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Morphine amplifies mechanical allodynia via TLR4 in a rat model of spinal cord injury

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Abstract

Central neuropathic pain (CNP) is a pervasive, debilitating problem that impacts thousands of people living with central nervous system disorders, including spinal cord injury (SCI). Current therapies for treating this type of pain are ineffective and often have dose-limiting side effects. Although opioids are one of the most commonly used CNP treatments, recent animal literature has indicated that administering opioids shortly after a traumatic injury can actually have deleterious effects on long-term health and recovery. In order to study the deleterious effects of administering morphine shortly after trauma, we employed our low thoracic (T13) dorsal root avulsion model (Spinal Neuropathic Avulsion Pain, SNAP). Administering a weeklong course of 10 mg/kg/day morphine beginning 24 hr after SNAP resulted in amplified mechanical allodynia. Co-administering the non-opioid toll-like receptor 4 (TLR4) antagonist (+)-naltrexone throughout the morphine regimen prevented morphine-induced amplification of SNAP. Exploration of changes induced by early post-trauma morphine revealed that this elevated gene expression of TLR4, TNF, IL-1 β , and NLRP3, as well as IL-1 β protein at the site of spinal cord injury. These data suggest that a short course of morphine administered early after spinal trauma can exacerbate CNP in the long term. TLR4 initiates this phenomenon and, as such, may be potential therapeutic targets for preventing the deleterious effects of administering opioids after traumatic injury.

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Keywords

TLR4; inflammasome; proinflammatory cytokines; (+)-naltrexone; interleukin-1 β ; NLRP3; tumor necrosis factor; neuropathic pain; rats

1. Introduction

Spinal cord injury (SCI) is the leading cause of central neuropathic pain (CNP) and is often intractable to treatment. Current therapies, including opioids, provide only ~50% pain relief in 1 out of 2–3 SCI patients (Finnerup et al., 2001). Although opioids are one of the most effective analgesics for CNP (Woller and Hook, 2013), recent rodent and clinical studies have shown that opioids administered soon after trauma can be detrimental to health and recovery in some circumstances (Grace et al., 2016; Hook et al., 2007; Hook et al., 2009; Loram et al., 2012; Salengros et al., 2010).

In order to study central neuropathic pain, we have developed and optimized a unilateral low thoracic (T13)/high lumbar (L1) dorsal root avulsion model of SCI that does not cause paralysis, urinary tract infections/retention, autotomy, or other non-pain relevant aspects that can complicate the study of this phenomenon. Our model, termed SNAP (Spinal Neuropathic Avulsion Pain), creates physical damage to the superficial laminae of the sensory dorsal horn and robust, reliable, below-level bilateral hindpaw mechanical allodynia for ~9 weeks (Ellis et al., 2014; Wieseler et al., 2010).

Toll like receptor 4 (TLR4) is a critical mediator of neuropathic pain arising from peripheral nerve injury, and is widely expressed in the nervous system, including by DRG neurons, endothelial cells, microglia and astrocytes (Due et al., 2012; Grace et al., 2014b; Nicotra et al., 2012). After trauma, endogenous danger signals are released by stressed, damaged, and dying/dead cells and signal via TLR4 (Kofler and Wiley, 2011; Nicotra et al., 2012). In addition, we have recently discovered that TLR4 is also activated by a range of clinically relevant yet structurally diverse classes of opioids (Grace et al., 2015; Hutchinson et al., 2011; Wang et al., 2012). This is important because virtually all SCI patients are treated with opioids soon after injury, whether *en route* to the hospital or during early care for acute trauma (Hook et al., 2011).

Given that both endogenous danger signals due to SCI and morphine could converge at TLR4, the first goal of this study was to determine whether morphine amplifies SNAP, and would recapitulate the multiple pre-clinical (Grace et al., 2016; Hook et al., 2007; Hook et al., 2009; Loram et al., 2012) and clinical (Hansen et al., 2005; Salengros et al., 2010; Trevino et al., 2013; van Gulik et al., 2012) examples of the deleterious effects of opioids given shortly after trauma. As TLR4 is widely expressed in the nervous system, we also assessed whether the combination of SNAP and morphine could amplify the expression of a range of neuroinflammatory markers that are downstream of TLR4, as an indication of engagement of this pathway. The second goal was to identify whether TLR4 was indeed a critical mediator of morphine-potentiated SNAP-allodynia.

