RNA in a sample, including mRNA, micro RNAs (miRNAs), and long non-coding RNAs (lncRNAs). This methodology was used first by Guan et al. (29) to determine the effect of traditional LR SCS on the CCI (see section SCS in the Chronic Constriction Injury Model above) and later by the same group (30) to study the effect of LR SCS on CIPN (see section SCS in the Chemotherapy-Induced Painful Neuropathy Model above). Ipsilateral L4–L6 SC segments were dissected and stored in a DNA/RNA shield solution. RNA was extracted and quantitated using standard methods. Five hundred ng of total RNA was used to build strand-specific sequencing libraries. These were built after polyadenyl [poly(A)] selection of mRNA using a commercial kit. Samples were barcoded using a kit that contains adapters and primers designed for high amplification efficiency. The RNA sequencing libraries were quantified using quantitative polymerase chain reaction (qPCR). Libraries were normalized, pooled, and sequenced in an Illumina HiSeq4000 to a depth of 33.6 million reads per sample.

Recently, Vallejo et al. (31) used RNA sequencing to study the effect of DTMP on gene expression in the stimulated ipsilateral cord compared with LR and HR SCS in SNI animals (see section SCS in the Spared Nerve Injury Model above). RNA from frozen dorsal ipsilateral quadrants of the L1-L2 SCs of 20 animals (4 per experimental group) stored in RNAlater was isolated using the TriZol commercial kit. RNA libraries were constructed using a commercial kit after poly(A) enrichment of mRNA from a 1 μg sample of total RNA. RNA was coded by chemically fragmenting the mRNA, annealing with random hexamers, and converting to double-stranded cDNA ligated to indexed adaptors. The cDNA was amplified, quantitated, and polled using qPCR. Pooled barcoded libraries were sequenced in an Illumina HiSeq 4000 and quality controlled using software algorithms that select and map mRNAs to the rat genome (NCBI Rnor_6.0 Annotation Release 106).

Gene Expression Using Reverse Transcription qPCR

Vallejo et al. (40) used quantitative reverse transcription PCR (RT-qPCR) to study the modulatory effects of phase polarity and extent of anodic charge of LR signals (50 Hz, 50 μs cathodic PW, the intensity at 66% of the MT) on a panel of 21 genes associated with glial-related processes. Vallejo et al. (28) and Guan et al. (29) also used RT-qPCR to validate their high throughput results. Transcripts from the genes of interest were identified in the rat genome to design sequence-specific primers using bioinformatic tools (41). Column-purified RNA extracted from experimental samples was reverse-transcribed into first-strand cDNA using a commercial kit. Quantitation was carried out by amplification of cDNA using qPCR. A mix of cDNA, reverse and forward primers, polymerase, deoxy-nucleotide triphosphates (dNTPs), and a fluorescent DNA-intercalation probe was thermally cycled under appropriate conditions. Gene expression levels were quantitated in triplicate by measuring the C_q values from the thermal amplification cycles using the $\Delta\Delta C_q$ method (42). An internal control gene was used to normalize the expression of genes of interest, obtaining a ΔC_q. Differential gene expression between experimental groups compared their corresponding ΔC_q values and obtaining a $\Delta\Delta C_q$.

Immunohistochemistry

Immunohistochemistry was used by Sluka et al. (27) as well as Guan et al. (38) to detect proteins associated with glial activation related to treatment with traditional LR SCS (see section SCS in the Spared Nerve Injury Model above). Anesthetized rats were transcardially perfused with heparinized saline (10%) followed by paraformaldehyde (4%) with 15% picric acid. SCs were dissected and fixed for 1 h in paraformaldehyde and frozen after immersion in 30% sucrose. Sections (20 μm thick) were frozen cut onto slides for staining. Before exposure to specific antibodies, slides were blocked with 3% goat serum followed by avidin-biotin. Overnight exposure to anti-mouse GFAP antibodies (1:5,000) and goat anti-Rabbit MCP-1 antibodies (1:500) were used to stain active astrocytes. GFAP and MCP-1 staining were developed by exposing slides to biotinylated goat anti-mouse IgG and goat and goat anti-rabbit IgG, respectively, followed by exposure to Streptavidin-488 for fluorescent detection. Active microglia were stained by exposure to anti-mouse OX-42 antibodies (1:2,500) and developed using the same process for developing astrocyte stains. Slides from five animals per group were imaged using fluorescence microscopy and analyzed using imaging software (ImageJ) to differentially quantify the amount of antibodies in stimulated and non-stimulated cords.

High-Throughput Proteomics and Phosphoproteomics

Vallejo et al. (43) pioneered high throughput protein profiling methods for studying the effect of traditional LR SCS on the SNI model of neuropathic pain. The global proteomic analysis used by this group identified and quantitated proteins in a sample using liquid chromatography and tandem mass spectrometry (LC/MS/MS). Proteins from SC tissues (see section SCS in the Spared Nerve Injury Model above) were separated, purified, and quantitated using standard procedures that used non-ionic buffers compatible with the LC method (44). Proteins were trypsinized after reduction and alkylation of cysteine residues. Tryptic peptides were purified and desalted, and labeled with isotopic tags (TMT 10plex) for simultaneous processing and quantitation. Labeled samples were equally mixed and separated using LC into 18 fractions. Each fraction was then subjected to LC/MS/MS in quadruplicate. Mass spectra of tagged peptides were searched against the Uniprot curated proteome of the rat to identify proteins based on unique peptide profiles using bioinformatics software (45). Identified proteins were quantified from normalized spectral intensities of their unique peptides.

Post-translational modifications of proteins serve as a diverse source of regulatory and signaling moieties. Protein phosphorylation by kinases is one such process. Phosphoproteomics has been used to investigate glia-mediated regulation of pain-related processes. Phosphorylated proteins are enriched from the total protein sample *via* reversed-phase solid-phase extraction, followed by phospho-enrichment using immobilized metal affinity chromatography (IMAC) (46) with iron-based magnetic beads (PTMScan® Fe-IMAC, Cell Signaling

Technology, Danvers MA). Unbound peptides were washed out, and immobilized phosphopeptides eluted with basic pH buffer. Reversed-phase purification was performed before LC/MS/MS analysis, carried out as described above for whole proteomics.

Bioinformatics and Statistical Analyses

Using high-throughput methods is the vast amount of data that needs to be analyzed to gain useful insight. Various algorithms have been created for mining the data using curated databases (47). Vallejo et al. (28, 31) have used weighted gene coexpression network analysis (WGCNA) (48) to catalog genes according to their expression trends based on the various treatment groups. The WGCNA groups genes in a hierarchical fashion in modules. Pairwise comparisons between expression patterns in a given module for treatment groups are based on an eigengene value, representing the degree of variance. Significance *P*-values for multiple comparisons of eigengenes were corrected using the false discovery rate (FDR) method (49).

To determine the biological relevance of genes in a module, individual modules were subjected to a gene ontology enrichment analysis (GOEA), emphasizing their involvement in biological processes curated into gene ontology terms. Various software options are used to carry out the GOEA (50). The method ranks the GO terms based on the number of genes in the module (i.e., experimental gene set) represented in a given curated GO term. Significance *P*-values for multiple comparisons based on the ranks were corrected using the FDR method.

Protein interaction network maps can be constructed using web-based bioinformatics tools, such as string-db (51), generating them from curated literature reports. Tools can also provide clustering based on connectivity indexing and GOEA and analysis based on Reactome and protein domains, which allow categorization of proteins based on their biological relevance. The FDR method is used to rank the results based on significance.

Experimental Designs

Experimental designs were well-controlled, including at a minimum a stimulation sham (SCS turned off). Others included naïve and injury sham (uninjured) animals. **Table 1** summarizes the experimental designs of the work cited in the previous sections.

RESULTS AND DISCUSSION

Modulation of Glia-Related Gene Expression by SCS

Transcriptomics and qPCR have been used to study the modulatory effects of SCS in SC and DRG tissues. In their transcriptomics work on the SNI, Vallejo et al. (28) showed that 72 h of continuous treatment with traditional LR SCS modulated gene expression in the SC related to neuroinflammation and immune response. This included glia-related genes such as *Lyz2*, *Cd68*, *Cd74*, *Cxcl16*, *RT1-Bb*, *RT1-Da*, *RT1-Db1*, *Tlr2*, *Itgb2*, *Aif1*, and *Tspo*. Some of these genes are markers of microglial activation (e.g., *Cd68*, *Cd74*, *Itgb2*) and astrocyte activation (e.g., *Tlr2*, *Cxcl16*), which are usually elevated by nerve injury and the inflammatory process. Interestingly, LR SCS elevated the expression of these genes. Furthermore, the increase occurs in the absence of injury for some of these genes, implying that LR SCS may activate glial cells. Transcriptomics work reported by Guan et al. (29) on the effect of LR SCS on the CCI are remarkably similar, despite the differences in pain models, their chronicity, stimulation times, and SC segments analyzed. This work found that traditional LR SCS upregulated immune- and inflammatory-related processes and that this treatment elevated expression of markers of astrocyte (*Gfap*, *Ccl2*) and microglia (*Cd68*, *Itgam*) activation in the SC, caudal to the stimulation site. This group followed up with one study (36) in which they measured microglia mRNA markers for specific M1-like (pro-inflammatory) and M2-like (anti-inflammatory) phenotypes in the L4–L6 SC. They found that expression levels of two M1-like markers (*Cd16* and *Cd32*) were significantly elevated by the CCI, while *Cd16* was further increased by low rate SCS. The expression levels of the M1-like marker *i-Nos* were elevated by treatment but were not by the CCI. None of the three M2-like markers (*Arg1*, *Cd163*, and *Tgfb*) were affected by either the CCI or SCS. These authors also showed that intrathecal administration of a low dose (.067 μg/μl) of the microglia inhibitor, minocycline, in conjunction with LR SCS, provided analgesic effect after acute (2 h) SCS. Cedeño et al. (32) analyzed a microglia-specific transcriptome (101 genes) and found that the SNI upregulated 79% of these genes relative to naïve animals and that LR SCS reversed the expression levels of only 23% of genes toward the naïve level, in contrast with what was obtained with DTMP and HR SCS. Indeed, they found that the expression profile of the microglia transcriptome associated with LR SCS treatment negatively correlated with the expression profile of naïve animals. This group followed up with a report (33) in which larger microglia transcriptomes associated with resting (1,569 genes), post-injury (3,706 genes), and neuroprotective (1,588 genes) states were analyzed. They found that expression levels relative to SNI in both post-injury and neuroprotective states upon LR SCS correlated weakly with those found for naïve animals. This was consistent with the fact that only ∼50% of genes in these transcriptomes returned toward naïve levels upon treatment with LR SCS. Glial activation induces the release of pro-inflammatory cytokines (such as *Tnfa*, *Il1b*, and *Il6*) and a reduction of anti-inflammatory ones (such as *Il10* and *Il4*). Tilley et al. (52, 53) measured some of these in stimulated SC and the L5 DRG of SNI animals and those treated with LR SCS using RT-qPCR. They found that the SNI elevated the expression level of *Tnfa* in the SC relative to injury-sham. Furthermore, they found that LR SCS further increased *Tnfa* expression in both injured and uninjured animals. Interestingly, changes in *Tnfa* correlated well with the changes observed in the microglial activation marker, *Itgam*, and the astrocyte activation marker, *Gfap*. *Itgam* was also increased in the L5 DRG due to the SNI and further increased by LR SCS, while the level of *Il1b* was significantly elevated in the DRG due to LR SCS. Shu et al. (38) also found that LR SCS significantly increased *il1b* in the L4-L6 SC of CCI animals. However, *Tnfa* levels were not changed. It is noteworthy to highlight the similarity of the findings by Tilley et al. in the DRG and those by Shu et al. in the L4-L6 SC, considering that

TABLE 1 | Summary of experimental designs in preclinical investigations of SCS.

Model	Tissues analyzed	SCS treatments	Controls	SCS time	References.
SNI (14d)	SC[a]	LR (4 Hz, 250 μs, 90% MT) LR (60 Hz, 250 μs, 90% MT) At T10-T12	No-SCS	6 h/day for 4 days	Sato et al. (27)
SNI (4d)	L1-L2 SC L5 DRG	LR (50 Hz, 20 μs PW, 70% MT) At L1-L2	No-SCS No-SNI (implanted) SNI (no implant) Sham for SNI (no implant)	72 h continuous	Vallejo et al. (28)
SNI (5d)	L1-L2 SC	DTMP (50 Hz/1.2 kHz, 50/150 μs PW, 70% MT) LR (50 Hz, 150 μs PW, 70% MT) HR (1.2 kHz, 50 μs, 70% MT) At L1-L2	No-SCS Naïve	48 h continuous	Vallejo et al. (31)
SNI (5d)	L1-L2 SC	LR (50 Hz, 50 μs PW cathodic, variable anodic PW, 66% MT) At L1-L2	No-SCS No-SNI	24 h continuous	Vallejo et al. (40)
CCI (36d)	L4-L6 SC	LR (50 Hz, 200 μs PW, 80% MT) At T10-T12	No-SCS	2 h/day for 3.5 days	Stephens (29)
CCI (18d)	L4-L6 SC	LR (50 Hz, 200 μs PW, 80% MT) At T10-T12	No-SCS	3 h per session (2 per day) for 3.5 days	Shu et al. (38)
CIPN	L4-L6 SC	LR (50 Hz, 200 μs PW, 80% MT) At T10-T12	No-SCS CIPN (no implant) Naïve	6-8 h/day for 14 days[b]	Sivanesan et al. (30)

[a] *Vertebral levels analyzed were not reported.*
[b] *Started preemptively 1 day before inducing the pain model.*

SCS was applied more rostrally. Thus, preclinical results based on transcriptomics suggest that traditional LR SCS may enhance microglial activation. Based on molecular biology, these reports complement the existing mechanisms of action of conventional SCS, which have mostly been centered on the effect of electrical stimulation on neuronal activity (10). The modulation of glial cell activity by SCS suggests that the mechanisms of action should also account for these cells in their interactions with neurons and their combined contribution to neuropathic pain.

Vallejo et al. (40) found that the expression levels of glia-related genes could be modulated by modifying the anodic content of the electrical signal. Although this may not have a direct clinical application due to the charge unbalanced signals, it illustrates that the properties of electrical signals may influence glial activation and, thus, its effects on neuroinflammation and neuropathic pain relief. **Figure 1** shows that an increase in the amount of anodic charge in a bipolar signal correlates strongly with a decrease in the expression levels of glial activation markers *Aif1*, *Gfap*, *Cd68*, *Tspo*, *Cd74*, and *Cxcl16*.

These findings are congruent with previous evidence demonstrating that glial cells respond to the application of electric fields. Roitbak and Fanardjian (54) showed that the membrane of cortex astrocytes of a cat could be depolarized by changing the parameters (intensity, polarity, and rate) of pulsed electrical signals. Lee et al. (23–25) also demonstrated that electrical stimulation of astrocytes in the brain of rodents induced the release of glutamate. This release is dependent on the properties of the electric signal, including rate, pulse width, intensity, and extent of charge balance. Yamazaki et al. (55) also demonstrated that electrical stimulation of oligodendrocytes could modulate conduction velocities in the

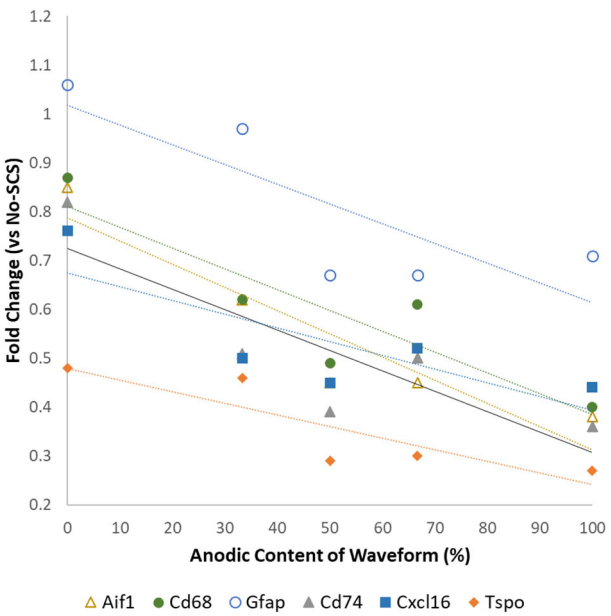

FIGURE 1 | Differential expression levels of mRNA associated with glial activation as a function of the anodic content of pulsed signals at a low rate (50 Hz, 50 μs PW cathodic, 66% MT). Data from Vallejo et al. (40).

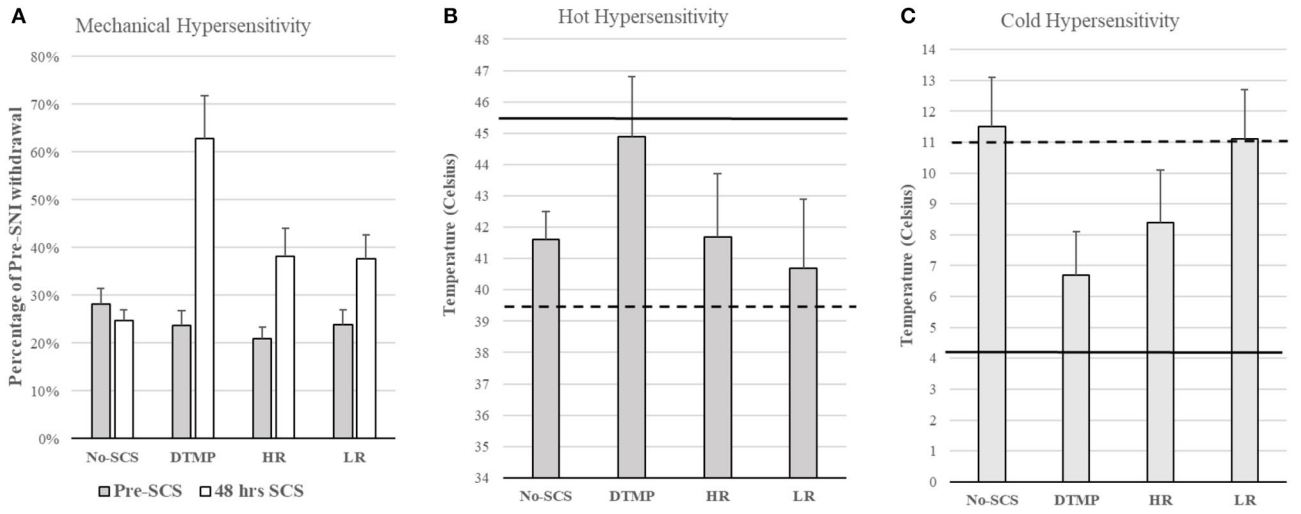

FIGURE 2 | (A) Mechanical hypersensitivity relative to Pre-SNI. **(B)** Hot hypersensitivity. The dashed line denotes the mean of all animals pre-SCS, Continuous line denotes the mean baseline of all animals (pre-SNI). **(C)** Cold hypersensitivity. The dashed line denotes the mean of all animals pre-SCS, Continuous line denotes the mean baseline of all animals (pre-SNI). Figures reproduced from Vallejo et al. (31). SNI, spared nerve injury; SCS, spinal cord stimulation.

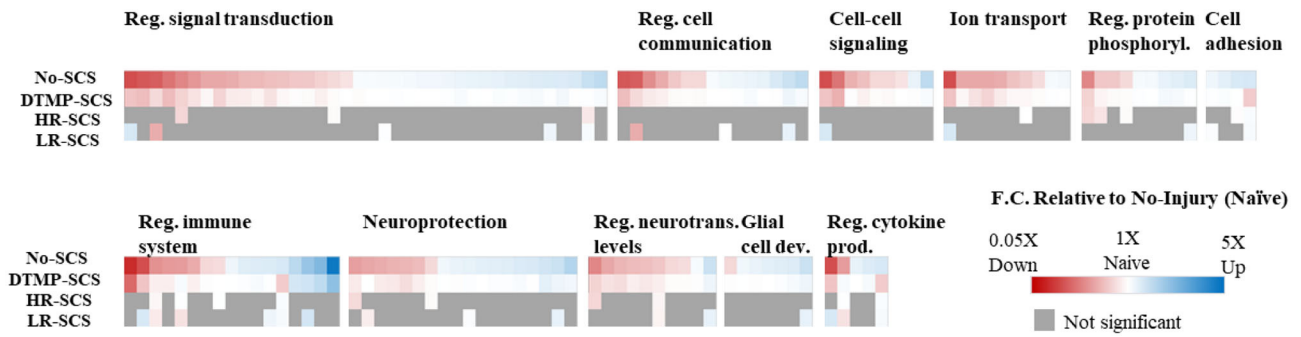

FIGURE 3 | Heat maps illustrating expression levels due to the effect of DTMP, LR, and HR SCS on genes enriched in biological processes that involve neuron–glial interactions, compared with the effect of the pain model (No-SCS). The white color indicates that the expression is that of a naïve animal. Reproduced from Vallejo et al. (31). DTMP, differential target multiplexed programming; HR, high rate; LR, low rate; SCS, spinal cord stimulation.

axons they myelinate. Another important fact is that glial cells are the most abundant cells in the spinal cord (56, 57). A recent human anatomical study showed about 13 glial cells per every neuronal soma in the gray matter of the dorsal horn closest to the SCS field in the T8-T11 levels (57).

Considering that glial cells are the most abundant in the spinal cord, play a fundamental role in chronic pain, and are electrically excitable, Vallejo et al. developed an SCS approach in which various pulsed electric signals are multiplexed in space and time to target glial cells and neurons differentially. They found that differential target multiplexed programming (DTMP) provided significant improvements in both mechanical and thermal hypersensitivity in SNI rats (see **Figure 2**) (31). Improvement in mechanical hypersensitivity was also significantly better than that obtained using LR or HR SCS.

Transcriptomics validated that, relative to naïve animals, the SNI upregulated hundreds of genes involved in regulating the immune system, inflammation, and signal transduction. DTMP modulated more of these genes than both HR and LR SCS. More importantly, DTMP significantly reversed the expression levels of 166 of such genes within 10% of the expression levels found in naïve animals. In contrast, HR SCS and LR SCS only modulated 70 and 91 of such genes, respectively, within 10% of the naïve expression levels. DTMP also reduced expression levels of genes associated with microglia and astrocyte activation (i.e., *Itgam* and *Gfap*, respectively), which the pain model had increased. This work also illustrated that DTMP provides a more substantial modulatory effect on genes associated with pain-related processes than HR and LR SCS (see **Figure 3**). These results indicate that DTMP may provide its analgesic effect through modulation of immune-related processes, synaptic

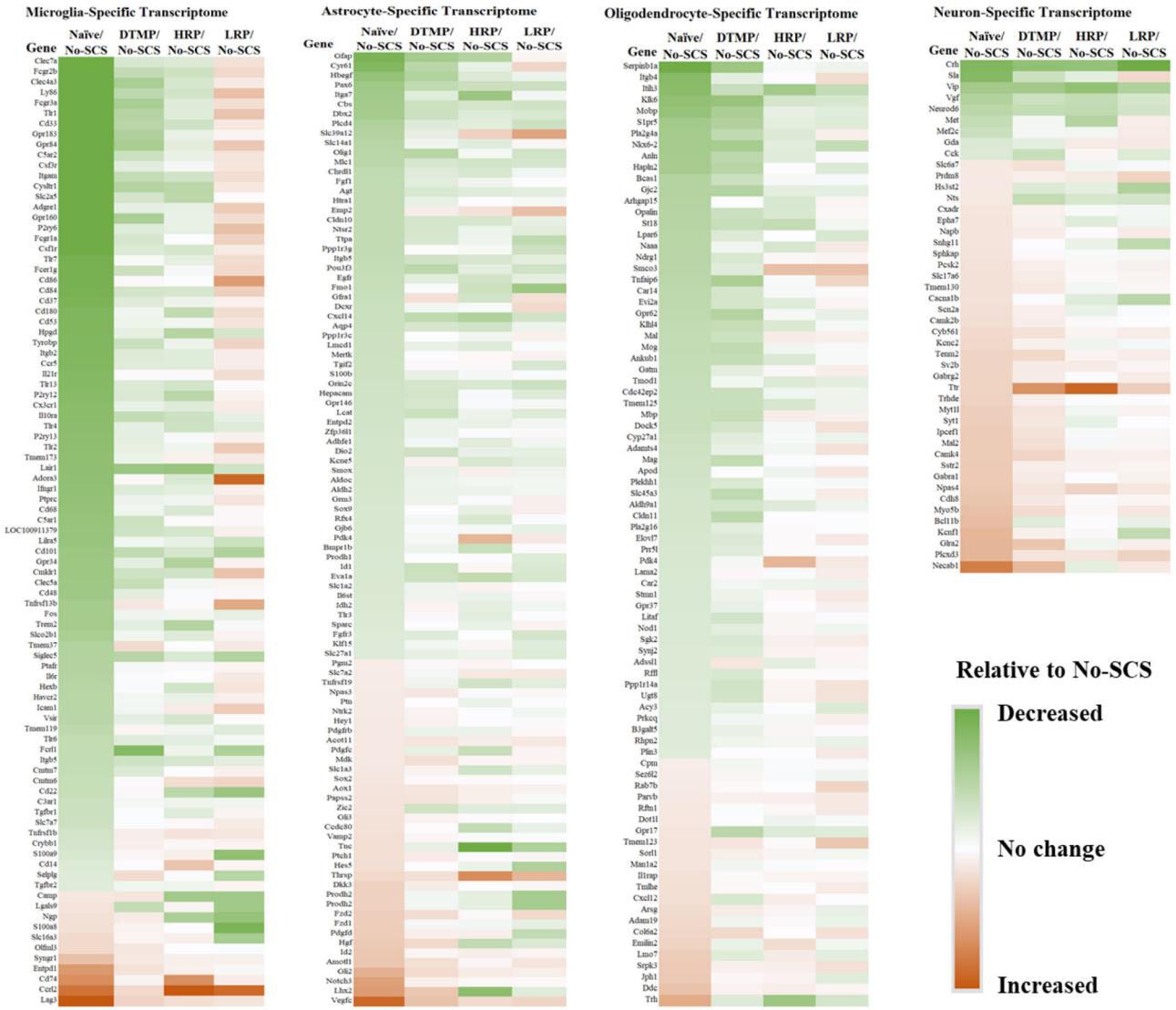

FIGURE 4 | Heat maps for cell-specific transcriptomes illustrating differential expression levels (i.e., fold changes) of genes in naïve, DTMP-treated, HR-treated, and LR-treated animals relative to untreated (No-SCS) animals. From left to right: microglia-specific, astrocyte-specific, oligodendrocyte-specific, neuron-specific transcriptomes. Reproduced from Cedeno et al. (32). DTMP, differential target multiplexed programming; HR, high rate; LR, low rate; SCS, spinal cord stimulation.

signaling, and neurotransmission and rebalancing neuronal-glial interactions that the onset of neuropathic pain had perturbed.

In an analysis of cell-specific transcriptomes, Cedeño et al. (32) demonstrated that DTMP is more effective at modulating neurons and glial cells (microglia, astrocytes, and oligodendrocytes) transcriptomes toward the gene profile found in naïve animals (**Figure 4**). For instance, neuron (72 genes), microglia (101 genes), astrocyte (188 genes), and oligodendrocyte (154 genes) transcriptomes of DTMP-treated animals significantly correlated positively and strongly with that of naïve animals ($R_{Pearson} \geq 0.65$). In contrast, for HR-treated animals, only the microglia transcriptome significantly correlated positively and strongly ($R_{Pearson} = 0.61$) with the naïve one, while the neuron transcriptome significantly correlated positively and moderately ($R_{Pearson} = 0.41$). Furthermore, no cell-specific transcriptome of LR-treated animals correlated strongly. Instead, the microglia transcriptome of LR-treated animals significantly correlated negatively and weakly ($R_{Pearson} = -0.20$) with the naïve profile, meaning that many microglia genes were further upregulated by LR SCS relative to the SNI effect.

This result is congruent with the previous findings on the effects of LR SCS on microglia activation. In further work, Smith et al. (33) reported on the effects of DTMP, HR, and LR on microglia transcriptomes associated with their resting state and states associated with pro-inflammatory processes (so-called M1-like) and neuroprotective processes (so-called M2-like). In agreement with Cedeño et al., it was found that DTMP provided the largest modulatory effect on the SNI. The

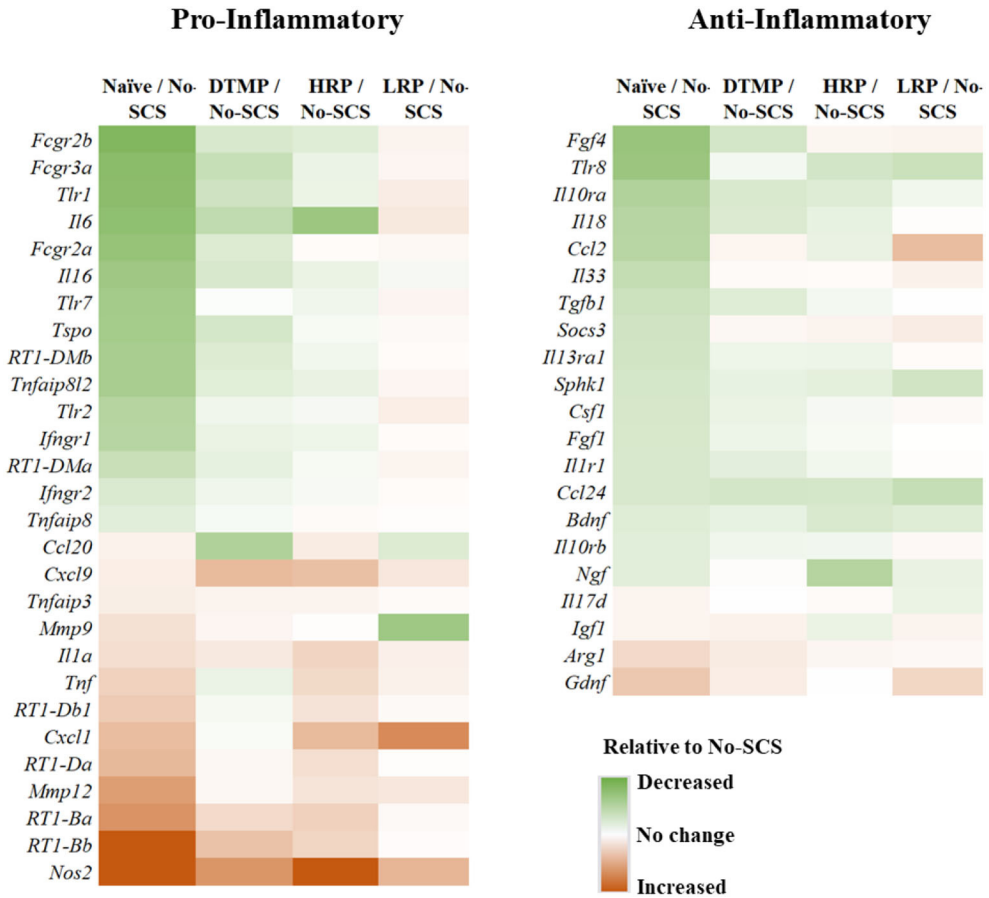

FIGURE 5 | Heat maps illustrating differential expression levels of microglia-related genes known to be involved in pro- and anti-inflammatory processes for naïve, DTMP-treated, HR-treated, and LR-treated animals relative to untreated animals (No-SCS). Reproduced from Smith et al. (33). DTMP, differential target multiplexed programming; HR, high rate; LR, low rate; SCS, spinal cord stimulation.

microglial transcriptomes of DTMP-treated animals significantly correlated positively and strongly ($R_{Pearson}$ = 0.58–0.65) with naïve profiles. HR treatment also produced significant positive correlations although moderate ($R_{Pearson}$ = 0.42–0.48). Although LR treatment also produced significant positive correlations, only the resting microglia transcriptome correlated moderately with the naïve profile ($R_{Pearson}$ = 0.39). Both the M1-like and M2-like transcriptomes correlated weakly ($R_{Pearson}$ = 0.17). A further look at selected microglia genes within these transcriptomes, which had been reported in the literature to be associated with pro- and anti-inflammatory processes, clearly indicated that treatments with DTMP and HR better match (**Figure 5**) the profile of naïve animals. On the other hand, LR treatment further upregulated expression levels of pro-inflammatory genes that the SNI had increased.

Another important result of Cedeño et al. (32) is that DTMP provided strong modulation of astrocyte-specific and oligodendrocyte-specific genes, with more than 65% of genes modulated back to within 15% of their naïve levels (with more than 78% expression recovery). These glial cell types are the most abundant in the spinal cord (57), constituting about 80% of the combined microglia, astrocyte, and oligodendrocyte populations. The role of astrocytes in chronic neuropathic pain is well-established (19). Thus, a reversal of expression levels by DTMP toward naïve levels indicates a rebalancing of neuron-astrocyte interactions at synapses. An understanding of the role of oligodendrocytes in neuropathic pain is emerging. Ablation of oligodendrocytes in murine spinal cords induced neuropathic pain-like behavior (20). For instance, SNI increased expression levels of *Mobp*, an oligodendrocyte marker. An increase in expression levels of the protein encoded by this gene (myelin oligodendrocyte basic protein) was found in patients with neuropathic pain associated with HIV infection (21). Expression levels of the gene *S1pr5*, which is only expressed by oligodendrocytes, were also increased by the SNI. This gene is associated with signaling via sphingosine-1-phosphate (S1P) that triggers the migration of OPCs. Both of these genes were significantly modulated by DTMP toward naïve expression levels (31).

