An investigation into the noradrenergic and serotonergic contributions of Diffuse Noxious Inhibitory Controls in a monoiodoacetate model of Osteoarthritis.

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Abstract

Osteoarthritis (OA) is a debilitating conditioning with pain as the major clinical symptom. Understanding the mechanisms that drive OA-associated chronic pain is crucial for developing the most effective analgesics. Although the degradation of the joint is the initial trigger for the development of chronic pain, the discordance between radiographic joint damage and the reported pain experience in patients, coupled with clinical features that cannot be explained by purely peripheral mechanisms, suggest there are often other factors at play. Therefore, this study considers the central contributions of chronic pain, using a monoiodoacetate model of OA. Particularly, this study explores the functionality of descending controls over the course of the model through assessing Diffuse Noxious Inhibitory Controls (DNIC). Early phase MIA animals have a functional DNIC system, while DNIC are abolished in late phase MIA animals, indicating a dysregulation in descending modulation over the course of the model. In early phase animals, blocking the actions of spinal α_2-adrenergic receptors completely abolishes DNIC, while blocking the actions of spinal 5-HT_7 receptors only partially decreases the magnitude of DNIC. However, activating the spinal α_2-adrenergic or 5-HT_7 receptors in late phase MIA animals restored DNIC-induced neuronal inhibition. This study confirms that descending noradrenergic signaling is crucial for DNIC expression. Furthermore, we suggest a compensatory increase in descending serotonergic inhibition acting at 5-HT_7 receptors as the model progresses, such that receptor activation is sufficient to override the imbalance in descending controls and mediate neuronal inhibition.

Keywords: Diffuse Noxious Inhibitory Controls, descending modulation, noradrenaline, serotonin.

New and Noteworthy

This study showed that there are both noradrenergic and serotonergic components contributing to the expression of Diffuse Noxious Inhibitory Controls (DNIC). Furthermore, while a tonic descending noradrenergic tone is always crucial for the expression of DNIC, variations in descending serotonergic signaling over the course of the model means this component plays a more vital role in states of sensitisation.

Introduction

Osteoarthritis (OA) is the most common rheumatic condition caused by degradation of the synovial joints. Despite pain being the major clinical symptom, current analgesics remain relatively ineffective. This may be due to analgesics principally concentrating on the peripherally driven aspects of joint pain, yet centrally driven aspects also contribute to the pain experience (Wieland et al. 2005). A subset of OA patients develop referred pain at sites distant to initial joint damage or suffer with chronic pain following total knee replacement surgery, indicating that the pain associated with OA cannot be considered purely peripheral and may be attributed to a long-term centrally dysfunctional amplification of the pain response (Malfait and Schnitzer 2014; Wylde et al. 2011).

The continuous barrage of peripheral nociceptive signaling due to joint damage means spinal neurons with joint input can become hyperexcitable, which can subsequently lead to an altered function of supraspinal structures (Schaible 2012). Indeed, in patients with hip OA, an increased activity within the Periaqueductal grey (PAG) was found when areas of referred pain were stimulated (Gwilym et al. 2009). Monoaminergic descending controls are coordinated in the brainstem and modulate spinal nociceptive processing. An enhanced descending serotonergic facilitatory drive has been reported in animal models of OA as contributing to the hyperexcitability of spinal neurons (Rahman et al. 2009). Therefore, adaptive changes in the brainstem and descending modulatory pathways are important features to be considered when treating OA associated chronic pain.

One method for assessing the functionality of descending controls is through measuring Diffuse Noxious Inhibitory Controls (DNIC) (Yarnitsky 2015). DNIC are a unique form of endogenous inhibitory control, whereby evoked activity of convergent neurons is

strongly inhibited by a concurrent noxious stimulus outside of the receptive field (Le Bars et al. 1979a; Cadden et al 1993; Bannister et al 2015). Conditioned Pain Modulation (CPM) is the human counterpart of DNIC and can be assessed in the clinic. DNIC cannot be observed in anesthetized animals with spinal cord transection, while CPM is lost in tetraplegics, indicating that both rely on the activation of supraspinal structures and functional descending controls (Le Bars et al 1979b; Roby-Brami et al 1987). Furthermore, CPM measurements in the clinic provide valuable insights into a patient's physiology, such as their likelihood of developing chronic pain or responding to drugs that restore descending inhibition (Yarnirsky et al. 2008, 2012).