2. Materials and Methods

2.1. Animals and Ethics

Pathogen-free male Sprague-Dawley rats (325–350g; Harlan Laboratories, Madison, WI, USA) were used for all experiments. The rats were allowed a minimum of 1 week to habituate to the colony room before initiating the experiment. Rats were pair-housed prior to surgery and then single-housed after surgery to avoid further spinal cord damage by a cagemate, given removal of overlying vertebral bone. Standard rat chow and water were available *ad libitum*. Housing was in a temperature-controlled room that was maintained at 23+/-2°C with a 12 hr light/dark cycle (lights on at 0700 hr). All procedures were performed during the light cycle, and are summarized in Figure 1. All protocols were approved by the University of Colorado Boulder Institutional Animal Care and Use Committee.

2.2. Drugs

Where applicable, drugs were prepared and are reported as free base concentrations. (-)Morphine sulfate was gifted by the NIDA Drug Repository (Research Triangle, NC, USA). Morphine was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories, Abbott Park, IL, USA) and administered at a dose of 10 mg/kg per subcutaneous (s.c.) injection, based on a previously reported analgesic dose for SCI-pain (Hook et al., 2007). Controls received s.c. equivolume (1 ml/kg) saline. (+)-Naltrexone (synthesized endotoxin-free and gifted by Kenner Rice, NIDA and NIAAA, Rockville, MD, USA) was dissolved in sterile endotoxin-free isotonic saline (Abbott Laboratories) and administered at 6 mg/kg per s.c. injection, as described previously (Ellis et al., 2014). Controls received s.c. equivolume (1 ml/kg) saline. (+)-Naltrexone was given systemically as it is known to cross the blood brain barrier (Hutchinson et al., 2008).

2.3. Spinal Neuropathic Avulsion Pain (SNAP) Surgery

Unilateral (left) T13 dorsal root avulsion was performed under isoflurane anesthesia, as previously described in detail (Wieseler et al., 2012). Briefly, laminectomy was performed at the T12 vertebral level and the dura mater was incised over the dorsal root entry zone. The T13 dorsal rootlets were carefully isolated and then clamped at the dorsal root entry zone and briskly pulled out (avulsed). Sterile saline-moistened surgical gelfoam was placed over the exposed spinal cord to protect it, the muscle was sutured in layers with sterile 3–0 silk, and the skin was closed with stainless steel wound clips. Sham surgery consisted of the laminectomy, but the dorsal roots were not disturbed. Immediately following surgery, rats were single-housed in a cage with foam padding for a few hours to protect their spinal cord from further trauma due to the brief ataxic period that follows recovery from anesthesia. Immediately after each morphine injection, rats were placed in hanging cages with access to food and water *ad libitum* for ~6 hr in order to ensure they do not choke on bedding and to make it easier to observe. Sham operated rats were treated identically, except for avulsing of the rootlets. Combi-Pen-48 antibiotic (0.2 ml; Bimeda, Inc., Le Sueur, MN, USA) was administered at the time of surgery and daily for 4 days after surgery.

2.4. Low Threshold Mechanical Allodynia Testing

Prior to surgery, rats were habituated to the testing environment for 4 consecutive days prior to recording of behavioral responses. All von Frey assessments were performed blind with respect to drug assignments. Assessment of von Frey thresholds occurred before surgery (baseline) and across a timecourse beginning two weeks after surgery. The von Frey test was performed on the plantar surface of each hind paw as previously described in detail (Milligan et al., 2001). A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (Stoelting) were sequentially applied to the left and right hind paws in random order, each for 8 s at constant pressure to determine the stimulus intensity threshold stiffness required to elicit a paw withdrawal response. Log stiffness of the hairs is determined by $\log_{10}$ (milligrams $\times 10$). The range of monofilaments used in these experiments (0.407–15.136 g) produces a logarithmically graded slope when interpolating a 50% response threshold of stimulus intensity (Chaplan et al., 1994). The stimulus intensity threshold to elicit a paw withdrawal response was used to calculate the 50% paw withdrawal threshold (absolute threshold) using the maximum-likelihood fit method to fit a Gaussian integral psychometric function (Harvey, 1986). This method normalizes the withdrawal threshold for parametric analyses (Harvey, 1986).

