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Kappa Opioid Signaling in the Right Central Amygdala Causes Hindpaw Specific Loss of Diffuse Noxious Inhibitory Controls (DNIC) in Experimental Neuropathic Pain

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Abstract

Diffuse noxious inhibitory controls (DNIC) is a pain inhibits pain phenomenon demonstrated in humans and animals. DNIC is diminished in many chronic pain states, including neuropathic pain. The efficiency of DNIC has been suggested to prospectively predict both the likelihood of pain chronification and treatment response. Little is known as to why DNIC is dysfunctional in neuropathic pain. Here, we evaluated DNIC in the rat L5/L6 spinal nerve ligation (SNL) model of chronic pain using both behavioral and electrophysiological outcomes. For behavior, nociceptive thresholds were determined using response to noxious paw pressure on both hindpaws as the test stimulus before, and after, injection of a conditioning stimulus of capsaicin into the left forepaw. Functionally, the spike firing of spinal wide dynamic range (WDR) neuronal activity was evaluated before and during noxious ear pinch, whilst stimulating the ipsilateral paw with von Frey hairs of increased bending force. In both assays, the DNIC response was significantly diminished in the ipsilateral (i.e., injured) paw of SNL animals. However, behavioral loss of DNIC was not observed on the contralateral (i.e., uninjured) paw. Systemic application of nor-Binaltorphimine (nor-BNI), a kappa opioid antagonist, did not ameliorate SNL-induced hyperalgesia but reversed loss of the behavioral DNIC response. Microinjection of nor-BNI into the right central amygdala (RCeA) of SNL rats did not affect baseline thresholds but restored DNIC both behaviorally and electrophysiologically. Cumulatively, these data suggest that net enhanced descending facilitations may be mediated by kappa opioid receptor signaling from the RCeA to promote diminished DNIC following neuropathy.

Keywords: neuropathic pain; Amygdala; Kappa opioid receptor; Diffuse Noxious Inhibitory Controls; conditioned pain modulation; Descending Pain Modulation

Introduction

Neuropathic pain is difficult to manage in a large number of patients [25; 37]. Mechanisms of underlying pathophysiology are multiple, likely contributing to relatively poor outcomes of present therapy [12]. Alterations in central nociceptive modulation, particularly in descending brainstem control systems, have been suggested to promote chronic pain [31; 32].

DNIC is a reliable measure of net descending inhibition. In humans, a correlate of DNIC called conditioned pain modulation (CPM) is assessed by the ability of a remotely applied 'conditioning' stimulus to inhibit the subject's pain response to a 'test' stimulus. In healthy controls, co-application of the 'conditioning' and 'test' stimuli elicits analgesia to the 'test' stimulus [42]. Interestingly, the CPM/DNIC response is decreased or lost in a number of chronic pain types [6; 20; 26], including neuropathic pain [11; 36; 38; 44]. Patients with the least efficient CPM response prior to a scheduled surgery showed the greatest likelihood of development of chronic pain [43] as well as the best responses to therapies that mimic descending pain inhibition, such as duloxetine [44].

The causes of dysfunctional CPM/DNIC in neuropathic pain states are unknown. Whilst the subnucleus reticularis dorsalis (SRD) and not the rostral ventral medulla (RVM), is a vital component of the DNIC response in naïve rats [3], inactivating the RVM restores DNIC in a rat model of functional pain [30]. Additionally, blocking 5-HT_3 mediated descending facilitations restored the DNIC response in a model of neuropathic pain [2]. Together, these studies suggest

that absent DNIC expression could result from facilitation of pain, which masks an apparently normal inhibitory response mediated by the SRD. However, mechanisms underlying DNIC above the brainstem remain virtually unknown and how facilitatory drive from higher centers may impinge on the RVM is unclear.