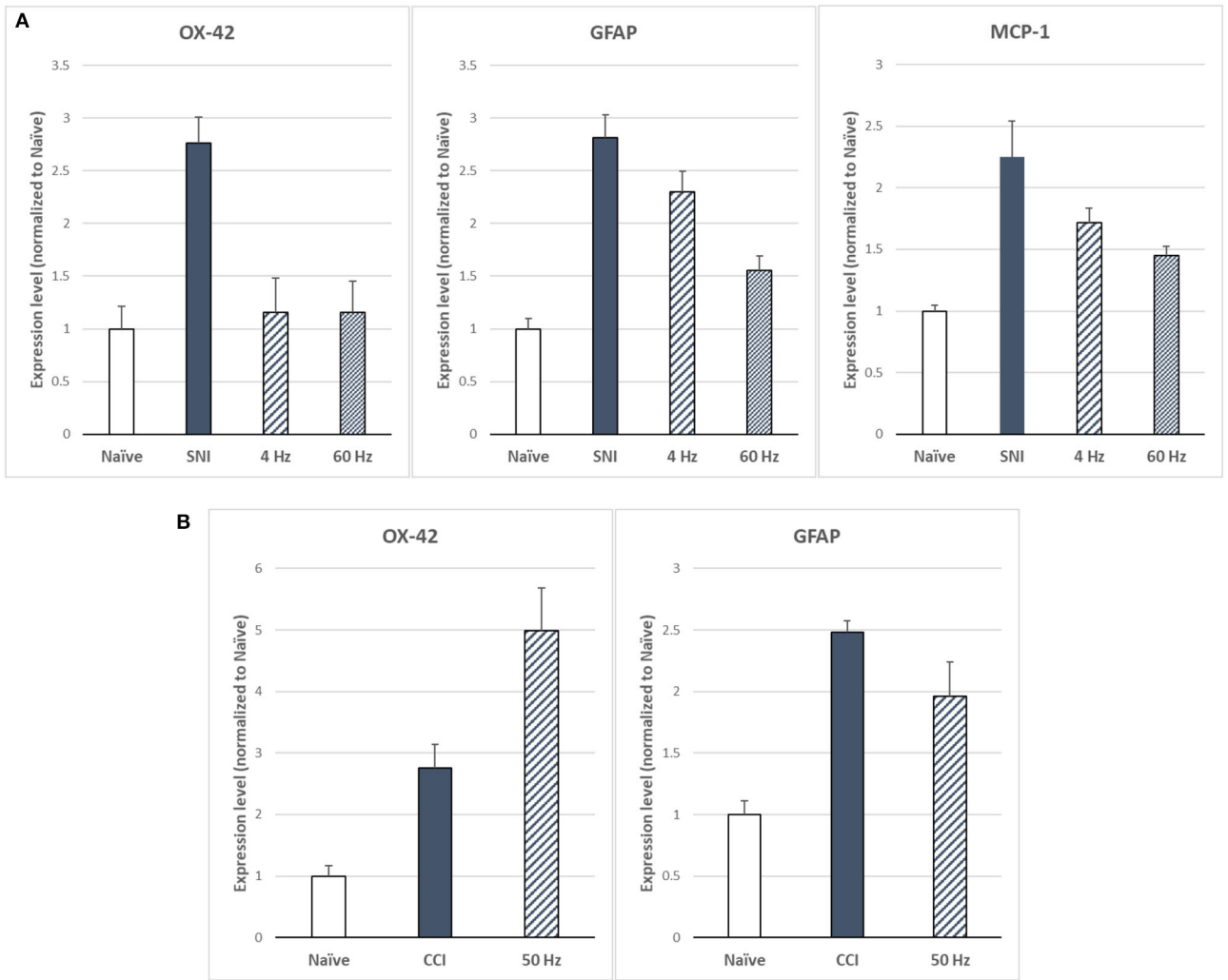

FIGURE 6 | (A) Expression levels of microglia marker OX-42 and astrocyte markers GFAP and MCP-1 as based on Sato et al. (27) for LR SCS at 4 or 60 Hz (250 μs PW, 90% MT, 6 h/day for 4 days) in the SNI model (14 days). **(B)** Expression levels of microglia marker OX-42 and astrocyte marker GFAP based on Shu et al. (38) for LR SCS at 50 Hz [200 μs PW, 80% MT, 3h (2× day) for 3.5 days] in the CCI model (18 days). SCS, spinal cord stimulation.

Modulation of Glia-Related Protein Expression by SCS

Sluka et al. (27) reported first on the effect of SCS on glia-related protein expression in the dorsal horn of SNI animals. They found that the expression levels of OX-42 (also known as ITGAM, a marker of microglial activation), MCP-1 (also known as CCL2), and GFAP (markers of astrocyte activation) were significantly elevated, relative to naïve animals, after 14 days of the SNI (**Figure 6A**). They found that LR SCS treatment for 6 h/day for 4 consecutive days at either 4 or 60 Hz significantly decreased expression levels of these markers. Interestingly, in a recent study, Shu et al. (38) found that although expression levels of GFAP and OX-42 were significantly elevated by the CCI (18 days) relative to naïve animals (**Figure 6B**), LR SCS treatment at 50 Hz (6 h/day in two 3 h sessions for 3.5 days) did not decrease the expression of these proteins. In contrast, they found that LR SCS significantly increased the expression of OX-42 relative to the expression in untreated animals. Lack of congruence on the effects of LR SCS with the previous work by Sato et al. was attributed, in part, to experimental differences (animal models, SCS protocols, and post-injury times). Preliminary results of a proteomics-based analysis in our laboratory show that continuous 48 h of DTMP significantly decreased expression levels of astrocyte markers GFAP and VIM, which had been significantly increased by the SNI (**Figure 7**). Besides these two proteins, the SNI also upregulated S100A8 and S100A9, calcium-binding proteins known to induce astrocyte differentiation in inflammatory processes, and CNTF, a protein expressed by astrocytes during gliosis. DTMP decreased their expression levels toward naïve levels. The study also found that the pain model increased expression levels of three phosphorylated isoforms of GFAP (p-GFAP at residues T31, S80, and T148) and 11

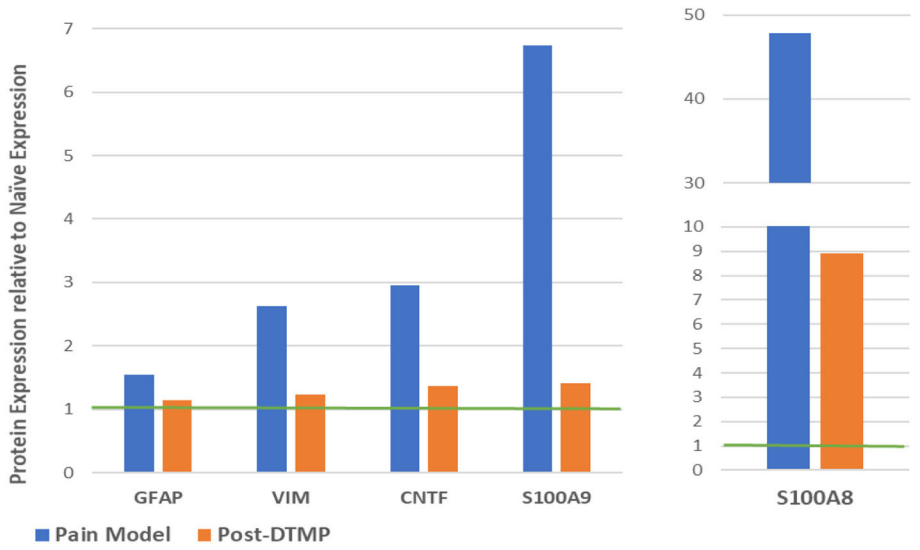

FIGURE 7 | Modulatory effect of DTMP on protein expression levels of astrocyte-related proteins in the spinal cords of SNI (pain model). The green line denotes the normalized expression level in naïve animals. DTMP, differential target multiplexed programming; SNI, spared nerve injury.

phosphorylated isoforms of VIM (p-VIM at residues S7, S10, S18, S39, S51, S56, S73, S325, S430, T436, and S549). DTMP decreased expression levels of the three p-GFAP isoforms and 10 of the p-VIM isoforms toward levels in naïve animals. Although the role of these phosphorylated isoforms has not been elucidated, phosphorylation and dephosphorylation of filament proteins such as GFAP and VIM may be associated with signaling processes in which the phosphorylated isoforms are intermediate states linked with processes tightly associated with it such as neurotransmission regulation (i.e., glutamate or GABA buffering) and calcium-mediating signaling of inflammatory pathways.

Astrocytes play an important role at the synaptic cleft, where they monitor the homeostatic balance of nutrients, ions, and neurotransmitters. An analysis of the proteomics of the effect of DTMP on the regulation of ion transport within the spinal cord of SNI animals found that proteins expressed by astrocytes are key elements in the establishment of neuropathic pain and the analgesic effect of DTMP. For example, KIR4.1 is a potassium ion (K^+) channel that allows entry of K^+ into astrocytes while inhibiting the release of BDNF mediated by astrocytic Na^+/K^+ ATPases such as ATP1A2 and ATP1B2. KIR4.1, ATP1A2, and ATP1B2 were found to be upregulated by DTMP. Buffering of K^+ into the astrocytes facilitates the activity of neuronal KCC2, a K^+/Cl^- symporter that maintains the homeostatic balance of chloride in neurons, which is known to play an important role in GABA-regulated post-synaptic inhibition. DTMP also reversed the effect of the SNI on the enzymes PHGDH and PSAT1, involved in the synthesis of L-serine, an essential amino acid in the production of neurotransmitters D-serine and glycine. Neurons cannot synthesize L-serine. The reduction of PHGDH levels has been previously associated with the reduction of L-serine and neuropathic pain. Thus, an increase of PHGDH and PSAT1 by DTMP treatment is congruent with the important role of glial synthesis of important nutrients that keep homeostatic balance in the synapsis. Further emphasis is on the modulating role of DTMP on astrocytes in regulating ionotropic and metabotropic glutamate (GLU) receptors. For instance, the SNI significantly decrease expression levels of the metabotropic glutamate receptor MGLUR5, which DTMP reversed. Neuropathic pain also affects intracellular second messenger pathways that involve calcium ions (Ca^{2+}). Large concentrations of intracellular Ca^{2+} in glial cells are associated with inflammatory pathways. The SNI increased the expression of IP3R1, a protein of the endoplasmic reticulum that aids the release of Ca^{2+} into the cytoplasm. DTMP significantly reversed its expression. The decrease of intracellular Ca^{2+} in astrocytes would reduce its release into gap junctions that link astrocytes to each other in propagating calcium waves, which is considered a key process in the sensitization of distal neural tissues. Further analyses of the role of calcium signaling in the SNI and the effects of DTMP are underway. The investigations into the modulatory role of DTMP on the NFkB signaling pathway in inflammation and neuroprotective role *via* modulation of the caspase-apoptosis pathway.

CONCLUDING REMARKS AND THE NEXT FRONTIERS

Recognizing the role of glial cells in chronic neuropathic pain is a relatively recent advancement in our understanding of its mechanism of action, particularly in a line of thought that focused on a doctrine that has placed neurons as the only active player. If this fact is accepted, it is also rational to think that treatments for chronic neuropathic pain must also account

for their presence and role. Until recently, the field of SCS was bound to a theory of pain that, although useful for its development and advancements, had ignored many fundamental processes related to the interactions of the neurons with their surrounding glial cells. The advent of molecular biology tools has also provided an opportunity to explore neural tissues beyond what electrophysiological measurements could tell us from the perspective of neuronal behavior. These tools have opened the door to a molecular understanding of biological processes associated with pain that can facilitate the optimization of SCS approaches, targeting both neurons and glial cells in such a way that their interactions, perturbed by the onset and persistence of chronic pain, can be rebalanced. The recent preclinical work highlighted in this report should encourage others to move into the next frontiers in our understanding of the mechanism of action of SCS and the value of this comprehension to improving clinical outcomes. At the preclinical level, we need to understand further the effects of the electrical signals applied in SCS at the cellular level and the development of chronic pain. Cell sorting techniques and single-cell RNA sequencing would provide more specificity. *In situ* proteomics and peptidomics of neural tissues, using laser-based desorption ionization techniques (such as MALDI) coupled with tandem MS/MS will also provide a way of "imaging" the spatial distribution of proteins in neural tissue and how pain models and treatments affect these. The investigation of other post-translational changes in proteins (acetylation, glycosylation, etc.) would also help understand the persistence of chronic pain since particular isoforms of modified proteins may drive this. Other interesting frontiers include the link between epigenetics and chronic pain, the potential role of genetic predisposition, and environmental factors in establishing and maintaining chronic pain. In a more practical sense, clinical validation of the hypotheses formulated by preclinical discoveries is perhaps the next frontier. This would require searching for suitable pain-related biomarkers that can be identified and quantitated in easily accessible fluid samples or using imaging techniques such as MRI.

AUTHOR CONTRIBUTIONS

DC and RV designed the outline of the manuscript. DC produced the first draft. All authors contributed equally to the final version of the manuscript.

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Role of Connexin 43 in an Inflammatory Model for TMJ Hyperalgesia

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Temporomandibular joint disorders (TMD) consist of a heterogeneous group of conditions that present with pain in the temporomandibular joint (TMJ) region and muscles of mastication. This project assessed the role of connexin 43 (Cx43), a gap junction protein, in the trigeminal ganglion (TG) in an animal model for persistent inflammatory TMJ hyperalgesia. Experiments were performed in male and female rats to determine if sex differences influence the expression and/or function of Cx43 in persistent TMJ hyperalgesia. Intra-TMJ injection of Complete Freund's Adjuvant (CFA) caused a significant increase in Cx43 expression in the TG at 4 days and 10 days post-injection in ovariectomized (OvX) female rats and OvX females treated with estradiol (OvXE), while TG samples in males revealed only marginal increases. Intra-TG injection of interference RNA for Cx43 (siRNA Cx43) 3 days prior to recording, markedly reduced TMJ-evoked masseter muscle electromyographic (MMemg) activity in all CFA-inflamed rats, while activity in sham animals was not affected. Western blot analysis revealed that at 3 days after intra-TG injection of siRNA Cx43 protein levels for Cx43 were significantly reduced in TG samples of all CFA-inflamed rats. Intra-TG injection of the mimetic peptide GAP19, which inhibits Cx43 hemichannel formation, greatly reduced TMJ-evoked MMemg activity in all CFA-inflamed groups, while activity in sham groups was not affected. These results revealed that TMJ inflammation caused a persistent increase in Cx43 protein in the TG in a sex-dependent manner. However, intra-TG blockade of Cx43 by siRNA or by GAP19 significantly reduced TMJ-evoked MMemg activity in both males and females following TMJ inflammation. These results indicated that Cx43 was necessary for enhanced jaw muscle activity after TMJ inflammation in males and females, a result that could not be predicted on the basis of TG expression of Cx43 alone.

Keywords: connexins, estrogen status, hyperalgesia, sex differences, temporomandibular joint, trigeminal ganglion

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INTRODUCTION

Temporomandibular joint disorders (TMD) represent a diverse group of conditions accompanied by pain in the temporomandibular joint (TMJ) region and muscles of mastication. TMD is the most common non-dental orofacial pain condition and is the main reason for TMD patients to seek medical treatment (1, 2). Although routine clinical examinations in TMD typically find little

evidence of tissue or nerve damage (3, 4), results from more invasive diagnostic methods such as synovial fluid sampling (5) or jaw muscle microdialysis sampling (6, 7) suggest that TMD is characterized as a persistent mild inflammatory condition. A second prominent feature of TMD is the higher prevalence in women than men (8, 9). Pressure pain thresholds are reportedly lower in female than male TMD patients (10) and vary over the menstrual cycle (11) to further suggest that estrogen status is a key factor for TMD pain in women.

Chronic pain conditions are thought to be driven and maintained by combination of peripheral and central neural mechanisms (12, 13). The TMJ and masticatory muscles are supplied by sensory neurons whose cell bodies lie within the trigeminal ganglion (TG) and dorsal root ganglia of the upper cervical spinal cord (14–16). Results from *in vitro* studies suggest that TMJ nociceptors are sensitized after local inflammation (17) and are further enhanced by estrogen treatment (18). Other studies have shown that ion channels in TG neurons associated with nociception are upregulated by TMJ inflammation and further enhanced by elevated estrogen conditions (19, 20). A key mechanism linking inflammation to sensitization of nociceptors involves activation of satellite glial cells (SGC), a class of non-neuronal cells that surround sensory neurons. SGCs serve a homeostatic function and amplify the effects of local inflammation on the excitability of nociceptors by releasing pro-nociceptive molecules (21–23). Inflammation of the TMJ region activates SGCs in the TG (24–27) resulting in an increase in coupling between SGCs and the formation of gap junctions (28). Connexin 43 (Cx43) is the most common gap junction protein in the TG and is mainly restricted to SGCs (29–32). Although Cx43 expression is regulated in a sexually-dimorphic manner in other tissues (33, 34), no previous studies have determined if sex differences and/or estrogen status alter Cx43 expression and function in an animal model for TMJ hyperalgesia.

The present study also was designed to address the key features of TMD in an animal model. Thus, we used an intra-TMJ injection of Complete Freund's Adjuvant (CFA) at a dose (10 μg) that produces minimal signs of tissue damage (35). Second, we monitored changes in a specific jaw-related muscle behavior, masseter muscle electromyography (MMemg), a signature activity that persists throughout the 10 day observation period following CFA injection. MMemg activity is a valid measure of jaw hyperalgesia since intra-TMJ injection of algesic agents evokes activity in a dose-dependent manner that correlates with pain reports in humans (36). Third, we determined if Cx43 expression and its role in TMJ hyperalgesia are sexually dimorphic and/or are dependent on estrogen status to address the issue that the vast majority of preclinical studies for pain have been conducted in male animals (37, 38).

MATERIALS AND METHODS

General Animal Preparation

A total of 133 adult male, ovariectomized females (OvX) and estradiol-treated OvX female (OvXE) rats (250–350 g, Sprague–Dawley, Harlan, Indianapolis, IN) were used in these experiments. OvX females were purchased commercially and used within 2 weeks of ovariectomy. OvXE rats were injected with estradiol (E2, 30 μg/kg, sc) 1 day prior to processing tissue for immunohistochemical or molecular analyses or for muscle recording. This dose of E2 results in a blood level of E2 consistent with the surge of E2 seen in the proestrous phase of cycling female rats (39). Vaginal lavage samples were taken on the day of the experiment to confirm the estrogen status of females. Samples from OvX rats had mainly small nucleated leukocytes, while samples from OvXE rats had mainly large nucleated epithelial cells consistent with the early diestrous and proestrous stages of the estrous cycle, respectively. Animals were housed in pairs and given free access to food and water. Climate and lighting were controlled (25 ± 2°C, 12:12-h light/dark cycle, light on at 7:00 A.M.). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (USA) and according to guidelines set by The National Institutes of Health guide for the Care and the Use of Laboratory Animals (PHS Law 99-158, revised 2015).

Complete Freund's Adjuvant Into TMJ

Rats were anesthetized with 5% isoflurane and the fur overlying the TMJ was shaved. A single dose of CFA (10 μg, 10 μl) was injected into the left TMJ via a 33-gauge needle inserted into the TMJ-capsule (∼3 mm in deep) and animals survived for 4 or 10 days prior to tissue collection or muscle recording. Controls received an injection of PBS. All rats received a single dose of carprofen (25 mg/kg, i.p) immediately after the intra-TMJ injection. It is not likely that carprofen affected these results since tissue collection and muscle recording were performed 10 days later.

Immunohistochemistry

Separate groups of males, OvX and OvXE female rats (sham, 4 day CFA, 10 day CFA, four rats per group) were anesthetized with pentobarbital sodium (50 mg/kg, i.p) and the depth of anesthesia was confirmed by the loss of hindlimb withdrawal reflex. Rats were perfused transcardially with heparinized phosphate buffered saline (PBS) followed by 10% buffered formalin. TGs were removed and postfixed overnight in 10% formalin. Transverse sections (30 μm) were cut on a vibratome and collected in 0.01 M PBS. Free-floating sections were incubated in blocking buffer (PBS, 0.1% Triton X-100, 1% secondary serum) for 1 h and then incubated with anti-mouse primary antibody for glial fibrillary acidic protein (GFAP, Abnova MAB107670, Walnut, CA) and anti-rabbit primary antibody for Cx43 (Cell Signaling 3512, Danvers, MA) at 1:1,000 in PBS with 0.1% Triton X-100 overnight at 4°C. Specificity of the antibody to Cx43 was determined previously (40). Sections were rinsed in PBS (x3) and then incubated with anti-mouse Cy2 secondary antibody (Jackson Immunoresearch 715228151 West Grove, PA) and anti-rabbit Cy5 secondary antibody (Jackson Immunoresearch 711175152 West Grove, PA) at 1:500 in PBS in the dark for 2–3 h. Sections were rinsed in PBS (x3), placed on slides and cover slipped with ProLong Gold with 4,6-diamino-2-phenyindole (DAPI, Invitrogen, Carlsbad, CA). Fluorescent-labeled sections were viewed on a Zeiss LSM 700 confocal microscope at 40X magnification. Images were taken at the level of the junction

of the maxillary and mandibular (5–7 images per rat). Staining of Cx43 was corrected for brightness without substraction for background, quantified by densitometry using NIH ImageJ Software and quantified without prior knowledge of treatment. Digital gain settings for Cx43 = 1.5 and for GFAP = 1.0. Statistical analyses of densitometry results were assessed by analysis of variance (ANOVA) and $p < 0.05$ was set as the level of significance without prior knowledge of treatment.

Real-Time Polymerase Chain Reaction

TGs (four per group) were removed from rats following perfusion with saline and RNA LATER solution (Molecular BioProducts, San Diego, CA). RNA was extracted using the Trizol method (Invitrogen, Carlsbad, CA). cDNA was synthesized using iScript cDNA kit (Bio-Rad, Hercules, CA). RT-PCR was performed in triplicate on 2 μL cDNA with QuantStudio 3 (Applied Biosystems) using iQ SYBRgreen supermix (Bio-Rad). Data was analyzed using the ΔΔCT method using UBC as a reference gene. Primer sets were UBC F-tcgtacctttctcaccacagtatctag, R- gaaaactaagacacctccccatca and CX43 F: 5′-taagtgaaagagaggtgccca-3′ R: 5′-gtggagtaggcttggaccttt-3′. 40 cycles were employed at 95°C for 15 s, 59°C for 30 s, and 72°C for 30 s.

Western Blot

TGs (four per group) were removed after saline perfusion, homogenized, and protein extracted using the Trizol method (Invitrogen, Carlsbad, CA). Protein concentration was determined with bicinchroic acid (BCA) assay (Pierce, Rockford, IL). A protein aliquot of 30 μg was separated on 4–15% polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane. Membranes were incubated with Cx43 antibody (3512, Cell Signaling, Danvers, MA), followed by Anti-rabbit IRDye 680 (1:15,000, LI-COR, Lincoln, NE). Proteins were visualized with an Odyssey infrared scanner (LI-COR) and arbitrary optical density was determined. Normalizing controls were utilized by simultaneous staining with glyceraldehyde 3-phosphat dehydrogenase (GAPDH) antibody (1:1,000, WH0002597M1, Sigma, St. Louis, MO) followed by goat anti-mouse IRDye 800 (1:15,000, LI-COR). Protein levels were quantified *via* densitometry using NIH ImageJ Software.

Interference RNA for Cx43

Animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p) and maintained with isoflurane (1–2%). The fur overlying the scalp was shaved and povidone-iodine was applied before surgery. Lidocaine gel (2%) was applied to scalp wound margins and the body temperature was maintained at 38°C with a heating blanket. The animals were placed in a stereotaxic apparatus and a small hole (3–4 mm) was drilled into the left parietal bone (3.5–4 mm anterior to the auricle and 3–4 mm lateral to the midline). The siRNA solution (600 μg, 200 nL, Stealth RNAi Gja1RSS351267, Invitrogen, Carlsbad, CA) was injected into the left TG 7 days after intra-TMJ injection of CFA *via* a 33-gauge needle inserted through a 26-gauge guide cannula positioned stereotaxically and was kept in position at least 10 min after the injection to minimize leakage. The wound margin was closed with sutures and povidone-iodine solution was applied to the surgical wound area. A single dose of carprofen (25 mg/kg, i.p) was injected in each animal to minimize post-surgical pain. Animals survived for 3 days after siRNA injection (i.e., 10 days after intra-TMJ injection of CFA). Sham controls for CFA received an intra-TMJ injection of PBS only with no further treatment and survived 10 days.

Masseter Muscle Electromyography Recording

Rats (5–6 rats per group) were anesthetized with urethane (1.5 g/kg) and maintained with supplemental isoflurane (1–2%). The animal was placed in a stereotaxic apparatus and a pair of copper electrodes was implanted in the left masseter muscle (0.12 mm diameter, 5 mm interpolar distance) with a 26-gauge needle. A skin incision was made just above the zygomatic process of the temporal bone and a 26-gauge guide cannula was positioned in the TMJ-capsule (∼3 mm in deep). At least 1 h elapsed after cannula placement and before recording. MMemg was recorded under two separate protocols. In the first series following siRNA treatment, MMemg was recorded in response to intra-TMJ injections (PBS, 0.01, 0.1, and 1 mM ATP, 20 μl) delivered *via* a 33-gauge needle inserted through the guide cannula over 30 s in a cumulative dose design at 20 min intervals. In the second series, GAP19 (10 mM, 200–300 nl, Tocris, Minneapolis, MN), a mimetic peptide and specific inhibitor of Cx43 hemichannel formation was injected as a single dose (10 mM, 0.2 μl) into the TG via a 26-gauge guide cannula and a 33-gauge injection cannula 10 min prior to repeated intra-TMJ injections of ATP (1 mM, 20 μl). In both series MMemg was recorded continuously for 6 min for each stimulus period; 3 min prior to each ATP test stimulus to establish the baseline activity and 3 min after test stimulus. The rationale for using ATP as a test stimulus was based on earlier studies demonstrating that ATP can be injected repeatedly without causing tachyphylaxis or sensitization (39).

At the end of the experiment the rat was given a bolus of urethane and perfused transcardially with heparinized PBS and RNase-Away like buffer (60 mL). TGs following MMemg recording sessions were removed and (4 TGs per group, ipsilateral to PBS or CFA injection) were processed for mRNA and protein levels of Cx43. The location of the TG injection site was verified histologically from 1 to 2 rats per group upon removal.

MMemg Data Analysis

MMemg activity was sampled at 1,000 Hz, amplified (×10 k), filtered (bandwidth 300–3,000 Hz), displayed and stored online for analyses. EMG activity was sampled continuously for 6 min, for 3 min prior to each TMJ stimulus and for 3 min after the stimulation was applied. Baseline activity was quantified as the total area under the curve (Total MMemg) for the 3 min epoch (μV-s per 3 min) sampled immediately prior to stimulation. TMJ-evoked MMemg activity was calculated as AUC post-ATP injection minus the baseline.

Statistical Analyses

Densitometry was assessed from 5 to 7 TG sections per rat (4 rats per treatment group) and expressed as average percent positive area (**Figure 1**). Sections were analyzed without prior knowledge of treatment. Values were compared by one-way ANOVA and individual group differences assessed by Neuman–Keuls. Total MMemg activity was assessed by three-way ANOVA and corrected for repeated measures on one factor (5–6 rats per group; **Figure 2**). Significant treatment effects were assessed by Newman–Keuls after ANOVA. The data were presented as mean ± SEM and the significant level set at $p < 0.05$. Based on results from previous studies (41, 42), it was calculated that a sample size of $n = 5$ per treatment group would provide 80% power at $p < 0.05$. Experiments were performed on sham and TMJ-inflamed rats selected in random order. Western blots were performed on TG samples collected from four rats per treatment group (**Figure 3**). Values were log transformed to reduce error variance and then compared by two-way ANOVA and between group differences assessed by Neuman–Keuls after ANOVA. Total MMemg activity was assessed by three-way ANOVA and corrected for repeated measures on one factor (**Figure 4**). Significant treatment effects were assessed by Newman–Keuls after ANOVA and included 5–6 rats per treatment group. The data were presented as mean ± SEM and the significance level set at $p < 0.05$. Experiments were performed on sham and TMJ-inflamed rats selected in random order.

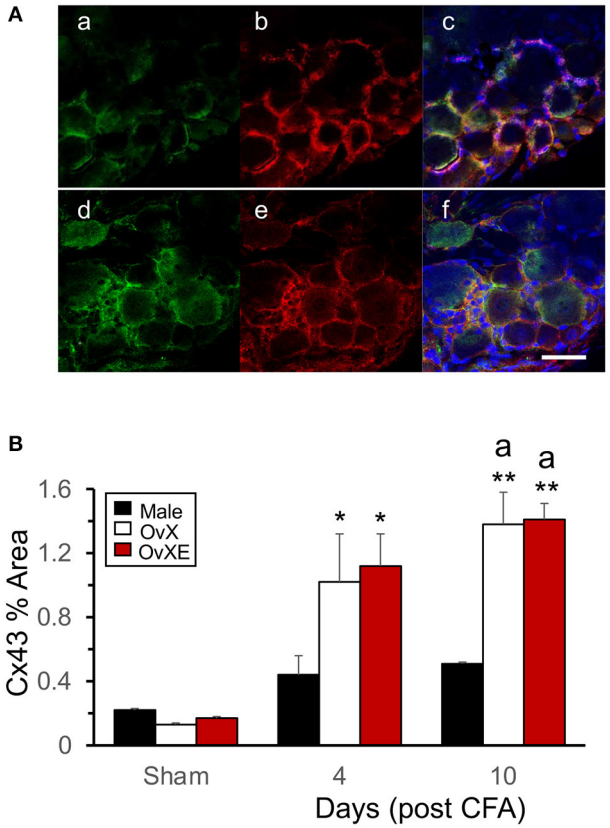

FIGURE 1 | (A) Expression of glial fibrillary acidic protein (GFAP) and connexin43 (Cx43) in the trigeminal ganglion of an OvXE (a–c) and male rat (d–f) 10 days after intra-TMJ injection of CFA. Scale = 30 μm. **(B)** Densitometry values expressed as percentage of positive area for Cx43 in male, OvX and OvXE rats under sham conditions and at 4 and 10 days after intra-TMJ injection of CFA based on an average of immunostaining as shown by the examples in panel **(A)**. *$p < 0.05$, **$p < 0.01$ vs. sham; $^{a}p < 0.05$ vs. males. Sample size = 4 rats per group, average of 5–7 images per rat.

RESULTS

Immunohistochemistry

Glial fibrillary acidic protein (GFAP) and Cx43 were often co-localized and appeared as stained elements surrounding small and moderate diameter TG neurons of TMJ-inflamed OvXE rats (**Figures 1A**a–c) and male rats (**Figures 1A**d–f). **Figure 1B** summarizes the percentage of Cx43 stained area in the TG of sham animals which was very low for males and females. By contrast, Cx43 displayed a marked and sex-dependent increase in Cx43 area at 4 days and 10 days after CFA [$F_{(8,27)} = 7.01$, $p < 0.001$]. Both OvX and OvXE groups displayed significant ($p < 0.01$) and similar increases in Cx43 staining after CFA, while Cx43 staining in CFA-treated males was not statistically different from sham males ($p < 0.1$).

MMemg and siRNA Cx43

To determine if TG expression of Cx43 altered TMJ-evoked MMemg activity, siRNA for Cx43 was microinjected into the left TG 3 days prior to the recording session. As seen in **Figure 2A**, sham males displayed small but significant increases in ATP-evoked MMemg activity [$F_{(3,51)} = 7.45$, $p < 0.001$] that were similar after siRNA knockdown of Cx43 [$F_{(3,51)} = 13.7$, $p < 0.001$]. By contrast, CFA-treated males displayed significant increases in ATP-evoked MMemg activity in the absence of siRNA [$F_{(3,51)} = 62.9$, $p < 0.001$] and much smaller after siRNA [$F_{(3,51)} = 10.5$, $p < 0.001$]. Treatment main effects revealed that siRNA reduced the evoked MMemg activity compared to rats without siRNA treatment [$F_{(3,17)} = 26.84$, $p < 0.001$]. **Figure 2B** revealed that OvX sham females displayed small but significant increases in ATP-evoked MMemg activity [$F_{(3,51)} = 7.66$, $p < 0.001$] that were similar siRNA knockdown of Cx43 [$F_{(3,51)} = 9.63$, $p < 0.001$]. CFA-treated OvX females (**Figures 2B**, **3B**) displayed large ATP-evoked MMemg responses [$F_{(3,51)} = 104$, $p < 0.001$] that were completely prevented by siRNA treatment [$F_{(3,51)} = 2.98$, $p > 0.1$]. Overall treatment main effects revealed that siRNA reduced the ATP-evoked MMemg responses in OvX rats compared to OvX rats without siRNA treatment [$F_{(3,17)} = 64.13$, $p < 0.001$]. **Figure 2C** revealed that OvXE sham females displayed large increases in ATP-evoked MMemg activity [$F_{(3,51)} = 10.2$, $p < 0.001$] that were marginally reduced by siRNA knockdown of Cx43 [$F_{(3,51)} = 2.89$, $p < 0.05$]. CFA-treated OvXE females displayed the greatest ATP-evoked MMemg responses [$F_{(3,51)} = 100$, $p < 0.001$] and were completely prevented by siRNA treatment [$F_{(3,51)} = 1.79$, $p > 0.1$]. Overall treatment main effects indicated that intra-TG siRNA treatment greatly reduced evoked MMemg activity

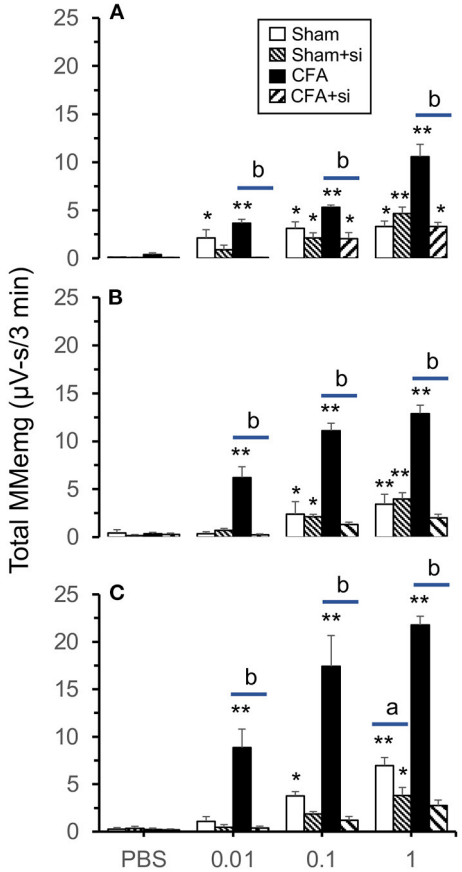

FIGURE 2 | siRNA for Cx43 inhibits intra-TMJ ATP-evoked MMemg activity in **(A)** male, **(B)** OvX and **(C)** OvXE females treated with CFA 10 days prior to recording. Note that responses to TMJ stimuli in sham (PBS-injected) rats were not affected. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. PBS stimulation; $^{a}p < 0.05$, $^{b}p < 0.01$ siRNA treated vs. untreated rats. Sample size = 5–6 rats per treatment group.