2.5. Hargreaves test for morphine analgesia

Testing was conducted blind with respect to group assignment. Rats received at least three 60-min habituations to the test environment on separate days before behavioral testing. Latencies for behavioral response to radiant heat stimuli applied to the plantar surface of each hind paw and tail were assessed using a modified Hargreaves test (Hargreaves et al., 1988). Nociceptive assessments were made 30 min after morphine delivery.

2.6. Processing of tissue for PCR

2.6.1 RNA isolation and cDNA synthesis—Total RNA from the 5 mm piece of the T13/L1 thoracic spinal cord was extracted using the standard phenol/chloroform extraction with TRIzol Reagent (Invitrogen) according to the manufacturer's guidelines. Total RNA was reverse transcribed into cDNA using Superscript II First-Strand Synthesis System (Invitrogen). First-strand cDNA was synthesized using total RNA, random hexamer primer (5 ng/ μ l) and 1 mM dNTP mix (Invitrogen) and incubated at 65°C for 5 min. After 2 min incubation on ice, a cDNA synthesis buffer (5 $\times$ reverse transcription (RT) buffer; Invitrogen) and dithiothreitol (10 mM) was added and incubated at 25°C for 2 min. Reverse transcriptase (Superscript II; 200 U; Invitrogen) was added to a total volume of 20 μ l and incubated for 10 min at 25°C, 50 min at 42°C, and deactivating the enzyme at 70°C for 15 min. cDNA was diluted twofold in nuclease-free water and stored at –80°C until real-time PCR (RT-PCR) was performed.

2.6.2. RT-PCR—Primer sequences were obtained from the GenBank at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and are displayed in Table 4.1. Primers were generated to span an intron to eliminate genomic interference. Amplification of the cDNA was performed, in a blinded procedure, using Quantitect SYBR Green PCR kit (QIAGEN) in iCycler iQ 96-well PCR plates (Bio-Rad) on a MyiQ single Color Real-Time

PCR Detection System (Bio-Rad). The reaction mixture (26 μ l) was composed of QuantiTect SYBR Green (containing fluorescent dye SYBR Green I, 2.5 mM MgCl₂, dNTP mix, and Hot Start Taq polymerase), 10 nM fluorescein, 500 nM each forward and reverse primer (Invitrogen), nuclease-free water, and 1 μ l of cDNA from each sample. Each sample was measured in duplicate. The reactions were initiated with a hot start at 95°C for 25 min, followed by 40 cycles of 15 s at 94°C (denaturation), 30 s at 55–60°C (annealing), and 30 s at 72°C (extension). Melt curve analyses were conducted to assess uniformity of product formation, primer–dimer formation, and amplification of nonspecific products. The PCR product was monitored in real-time, using the SYBR Green I fluorescence, using the MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Threshold for detection of PCR product was set in the log-linear phase of amplification and the threshold cycle (*CT*) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the comparative *CT* (*CT*) method. The expression of GAPDH was not significantly different between treatments.

2.7. Enzyme linked immunosorbant assay (ELISA)

IL-1 β protein in rat T13/L1 ipsilateral dorsal spinal cord was analyzed using a commercially available ELISA kit specific for rat IL-1 β (R&D Systems, Minneapolis, MN, USA). Total protein was corrected using a Bradford assay. The lower limit of sensitivity of this assay is 5 pg/mL.

2.8. Western Blot

Whole spinal cord from Ipsilateral T13/L1 was sonicated in a mixture containing extraction buffer (Invitrogen) and protease inhibitors (Sigma). Ice-cold tissue samples were centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was removed, and the protein concentration for each sample was quantified using the Bradford method. Samples were heated to 75°C for 10 min and loaded into a standard polyacrylamide Bis-Tris gel (Invitrogen). SDS-PAGE was performed in 3-(*N*-morpholino)-propanesulfonic acid running buffer (Invitrogen) at 175 V for 1.25 h. Protein was transferred onto a nitrocellulose membrane using the iblot dry transfer system (Invitrogen). The membrane was blocked with Odyssey blocking buffer (LI-COR Biosciences) for 1 h and incubated with a primary antibody in blocking buffer overnight at 4°C. The following day, the membrane was washed in 1 $\times$ PBS containing Tween 20 (0.1%) and then incubated in blocking buffer containing either goat anti-rabbit (NLRP3) or goat anti-mouse (B-actin) (LI-COR) IRDye 800CW secondary antibody at a concentration of 1:10,000 (LI-COR) for 1 h at room temperature. Protein expression was quantified using an Odyssey Infrared Imager (LI-COR) and expressed as a ratio to their housekeeping protein. Primary antibodies included rabbit anti-rat NLRP3 monoclonal antibody (1:1000; Abcam), and mouse anti-rat β -actin (1:200,000; Sigma-Aldrich).