Increased endogenous kappa opioid receptor (KOR) signaling in the brain is part of the canonical stress response [18; 35; 39]. In a rat model of functional pain, stress-induced allodynia and loss of DNIC was blocked by nor-BNI, a KOR antagonist given systemically or in the right central amygdala (RCeA) [27], suggesting that KOR signaling in the RCeA drives facilitation of pain, and loss of DNIC, in uninjured but sensitized states. However, when allodynia is present in the absence of an external psychological stressor, as is the case in neuropathic pain, it is unknown whether the loss of the DNIC response is similarly caused by increased RCeA KOR signaling. There is sustained, increased neuronal activity in the RCeA in neuropathic pain animals [9] supportive of a role of this region in neuropathic pain pathology. Additionally, unlike functional pain, some reports suggest that the loss of DNIC in neuropathic pain might be localized to particular areas of the body associated with injury [10; 38]. Whether and how this may occur in experimental models is unknown.

Here, the effect of systemic nor-BNI on DNIC was investigated behaviorally on both the ipsilateral- and contralateral hindpaw of SNL rats. Effects of microinjection of nor-BNI in the RCeA on DNIC was also investigated with both behavioral and electrophysiological analyses.

Methods

Animals

Male, Sprague Dawley rats, 250g to 350g at time of testing (Envigo, Indianapolis, IN) or 120-140g (UCL Biological Services, London, UK) were used for behavioral and electrophysiological experiments, respectively. Rats were group housed on a 12-hour light-dark cycle (lights on at 0700) with food and water ab libitum. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona or the Home Office, UK with adherence to the Animal (Scientific Procedures) Act 1986. All experiments were in accordance with guidelines from the International Association for the Study of Pain and animals were monitored throughout studies to reduce unnecessary stress or pain.

Surgeries

Spinal Nerve Ligation (SNL)

L5/L6 ligation were performed as described by Kim and Chung [15]. Briefly, under anesthesia (5% induction, 2% maintenance isoflurane at 2L/min) an approximately 2 cm incision was made to the left of the midline and the L5 and L6 nerves of the left hindlimb were exposed and tightly ligated with 4-0 or 6-0 silk sutures (Henry Shein Inc., USA). For behavioral experiments, muscle was closed with 4-0 silk suture (CP Medical, USA) and the skin closed with wound clips (MikRon Precision Inc, USA). For electrophysiolgical experiments, 3-0 absorbable sutures were used for both muscle and skin closure. Sham surgery was the same, except the L5 and L6 were not ligated. Post-surgery all behavioral animals were given one dose of gentamycin (8 mg/kg, s.c., VetOne, USA).

Cannulas

For behavioral studies, stereotaxic surgeries were performed on the same day as sham/SNL. Animals were anesthetized by i.p. ketamine/xylazine (80/12 mg/kg; Western Medical Supply, USA/ Sigma-Aldrich, USA) and the head was fixed in ear bars. Cannula (26 gauge, PlasticsOne, USA) were inserted into the right central amygdala (RCeA) using brain loci coordinates obtained from Paxino and Watson's brain atlas [33] (-4.0mm ML, -2.0mm AP, and -7.0mm DV from Bregma). Rats were allowed to recover for at least 10 days before behavioral testing. Electrophysiology studies microinjected drug on the day of testing.

All cannula placements were verified post mortem and animals with incorrect placement were excluded from all analysis. Verification entailed injection of black dye, harvesting of brain and then fixation in 10% formalin for at least 24 hours with 30µm sections cut in the area of interest and placement verified with reference to Paxinos and Watson's brain atlas [33].

Diffuse Noxious Inhibitory Controls (DNIC)

Behavioral

Nociceptive thresholds were determined pre- and 2-3 weeks post-surgery using Randall Selitto (Ugo Basile, Italy). After acclimatization in the laboratory for approximately 30 minutes, animals were lightly restrained in a cloth, which covered them entirely, and increasing pressure was applied to the plantar surface of each hind paw. The threshold was determined as the point at which the animal either withdrew its paw, significantly flinched or vocalized. The mean of three readings on each hindpaw was taken as the threshold.

For DNIC experiments nor-BNI (s.c. or microinjection into the RCeA) was given on day 1 at least 20 hours before the DNIC time course. On day 2, post nor-BNI threshold was determined

and then rats were briefly anesthetized (induction 5%, maintenance 2.5%, 2L/min) and capsaicin (125 µg in 50 µL, subdermal) was injected into the left forepaw as described previously [8; 27]. Nociceptive thresholds were then measured at 20, 40, 60 and 90 minutes post-capsaicin.