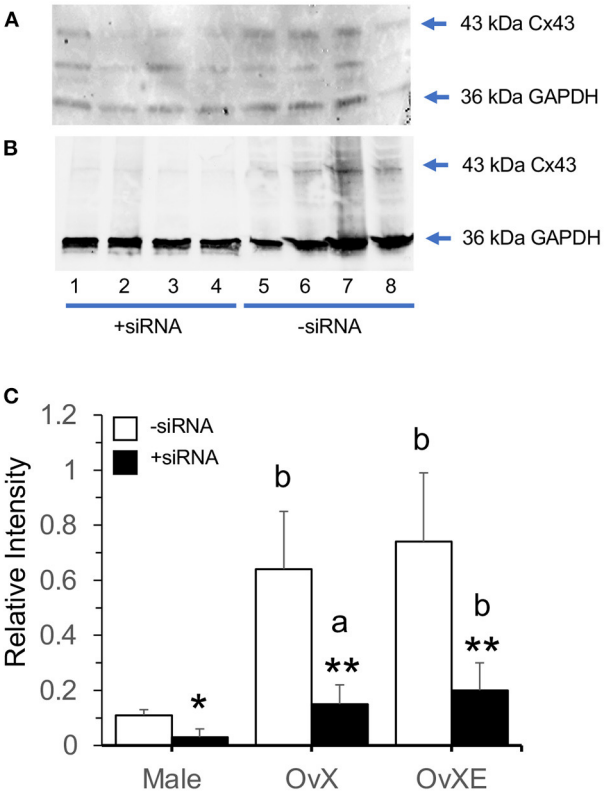

FIGURE 3 | Western blots for Cx43 of TG tissue from OvXE **(A)** and male rats **(B)** at 10 days after CFA and treated with siRNA for Cx43 or with PBS by intra-TG injection 3 days prior to tissue collection. **(C)** Summary of the effects of siRNA for Cx43 on protein levels in the TG of male, OvX, and OvXE females. $^{*}p < 0.05$; $^{**}p < 0.01$ vs sham controls; $^{a}p < 0.05$, $^{b}p < 0.01$ vs males vs. sham controls. Sample size = 4 rats per group.

compared to rats without siRNA treatment [$F_{(3,17)} = 69.64$, $p < 0.001$]. RT-PCR analyses of TG samples revealed no significant sex differences for Cx43 among siRNA-injected sham animals (ΔCT: male = −5.37 ± 2.25; OvX = −5.58 ± 3.7; OvXE = −5.58 ± 1.15, mean ± SD) or at 10 days after CFA (ΔCT: male = −6.06 ± 0.83; OvX = −6.92 ± 5.31; OvXE = −4.53 ± 1.8, mean ± SD). **Figures 3A,B** displays the western blot for males and OvXE females at 10 days post-CFA, respectively, with and without siRNA knockdown of Cx43. The results for western blots for all groups are summarized in **Figure 3C** revealing that siRNA for Cx43 significantly reduced TG expression of Cx43 in both males and females [$F_{(1,18)} = 10.11$, $p < 0.001$].

MMemg and Pharmacological Blockade of Cx43 Formation by GAP19

To determine if acute blockade of Cx43-dependent hemichannel formation affected ATP-evoked MMemg responses, the peptide mimetic inhibitor of Cx43, GAP19, was microinjected into the left TG of sham male, OvX and OvXE rats and in rats at 10 days after CFA treatment. As seen in **Figure 4**, CFA-induced enhancement of TMJ-evoked MMemg activity was significant for males and female groups [overall response

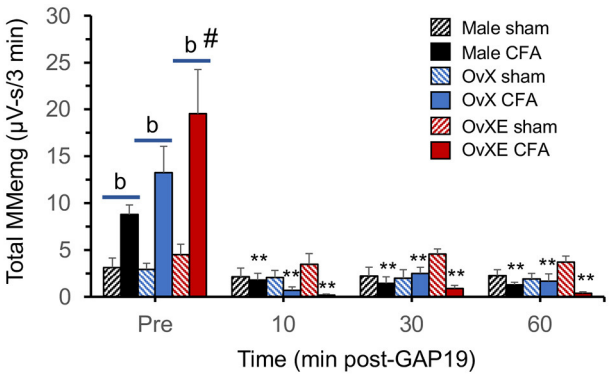

FIGURE 4 | Acute microinjection of GAP19 into the TG reduces the enhanced TMJ-evoked MMemg activity of 10 day CFA-treated males, OvX and OvXE females, while responses in sham rats were not affected. $^{**}p < 0.01$ vs. pre-injection response; $^{b}p < 0.01$ sham vs. CFA groups; $^{\#}p < 0.01$ vs. all other groups. Sample size = 5 rats per group.

main effects $F_{(3,72)} = 44.19$, $p < 0.001$]. GAP19 injection did not significantly affect the ATP-evoked MMemg responses in sham males, OvX or OvXE females [$F_{(3,72)} = <1.0$, $p > 0.1$]. By contrast, ATP-evoked responses in CFA-treated males [$F_{(3,72)} = 8.55$, $p < 0.001$], OvX females [$F_{(3,72)} = 22.2$, $p < 0.001$] and OvXE females [$F_{(3,72)} = 58.7$, $p < 0.001$] all displayed marked decreases in evoked MMemg activity following GAP19 administration.

DISCUSSION

These results revealed a significant increase in Cx43 expression in the TG of OvX and OvXE females that persisted for at least 10 days after mild inflammation of the TMJ, while Cx43 expression in males displayed only marginal increases. Two different approaches were used to assess the functional contributions of Cx43 to TMJ-evoked hyperalgesia. First, small interference mRNA for Cx43 was injected into the TG to silence Cx43 expression in sham and 10 day CFA-treated rats. This resulted in a significant reduction in TMJ-evoked MMemg activity in males and both female groups after TMJ inflammation, but not in sham animals, that was matched by a corresponding decrease in Cx43 protein in TG samples. Second, the mimetic peptide, GAP19, a specific inhibitor of hemichannel formation in nervous tissue (43), was injected acutely into the TG of sham and CFA-inflamed rats. This approach also caused a marked decrease in TMJ-evoked MMemg activity in all CFA-treated animal groups, while evoked activity in sham rats was not affected.

Despite numerous preclinical studies directed at understanding the underlying mechanisms for TMJ hyperalgesia, little progress has been made in developing new pharmacological treatments that are specific for TMD pain (1, 44, 45). Several reasons may contribute to this apparent lack of progress; however, one limitation may be the mismatch between the features of animal models for TMJ nociception and the clinical signs in TMD patients. The present study was designed to minimize these mismatches. TMD patients display few overt signs of tissue damage or inflammation yet often present with fluctuating bouts of pain in a non-progressive manner (46–48). By contrast, rodent models for TMJ hyperalgesia often involve treatments that cause significant tissue damage. Indeed, an intra-TMJ injection of even a dose of CFA as low as 10 μg is sufficient to elevate TMJ tissue levels of proinflammatory cytokines and to increase meal duration in rats (35), while CFA doses of 25 μg or greater cause soft tissue damage and progressive bone erosion (49, 50). A second feature of a valid model for TMJ hyperalgesia is the ability to monitor a surrogate measure of TMJ hyperalgesia. The present study monitored MMemg activity which is a behavior that specifically assesses jaw function and persists throughout the 10 day observation period following CFA treatment. Other direct measures of TMJ hyperalgesia in awake rats such as a decrease in gnawing behavior (51, 52) or bite force (53, 54) and an increase in grimace scale values (52) are seen following intra-TMJ injection of CFA; however, changes in these behaviors are transient and often only a few days. Increased meal duration has been shown to persist for many days after CFA in rats (55); however, this required much larger doses of CFA than that used in the present study (250 μg vs. 10 μg). A third feature of the present model was the comparison of results in males vs. females under high and low estrogen status. Despite the higher prevalence of TMD in females than males (8, 9), few preclinical studies have directly compared responses of males and females for TMJ hyperalgesia. The rationale for using ATP as a test stimulus to evoke MMemg activity was based on two lines of evidence. First, earlier we determined that a 1 mM concentration of ATP reliably evoked trigeminal brainstem activity and could be injected repeatedly at 20 min intervals within the TMJ without causing persistent sensitization or tachyphylaxis (56) and secondly, that ATP is a normal constituent of synovial fluid and evokes increases in pain intensity in a dose-dependent manner (57).

A critical unresolved issue in chronic TMD is the relative contribution of peripheral sensitization of nociceptors in driving long-term changes in central neural processing. Although synovial fluid sampling in TMD patients reveal increased levels of pro-nociceptive molecules such as serotonin and glutamate, the levels of molecules and the magnitude of pain intensity are not well-correlated (5). It is widely accepted that both peripheral and central neural mechanisms contribute to most chronic pain conditions (12, 13). The inhibitory effects of local knockdown of Cx43 within the TG by siRNA or by acute blockade of Cx43-dependent hemichannel formation on TMJ-evoked MMemg activity suggest that Cx43 contributes to a persistent peripheral driving force to enhance TMJ hyperalgesia after inflammation. Cx43 is the most abundant of several connexins expressed in the TG (30). Previous studies have reported that Cx43 expression in the TG was elevated at 8–10 days after trigeminal nerve injury (58, 59), at 3 days after tooth pulp inflammation (31) and 1 day after TMJ inflammation (32) in male rats. Garrett and Durham (30) reported increases in Cx26, Cx36, and Cx40 at 3 days after TMJ inflammation in male rats with no increase in Cx43 in the TG. Interestingly, we also found only marginal increases in Cx43 in the TG of male rats at 4 and 10 days after CFA, while marked increases in Cx43 were seen for OvX and OvXE female groups. This finding underscores the necessity of performing preclinical studies on female as well as male animals. There may be several reasons for the apparent mismatch between the marginal increase in Cx43 expression in the TG after TMJ inflammation and the significant reduction in TMJ-evoked MMemg activity and the reduction in Cx43 protein after siRNA in males. First, we cannot exclude that testosterone offers some level of protection to developing TMJ hyperalgesia after inflammation as has been suggested previously (60–62). Second, TG neurons that drive the TMJ-evoked MMemg activity in males may be more sensitive to increases in Cx43 compared to females and may require only minimal changes to be effective. Third, estrogen reduces the degradation of Cx43 in cardiac tissue (63) and thus, due to its rapid turnover (64), Cx43 protein may remain elevated for longer times in females. The short half-life of Cx43 may also explain the lack of change in mRNA at 3 days after siRNA injection. Fourth,

it is possible that post-translation requirements such as the rate of phosphorylation may be different in males and females (64). Indeed, earlier we reported that estrogen status and inflammation interact through kinase-dependent mechanisms to enhance TMJ hyperalgesia (65).

The present study used a model for TMJ hyperalgesia that addressed several of the features typically seen in TMD patients to conclude that Cx43 plays a critical role in maintaining TMJ homeostasis after low levels of inflammation. Inhibition of Cx43 by siRNA or by acute blockade of Cx43-dependent hemichannel formation by GAP19 caused a significant decrease on TMJ-evoked MMemg, a valid surrogate measure of TMJ hyperalgesia, in both males and females. Lastly, we found similar changes in Cx43 expression in the TG and inhibitoion of response magnitudes to siRNA or GAP19 on TMJ-evoked MMemg in OvX and OvXE females. These results suggest that that estrogen status alone is not a significant determinant of the influence of Cx43 on TMJ hyperalgesia. However, the fact that inhibition of Cx43 function significantly reduced the effects on TMJ hyperalgesia in both males and females suggest that approaches that target Cx43 may be a novel therapeutic approach to manage TMD pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC, University of Minnesota.

AUTHOR CONTRIBUTIONS

FA, MR, and RT performed experiments and collected and analyzed data. MR and DB designed experiments. DB analyzed data and prepared the manuscript. All authors contributed to the article and approved the submitted version.

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Identifying Motor Control Strategies and Their Role in Low Back Pain: A Cross-Disciplinary Approach Bridging Neurosciences With Movement Biomechanics

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Persistent low back pain (LBP) is a major health issue, and its treatment remains challenging due to a lack of pathophysiological understanding. A better understanding of LBP pathophysiology has been recognized as a research priority, however research on contributing mechanisms to LBP is often limited by siloed research within different disciplines. Novel cross-disciplinary approaches are necessary to fill important knowledge gaps in LBP research. This becomes particularly apparent when considering new theories about a potential role of changes in movement behavior (motor control) in the development and persistence of LBP. First evidence points toward the existence of different motor control strategy phenotypes, which are suggested to have pain-provoking effects in some individuals driven by interactions between neuroplastic, psychological and biomechanical factors. Yet, these phenotypes and their role in LBP need further validation, which can be systematically tested using an appropriate cross-disciplinary approach. Therefore, we propose a novel approach, connecting methods from neuroscience and biomechanics research including state-of-the-art optical motion capture, musculoskeletal modeling, functional magnetic resonance imaging and assessments of psychological factors. Ultimately, this cross-disciplinary approach might lead to the identification of different motor control strategy phenotypes with the potential to translate into clinical research for better treatment options.

Keywords: low back pain, kinematics, pain-related fear, motor control, functional magnetic resonance imaging

INTRODUCTION

Low back pain (LBP) is one of the most common conditions regarding years living with a disability throughout the world (1). The prevailing form of LBP does not have a clearly identifiable nociceptive source and is termed non-specific LBP (2). While many of these cases resolve within the first year, some still experience pain 1 year after onset, i.e., they develop a recurrent or chronic form, resulting in an enormous individual, economic and societal burden (1, 3). The clinical management

of LBP is often limited to symptomatic interventions addressing the pain and its consequences, whereby effect sizes for these interventions are only low to moderate (2, 4, 5). This spurs a call for re-examining and identifying novel mechanisms associated with the development and persistence of LBP.

So far, research on LBP has identified several pathogenic mechanisms involving biophysical, genetic, social and psychological contributors (6). Research on LBP-related factors has revealed both biological and behavioral changes. On a biological level, LBP has been linked to disc degeneration, inflammation, and atrophy, fat infiltration and fiber type transition of paraspinal muscles (7–9). On a behavioral level, LBP has been shown to be associated with changes in movement, which can be described as changes in motor control (thereby affecting spine posture, stability, and movement) observed at the level of the nervous system [spinal- (10) and supraspinal (11) processes] as well as the musculoskeletal system (biomechanical mechanisms including muscle activity and kinematics) (12). Furthermore, psychological factors constitute important and non-negligible risk factors for the development and persistence of LBP (13).

However, as recently stated, research on these different pathomechanisms of LBP is often limited by significant knowledge gaps arising from siloed research within different research disciplines, highlighting the need for cross-disciplinary approaches that have the potential to identify important interactions between different mechanisms contributing to LBP (14). This becomes particularly evident when considering new theories about the role of subject-specific motor control strategies in LBP (movement behavior phenotypes which can predispose to and result from pain/injury) with potential long term consequences (12, 15, 16). In this context, LBP-associated changes in motor control are suggested to exert polydirectional and pain-provoking effects, involving interactions between neuroplastic, psychological and biomechanical factors that have not yet been systematically validated (15–17). Hence, to study such interactions and their role in the development and persistence of LBP, an appropriate cross-disciplinary approach that incorporates methods from neuroscience and movement biomechanics research is required.

Therefore, after a summary of the relevant literature, we present a novel cross-disciplinary approach combining neuroscientific and movement biomechanics research methods with the aim of identifying different motor control strategy phenotypes and their role in LBP as well as their underlying supraspinal, psychological, and biomechanical features. Ultimately, this approach might help to fill important knowledge gaps in LBP research with the potential to translate into clinical research for better treatment options.

BIOMECHANICAL MECHANISMS

Numerous studies have investigated biomechanical alterations in LBP, mainly by observing spine/trunk kinematics and muscle activity during functional activities as well as during steadily held postures with and without experimentally induced perturbations. Investigations of functional activities in LBP patients compared to healthy controls indicate trends toward a reduced lordotic posture and range of motion (RoM) in the lumbar spine during activities such as standing, walking, running, chair rising or picking up an object (18–20). In terms of muscle activity, studies show less clear trends, but instead a large variety of muscle activity patterns, ranging from higher lumbar extensor muscle activity to no differences or even lower activity in LBP patients compared to healthy controls (21). Studies combining kinematic and electromyographic experiments with musculoskeletal modeling report higher lumbar spine loading in LBP patients, which can be mainly explained by postural adaptations and increased trunk muscle activity (22, 23). Postural control studies with LBP patients revealed a delay in trunk muscle activity onset in response to both predictable and unpredictable perturbations (24, 25). These findings indicate that LBP patients experience a variety of motor control impairments, likely due to interaction deficiencies between sensory and motor systems that are responsible for goal-oriented spine posture, stability and movement (26, 27). Due to the large inter-individual variation, especially in terms of muscle activity patterns, van Dieën et al. (12) suggested that this might reflect the existence of multiple motor control strategies along a spectrum between two distinct phenotypes, resulting from adaptations in motor control to LBP and interference of LBP with motor control. Although not systematically tested yet, the "tight control" phenotype is suggested to involve increased trunk muscle excitability to provide tight control over trunk movements at the cost of higher tissue loading, whereas the "loose control" phenotype is characterized by a reduced excitability of trunk muscles to avoid high tissue loading at the cost of loose control over movement (12). Both motor control phenotypes might also be associated with supraspinal adaptions (e.g., cortical reorganization) (16), due to e.g., less dynamic motor behavior and impaired sensory feedback.

SUPRASPINAL PROCESSES

More than 20 years ago and using magnetencephalography, researchers detected a shifted sensory representation of tactile input from the back in chronic LBP patients in the primary somatosensory cortex (28). Moreover, changes of paraspinal muscle representations in the primary motor cortex have been observed in chronic LBP patients, i.e., the motor cortex representations of the longissimus and deep multifidus muscles showed increased overlap compared to healthy controls, suggesting less fine-grained ("smudging") cortical representations of paraspinal muscles (29). Such changes in the cortical organization of paraspinal muscles have also been shown to be associated with delayed activation of the transversus abdominis during rapid arm movements in patients with recurrent LBP, indicating a relationship between brain changes and motor control in LBP (11). However, it is still unclear whether the observed cortical sensorimotor changes in chronic LBP represent an epiphenomenon, simply triggered by altered sensory input [in particular from muscle spindles, the main

transmitters of proprioceptive information (30)] and altered motor output, or if they are causally involved in the occurrence of recurrent and chronic LBP. The primary somatosensory cortex is well-known for encoding sensory aspects of pain (31) and recent research indicates that this region is hyperactive in chronic pain conditions, potentially driven by long-lasting disinhibition as shown in animal models of chronic pain and in humans (32, 33) Hence, the alterations in the primary somatosensory cortex in chronic LBP patients could be causally related to the experience of persistent LBP. Alternatively, the observed cortical sensorimotor changes might indirectly provoke persistent LBP by a reduced ability to (top-down) control paraspinal muscles. This might limit trunk movement variability and therefore spinal load distribution with unfavorable biomechanical and pro-nociceptive consequences such as increased loading on spinal tissues (12, 15). Indeed, current evidence suggests an association between brain changes and altered motor control in chronic LBP (34), which should be further explored to disentangle potential clinically relevant interactions between brain mechanisms and dysfunctional motor control strategies in LBP. Yet, while extensive knowledge exits about the cortical representation of various body parts and their potential reorganization based on environmental changes [e.g., the somatotopic representation of the hand and digits (35) and their cortical arrangement based on everyday hand use (36)], very little is known about a potential cortical topographic organization of sensory afferents from the back (e.g., along the thoracolumbar axis). In 2018, intra-cortical stimulation of the primary somatosensory cortex revealed the sensory representations of the thorax and abdomen (37) but still, the cortical representation of the back along the thoracolumbar axis, and in particular of proprioceptive afferents, is unclear. With regards to this, reorganization of proprioceptive input from paraspinal muscles is likely to be more important pathophysiologically for the chronification of LBP [compared to tactile input (38)], but the cortical somatotopy of proprioceptive input from the back has not yet been studied. Detailed cortical maps of paraspinal afferent input might therefore be of major importance to further explore potential relationships between brain changes and unfavorable motor control strategies (e.g., tight control strategy) in LBP.

PSYCHOLOGICAL FACTORS

Pain-related fear and associated avoidance behavior as well as depression and anxiety have received extraordinary attention in the last two decades because they were empirically identified as important psychological factors in the development and persistence of LBP (3, 8, 39, 40). According to the Fear Avoidance model (41), misinterpretations of pain as a sign of harm in combination with negative affectivity and pain catastrophizing can lead to pain-related fear and avoidance behavior which might further aggravate pain, disability and depression (8). Indeed, positive relationships between pain-related fear, LBP intensity and disability have been found in systematic reviews and meta-analyses (39, 42), and fear avoidance beliefs have been shown to be associated with poor treatment outcome in patients suffering from LBP within a time period of <6 months (43). However, the predictive value of pain-related fear regarding the development of LBP is limited (39) and psychological factors in general (when considered in isolation) explain only a small proportion in outcomes such as pain intensity (44, 45). Yet, recent research has shown an association between pain-related fear and dysfunctional motor behavior in LBP patients and healthy individuals (46–48), indicating significant interactions between psychological factors and motor control (psychomotor interactions), which can promote potential clinically relevant consequences such as limited motor variability, increased paraspinal muscle co-contraction and loading on spinal tissues (15). Research on the role of pain-related fear in LBP should therefore systematically involve measures of motor control (such as spinal movement biomechanics) to identify potential pain-provoking interactions. With regards to this, a recently published meta-analysis including 52 studies found that higher levels of pain-related fear, catastrophizing and depression were significantly associated with reduced amplitudes of spinal movement and larger muscle activity, independently from pain intensity (49). Due to rather small effect sizes, however, it was concluded that more experimental studies with more specific and individualized measures of psychological factors, pain intensity, and spinal motor behavior are needed to better understand the underlying psychomotor interactions and to inform current treatment strategies.

BUILDING BRIDGES: A CROSS-DISCIPLINARY APPROACH

To investigate potential interactions between psychological factors, biomechanical mechanisms and supraspinal processes in LBP (**Figure 1**), we propose a cross-disciplinary approach, aiming at bridging between the "silos" neurosciences and movement biomechanics. The methodological basis comprises the assessment of psychological factors through questionnaires, biomechanical assessments of movement during functional activities based on high-resolution optical motion capturing and musculoskeletal modeling as well as the establishment of cortical topographic maps of paraspinal afferent input using functional magnetic resonance imaging (fMRI).

Questionnaires

To assess pain-related fear, self-reports are an adequate direct measure of subjective feelings of fear that are easily accessible for clinicians and researchers (50). The most common self-reporting tools for assessing pain-related fear are questionnaires based on psychological constructs such as fear of movement/(re)injury [Tampa Scale for Kinesiophobia, TSK (51)], perceived harmfulness of daily activities [Photograph Series of Daily Activities, PHODA (52)] or fear avoidance beliefs [Fear Avoidance Beliefs, FABQ (53)]. However, it must be noted that even though recent neuroscientific and biomechanical evidence supports the diversity of pain-related fear constructs (46, 48, 54), it is still unclear how specific the different questionnaires are in assessing the various psychological constructs (55). Combining

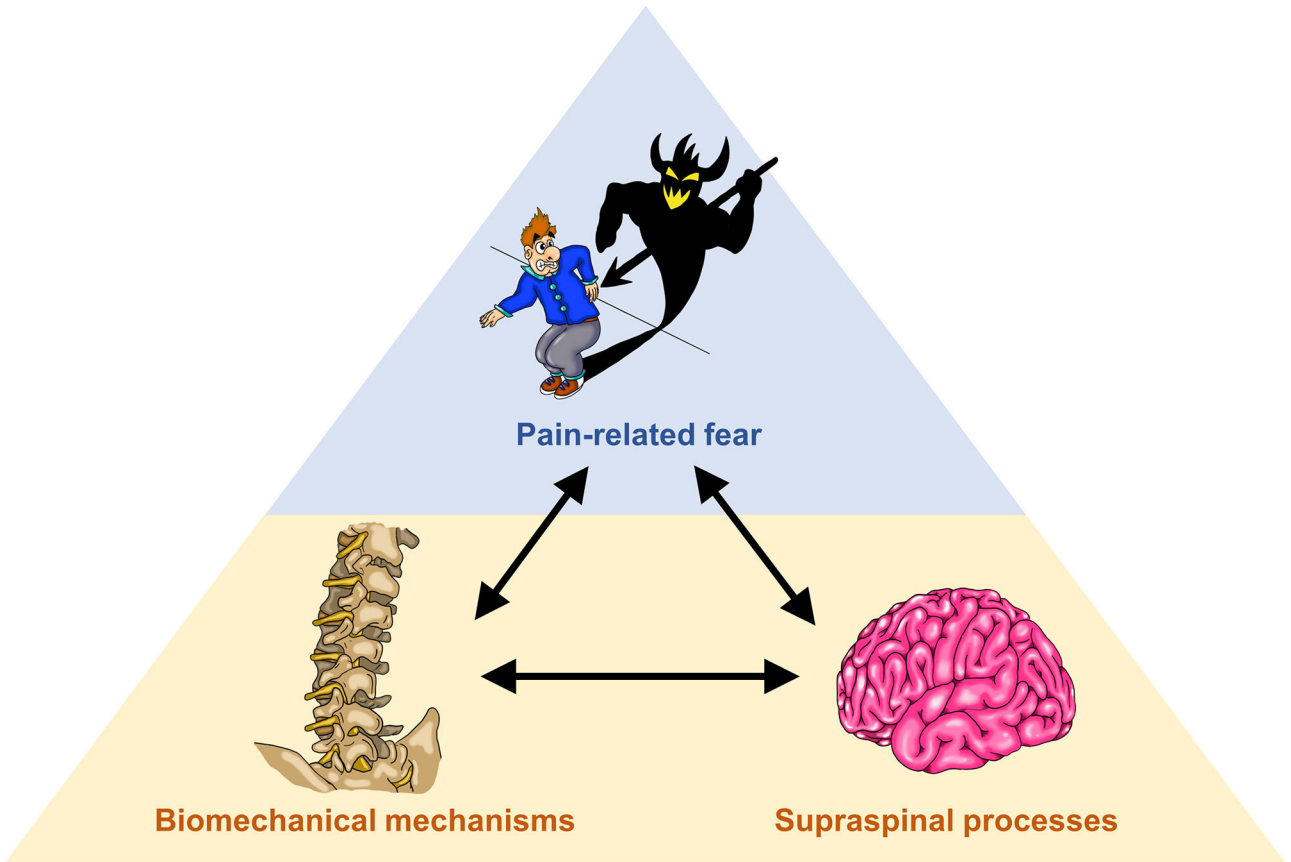

FIGURE 1 | Illustration of interactions between pain-related fear (upper blue shaded area), biomechanical mechanisms and supraspinal processes (motor control; lower orange shaded area).

these questionnaires with biomechanical and neuroscientific measures might lead to a better understanding of the underlying psychological constructs. In addition, to reveal potential commonalities or differences between pain-related fear and general anxiety, the State-Trait Anxiety Inventory questionnaire (STAI) will be used to assess the participants' current level of anxiety (S-Anxiety) as well as aspects of "anxiety proneness" in general (T-Anxiety) (56, 57). To assess depressive symptoms, the Patient Health Questionnaire (PHQ-9) will be used (58).

Assessing the Biomechanics of Spinal Movement

The functional biomechanics of the spine are investigated using a comprehensive non-invasive experimental and computational approach, which combines state-of-the-art optical motion capture with advanced musculoskeletal modeling. Motion data are collected in a motion analysis laboratory, where participants are equipped with 58 retro-reflective skin markers according to a previously developed configuration (59) (**Figure 2**) and asked to perform various activities of daily living. These include walking and running on a level ground, climbing up and down a 5-step staircase, standing up from and sitting down on a chair, lifting up and putting down a 5 kg-box as well as performing vertical jump maneuvers. A 27-camera Vicon motion capture system and several force plates are used to record three-dimensional marker trajectories and ground reaction forces (GRFs), respectively (**Figure 2**). The suitability of this method for quantifying spinal motion during functional activities, which was previously used to investigate three-dimensional spinal kinematics in healthy populations as well as various patient populations including non-specific chronic LBP (20) was supported by comprehensive investigations of validity as well as within- and between-session reliability (60, 61).

For estimating intersegmental kinematics and spinal loading, we developed male and female musculoskeletal full-body models with a highly detailed spine (**Figure 2**) using the OpenSim modeling environment (62). To account for individual subject characteristics, the models are adjusted for each participant by considering segmental lengths and masses as well as sagittal plane spinal shape derived from the skin markers. Simulations are driven by the marker trajectories and GRFs collected in the motion analysis laboratory. Initial predictions of spinal loading in healthy pain-free individuals showed high consistency with reported *in vivo* measurements (62), supporting the suitability of this approach for accurately investigating LBP-related biomechanical adaptations in large patient populations.

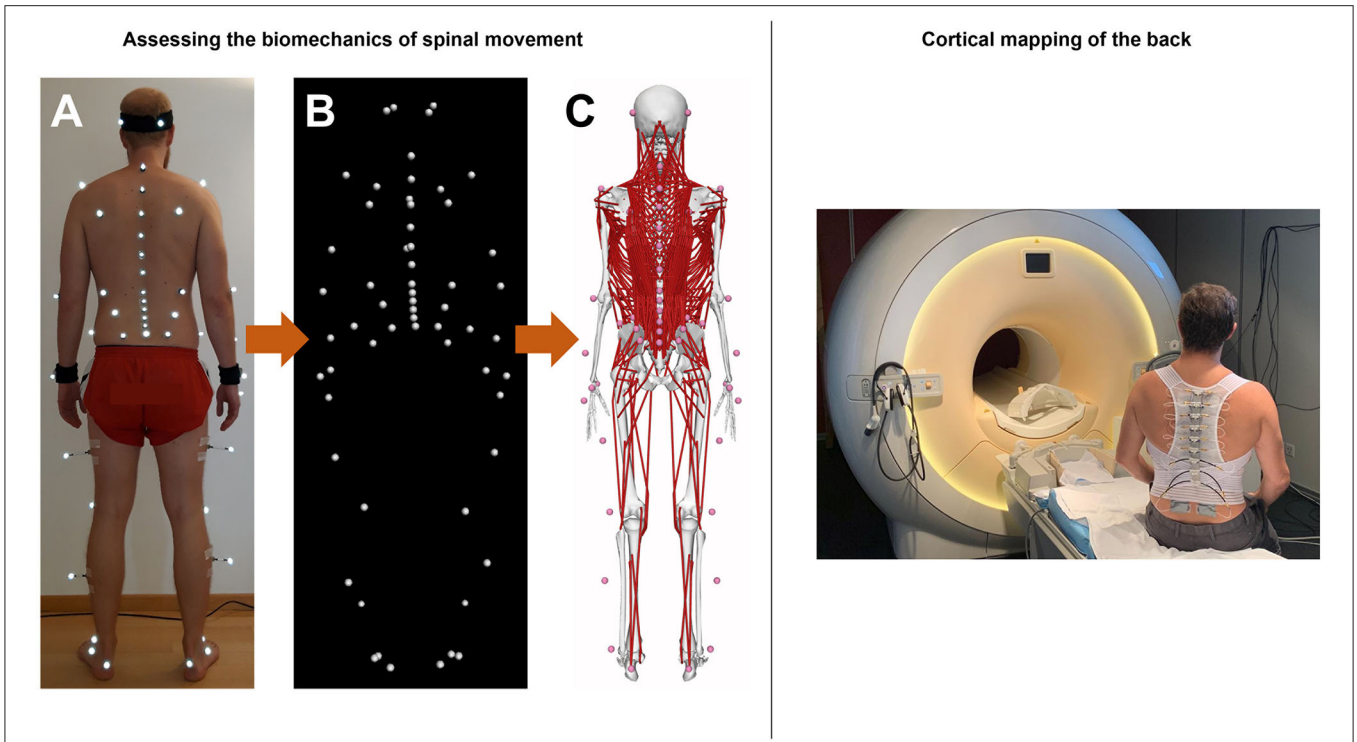

FIGURE 2 | Left: Experimental and computational approach for quantifying movement biomechanics during functional activities. **(A)** Application of retro-reflective skin markers in a full-body configuration. **(B)** Capturing marker trajectories using infrared camera-based motion capture system. **(C)** Motion data-driven musculoskeletal full-body model with a detailed thoracolumbar spine. Right: Illustration of a subject wearing pneuVID elements in a MR environment. PneuVID can apply computer-controlled vibrotactile stimuli between 10 and 150 Hz (and amplitudes 0.5–1mm) to a customizable stimulation area between 1 and 4 cm^2 of each vibration unit. The vibration device controller (not shown) allows bilateral or unilateral vibrotactile stimulations of different body parts, including paraspinal tissue, in various stimulation settings.

To account for LBP-related changes in muscle activity, we are planning to include electromyographic (EMG) measurements of the main trunk stabilizers and to use this information as additional input for our models. This will further increase prediction accuracy, especially when participants present activity patterns such as increased antagonistic muscle coactivation, which was shown to have direct implications on spinal loading (22, 23).

Cortical Mapping of the Back

Non-invasive human brain imaging techniques such fMRI with its high spatial resolution provide suitable tools for the investigation of the cortical representation of different body parts (63). We developed a novel MR-compatible vibration device (pneumatic spinal vibration device, pneuVID, **Figure 2**), which can apply computer-controlled vibrotactile stimuli between 10 and 150 Hz to different thoracolumbar segmental levels. This is the first apparatus specifically designed for paraspinal muscle vibration on different segmental levels in an MR environment. The pneuVID has been successfully tested for MR compatibility and permits MR measurements in supine position to allow better and more comfortable subject positioning (using special pillows for the back to embed the vibration units) and head fixation.

Using the pneuVID in combination with high spatial resolution fMRI (3 or 7 Tesla), detailed cortical maps of paraspinal afferent input can be explored using different vibration frequencies: Applying vibratory stimulation at frequencies between 60 and 80 Hz and amplitudes of 0.5–1 mm on paraspinal muscles has been shown to be a potent stimulus for muscle spindle activation (and therefore proprioceptive signaling) (26). In contrast, stimulus frequencies around 20 Hz will primarily activate receptors in superficial skin layers (e.g., Meissner's corpuscles) (64). Thus, by using randomized fMRI stimulation protocols including different vibration frequencies at various thoracolumbar segmental levels, the current approach has the potential to identify and differentiate cortical proprioceptive somatotopic maps from tactile somatotopic maps of the back and compare them between healthy controls and LBP patients of different symptom durations. It must be noted, however, that it is currently unclear which trunk muscle spindles are affected in their activation profiles by pneuVID stimulation. We assume that mainly superficial muscles along the thoracolumbar axis (i.e., longissimus and spinalis muscles) are targeted. Nonetheless, since the stimulation sites are also located over the rotatores and multifidi muscles, these structures, which are important in providing proprioceptive information [with the rotatores breves

muscles having the highest density of muscle spindles of the lumbar and thoracic muscles (65)] might also be affected.

