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AN IL-1 RECEPTOR ANTAGONIST BLOCKS A MORPHINE-INDUCED ATTENUATION OF LOCOMOTOR RECOVERY AFTER SPINAL CORD INJURY

Michelle A. Hook¹, Stephanie N. Washburn¹, Georgina Moreno², Sarah A. Woller¹, Denise Puga¹, Kuan H. Lee¹, and James W. Grau¹

¹Department of Psychology, Texas A&M University

²Department of Neurology, University of Iowa

Abstract

Morphine is one of the most commonly prescribed medications for the treatment of chronic pain after a spinal cord injury (SCI). Despite widespread use, however, little is known about the secondary consequences of morphine use after SCI. Unfortunately, our previous studies show that administration of a single dose of morphine, in the acute phase of a moderate spinal contusion injury, significantly attenuates locomotor function, reduces weight gain, and produces symptoms of paradoxical pain (Hook et al., 2009). The current study focused on the cellular mechanisms that mediate these effects. Based on data from other models, we hypothesized that pro-inflammatory cytokines might play a role in the morphine-induced attenuation of function. Experiment 1 confirmed that systemic morphine (20 mg/kg) administered one day after a contusion injury significantly increased expression levels of spinal IL-1 β 24 hrs later. Experiment 2 extended these findings, demonstrating that a single dose of morphine (90 μ g, i.t.) applied directly onto the spinal cord increased expression levels of spinal IL-1 β at both 30 min and 24 hrs after administration. Experiment 3 showed that administration of an interleukin-1 receptor antagonist (IL-1ra, i.t.) prior to intrathecal morphine (90 μ g), blocked the adverse effects of morphine on locomotor recovery. Further, pre-treatment with 3 μ g IL-1ra prevented the increased expression of at-level neuropathic pain symptoms that was observed 28 days later in the group treated with morphine-alone. However, the IL-1ra also had adverse effects that were independent of morphine. Treatment with the IL-1ra alone undermined recovery of locomotor function, potentiated weight loss and significantly increased tissue loss at the injury site. Overall, these data suggest that morphine disrupts a critical balance in concentrations of pro-inflammatory cytokines in the spinal cord, and this undermines recovery of function.

Keywords

Contusion; inflammation; opioid; neuropathic pain; cytokine

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Address for correspondence: Michelle A. Hook, Ph.D., Department of Psychology, Texas A&M University, College Station, TX 77843-4235, Tel: (979) 458-1122, Fax: (979) 845-4727, michellehook@tamu.edu.

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Conflict of Interest Statement

All authors declare that there are no conflicts of interest.

1. Introduction

Our previous studies suggest that morphine, administered in the acute phase of a spinal contusion injury, significantly undermines recovery of function and increases the expression of paradoxical pain symptoms in the chronic phase of injury (Hook et al., 2007; Hook et al., 2009). Despite producing analgesia, a single dose of *systemic* morphine administered one day after a moderate contusion injury, led to allodynic responses to innocuous sensory stimuli (three weeks later), decreased sensory function, decreased weight gain, and increased lesion size when compared with vehicle-treated controls (Hook et al., 2007). *Intrathecal* morphine significantly attenuated the recovery of locomotor function, decreased weight gain, increased the incidence of autophagia, and increased lesion size rostral to the injury site (Hook et al., 2009). These data suggest that caution is warranted when administering morphine in the acute phase of a spinal cord injury (SCI). For SCI patients faced with a lifetime of intractable pain, however, simply removing morphine as a potential analgesic is not an option. It is essential that we further our understanding of the consequences of, and molecular mechanisms engaged by, commonly used opiate analgesics.