Electrophysiology

In vivo electrophysiology experiments were conducted on postoperative days 14 to 18 (sham and SNL-operated animals) or on weight-/age-matched naive rats as previously described [1; 2]. Briefly, animals were anesthetized and maintained for the duration of the experiment with isofluorane (1.5%) delivered in a gaseous mix of N_2O (66%) and O_2 (33%). A laminectomy was performed to expose the L4 and L5 segments of the spinal cord. Extracellular recordings were made from deep dorsal horn neurons (laminae V–VI) using parylene-coated tungsten electrodes (A-M Systems, Sequim, WA). All the neurons recorded were wide dynamic range (WDR) and responded to natural stimuli in a graded manner with coding of increasing intensity. The peripheral receptive field was stimulated using punctate mechanical stimuli (von Frey filaments: 8, 26, and 60g), and the number of action potentials fired in 5 seconds was recorded. Data were captured and analyzed by a CED 1401 interface coupled to a Pentium computer with Spike2 software (Cambridge Electronic Design; rate functions). Three baseline responses to mechanical stimuli were characterized for each neuron before DNIC and subsequent pharmacological assessment (a drug study was conducted on 1 neuron per animal only). Concisely, extracellular recordings were made from 1 WDR neuron per animal by stimulating the hindpaw peripheral receptive field and then repeating in the presence of ear pinch. The number of action potentials fired in 5 seconds was recorded for each test. Baseline responses were calculated from the mean of 2 trials. Each trial consisted of 3 consecutive stable responses to 8, 26, and 60g von Frey filaments applied to the hindpaw (where all neurons met the inclusion criteria of 10% variation

in action potential firing for all mechanically evoked neuronal responses). This was then followed by consecutive responses to the same mechanical stimuli (8, 26, and 60g von Frey filaments) in the presence of the conditioning stimulus.

DNIC was induced using a noxious ear pinch (15.75 3 2.3 mm Bulldog Serrefine; InterFocus, Linton, United Kingdom) on the ear ipsilateral to the neuronal recording, whilst concurrent to this, the peripheral receptive field was stimulated using the von Frey filaments listed. A DNIC response was quantified as an inhibitory effect on neuronal firing during ear pinch. A 1-minute non-stimulation recovery period was allowed between each test in the trial. After this, for pre-drug neuronal recordings, a 10-minute non-stimulation recovery period was allowed before the entire process was repeated and data for control trial number 2 were collected.

Drugs

Nor-BNI (Tocris, UK) was dissolved in saline and administered either subcutaneously at 3 mg/kg, 1 ml/kg dose/volume or 5 µg/µL into right central amygdala (2.5 µg in a volume of 0.5 µL) as previously described [27; 40]. Capsaicin (Sigma-Aldrich, USA) was dissolved in 1:1 Tween 80 (Sigma-Aldrich, USA) and 100% ethanol (Decon, USA) to make a stock solution of 50 µg/µL. This was diluted in 0.9% saline to a final concentration of 2.5 µg/µL and 50 µL (125 µg) per rat was injected subdermally into the left forepaw (as previously described [8; 24; 27]). This solution was made 40 minutes prior to injection and stored at -20ºC until use.

Statistical Analysis

All graphs were created and one-way analysis of variance (ANOVA) and t-tests were performed in GraphPad Prism (7.0, USA). For repeated measures ANOVA, SPSS Statistics (24, IBM) was used. Between subject factor was GROUP and within subject was TIME and/or TREATMENT.

Where significance was seen a Bonferroni post hoc test was performed. All data are shown as mean ± SEM and significance was set at $p<0.05$.