DNIC rely on the conditioning stimulus activating descending noradrenergic inhibitory controls, which activate α_2-adrenergic receptors in the spinal cord to mediate neuronal inhibition, as blocking these receptors abolishes DNIC in naïve animals (Bannister et al. 2015). There is also a serotonergic component to DNIC, but its role is more difficult to unravel as descending serotonergic signaling mediates both facilitatory and inhibitory actions on nociceptive processing (Bannister et al. 2017). Serotonergic actions at spinal 5-HT_3 receptors mediate facilitatory actions, and an enhancement of this pathway contributes to the loss of DNIC in models of nerve injury (Bannister et al. 2015). Yet, increasing the synaptic content of serotonin in the spinal cord with serotonin reuptake inhibitors (SSRIs) in a rat model of neuropathy restored neuronal inhibition induced by DNIC (Bannister et al. 2017). Interestingly, spinal 5-HT_7 receptors mediate antinociception, and it was demonstrated to be the activation of this receptor via SSRIs that restored DNIC (Dogrul et al. 2009, Brenchat et al. 2012). Furthermore, an increase in the number of 5-HT_7 receptors has been reported in the ipsilateral dorsal horn of neuropathic rats, indicating that sensitizing conditions may regulate serotonergic receptor expression (Brenchat et al. 2010).

We used a monoiodoacetate (MIA) model of OA, as variations in descending monoaminergic controls have been reported as the model progresses (Burnham and Dickenson 2013). We investigated the expression of DNIC in both early and late phases of the MIA model, and pharmacologically manipulated the α_2-adrenergic and 5-HT_7 receptor to better understand their role in DNIC expression in sensitised states.

Methods

Animals

In all experiments, male Sprague Dawley rats were used. Food and water were provided ab libitum, with cages kept in a 12 hour light/dark cycle. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986.

The MIA model

Male Sprague-Dawley rats (190-210g for early phase and 120-140g for late phase) were anaesthetized with isofluorane and arthritis was induced in the left knee with an intrarticular injection of 2mg MIA (Sigma, UK) in 25μL of 0.9% saline. Sham animals received an intrarticular injection of 25μL 0.9% saline only.

Electrophysiology

Electrophysiological experiments were carried out 2-6 days post MIA injection for early phase animals and 14-20 days post MIA injection for late phase animals as previously described (Urch and Dickenson 2003). Briefly, animals were anesthetized for the duration of the experiment with isofluorane (1.5%) delivered in a gaseous mix of O_2 (33%) and N_2O (66%). A laminectomy was performed to expose the L4-L5 segments of the spinal cord. Extracellular single unit recordings were made from deep dorsal horn WDR neurons (Lamina V-VI) using parylene coated tungsten electrodes (A-M systems). All WDR neurons used in this study responded to both innocuous and noxious stimulations in a graded manner coding intensity. Data was captured and analysed by a CED 1401 interface coupled to a computer running Spike2 software (Cambridge Electronic Design; rate functions).

DNIC study design

Firstly, the pre-conditioned mechanically evoked neuronal firing rates were quantified in response to 8g, 26g and 60g von Frey filament stimulation applied to the hind paw. This was repeated 3 times to obtain a stable pre-conditioned response (where all neurons met the inclusion criteria of <10% variation in action potential firing). For the DNIC response, the same von Frey filaments were applied to the hind paw receptive field with a concurrent noxious ear pinch (15.75 x 2.3 mm Bulldog Serrefine, Interfocis, Linton). This trial was repeated and pre-conditioned and DNIC responses were calculated as the mean from the two trials. A DNIC response was quantified as an

inhibition on mechanically evoked neuronal firing in presence of the conditioning noxious ear pinch. A 1-minute nonstimulation recovery period was allowed between each test, while a 10-minute nonstimulation recovery period was allowed between each trial to ensure neuronal responses had returned to baseline.

Drug administration

Firstly, two DNIC trials were carried out to collect pre-drug baseline controls. Each individual drug dose was then administered and the neuronal response was followed for one hour, with tests carried out at 20 and 40 minutes (one neuron per animal). For each time point another DNIC trial was conducted, which consisted of pre-conditioned responses to 8g, 26g and 60g mechanical stimulations repeated 3 times to obtain stable responses, followed by a DNIC response with a concurrent noxious ear pinch. For post-drug effects, the maximal changes for pre-conditioned and DNIC responses are presented in the graphs for Fig 1-4.