Recent studies have implicated the immune system in the development of the ‘paradoxical’ pain observed with morphine administration (Watkins et al., 2005; Watkins et al., 2007; Scholz & Woolf, 2007). These studies have shown that repeated morphine administration activated microglia and astrocytes (Song & Zhao, 2001; Cui et al., 2006; Raghavendra et al., 2002; Tai et al., 2006), and increased TNF α , IL-1 β and IL-6 expression in the spinal cord (Johnston et al., 2004; Raghavendra et al., 2002; Tai et al., 2006). These pro-inflammatory cytokines block the analgesic effects of opioids (Gul et al., 2000; Szabo et al., 2002), and have been linked to the development of paradoxical pain symptoms. Indeed, at a cellular level, IL-1 β has been shown to facilitate substance-P release from primary afferent neurons in the spinal dorsal horn (Inoue et al. 1999), increase tyrosine phosphorylation of the NMDA receptor NR2B subunit (Viviani et al. 2003), increase surface expression of AMPA receptors (Stellwagen et al., 2005), and has been linked to inhibition of glutamate transporters such as GLT-1 and GLAST (Tai et al., 2006; Prow & Irani, 2008). Subsequent increases in neuroexcitability, with the potentiation of the glutamate signal, underlies the development of central sensitization, a mechanism that results in increased pain reactivity and may exacerbate the secondary neuronal death seen after spinal injury. Also, the pro-inflammatory cytokines, TNF α and IL-1 β , activate NF- κ B, which is the transcriptional regulator of the synthesis of the inducible form of nitric oxide synthase (iNOS) (Conti et al., 2007). Increased iNOS activity elevates basal nitric oxide (NO) levels, which may also exacerbate the secondary neuronal death seen after spinal injury (Conti et al., 2007). Clearly, the changes in pain reactivity, increased lesion size, and the decreased recovery of function observed with morphine administration may be due to elevations of pro-inflammatory cytokine levels.

The effects of morphine on the immune system are particularly relevant in a spinal contusion injury, which is characterized by inflammation. Pro-inflammatory cytokine levels are significantly upregulated 1, 3, and 6 hrs after a contusion injury, returning to background levels after 1–3 days (Yang et al., 2004; Wang et al., 2005; Yang et al., 2005; Pineau & Lacroix, 2007). In the early stages of injury, pro-inflammatory cytokines appear to have beneficial effects as they are involved in the regulation of leukocyte recruitment and microglial activation. Conversely, they have also been linked to cytotoxic effects (Merrill & Benveniste, 1996; Gruol & Nelson, 1997; Knoblach et al., 1999; Nesic et al., 2001). Indeed, Yang et al. (2005) suggest that there is a concentration-dependent relationship, and critical balance, between the beneficial and toxic effects of pro-inflammatory cytokines. As morphine increases the expression of pro-inflammatory cytokines, we hypothesize that superimposing this analgesic on the vulnerable contusion site may push the injured system

past an adaptive inflammatory response to cytotoxic levels thereby increasing the expression of paradoxical pain symptoms and cell death. To test this hypothesis The initial experiments confirmed that acute morphine administration increased pro-inflammatory cytokine levels at the site of injury. This effect was observed with both systemic (Experiment 1) and intrathecal (Experiment 2) morphine administration. Experiment 3 then examined whether co-administration of an IL-1 receptor antagonist (IL-1ra) and morphine would block the morphine-induced attenuation of recovery of function. In Experiment 3, both morphine and the IL-1ra were applied directly onto the spinal cord, thereby focusing the loci of changes on the vulnerable contusion site. We found that the IL-1ra did prevent the effects of morphine on recovery of locomotor function and blocked the development of at-level neuropathic symptoms. However, subjects treated with the IL-1ra alone also displayed a dose-dependent attenuation of weight gain after injury, and increased tissue loss across the extent of the lesion.

2. Methods

2.1 General Methods

2.1.1 Subjects—The subjects were male Sprague-Dawley rats obtained from Harlan (Houston, TX). They were approximately 90–110 days old (300–350 g) and were individually housed in Plexiglas bins [45.7 (length) × 23.5 (width) × 20.3 (height) cm] with food and water continuously available. To facilitate access to the food and water, extra bedding was added to the bins after surgery and long mouse sipper tubes were used so that the rats could reach the water without rearing. Subjects were weighed on the same days that they were assessed for locomotor function, and were checked daily for signs of autophagia and spasticity. A subject was classified as having spasticity if the limb was in an extended, fixed position and was resistant to movement. Bladders were manually expressed in the morning (8:00–9:30 hrs) and evening (18:00–19:30 hrs) until subjects regained bladder control, which was operationally defined as three consecutive days with an empty bladder at the time of expression. The rats were maintained on a 12 hr light/dark cycle and tested during the last 6 hrs of the light cycle.