2.9. Pharmacological Manipulations

2.9.1. Effect of a weeklong course of morphine, beginning 24 hr post-surgery SNAP allodynia—Morphine (10 mg/kg) or equivolume vehicle (saline) was administered

once daily for 7 days beginning 24 hr post-SNAP ($n = 6$ per group). Sham surgery is not the most appropriate control in this particular study because pain resulting from tissue trauma inherent in the sham surgery may also be potentiated by morphine, thereby confounding interpretation of results. Instead, surgically naïve rats were given the same course of morphine as the SNAP rats and placed in the hanging cages at the same time across 7 days of morphine treatment. Beginning 2 weeks post-surgery, rats were behaviorally tested to define their mechanical response thresholds once a week for 5 weeks post-surgery.

2.9.2. Effect of a week-long course of morphine, beginning 24 hr post-surgery on TLR4, TNF, IL-1 β , and NLRP3 mRNA—After recording behavioral responses at the 1 week or 4 week post-drug treatment timepoint, 4 groups of rats ($n=6-8$ /group) per timepoint were overdosed with a lethal dose of sodium pentobarbital and transcardially perfused with ice-cold 0.9% saline for 2 min: (i) SNAP rats that received morphine (10 mg/kg s.c. per day), (ii) SNAP rats that received vehicle, (iii) surgically naïve rats that received the same course of morphine as the SNAP rats (10 mg/kg s.c. once a day for 7 days) and placed in the hanging cages, and (iv) surgically naïve rats that received no manipulation, remained in their home cage, and were not behaviorally tested. The spinal cord was dissected and a 5 mm section of ipsilateral dorsal spinal cord, with the meninges removed, was dissected free to include the T13/L1 spinal level. All tissue was flash frozen in liquid nitrogen. Samples were then stored at -80°C until analysis.

2.9.3. Effect of a weeklong course of morphine, beginning 24 hr post-surgery on IL-1 β and NLRP3 inflammasome proteins—After recording behavioral responses at the 1 week post-drug treatment (2 weeks post-SNAP) timepoint, SNAP rats that received morphine (10 mg/kg s.c.) or vehicle and a group of surgically and drug naïve rats ($n = 6$ per group), were overdosed with a lethal dose of sodium pentobarbital and transcardially perfused with ice-cold 0.9% saline for 2 min. The spinal cord was dissected and a 5 mm section of ipsilateral dorsal spinal cord, with the meninges removed, was dissected free to include the T13/L1 spinal level. All tissue was flash frozen in liquid nitrogen. Samples were then stored at -80°C until ELISA and western blot analysis.

2.9.4. Effect of a weeklong course of morphine co-administered with the TLR4 antagonist (+)-naltrexone, beginning 24 hr post-surgery on SNAP allodynia—For this study, SNAP rats were used for all groups. (+)-Naltrexone (6 mg/kg s.c.) or equivolume vehicle (saline) was administered three times a day at approximately 0900, 1200, and 1500 hr for 7 days beginning 24 hr post-surgery. Immediately after the first (+)-naltrexone injection each day, morphine was injected once a day for 7 days at 10 mg/kg s.c. beginning 24 hr post-surgery. Beginning 2 weeks post-surgery, rats were behaviorally tested to define their mechanical response thresholds once a week for 10 weeks post-surgery.