The 5-HT_7 receptor antagonist SB-269970 (Tocris) was dissolved in saline and applied topically to the spinal cord (100µg/50µL) of early phase MIA animals. The α2-adrenoceptor antagonist atipamezole (Sigma) was dissolved in vehicle (97% saline, 2% cremaphor, 1% DMSO) and applied topically to the spinal cord (100µg/50µL) of early phase MIA animals. Tapentadol, a noradrenaline reuptake inhibitor and µ-opioid receptor (MOR) agonist (Grünenthal) was dissolved in saline and delivered via a subcutaneous injection at a dose of 2mg/kg in late phase MIA animals. The 5-HT_7 receptor agonist AS-19 (Tocris) was dissolved in vehicle (97% saline, 2% cremaphor, 1% DMSO) and applied topically to the spinal cord (100µg/50µL) of late phase MIA animals.

qPCR

Animals were terminally anaesthetised with an overdose of isoflourane and the ipsilateral lumbar dorsal horn and L3-L5 DRGs were dissected, snap frozen in liquid nitrogen and stored at -80°C. RNA was extracted from homogenized tissue using a RNAse microkit (Qiagen). First strand cDNA synthesis was performed on 500ng RNA using a Superscript III Reverse Transcriptase kit (Invitrogen) according to manufacturers instructions with deoxynucleotide-triphosphates (Promega), and random primers (Promega). mRNA levels of the α_2-adrenergic and 5-HT_7 receptor were

measured with quantitative PCR using specific primers (Table 1) and LightCycler® 480 SYBR Green I master mix (Roche, UK). The mRNA levels were normalized to GAPDH and expressed relative to sham controls.

Statistical Analysis

Statistical analyses were performed using SPSS v22 (IBM, Armonk). All data plotted represents the mean ± SEM. For electrophysiology, statistical differences in neuronal responses with noxious conditioning ear pinch, or following drug application were determined using a two-way repeated-measures ANOVA with Bonferroni post hoc test. For analysis of percentage change for conditioned responses before and after drug application a Kurskall-wallis independent samples one-way ANOVA was used. For qPCR, statistical differences in mRNA expression levels between MIA and sham controls were determined with a Kurskall-wallis independent samples one-way ANOVA. Asterisks denote statistically significantly differences (*P<0.05, **P<0.01, ***P<0.001).

Results

3.1 DNIC expression in early and late phase MIA animals

Throughout this study, DNIC was induced by a concurrent noxious ear pinch ipsilateral to the WDR neurons being recorded. The noxious conditioning ear pinch produced a consistent and substantial reduction in mechanically evoked neuronal firing in both early phase MIA (n=19) and sham animals (n=27) (Figure 1A + C). The degree of neuronal inhibition induced by the conditioning noxious ear pinch in early phase animals was 44%, 31% and 33% for 8g, 26g and 60g respectively (Figure 1A), while the degree of neuronal inhibition in sham controls was 45%, 38% and 34% for 8g, 26g and 60g respectively (Figure 1C), indicating that the magnitude of DNIC induced inhibition is comparable between these groups. The significant difference between pre-conditioned neuronal firing and neuronal firing with a concurrent conditioning ear pinch in early phase MIA and sham animals confirms the presence of DNIC and functional descending controls in these groups (Early phase MIA: $p<0.001$ for all mechanical forces, Sham: 8g: $p<0.001$, 26g: $p<0.01$, 60g: $p<0.001$). On the other hand, the conditioning ear pinch produced no reduction in mechanically evoked neuronal firing in late phase MIA animals (n=42), indicating a loss of DNIC and a dysfunctional endogenous inhibitory system ($p>0.05$ for all mechanical forces).

3.2 Descending noradrenergic controls and the expression of DNIC in MIA animals

As it has been demonstrated that DNIC are reliant upon descending inhibitory noradrenergic controls acting at α_2-adrenergic receptors in the spinal cord, we assessed the consequences of blocking this receptor on DNIC expression in early phase MIA animals (Bannister et al. 2015, 2017). Following the spinal application of the selective α_2-adrenergic receptor antagonist atipamezole, there was no longer a reduction in neuronal firing with a concurrent noxious ear pinch, indicating that blocking spinal noradrenergic actions results in a complete loss of DNIC (Figure 2A) ($p>0.05$ for all mechanical forces, n=5). The spinal application of atipamezole also resulted in a facilitated pre-conditioned response, with a significant increase in neuronal firing in response to 8g ($p<0.01$), 26g ($p<0.05$) and 60g ($P<0.05$).