All of the experiments were reviewed and approved by the institutional animal care committee at Texas A&M and all NIH guidelines for the care and use of animal subjects were followed.

2.1.2. Surgery—Subjects were anesthetized with isoflurane (5%, gas). Once a stable level of anesthesia was achieved, the inspired concentration of isoflurane was lowered to 2–3% and an area extending approximately 4.5 cm above and below the injury site was shaved and disinfected with iodine. A 7.0 cm incision was made over the spinal cord. Next, two incisions were made on either side of the vertebral column, extending about 3 cm rostral and caudal to the T12–T13 segment. The dorsal spinous processes at T12–T13 were removed (laminectomy), and the spinal tissue exposed. The dura remained intact. For the contusion injury, the vertebral column was fixed within the MASCIS device (Constantini & Young, 1994; Gruner, 1992) and a moderate injury was produced by allowing the 10 g impactor (outfitted with a 2.5 mm tip) to drop 12.5 mm. The wound was then closed with Michel clips.

In Experiments 2 and 3, an intrathecal cannula was also inserted into the subarachnoid space immediately after the contusion injury. For this procedure, a 15 cm long polyethylene (PE-10) cannula, fitted with a 0.23 mm (diameter) stainless steel wire (SWG-X-090, Small Parts), was threaded 2 cm under the vertebrae immediately caudal to the injury site. The cannula tip terminated over the S2–S3 spinal segments, so that morphine was delivered to the lumbosacral regions mediating the hindpaw and pain reactivity tests. To prevent cannula

movement, the exposed end of the tubing was secured to the vertebrae rostral to the injury using an adhesive (Cyanoacrylate). The wire was then pulled from the tubing and the wound was closed using Michel clips.

To help prevent infection, subjects were treated with 100,000 units/kg Pfizerpen (penicillin G potassium) immediately after surgery and again 2 days later. For the first 24 hrs after surgery, rats were placed in a recovery room maintained at 26.6° C. To compensate for fluid loss, subjects were given 2.5 ml of saline after surgery. Michel clips were removed 14 days after surgery.

2.2. Experiment 1

Experiment 1 examined molecular changes that might underlie the morphine-induced attenuation of recovery after SCI. Sixteen male Sprague Dawley rats served as subjects. Twenty four hours after a contusion surgery, subjects were injected with morphine ((C¹⁷H¹⁹NO³)² H²SO⁴·5H²O), 20 mg/kg (equivalent to 15.04 mg/kg morphine base); i.p., Mallinckrodt, Hazelwood, MI) or vehicle. We have previously shown that this high dose of morphine is required to achieve strong antinociception and block behavioral reactivity to nociceptive stimulation after spinal cord injury, a dose that is much higher than that needed for sham controls (10 mg/kg; Hook et al. 2007). Locomotor ability was assessed with the BBB scale (Basso et al., 1995) prior to and after drug treatment. This behavioral index was used to ensure that injury severity was balanced across groups prior to drug treatment.

2.2.1 Drug administration—One day after surgery, and after baseline locomotor assessments, the rats were injected with 20 mg/kg i.p. of morphine sulfate or vehicle.

2.2.2. Locomotor recovery—Locomotor behavior was assessed using the Basso, Beattie and Bresnahan (BBB) scale (Basso et al., 1995), in an open enclosure (99 cm diameter, 23 cm deep). This 21-point scale is used as an index of hindlimb functioning after a spinal injury. Using this scale, no movement of the hindlimbs (ankle, knee or hip) is designated a score of 0, and intermediate milestones include slight movement of one joint (1), extensive movement of all three joints (7), occasional weight supported stepping in the absence of coordination (10), and consistent weight supported stepping with consistent FL-HL coordination (14). Higher scores reflect consistent limb co-ordination and improved fine motor skill. Baseline motor function was assessed on the day following injury and prior to drug treatment. Because rodents often freeze when first introduced to a new apparatus, subjects were acclimated to the observation fields for 5 min per day for 3 days prior to surgery. Each subject was placed in the open field and observed for 4 min. Care was taken to ensure that the investigators' scoring behavior had high intra- and inter-observer reliability (all *r*'s > 0.89) and that they were blind to the subject's experimental treatment.