2.10. Statistical Analysis

All data were analyzed using GraphPad Prism versions 5.01–5.04 for Windows Vista (GraphPad Software, San Diego, CA, USA). Ipsilateral and contralateral behavioral data were analyzed individually. Behavioral measures were normalized as described above and group differences were analyzed by repeated measures two-way ANOVA with Bonferroni

post hoc test, and by comparing area under the curve (AUC), as previously described (Jones and Sorkin, 2004). AUC values were calculated from absolute threshold values, from 3.56 (the lowest threshold response value in the data set) up to the threshold response of each rat across time. Then a one-way ANOVA was performed on the AUC values for each group. Decreased AUC reflects an increase in mechanical allodynia. For all studies, the AUC measures across time were collapsed into a single timepoint for each animal, thus there is not a repeated measurement. For baseline measurements, a one-way ANOVA at that single timepoint was the statistic used. The RT-PCR, western blot, and ELISA data were analyzed using a one-way ANOVA. Bonferroni *post hoc* tests were used where appropriate, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Administering a weeklong course of morphine beginning 24 hr post-surgery amplifies SNAP allodynia

In order to determine if a short course of morphine early in the post-trauma period would amplify SNAP allodynia, morphine (10 mg/kg s.c.) or vehicle was administered once a day for one week beginning 24 hr post-surgery. No baseline (BL) differences were observed between groups in either ipsilateral (Fig. 2A) or contralateral (Fig. 2B) paw withdrawal thresholds. Morphine significantly enhanced SNAP allodynia for both the ipsilateral (Fig. 2A; time x treatment: $F_{12,75} = 0.62$, $p=0.8$; time: $F_{3,75} = 0.64$, $p=0.6$; treatment: $F_{4,25} = 14.6$, $p<0.001$), and contralateral paws (Fig. 2B; time x treatment: $F_{12,75} = 1.03$, $p=0.4$; time: $F_{3,75} = 0.06$, $p=0.8$; treatment: $F_{4,25} = 11.2$, $p<0.001$). This was also reflected in the AUC analysis, with a significant effect of treatment in both the ipsilateral ($F_{2,15} = 31.21$; $p<0.0001$; Fig. 2C) and contralateral ($F_{2,15} = 25.15$; $p<0.001$; Fig. 2D) hindpaws. Morphine analgesia was verified across the week-long timecourse (Fig. S1; time x treatment: $F_{2,14} = 1.45$, $p=0.3$; time: $F_{2,14} = 3.63$, $p=0.054$; treatment: $F_{1,7} = 23.2$, $p<0.01$).

3.2 SNAP-induced pro-inflammatory gene expression is enhanced by morphine

Since we hypothesized that both endogenous danger signals due to SCI and morphine could converge at TLR4, we first examined the consequences of morphine treatment after SNAP on mRNA expression of TLR4 and its downstream signaling pathway.

TLR4 mRNA was significantly upregulated by treatment at both the 1 week ($F_{3,20} = 18.67$; $p<0.0001$; Fig. 3A) and 4 weeks ($F_{3,21} = 3.469$; $p<0.05$; Fig. 3B) after the final morphine injection. Morphine significantly amplified TLR4 expression induced by SNAP at only at the 1 week timepoint ($p<0.05$). As TLR4 activation can result in production of pro-inflammatory cytokines, we quantified the mRNA expression of TNF and IL-1 β , which have a well-defined role in neuropathic pain (Grace et al., 2014a). TNF mRNA was significantly upregulated by treatment at both 1 week ($F_{3,22} = 4.736$; $p<0.05$; Fig. 3C) and 4 weeks ($F_{3,20} = 7.241$; $p<0.01$; Fig. 3D) after the final morphine injection. Morphine significantly amplified TNF expression induced by SNAP at only at the 1 week timepoint ($p<0.05$). IL-1 β mRNA was significantly upregulated by treatment at both the 1 week ($F_{3,21} = 10.93$; $p<0.001$; Fig. 3E) and 4 week ($F_{3,21} = 5.692$; $p<0.01$; Fig. 3F) timepoints. Morphine significantly amplified IL-1 β mRNA induced by SNAP at only at the 1 week timepoint

($p < 0.05$). Since IL-1 β can require proteolytic activation by inflammasomes, expression of NLRP3 was also assessed (Latz et al., 2013). NLRP3 mRNA was significantly elevated by treatment at both the 1 week ($F_{3,21} = 11.31$; $p < 0.0001$; Fig. 3G) and 4 weeks ($F_{3,19} = 12.95$; $p < 0.0001$; Fig. 3H) timepoints. Morphine significantly amplified NLRP3 expression induced by SNAP at both timepoints ($p < 0.05$). There was no significant difference between the naïve groups for any markers, at any timepoints.