FILLING THE GAPS

Using the methodologies spanning different research disciplines as described above, the current approach has the potential to address important questions in LBP research:

(1) Do loose/tight motor control strategy phenotypes indeed exist and/or do other motor control strategies exist? Biomechanical assessments of dynamic movement tasks, involving subject-specific spine kinematics, segmental loadings and paraspinal muscle forces during daily activities (lifting, walking running etc.), will be performed to investigate potential relationships with LBP duration, disability, and psychological factors. Relevant features will be extracted for subsequent data analysis (e.g., unsupervised cluster analysis) with the goal of classifying different motor control strategy phenotypes that are possibly associated with different LBP symptom durations (acute, subacute, and chronic stages).

(2) Can a topographic cortical organization of thoracolumbar sensory input be identified? How does this cortical organization relate to the identified motor control strategy phenotypes in LBP? For example, it is plausible that degraded paraspinal proprioceptive feedback (e.g., provoked by a tight control strategy) is causally linked to LBP-provoking alterations in motor control *via* neuroplastic cortical changes (e.g., "smudging" of cortical maps of paraspinal afferent input) (16). For the first time, we therefore aim to test whether cortical maps of thoracolumbar afferent input demonstrate a relationship with spinal movement patterns, LBP duration and psychological factors. Novel insights into these relationships would pave the way for future investigations of causal interactions between cortical changes and motor control strategies using longitudinal study designs.

As recently stated, a better understanding of musculoskeletal pain depends on reconnecting the brain with the rest of the body (14). Our approach including investigations of potential interactions between supraspinal processes and biomechanical mechanisms contributes to this reconnection and could facilitate a transfer of the knowledge generated within the past 20 years of research on motor control related neuroplasticity into clinical practice.

CLINICAL IMPACT

Provided that the suggested motor control strategy phenotypes can be reliably identified using the approach described in this article, the knowledge generated might lead to important implications for clinical research and interventions. For example, it has been proposed that a persistent "tight control strategy" may be specifically targeted by reducing muscle excitability and co-contraction while increasing movement variability in motor control exercise (12). With regards to this, our approach might provide promising behavior- and neuroimaging-based outcomes to test the potential therapeutic effect of individualized motor control exercises and how they compare to other treatment approaches.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SS and MM wrote the first draft of the manuscript. CB and PS critically revised the manuscript and contributed additional text parts. All the authors approved the version to be published.

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BRIEF RESEARCH REPORT
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Prevalence of Musculoskeletal Pain and Its Relation With Weight of Backpacks in School-Going Children in Eastern India

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Background: Recently, heavy school backpacks have become a significant concern among parents and health professionals, as well as the media, but evidence for the same is limited in the Indian context.

Aim: To find the prevalence of musculoskeletal pain among school-going children and its relationship with backpack weight.

Design: Cross-sectional study.

Method: This study was carried out among school-going children from grade 6 to 10 with age of 10 to 16 years from an urban and rural location. Schools were selected randomly from all enlisted schools in the district of Khurdha, Odisha state of India. A structured questionnaire was administered to assess symptoms of musculoskeletal pain. Anthropometric measurements along with backpack weight were taken.

Statistical Analysis: Chi-square test was performed for categorical variables and Student's *t*-test for continuous variables. Multivariate regression analysis was performed to identify factors with maximum effect on musculoskeletal pain.

Results: The prevalence of musculoskeletal pain was 18.8% in the preceding year. Backpacks weights were higher among children of urban schools as compared with rural areas. Children from urban schools were more likely to have pain than those from rural schools (OR 1.88, 95% CI 1.41–2.49). Those children with a backpack weight more than 10% of body weight had almost twice the risk of musculoskeletal pain compared to backpack weight less than 10% (OR 1.91, 95% CI 1.4–2.6) in univariate analysis where as no significant association was found on multivariate analysis.

Conclusion: The prevalence of musculoskeletal pain was high in school-going children. In children, carrying higher backpack weight, and a higher percentage of the backpack to bodyweight had a significant association with musculoskeletal pain. Gender, height, body mass index, and backpack weight to body weight > 10% had no association with musculoskeletal pain.

Keywords: backpack, back pain, musculoskeletal pain, bag weight, school bag

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INTRODUCTION

The age of school-going children ranges between 6–16 years, and these children go through various physical and psychosocial developmental stages and phases of accelerated growth of skeletal and soft tissue, especially during puberty. Any repetitive stress in this age group may have far-reaching consequences into adulthood. Recently, heavy school backpacks have become a great concern among parents, health professionals, and the media (1, 2). Some studies suggest that with the use of heavy school backpacks, there is an increase in the incidence of low back pain, abnormal posture, and other musculoskeletal problems (3–8). Children with Backpack weight > 10% of their body weight has higher prevalence musculoskeletal pain than those with backpack weight < 10% (9). On the contrary, some other studies suggest that although there is a high prevalence of back pain among children, it has no association with the weight of backpacks carried and may be due to other factors than mechanical ones (10–13).

Many countries, such as India, have tried to ascertain a specific percentage of body weight to restrict the load carried by children in their school backpacks (14, 15). The European countries have backpack limits of 10% of body weight, while American occupation therapy recommends a limit of 15% body weight, but these recommendations are also highly diverse and there are no set international guidelines on this subject. Some studies have also claimed that there is a gender variation and that the body mass index (BMI) plays an essential role in the causation of low back pain among school-going children, although there is not enough concrete evidence to justify this (5, 6). In India, the Ministry of Human Resource Development has recommended a bag weight of up to 5 kg for children in grade 10, 4.5 kg for grades 8 and 9, and 4 kg for grades 6 and 7. Previous studies have reported having a higher backpack for private schools as compared to public schools. The impact of the backpack on musculoskeletal pain (MSP) with respect to public and private schools has not been well-established though. Thus, with such varied literature, it is perplexing to healthcare professionals and others alike if there is a need for putting limitations of weights that children can carry in their backpacks and what amendments should be included in the limitations that should be put depending on the demographic profile of these children. There is limited literature on the burden of MSP and its associated factors in India, specifically in rural areas from the eastern part of India. This study aimed to determine the prevalence of musculoskeletal pain in school-going children and its relation to backpack weight.

METHODS

A cross sectional study on school-going children from urban and rural locations was conducted. Participating schools were selected randomly from the enlisted schools in the district of Khurdha, Odisha state of India. Every school within the Bhubaneswar Municipal Corporation limits was classified as urban and the others as rural. A number was assigned to all schools, and selection was done randomly. After ethical clearance by the institute ethics committee and permission from the school authorities and parents was obtained, students from the 6 to 10 grade willing to participate in the study were included. Children were excluded if they had any of the following: (i) any evidence of congenital or inherited musculoskeletal disorders (ii) using other forms of school bags other than backpacks like a bag with wheels etc. After explaining the details of the study, a structured questionnaire was administered, and anthropometric measurements were taken. The questionnaire was explained to the students, and their understanding was checked by asking them their understanding of each question. Investigators helped the students by simplifying the questions and clarifying doubts that arose.

Sample Size

A previous Indian study found the prevalence of low back pain in the pediatric population to be 53.9% (12). Keeping this in mind, we calculated the sample size by the formula $Z^2 * p * (1 - p)/I^2$; where Z is the 95% confidence interval, p is the prevalence taken (53.9%), and I is the relative error (taken as 10% in this study) that is 5.39. So, the sample size calculated was 329. Because of the cluster sampling technique, a design factor of 4 was multiplied to reach the final sample size of 1,316.

Data Collection

Demographic details, such as name, date of birth (DOB), gender, and grade and section were collected. Anthropometric measurements including height, weight of child, and weight of child with backpack and other accessories were obtained. A digital electronic scale with an accuracy of 0.01 kg calibrated over a range of known weights, and a stadiometer accurate to 0.1 cm were used for anthropometric measurements. The weight of each student was measured first without schoolbag, then after, carrying his schoolbag, which also included a tiffin box and water bottle inside it, to obtain the total weight. The difference of the two weights was recorded as the schoolbag weight, and then, the schoolbag weight percentage compared with the body weight was calculated. Similarly, height was determined using the stadiometer with students standing straight on it without shoes and looking at Frankfurt plane with heel, buttock, shoulder and occiput touching the stadiometer. BMI was calculated using formula weight (kg)/(height in mt)^2.

To assess for musculoskeletal symptoms, the Modified Nordic Musculoskeletal Disorders Questionnaire was used (16). This validated questionnaire has a clear image showing different body areas labeled for easy understanding especially for children.

Data were analyzed using the STATA software, Chi-square test for categorical variables, and student's *t*-test for continuous variables. Multivariate regression analysis was performed to identify factors having a maximum effect on musculoskeletal pain. A *p*-value of <0.05 was considered significant.

RESULT

We collected data of 1,329 children across four different (two urban and two rural) categories of schools from 10 to16 years and between grades 6 and 10. Out of these 1,329 children, 685

TABLE 1 | Demographic details of school children.

Demographic details	Urban (mean ± SD)	Rural (mean ± SD)	*P*-value
Number of children	685	644	
Age	14.37 ± 1.16	13.17 ± 1.34	
Sex			
Boy	353	310	
Girls	332	334	
Type of school			
Public	348 (26%)	332 (25%)	
Private	337 (25%)	312 (24%)	
Weight (kg)	52.5 ± 13.6	44.8 ± 11.7	<0.05
Height (mt)	1.57 ± 0.09	1.52 ± 0.09	
BMI (kg/m^2)	21.01 ± 4.43	19.26 ± 4.90	
Weight of backpack (kg)	4.62 ± 1.73	3.49 ± 1.26	<0.05
Percent backpack to body wt (%)	7.55 ±3.06	8.27 ±2.73	<0.05

TABLE 2 | Association of backpack weight among urban vs. rural, public vs. private schools and various grades.

Characteristics	Weight of backpack (kg) (mean ± SD)	*P*-value
Residence		
Urban	4.62 ± 1.74	<0.05
Rural	3.5 ± 1.26	
Type of school		
Public	3.42 ± 1.33	<0.05
Private	4.76 ± 1.63	
Grade		
6th	4.08 ± 1.309	
7th	4.43 ± 1.64	
8th	4.05 ± 1.76	0.012
9th	4.09 ± 1.47	
10th	3.86 ± 1.77	

TABLE 3 | Association of backpack weight among public vs. private schools across grades.

School grades	Weight of backpacks (kg) Public school (Mean ± SD)	Weight of backpacks(kg) Private school (Mean ± SD)	*P*-value
6th	3.91 ± 1.38	4.13 ± 1.29	0.40
7th	3.72 ± 1.41	5.05 ± 1.56	<0.05
8th	3.26 ± 1.42	5.06 ± 1.63	<0.05
9th	3.56 ± 1.09	5.06 ± 1.63	<0.05
10th	3.23 ± 1.36	5.16 ± 1.89	<0.05

children belong to an urban area, whereas 644 children were from a rural area. We collected data from both public (680 children) and private (649 children) schools. Thus, a total of four categories of children were accounted for—urban public, urban private, rural public, and rural private schools. The number of children belonging to urban private, urban public, rural private, and public school are mentioned in above (**Table 1**).

The mean age of urban school children was 14.4 years whereas that of rural school was 13.4 years. The mean weight of children of urban schools was 52.5 kg (±13.6) kg, and that of rural schools was 44.8 kg (±11.7).

We measured the backpack weight of both urban and rural children and looked for the association of backpack weight with musculoskeletal manifestation in the form of pain in shoulder joint and elbow joint, neck and back. The mean weight of backpacks of the children in the urban schools was 4.62 (±1.74) kg, whereas that in the rural school was 3.5 (±1.26) kg (**Table 2**). The mean backpack weight difference among children from private (4.76 ± 1.63 kg) and public (3.42 ± 1.33 kg) schools was statistically significant. When backpack weight was calculated in terms of percentage of body weight, the urban school children (7.55 % ± 3.06) had a lower percentage as compared with the rural school children (8.27% ± 2.73) (**Table 1**). This probably was secondary to higher body weight in urban school children as compared with rural schools. When compared among various grades in private and public schools, the difference was significant except for the 6 grade (**Table 3**).

The frequency of pain in different groups of children in urban and rural areas is given in **Table 4**. There were 250 (18.8%) children who experienced any degree of pain the preceding year. The major sites of pain reported in this study were the shoulder joints (39.2%), knee (19.6%), back (18%), and neck (10.4%). Children from urban and private schools had a higher prevalence of pain (**Table 4**); 175 out of 1,056 children (16.6%) with backpack weight < 10% of body weight has musculoskeletal pain whereas 75 out of 273 children (27.5%) with backpack weight more than 10% of bodyweight has musculoskeletal pain. Mean backpack weight, percentage of backpack weight to body weight, backpack weight more than recommended weight, and backpack weight more than 10% of body weight are significantly associated with musculoskeletal pain in univariate analysis (**Table 4**).

A multivariate regression analysis was performed to assess the relationship between back pain and various independent variables such as gender, school type (private vs. public school), backpack weight, percentage backpack, obesity, and back pack weight more than recommended weight. In logistic regression, a significant association was found between the weight of backpack and musculoskeletal pain in children. Gender had no association with pain. Children having backpack weight higher than that recommended (as per HRD ministry), or with backpack weight more than the recommended weight or higher percentage of backpack to body weight did not have any association with pain in multivariate analysis (**Table 4**).

DISCUSSION

There is a growing concern among parents regarding the increase in school bag weight in children of school-going age. There is very

TABLE 4 | Risk factors associated with musculoskeletal pain.

Parameter	Musculoskeletal pain—present *N* (%)	Musculoskeletal pain—absent *N* (%)	Odds ratio (OR) 95% CI	Adjusted odds ratio 95% CI	*P*-value
Resident					
Urban	160 (64%)	525 (48.7)	1.88	0.93	<0.001
Rural	90 (36%)	554 (51.3)	(1.41-2.49)	(0.42-2.03)	
School					
Public	117 (46.8%)	563(52)	0.81	0.82	0.12
Private	133 (53.2%)	516 (48)	(0.61-1.06)	(0.58-1.05)	
School type					
Urban public	64 (25.6%)	284 (26.3)	N/A	N/A	N/A
Urban private	96 (38.4%)	241 (22.3)			
Rural public	53 (21.2%)	279 (25.9)			
Rural private	37 (14.8%)	275 (25.5)			
Sex					
Girls	136 (54.4%)	530 (51)	1.24		0.13
Boys	114 (45.6%)	549 (49)	(0.94-1.63)		
School grade					
6th	13 (5.20%)	118 (10.9)	N/A	N/A	N/A
7th	28 (11.20%)	142 (13.1)			
8th	45 (18.00%)	287 (26.6)			
9th	117 (46.80%)	306 (28.4)			
10th	47 (18.80%)	226 (21.0)			
Obese vs. Non-obese					
Non-obese	195 (78)	874 (81)	0.83	1.00	0.28
Obese	55 (22)	205 (19)	(0.59-1.16)	(0.67-1.50)	
Recommended wt for grades (HRD Ministry)					
Below	124 (49.6)	728 (67.5)	0.47	0.78	<0.001
Above	126 (50.4)	351 (32.5)	(0.36-0.63)	(0.61-1.55)	
Backpack % to body weight					
<10%	175 (30)	881 (18)	1.91	0.95	<0.001
>10%	75 (70)	198 (82)	(1.4-2.6)	(0.62-1.45)	
BMI					
Overall	20.53 ± 4.44	20.07 ± 4.8	N/A	N/A	0.16
Urban	21.59 ± 4.32	20.83 ± 4.4			0.05
Rural	18.85 ± 4.04	19.35 ± 5.02			0.21
Mean weight of backpack (kg)	4.74 ± 1.83	3.92 ± 1.58	N/A	N/A	<0.05
Mean backpack % to body weight	8.67 ± 3.23	7.72 ± 2.8	N/A	N/A	<0.05

Risk factor associated with pain after univariate (OR) and multivariate analysis (Adjusted OR). OR, Odds ratio; CI, Confidence interval; N/A, Not applicable.

limited evidence on excessive backpack weight causing health issues in children. There are few studies published that have addressed this growing concern. It has been found in studies that backpack weight significantly contributes to musculoskeletal pain in the form of back pain, shoulder pain, and neck pain. The data from India are further limited. Although the Ministry of Human Resources and Development (HRD) has recent recommendations for backpack weight for various grades, its effect on the musculoskeletal system is not well-studied.

In this study, we found the prevalence of musculoskeletal pain among school children to be 18.8%, whereas the prevalence was reported to be 35.4% in a study from Uganda (3) while that was documented to be 40% in school children in a meta-analysis done by Calvo-Munoz et al. (17). The backpack weight is significantly high in children from private schools and among urban schools. This result is similar to that of a study from Uganda conducted by Mwaka et al. (3). This may be due to students of urban schools carrying more textbooks along with tiffin and lunch box and water bottles as compared with their rural counterparts in public schools. As per this study, shoulder, knee, and neck are the commonly reported sites of pain by children, which is similar to a study published by Parthibane et al. (18). We did not find any significant gender difference in the prevalence of musculoskeletal pain in school children as compared with previous studies where boys were reported to have a lower risk of pain than girls (19, 20). Boys perhaps reported a higher

prevalence of pain in view of greater exposure to sports and intense activities, whereas Akbar et al. had reported a higher prevalence in young females (21). This may be due to a higher perception of pain as reported by Kovacs et al. (22). We did not find BMI to be a risk factor for developing pain in school children, although previous studies have reported an association (7). Children using a heavier backpack were at a higher risk of having musculoskeletal pain. This was unlike that reported previously, where no association was found between backpack weight and pain (23). Similarly, we did not find any association between the percentage of backpack weight to body weight with musculoskeletal pain. Although many professional occupational bodies worldwide, along with the American Academy of Pediatrics, recommend backpack weight not to exceed 11-15% of body weight, we did not get any significant association in this study. Similarly, we also could not find any association between musculoskeletal pain and the maximum recommended backpack weight by the HRD ministry. Cohort studies will be better to investigate such association of backpack weight and musculoskeletal pain.

Limitation

We collected data from the students themselves; parents were not involved in data collection although consent was taken *a priori*. We did not conduct a detailed clinical examination, nor did we take a detailed history of injury, lifestyle, sitting posture, etc. as other factors contributing to the pain.

CONCLUSION

The prevalence of musculoskeletal pain among school children was found to be 18.8%. Children carrying a heavier backpack and with a higher percentage of backpack to body weight had a significant association with MSP. Gender and backpack weight to body weight > 10% had no association with musculoskeletal pain. The current HRD ministry-recommended backpack weight for each grade was noted to have a higher risk of having pain as per our study. Hence, more studies are needed to determine the appropriate maximum backpack weight for school children of different grades.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute Ethics Committee, AIIMS Bhubaneswar. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SS and SP were involved in data collection and entry, participated in data analysis, and wrote the first draft of the manuscript. JJ conceptualized the study, coordinated the execution of the project, critically reviewed the draft manuscript, and acted as guarantor. RD and AS supervised data collection, interpreted the data, and critically reviewed the draft manuscript. All the authors have approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2021.684133/full#supplementary-material

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HYPOTHESIS AND THEORY
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Impaired Lymphatic Drainage and Interstitial Inflammatory Stasis in Chronic Musculoskeletal and Idiopathic Pain Syndromes: Exploring a Novel Mechanism

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A normal functioning lymphatic pump mechanism and unimpaired venous drainage are required for the body to remove inflammatory mediators from the extracellular compartment. Impaired vascular perfusion and/or lymphatic drainage may result in the accumulation of inflammatory substances in the interstitium, creating continuous nociceptor activation and related pathophysiological states including central sensitization and neuroinflammation. We hypothesize that following trauma and/or immune responses, inflammatory mediators may become entrapped in the recently discovered interstitial, pre-lymphatic pathways and/or initial lymphatic vessels. The ensuing interstitial inflammatory stasis is a pathophysiological state, created by specific pro-inflammatory cytokine secretion including tumor necrosis factor alpha, interleukin 6, and interleukin 1b. These cytokines can disable the local lymphatic pump mechanism, impair vascular perfusion via sympathetic activation and, following transforming growth factor beta 1 expression, may lead to additional stasis through direct fascial compression of pre-lymphatic pathways. These mechanisms, when combined with other known pathophysiological processes, enable us to describe a persistent feed-forward loop capable of creating and maintaining chronic pain syndromes. The potential for concomitant visceral and/or vascular dysfunction, initiated and maintained by the same feed-forward inflammatory mechanism, is also described.

Keywords: interstitial inflammatory stasis, cytokines, lymphatic dysfunction, counterstrain techniques, myofascial pain and dysfunction, idiopathic diseases, fascia

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INTRODUCTION

Chronic pain is the leading cause of disability with up to 49% of the population experiencing pain <3 months duration. The estimated cost of chronic pain and associated opioid use disorder in the USA is currently between $560 and 635 billion annually (1). Chronic pain is positively correlated with age (2) and, given the rapidly aging demographic, the burden of chronic pain will continue to impose significant challenges to our healthcare system.

Myofascial pain syndrome (MPS) is among the most common, yet least understood forms of chronic musculoskeletal pain, and is a frequent cause of primary care physician and pain clinic visitation (1, 2). Few people live without experiencing muscle pain following injury, overuse, strain, or trauma. Although pain associated with MPS frequently resolves in a few weeks, in some cases it can persist long after the inciting event and/or spread to distant, uninjured tissues (3, 4). Although MPS is typically characterized by the expression of pain localized to myofascial tissues, it is also associated with a broad and growing profile of *non-musculoskeletal* symptoms including fatigue, sleep disturbance, and visceral pain syndromes (5). These associations suggest a shared pathophysiology between MPS and several common idiopathic conditions (e.g., visceral pain syndromes). The pathophysiological mechanisms underlying this association, however, are not fully understood and remain largely undescribed.

It is well-established that persistent, peripheral nociceptive sources can initiate, maintain, and perpetuate chronic pain states. This occurs, in part, through central mechanisms including retrograde inflammation produced by dorsal root reflexes (6), and/or areas of secondary hyperalgesia produced by glial cell neuroinflammation (3). However, in idiopathic *peripherally generated* chronic pain, our understanding of the pathological processes that generate and maintain ongoing nociceptive input is limited. Examples include whiplash associated disorders which present with pain, proprioceptive and autonomic-linked symptoms despite a lack of correlative pathological evidence on computer tomography and/or magnetic resonance imaging [for review see (7–10)]. Additionally, existing pain hypotheses are limited in their ability to address many of the pathophysiological findings common to both chronic pain and idiopathic visceral/vascular syndromes. This includes elevated levels of plasma and interstitial pro-inflammatory cytokines in myofascial (11, 12) and visceral pain syndromes (13), and evidence of sympathetic nerve activation (SNA) in MPS (14–16), visceral disease (17, 18), and vascular disorders (19). Microvascular disturbances and impaired lymphatic function have also been identified in both MPS (20) and visceral disease (21), supporting the concept of a shared pathophysiology.

Considering the limitations in current understanding, we hypothesize that elevated pro-inflammatory cytokine levels, through specific pathophysiological mechanisms, adversely impact vascular hemodynamics and lymphatic function in the extracellular compartment. Impaired venous and lymphatic drainage can create a state of *inflammatory interstitial stasis* (IIS), which results in ongoing nociceptive bombardment of the dorsal horn (central sensitization). Recent anatomical discovery and advances in pre-clinical and clinical research, enable us to further elucidate the potential pathophysiological factors involved in this process. This includes contraction of fascial myofibroblasts following local TGF-b1 expression (22) which we hypothesize can cause pre-lymphatic/lymphatic vessel contraction and/or fibrosis. And the effect of specific pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-a), interleukin-6 (IL-6) and interleukin (IL-1b) in cessation of the normal lymphatic pump mechanism (23), the development of chronic pain states (24) and the creation of long-term microvascular disturbance following stimulation of segmentally linked somato/visceral-sympathetic reflexes (23, 24).

These concepts, including others critical to our IIS hypothesis, will be described in the sections to follow and presented schematically in flowchart format. **Figure 1** (flowchart 1) specifically highlights the fascial, sympathetic, and lymphatic pathophysiological mechanisms related to IIS. **Figure 2** is a *comprehensive* flowchart which incorporates the concepts in **Figure 1** and additional, previously documented mechanisms, that may contribute to the development of IIS.

Pro-Inflammatory Mediators and Peripheral Afferent Nociceptors

Tissue injury and/or inflammation leads to the local release of algogenic substances including glutamate, serotonin, bradykinin, adenosine triphosphate, protons (low pH), Substance P, nerve growth factor (NGF), and norepinephrine (NE) all of which are transmitted to the central nervous system by primary afferent nociceptors (nociceptors) [for review see Willard (25)]. Nociceptors have unmyelinated free nerve endings that terminate peripherally in the extracellular matrix (ECM), and respond to both inflammatory and mechanical stimuli (26, 27). Virtually all tissues are innervated by nociceptors including fascia (28), tendons (29), blood vessels (30, 31), nerve sheaths (32), ligaments, menisci, synovium, bone (33), visceral tissues or capsules in the case of solid organs (34), vertebral discz (35) and meninges (36). Primary afferent nociceptors enter the dorsal root segmentally, where they trifurcate forming ascending, descending and segmental level fibers. Thus, these small caliber fibers can influence the segmental level of entry and several segments above and below (37). This anatomical structure enables singular activated nociceptors to have heterosegmental nociceptive and reflexive impact. Research specifically highlights the role of visceral afferents in pain production as their activity is synaptically transmitted deep in the dorsal horn to convergent viscero-somatic neurons, which receive nociceptive input from the skin and deep somatic tissues of the corresponding dermatomes, myotomes and sclerotomes (38). Additionally, injury and/or immune responses will result in the production of pro-inflammatory cytokines from various cells, including endothelial cells, macrophages, dendritic cells, and fibroblasts. These substances lower nociceptor activation thresholds in the periphery (25) and, if persistent, can create structural and/or functional changes in the spinal cord including central sensitization (39). Thus, clinical consideration must be given to viral, infectious, traumatic, post-surgical and/or overuse histories as each can facilitate the cellular release of pro-inflammatory cytokines that result in nociceptor activation.

Neurogenic Inflammation

Persistent nociceptive bombardment of the dorsal horn leads to primary afferent depolarization of convergent somatosensory pathways (40) and dorsal root reflexes which result in neurogenic inflammation or the retrograde release of proinflammatory neuropeptides including substance P and calcitonin gene-related peptide CGRP, into peripheral tissues (6).

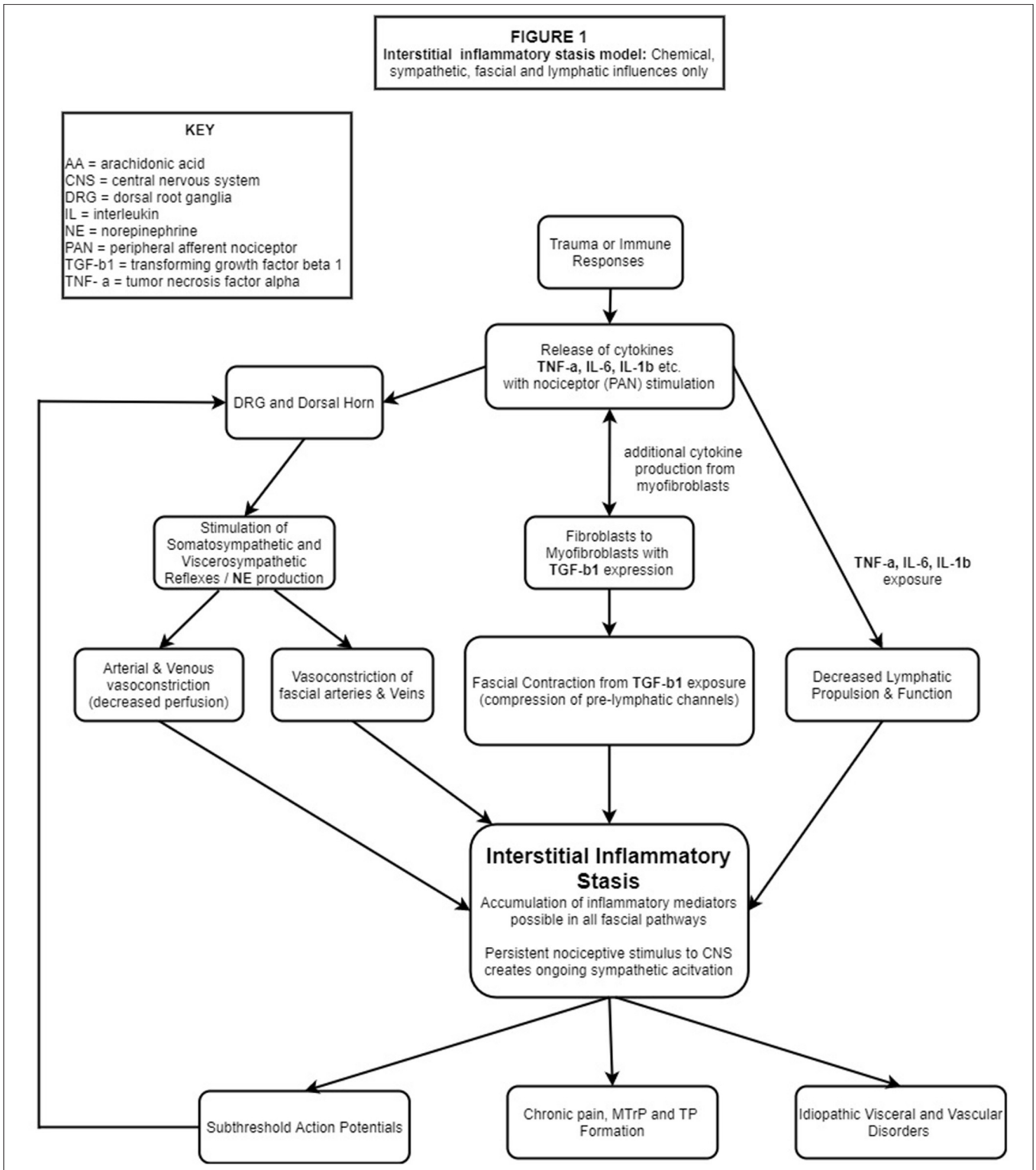

FIGURE 1 | Trauma and/ or immune responses lead to PC production, most notably IL-1β, IL-6 and TNF-α. PANs of multiple tissues embedded in the ECM are stimulated, transporting these substances to the DRG and DH where glial cells are stimulated leading to central and peripheral neuroinflammation/sensitization. Nociceptive bombardment stimulates somato/visceral-sympathetic reflexes causing the release of NE, resulting in peripheral vasoconstriction (including fascial vasculature) while the cytokines IL-1β, IL-6, TNF-α which deactivate the local lymphatic pump mechanism and simultaneously stimulate fibroblasts to differentiate into myofibroblasts. TGF-b1 released by fibroblasts & myofibroblasts, causes contraction of fascial tissues compressing pre-lymphatic pathways. Impaired hemodynamics from vasoconstriction, deactivation of the lymphatic pump mechanism and compression of pre-lymphatic pathways create areas of hypoxia and IIS. Continued PAN stimulation results in a pathophysiological feed-forward loop of lymphatic stasis, nociceptor stimulation and sympathetic activation which manifests in chronic pain, sub-threshold action potentials and idiopathic visceral/vascular dysfunction.

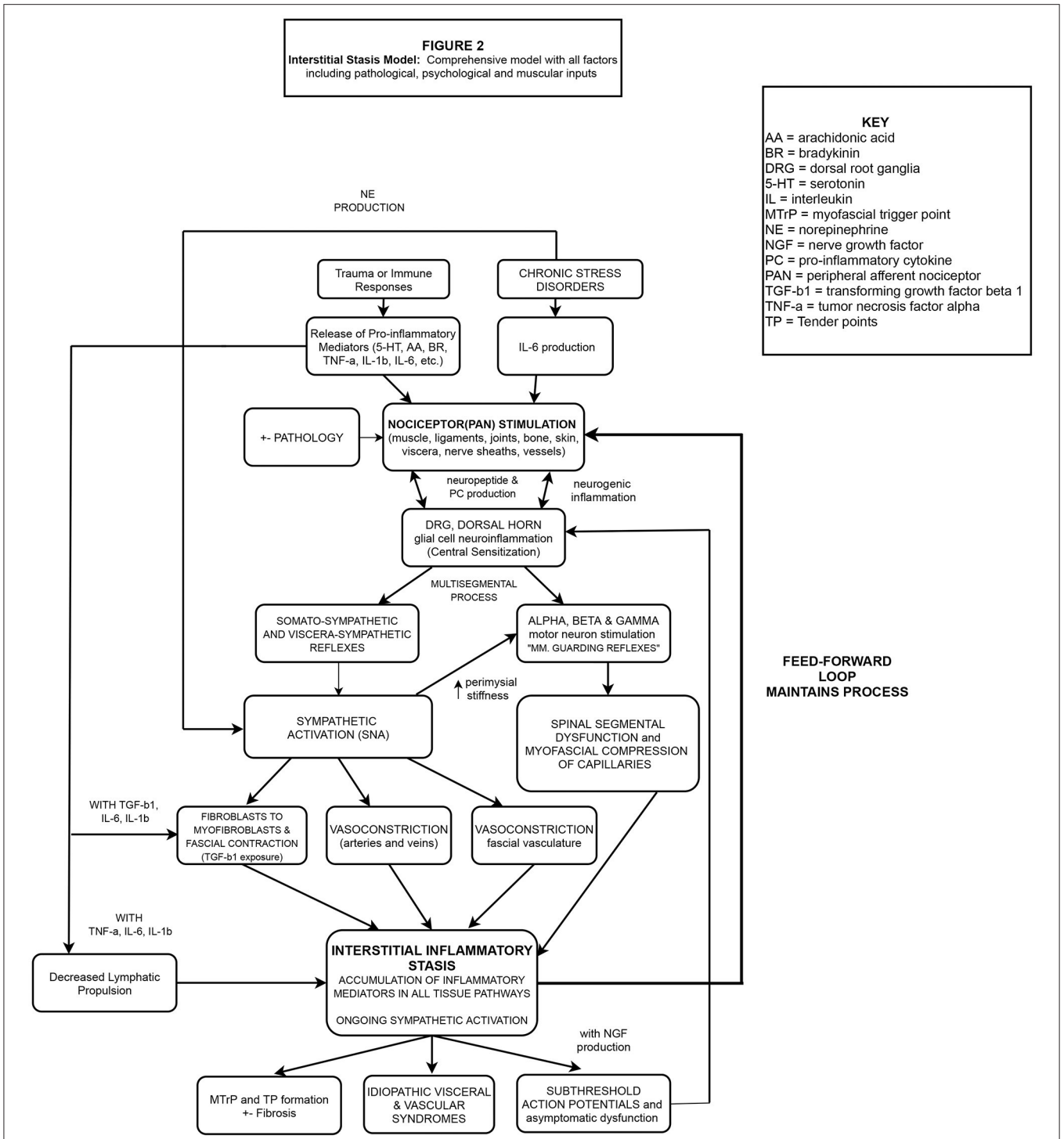

FIGURE 2 | Nociceptive bombardment (various sources) produces PCs including IL-1β, IL-6, TNF-α etc. PANs of multiple tissues embedded in the ECM are stimulated, transporting these substances to the DRG, causing antidromic release of neuropeptides from the DRG into the injured and neurosegmentally linked tissues, exacerbating the response beyond the region of primary hyperalgesia. Glial cells in the DH are stimulated leading to central and peripheral neuroinflammation/sensitization. PAN entry into the DH at multiple levels alters the activity of alpha and gamma motor neurons, creating multi-segmental muscle guarding reflexes, and myofascial compression of pre-lymphatic pathways. Simultaneously, somato/visceral-sympathetic reflexes are stimulated, causing the release

(Continued)

FIGURE 2 | of NE, resulting in peripheral vasoconstriction (including fascial vasculature) while cytokines IL-1β, IL-6, TNF-α deactivate local lymphatic propulsion and stimulate fibroblasts to differentiate into myofibroblasts. TGF-b1 released by fibroblasts & myofibroblasts, creates local fascial contraction, perimysial stiffness (gamma motor activation) and compression of pre-lymphatic pathways. Due to the combined mechanisms, areas of hypoxia and inflammatory stasis develop which continuously stimulate local PANs. A pathophysiological feed-forward loop of lymphatic stasis, nociceptor stimulation and SNA manifests in chronic pain, sub-threshold action potentials and idiopathic visceral/vascular dysfunction.