Having shown that blocking the actions of α_2-adrenergic receptors caused a loss of DNIC in early phase animals, we investigated if enhancing this descending inhibitory noradrenergic system could restore DNIC in late phase MIA animals. Tapentadol, which acts as both a Noradrenaline Reuptake Inhibitor (NRI) and μ-opioid receptor agonist, restored neuronal inhibition induced by a noxious conditioning ear pinch (n=6) (Figure 2B). Following systemic tapentadol, a concurrent noxious ear pinch resulted in a significant reduction in mechanically evoked neuronal firing, with the magnitude of neuronal inhibition of 21%, 31% and 31% for 8g ($p<0.01$), 26g ($p<0.05$), 60g ($p<0.01$) respectively. Additionally, tapentadol significantly inhibited pre-conditioned mechanically evoked neuronal firing (8g: $p<0.01$, 26g: $p<0.05$, 60g: $p<0.01$).

3.3 The role played by spinal 5-HT_7 receptors in DNIC expression in MIA animals

In early phase MIA animals, the 5-HT_7 receptor antagonist SB-269970 reduced the magnitude of DNIC induced neuronal inhibition in the presence of a concurrent noxious ear pinch but did not abolish it. Indeed there was no significant difference in neuronal inhibition before and after drug administration (Figure 4A). Following spinal application of SB-269970, the degree of neuronal inhibition induced by a conditioning ear pinch fell from 45%, 33% and 32% to 15%, 21% and 19% for 8g, 26g and 60g mechanical stimulations respectively (n=6) (Figure 3A). Therefore, the neuronal inhibition was reduced by 12-30% when the actions of serotonin acting at 5-HT_7 receptors in the spinal cord was blocked, yet DNIC remained as there was still a significant reduction in

neuronal firing with a conditioning ear pinch compared to pre-conditioned responses (26g: $p<0.05$, 60g: $p<0.05$).

In late phase MIA animals, the spinal application of the 5-HT_7 agonist AS-19 restored DNIC such that a significant reduction in mechanically evoked neuronal firing was achieved with a concurrent noxious ear pinch in response to 26g and 60g stimulations ($p<0.05$, n=6) (Figure 3B). Following the spinal application of AS-19, the degree of DNIC induced neuronal inhibition was 28%, 34% and 32% in response to 8g, 26g and 60g stimulations, which is comparable to that we would expect to see in naïve animals and sham controls (Bannister et al 2015). Furthermore, preconditioned mechanically evoked neuronal firing was significantly inhibited by AS-19 (8g: $p<0.01$, 26g: $p<0.01$, 60g: $p<0.001$).

3.4 The function of noradrenaline and serotonin in DNIC in the MIA model

In agreement with previous studies, blocking the actions of spinal α_2-adrenergic receptors completely abolishes DNIC while enhancing the descending noradrenergic inhibitory system restores DNIC to similar levels observed in naïve animals, which suggests that a tonic noradrenergic tone is crucial for the expression of DNIC (Bannister et al. 2015, 2017) (Figure 4). Meanwhile, blocking the actions of spinal 5-HT_7 receptors did not abolish DNIC in early phase MIA animals where descending controls are functional, likely because the conditioning stimulus still activates descending noradrenergic signaling to mediate neuronal inhibition. However, when DNIC are absent in late phase MIA animals, activating spinal 5-HT_7 receptors is sufficient to restore neuronal inhibition to similar levels observed in naïve animals (Figure 4). Interestingly, spinal application of AS-19 had limited inhibitory effects on either pre-conditioned or DNIC responses in sham controls (data not shown), which indicates the function or activation properties of spinal 5-HT_7 receptors may be modulated in the sensitised state.

3.5 The expression of α_2-adrenergic and 5-HT_7 receptors in MIA animals

The absence of DNIC in late phase MIA animals strongly suggests an imbalance in descending inhibitory and facilitatory controls exists, specifically a reduction in noradrenergic controls acting at α2-adrenergic receptors and enhanced serotonergic controls acting at 5-HT_3 receptors in the spinal cord. One mechanism through which this imbalance may be mediated could be an increase or decrease in the expression of

receptors in the dorsal horn, such that the release of monoaminergic neurotransmitters into the spinal cord will subsequently produce larger or smaller effects. However, we found no significant difference in the mRNA expression of α2-adrenergic and 5-HT_7 receptors between MIA and sham groups in either the lumbar DRGs or dorsal horn, suggesting the imbalance in descending controls is not a due to an up- or down-regulation of these receptors (Figure 5).