Results

SNL produced an ipsilateral loss of DNIC that was restored by systemic blockade of KOR without any effect on baseline mechanical threshold

Previous work from our laboratory, has shown that systemic nor-BNI has no effect on mechanical allodynia in the ipsilateral paw [28]. To determine if this was also the case for mechanical hyperalgesia, vehicle or nor-BNI were administered 24 hours prior to post-surgery and baseline responses to noxious pressure applied to the paw, which were measured using the Randall Selitto test. Post-surgery, SNL animals had a significantly lower nociceptive threshold on the ipsilateral paw in the Randall Selitto test compared to sham, as there was a significant main effect of TIME*GROUP ($F(1.59,35.0)=1.43$ $p<0.001$, Figure 1a). This was not alleviated by nor-BNI (3 mg/kg, s.c.), administered 24 hours prior, as there was no significant TIME*GROUP*TREATMENT effect. To test the DNIC response, the TRPV1 agonist capsaicin was injected into the left forepaw as a 'conditioning stimulus' to induce pain at a distinct site from the hindpaw test area. There was a significant effect of TIME*GROUP*TREATMENT ($F(4,88)=4.11$, $p=0.004$) in the hindpaw. In the sham-vehicle group, capsaicin induced a significant increase in nociceptive thresholds with a significant effect at 20 minutes post-capsaicin ($p<0.001$, Figure 1b) indicating a DNIC response. We then evaluated the DNIC response in the presence of nor-BNI to determine if kappa opioid signaling had any effect on DNIC under control conditions. DNIC in sham controls was not affected by nor-BNI (s.c)

administered approximately 24 hours prior to testing, as there was a similar significant effect at 20 minutes ($p<0.001$) and no significant difference between vehicle and nor-BNI treated animals at this time point (Figure 1b,c).

In SNL animals there was a significant loss of DNIC in the ipsilateral (left, injured) paw, as post-capsaicin there was no significant increase in hindpaw threshold at any measured time-point (Figure 1b). To determine if kappa opioid signaling is involved in this loss of DNIC, SNL animals were pre-treated with either vehicle or nor-BNI (s.c.). Nor-BNI significantly restored the DNIC response, as at the 20 minute time point nor-BNI treated SNL rats had a significantly higher threshold than vehicle treated SNL rats ($p=0.02$, Figure 1b) and there was no significant difference in threshold between sham and nor-BNI treated SNL.

There was no loss of DNIC in the contralateral paw of SNL animals

While DNIC is defined as "diffuse noxious inhibitory controls", the loss of DNIC has been shown in some reports to be specific to the site of pain [38]. To test if this is the case in the SNL model, the contralateral paw was also tested. There was no significant effect of surgery or nor-BNI on the contralateral paw baseline thresholds (Figure 1d). In contrast to the ipsilateral paw, there was a significant DNIC response in the contralateral paw of both sham and SNL animals. There was a significant main effect of TIME ($F(2.90, 63.70)=39.14$, $p<0.001$) but no significant interaction between GROUP or TREATMENT (Figure 1e). In all groups there was a significant increase in paw withdrawal threshold at 20 minutes compared to baseline, indicating a robust DNIC response (sham-vehicle $p<0.001$, sham-nor-BNI $p=0.04$, SNL-vehicle $p=0.004$, SNL-nor-BNI $p<0.001$, Figure 1e, f).

Loss of behavioral DNIC in SNL was restored by nor-BNI administered into the RCeA without effect on baseline mechanical hyperalgesia

Sustained increase in neuronal excitability in the RCeA has been reported in animal models of neuropathic pain [9] and is indicative of contributions to the maintenance of pain. Prior work in our laboratory has shown that, similar to systemic administration, microinjection of nor-BNI into the RCeA had no effect on mechanical allodynia in the von Frey test [28]. Here, it was determined if this was also the case for mechanical hyperalgesia. On the ipsilateral hindpaw, there was a significant effect of TIME ($F(2,36)=50.48$, $p<0.001$) with a significant reduction in mechanical threshold post-surgery, in SNL only ($p<0.001$, Figure 2a) demonstrating that SNL had significant hyperalgesia. Post-surgery, approximately 24 hours post-injection, there was no significant effect of microinjection of nor-BNI into the RCeA on mechanical paw withdrawal threshold of either sham or SNL (Figure 2a). Therefore, nor-BNI administered into the RCeA does not significantly affect either innocuous [28] or noxious mechanical thresholds on the ipsilateral paw at baseline.