3.3. SNAP-induced IL-1 β and NLRP3 protein expression is enhanced by morphine

Next we examined protein expression of NLRP3 and IL-1 β . The tissue collection was performed at 1 week after the last morphine injection given that our most robust effects in gene expression occurred at this timepoint. Since the naïve+morphine group in the above studies was not statistically different from the naïve group for any marker analyzed, only a naïve group was included here. IL-1 β protein was significantly upregulated by treatment ($F_{2,15} = 14.54$; $p < 0.001$; Fig. 4A). Morphine significantly amplified IL-1 β expression induced by SNAP ($p < 0.05$). NLRP3 protein was significantly upregulated by treatment ($F_{2,20} = 33.3$; $p < 0.0001$; Fig. 4B). Morphine significantly amplified NLRP3 mRNA expression induced by SNAP ($p < 0.05$).

3.4. Co-administering a week-long course of morphine and the TLR4 antagonist (+)-naltrexone beginning 24 hr post-surgery blocks morphine amplification of SNAP allodynia

The final experiment set out to determine if signaling at TLR4 during morphine administration was responsible for amplifying SNAP-induced allodynia. The TLR4 antagonist (+)-naltrexone (6 mg/kg s.c.) or vehicle was administered 3 times a day, with the first injection of the day co-administered with the once-daily morphine or vehicle. All drugs were administered for one week beginning 24 hours post SNAP surgery. No baseline (BL) differences were observed between groups in either the ipsilateral (Fig. 5A) or contralateral (Fig. 5B) paws. (+)-Naltrexone significantly attenuated morphine-potentiated SNAP allodynia for both the ipsilateral (Fig. 5A; time x treatment: $F_{16,120} = 0.81$, $p = 0.7$; time: $F_{8,120} = 11.9$, $p < 0.001$; treatment: $F_{2,15} = 13.4$, $p < 0.001$), and contralateral paws (Fig. 5B; $F_{16,120} = 1.78$, $p < 0.05$; time: $F_{8,120} = 17.0$, $p < 0.001$; treatment: $F_{2,15} = 5.59$, $p < 0.05$). This was also reflected in the AUC analysis with a significant reduction due to morphine treatment, which was normalized by (+)-naltrexone in both the ipsilateral ($F_{2,15} = 9.892$; $p < 0.01$; Fig. 5C) and contralateral ($F_{2,13} = 6.679$; $p < 0.05$; Fig. 5D) hindpaws.

4. Discussion

Although opioids have long been accepted as one of the most effective analgesics, there is emerging evidence that opioids may also be detrimental for pain. While opioids create pain suppression via activation of classical opioid receptors on neurons, opioids simultaneously induce pain enhancement via activation of TLR4 receptors (Grace et al., 2015; Watkins et al., 2009). Together with the release of endogenous danger signals after SCI, opioids may interact to create enduring enhancement of pain via TLR4. In the present set of studies, we provide evidence that a weeklong course of morphine starting 24 hours after T13 dorsal root avulsion SCI (SNAP) amplifies mechanical allodynia for at least 4 weeks following cessation of opioid dosing. This amplification of mechanical allodynia was prevented by co-

administering the *non*-opioid TLR4 antagonist (+)-naltrexone throughout the period of morphine exposure. This effect of (+)-naltrexone is strikingly different than its effect on reversing SNAP-induced mechanical allodynia, in the absence of morphine. In this case, a course of (+)-naltrexone to treat SNAP-induced allodynia fully reverses this mechanical allodynia (Ellis et al., 2014). However, upon cessation of (+)-naltrexone, SNAP-induced allodynia fully returned within a few days, a strikingly different temporal profile than revealed in the present studies. Morphine also potentiated allodynia resulting from sham surgery, which suggests a general effect of morphine on pain enhancement. This is supported by our previous work demonstrating the morphine can potentiate pain resulting from peripheral nerve injury and inflammation (Grace et al., 2016; Loram et al., 2012). However, morphine administration in naive rats does not induce mechanical allodynia and neuroinflammation; there must already be a reactive and primed environment in order to observe morphine-potentiated effects.