In support of this concept, IL-1β injections into the dorsal root ganglia (DRG) and dorsal horn are able to induce secondary hyperalgesia, via retrograde inflammation, in the intraperitoneal, intracerebroventricular and intra-plantar tissues of rats (41, 42). This central to peripheral mechanism is a separate phenomenon from spinal glial cell neuroinflammation and expands the inflammatory process into contiguous, non-injured peripheral tissues, creating regions of secondary hyperalgesia (pain experienced outside the original injury site) (3). Glial cell neuroinflammation occurs from nociceptive signals derived from muscle (43), joint (44) and/or visceral (45) tissues and can initiate the transition from acute to chronic pain states following central sensitization (46).

Pro-inflammatory cytokines generated by trauma or immune responses can also be transported *from the periphery*, *via* axonal or non-axonal mechanisms, to the DRG and dorsal horn, facilitating the induction of central sensitization (47), which has important implications to the concept of IIS. Additionally, Xie 2006, demonstrated that, once inflamed, the DRG not only produces pro-inflammatory cytokines but also decreases its production of anti-inflammatory cytokines (48), which further exacerbates the peripheral inflammatory process. Importantly, studies indicate that specific pro-inflammatory cytokines including IL-1β, IL-6, and TNF-α, are particularly associated with glial cell neuroinflammation and chronic pain states (49).

The Role of Muscle Guarding Reflexes in the Pathophysiology of Trigger Points

Muscle guarding reflexes are the body's protective, involuntary motor responses to reduce nociception (50). Stimulation of sensitized dorsal horn nociceptive neurons is known to alter the activity of the alpha motor neuron pool, thus creating one type of the muscle guarding reflex (51, 52). For example, stimulation of the kidney, ureter, or colon in rabbits induces variable, paravertebral muscle responses depending on the organ stimulated (53). Additionally, biochemical stimulation of nociceptors via bradykinin, and serotonin, can activate the gamma motor neuron system (54), which excites segmental stretch reflexes, limits muscle flexibility, and can contribute to formation/perpetuation of myofascial trigger points (MTrPs) (55). MTrPs, the hallmark of MPS, are defined as hyperirritable nodules in a taut band of skeletal muscle. They are the principal cause of musculoskeletal pain and are characterized as either active or latent (56). Active MTrPs are known to produce spontaneous local and or referred pain at rest, whereas latent MTrPs do not. Latent MTrPs are typically considered the "dormant," subthreshold state, of the active MTrP (57). Tender points (TPs) on the other hand, are described as areas of tenderness occurring in muscle, the muscle-tendon junction, bursa, or fat pad, and are typically considered characteristic of fibromyalgia syndrome (58). Although it is to be emphasized that MTrP and TP are separate entities, recent research utilizing intramuscular electromyographic registration of spontaneous electrical activity, has demonstrated that most fibromyalgia TP sites are located *inside* the local and or neurological referred pain patterns of active MTrPs (59) and almost all fibromyalgia TP sites, as specified by the American College of Rheumatology criteria are known MTrP locations (60). This clinically observed overlap in referred pain patterns suggests some degree of shared pathophysiology.

It is important to note that tissue texture abnormalities including MTrPs are not always confined to a single segmental level as activated nociceptors can also expand receptive fields to non-contiguous areas, contributing to the development of MTrPs in distant locations (4). Neurogenic inflammation, muscle guarding reflexes and the potential impact of MTrPs on central sensitization are important concepts in the pathogenesis of MPS according to existing pain hypotheses.

RECENT MYOFASCIAL PAIN HYPOTHESES

The Integrated Hypothesis

Included among the three most prevailing MPS models, is an expansion of Simons' Integrated Hypothesis described by Gerwin et al. in 2004. It proposes that MTrPs are initiated by local acute or chronic myotendinous injuries including unaccustomed eccentric exercise and or sustained work-related strain (61). Sustained muscle contraction, if persistent, theoretically leads to hypoxia "possibly by the development of high pressures within the contracting muscles" which may explain the significant elevation of vasoactive, inflammatory, and algogenic substances demonstrated in active MTrPs (62). Lowered tissue pH, which inhibits the activity of acetylcholinesterase, combined with the release of calcitonin gene-related peptide (causing increased acetylcholine release), would theoretically contribute to the observed increase in motor end plate activity and focal hypertonicity associated with MTrPs (63). This cycle, if combined with "other factors that predispose to focal hypoperfusion" including "sympathetic nervous system involvement" could be self-sustaining, and unless interrupted, could lead to the initiation and perpetuation of active MTrPs. For multiple reasons, including those discussed in the next section, the integrated hypothesis is no longer considered well-supported by the existing literature.

The Neurogenic Hypothesis

Theoretical and clinical limitations related to the integrated hypothesis led to the development of the Neurogenic Hypothesis in 2010 (63). Srbely also published a second paper (62) contrasting the Neurogenic Hypothesis with the Integrated Hypothesis as it relates to MPS. Srbely noted that MTrPs are linked to non-musculoskeletal conditions and exist in the absence of precipitating mechanical injury. This includes urogenital syndromes (64), and a documented case of herpes zoster infection in which the MTrP resolved following antibiotic therapy (65). Additionally, infectious, psychogenic, and endocrine causes have been attributed to MTrP formation (66), which cannot be adequately explained by the persistent release of acetylcholine or increased motor endplate activity that follows mechanical injury. He hypothesized that MTrPs are neurogenic expressions of central sensitization, potentially evoked and maintained by an underlying primary pathology (e.g., osteoarthritis or visceral disease) located within the common neurologic segment of the MTrP. The local, anatomic, and physiologic changes observed at MTrP sites, he argued, are due to neurogenic inflammation, triggered by segmentally linked, central sensitization. Srbely states that these neurogenic and inflammatory mechanisms could also account for some of the biochemical changes documented in MTrPs during interstitial sampling studies, including decreased pH and increased concentrations of SP (67). The autonomic effects related to MTrPs, he postulated, may also be attributed to central sensitization (68). Subsequently, the Neurogenic Hypothesis expanded the potential causes of MPS to include pathological non-muscular tissues (e.g., degenerative joints) and visceral structures owing to the anatomic convergence of sensory pathways in the dorsal horn. The importance of chronic primary pathologies in driving this pathophysiologic process highlights the need to understand the mechanisms and origins of potential nociceptive sources that contribute to the maintenance of an *ongoing state* of central sensitization. Although the Neurogenic Hypothesis integrates well-established physiologic mechanisms (central sensitization and neurogenic inflammation) to characterize the pathophysiology of chronic inflammatory muscle disease, it currently lacks sufficient supporting evidence in human models.

The Neuro-Fasciagenic Model of Somatic Dysfunction

Fascia has also been implicated in the formation and maintenance of chronic pain states. For example, Tozzi, in 2014 published an article describing the structural, functional, and neurological properties of fascia arguing that a purely fascial-based rationale could be developed to explain the palpable features (tissue texture changes, asymmetry, restriction of motion, and tenderness) associated with somatic dysfunction (69). He reviewed the existing literature describing over 50 fascial-based factors including neuromuscular, structural, mechanical, fluid, electromagnetic and hormonal influences that may combine "through various types of interactions" to create somatic dysfunction. This manuscript highlighted the considerable body of research which suggests that fascia may be involved in the development of somatic dysfunction and chronic pain states. However, the *specific mechanism* by which fascia contributes to the creation of an ongoing nociceptive source, was not described by Tozzi.

INTERSTITIAL INFLAMMATORY STASIS HYPOTHESIS

Somatosympathetic Reflexes

For our IIS hypothesis it is important to recognize the role the sympathetic nervous system (SNS) plays in the generation and maintenance of chronic pain states and idiopathic visceral/vascular dysfunction. Stimulation of A delta and C small fiber, unmyelinated nociceptors from virtually all tissue types, reach lamina I and deep into the dorsal horn where they can produce varying degrees of pre and post-ganglionic sympathetic responses termed *somato-sympathetic and/or visceral-sympathetic reflexes* (70). Activation of these reflexes results in the release of NE from postganglionic neurons, which generally elicits peripheral vasoconstriction responses (23), which have been shown to reduce local muscle blood flow (perfusion) by up to 25% (24). These reflexes are the neurological link between peripheral nociceptors and the SNS, and involve segmental, medullary, and/or supramedullary structures (70–72). Experimental evidence supporting SNS involvement in chronic pain states includes reduced muscle perfusion demonstrated in chronic myalgia patients (14), and decreased (improved) spontaneous electromyographic activity recorded from MTrPs, after local injection of a sympathetic antagonist (15). Additionally, the electrical activity in a MTrP locus was shown to increase after emotional stress and was also successfully abolished following local, alpha-adrenergic blockade (16).

It is known that muscle tissue receives both vasoconstrictive and vasodilatory innervation; however, neurogenic vasodilation *has not* been demonstrated in *resting human muscle* tissue [for review see (7, 73)]. Therefore, following nociceptor stimulation and NE exposure (from somato/visceral-sympathetic reflex activity), peripheral vasoconstriction will override the effects of any local vasodilatory neuropeptides (e.g., from neurogenic inflammation). The resultant sympathetic nerve activation (SNA) may lead to disturbances in arterial and venous microcirculation which have been documented in myalgia patients (74, 75) with observed morphological changes including swollen endothelial cells and the local destruction of myofilaments (76). These microcirculatory disturbances have also been identified in MPS patients, within the MTrP locus (77) and are specifically characterized by local muscle hypoxia and reduced washout of inflammatory substances (7). Additional confirmation of vasoconstriction at active MTrP sites was documented utilizing diagnostic ultrasound to analyze the vascular environment surrounding MTrPs. The study concluded that active MTrPs have a constricted vascular bed including an enlarged overall vascular volume indicating venous stasis (20). In this state of impaired venous return, peripheral pro-inflammatory cytokine concentrations can reach the threshold necessary to further

stimulate local, chemosensitive nociceptors, establishing a vicious feed-forward cycle which facilitates MTrP formation via nociceptor/DRG sensitization, and continued activation of somato-sympathetic reflexes. The mechanism described is a reflexive, segmental phenomenon that does not require *supraspinal* sympathetic activation and can act as a primary factor in the development of IIS.

In addition to local SNA from somato/visceral-sympathetic reflexes, psychological considerations are also critical to the development of an accurate and comprehensive pain model (**Figure 2**) as chronic stress may lead to increased peripheral inflammation and NE production. Transient activation of the hypothalamus-pituitary-adrenal axis (HPA) is the body's normal response to an acutely stressful or traumatic event. However, a *chronic* trauma/stress related disorder like post-traumatic stress disorder is associated with dysregulation of the HPA with resultant elevations of plasma NE (78), cerebral spinal fluid IL-6 levels (79), and cerebral spinal fluid substance P levels (80). The additional long term physiological effects of chronic stress include decreased cortisol production with a subsequent elevation of plasma IL-6 levels (81, 82), which may increase the risk of developing cardiovascular disease and or autoimmune disorders (83–85). Elevated levels of IL-6, IL-17A and a dysregulated HPA have also been observed in fibromyalgia patients demonstrating additional overlap between chronic pain states and idiopathic organ/endocrine dysfunction (11, 86). Taken together, these findings highlight the potential contribution of psychological factors in the development of systemic inflammation. In the context of this manuscript, the known correlation between chronic pain and post-traumatic stress disorder may be of significance as elevated levels of IL-6 (inflammation) and NE (vasoconstriction) would create the ideal interstitial environment for the development of IIS.

Sampling Studies of Chronic Myofascial Pain Patients

In direct support of the IIS concept, sampling studies of interstitial fluid have identified catecholamines and various algesic substances in MPS patients. Microdialysis sampling of interstitial fluid in the locus of active MTrPs has demonstrated lower pH levels and elevated levels of inflammatory mediators including bradykinin, substance P, TNF-a, IL-1b, IL-6, interleukin-8, serotonin, and NE when compared to latent MTrPs and/or controls (12). Elevated NE levels are direct evidence of SNA and local vasoconstriction in active MTrPs. With regards to fibromyalgia, a review of 25 selected studies revealed higher serum levels of IL-6 vs. controls (11). Neuropeptides have also been identified in cerebrospinal fluid (CSF) in response to noxious stimuli. For example, elevated levels of substance P were observed within the CSF of fibromyalgia patients (87) at levels up to three-times greater than healthy controls (88). Additionally, fibromyalgia patients have been found to have significantly increased CSF concentrations of NGF (89), interleukin-8 (90) and intrathecal glutamate (91). Sensitization of the dorsal horn due to nociceptor activation following IIS can result in glial cell activation, and subsequent release of pro-inflammatory mediators in the CSF, mediating the transition from acute local pain to chronic widespread pain. For example, the potential contribution of IIS to CSF inflammation specifically offers a pathophysiological rationale for post-traumatic fibromyalgia syndrome (92). We emphasize that the theoretical contribution of IIS to CSF inflammation in fibromyalgia patients does not preclude additional sources of CNS inflammation in fibromyalgia including neuroendocrine contributions. The potential relationship between IIS and elevated levels of NGF in fibromyalgia patients will also be covered in the *subthreshold endplate potential* section to follow.

Newly Identified Interstitial Pre-Lymphatic Pathways

In 2018 (93), researchers utilizing confocal laser endomicroscopy, identified previously undescribed interstitial, *pre-lymphatic* sinuses or pathways in the dermis, vascular adventitia, submucosa of the viscera, bronchi, adipose tissue and in *all* fascial tissues of the musculoskeletal system. These macroscopically visible, fluid-filled spaces were confined by thick, well-organized, collagen bundles and have no previously described anatomical correlate. It was further described as a "compressible and distensible" interstitial space in which interstitial fluid or *pre-lymphatic fluid* accumulates and flows. Interestingly, the pathways were primarily associated with tissues involved in frequent movement such as the musculoskeletal system, lungs, and/or digestive tract. The peristaltic nature of these tissues would ostensibly augment the normal movement of interstitial flow created by the circulatory system. Notably, the authors stated that these pre-lymphatic pathways would have important implications in tissue function and pathology including edema, metastasis, disease, and fibrosis. They cited specific examples of impaired interstitial flow, the pathophysiology of which could be explained by occlusion of these pre-lymphatic channels including, characteristic duct edema present in acute bile duct obstruction, and the enlarged extracellular spaces noted in keloid scarring (94). With regards to our hypothesis, if lymphatic pathways are impeded by scarring or tissue contraction (discussed in sections to follow), areas *inflammatory stasis*, may be created capable of continuously stimulating chemosensitive nociceptors (e.g., visceral, vascular, musculoskeletal), and thus act as an ongoing nociceptive source to the CNS.

The Lymphatic Pump (Intrinsic) Mechanism and Interstitial Inflammatory Stasis

In the lymphatic system, pre-lymphatic channels connect to initial lymphatics vessels which are composed of a thin layer of endothelial cells, although completely lack muscle cells (95). They are physically tethered to the surrounding tissue structure through anchoring filaments (96) thus can be impacted by tensions in the surrounding extracellular compartment. More proximally, lymph fluid empties into collecting lymphatic vessels which contain smooth muscle cells and contain unidirectional valves to prevent retrograde flow. The primary mechanism of lymphatic propulsion is provided by *lymphangions*

which are the specialized, contractile segments of lymphatic collecting vessels. Therefore, lymphatic fluid is independently and actively driven by rhythmic, phasic, heart-like contractions of successive lymphangions (defined as the muscle segment between successive valves) eventually emptying into the venous circulation (97). Lymphatic vessels are critically modulated by fluid pressure and inflammatory mediators. As such, lymphatic vessels act to resolve the *inflammatory process* by increasing lymphangion contractile frequency in response to inflammation (98). This lymphatic, homeostatic, clearing mechanism, has been demonstrated in response to multiple inflammatory mediators including substance P, CGRP, neuropeptide Y, vasoactive intestinal polypeptide, prostaglandins, IL-1b and TNF-a (99–101).

Despite the action of this intrinsic anti-inflammatory mechanism, pathophysiological disruption of normal lymphatic propulsion is known to occur, leading to excess inflammation in the extracellular compartment. For example, lymphatic dysfunction has been identified in human patients suffering from inflammatory bowel disease (e.g., Crohn's and ulcerative colitis) as evidenced by lymphatic vessel obstruction, dilation, and submucosal edema (21). Notably, surgical resection of diseased areas, returns the morphological appearance of lymphatic vessels to normal supporting the concept of a *functional* lymphatic disturbance (102). Recent research has shed light on this phenomenon as it is now known that specific cytokines, namely IL-1b, IL-6 and TNF-a, can actually *disable* the normal lymphatic pump mechanism during acute inflammatory events, creating a "dramatic, rapid reduction in lymphatic propulsive flow and frequency (22)." This may occur to prevent the spread of infectious and/or inflammatory agents beyond the region needed for a localized immune response; however, it results in lymphatic stasis. The importance of this research to our IIS hypothesis will become apparent in the following sections as these specific cytokines, trapped in the interstitium, may facilitate the transition from acute to chronic pain by long-term impairment of the local lymphatic pump mechanism.

Fascial Contractility

The Foundation of Osteopathic Research and Clinical Endorsement or FORCE group has recently written several articles intended to develop a modern definition of fascia. "The fascia is any tissue that contains features capable of responding to mechanical stimuli. The fascial continuum is the result of the evolution of the perfect synergy among different tissues, liquids, and solids, capable of supporting, dividing, penetrating, feeding, and connecting all the districts of the body: epidermis, dermis, fat, blood, lymph, blood and lymphatic vessels, the tissue covering the nervous filaments (endoneurium, perineurium, epineurium), voluntary striated muscle fibers and the tissue covering and permeating it (epimysium, perimysium, endomysium), ligaments, tendons, aponeurosis, cartilage, bones, meninges, involuntary striated musculature and involuntary smooth muscle (all viscera derived from the mesoderm)" (103). Fascia is composed of cells including macrophages and mast cells (104); however, its foundational cell is the fibroblast which is the principal cell responsible for production of the ECM. As cited previously, virtually all fascial tissues (viscera, ligaments, nerves, disc tissue etc.) contain unmyelinated nociceptors, and thus have the potential to become primary nociceptive sources. Cytokines, including the IL-6, TGF-β1, and IL-1β have a significant impact on fibroblasts, stimulating them to differentiate into *myofibroblasts*, a contractile form expressing α-smooth muscle actin. These contractile cells are associated with pathological conditions including palmar fibromatosis and hypertrophic scarring (105). Importantly, myofibroblasts have also been identified in normal, *non-pathological* tissues including the fascia cruris (106), ligaments (107), tendons (108), bronchial connective tissues (109), organ capsules (110), and several other collagenous connective tissues (111). Following inflammatory exposure, myofibroblasts are known to secrete additional cytokines including TGF-β1, IL-1β, etc. which may increase the rate of ECM synthesis, creating fibrosis (112). The production of the cytokine TGF-β1 by fibroblasts takes on additional clinical significance as *normal* (non-pathological) fascia samples were recently demonstrated to contract following TGF-β1 exposure. Both rat and human samples of the lumbar fascia, plantar fascia, and sections of the fascia lata were analyzed and found to contain significant numbers of myofibroblasts. Following application of TGF-β1 to the lumbar fascia, tissue contractions were measured and calculated to be at an estimated force of 2.63N (113). The potential clinical impact of this contraction is below the threshold for mechanical spinal stability; however, is above the threshold for mechanosensory stimulation impacting gamma motor neuron activity and therefore musculoskeletal function (114). Adding additional support to this concept, Schleip found a strong positive correlation between myofibroblast cell density and contractile response, with a generalized increase in myofibroblast density in perimysial tissues (where most spindle capsules are embedded) (113). This corresponds with previous research demonstrating perimysial changes in myofascial pathologies (115) and supports the hypothesis of Stecco et. al. that MPS could be influenced by abnormal perimysial fascial stiffness (116). Important to our hypothesis, the fascia-myofibroblast contractile responses measured following TGF-b1 expression may be capable of partially or fully occluding pre-lymphatic flow through initial lymphatic vessels and/or "compressible" interstitial pathways as described previously by Benias et al. (93). This process may act as an independent, purely *fascial-based mechanism*, capable of disrupting interstitial lymphatic drainage, creating localized regions of IIS.

Inflammatory Stasis and Fibrosis

Fibrosis or scar tissue formation is defined as thickening of the ECM that is preceded by inflammation or physical tissue injury. Since the same pro-inflammatory cytokines (IL-6, TGF-β1 etc.) involved in our proposed interstitial stasis model are also the exact cytokines described in the process of excessive, non-physiological scar tissue formation (fibrosis), it is plausible there is a shared pathophysiology. Increased ECM synthesis by fibroblasts in response to inflammation is known to cause fibrosis but may also cause the formation of fibrotic clusters called *fibrotic foci* (117) which are associated with idiopathic

lung fibrosis. The overproduction of collagen I by myofibroblasts, severely impairs regional tissue architecture and is considered the key component in all types of organ fibrosis (118). In fact, a similar mechanism (to our described hypothesis) has been previously observed in the scarring process citing excessive neuroinflammatory stimuli, prolonged production of growth factor TGF-b1 and overproduction of the ECM (119–121). Since chronic inflammation is the driving force behind myofibroblast proliferation, interruption of the inflammatory process can resolve the fibrotic process (118). For example, viral clearance by interferon, prevents associated liver fibrosis in viral hepatitis patients. Unfortunately, many forms of fibrosis following injury and/or infection are idiopathic (e.g., idiopathic pulmonary fibrosis and/or kidney fibrosis) and intractable as the source of chronic inflammation is unknown. Recognition and resolution of the feed forward, multi-tissue, hypothetical IIS mechanism we describe, by manipulative or pharmacological interventions, may help resolve the symptoms associated with non-physiological scarring and potentially interrupt the process of idiopathic organ fibrosis.

The Role of Inflammatory Interstitial Stasis in the Generation of Subthreshold Potentials

Subthreshold action potentials (SAPs) generated from areas of IIS may also play a significant role in the process of central sensitization, chronic pain, and the development of *latent* MTrPs. Pro-inflammatory cytokines (including IL-1b and TNF-a), released in response to tissue stressors and/or immune responses, strongly induce nerve growth factor (NGF) synthesis (122). NGF receptor activation and signaling alters nociception via direct nociceptor sensitization at the site of injury and can change gene expression in the DRG, which collectively increases nociceptive signaling from the periphery to the CNS (123). Considering that NGF production is related to peripheral cytokine exposure, NGF production would logically be more likely to occur in zones of IIS. Elevated pro-inflammatory cytokines concentrations would induce NFG production in dorsal horn glial cells, creating SAPs (124). This may have clinical significance as elevated levels of NGF have been identified in the CSF of fibromyalgia patients (89). Therefore, latent MTrPs may be clinical manifestations of *sub threshold* pro-inflammatory cytokine concentrations in areas of IIS. Although latent MTrPs are not associated with spontaneous pain, they can cause local and even referred pain upon deep palpation. Mense hypothesized that latent MTrPs send nociceptive, subthreshold signals toward the dorsal horn of the spinal cord (125), which would effectively cause central sensitization *without* the perception of pain. He emphasized that latent MTrPs may be of particular importance in chronic myalgia as pathological changes in muscle tissue are typically associated with subthreshold input and low frequency activation of nociceptors (125).

To summarize, latent MTrPs may be related to SAPs generated in response to low level pro-inflammatory cytokine exposure in lesser areas of IIS. These nociceptive signals could both initiate and/or maintain central sensitization. The involved tissues and neuromeric fields would logically be prone to injury and or may become symptomatic (suprathreshold) following any additional trauma and/or inflammatory insult.

Hypothesis Summary (Interstitial Inflammatory Stasis)

Based on the research presented, a novel lymphatic and fascial-based hypothetical mechanism can be described, having major implications in chronic pain states and idiopathic organ syndromes. Tissue injury and/or inflammation from immune responses causes the release of cytokines into neighboring tissues. This inflammatory reaction triggers fibroblasts to release additional cytokines including IL-1b, IL-6 and TGF-β1 thereby exacerbating the local nociceptive and inflammatory processes. These specific cytokines simultaneously disable the local, lymphatic pump mechanism. If interstitial concentrations of IL-1b, IL-6 and or TNF-a reach the threshold necessary to cause significant, local expression of TGFb-1, lymphatic propulsion may become impaired due to fascial (myofibroblast) contraction and or vessel fibrosis. These specific algogenic cytokines, now trapped locally in the interstitium, may continuously stimulate chemosensitive nociceptors and facilitate the transition from acute to chronic pain by long-term impairment of the *local lymphatic pump mechanism.* This is despite eventual recovery of the *systemic lymphatic pump*. The resultant cytokine exposure will also activate local somato/visceral-sympathetic reflexes, impairing regional vascular perfusion. Therefore, this hypothetical, pathophysiological hemodynamic process is due to a combination of impaired vascular perfusion and long-term disruption of the *local lymphatic pump* mechanism. The areas of interstitial stasis generated may exist in any one of the newly identified musculoskeletal, visceral, adventitial and/or dermal interstitial pathways. The resultant stasis and elevated interstitial cytokine concentrations may create a feed-forward nociceptive loop, which results in continuous stimulation of musculoskeletal and or non-musculoskeletal nociceptors, maintaining the process. The hypothesis is not selectively dependent on pathology and or any specific source of inflammation, as multiple tissue nociceptors are capable of initiating and maintaining IIS.

Applying the IIS hypothesis to musculoskeletal pain research, the cytokines (IL-1b, IL-6, TNF-a) shown to disable the lymphatic pump mechanism are the *exact cytokines* found to be primarily involved in the perception of pain (24), fascial contraction following TGF-b1 production (22, 105), and were also among those elevated in active MTrP sampling studies (11, 12). Additional experimental support for the IIS hypothesis is the fact that long-term, impaired lymphatic drainage was recently identified in the pathogenesis of lymphedema. Histological examination of lymphatic vessels in 29 secondary lymphedema patients demonstrated "contracted-type" and "sclerotic-type" collecting vessels in areas of lymphatic stasis. These vessels were found to have occluded lumens causing impairment of the normal lymphatic-pump mechanism. Most significantly, many of the contractile cells responsible for impairing lymphatic drainage were identified as *myofibroblasts,* not vascular smooth muscle cells, and were characterized by increased ECM synthesis (126).

Also in support of the IIS hypothesis is a finding of Asano et al. who demonstrated increased levels of inflammatory cytokines, namely TNF-a and IL-1b, in the walls of dysfunctional lymphatic collecting vessels (127). This lead Carthy to suggest that the *transformation into myofibroblasts* may have been triggered by local inflammation (128). Considered collectively, these recent research findings from the field of lymphedema, lend support to the hypothesis that ongoing impairment of the normal lymphatic pump mechanism (IIS) being involved in the generation and maintenance of chronic pain states.

Using irritable bowel syndrome as a *non-musculoskeletal* example, compelling evidence exists that increased inflammation in the enteric mucosa or neural plexuses may initiate the development of IBS-like symptoms (129). In a recent study of acute gastroenteritis infection patients, 23% were found to develop IBS-like symptoms within 3 months after infection. Altered gut physiology including evidence of chronic inflammation was still present at 3 months in both the symptomatic and asymptomatic groups, implicating post-infectious *peripheral inflammation* as a contributing factor. When the symptomatic and asymptomatic patients were compared based on psychosocial factors, elevated stress profiles (potentially, HPA dysregulation) were strongly associated with those who would eventually develop IBS-like symptoms (13). In this example, elevated sympathetic drive from stress would feed into the peripheral stasis mechanism we describe. As the associated elevation in NE and IL-6 levels (related to chronic stress) may induce vasoconstriction, reduce lymphatic propulsion, increase fibroblast to myofibroblast differentiation and create nociception. This emphasizes the fact that both peripheral and central factors must be considered in idiopathic pain states.

Based on the findings presented, we propose the following feed-forward pathophysiological hypothesis for MPS, and certain idiopathic visceral/vascular conditions. **Figure 1** is a simplified view of our IIS hypothesis including only biochemical, sympathetic, fascial, and lymphatic influences. **Figure 2** is a *comprehensive pain model* detailing multiple factors (including those described in **Figure 1**) related to the development of IIS and the subsequent pathophysiological outcomes.

DISCUSSION

Our proposed hypothesis expands existing pain models by highlighting the mechanisms by which IIS may be initiated and act as an ongoing peripheral nociceptive source. Via this mechanism, fascial, visceral, vascular and or neural pre-lymphatic pathways may entrap inflammatory mediators, which would continually stimulate local nociceptors, contributing to central sensitization, chronic pain, and sympathetic activation. Importantly, algesic substances trapped in the interstitium (not blood stream), have the potential to create a state of recalcitrant, *non-healing* pain, that may be resistant to pharmacological intervention.

As **Figure 2** demonstrates, the precipitating factors and pathophysiological mechanisms behind each patient's symptoms are unique and may often occur in combination within the same neuromeric field. Therefore, the neurological concepts of temporal and spatial summation would have important implications in the proposed model as pain can be initiated by a single repeated stimulus over time (temporal summation) or by multiple different pain generating mechanisms *converging* onto the dorsal horn (spatial summation). Even in cases of known pathology, patients may be asymptomatic (e.g., the single nociceptive source fails to override the inhibitory pain system) or may *become* asymptomatic following successful treatment of convergent, non-pathological, nociceptive sources. Therefore, assessment and treatment of all potential pain-producing tissues and mechanisms, as suggested by the proposed model, improves the likelihood of a patient reaching the goal of pain free function. Additionally, an argument could also be made for the treatment of *latent* MTrPs which would reduce central sensitization related to SAPs, helping to maintain pain-free function.

As stated previously, the proposed model is not exclusive to peripherally generated chronic pain as IIS may also offer a physiological rationale for idiopathic visceral and vascular dysfunction. Neurovascular bundles from all spinal segments also innervate vertebral and spinal cord vessels, making them capable of inducing *spinal* vasospasm by activating SNA (130, 131). Vasoconstriction of spinal arteries and veins may contribute to the pathophysiology of common disorders including radiculopathies, myopathies, idiopathic neuropathies, degenerative disc disease and/or degenerative joint disease. Theoretically, SAPs (produced by NGF) may also induce segmentally linked vasoconstriction following sympathetic activation which could serve as a possible explanation for the high incidence of spinal degenerative changes in *asymptomatic* individuals (over 73% of subjects tested) (132, 133). Specifically, subthreshold nociceptive signals generated by IIS could create vasoconstriction of segmental arteries that supply the vertebrae, leading to asymptomatic or *silent* spinal degeneration over time.

Persistent nociceptive input from IIS may also directly impact cranial tissues innervated by the spinal trigeminal nucleus which receives afferent input from the upper 3 cervical segments. In support of this concept, nociceptive inputs into the spinal trigeminal nucleus, including those produced by MTrPs, have been implicated in tension-type headache (19) and may logically, via SNA, contribute to other idiopathic cranial disorders including post-concussion syndrome. Additionally, second order nociceptive neurons projecting to higher centers through the dorsal column, can activate the "brain-gut axis" which links the autonomic nervous system to the neuroendocrine, immune, and enteric nervous systems (134). This could interfere with the normal efferent innervation of the viscera causing abnormal hormonal secretion and/or disruption of gastrointestinal motility (17). Collectively, these findings suggest that alleviating ongoing nociceptive sources related to interstitial stasis may be able to resolve the underlying pathophysiological mechanism responsible for idiopathic spinal, digestive, endocrine and cranial disorders.

Potential Non-pharmacological Interventions (Related to the Proposed Model)

The need for effective non-pharmacological interventions for pain is increasing with efforts to reduce opioid addiction. One promising intervention purported to deactivate nociceptors and alleviate tissue inflammation is *Counterstrain* (previously called Strain and Counterstrain) (135). Counterstrain utilizes cutaneous TPs/MTrPs to diagnose and treat MPS and idiopathic conditions. Once a TP is located, the body is gently placed into specific positions of ease that have been clinically identified to alleviate TP tension and tenderness. Tissue decompression (through positioning or local tissue manipulation) is believed to silence activated nociceptors, reducing the afferent barrage to the dorsal horn. Reduced nociception, deactivates segmental muscle guarding reflexes, reducing myofascial tension and capillary pressure. The treatment position is then maintained for up to 90 s to allow regional inflammation (interstitial pro-inflammatory cytokines) to gradually dissipate. Based on our hypothetical model, the associated reduction in interstitial NE concentrations during the release would also deactivate somato/visceral-sympathetic reflexes, helping to restore arterial and venous perfusion. Simultaneous reductions in IL-1b, IL-6 and TGF-b1 concentrations would normalize lymphatic propulsion and reduce myofibroblast (facial) contraction blocking pre-lymphatic pathways.