Sham-operated animals had a significant DNIC response. On the ipsilateral paw, there was a main effect of TIME (ipsilateral ($F(4, 72) = 93.73$), $p<0.001$) and post-hoc analysis revealed a significant increase in hindpaw withdrawal threshold at 20 minutes after forepaw capsaicin injection in sham-vehicle and sham-nor-BNI animals (both $p<0.001$ Figure 2b). The magnitude of this was unaffected by nor-BNI administration into the RCeA as there was no significant difference between vehicle and nor-BNI treated animals at any measured time-point in either paw. This suggests that antagonism of kappa opioid signaling in the RCeA has no effect on DNIC under control conditions.

Replicating our systemic results, there was a significant loss of DNIC in SNL animals that was localized to the ipsilateral and not contralateral paw. There was a significant effect of TIME*SURGERY*TREATMENT on the ipsilateral paw ($F(4,72)=13.96$, $p<0.001$). To determine if kappa opioid signaling in the RCeA is responsible for this loss of DNIC, SNL animals were microinjected with nor-BNI into the RCeA, approximately 24 hours prior to DNIC testing. This significantly restored the DNIC response in SNL rats. This was seen as a significant increase in paw withdrawal threshold in SNL-nor-BNI animals at 20 mins ($p<0.001$) and a trend to still be increased at 40 mins ($p=0.055$) without any significant effect of capsaicin on SNL-vehicle at any time-point (Figure 2b). SNL-nor-BNI had significantly greater thresholds than SNL-vehicle at 20 mins ($p<0.001$, Figure 2b, c) and 40 mins ($p=0.047$, Figure 2b). This suggests that heightened kappa opioid signaling in the RCeA promotes, in part, the loss of DNIC in neuropathic pain. Although, unlike systemic dosing, the DNIC response in SNL-nor-BNI was significantly lesser than sham-nor-BNI animals (one way ANOVA $F(3,18)=47.89$, $p<0.0001$, sham-nor-BNI compared to SNL-nor-BNI $p=0.035$, Figure 2c).

There were no significant effects of surgery or nor-BNI on contralateral baseline mechanical thresholds in either sham or SNL animals (Figure 2d) and again no loss of DNIC was seen on the contralateral paw, with a significant effect of TIME ($F(4,71)=171.14$), $p<0.001$) and an increase in mechanical threshold at 20 mins in both SNL-vehicle ($p<0.001$) and SNL-nor-BNI ($p<0.001$) (Figure 2e,f).

Blocking kappa opioid receptor activity in the RCeA did not affect baseline sensory thresholds or response profiles of WDR neurons

DNIC was originally described in wide dynamic range (WDR) neurons [19]. To determine if the effects seen behaviorally were reflected in WDR responses, we recorded baseline WDR

responses, responses of WDR neurons to a test stimulus in the presence of a conditioning stimulus (i.e., the DNIC response) and subsequently the effect of nor-BNI micro-injection on baseline and DNIC WDR responses. Micro-injection of nor-BNI into the RCeA did not impact baseline spinal WDR neuronal firing rates to innocuous or noxious mechanical, thermal or electrical stimulation (Figure 3).

Subsequently the DNIC response was tested in these animals using concomitant stimulation of the peripheral receptive field (hindpaw) with von Frey filaments of increasing bending force (8, 26 and 60g) and application of the conditioning stimulus (noxious ear pinch). In control (grouped naïve and sham-operated) animals, as observed previously [2], application of the conditioning stimulus caused a significant reduction in WDR neuronal firing in response to stimulation of the hindpaw for all forces applied ($F(1,4) = 22.92$, $p=0.002$; 8g $p = 0.0061$, 26g $p = 0.026$, 60g $p = 0.044$, Figure 4a). A similar effect was seen in control animals pretreated with nor-BNI ($F(1,4)=303.92$ $p=0.004$; 8g $p=0.01528$, 26g $p= 0.0199$, 60g $p= 0.01199$, Figure 4b) suggesting there was no significant effect of nor-BNI on DNIC in this group.