These results extend the work of Hook et al. (2007, 2009), who first described the deleterious effects of morphine administration shortly after SCI in rodents. While a single intrathecal injection of morphine 24 hours following contusion SCI attenuated weight gain and locomotor function and increased tissue loss around the SCI lesion site (Hook et al., 2009), there was no effect on mechanical thresholds at day 21. However, enhancement of allodynia may be dependent on the multi-day course of morphine used here. Therefore, this is the first demonstration that chronic opioid administration restricted to the early post-trauma period potentially amplifies mechanical allodynia for weeks after SCI.

The present studies also suggest that neuroimmune signaling accompanies morphine-enhanced SNAP allodynia. Here we show that spinal TLR4 and the pro-inflammatory cytokines TNF and IL-1 β are increased in response to spinal cord injury 2 weeks post-surgery and remain elevated 5 weeks post-surgery. Furthermore, NLRP3, a protein responsible for the proteolytic activation of IL-1 β , was elevated after injury. While morphine enhanced these markers at the week 1 timepoint, the upregulation did not persist out to 4 weeks. This suggests that these neuroimmune processes may contribute to the initiation, but not the long-term maintenance of morphine amplification of SNAP-induced mechanical allodynia. While our previous work has implicated microglia in potentiation of allodynia by morphine (Grace et al., 2016), the cell type(s) responsible for production of pro-inflammatory mediators in the current study requires further investigation; TLR4 is widely expressed in the nervous system, including by DRG neurons, endothelial cells, microglia and astrocytes (Due et al., 2012; Grace et al., 2014b; Nicotra et al., 2012).

Previous studies from our lab have shown that mechanical allodynia arising from both peripheral (chronic constriction injury) and central (SNAP) injuries can be reversed by the TLR4 antagonist (+)-naltrexone (Ellis et al., 2014; Hutchinson et al., 2008). In the present study, we extended the findings with SNAP by demonstrating that (+)-naltrexone was also able to block amplification of SNAP allodynia by peri-trauma morphine. This provides evidence that TLR4 is critically involved not only in SNAP-induced allodynia but additionally in the mechanism underlying morphine amplification of spinal cord injury pain. Given evidence that (+)-naltrexone potentiates the acute analgesic effects of morphine (Hutchinson et al., 2008), the combined data suggest that co-administering TLR4

antagonists with morphine in the early post-trauma period will improve the clinical utility of opioids for acute pain control while obviating the longer term negative consequences of opioid exposure.

The proinflammatory cytokine IL-1 β is a key mediator of the inflammatory cascade and is therefore very tightly regulated (Dinarello, 2011). IL-1 β is up-regulated by SCI (Pan et al., 2002; Pineau and Lacroix, 2007; Wang et al., 1997), and mediates morphine-induced attenuation of locomotor recovery after SCI (Hook et al., 2011). IL-1 β protein was increased by SNAP, and further amplified by morphine. Notably release of mature IL-1 β requires cleavage from the precursor protein pro-IL-1 β . Cleavage of pro-IL-1 β is achieved via caspase-1, which is activated following the formation and activation of a group of intracellular proteins collectively called the inflammasome. NLRP3 is expressed by microglia, and has previously been implicated morphine potentiation of neuropathic pain (Grace et al., 2016). Indeed, NLRP3 expression was significantly increased after SNAP, and further enhanced by morphine. In order for NLRP3 inflammasome formation to occur, the NLRP3 gene must first be transcribed and translated into protein, which is thought to require TLR4 ligation (Latz et al., 2013). Therefore, blockade of TLR4 signaling with (+)-naltrexone may have disrupted NLRP3 inflammasome activation, and will be tested in future studies.

Whether opioids can exacerbate chronic pain in humans is a subject of ongoing controversy. Several studies have demonstrated that post-operative pain is prolonged following remifentanyl anesthesia (Hansen et al., 2005; Salengros et al., 2010; van Gulik et al., 2012). Furthermore, trauma patients treated with a chronic opioid regimen had significantly higher pain 4 months after the original injury compared to those who never received opioids (Trevino et al., 2013). However, none of these studies have investigated central neuropathic pain, and long-term studies clinical studies still need to be performed.