The impact of Counterstrain on inflammation has been investigated at the cellular level, demonstrating improvements in tissue morphology. Researchers repetitively strained human fibroblasts for 8 h in a two-dimensional tissue matrix while measuring the effects on fibroblasts, including cytokine production. A 60-second Counterstrain (or indirect osteopathic manipulative treatment) was then applied which produced beneficial effects on fibroblast morphology, reversing the inflammatory effects (46% reduction in fibroblast IL-6 production after 24 h) when compared to control (136). Recently Counterstrain has been renamed *Fascial Counterstrain* and expanded to include over 800 anatomically named structures, treatments, and diagnostic TPs. This pain-free, non-invasive treatment warrants further investigation as it may have the capacity to alleviate microvascular stasis in all tissues, breaking the feed-forward cycle that creates myofascial pain and potentially idiopathic visceral/vascular syndromes.

Acupuncture, unlike Counterstrain, does not directly target peripheral inflammation (IIS) but is purported to work by dampening nociceptive input to the dorsal horn. Melzack and Wall's gate theory (137) proposes that the superficial dorsal horn of the spinal cord can be excited or *opened* by nociceptors and *closed* by stimulation of large A-beta nerve fibers. Since electroacupuncture is known to stimulate A-beta fibers (138) it is presumed that acupuncture works by activating this pain-gating mechanism. Alternatively, manual acupuncture is known to stimulate A-delta fibers (139) that synapse directly with inhibitory interneurons within the dorsal horn and can inhibit central pain transmission through enkephalin-dependent mechanisms (140). Recent studies of a similar intervention, termed dry needling, have demonstrated antinociceptive effects when treatments were targeted segmentally to discrete MTrP locations as compared to non-MTrP sites (141, 142). Dry needling may also be effective in reducing nociception generated by IIS.

Although the underlying mechanisms driving these interventions remain unclear, it is likely that local and segmentally targeted therapies will be of value in the treatment of chronic pain states generated peripherally by IIS.

Experimental Validation of the Proposed Model

A central tenet to this hypothesis is the development of a functional disturbance in the lymphatic pump mechanism. The current gold standard for quantifying lymphatic flow includes lymphangiography and lymphoscintigraphy, which have been previously employed to investigate disturbances in the lymphatic pump mechanism including blockage of lymphatic flow (143). More recent technologies, including near-infrared fluorescent optical imaging and/or transit-time ultrasound technique, provide *real-time* quantitative measures of lymphatic flow which could also be employed to identify functional lymphatic disturbances in somatic and/or visceral tissues.

Initial cross-sectional studies comparing clinical cohorts to healthy controls may also be useful in highlighting differences in lymphatic propulsion in support of our hypothesized reduction in lymphatic flow in chronic MPS. We would expect to observe decreased lymphatic flow localized within the region of hyperirritable MTrPs, in contrast to normal tissue. The role of fascial contractures in this mechanism may be further studied by examining for evidence of fibroblast activation biopsied from muscle tissue of fibromyalgia patients (specifically in tissues found to have lowered pain-pressure thresholds). This includes excess TGF-β1 expression, elevated levels of inflammatory mediators, increased myofibroblast concentrations and/or evidence of excess ECM secretion.

These human studies could be followed by controlled animal injury studies to investigate the causal relationships between cytokine accumulation and altered lymphatic flow. Previous animal models have been developed to assess the effect of lipopolysaccharide (LPS) induced production of TNF-a, IL-6 and IL-b (144). These could be used to assess lymphatic stasis utilizing near-infrared fluorescent optical imaging. Immunohistochemistry can be employed to detect TGF-b1 expression (145), which would introduce the potential for fascial contraction and/or fibrosis related to the production of the specific cytokines theoretically associated with IIS. Histological analyses of the fascial tissues could be performed to confirm the presence of fibrotic changes and fascial contractions.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

BT was responsible for conceptualization, design, writing, and editing of manuscript. JS and MV was responsible for design, writing, and editing. GR was responsible for design and writing. JS was responsible for design, writing, and editing of manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mediators of Neuropathic Pain; Focus on Spinal Microglia, CSF-1, BDNF, CCL21, TNF-α, Wnt Ligands, and Interleukin 1β

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Intractable neuropathic pain is a frequent consequence of nerve injury or disease. When peripheral nerves are injured, damaged axons undergo Wallerian degeneration. Schwann cells, mast cells, fibroblasts, keratinocytes and epithelial cells are activated leading to the generation of an "inflammatory soup" containing cytokines, chemokines and growth factors. These primary mediators sensitize sensory nerve endings, attract macrophages, neutrophils and lymphocytes, alter gene expression, promote post-translational modification of proteins, and alter ion channel function in primary afferent neurons. This leads to increased excitability and spontaneous activity and the generation of secondary mediators including colony stimulating factor 1 (CSF-1), chemokine C-C motif ligand 21 (CCL-21), Wnt3a, and Wnt5a. Release of these mediators from primary afferent neurons alters the properties of spinal microglial cells causing them to release tertiary mediators, in many situations *via* ATP-dependent mechanisms. Tertiary mediators such as BDNF, tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and other Wnt ligands facilitate the generation and transmission of nociceptive information by increasing excitatory glutamatergic transmission and attenuating inhibitory GABA and glycinergic transmission in the spinal dorsal horn. This review focusses on activation of microglia by secondary mediators, release of tertiary mediators from microglia and a description of their actions in the spinal dorsal horn. Attention is drawn to the substantial differences in the precise roles of various mediators in males compared to females. At least 25 different mediators have been identified but the similarity of their actions at sensory nerve endings, in the dorsal root ganglia and in the spinal cord means there is considerable redundancy in the available mechanisms. Despite this, behavioral studies show that interruption of the actions of any single mediator can relieve signs of pain in experimental animals. We draw attention this paradox. It is difficult to explain how inactivation of one mediator can relieve pain when so many parallel pathways are available.

Keywords: central sensitization, dorsal horn, nerve injury, neuropathy, cytokine, chemokine, growth factor, synaptic transmission

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INTRODUCTION

This review outlines aspects of the etiology of neuropathic pain at both the spinal and peripheral level. A variety of chemical mediators effect communication between the various cell types involved in the generation of pathological pain. We focus on mediators that affect spinal microglia, mediators released from microglia and their actions on their target cell types.

Peripheral nerve trauma, post herpetic neuralgia, spinal cord injury, traumatic brain injury, stroke and neuropathies associated with chemotherapy, diabetes or HIV infection can give rise to intractable neuropathic pain (1–13). Neuropathic components also contribute to pain associated with COVID-19, multiple sclerosis, fibromyalgia, migraine, osteoarthritis, rheumatoid arthritis, autoimmune disease, and complex regional pain syndromes (14–23). Although the signs and symptoms of neuropathic pain are similar in males and females, it is now well-established that the underlying cellular mechanisms are very different (24–33). Unlike nociceptive pain, which signals and protects an individual from tissue injury, neuropathic pain persists long after tissue healing and recovery has taken place (2). It is therefore maladaptive and serves no obvious biological purpose (5, 34, 35).

Many of the investigations into the etiology of neuropathic pain involve controlled, traumatic perturbations leading to defined and reproducible injuries to the spinal cord or peripheral nerves. Surgical, chemical or genetically-induced lesions to rodent peripheral neurons are followed by *in vivo* or *ex vivo* investigations of the properties of primary afferent, spinal or supra-spinal neurons. These are correlated with behavioral studies that seek to assess pain intensity by indices such as thermal or mechanical allodynia and hyperalgesia (36–40). Improvements in behavioral approaches within the last 15 years have focused on assessing pain in terms of its accepted definition as "An unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage," (41). Thus, contemporary operant models seek to provide quantification of pain *per se* as opposed to nociception (39). For example, rodents may be required to make a conscious choice between being in a pain-inducing environment and an otherwise undesirable environment such as a brightly illuminated space (4, 42–44). The time spent in the undesirable environment gives an index of the pain the animal is experiencing. A complementary approach to pain quantification involves assessment of behaviors such as social interaction, nest-building, ultrasonic vocalization, burrowing behavior and "facial grimace score" (45–47).

Regardless of the methodology used to assess the behavioral consequences of peripheral nerve injury, it is generally accepted that;

1. Peripheral nerve injury promotes Wallerian degeneration of severed axons, macrophage, neutrophil and T-lymphocyte invasion, Schwann cell, fibroblast, mast cell, and epithelial cell activation and the generation of an "inflammatory soup" containing **primary mediators** such as chemokines, cytokines, Wnt ligands, neuropeptides, and growth factors (see **Table 1** and **Figure 1**).
2. Primary mediators sensitize sensory nerve endings, attract additional macrophages and lymphocytes, alter gene expression, promote post-translational modification of proteins, and alter ion channel function in primary afferent neurons. This leads to increased excitability, spontaneous activity and the generation of secondary mediators (see **Table 2** and **Figure 1**).
3. **Secondary mediators** such as colony stimulating factor 1 (CSF-1), chemokine (C-C motif) ligand 21 (CCL21), and wingless-type mammary tumor virus integration site family, member 5A (Wnt5a) are released from primary afferent terminals in the spinal dorsal horn. They affect the properties of spinal microglial cells causing them to release tertiary mediators. In this way, spinal microglia can detect and respond to peripheral nerve injury.
4. Microglial-derived **tertiary mediators** such as BDNF, TNF-α, and IL-1β (Brain derived neurotrophic factor, tumor necrosis factor alpha, and interleukin-1β) increase excitatory transmission and attenuate inhibitory synaptic transmission in the superficial dorsal horn (see **Table 3** and **Figure 1**).
5. This and other aspects of synaptic plasticity facilitate the transfer of nociceptive information and promote misprocessing of sensory information leading to central sensitization at both the spinal and supra-spinal level.
6. Although it was once believed that altered microglial function was transient and confined to the onset phase of neuropathic pain, newer data implicates sustained alteration of microglial function in its long term maintenance. This is associated with long-term changes in astrocyte function.
7. Cell type involvement is sex dependent. Whereas, microglia play a predominant role in central sensitization in males, this is effected by macrophages and T-lymphocytes in females.
8. In addition to release of mediators, recent evidence suggests that cell to cell communication may be affected by the transfer of materials such as microRNA's in secreted extracellular vesicles or exosomes.

Each of these steps will be discussed below with special emphasis on the actions of **secondary mediators** on microglial activity and the release and actions of **tertiary mediators** in the spinal dorsal horn (**Figure 1**). Cytokine/chemokine/growth factor/glial cell interactions are also involved in modulation of sensory information in supraspinal structures following peripheral nerve injury. This includes the mesolimbic system (185) thalamus, sensory cortex, and amygdala (186–188). Interestingly, microglial activation appears on the contralateral side following nerve injury thus reflecting the projections of ascending tracts. Activation is not seen in areas which are not involved in pain processing such as the motor cortex (186). This implies that microglial activation in higher centers is not simply the result of diffusion of messengers *via* the cerebrospinal fluid (CSF). The present review will however focus on microglia activity within the spinal dorsal horn.

TABLE 1 | Primary mediators from site of nerve injury.

Primary mediator	Generated and/or released by injured peripheral tissue	Mimicking neuropathic pain *in vivo*	Alleviation of neuropathic pain in knockouts or by antagonists etc. *in vivo*	Demonstrated effect on dorsal root ganglion neurons
IL-1β	(48–54)	(55, 56)	(48, 57–60)	(61–65)
IL-15	(66)	(67)*		
IL-17	(68, 69)	(21, 70)	(21, 71, 72)	(21)
IL-18			(73)	
LIF	(74, 75)	(75)		(76–78)† (79)††
TNF-α or β	(48, 50, 51, 54, 80–82)	(48, 54, 55, 80, 83)	(48, 52, 83–87)	(83, 88–92)
Prostaglandins and other eicosanoids	(54, 93, 94)** (95)***	(96) (95)***	(94)	(97, 98) (95)***
NGF	(99)	(100, 101)	(99, 100, 102, 103)	(101, 104)
Substance P	(105, 106)	(107)	(108, 109)	(110–112)
MCP-1/CCL2	(49, 52, 113–116)	(116)	(52, 116, 117)	(116, 118, 119)
CXCL-1	(120, 121)			(122–124)
CXCL-4	(125)		(123, 125, 126)	(125)
Histamine	(127)	(128)	(128–130)	(128, 130)
Wnt3a	(131–134)	(133)	(131, 133)	(131, 133)
Wnt5a	(135)			

Released from Site of injury and affect primary afferent neurons.

**Implied from observations on osteoarthritis patients.*

***Measured increased cyclo-oxygenase 2 (COX 2) levels.*

****This work addresses the actions of the novel eicosanoid 5,6 epoxyeicosatrienoic acid (5,6 EET).*

† *These 3 papers show LIF promotes sprouting of perivascular sympathetic axons in DRG.*

†† *This paper demonstrated a direct action of LIF on DRG neurons.*

NERVE INJURY, WALLERIAN DEGENERATION, INFLAMMATION AND GENERATION OF PRIMARY MEDIATORS

Wallerian degeneration of injured peripheral nerves is associated with neutrophil, macrophage and T-lymphocyte infiltration, mast cell, endothelial cell, keratinocyte and fibroblast activation and alteration of Schwann cell properties (2, 54, 68, 80, 98, 189–196). All of these cell types produce and release a variety of inflammatory mediators and a few anti-inflammatory agents at the site of injury (2, 190, 197) and **Table 1**. These **primary mediators** include pro-inflammatory agents such as interleukin 1β (IL-1β) (48–50, 55, 57–59, 141), leukemia inhibitory factor (LIF) (74–76, 79, 198), interleukin 15 (IL-15) (66), interleukin 17 (IL-17) (21, 68, 70), interleukin 18 (IL-18) (199) tumor necrosis factor (TNF-α) (48, 51, 80, 83, 85–88, 200–203), monocyte chemoattractant protein 1 (MCP-1/CCL2) (49, 113–115, 118), chemokine (C-X-C motif) ligand 1 (CXCL1) (120–124) and CXCL4 (125), histamine (127–130), and the secreted glycoproteins Wnt3a (wingless-type mammary tumor virus integration site family, member 3A) and Wnt5a (133, 135). For a more complete list see Moalem and Tracey (54).

As discussed below, most of these mediators excite peripheral nerve endings as well as the cell bodies of primary afferent fibers in the dorsal root ganglion (DRG) (53). Release of pro-inflammatory **primary mediators** both at the site of injury and within the DRG provokes changes in the cell bodies, axons and peripheral endings of both injured and uninjured primary afferent axons (141, 204–206).

Satellite glial cells that surround the cell bodies of dorsal root ganglia (DRG) neurons represent an additional source of primary inflammatory mediators (2, 78, 142, 207–209). IL-1β may also be derived from macrophages that invade DRG after injury (141) as well as from sensory neuron resident macrophages (210). Peripheral nerve injury causes extensive satellite glial cell activation (as defined by glial fibrillary acidic protein [GFAP] immunoreactivity). This is prevented by local perfusion of TTX or bupivacaine. Na^+ channel block also reduces levels of NGF at a time when activated glia (Schwann cells) are an important source of NGF. This implicates injury-induced increased spontaneous activity in primary afferents in the activation of satellite glial cells (211). This aligns with the general concept of "neurogenic neuroinflammation" whereby intense neuronal activity can orchestrate immune cell activation (212).

In addition to the interactions of inflammatory mediators with neurons, many of them promote plasma extravasation and exhibit chemoattractant properties, both of which enable the recruitment of immunocompetent leucocytes and lymphocytes to the site of injury (54, 66, 68, 194). As already mentioned, these myeloid and lymphoid cells themselves release a host of cytokines and chemokines thereby instigating a positive feedback process in the initiation of neuroinflammation.

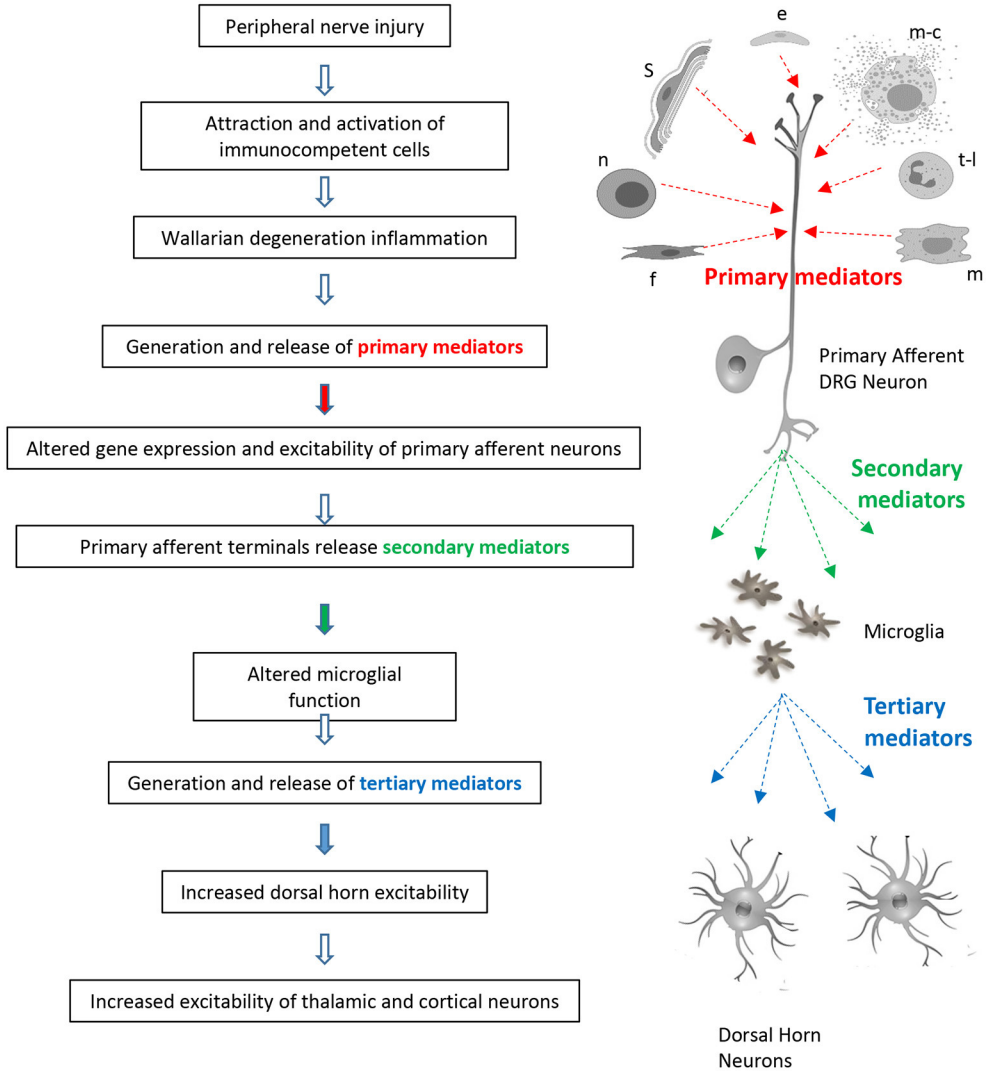

FIGURE 1 | Sites of action of primary, secondary, and tertiary mediators in signaling of neuropathic pain. Sources of primary mediators include Schwann cells (s), epithelial cells (e), mast cells (m-c), t-lymphocytes (t-l), macrophages (m), fibroblasts (f), and neutrophils (n).

TABLE 2 | Secondary mediators released from primary afferents.

Secondary mediator	Generated and/or released by DRG neurons	Mimicking neuropathic pain *in vivo*	Alleviation of neuropathic pain in knockouts or by antagonists etc. *in vivo*	Demonstrated effect on microglia
CSF-1	(136–142)	(138)	(138, 139)	(138–140) (143)*
CCL21	(144–146)	(144, 147)	(144, 147–149)	(144, 148)

Released from primary afferents to affect spinal microglia†.

**This paper provides indirect evidence, CSF-1 releases BDNF from microglia as monitored by increased dorsal horn excitability, some of the effect of CSF-1 on excitability is abrogated by BDNF binding protein.*

†*Wnt3a may be released from primary afferent terminals after nerve injury but is thought to signal directly to dorsal horn neurons without the intervention of microglia (134).*

Although inflammation is a primary response to tissue injury, it should be noted that some of the primary mediators associated with neuropathic pain also serve to initiate neuronal recovery and repair (213). Thus, production of NGF at the site of nerve injury (99, 100, 102, 103, 214) may be viewed as both an initiator of inflammation and an activator of neuronal regeneration and

TABLE 3 | Tertiary mediators produced by microglia to affect spinal dorsal horn neurons.

Tertiary mediator	Generated and/or released by microglia	Mimicking neuropathic pain *in vivo*	Alleviation of neuropathic pain in knockouts or by antagonists etc. *in vivo*	Demonstrated effect on spinal dorsal horn neurons
BDNF	(150, 151)	(152–154)	(150, 151, 153, 155)	(4, 143, 150, 154, 156–165)
IL-1β	(166–168)	(166, 169) (55)*	(2, 54, 57–60, 166, 170–174)	(175–179)
TNF-α	(54, 180)	(40, 181)	(84, 203, 277, 386).	(175, 182–184)

Microglia to spinal dorsal horn neurons.

**These experiments involved injection of IL-1β into peripheral nerve, thus its ability to produce allodynia most likely reflected its peripheral role as a primary mediator.*

repair. Moreover, functional recovery after peripheral nerve injury may depend on the pro-inflammatory cytokines IL-1β and TNF (48).

The situation with GDNF family ligands such as artemin is complex, whilst some reports describe its pro-inflammatory action and possible involvement in neuropathic pain, others suggest that artemin may be anti-inflammatory and activation of its receptors provide pain relief (215–219).

Interleukin 4 (IL-4) produced by peripheral nerve injury has exclusive anti-inflammatory and anti-nociceptive actions (220). These findings relate to the generalization that both inflammatory and anti-inflammatory mediators are released by nerve injury and it is disturbance of the balance between these two processes that can lead to pain (197).

Downstream Effectors of Mediator Actions

Although receptors for individual cytokines are selective for their respective ligands, the downstream transduction pathways often converge, resulting in translocation of transcription factors to the nucleus and transcription of additional downstream mediators. Common signaling pathways activated following cytokine receptor activation include (1) nuclear factor-κB (NF-κB), (2) the mitogen-activated protein kinases (MAPKs), (3) the janus kinase (JAK) and signal transducer and activator of transcription (STAT), and (4) the Smad family signaling pathways (50, 187).

By contrast, chemokines, histamine and neuropeptides such as substance P signal *via* heptahelical G-protein coupled receptors (221, 222).

At least some of the actions of inflammatory cytokines involve activation of cyclo-oxygenase 2 (105, 223, 224) and products such as prostaglandins (93, 94, 167, 180, 225, 226) and prostacyclin (227).

Wnt ligands (Wnt; wingless-type mammary tumor virus integration site family) are a family of 19 secreted glycoproteins that are important and versatile mediators of cell–cell communication, cell morphology and development. Ligands signal by the canonical Wnt pathway, the non-canonical planar cell polarity pathway, and the non-canonical Wnt/calcium pathway (133, 228). Wnt3a acts through the canonical pathway which involves β catenin. Wnt5a acts through the non-canonical β catenin independent planar cell polarity pathway and the Ryk (134).

The downstream mediators of BDNF activation of TrkB and NGF activation of TrkA are well-characterized and include the phosphatidyl inositol-3 (PI3)-kinase (also known as Akt or protein kinase B), phospholipase C-γ1 and the ras-MAPK pathway, also known as the extracellular receptor kinase (ERK) pathway (229). Since ras-MAPK is a mediator of both neurotrophin and cytokine receptor activation, there is considerable interest in its potential as a drug target (230–232).

EFFECTS OF PRIMARY MEDIATORS ON PRIMARY AFFERENT NEURONS

Gene array analysis of perturbations in primary afferent neurons following nerve injury have identified marked changes in genes coding for neuropeptides, cytokines, chemokines, receptors, ion channels, signal transduction molecules and synaptic vesicle proteins (146, 233) as well as changes in expression of long non-coding RNA's (234) and microRNA's (235–238). The latter post-transcriptionally regulate the protein expression of hundreds of genes in a sequence-specific manner (239). For example the microRNA (miRNA-let-7b) can be released from DRG neurons by neuronal activation. It acts in a paracrine function to induce rapid inward currents and action potentials in other DRG neurons by inducing toll like receptor 7 (TLR7)/TRPA1-dependent single-channel activities. Intraplantar injection of miRNA-let-7b elicits rapid spontaneous pain *via* TLR7 and TRPA1 (240). These observations again align with the concept of neurogenic neuroinflammation (212).

In addition, miR-21-5p which is released in the exosomal fraction of cultured DRG neurons, may be involved in neuron-macrophage communication after nerve injury (238, 241). The concept of cell-to-cell transport of material *via* exosomes or extracellular vesicles represents an exciting new direction for pain research (238, 241–245). A recent review focussed on release of extracellular vesicles from microglia (246).

Changes in DRG Excitability and Ion Channel Function

Recordings from rodent DRG neurons both *ex vivo* and *in vitro* confirmed that peripheral nerve injury increases their excitability and may provoke spontaneous discharge of action potentials (247–253). This peripheral sensitization and ongoing, aberrant spontaneous activity is a well-established harbinger of

central sensitization and chronic pain (5, 7, 9, 251, 252, 254–260). Spontaneous activity is also known to promote activation of spinal microglia and astrocytes (211, 212). Suppression of this activity *in vivo* by either pharmacological (257, 261) or optogenetic methodologies (262) leads to abatement of injury-induced allodynia and attenuation of hyperalgesia.

Increased DRG excitability is driven by increased expression and/or function of voltage-gated Na^+, Ca^{2+} and hyperpolarization activated cyclic nucleotide gated channels (HCN channels) (263–265) as well as decreased expression and/or function of K^+ channels (260) and altered expression, modulation and function of acid sensing ion channels (ASIC channels) and transient receptor potential (TRP) channels including TRPV1, TRPA1, and TRPM8 (215, 266–268).

Acute and/or long term exposure of DRG neurons to pro-inflammatory primary mediators such as IL-1β (interleukin 1β), IL-17 (interleukin 17), TNF (tumor necrosis factor), MCP-1/CCL-2 (monocyte chemoattractant protein-1/chemokine ligand 2), stromal cell-derived factor 1 (CXCL12), Wnt3a or prostaglandin E2 increases their excitability (21, 61, 64, 65, 88, 91, 116, 118, 119, 123, 125, 131, 133, 269, 270).

In general, the effects of primary mediators on cation channel function parallel the changes provoked by peripheral nerve injury (62, 63, 92, 125, 184, 271, 271–274) and it is now well-established that these excitatory actions play an indispensable role in the development and/or persistence of neuropathic pain. For example, administration of antibodies to interleukin I-receptor (IL-lR) or its genetic deletion or overexpression of interleukin receptor antagonist (IL-RA) reduce pain behavior in mice with experimental neuropathy thereby implicating IL-1β in the onset of neuropathic pain (2, 54, 57, 58, 202). Although IL-1β is involved at several points in the sensory system following nerve injury (176, 179, 187, 275, 276), its peripheral actions are underlined by the observation that local microinjection of recombinant IL-1β at the site of sciatic nerve injury in IL-1β-knock-out mice lowers mechanical pain thresholds to levels observed in injured wild-type animals (48).

The role of IL-17 has been studied in the paclitaxel model of chemotherapy induced pain. In addition to increasing DRG excitability, both IL-17 and paclitaxel facilitate sEPSC activity and attenuate sIPSC activity in the lamina II outer of the mouse dorsal horn. Selective knockdown of IL-17R in certain dorsal horn cells reduces paxlitaxel-induced hypersensitivity. Taken together these findings provide strong support for a role for IL-17 in this type of chronic pain (21).

Actions and involvement of TNF-α as a primary mediator very much parallel those of IL-1β Levels of TNF-α are elevated in sciatic nerve after injury (82, 85) and Nadeau et al. (48) showed that microinjection of TNF-α into TNF-knock-out mice lowered mechanical pain threshold in a similar fashion to IL-1β TNF-α also upregulates $Na_v1.7$ in DRG (89) and inhibition of TNF-α signaling results in attenuation or accelerated recovery from injury induced neuropathic pain (52, 84, 277). TNF-α receptors are also upregulated (84). Unlike IL-1β, TNF-α does not appear to participate in macrophage to DRG neuron signaling (141) but like IL-1β actions of TNF-α are not confined to the peripheral nervous system (180, 187, 277).

Although IL-6 is markedly upregulated in the peripheral and central nervous systems following nerve injury (50–52, 278, 279) and is released by macrophages at the site of nerve injury (51, 279), it fails to affect DRG excitability (53) yet has been reported to attenuate peripheral nociceptive transmission (280). This contradicts the finding that sciatic chronic constriction injury (CCI) failed to induce hypersensitivity to cutaneous heat and pressure in mice with a null mutation of the IL-6 gene (281). Its potential role as a primary mediator thus remains to be resolved. One possibility is that IL-6 serves as an "off signal" to ensure the transient nature of injury-induced neuroinflammation. It may fulfill this function in the spinal cord where it promotes a desensitized phenotype of microglia (282). Some lines of evidence implicate IL-15, IL-17, and IL-18 as primary mediators in the generation of neuropathic pain (**Table 1**).

Wnt3a also increases sensory neuron excitability *via* upregulation of P2X3 and TRPA1 receptor channels and stimulates production of inflammatory cytokines such as TNF-α and IL-18. Intraplantar injection promotes mechanical hypersensitivity and thermal hyperalgesia. These effects are prevented by inhibition of disheveled; one of the downstream effectors of Wnt3a action (133). Nerve injury also provokes the release of Wnt5a from Schwann cells and since its cognate receptors are upregulated in DRG neurons (135), it, like Wnt3a, may serve as a primary mediator in the onset of neuropathic pain.

Appearance of ectopic excitatory α-adrenoceptors and sprouting of perivascular sympathetic axons both within DRG and on nerve terminals at the site of injury is yet another means by which primary afferent excitability is increased (283–287), leading to signs of neuropathic pain in animal models (288). Sympathetic-sensory interaction is a characteristic feature of complex regional pain syndromes in humans (289). This may reflect a neurotrophic action of LIF or NGF on noradrenergic perivascular axons (76–78) and/or may be a consequence of spontaneous afferent activity (290).

Changes in Expression of Cytokines, Wnt Ligands, and Neuropeptides in Primary Afferent Neurons; Primary Mediators Promote Production of Secondary Mediators

Neuropeptides

Nerve injury alters expression of neuropeptides and their cognate receptors in DRG cell bodies (291–293). Studies have focussed on galanin, NPY, calcitonin gene related peptide (CGRP) and substance P. Since there is evidence for a role of a diffusible substance in soma—soma interactions (294), neuropeptides may play a role in controlling DRG excitability (295). For example, substance P is released in a Ca^{2+} dependent manner from DRG cell bodies (296) and its expression is increased after nerve injury (106, 297). Because large DRG neurons start to express excitatory substance P receptors after nerve injury, it may well play a role in pain etiology (298). This is because alterations in the properties of large DRG neurons and their associated low threshold Aβ fiber axons play major role in neuropathic pain (249, 299–303).

CGRP is also released in DRG where it may fulfill an excitatory autocrine and/or paracrine function in a similar fashion to substance P (122, 295, 304, 305).

Chemokines, Cytokines, and Wnt Ligands

Nerve injury upregulates mRNA and/or protein for a variety of secreted proteins, including chemokines, Wnt ligands, and cytokines and/or their receptors in primary afferent neurons. This includes IL-6 and its receptor (209, 278), MCP-1/CCL2 and CC chemokine receptor 2 (CCR2) (270, 306–308), TNF-α (309), IL-1β and IL-10 (306, 310), CCL-21 (146), and Wnt5a (134). As will be discussed below, several of these substances are released from primary afferent nerve terminals and serve as secondary mediators in the dorsal horn; conveying altered peripheral activity to microglia and/or to dorsal horn neurons. The weight of the evidence supports a secondary mediator role for CSF-1 and for the chemokine CCL-21 (**Table 2**).

SECONDARY MEDIATORS FROM PRIMARY AFFERENT TERMINALS ALTER FUNCTION OF SPINAL MICROGLIA

Signaling Between Injured Peripheral Nerve and Spinal Microglia

Following nerve injury, several substances generated in and released from primary afferents serve as **secondary mediators** that influence the properties of spinal microglia (238). In this way microglia can detect and mount a response to peripheral nerve injury.

Secondary Mediator Role of CSF-1

Injury-induced release of inflammatory mediators such as interleukin 1β from satellite glial cells and invading macrophages in DRG induces *Csf1* in the cell bodies of primary afferent neurons (136, 137, 141, 142). mRNA for colony stimulating factor (CSF-1) is also upregulated by nerve injury as is mRNA for the CSF-1 receptor in spinal microglia (138). Intrathecal injection of recombinant CSF-1 induces microglial proliferation and renewal as well as mechanical allodynia in naïve animals (138–140). When *Csf1* gene expression is selectively depleted from sensory neurons, nerve injury-induced CSF-1 expression and the development of mechanical hypersensitivity are prevented as is the injury-induced microglial activation and proliferation (141).

Release of CSF-1 from primary afferent terminals transforms the phenotype of resting microglia such that they expresses the ionotropic ATP receptor, P2X4R (138, 139, 143). The membrane adaptor protein DAP12 is required for nerve injury-induced upregulation of P2X4R but not for microglial proliferation. Taken together, with the observation that long term exposure of dorsal horn neurons to CSF-1 increases their excitability (143), these data support its role as a secondary mediator signaling between injured primary afferents and microglia which then release **tertiary mediators** such as BDNF and IL-1β (150, 157, 311).

ATP derived from dorsal horn neurons activates P2X4 receptors on microglia, promoting Ca^{2+} influx and BDNF release (151, 312–318). As will be discussed below, this mechanism is crucial to glial signaling and the development of central sensitization in males (313, 319) but not in females (27, 320).