BBB scores were transformed, as described in Ferguson et al. (2004), to help assure that the data were amenable to parametric analyses. Briefly, this transformation pools BBB scores 2–4, removing a discontinuity in the scale. The transformation also pools scores from a region of the scale (14–21) that is very seldom used under the present injury parameters. By pooling these scores, we obtain an ordered scale that is relatively continuous with units that have approximately equivalent interval spacing. Meeting these criteria allows us to apply metric operations (computation of mean performance across legs), improves the justification for parametric statistical analyses, and increases statistical power.

2.2.3. Molecular analyses—In Experiment 1, the subjects were euthanized (100 mg/kg of pentobarbital, i.p.) 24 hrs after drug treatment. A 5 mm segment of spinal cord was collected from the injury site, snap frozen in liquid nitrogen, and stored at –80 °C until

further analysis. At the time of analysis, the tissue was thawed in T-per (Pierce, Rockford, IL) containing protease inhibitor cocktail (Sigma, St. Louis, MO) and homogenized in a microcentrifuge tube using a pestle. Samples were then centrifuged at $4500 \times g$ for 15 min at 4°C and the supernatants were collected and stored at -80°C . Total protein concentrations were assessed using a BCA Protein Assay Kit (Pierce, Rockford, IL) and spectrophotometer (Biomate 3, Thermo Electron Corporation, Waltham, MA) according to the manufacturer's instructions.

The pro-inflammatory cytokines IL-1 β and IL-6 were analyzed in tissue homogenates using ELISA kits (BioSource, Carlsbad, CA, sensitivity < 3 and 8 pg/ml , respectively) according to the manufacturer's instructions. Samples were read at a wavelength of 450 nm using a microplate reader (Wallac Victor2 1420 Multilabel Counter, PerkinElmer, Waltham, MA).

2.3. Experiment 2

Using the same molecular procedures as outlined in Experiment 1, Experiment 2 examined the impact of intrathecal morphine administration on spinal levels of IL-1 β and IL-6. Thirty-two male Sprague Dawley rats served as subjects. Twenty four hours after a contusion surgery, subjects were injected with morphine ($90\text{ }\mu\text{g i.t.}$, (equivalent to $67.68\text{ }\mu\text{g}$ morphine base); Mallinckrodt, Hazelwood, MI) or vehicle. This dose of morphine was chosen based on previous studies that demonstrated that this high dose is required to achieve strong antinociception and block behavioral reactivity to nociceptive stimulation after SCI, as well as being detrimental to long-term recovery of locomotor function (Hook et al. 2009). Locomotor ability was assessed with the BBB scale (described in Experiment 1, Basso et al., 1995) prior to and after drug treatment. This behavioral index was used to ensure that injury severity was balanced across groups prior to drug treatment

2.3.1 Drug administration—On the day after surgery, and after baseline locomotor assessments, the rats were injected with $90\text{ }\mu\text{g i.t.}$ of morphine sulfate (Mallinckrodt, Hazelwood, MI) or vehicle.

2.3.2. Molecular analyses—Subjects were euthanized (100 mg/kg of pentobarbital, i.p.) 30 minutes or 24 hrs after drug treatment. A 5 mm segment of spinal cord was collected from the injury site, snap frozen in liquid nitrogen, and stored at -80°C until further analysis. Then using the methods described for Experiment 1 (2.2.3. *Molecular analyses*) we extracted the protein from the individual samples, assessed protein concentrations, and levels of both IL-1 β and IL-6 using ELISA kits (BioSource, Carlsbad, CA, sensitivity < 3 and 8 pg/ml , respectively), and according to the manufacturer's instructions.

2.4. Experiment 3

To examine the role of spinal IL-1, an IL-1ra and morphine were applied directly onto the spinal cord. After baseline assessments of locomotor and sensory function, 60 rats were assigned to one of three IL-1ra dose conditions ($0, 1$ or $3\text{ }\mu\text{g i.t.}$). Subjects received an assigned dose of IL-1ra dissolved in $2\text{ }\mu\text{L}$ of distilled water. Fifteen minutes later, half of the subjects in each IL-1ra condition ($n=10$) were treated with morphine sulfate ($90\text{ }\mu\text{g, i.t.}$). The remaining subjects were treated with vehicle.