Discussion

This study demonstrates that assessing DNIC responses provides a valuable insight into the functionality of descending controls in chronic pain states. Previous studies have demonstrated that the 2mg MIA model produces mechanical hypersensitivity at the hind paw; therefore this site of secondary hyperalgesia was stimulated during electrophysiological experiments. The presence of DNIC was confirmed in early phase MIA animals by a reduction in mechanically evoked neuronal firing in the presence of a noxious conditioning ear pinch, indicating that descending controls remained functional. On the other hand, DNIC are absent in late phase MIA animals, which is in agreement with pre-existing evidence that central changes and variations in descending controls develop as the model progresses (Burnham and Dickenson 2013). Firstly, although NSAIDs prove effective at relieving pain in early phase MIA animals, as the MIA model progresses animals develop NSAID resistant pain, which reflects the situation in the clinic as targeting the inflammation in the joint only provides pain relief in a portion of patients (Fernihough et al. 2004; Havelin et al. 2016). Furthermore, a reduction in descending noradrenergic inhibition and an enhanced descending serotonergic facilitation acting at spinal 5-HT_3 receptors have been demonstrated in a late phase 2mg MIA model (Rahman et al. 2009; Burnham and Dickenson 2013). This dysregulation in descending inhibitory and facilitatory controls has been proposed to the cause of a loss of DNIC in an animal model of neuropathy (Bannister et al. 2015). Specifically, the authors found they could reveal DNIC by blocking spinal 5-HT_3 receptors with the antagonist ondansetron, and suggest that an enhancement of descending faciliatory serotonergic actions coupled with a reduced noradrenergic control compromises the ability to induce DNIC (Bannister et al. 2015). A clinical study found patients with severe knee OA pain had significantly less CPM than healthy controls; therefore it is important to understand the pharmacological basis of DNIC/CPM and how this may be manipulated to restore effective endogenous analgesia in OA patients that do not respond to traditional analgesics (Arendt-Nielsen et al. 2010).

Blocking the actions of spinal α_2-adrenergic receptors completely blocked neuronal inhibition induced by a noxious conditioning ear pinch in early phase MIA animals, which indicates that DNIC expression is reliant upon inhibitory actions of noradrenaline in the spinal cord. An enhanced descending noradrenergic inhibitory drive has been reported in early phase MIA animals, which may explain why the spinal application of atipamezole significantly facilitated pre-conditioned mechanically evoked neuronal responses (Burnham and Dickenson 2013). We also found that Tapentadol restored DNIC in late phase MIA animals. Tapentadol has a dual mode of action, acting as both an NRI and MOR agonist, but previous studies have demonstrated that the NRI contributions of tapentadols actions are predominant in sensitised states (Tzschentke et al. 2007; Schroder et al. 2010; Bee et al. 2011). Therefore, although DNIC are thought to function through a partially opioidergic mechanism, we propose that tapentadol can restore DNIC predominantly through its ability to increase the synaptic content of noradrenaline, which can subsequently activate spinal α_2-adrenergic receptors to mediate neuronal inhibition (Le Bars et al. 1981). Overall, our study confirms that variations in descending noradrenergic inhibitory tone over the course of the MIA model play a principal role in the resultant expression of DNIC.

DNIC is thought to have a serotonergic component as serotonin transporter gene polymorphisms affect the magnitude of CPM in healthy subjects (Lindstedt et al. 2011). However, the predominant inhibitory or faciliatory actions of serotonin are much more complex to unravel due to the myriad of spinal receptors that can be activated. The 5-HT_7 receptor has been demonstrated to play an inhibitory role in uninjured animals, as blocking the actions of spinal 5-HT_7 receptors blocked the antinociceptive effects observed following both a systemic or RVM microinjection of morphine (Dogrul and Seyrek 2006; Dogrul et al. 2009). We found that while the selective 5-HT_7 receptor antagonist SB-269970 reduced the degree of neuronal inhibition induced by a noxious conditioning ear pinch, it did not abolish DNIC expression in early phase MIA animals. However, in late phase MIA animals where DNIC are absent the spinal application of the selective 5-HT_7 receptor agonist AS-19 restored DNIC induced neuronal inhibition to a similar degree observed in sham controls. Interestingly, a previous study demonstrated that the spinal application of selective serotonin reuptake inhibitors (SSRIs) restored the expression of DNIC in an animal model of neuropathy through inhibitory actions at spinal 5-HT_7 receptors (Bannister et al. 2017). Yet, when the spinal actions of noradrenaline acting at α_2-adrenergic receptors were completely blocked with