MCP-1/CCL2 Plays a Neuromodulatory Role Within Injured DRG but Is Unlikely to Function as a Secondary Mediator Between Nerves and Microglia

Mice lacking the CCR2 receptor for the chemokine MCP-1/CCL-2 fail to develop signs of neuropathic pain following nerve injury (118, 321), a MCP-1/CCL2 antagonist blocks paclitaxel-induced neuropathic pain (52) and over expression of CCR2 enhances nociceptive responses (322). MCP-1/CCL2 is not found in undamaged peripheral nerves but is strongly upregulated following injury (221, 323). This may be a consequence of the action of TNF-α and spontaneous neural activity (118, 324). MCP-1/CCL2 is expressed in vesicles in DRG soma (117, 270) and is released from DRG cell bodies in a Ca^{2+} dependent manner (270). This evoked release is increased under neuropathic conditions (115, 325). Injury has also been reported to increase immunoreactivity for CCR2 in dorsal horn microglia (326) and spinal administration of CCL2 promotes microglial activation (325, 327). Although these findings might be expected if MCP-1/CCL2 serves as a secondary mediator between primary afferents and spinal microglia, recent work casts doubt on this conclusion. For example, Jung et al. (117) did not detect MCP-1/CCL2 in primary afferent terminals and other studies of microglia *in vivo* failed to confirm the presence of CCR2 either before or after nerve injury (117, 146, 328). Now that more specific biomarkers for cell types are available, one possible explanation for this discrepancy is that CCR2 may be expressed on infiltrating monocytes or on astrocytes rather than on microglia (221, 329, 330).

Rather than functioning as a secondary mediator between primary afferents and spinal microglia, MCP-1/CCL2 may fulfill an autocrine or paracrine function within DRG (118, 270). This possibility is supported by the aforementioned observation that MCP-1/CCL2 is released from DRG cell bodies in a Ca^{2+} dependent manner (270). It has also been shown to excite injured DRG neurons by transactivation of TRPA1 and TRPV1 channels (115, 118). MCP-1/CCL2 may thus stimulate first order neurons in the pain cascade and/or carry out its classical chemokine function to attract CCR2-expressing peripheral monocytes/macrophages to the spinal cord (117, 146). MCP-1/CCL2 may also promote the release of the excitatory neuropeptide CGRP within DRG (122).

Secondary Mediator Role for CCL-21

Intrathecal administration of chemokine (C-C motif) ligand 21 (CCL21) rapidly induces pain-like behavior in naive mice whereas CCL21 neutralizing antibodies or blockade of its cognate CXCR3 receptors with (+/−)-NBI-74330 diminishes pain-related behavior in nerve injured animals (147). The failure of CCL21 deficient mice to display tactile allodynia following nerve injury (148) has been ascribed to the failure of microglia to upregulate the P2X4 receptor for ATP (144, 146). CCL21 is upregulated in DRG following nerve injury, vesicles containing CCL21 are preferentially transported into axons (145), CCL21 affects microglial function (148) and it can be released from terminals

of injured or "endangered" neurons (149, 331). Taken together, these findings suggest that CCL21 is more likely than MCP-1/CCL2 to function as a secondary mediator between primary afferents and microglia following injury (146, 221). CCL21 has also been reported to signal to astrocytes (332).

What Is the Role of Stromal Cell-Derived Factor-l Alpha (CXCL-12/SDF-lα)?

Stromal cell-derived factor-l alpha (SDF-lα) also known as C-X-C motif chemokine 12 (CXCL12), and its cognate receptor CXCR4, are constitutively present in DRG neurons and satellite glia, spinal astrocytes and microglia (333, 334). Peripheral nerve injury upregulates both CXCL12 and CXCR4 in DRG and/or spinal cord (123, 221, 333, 335, 336) as a possible consequence of the action of TNF-α (336). The functional significance of these changes is demonstrated by the observation that CXCL12-induced Ca^{2+} response in DRG neurons is enhanced in nerve injured animals (123). Intrathecal administration of CXCL12 induces hypersensitivity in naive rats in a CXCR4 dependent manner (333, 333). In addition intrathecal injection of CXCL12 neutralizing antibody or the CXCL12 antagonist, AMD3100 transiently reverses allodynia after peripheral nerve injury (123, 336).

CXCL12 has been implicated in pain signaling following spinal cord injury (337) and may be involved in hyperalgesic priming (338). In view of this and the findings presented above, it is clear that the CXCL12–CXCR4 system has an important role in modulation of neuropathic pain. It may be particularly involved in astrocyte signaling and long term pain maintenance (333). Despite this, we could find no reports that CXCL12 is released from injured primary afferents to affect microglia. It thus remains to be determined whether CXCL12 functions as a *bona fide* secondary mediator.

What Is the Role of Fractalkine (CX3CL1)?

Fractalkine (CX3CL1) is produced constitutively by spinal cord neurons (339, 340) and its receptors (CX3CR1) are primarily expressed by dorsal horn microglia (340, 341). These are upregulated after nerve injury *via* an IL-6 dependent mechanism (342). Intrathecal injection of fractalkine produces mechanical allodynia and thermal hyperalgesia whereas injection of a neutralizing antibody raised against CX3CR1 delays the onset of mechanical allodynia and/or thermal hyperalgesia in two different neuropathic pain models (341). This is consistent with the observation that mice lacking CX3CR1 do not display allodynia following peripheral nerve injury (343).

Fractalkine exists in both a membrane tethered form and as a soluble protein (344). Nerve injury increases the level of soluble fractalkine in cerebrospinal fluid (345) and this release by cathepsin S appears obligatory for the expression of neuropathic pain (221, 346). Soluble fractalkine promotes microglia activation and the generation of tertiary mediators including IL-1β and TNF (167, 341).

Cathepsin S is itself released from microglia by an ATP-P2X7 dependent mechanism (347). Since fractalkine immunoreactivity does not localize with CGRP, IB4 or NF200 in the dorsal horn, it has been suggested that under neuropathic conditions, stimulation of primary afferent fibers induces release of cathepsin S from microglia, which liberates soluble fractalkine from dorsal horn neurons, thereby contributing to the amplification and maintenance of chronic pain (345). Since production of soluble fractalkine requires prior release of cathepsin S from already activated microglia, it cannot be regarded as a straightforward secondary mediator, signaling between neurons and microglia in the same way as CCL21 or CSF-1.

Because antibodies raised against CX3CR1 reduce nociceptive responses when administered 5–7 days after CCI, the prolonged release of fractalkine may contribute to the maintenance as opposed to the onset of neuropathic pain. This may relate to the observation that nerve injury provokes *de novo* expression of CX3CL1 in dorsal horn astrocytes (340).

Fractalkine signaling has also been implicated in synaptic degeneration seen in HIV patients who experience painful neuropathy (8). This can be modeled in mice by intrathecal injection of the viral coat protein gp120. This upregulates fractalkine and knockout of its cognate receptor CX3CR1 protects synapses from gp120-induced toxicity. Inhibition of the Wnt/β-catenin signaling blocks both gp120-induced fractalkine upregulation and synaptic degeneration. Injection of gp120 stimulates Wnt/beta-catenin-regulated fractalkine expression *via* NMDA receptors and the NMDA antagonist APV, Wnt/beta-catenin signaling suppressor DKK1, or knockout of CX3CR1 alleviate gp120-induced mechanical allodynia. Taken together the results suggest that HIV-1 gp120 provokes synaptic degeneration in dorsal horn by activating microglia *via* Wnt3a/beta-catenin-regulated fractalkine expression.

What Is the Role of Interferon Gamma?

Several lines of evidence implicate interferon gamma (IFN-γ) in the etiology of neuropathic pain. Spinal microglia in naive animals express the appropriate receptor (IFN-γR) and stimulation with IFN-γ induces both tactile allodynia and altered microglia function. Genetic ablation of IFN-γR impairs nerve injury-evoked activation of ipsilateral microglia and tactile allodynia (348). The purinergic P2X4 receptor is upregulated in IFN-γ stimulated—microglia and, as will be discussed below, the appearance of such receptors plays a crucial role in the onset of neuropathic pain in males (151, 312, 314, 316, 317). IFN-γ has also been shown to increase dorsal horn excitability (349, 350) and to facilitate synaptic transmission between C-fibers and Lamina 1 neurons *via* a microglial dependent mechanism (351). Although the level of IFN-γ is increased in spinal cord following peripheral nerve injury (352), this may originate from invading T-lymphocytes. This implies that IFN-γ does not have a major role as a secondary mediator to effect communication between injured primary afferents and microglia.

Microglial-Independent Signaling Between Primary Afferents and Dorsal Horn Neurons

Apart from the role of glutamate and its involvement in long term potentiation (353), there are several situations where secondary messengers generated in, and released from primary afferents exert direct long term effects on dorsal horn neurons. For example, the primary mediator role of the secreted glycoprotein

Wnt3a has already been alluded to Simonetti et al. (133). Recent evidence suggests that Wnt3a promotes the release of another ligand, Wnt5a from primary afferents which in turn promotes dendritic retraction of dorsal horn neurons (134). This occurs without the intervention of microglial signaling.

The secondary mediator CSF-1 decreases excitatory drive to inhibitory neurons in dorsal horn *via* a BDNF independent process (143). Since the presence of CSF-1 receptors on neurons has been questioned (354), it remains to be determined whether this reflects a direct effect of CSF-1 on neurons or whether other microglial derived tertiary mediators are recruited.

RELEASE OF TERTIARY MEDIATORS FROM MICROGLIAL CELLS

Release of BDNF in the Spinal Dorsal Horn

Initial studies on the release and actions of BDNF were predominantly done on male rodents in an attempt to avoid possible complications imposed by the female oestrous cycle. More recent data strongly suggest major differences in the mechanism of central sensation in females compared to males; microglial derived BDNF is probably not involved in females (24, 26, 27, 313, 320, 355). In males however, numerous lines of behavioral and cellular data strongly implicate the release of BDNF from spinal microglia in the etiology of neuropathic pain (4, 143, 150, 152, 154, 156, 157, 161, 164, 315, 356–359).

As already mentioned, the secondary mediator CSF-1 is released from injured primary afferents and interacts with its receptors on microglial cells (137). This leads to the up regulation of several genes that are implicated in the development of neuropathic pain. This includes *Itgam* (encoding CD11b), *Cx3cr1* (encoding the fractalkine/CX3CL1 receptor, CX3CR1), *Bndf* (encoding BDNF), and *Ctss* (encoding cathepsin S) (139). BDNF which acts by increasing dorsal horn excitability, is a major tertiary mediator in the development of central sensitization (4, 143, 150, 151, 156, 157, 163, 314, 315).

Long-term exposure of dorsal horn neurons to CSF-1 also increases their excitability and this effect is abrogated by the BDNF binding protein TrkB-fc (143). These findings underline the importance of a sensory neuron—CSF-1—microglia—BDNF signaling process in the onset of neuropathic pain (4, 9, 139, 238, 360).

Role of ATP and P2X4 in BDNF Release

Although stimulation of primary afferents releases ATP and generates P2X mediated EPSC's in a subpopulation of lamina II neurons (361), primary afferent neurons do not appear to be the main source of ATP following peripheral nerve injury. It may rather derive from neurons in the superficial dorsal horn itself (362). BDNF release from microglia is brought about by ATP activation of upregulated P2X4R (151, 168, 312, 314, 316–318). This release is biphasic. An early phase occurs within 5 min, whereas a late phase peaks 60 min after ATP stimulation. The late phase of release is associated with an increased level of BDNF within the microglia. Both phases of BDNF release are dependent on extracellular Ca^{2+} but the late phase of release and accumulation is dependent on transcription and translation. This suggests that activation of P2X4R initially releases a pre-existing pool of BDNF and subsequently promotes *de novo* synthesis of BDNF. This vesicular release of BDNF is abolished by inhibiting SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor)-mediated exocytosis and the P2X4R-evoked release and synthesis of BDNF are dependent on activation of p38-mitogen-activated protein kinase (MAPK) (312, 314–317).

Activation of P2X4 on microglia and release of BDNF are involved in the onset of neuropathic pain in males, but as already mentioned, not in females. This is congruent with the observation that spinal microglia from female rodents do not express P2XR (26).

Role of ATP and Metabotropic P2Y Receptors in BDNF Release

There is also evidence for a role for metabotropic P2Y receptors in microglial activation and the onset of neuropathic pain (363–365). This involves P2Y6, 11, 12, 13, and 14 (366–369). Whilst P2Y6 signals through $G_{q/11}$ and P2Y12, 13, and 14 signal through G_s, P2Y11 signals through both G_q and G_s (222).

P2Y12 mRNA and protein are increased in microglia after peripheral nerve injury and intrathecal injection of a P2Y12 antagonist or antisense knockdown of P2Y12 expression suppresses the development of injury-induced pain behaviors and the phosphorylation of microglial p38 MAPK. By contrast, intrathecal infusion of a P2Y12 agonist into naive rats mimics the nerve injury-induced activation of microglial p38 and increases pain behaviors (366). Since phosphorylation of p38MAPK by P2X4 agonists has been implicated in BDNF release (314) this may also be affected by P2Y12 activation.

Spared nerve injury also induces a p38MAPK-dependent increase in P2Y6, 13, and 14 mRNA in microglia. This is thought to depend on activation of ROCK Rho-associated coiled-coil-containing protein kinase (370). Since intrathecal injection of the specific P2Y6 antagonist MRS2578, the specific P2Y13 antagonist MRS2211 or P2Y14 antisense, attenuate mechanical pain hypersensitivity, these three receptors may function as downstream effectors that mediate some of the actions of ATP in microglia (367, 371).

Wnt Signaling and BDNF Release

Wnt signaling can also promote BDNF release (359, 372). This phenomenon has been examined in models of HIV pain which involve exposure of sensory neurons to viral coat proteins such as gp120 (12, 372). Intrathecal injection of gp120 produces mechanical allodynia and increases expression of Wnt3a, β catenin and microglial BDNF in the murine spinal cord. Blockade of Wnt or BDNF signaling alleviates mechanical allodynia as does inhibition of microglial activation with minocycline (12).

Zhang et al. (359) have suggested a mechanism whereby Wnt signaling provides an important link between increased neuronal activity and BDNF expression. Increased glutamatergic neuronal activity activates NMDA receptors and increases the level of intraneuronal Ca^{2+} This promotes Wnt protein synthesis and release *via* MAPK/CREB signaling (373, 374). Activation of frizzed receptors on microglia promotes Wnt signaling *via* β catenin leading to increased BDNF expression and release.

This is a further illustration of the concept of "neurogenic neuroinflammation" whereby intense neuronal activity promotes immune cell activation (212).

BDNF in Inflammatory vs. Neuropathic Pain

Inflammatory pain as induced by formalin or carrageenan exposure is attenuated using the Cre-loxP system to *selectively* delete BDNF from nociceptive sensory neurons. Despite this, these animals display normal signs of neuropathic pain following nerve injury (375). Whilst BDNF thus appears to be involved in both inflammatory and neuropathic pain (376), in the first case it is derived from peripheral nociceptors whereas in the second case it is derived from ATP-activated microglia.

Time Course of Microglia Activation and Long-Term Effect of BDNF

Whereas, early studies of microglia activation in response to peripheral nerve injury focussed on short term changes (312), more recent work has shown that microglial activation in rodent dorsal horn persists for more than 3 months after injury (377). Activation even persists beyond the known involvement of pro-inflammatory cytokine signaling. Thus, selective depletion of spinal microglia with the targeted immunotoxin Mac1-saporin or sequestration of BDNF with the selective binding agent TrkBFc almost completely reversed thermal and mechanical alloynia in both the acute (2 week) and chronic (3 month) phase after injury. By contrast, neutralizing cytokine signaling using intrathecal injection of a cocktail of antibodies against IL-β, TNF-α, and IL-6 significantly attenuated tactile and cold allodynia at 2 weeks but not at 3 months after injury. These findings may have therapeutic relevance as they suggest different mediators should be targeted in the management of acute vs. chronic neuropathic pain (377).

BDNF, TrkB, and Antidepressants

It has recently been reported that some antidepressants bind to TrkB and augment BDNF signaling (378). Since the many lines of evidence outlined above implicate BDNF in central sensitization, augmentation of TrkB signaling by antidepressants would be expected to exacerbate pain. Despite this, tricyclic antidepressants and serotonin-noradrenaline reuptake inhibitors are first line treatments in neuropathic pain management (379). The relationship between these disparate observations remains to be studied and resolved.

Release of IL-β in the Spinal Dorsal Horn

IL-1β plays a modulatory or effector role in nociception in the periphery, dorsal root ganglia, spinal cord and higher centers. These effects assume particular importance in the etiology of neuropathic pain. Corroborative evidence for a role of IL-1β neuropathic pain comes from the observation that inhibition of matrix metalloproteases responsible for IL-1β processing leads to attenuation of pain in a rodent model (170).

Whilst the CSF-1, P2X4-microglia-BDNF pathway is well-characterized, less is known about the release of IL-1β. In the spinal cord, it is produced and released from macrophages, astrocytes and microglia (2, 380, 381). Release from microglia is a consequence of activation of P2X7 receptors (166, 168, 311, 319) and may be provoked by the action of fractalkine (167). In agreement with this, it has recently been reported that the Ca_v1 channel blocker, cilnidipine blocks microglial P2X7 receptors, impairs IL-1β release and reverses nerve injury-induced mechanical hypersensitivity (173). It has also been suggested that P2X4R interact intracellularly with P2X7R to augment P2X7R-mediated IL-1β release (168).

Release of IL-1β is unlikely to reflect a SNARE dependent process as has been suggested for BDNF (314). IL-1β is known to be processed intracellularly from its inactive pro-form by caspase-1 into its mature bioactive form (382). Release from macrophages and dendritic cells and partially from neutrophils, may be brought about *via* the formation of gasdermin D pores in the cell membrane (382–384). One recent report implicates gasdermin D in IL-1β release from microglia in *Toxoplasma gondii* (parasitic protozoan) infections (385) but it remains to be determined whether a similar mode of release is engaged in neuropathic pain. In this situation, IL-1β release may involve its excocytosis *via* panexin channels (166).

Release of TNF-α in the Spinal Dorsal Horn

The role of TNF-α as a peripheral primary mediator has already been alluded to and several studies have shown that signs of neuropathic pain may be alleviated by perturbation of TNF-α signaling (84, 203, 277, 386). Several lines of evidence also support a role of TNF-α as a tertiary mediator responsible for signaling between microglia and dorsal horn neurons.

Nerve injury increases levels of TNF-α mRNA in spinal microglia and microglia derived cytokine induces COX2 and PGI2 synthase expression in endothelial cells suggesting that a TNF-α mediated glia-endothelial cell interaction is involved in the generation of neuropathic pain (180).

Release of Wnt 5a in the Spinal Dorsal Horn

Wnt proteins are upregulated in the spinal cord of various pain models (3, 11, 134, 199). In a very consistent manner as seen in the pathogenesis of HIV-associated pain, Wnt ligands (e.g., Wnt5a) are specifically upregulated in the SDH of "pain-positive" HIV patients (11). By regulating the pathogenesis of gp 120—induced pain, Wnt5a sensitizes pain-processing SDH neurons through the JNK/TNF-α signaling pathway.

ACTIONS OF THE TERTIARY MEDIATOR BDNF IN THE DORSAL HORN

In male rats, intrathecal administration the BDNF binding protein TrkB-Fc prevents the development of mechanical allodynia after spared nerve injury (387). Several cellular mechanisms have been implicated in the excitatory actions of microglial-derived BDNF that lead to central sensitization.

BDNF Increases Excitatory Drive to Excitatory Neurons and Decreases That to Inhibitory Neurons

In rat spinal organotypic cultures, 5–6 d exposure to BDNF increases excitatory synaptic drive to excitatory lamina II neurons

whilst decreasing excitatory drive to inhibitory neurons (157, 356). In mice, effects of BDNF are dominated by increased excitatory drive to excitatory neurons. Whereas, presynaptic TrkB and p75 neurotrophin receptors are involved, postsynaptic effects are mediated exclusively by TrkB (143). Whilst the passive and active properties of lamina II neurons such as rheobase, resting potential, input resistance and excitability are little affected (143, 157, 356), the altered synaptic activity is capable of increasing spontaneous action potential discharge in excitatory neurons whilst reducing it in inhibitory neurons (356). Three observations show that these actions of BDNF are relevant to injury-CSF-1-microglia evoked central sensitization. Firstly BDNF—induced changes in synaptic transmission and its lack of effect on the intrinsic excitability of lamina II neurons very much parallel those invoked by peripheral nerve injury (157, 388, 389). Secondly, Ca^{2+} responses evoked by neuronal depolarization are enhanced by BDNF and also by conditioned medium from lipopolysaccharide-activated microglia. The effect of this conditioned medium is attenuated by sequestering BDNF with TrkBd5 (157). Thirdly, the putative microglial modulator CSF-1 increases synaptic excitation of excitatory lamina II neurons in mice and this effect is abrogated by sequestering BDNF with TrkBfc (143) whereas, as already mentioned, CSF-1 reduces excitation of putative inhibitory neurons in a BDNF-independent mechanism, suggesting that injured primary afferents can also signal directly to dorsal horn neurons without the involvement of microglia (143).

BDNF and NMDA Receptor Function

Effects of BDNF on Postsynaptic NMDA Receptors

The BDNF effects alluded to above relate primarily to AMPA receptor mediated transmission as neurons were studied at a holding potential of −70 mV (143, 157, 356, 388, 389). There is however a considerable body of evidence to support a role for altered NMDA receptor function in the etiology of pathological pain. This is supported by the occasional success realized with NMDA blockers such as ketamine in the clinic (390, 391). The link between NMDA receptor function and BDNF was established over 20 years ago by the observation that it enhances excitatory responses to NMDA in rat spinal cord *in vitro* (392). BDNF phosphorylates GluN1 *via* ERK and PKC (393). It also acts through TrkB to phosphorylate the GluN2B subunit by the Src-family kinase Fyn and thereby potentiates excitatory NMDA receptor-mediated currents (165). Interestingly, this potentiation appears to require the coincident BDNF mediated Cl^- disinhibition. The exact molecular mechanism of this interaction remains to be elucidated as it does not appear to reflect increased NMDA receptor availability as a result of GABA-induced depolarization (165).

Effects of BDNF on Presynaptic NMDA Receptors

BDNF also acts *via* TrkB and a Src-family kinase to potentiate the function of presynaptic NMDA receptors on primary afferent terminals (394). It has been reported that presynaptic NMDA receptors only potentiate glutamate release from primary afferents after nerve injury (395). This further underlines the presynaptic BDNF effect in the development of central sensitization.

BDNF Decreases Inhibition by Perturbation of Chloride Gradients

Peripheral nerve injury reduces expression of the potassium-chloride exporter (KCC2) in spinal lamina 1 neurons (396, 397). The resulting accumulation of intracellular Cl^- causes normally outward, inhibitory GABAergic synaptic currents mediated by Cl^- influx to become inward excitatory currents mediated by Cl^- efflux (396–398). Since the knockdown of spinal KCC2 in non-injured rats reduces pain thresholds and induces neuropathic pain behaviors, these changes contribute to the pathophysiology of central sensitization (150, 396).

In male rats, BDNF mediates this downregulation of KCC2 (164). Thus, administration of ATP activated microglia, but not control microglia, reproduces the shift in anion gradient seen after nerve injury as does application of BDNF. Also, blocking TrkB or using interfering RNA against BDNF reverses both injury induced pain behaviors and the shift in anion gradient (150). Further analysis of this phenomenon reveals that changes in KCC2 expression in deep dorsal horn neurons are confined to nociceptive neurons that project *via* the spinothalamic tract whereas wide dynamic range (WDR) neurons that are activated by a variety of sensory modalities are unaffected (399). It has also been shown that neurons in lamina I are more susceptible to changes in Cl^- gradient than those in lamina II (397) and biophysical and modeling analysis shows this loss is especially effective in promoting increased neuronal firing (400). These are important observations as lamina I and deep dorsal horn nociceptive neurons are the most important sites for relay of nociceptive information to the brain (303, 401, 402). Since loss of GABAergic inhibition enables non-noxious Aβ fiber-mediated excitatory transmission to acess the superficial spinal dorsal horn, this process plays a major role in the establishment of allodynia (300, 403, 404).

Reversal of the Cl^- gradient may rationalize the observation that BDNF increases GABA release in the dorsal horn (159, 161, 405). Under these conditions GABA produces inward currents (396) which would be enhanced and therefore strongly excitatory.

BDNF and Induction of Long-Term Potentiation

Long term potentiation (LTP) of synaptic transmission contributes to central sensitization in the dorsal horn (353, 406–408). LTP of C-fiber responses can also be augmented by BDNF (387) and LTP induced by high frequency nerve stimulation is occluded by BDNF treatment (409). This reflects functional upregulation GluN2B subunits of NMDA receptors by activation of the tyrosine phosphatase SHP2 (409) or Fyn kinase-mediated phosphorylation of GluN2B subunit at tyrosine 1472 (387). These authors also showed intrathecal administration of BDNF scavenger TrkB-Fc prior to surgery could prevent the nerve injury-induced increase of both phosphorylated Fyn and phosphorylated GluN2B expression and as mentioned above it also prevented the development of mechanical allodynia

after spared nerve injury. The importance of these effects was recently underlined by the observation that spinal LTP induced by high frequency stimulation as well as microglial activation and upregulation of BDNF are inhibited by antibodies to CSF-1. This strongly implicates CSF-1/nerve injury driven microglial derived BDNF in the generation of spinal LTP (408).

BDNF, Intracellular Ca^{2+} Oscillation, and Spontaneous Bursting Activity

Manipulations that increase neuronal excitability can induce synchronous waves in the level of cytosolic Ca^{2+} that propagate across the whole dorsal horn (410–412). Similarly, K^{+}-induced depolarization invokes oscillatory activity as monitored by spontaneous field potentials (413). It has also recently been shown that action potential discharge encodes cytosolic Ca^{2+} levels in lamina 1 neurons and even a single action potential can provoke a measurable Ca^{2+} response (414). This implies that spontaneous bursting activity and oscillations of cytosolic Ca^{2+} level may be closely related. Although long term application of BDNF does not change the resting membrane potential, input resistance of rat dorsal horn neurons in organotypic culture (158) it promotes oscillations in the level of intracellular Ca^{2+} in some neurons whilst depressing it in others (163). There appear to be several mechanisms whereby oscillations may be set up, for example those observed by Alles et al. (163) and Chapman et al. (411) were prevented following blockade of AMPA glutamate receptors whereas those by Asghar et al. (413) were merely attenuated. The oscillations recorded by all three groups were however blocked by TTX, again underlining the tight assocaition between action potential activity and Ca^{2+} signalling which in turn may enable Ca^{2+}-dependent gene expression. Whilst the oscillations appeared to be primarily originating from neurons the possible contribution of signal from astrocytes cannot completely be ruled out. Although any direct relationship between these oscillations and neuropathic pain mechanisms remains to be established, sciatic nerve injury has been reported to induce spontaneous bursting activity in a subgroup of dorsal horn neurons *in vivo* (415). MRI studies have also revealed oscillatory activity in the spinal cord of neuropathic pain patients (416). It may be posited therefore that oscillations in Ca^{2+} level and spontaneous bursting activity contribute to the bouts of spontaneous "electric shock like" pain experienced by those afflicted with painful neuropathies (163).

BDNF in Injury-Induced Synaptic Reorganization in Dorsal Horn Neurons

As already mentioned, peripheral nerve injury produces neuron type specific effects on synaptic transmission in the dorsal horn; excitation of excitatory neurons is increased whereas excitation of inhibitory neurons is decreased (143, 156–159, 356, 388, 389). In addition to altered neurotransmitter release and alterations in postsynaptic sensitivity, connectivity is lost at some synapses (8, 417, 418) but new connections and/or reorganization of dendritic spines occurs at others (408, 419).

Microglia are clearly capable of releasing mediators which promote neuronal loss in an animal model of multiple sclerosis (140) and synaptic degeneration in a model of HIV pain (8). This process of microgliosis is also seen following peripheral nerve injury (420, 421). As discussed below, these processes are likely to reflect the action of microglia-derived BDNF and in the case of HIV pain may reflect phagocytosis of damaged synapses by activated microglia (8).

Is BDNF Involved in Injury-Induced Loss of Primary Afferent Terminals Onto Inhibitory Neurons?

Peripheral nerve injury promotes transient loss of glutamatergic excitatory terminals from non-peptidergic IB-4 positive nociceptive fibers in the *substantia gelatinosa* (418, 422). These fibers form the synaptic terminals of the "type 1" synaptic glomeruli (423) which contact GABAergic neurons (402, 424). Morphological changes may therefore contribute to injury-induced reductions in the amplitude and frequency of spontaneous and miniature EPSCs in tonic firing, putative inhibitory neurons (388). This attenuation of excitatory drive to inhibitory neurons would be expected to contribute to an overall increase in dorsal horn excitability (158). Since BDNF also reduces mEPSC amplitude and frequency in putative inhibitory neurons in rat dorsal horn (356) it is possible that BDNF accounts for loss of primary afferent terminals (418, 422). This possibility requires further investigation as BDNF stimulates overall axon growth and regeneration in the spinal cord (425, 426).

This differs from the situation in mice where BDNF does not affect excitatory drive to inhibitory neurons (143). It remains to be determined whether this simply reflects a species difference or whether it is a consequence of the more rigorous criteria to define inhibitory neurons in mice (143, 412) compared to rats (157, 356).

BDNF is not involved in injury-induced loss of GABA terminals. Nerve injury also promotes loss of GABAergic inhibitory terminals in laminae I and II of the dorsal horn (422, 427). Because BDNF enhances GABAergic transmission at various synaptic loci in the dorsal horn (158, 159, 161), the nerve injury-induced loss of inhibitory terminals is unlikely to involve BDNF.

BDNF May Increase Primary Afferent Terminals on Excitatory Neurons

In rats, both nerve injury and BDNF increase excitatory synaptic drive to putative excitatory neurons (157, 356, 388, 389, 428) and a similar effect of BDNF is seen in mice. CSF-1 also increases synaptic drive in a BDNF dependent fashion (143). These observations parallel the observation that both BDNF and CSF-1 increase CGRP containing terminals in response to nocigenic high frequency stimulation (408) as these terminals primarily innervate excitatory neurons (402).

BDNF and Astrocyte Activation

In addition to its actions on neurons as described above, BDNF also activates astrocytes such that they release additional mediators that participate in the establishment of central sensitization (429).

ACTIONS OF THE TERTIARY MEDIATOR INTERLEUKIN 1β IN THE DORSAL HORN

IL-1β levels are increased in the cerebrospinal fluid (CSF) of patients with complex regional pain syndrome (275) and in spinal cords obtained post-mortem from patients with painful HIV related neuropathy (3). Although there are several reports of the effectiveness of IL-1β antagonists and genetic impairment of cytokine function in animal models of neuropathic pain (57–59, 171) studies of the effectiveness of the modified human interleukin 1 receptor antagonist protein (anakinra) in the clinic have been limited by the pharmacokinetic issues imposed by the blood brain barrier (171).

As mentioned above, release of IL-1β from microglia is primarily affected by activation of P2X7 receptors (166, 173, 311, 319) and/or by the action of fractalkine (167). In a similar fashion to BDNF, IL-1β increases overall dorsal horn excitability, glutamate release from primary afferents and excitatory synaptic transmission between primary afferent C-fibers and lamina 1 neurons (167, 176, 430).

Effects of IL-1β on Synaptic Transmission in the Spinal Dorsal Horn

Like BDNF, IL-1β does not affect the membrane potential or rheobase of lamina II neurons, suggesting that most of its effect on dorsal horn excitability can be ascribed to changes in synaptic transmission (175, 176). We found that exposing organotypic cultures of rat spinal cord to 100 pM IL-1β for 6–8 d increased the amplitude of spontaneous EPSC's (sEPSC) in putative excitatory "delay" neurons, and decreased the frequency of spontaneous IPSC's (sIPSC). These are somewhat similar to those seen with peripheral nerve injury (388, 389). IL-1β would therefore be expected to increase dorsal horn excitability and to facilitate the transfer of nociceptive information. This was confirmed by the observation that Ca^{2+} responses evoked by exposure of neurons to 20 mM K^+ were augmented by IL-1β exposure (176). However, other actions of IL-1β included disinhibition of putative inhibitory "tonic" neurons and although the frequency of sIPSC's in putative excitatory "delay" neurons was decreased, their amplitude was increased. The latter observations may be rationalized if GABA assumes an excitatory role in the injury situation due to perturbation of Cl^- gradients by BDNF (150).

We used long-term application of IL-1β to parallel the time course of injury-induced changes in spinal cytokine levels (48, 176). Our findings are paralleled by the observations that acute application of IL-1β increases the amplitude of AMPA and NMDA currents in the spinal dorsal horn (178) and increases glutamate release *via* an interaction with presynaptic NMDA receptors (430). Acute cytokine application also enhances both the frequency and amplitude of sEPSCs in unidentified lamina II neurons (175). These authors reported a reduction in the frequency and amplitude of sIPSCs. The differences between this work and ours may not only represent the different time course of cytokine activation as Kawasaki et al. used 600 pM IL-1β in their work whereas we used a somewhat lower concentration of 100pM and observed differential actions on excitatory vs. inhibitory neurons.

Further analysis of fractalkine—microglia—IL-1β signaling led Clark et al. (167) to propose the following sequence of events. Soluble fractalkine activates CX3CR1 on microglial cells leading to the rapid release of IL-1β. IL-1β activates IL-1r on lamina 1 neurons and modulates function of postsynaptic NMDA receptors such that Ca^{2+} influx is increased when they are activated by glutamate. Elevated levels of intracellular Ca^{2+} in lamina I neurons activates phospholipase A2 leading to the liberation of arachidonic acid and the generation of prostaglandins. Within a few minutes of fractalkine application, prostaglandins increase transmitter release from primary afferents both directly and indirectly *via* iNOS activation and release of NO from microglia.

Presynaptic NMDA receptors have also been implicated in spinal actions of IL-1β where signaling between IL-1r and NMDA may be affected by the sphingomyelinase/ceramide signaling pathway to enhance glutamate release from the primary afferents in neuropathic rats (395, 430). IL-1β enhances endocytosis of glial glutamate transporters in the dorsal horn astrocytes through activating protein kinase C (431), the resultant augmentation of glutamate responses represents a complementary mechanism where cytokine enhances excitatory synaptic transmission.

ACTIONS OF THE TERTIARY MEDIATOR TNF-α IN THE DORSAL HORN

Acute activation of TNF receptor 1 by TNF-α inhibits the excitability of a subset of spinal GABAergic neurons. This effect involves p38 mitogen-activated protein kinase dependent suppression hyperpolarization-activated cation current (I_h) (182). These effects have been reported to diminish with time suggesting TNF-α may be primarily involved with the induction rather than the persistence of neuropathic pain (40).