In contrast, in SNL-vehicle treated animals, there was no significant DNIC response as no decrease in WDR neuronal response was observed during application of the conditioning stimulus (Figure 4c). However, when pre-treated with micro-injection of nor-BNI in the RCeA, a significant decrease in WDR neuronal firing was recorded upon application of the conditioning stimulus. There was a significant effect of TIME ($F(1, 5) = 135.78$, $p<0.001$) and post hoc tests showed a significant reduction in WDR neuronal firing at all forces under DNIC conditions (8g $p=0.0007$, 26g $p= 0.0002$, 60g $p=0.002$, Figure 4d). This suggests that kappa opioid signaling in the RCeA of SNL animals contributes to diminished DNIC expression (as recorded in our behavioral assays) via WDR neuronal activity.

Discussion

The relationship between stress and pain is evident in functional pain disorders, in which stressful episodes result in pain seemingly without prior noxious input/damage [4; 13; 17; 22; 34]. Yet in disorders of known etiology, such as neuropathic pain, the impact of the ongoing *physiological* stress of pain is unclear. Data presented here suggests that part of the canonical stress response, an increase in kappa opioid signaling in the CeA [18; 35; 39], underlies loss of DNIC in the ipsilateral paw of an animal model of neuropathic pain in the absence of an external psychological stressor. As CPM/DNIC has been postulated to represent 'the endogenous analgesic capacity of the individual' [41], this suggests that kappa opioid signaling in the RCeA may promote ongoing neuropathic pain through reduction of this analgesic capacity, by altering the balance to favor descending facilitation over inhibition.

The mechanism underlying loss of DNIC in neuropathic pain is poorly understood despite a number of clinical studies reporting dysfunctional CPM/DNIC in patients [10; 11; 29; 36; 44]. DNIC inhibits pain through a spino-bulbo-spinal loop [41], with a key nucleus being the SRD in the medulla and not the RVM [3]. Yet in functional pain models, inactivation of the RVM restores the DNIC response [30] suggesting competing descending modulation with facilitation and inhibition arising respectively from the RVM and SRD [30]. Whether this is the case in neuropathic pain remains unclear. One study suggests that impaired descending inhibition is the cause of the loss of DNIC in SNL animals, with lesser activation of the locus coeruleus, and subsequently lower levels of noradrenaline in the spinal cord, compared with sham controls during the DNIC [16]. This would fit well with another study that suggests a role for an appropriate balance of descending facilitation and inhibition. In this study blocking descending facilitation with ondansetron, the 5-HT_3 antagonist, restored DNIC in SNL rats [2]. DNIC was

also restored by increasing descending inhibition, with inhibitors of noradrenaline uptake [2], thus implicating a role of balance between descending inhibition and facilitation [1; 2].

A difference between the loss of DNIC observed in experimental neuropathic versus functional pain is the need for an external stressor (such as bright lights) prior to testing DNIC and allodynia in the injury-free model [27]. Stress is known to activate kappa opioid signaling through release of endogenous dynorphin in the brain, including the amygdala [18; 35]. The RCeA is also a key locus in chronic pain, evident by ongoing excitability in neuropathic pain [9] and enlarged receptor field sizes after the induction of arthritis in either hind leg in an animal model [14]. As pain may involve activation of a number of physiological stress responses [4], it was hypothesized that kappa opioid signaling in the RCeA could also underlie the loss of DNIC in SNL animals. This possibility was supported by previous studies of reduced DNIC in ethanol and oxiplatin induced neuropathic pain that was restored by adrenal medullectomy, a treatment that had no effect in uninjured animals [8]. Neuropathic pain may therefore induce a tonic level of physiological stress, or engagement of similar neural circuits, that could contribute to the loss of DNIC.

In this present study, a loss of DNIC was seen in SNL animals on the ipsilateral paw. This was restored to sham control levels by systemic administration of nor-BNI, suggesting a role for kappa opioid receptors in the mechanism underlying dysfunctional DNIC. Furthermore, both behaviorally and functionally, as seen by the Randall-Selitto test and by the activity of WDR neurons respectively, there was a restoration of DNIC following microinjection of nor-BNI into the RCeA. This suggests that the RCeA is a key, but not necessarily the only, locus for the effect of kappa opioid signaling, promoting a loss of the DNIC response likely via a descending

pathway to the brainstem. Other brain regions and mechanisms underlying loss of DNIC above the RVM are still relatively unknown and further investigation of is needed.