atipamezole, SSRIs could no longer reveal neuronal inhibition (Bannister et al. 2017). These findings appear to agree with our study as in early phase MIA animals, which are thought to have functional descending noradrenergic inhibitory controls, blocking the actions of 5-HT_7 receptors cannot abolish DNIC as the noxious conditioning stimulus can still activate descending noradrenergic signaling to activate spinal α_2-adrenergic receptors and mediate neuronal inhibition. However, in late phase MIA animals, where the descending noradrenergic inhibitory system is reported to be reduced, the activation of spinal 5-HT_7 receptors is sufficient to override the imbalance in descending controls and produce DNIC induced neuronal inhibition.

The 5-HT_7 receptors in the dorsal horn are localized to opiodergic and GABAergic interneurons, suggesting the release of serotonin in the spinal cord may mediate neuronal inhibition through the activation of inhibitory interneurons (Brenchat et al. 2010; Viguier et al. 2012). A previous study with the MIA model found that the antinociceptive effects of the Serotonin Noradrenaline Reuptake Inhibitor (SNRI) milnacipran can be preferentially mediated by either noradrenergic or serotonergic signaling depending upon the stage of the MIA model, with the late phase acting through an increased descending serotonergic inhibitory drive acting at 5-HT_7 receptors (Burnham and Dickenson 2013). The fact that blocking the actions of 5-HT_7 receptors only has a small effect in early phase MIA animals yet can completely restore DNIC in late phase animals suggests that a compensatory upregulation of 5-HT_7 receptor mediated inhibition may occur when noradrenergic inhibitory controls are reduced in states of sensitisation.

One mechanism through which an imbalance in descending controls may be mediated is through alterations in the expression levels of noradrenergic or serotonergic receptors in the dorsal horn, such that the release of monoaminergic neurotransmitters into the spinal cord subsequently produces larger or smaller effects. Indeed, an increase in 5-HT_7 receptor density was demonstrated in the ipsilateral dorsal horn of mice with partial sciatic nerve ligation, suggesting a compensatory mechanism whereby receptor expression was increased to mediate inhibition (Brenchat et al. 2010). However, we found no significant differences in the mRNA expression of α_2-adrenergic or 5-HT_7 receptors in the ipsilateral lumbar dorsal horn or DRGs in either early or late phase MIA animals. The lack of changes in mRNA expression suggests that the imbalance in descending inhibitory and facilitatory controls resulting in a loss of DNIC are not necessarily a result of an altered number of receptors in the spinal cord. As the

monoaminergic descending controls modulate nociceptive transmission at the level of the spinal cord mainly through volume transmission, the most likely mechanism for the imbalance in descending controls is alterations in supraspinal structures and the subsequent decreased release of noradrenaline and an increased release of serotonin from the brainstem (Todd 2010). However, although this study indicates there are no changes in the mRNA receptor expression, other post-translational changes may occur and influence the binding affinity or down-stream signaling of receptors at the level of the spinal cord. It should also be noted that changes in mRNA expression may occur in specific areas of the dorsal horn, and that neuronal cells only constitute a portion of the cellular composition of the dorsal horn and DRGs, and therefore subtle changes in receptor expression may be missed with this technique (Thakur et al. 2014).