This study demonstrates that morphine can enhance allodynia after SCI, and is mediated by TLR4 signaling. Whether these data have relevance for the clinical management of SCI-pain remains to be determined. However, our data suggest that then targeting TLR4 and/or neuroimmune signaling may prevent amplified allodynia, while retaining morphine analgesia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Morphine administered shortly after spinal cord injury amplifies mechanical allodynia
- Morphine increases spinal injury mRNA expression of TLR4 and TNF
- Morphine increases spinal cord injury-induced IL-1 β mRNA and protein
- TLR4 antagonism blocks morphine-induced enhancement of mechanical allodynia

Figure 1.
Intervention timeline.

Figure 2.

Assessment of the effects of morphine on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (A) and contralateral (B) hindpaw. SNAP rats that received morphine (10 mg/kg, s.c.) for 7 days beginning 24 hours after surgery were significantly more allodynic than SNAP rats that received vehicle and naïve rats that received morphine in both the ipsilateral (C) and contralateral (D) hindpaw. Data are presented as mean $\pm$ SEM, $n=6$ per group. * $p<0.05$ compared to all other groups, + $p<0.05$ compared to Naïve+Morphine group.

Figure 3.

Assessment of the effects of administering morphine on TLR4, TNF, and IL-1 β mRNA gene expression in the T13/L1 ipsilateral dorsal spinal cord. SNAP significantly increased TLR4 mRNA at both the 1 week (A) and 4 week (B) timepoint post-final morphine injection, but morphine only amplified TLR4 mRNA at the 1 week timepoint. Only morphine significantly increased TNF mRNA at the 1 week (C) timepoint post-final morphine injection, while both SNAP and morphine increased TNF mRNA at the 4 week (D) timepoint post-final morphine injection. Morphine did not further amplify TNF mRNA at the 4 week timepoint. SNAP significantly increased IL-1 β mRNA at both the 1 week (E)

and 4 week (F) timepoint post-final morphine injection, but morphine only amplified IL-1 β mRNA at the 1 week timepoint. SNAP significantly increased NLRP3 mRNA at both the 1 week (G) and 4 week (H) timepoint post-last injection, and morphine furthered amplified NLRP3 mRNA at both timepoints. Data are presented as mean $\pm$ SEM, n= 6 to 7 per group. *p<0.05, **p<0.01, ***p<0.001 compared to Naïve+Morphine group; #p<0.05 compared to SNAP+Vehicle.

Figure 4.

Assessment of the effects of administering morphine on IL-1 β and NLRP3 protein in the T13/L1 dorsal spinal cord. SNAP significantly increased IL-1 β (A) and NLRP3 (B) protein, which were both further amplified by morphine. Data are presented as mean $\pm$ SEM, n= 6 to 8 per group. *p<0.05, **p<0.01, ***p<0.001 compared to Naïve+Morphine group; #p<0.05 compared to SNAP+Vehicle.

Figure 5.

Assessment of the effects of morphine + (+)-naltrexone on SNAP. Rats were tested for mechanical allodynia across a timecourse on both the ipsilateral (**A**) and contralateral (**B**) hindpaw. SNAP rats that received (+)-naltrexone (3× 6 mg/kg, s.c.) + morphine (10 mg/kg, s.c.) for 7 days beginning 24 hours after surgery were significantly less allodynic than SNAP rats that received morphine + saline and were not significantly different than SNAP rats that received vehicle + vehicle in both the ipsilateral (**C**) and contralateral (**D**) hindpaw. Data are presented as mean ± SEM, n=6 per group. *p<0.05 compared to vehicle.

Table 1

Primer Sequences

Gene	Primer Sequence (5'-3')	GenBank accession no.
<i>Gapdh</i>	TCTTCCAGGAGCGAGATCGC (forward) TTCAGGTGAGCCCCAGCCTT (reverse)	NG_028301.1
<i>Tlr4</i>	TCCCTGCATAGAGGTACTTC (forward) CACACCTGGATAAATCCAGC (reverse)	NM_019178.1
<i>Tnf</i>	CAAGGAGGAGAAGTTCCCA (forward) TTGGTGGTTTGCTACGACG (reverse)	NM_012675.3
<i>Il-1β</i>	CCTTGTGCAAGTGTCTGAAG (forward) GGGCTTGGAAGCAATCCTTA (reverse)	NM_031512.2
<i>Nlrp3</i>	AGAAGCTCCCCTTGGTGAATT (forward) GTTGTCTAACTCCAGCATCTG (reverse)	NM_001191642.1