Interestingly, the loss of behavioral DNIC after neuropathy was localized to the allodynic ipsilateral, but not contralateral, paw. This is similar to a study in neuropathic pain patients, in which the DNIC response at 'pain' and 'pain-free' areas was examined. In response to a 'conditioning' pain of ischemia in the upper arm or thigh region, there was no DNIC response on brush-evoked pain in the allodynic area [38].

The reasons for the localization of the loss of DNIC are unclear. One possibility is that the loss of the DNIC response in the injured hindlimb could result from a reduction in the input to the spinal cord from the test stimulus due to nerve injury, thus leading to a smaller DNIC response. On the other hand, some observations suggest that this may not be the case. First, following SNL, there is decreased WDR neuronal firing, observed only at the noxious levels of stimulation, i.e., greater than 25 g during baseline testing in animals 14-17 days post-surgery [5]. No changes in WDR neuronal firing are observed using the 8 g force following SNL surgery. However, we see a loss of DNIC in terms of WDR neuronal firing at all forces tested including 8 g, which at baseline evokes the same amount of WDR firing in both sham and SNL rats. This suggests that there is a similar amount of signal transduced from the injured paw as before, so this is unlikely to explain the loss of DNIC. Second, if enhanced descending facilitation is causal in the loss of DNIC [30], possible reduction in peripheral or central signaling would be expected to have little effect. Third, the full magnitude of the DNIC response may not have been determined in our study as ethical considerations dictate a cut-off point of 500 g to prevent tissue damage and often both sham and SNL animals reached this cut-off during DNIC testing. It is therefore possible that at higher forces, a greater DNIC response may be seen in the contralateral paw of sham

compared to SNL. Finally, we note that our previous study in WDR neurons of SNL animals found a loss of DNIC in both ipsilateral and contralateral paws [2]. The reasons for the differences observed in the behavioral and functional output measures are not clear. In a model of osteoarthritis in which DNIC is lost ipsilaterally, we found that the degree of contralateral DNIC depends on the strength of the conditioning stimulus [21]. This may explain some of the differences seen here, since capsaicin is likely to be a stronger stimulus than ear pinch.

We further note that localization of loss of DNIC could be due to other factors that were not examined in the present study including time dependency. Evaluation of DNIC at later time points following SNL surgery could demonstrate a loss of DNIC on the contralateral hindpaw. In this regard, previous work has shown no loss of DNIC on the contralateral paw at 2 weeks, a partial loss at 4 weeks then a full loss at 6 weeks post-SNL surgery [16]. Data from the current study is consistent with this timeline as DNIC was investigated at 2-3 weeks post-surgery. These factors highlight the role of multiple factors in the DNIC response and may help to explain the observation that DNIC and its reduction can be variable in humans. To date, there have been no studies that have investigated CPM/DNIC as a function of time in non-allodynic areas in neuropathic pain patients. This question awaits further investigation. A complication in such studies in patients is that when CPM is tested in non-painful areas, three stimuli are present – the painful area as well as the test and conditioning stimulus.

Despite the restoration of DNIC in the ipsilateral paw, nor-BNI, when administered either into the RCeA or systemically, had no effect on baseline allodynia either on mechanical threshold responses as measured in the Randall Selitto test nor on WDR neuronal firing to natural and electrical stimuli at the 24-hour time-point. In agreement with this, previous data from our laboratory showed no effect of nor-BNI on tactile thresholds in von Frey testing at any time-

point over 24 hours [28]. The lack of effect of nor-BNI on baseline allodynia may be interpreted as a failure to reduce pain. Yet, the restoration of DNIC and our prior finding of relief of the aversive component of pain using evaluation of motivated behavior (conditioned place preference) [28] suggest that blocking kappa opioid signaling may alleviate ongoing pain whilst preserving baseline thresholds. This is advantageous when it comes to consideration of kappa opioid antagonists as therapy for chronic pain, because response to acute pain is physiologically necessary [7] and so preservation of baseline pain thresholds is advantageous for drug therapy. Kappa opioid antagonists may, therefore, be a viable therapy for treating aspects of ongoing neuropathic pain in patients. Also, as kappa opioid antagonists have been advanced to human trials [23] there is potential for relatively quick translation from 'bench to bedside'.