Importantly, our study confirms that DNIC and CPM share similar pharmacology. Clinical studies in patients with diabetic neuropathy have reiterated that CPM rely upon noradrenergic and serotonergic signaling. Firstly, similarly to our study, tapentadol activated abolished CPM responses while providing significant pain relief in patients (Niesters et al. 2014). Secondly, patients CPM responses were found to predict the likelihood of them responding to the serotonin noradrenaline reuptake inhibitor (SNRI) duloxetine, such that duloxetine was most effective in patients with an inefficient CPM (Yarnitsky et al. 2012). These findings are in agreement with our study, that analgesics that can activate reduced descending inhibitory pathways may prove the most effective in patients with central changes. Interestingly, SSRIs have proved relatively ineffective in the clinic, which may be due to serotonin mediating both inhibitory and facilitatory actions. As our study indicates that noradrenaline signaling is critical for DNIC expression, selective NRIs or SNRIs may prove the most effective at restoring endogenous inhibition. Furthermore, these clinical studies highlight how valuable insights into a patients physiology can be obtained from assessing CPM responses, and could lead the way towards patient segmentation and a personalised medicine approach such that through extra diagnostic tests could ensure each individual patient receives the most appropriate analgesic.

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Figure 1 – DNIC expression in early and late phase MIA animals and sham controls. A) A conditioning noxious ear pinch produced a significant reduction in mechanically evoked neuronal firing in ipsilateral WDR neurons in early phase MIA animals (n=19). B) In late phase MIA animals a conditioning noxious ear pinch no longer produces a reduction in mechanically evoked neuronal firing in ipsilateral WDR neurons (n=42). C) A conditioning noxious ear pinch produced a significant reduction in mechanically evoked neuronal firing in ipsilateral WDR neurons in sham controls (n=27). D-F) A representative tract from ipsilateral WDR neurons in early phase MIA, late phase MIA and sham animals, showing 3 baseline pre-conditioned responses and a DNIC response with a concurrent noxious ear pinch. Two-way ANOVA with Bonferroni correction. **P<0.01, ***P<0.001.

Figure 2 –The pre-conditioned and Diffuse Noxious Inhibitory Controls response profiles of ipsilateral WDR neurons before and after spinal application of atipamezole (100μg/50μL) or subcutaneous tapentadol (2mg/kg) in early and late phase MIA animals respectively. A) In early phase MIA animals, the spinal application of the α_2-adrenergic receptor antagonist atipamezole stopped the noxious conditioning ear pinch from producing a reduction in mechanically evoked neuronal firing, and also facilitated pre-conditioned neuronal responses (n=5). B) In late phase MIA animals, a subcutaneous injection of tapentadol restored DNIC induced neuronal inhibition, such that there was a significant reduction in mechanically evoked neuronal firing with a

concurrent noxious ear pinch (n=6). Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

Figure 3 - The pre-conditioned and Diffuse Noxious Inhibitory Controls response profiles of ipsilateral WDR neurons before and after spinal application of SB-269970 (100µg/50µL) or AS-19 (100µg/50µL) in early and late phase MIA animals respectively. A) In early phase MIA animals, the spinal application of the 5-HT_7 receptor antagonist SB-269970 reduced the magnitude of neuronal inhibition induced by a conditioning noxious ear pinch but both before and after application there remained a significant reduction in mechanically evoked neuronal firing with concurrent ear pinch (n=6). B) In late phase MIA animals, the spinal application of the 5-HT_7 receptor agonist AS-19 restored DNIC induced neuronal inhibition, such that a noxious conditioning ear pinch produced a significant reduction in mechanically evoked neuronal firing (n=6). Two-way ANOVA with Bonferroni correction. *P<0.05, **P<0.01, ***P<0.001.

Figure 4 – Conditioned responses (as a percentage of the baseline) in early and late phase animals. A) In early phase MIA animals, the spinal application of atipamezole completely abolished DNIC induced neuronal inhibition while the spinal application of SB-269970 only partially reversed it (atipamezole n=5, SB-269970 n=6). B) In late phase MIA animals, both the subcutaneous injection of tapentadol and the spinal application of AS-19 significantly restored neuronal inhibition to levels comparable to that observed in sham controls (tapentadol n=6, AS-19 n=6). Kruskall-Wallis. *P<0.05, **P<0.01, ^P<0.05, ^^P<0.01.

Figure 5 – The mRNA expression of the α_2-adrenergic and 5-HT_7 receptors in the ipsilateral lumbar dorsal horn and DRGs from early and late phase MIA animals (EP n=4, EPS n=4, LP n=4, LPS n=4). A) In the dorsal horn, there is no significant change in the mRNA expression levels of the α_2-adrenergic or 5-HT_7 receptors between groups. B) In the L3-L5 DRGS, there is no significant change in the mRNA expression levels of the α_2-adrenergic or 5-HT_7 receptors between groups. Kruskall-Wallis.