2.4.1. Drug administration—To ensure that the opioid-induced changes in recovery were due to an action at the level of the spinal cord, both morphine and an IL-1ra were applied intrathecally in Experiment 3. Twenty-four hours after injury, the rats were given an intrathecal infusion of IL-1ra ($0, 1$ or $3\text{ }\mu\text{g}$ recombinant rat IL-1ra, R&D Systems, Minneapolis, MN) dissolved in $2\text{ }\mu\text{L}$ of distilled water. The drug injection was followed by a $20\text{ }\mu\text{L}$ injection of saline, to flush the catheter. Fifteen minutes later, half of the subjects in

each IL-1ra condition were treated with morphine sulfate (90 µg i.t.) dissolved in 2 µL of distilled water. The remaining subjects were treated with 2 µL of vehicle. These drug injections were also followed by a 20 µL injection of saline, to flush the catheter.

2.4.2. Antinociceptive efficacy of morphine—For the assessment of morphine efficacy, with and without the IL-1ra, nociceptive reactivity was assessed immediately before and 30 minutes after intrathecal morphine administration. A change from baseline score (reactivity after morphine minus reactivity before morphine) was calculated and used as an index of morphine efficacy. Antinociception was measured using radiant heat and incremented shock (independent variables), and both tail-flick (spinally-mediated motor response) and vocal (supraspinal-mediated response) reactivity thresholds were recorded. Reactivity was assessed with radiant heat, as described in prior studies (e.g., King et al., 1996; McLemore et al., 1999; Crown et al., 2000). Subjects were placed in the restraining tubes with their tail positioned in a 0.5 cm deep groove that was cut into an aluminum block. Next, subjects were allowed to acclimate to the apparatus for 15 min. Thermal and incremented shock thresholds were then assessed 2 times using the tail-flick test. These tests occurred at 2 minute intervals, and the second tests were recorded as baseline tail-flick latencies. To confirm that subjects did not respond in the absence of the stimuli, blank trials were also performed. A ‘false alarm’ was recorded if subjects made a motor or vocalization response during the blank tests. The blank trials were performed 1 min before or after each sensory test (in a counterbalanced fashion). No false alarms were recorded.

Thermal reactivity was tested using a 375-W movie light that was focused onto the rat's tail with a condenser lens positioned 8 cm below the light source. For testing, a wire hook that was 10 cm long and covered with heat shrink tubing was taped to the last 2.5 cm of the tail. The hook was placed over an elastic band located 11 cm behind the aluminum block. The flexibility of the elastic band allowed for a tail-flick response while maintaining the rat's tail under the heat source. The latency to vocalize was then assessed. Shock thresholds were assessed using a manual shocker (BRS/LVE, Model SG-903) that allowed continuous variation of shock intensity between 0 and 2 mA (AC, constant current). Test shocks were applied 7 cm from the base of the tail through electrodes constructed from lightweight fuse clips. Test shock intensity was gradually incremented at a rate of 0.05 mA every 3 s. After both movement and vocalization responses were detected, the shock (or heat) was terminated. If a subject failed to respond, the test trial was automatically terminated after 8 s of heat exposure or after shock intensity reached 1.2 mA.

2.4.3. Locomotor recovery—Using the methods described for Experiment 1, locomotor behavior was assessed with the BBB scale for 21 days post injury (Basso et al., 1995). Baseline motor function was assessed on the day following injury and prior to drug treatment. Locomotor behavior was then scored once per day for 1 week (Days 2–7), every other day from Day 9 to Day 15 and then every 3rd day until Day 21. Pre-treatment locomotor performance (baseline assessment on Day 1, prior to morphine treatment), assessed with the BBB scale, accounted for 56% of the variance in recovery across subjects. By using Day 1 as a covariate in an analysis of covariance (ANCOVA), we substantially reduced unexplained variance and thereby increased statistical power.