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The authors have no conflicts of interest to declare.

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Figure 1: Ipsilateral Specific Loss of DNIC in SNL is restored by systemic administration of Nor-BNI, without effect on baseline mechanical thresholds (A) Ipsilateral paw withdrawal thresholds pre-surgery, post-surgery and 24 hours post nor-BNI (3mg/kg, s.c.). (B) Time-course of DNIC experiment in the ipsilateral paw. A significant loss of DNIC was seen at 20 minutes post-capsaicin in SNL-vehicle but not in SNL-nor-BNI treated animals. (C) Summary of ipsilateral DNIC response at 20 minutes as a percentage of sham-vehicle. (D) Contralateral paw withdrawal thresholds pre-surgery, post-surgery and 24 hours post nor-BNI (3mg/kg, s.c.). (E) Time-course of DNIC in the contralateral paw. (F) Summary of contralateral DNIC response at 20 minutes as a percentage of sham-vehicle **p<0.01 SNL-vehicle versus SNL-nor-BNI, ## p<0.01 compared to pre-surgery baseline (within group) ++ P<0.01 Sham-vehicle versus SNL-vehicle, PWT: paw withdrawal threshold. Sham-vehicle n=6, Sham-nor-BNI n=5 (1 animal excluded due to experimental error), SNL-vehicle n=7, SNL-nor-BNI n=8 (1 animal excluded for lack of hyperalgesia post-surgery). SNL: spinal nerve ligation, nor-BNI: Norbinaltorphimine

Figure 2: Ipsilateral Specific Loss of DNIC in SNL is restored by administration of Nor-BNI into the RCeA, without effect on baseline mechanical thresholds (A) Ipsilateral paw withdrawal thresholds pre-surgery, post-surgery and 24 hours post nor-BNI (2.5µg/0.5µL in the RCeA). (B) Time-course of DNIC experiment in the ipsilateral paw. A loss of DNIC was seen at

20 minutes post-capsaicin in SNL-vehicle, but was significantly restored in SNL animals pre-treated with Nor-BNI in the RCeA (C) Summary of ipsilateral DNIC response at 20 minutes as a percentage of sham-vehicle. (D) Contralateral paw withdrawal thresholds pre-surgery, post-surgery and 24 hours post nor-BNI (2.5µg/0.5µL in the RCeA) (E) Time-course of DNIC in the contralateral paw. (F) Summary of contralateral DNIC response at 20 minutes as a percentage of sham-vehicle ****p<0.001 SNL-vehicle versus SNL-nor-BNI, #### p<0.001 compared to pre-surgery baseline (within group) ++++ P<0.001 Sham-saline versus SNL-saline, & p<0.05 sham-nor-BNI versus SNL-nor-BNI, PWT: paw withdrawal threshold. n=6 for sham and n=5 for SNL groups. SNL: spinal nerve ligation, nor-BNI: Norbinaltorphimine

Figure 3: Administration of Nor-BNI into the RCeA did not have any effect on baseline WDR thresholds. (A) Control mechanical thresholds (B) Control thermal thresholds (C) Control electrical thresholds (D) SNL mechanical thresholds (E) SNL thermal thresholds (F) SNL electrical thresholds. n=4 for all groups. SNL: spinal nerve ligation, nor-BNI: Norbinaltorphimine

Figure 4: There was a significant loss of the DNIC response in spiking of WDR neurons at all forces applied to the ipsilateral paw. This was restored by microinjection of nor-BNI (A) Control-vehicle (B) Control-Nor-BNI (C) SNL-vehicle (D) SNL nor-BNI *p<0.05 ** p<0.01, ***p <0.001, when DNIC is compared to baseline values. n=4 for naïve groups and n=6 for SNL. BL: baseline, DNIC: diffuse noxious inhibitory control, SNL: spinal nerve ligation, nor-BNI: Norbinaltorphimine