Although fractalkine action on microglia and potentiation of synaptic transmission in the dorsal horn involves IL-1β but not TNF-α (167), it does appear to facilitate long term potentaition (183). This has led to the suggestion that the differential contributions of TNF-α and IL-1β to fractalkine-induced enhancement of synaptic transmission may reflect the well-characterized phenotypic diversity of microglia (432). Thus, activation of microglia by different secondary mediators may result in release of specific mixtures of tertiary mediators which in turn promote diverse effects on synaptic transmission (183).

GENERAL COMMENTS REGARDING INJURY-INDUCED SIGNALING IN THE SPINAL DORSAL HORN

Role of Astrocytes; Initiation and Maintenance of Neuropathic Pain

Astrocytes become rapidly and persistently activated after peripheral nerve injury, suggesting they play a role in both the onset and maintenance of central sensitization (3, 433–435). As mentioned above, recent evidence also implicates microglial

function in the long-term maintenance of neuropathic pain in animal models (377) but this may not be the case in all types of neuropathic pain in the clinic (3).

It is well-established that IL-1β from microglia stimulates astrocytic production of TNF–α and IL-6 as well as IL-1β itself (381, 434) thereby amplifying the initial IL-1β signal. Microglial derived IL-1β reduces the capacity of astrocytes to take up glutamate (179, 430) as a result of internalization of the astrocytic glutamate transporter (EAAT2) (179). Loss of EAAT2 function induces hyperalgesia, augmentation of glutamatergic synaptic responses and increased sensitivity of dorsal horn neurons to primary afferent stimulation (436, 437). Activated astrocytes have also been reported to release the NMDA receptor co-agonist D-serine (438) thereby augmenting overall dorsal horn excitability. Evidence for astrocyte involvement in the clinic has been obtained by post-mortem studies of HIV-patients with painful neuropathy (3). These authors showed that expression levels of the microglial markers CD11b and Iba1 were not elevated whereas the astrocytic markers GFAP and S100 beta were clearly increased. This was accompanied by increased levels of TNF-α and IL-1β, as well as components of MAPK signaling pathway, including pERK, pCREB, and c-Fos.

Since astrocytes are not the primary focus of this review, readers are directed to the recent review by Ji et al. (435) which underlines the role of astrocytic gap junctions and astrocyte derived chemokines in pathological pain. Several other comprehensive reviews have appeared (439–441) and recent work has underlined the role of astrocyte derived IL-17 in paxlitaxel induced pain (21).

Ubiquitous Nature of Mediator Release and Effect

We have used the term primary mediator to cover substances released from the site of nerve injury, secondary mediator to describe substances released from primary afferent terminals and tertiary mediators to define substances released from microglia (**Figure 1**). Whereas, BDNF selectively released from microglia can be described as a tertiary mediator, production and effect of cytokines and chemokines is far more widespread. For example, IL-1β which is a classical macrophage derived signal, can be released from Schwann cells, microglia, astrocytes, neutrophils, granulocytes, mast cells and endothelial cells (2, 190, 381, 434, 442, 443) it would thus be classified both as a primary and tertiary messenger. In general it can be said that cytokines such as IL-1β can be released from more or less any cell type in response to an appropriate stimulus. IL-17 appears to be a primary mediator which is also released from spinal astrocytes in a model of chemotherapy pain (21).

Opening of the blood brain barrier is a well-known correlate of nerve injury induced allodynia (50, 444) and this may be initiated by aberrant afferent nerve activity (445). This enables lymphocyte and macrophage invasion of neural tissue. In addition, mediators generated in damaged nerves, microglia, Schwann cells or astrocytes might be expected to enter the circulation and exert actions throughout the body. This is supported by the observation that plasma levels of IL-1β are elevated in rodents subjected to spared nerve injury (446) or exposure to paclitaxel which models chemotherapy pain (52).

Mediators generated in the spinal cord would also be expected to have access to other brain regions *via* the CSF. IL-1β levels are increased in the CSF of patients with complex regional pain syndrome (275) and with thoracic disc herniation (447). Inflammatory mediators may also be elevated in the CSF of osteoarthritis patients (15).

Taken together these finding suggest that the diffusion of spinally and DRG generated mediators may gain access to other brain regions *via* both the CSF and systemic circulation. This may lead to mirror image pain following unilateral nerve injury (448) and/or mediator actions in higher brain regions that contribute to the analysis of nociceptive phenomena, the affective components of pain, sickness syndrome and formation of memory traces (446, 449). For example, microglia activation and BDNF release in the mesolimbic reward circuitry may contribute to the negative affect associated with chronic pain (185). With the possible exception of BDNF, all of the mediators described (cytokines, chemokines and Wnt ligands) can be released from multiple cell types and as such may play a role in the initiation or maintenance of neuropathic pain throughout the nervous system. Discussion of the actions of mediators in higher brain centers is outside the scope of this review.

Sex

Neuropathic pain is seen more frequently in women than in men (29) and it is now recognized that understanding of divergent pain mechanisms in males vs. females is crucial to the development of appropriate therapeutic approaches (28, 33, 450, 451).

Investigations over the last 15 years or so have started to unravel cellular and molecular mechanisms that may contribute to this difference (29, 31–33, 452, 453). For example, microglia are not required for mechanical sensitivity to pain in female mice which require activation of adaptive immune cells such as T-lymphocytes (27, 320). The difference may result from a lack of P2X4 receptors in the microglia of females (26, 313). Despite this, behavioral responses to nerve injury in female rats are similar to those seen in males and both involve downregulation of KCC2 and perturbation of Cl- gradients (25). Because BDNF is not necessary for the development of allodynia in female rodents (27), the mediator released from adaptive immune cells remains to be determined. Similar findings have been found in the Freund's adjuvant *in vivo* model of inflammatory pain in rodents and confirmed in human neurons (33). These authors also showed that *ex vivo* BDNF enhanced synaptic NMDA receptor responses in lamina I neurons from males but not from females and that ovariectomy eliminated these differences. Importantly, the findings illustrate how sexual convergence onto shared cellular and behavioral endpoints, such as allodynia, pain sensitivity or KCC2 downregulation, may mask sex differences in underlying molecular and cellular mechanisms (28). Other recent work has shown that macrophage invasion of DRG is predominant in males and not in females although both show similar amounts of allodynia following peripheral nerve injury (454).

The realization that different mechanism are engaged to generate neuropathic pain in males vs. females has obvious therapeutic implications. For example, blockade of $Na_v1.8$ channels in the peripheral nervous system with A-803467 is more effective in females than in males in a rodent model of joint neuropathic pain (455). Might blockers of $Na_v1.8$ be more effective in women than in men? On the other hand, restoration of KCC2 function (456) may be effective in both males and females?

Multiplicity of Signaling Processes

Different Injuries Different Mediators?

It is well-known that different types of nerve injury provoke different types or behavioral or physiological response. Thus, while mechanical allodynia produced by spared nerve injury persists for many weeks, that produced by chronic constriction injury is short-lived and recovery is seen in about 4 weeks (38, 142). Similarly, changes in synaptic transmission in the superficial dorsal horn are more robust after sciatic CCI than after complete sciatic nerve section (axotomy) (389). These findings may be consistent with an earlier observation that CCI promotes stronger and more long lasting upregulation of the primary mediators TNF-α, IL-1β, IL-10, MCP-1/CCL-2 in nerve stumps than nerve crush (306). Whilst neuropathic pain associated with multiple sclerosis is characterized by loss of spinal neurons (140), this effect is not produced with CCI (457, 458).

Recent work has shown how the nature of peripheral injury dictates the precise spinal circuitry involved in the generation of mechanical allodynia (459). Thus, neuropathic injuries generate allodynia by activation of excitatory protein kinase C gamma positive (PKCγ) neurons at the lamina II/III interface (460) whereas mechanical allodynia induced by inflammation involves excitatory calretinin positive neurons in inner lamina II (461). Cholecystokinin (CCK) positive neurons in laminae III-IV are important in both situations. Peirs et al. (459) also distinguished punctate allodynia (as produced by Von Frey filaments) from dynamic allodynia (produced by brushing a cotton swab across the hindpaw skin). This allowed them to identify a subset of CCK neurons which expressed the musculoaponeurotic fibrosarcoma oncogene homolog (Maf) and the transient vesicular glutamate transporter 3 (tVGLUT3), which are primarily involved in conveying dynamic rather than punctate allodynia.

Other work using knockout mice has shown that deficiency of CCL19/21 attenuates nerve injury evoked pain but not the hyperalgesia evoked by the autoimmune encephalomyelitis model of multiple sclerosis (149).

The above findings point to the possibility that different types of injury provoke the generation of different sets of mediators (276). This may be due to differential damage to various subsets of primary afferent fibers.

A Paradox

The above sections outline the actions of many of the proposed primary, secondary and tertiary mediators involved in the development and persistence of neuropathic pain. There are several pathways by which a peripheral nerve injury can lead to pain but as shown in **Tables 1**–**3**, interruption of the actions of any single mediator seems to be capable of alleviating pain. For example, ATP activation of P2X7 receptors on microglia promotes release of IL-1β and activation of P2X receptors promotes release of BDNF. This would imply that it would be necessary to prevent the action of both IL-1β and BDNF to prevent the development of allodynia but it is known that inhibition of the actions of either individual mediator is effective. In other words if BDNF is inhibited why can't pain be initiated by IL-1β If IL-1β is inhibited why can't pain be initiated by BDNF? Also as mentioned above the actions of inflammatory mediators are mediated by a limited number of downstream signaling processes: ERK-MAPK signaling seems particularly important in this regard. If one signaling cytokine is blocked or knocked out why aren't its downstream effector mechanisms activated by other cytokines?

A better understanding of the interactions between mediators and their receptors and downstream effectors is clearly required for a more complete understanding of mechanisms underlying neuropathic pain in animal models that will lead to a better understanding of pain etiology in individual patients. This in turn may enable the application of personalized medicine approaches to pain management (459, 462).

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Low back pain and its determinants among wait staff in Gondar town, North West Ethiopia: A cross-sectional study

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Background: Low back pain is a common public health issue in the working population and one of the leading causes of disability. It is the leading cause of work-related conditions and the most common reason for filing a workers' compensation claim in low- and middle-income countries. Ethiopia is a developing country; there is a shortage of working materials, skilled labor, and a lack of awareness of ergonomics posture, which lead to lifting heavy objects, long periods of standing, repetitive twisting, and same sustained posture for long periods of time without a shift. As a result, the purpose of this study was to assess the prevalence and associated factors of work-related low back pain among restaurant wait staff in Gondar, Ethiopia, in the year 2019.

Methods: Institution-based cross-sectional study, including 420 restaurant wait personnel, was undertaken from 1 March to 30 April 2019. A simple random sampling procedure was used to choose the restaurants and wait staff. A standardized Nordic questionnaire was used to collect data. Data were entered into Epi Info 7 and analyzed in SPSS version 20. The univariate and multivariate logistic regression analyses were calculated. The significance of associations was reported by a *P*-value of < 0.05 and an adjusted odds ratio (AOR). The model fitness checked by the Hosmer–Lemeshow goodness of fit test was used.

Result: In this study, a total of 420 participants (99.53% response rate) ranging in age from 17 to 53 years old participated, with 184 (43.8%) participants reporting low back pain at some point in the past 12 months. Female participants had a higher prevalence of 130 (70.6%). Sex (AOR = 2.98; 95% CI: 1.07–8.30), frequent exercise (AOR 0.47; 95% CI: 0.24, 0.93), extended standing (AOR 8.82; 95% CI: 3.30, 20.32], and repetitive tasks (AOR 7.49; 95% CI: 4.29, 13.19) were all found to be significant predictors in low back pain.

Conclusion: More than two-fifth of waitresses and waiters reported low back discomfort at some point in the past 12 months. Predisposing factors for low back discomfort among restaurant wait staff included being female, standing

for long periods of time while serving, and performing repetitive tasks. Regular exercise was found to be a protective factor against low back pain in wait staff. Delivering ongoing safety training is among the most potent essential measures required in preventing low back pain.

KEYWORDS

low back pain, wait staff, waiters, waitress, Gondar, Ethiopia

Introduction

Musculoskeletal injuries are a broad term that refers to a variety of inflammatory, degenerative diseases, and disorders that cause pain and functional impairments in people who are exposed to work activities and conditions that contributed to the development or exacerbation of the condition but did not act as the sole cause (1). Despite the identification of several associated factors (such as work posture, long periods of standing, moving heavy objects, repetitive twisting forward and backward, obesity, and aging), the reasons for low back pain remain unknown, making diagnosis challenging (2).

According to the WHO, 50–70% of workers suffer from work-related musculoskeletal disorders (WMSDs). WMSDs afflict around 317 million people each year, with 6,300 people dying every day (3). According to the United States Bureau of Labor Statistics, back injuries account for 20% of all workplace injuries and illnesses and nearly 25% of annual workers' compensation payouts. Based on a recent assessment by the United States National Safety Council, overexertion is the most common cause of occupational injury, accounting for 31% of all injuries (4). In many parts of the world, low back pain is the leading cause of activity limitation and work absence, imposing a substantial financial load on individuals, families, and governments (5).

Low back pain is described as "pain and discomfort, situated below the costal edge and above the inferior gluteal folds, with or without leg pain," according to European standards for preventing low back pain (6). Low back pain (LBP) is a common health concern among the general public, and it is one of the leading causes of disability, negatively impacting work performance and wellbeing. Work-related low back pain (WLBP) is a musculoskeletal condition that is described as any back pain thought to be induced by occupational exposures. This illness is also known as overuse syndrome, repetitive strain injury, or cumulative trauma disorder (7). WLBP is a type of low back pain that occurs due to work and is clinically determined to have been caused, at least in part, or exacerbated by the work environment (8).

In affluent countries, a variety of initiatives have been implemented to mitigate the impact. As a result, the severity and cost of lower back pain are decreasing, absenteeism from work and medical costs are falling, working conditions are improving, and many factors that lead to the development of lower back pain are being discovered (8). However, the burden of low back pain was exacerbated in developing countries because the types of work, working conditions, and other factors contributing to the development of lower back pain among different working groups, including restaurant wait staff, were unknown (9).

In Ethiopia, the tourism industry is occasionally booming and hiring a large number of people in the hotel and other sectors. However, the working environment is hazardous to the worker, and health and safety systems are inadequately implemented. Furthermore, most working materials and skilled manpower are insufficient, thus the behavior of work in wait staff requires lifting heavy objects, long periods of standing, repetitive twisting, and the same sustained posture for long periods of time without a shift. These can be the leading causes of low back pain in Ethiopia, and there was a lack of information on the prevalence and associated factors of low back pain among waiters and waitresses in Ethiopia, particularly in Gondar town. As a result, the purpose of this study was to determine the prevalence and associated factors of low back pain.

Materials and methods

Study design, area, and period

A cross-sectional study was conducted among restaurant wait staff in Gondar, Ethiopia. The research was carried out from 1 March to 30 April 2019 in Gondar, a town in northern Ethiopia. It is located 750 km from Addis Ababa, Ethiopia's capital city. In Gondar, there are 101 restaurants, and 1,309 restaurant workers serve customers in food preparation, cooking, distribution, food hygiene, service cleaning, and cashier positions.

Source population and study population

All the restaurant wait staff working at restaurants (hotels) and the selected restaurants in Gondar town were the source and study population of this study, respectively.

Inclusion and exclusion criteria

All the restaurant wait staff working at Gondar town restaurants for at least 12 months were included in this study, whereas restaurant wait staff with physical deformities (such as excessive lumbar lordosis, increased thoracic kyphosis, and scoliosis) (10), a history of traumatic low back pain, back surgery, or medically diagnosed low back pain were excluded.

Sample size determination

The sample was determined by using a single population proportion formula on the following assumption (11). Level of significance (α): 5% (with a confidence level of 95%), marginal error: 5% P: is the prevalence of low back pain among waiters that is 50% because no studies were conducted in this area in our country.

The Z-value of 1.96 was used at 95% CI (n: sample size, P: proportion, d: marginal error).

$$\frac{(Z_{a/2})^2 * P(1-P)}{d^2}$$

$$\frac{(1.96)^2 * 0.5(0.5)}{0.05^2}$$

$$n = 384$$

The total sample size (n) with a 10% nonresponse rate becomes 422.

Sampling procedure

A simple random sampling was used to select the study subjects. The study participants were selected from 40 restaurants in Gondar town. Each restaurant consisted of an average of 13 waiting staff. To ensure representativeness, first, a proportional allocation of the participants was done for each restaurant, and then waiters and waitresses from those restaurants were selected using a simple random sampling approach.

Operational definition

Body mass index

Weight in kilogram divided by the square of the height in meters (kg/m^2); **underweight** < 18.50 kg/m^2, **normal** 18.50–24.99 kg/m^2, and **overweight** ≥ 25 kg/m^2 (12).

Low back pain

A pain and discomfort, localized below the costal margin and above the inferior gluteal folds, with or without leg pain (13).

Nonspecific low back pain

A type of low back pain not attributed to recognizable, known specific pathology (14).

Repetitive task

Workers put to repetitive tasks that recur every 30 s in the same direction in < 30 s (15).

Regular physical exercise

Performing any type of physical exercise for 30 min at least two times each week (8).

Prolonged standing

Standing for more than 4 h (16).

Data collection instrument

Face-to-face interviews were used to gather information. The study participants' low back pain was assessed using the standardized Nordic questionnaire for the evaluation of musculoskeletal symptoms. The questionnaire was designed to determine the prevalence of musculoskeletal issues in a certain population while also considering where they occur in the body (17). The questionnaire had four components, which are sociodemographic, personal and psychological, occupational and ergonomic, and low back pain-related questions (Supplementary File I).

Data quality control

The questionnaire was written in English, translated into Amharic, and then back into English by language experts. The questionnaire's Amharic translation was pretested in Bahir Dar town's eateries with 5% of the total sample size and required corrections were made based on the results. Three data collectors were in charge of data collection. The principal investigator (ES) provided the data collectors with a 2-day comprehensive training on how to approach study participants, how to use the questionnaire and guidelines, and data collection procedures. The investigators kept a close eye on the data collection technique and evaluated the obtained questionnaire on a regular basis for accuracy, completeness, and consistency.

Data management and analysis

The obtained data were coded and reviewed for completeness, missing values, and clarity by the primary investigator and supervisor at the time of data entry. The Epi Info 7 was used to enter the coded data, which was then exported, processed, and analyzed using SPSS version 20. Frequency, mean, SD, and tables were used to present the findings of descriptive statistics. Binary logistics regression was conducted to identify the associations between dependent and independent variables. Independent variables with *p*-values of 0.2 in the univariate analysis were taken to the multivariate logistic regression analysis to control the effects of potential confounders.

A *p*-value of 0.05 (95% CI) and an adjusted odds ratio (AOR) were used to determine the significance of the associations. The model fitness was checked by the Hosmer–Lemeshow goodness of fit test, with a *p*-value > 0.05.

Results

Sociodemographic characteristics of study participants

A total of 420 participants aged 17–53 years participated in this study. This is a 99.53% response rate and is beyond the power calculated sample size ($n = 384$).

Out of the 420 respondents interviewed, 257 (61.2%) of the participants were female participants. Two-thirds (63.3%) of the participants were aged 17–24 years. The mean (SD) year of experience of the waiters was 1.9 (0.6) years. Two-thirds (65%) of the respondents had work experience of 2–5 years. Three-fourths (70.7%) of participants had part-time jobs in addition to their waiting jobs. More than half (63.3%) of the participants' work conditions were during the daytime (Table 1).

TABLE 1 Socio-demographic characteristics of restaurant wait staff in Gondar town, Ethiopia, 2019 ($n = 420$).

Variable	Category	Frequency (n)	Percent (%)
Age	17–24	265	63.1
	25–34	137	32.6
	35–53	18	4.27
Sex	Female	257	61.2
	Male	163	38.8
Marital status	Currently unmarried	298	71.0
	Currently married	122	29.0
Religion	Orthodox	359	85.5
	Protestant	29	6.9
	Muslim	24	5.7
	Catholic	7	1.7
Education level	Can't read and write	8	1.9
	Can read and write	22	5.2
	Primary school	82	19.5
	Secondary school	220	52.4
	Collage and above	88	21.0
Work condition status	Day	266	63.3
	With shift day and night	137	32.6
	Night	17	4.1
Year of experience	0–1	102	24.3
	2–5	273	65.0
	6–10	40	9.5
	≥11	5	1.2
Additional job	Yes	297	70.7
	No	123	29.3

Individual and behavioral characteristics of the participants

Out of 420 respondents interviewed, 200 (47.6%) participants had a BMI of 18.50–24.99 kg/m^2. More than two-fifths (43.1%) of the participants took ergonomic training, one-third (28.1%) of the participants had knowledge about lower back ergonomics, and more than one-third (39.7%) of the participants never had regular exercise before. Three-fourths (74.7%) of the participants were satisfied with their comfortable daily activity. A total of 132 (71.7%) waiters felt happy at work, but 117 (27.9) waiters were bothered by feeling senseless and under little pressure due to their work, and also 259 (61.7%) waiters felt fatigued due to their workload (Table 2).

Occupational and ergonomics factors of the restaurant wait staff

Out of 420 participants, 84.5% of the participants felt LBP while bending or twisting. Nearly, three-fourths of the participants (69.3%) complain about LPB during standing. Almost all of the participants (92.4%) did not complain about LBP during sitting position (Table 3).

Low back pain prevalence among restaurant wait staff

Of 420 respondents, 184 (43.8%) respondents experienced low back pain throughout their job careers. Of the respondents with LBP in the last 6 months, 52 (12.4%) respondents were absent from their work due to LBP. In this study, the prevalence of LBP was higher among female waiters (70.6%) than among

TABLE 2 Personal and psychological characteristics of restaurant wait staff in Gondar town, Ethiopia, 2019 ($n = 420$).

Variables	Category	Frequency (*n*)	Percent (%)
BMI	<18.50	155	37
	18.50–24.99	200	47.6
	>25	65	15.4
Ergonomic training	Yes	181	43.1
	No	239	56.9
Knowledge of back ergonomics	Yes	118	28.1
	No	302	71.9
Habit of doing regular exercise	Never exercise	166	39.5
	Sometimes	180	42.9
	Usually	74	17.6
Are you satisfied for being waiter	Yes	314	74.8
	No	106	25.2
Comfortable with daily activity	Yes	334	79.5
	No	86	20.5
Mental stressed being waiter	Yes	142	33.8
	No	278	66.2
Sleep disturbance	Yes	99	23.6
	No	321	76.4
Feeling senseless and little pleasure	Yes	117	27.9
	No	303	72.1
Fatigue because of daily workload during	Yes	259	61.7
	No	161	38.3
Satisfied with income	Yes	179	42.6
	No	241	57.4
Satisfied with work	Very dissatisfied	158	37.6
	Dissatisfied	80	19
	Neutral	51	12.1
	satisfied	122	29.0
	Very satisfied	9	2.1

TABLE 3 Occupational and ergonomics factors of restaurant wait staff in Gondar town, Ethiopia, 2019 ($n = 420$).

Variable	Category	Frequency (*n*)	Percent (%)
Bending / twisting	Yes	356	84.8
	No	64	15.2
Lifting	Yes	72	17.1
	No	348	82.9
Standing	Yes	291	69.3
	No	129	30.7
Sitting	Yes	32	7.6
	No	388	92.4
Forming repetitive tasks	Yes	115	27.4
	No	305	72.6
Working in an awkward / cramped position	Yes	25	6
	No	395	94
Working when physically fatigued	Yes	23	5.5
	No	397	94.5
Feel pain on your low back more at night shift different from the day	Yes	77	18.3
	No	343	81.7

male waiters [54 (29.3%)]. Among the BMI group of waiters, a higher prevalence of LBP was observed in the lowest BMI groups (<25) of waiters. It is also higher among waiters who had sleeping disturbance [127 (69.1%)] than those who had no sleeping disturbance [57 (30.9%)].

Factors associated with low back pain

In the multivariate logistic regression analysis, sex, regular exercise, prolonged standing, and repetitive tasks were variables that were significantly associated with low back pain among restaurant wait staff.

Female restaurant wait staff had 2.98 times more low back pain than male wait staff [adjusted odds ratio (AOR): 2.98 (1.07–8.30)]. Restaurant wait staff who exercised on a regular basis was 53% less likely to have low back pain than restaurant wait staff who did not do exercise on a regular basis [AOR): 0.47 (0.24–0.93)]. The odds of having low back pain were 8.82 times higher for restaurant wait staff who had prolonged standing than for restaurant wait staff who did not have prolonged standing [AOR: 8.82 (3.30–20.32)]. Restaurant wait staff who had a repetitive task had 7.49 more low back pain than restaurant wait staff who did not have a repetitive task [AOR: 7.49 (4.29–13.19)] (Table 4).

Discussion

The purpose of this study was to determine the prevalence of low back pain and its associated factors among restaurant wait staff in Gondar, Ethiopia. The overall prevalence of low back pain among restaurant wait staff was 43.8%, and variables such as sex, regular exercise, prolonged standing, and repetitive tasks were significantly associated with low back pain among restaurant wait staff.

The magnitude of work-related low back pain in this study is lower than in research, which was conducted in Taiwan (52.7%) (18). This difference observed in the prevalence rate of LBP could be due to the difference in the study setting, sample size, and the study participant's characteristics. The Taiwan study was conducted among 905 restaurants and hotel workers with a large sample size when compared to the present study. Another possible reason might be the difference in

TABLE 4 Bi-variable and multivariable logistic regression analysis on factors associated with low back pain among restaurant wait staff in Gondar town, Ethiopia, 2019 ($n = 420$).

Variables		LBP		Crude OR (95% CI)	Adjusted OR (95% CI)	*p*-value
		Yes	No			
Sex	Female	130 (50.6%)	127 (49.4%)	3.15 (2.04–4.85)	2.98 (1.07–8.30)	0.04*
	Male	40 (24.5%)	123 (75.5%)	1	1	
Marital status	Not Currently married	107 (35.9%)	191 (64.1%)	1	1	
	Currently married	63 (51.6%)	59 (48.4%)	1.90 (1.24–2.91)	1.11 (0.65–1.87)	0.70
Additional part of job	Yes	124 (67.4%)	173 (73.3)	0.75 (0.49,1.15)	0.90 (0.42–1.94)	0.79
	No	60 (32.6%)	63 (26.7)	1	1	
Regular exercise	Never exercise	70 (42.2%)	96 (57.8%)	1	1	
	Sometimes	60 (33.3%)	120 (66.7%)	0.69 (0.44–1.06)	0.62 (0.31–1.27)	0.19
	Usually	40 (54.1%)	34 (45.9%)	1.61 (0.93–2.8)	0.47 (0.24–0.93)	0.03*
Prolonged standing	Yes	149 (51.2%)	142 (48.8%)	5.39 (3.20–9.08)	8.82 (3.30–20.32)	0.00*
	No	21 (16.3%)	108 (83.7%)	1	1	
Forming repetitive tasks	Yes	87 (75.7%)	28 (24.3%)	4.77 (3.07–7.40)	7.49 (4.29–13.19)	0.00*
	No	83 (27.2%)	222 (72.8%)	1	1	

1, reference category; AOR, Adjusted odds ratio; CI, confidence interval; COR, crudes odds ratio; *statistically significant at $p < 0.05$.

the study characteristics between the study participants. The Taiwan study assesses the work-related LBP pain with their pain intensity, while the present study did not assess the pain intensity and excluded the study participants who presented a previous history of LBP. In addition, a study done in Iran among steel workers found that 63.81% had experienced LBP (19). The main difference is that this study is conducted on restaurant workers, while the Iranian study was conducted among steel construction workers, which need high force and different ergonomic postures. The job of the wait staff is also manual. However, maybe a more plausible explanation could be the relatively higher intensity/level of manual work is higher among the steel industry workers. Furthermore, the study done in Ethiopia among teachers found that 57.5% had low back pain (16). This variation could be attributed to the sample size and the population studied in the preceding study, which included teachers suffering from low back pain. Similarly, in the study done in Ethiopia, Gondar, work-related low back pain among low-wage workers was 58.1% (10). The possible reason for this variation could be the variation in work nature, working time, and level of understanding of the ergonomics position. Teachers most commonly work in a standing position, while wait staff uses their back during bending and lifting.

In contrast, the prevalence of work-related low back pain among restaurant wait staff is higher than in studies conducted in the United States, at 18% among restaurant wait staff (20). The possible explanation for the variation in the current study may be that there is low access to information about occupational health and safety practices (21). Furthermore, this study's results are much higher than the studies done on first-class restaurant workers in Turkey (26%) (22). This variation might be due to the difference in the study participant and ways of the assessment procedure. The Turkey study was conducted in the selective study population with pain intensity and pain coping mechanism assessment among the first class wait staff, but our study was conducted among the whole wait staff which is not categorized by classes and underground mine workers in Zambia (24%) (23). The possible explanation for variation could be the difference in the sample size and sampling method. The Zambian study was conducted among 202 mining workers recruited with a stratified sampling technique, while this study was conducted with a large sample increased by double among wait staff with a simple random technique.

The findings of this research revealed that sex is significantly associated with work-related low back pain, which means a female is 3 times [AOR (1.07–8.30)] more likely to have work-related low back pain than compared to a male. This result was in line with the study, which was conducted in Iran (24), a literature review done in (25), and a systematic review done in Africa (26), Gondar, Ethiopia (16). One possible explanation is that women are more obese than men, which cause low back pain. Another possible explanation is that men exercise more frequently than women. Furthermore, women have a lower pain tolerance than men, and they are more likely to report any pain condition. Osteoporosis, menstruation, pregnancy, and childbirth may all play a role in the increased occurrence of LBP in women (16, 27).

The other variable that was significantly associated with work-related low back pain was regular physical exercise. Waiters/waitresses who exercised on a regular basis were 53% [AOR (0.24–0.93)] less likely to develop LBP than those who did not do regular exercise. This is similar to the research conducted in the United States (28), a systematic review done on leisure time physical activity and low back pain (29), Addis Ababa, Ethiopia (8), Gondar, Ethiopia (16). The possible explanation might be that shortened and weak muscles can cause LBP as they can cause misalignment of the spine. Exercises can

strengthen, lengthen, and make the muscles of the back strong to support and keep the spine in perfect alignment for proper functioning (30).

Regarding prolonged standing, it was one of the associated factors of low back pain. In our research, it was about 9 times [AOR (3.30–20.32)] more likely to develop low back pain than not standing for a prolonged time. This is in line with the study conducted in Ethiopia, Addis Ababa (8), and Gondar, Ethiopia (16). Standing for extended periods of time places an undue strain on the lumbar spine and other anatomical systems, which can result in LBP.

In our study, performing repetitive tasks was one of the associated factors with low back pain in our study, and it was 7 times [AOR (4.29–13.19)] more likely to cause low back pain than not doing repetitive tasks. This result is the same as the study done in Taiwan (31). The repetition of identical motions, but also the repetition of multiple activities with motions that are quite similar utilize the same muscles and tissues. As a result, joints and muscles are vulnerable to repetitive motion injuries, and muscles may not have enough time to recover from the strain before the motion is repeated. There is additional data that show a strong link between repeated work and lower back pain (8). The organization should facilitate the wait staff's frequent resting and create an environment for regular exercise. The wait staff should avoid prolonged standing and practice ergonomic health and safety procedure to prevent work-related low back pain.

Conclusion

More than two-fifths of waitresses and waiters reported low back discomfort at some point within 12 months. Waitresses with low back discomfort were more likely to be female, stand for lengthy periods of time while serving, and do repetitive tasks. Regular exercise was found to be a protective factor against low back pain in restaurant waiter employees. It is preferable to provide waiters/waitresses with ergonomic training in regard to prolonged standing, repetitive tasks, and exercise recommendations. Adjusting organizational measures, promoting and practicing frequent rest breaks, regular exercising, avoiding prolonged standing, and the formation of repetitive tasks delivering ongoing safety training is among the most potent essential measures required in preventing low back pain. The organization should implement and follow occupational health and safety service protocols.

Strength and limitations of the study

This study assessed the burden of LBP among wait staff with a large sample size. Despite this, this study has certain limitations. The cross-sectional form of this study precludes a follow-up, which would have provided a better design for discovering variables connected to low back pain. Patients' self-reported data were also used to attain the results. This could have been influenced by recollection bias. Another possible limitation could be the absence of the control group, which makes it difficult to identify the actual proportion of low back pain resulting from the work condition.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by university of Gondar Ethical Committee board. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MG, ESY, and KSA devised the initial concept, assisted in the writing of the proposal, created the study, and were involved at every stage of the project's execution. ESY and MG handled the data analysis, the first draft of the manuscript, and in charge of data analysis. ESY had double-checked, reran the data analysis, and considerably reworked the content before submission. KSA, ESY, MG, and AK conceptualized the basic idea, wrote the proposal, and critically revised the manuscript for essential intellectual content. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpain.2022.964297/full#supplementary-material

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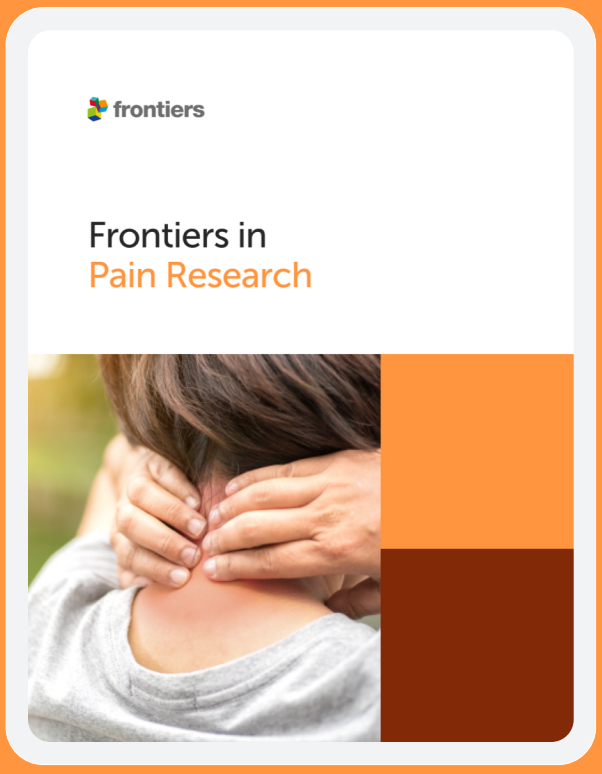

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