2.4.4. Sensory function—Sensory function was re-assessed after Day 21. We assessed sensory reactivity using the nociceptive stimuli (a gradually incremented shock and radiant heat) and the procedures described previously (2.4.2. *Antinociceptive efficacy of morphine*). On an alternate day, tactile reactivity to von Frey stimulation was assessed. Subjects were placed into Plexiglas tubes [7.0 cm (internal diameter) × 20 cm (length)] that had 6 (length) × 1.7 (width) cm notches removed from the sides of the base. These slots allowed the hindlegs to hang freely below the tube. Progressively stronger tactile stimuli (von Frey

stimuli formed from nylon monofilaments, Semmes-Weinstein Anesthesiometer; Stoelting Co., Chicago, IL) were applied sequentially to the plantar surface of the paw at approximately 2 s intervals until subjects exhibited a paw withdrawal (motor response) and vocalization. If one or both responses were not observed, testing was terminated at a force of 300 g. Each subject was tested twice on each foot in a counterbalanced ABBA order. Test sequences were spaced 2 min apart. Stimulus intensity was reported using the formula provided by Semmes-Weinstein: $\text{Intensity} = \log_{10}(10,000 * \text{g force})$. At-level pain was assessed using a girdle reactivity test (Christensen & Hulsebosch, 1997). The girdle region was shaved and grid map of the girdle zone for allodynic responding was made on the rats using an indelible marker (44 squares). The subjects were gently restrained in a towel for testing. They were acclimated to handling and the towel prior to testing. To ensure that the rats remained calm for testing, they were handled for 5 minutes immediately prior to beginning the girdle test. A von Frey hair with bending force of 204.14 mN (26 g force) was then applied to each point on the grid, and vocalization responses were recorded and mapped onto a grid map of that animal. Since animals do not normally vocalize with this stimulus, a vocalization response indicates that a noxious stimulus was experienced. In mapping the area of response, the number of vocalizations are recorded (N_v) and normalized by the following formula: $(N_v \times 100)/\text{total number of applications (44)}$, indicating the percent vocalizations out of the total number of applications.

2.4.5. Histology—At the end of behavioral testing, subjects were deeply anesthetized (100 mg/kg of pentobarbital, i.p.) and perfused (intracardially) with 4% paraformaldehyde. A 1 cm long segment of the spinal cord that included the lesion center was taken and frozen for sectioning. The tissue was sectioned coronally in 20 μm thick sections and every 10th slice was preserved for staining. All sections were stained with cresyl violet for Nissl substance and luxol fast blue for myelin (Beattie, 1992; Behrmann et al., 1992).

The total cross-sectional area of the cord and spared tissue was assessed at the lesion center using MicroBrightField software. Sections ± 600 , 1200, and 1800 μm from the lesion center (rostral and caudal) were also traced and analyzed. Assessments were made by an experimenter who was blind to the subject's treatment condition. Four indices of lesion magnitude were derived: lesion, residual gray matter, residual white matter, and width. To determine the area of lesion, an observer who was blind to the experimental treatments traced around the boundaries of cystic formations and areas of dense gliosis (Basso et al., 1995). Nissl-stained areas that contained neurons and glia of approximately normal densities denoted residual gray matter. White matter was judged spared in myelin-stained areas lacking dense gliosis and swollen fibers. The total area of each cross-section was derived by summing the areas of damage, gray and white matter. Width was determined from the most lateral points along the transverse plane. These analyses yielded six parameters for each section: white matter area, gray matter area, spared tissue (white + gray), damaged tissue area, net area (white + gray + damage), and section width.

To control for variability in section area across subjects, we applied a correction factor derived from standard undamaged cord sections, taken from age-matched controls. This correction factor is based on section widths and is multiplied by all area measurements to standardize area across analyses (see Grau et al., 2004). By standardizing area across sections we were able to estimate the degree to which tissue is 'missing' (i.e., tissue loss from atrophy, necrosis, or apoptosis). An accurate assessment of the degree to which a treatment has impacted, or lesioned, the cord includes both the remaining 'damaged' tissue as well as resolved lesioned areas. When we sum the amount of 'missing' tissue and the measured 'damaged' area we derive an index of the relative lesion (% relative lesion) in each section that is comparable across sections. We can also compute the relative percent of gray and white matter remaining in each section, relative to intact controls.

2.5. Data Analyses

The results were analyzed using analysis of variance (ANOVA) and trend analyses. In experiments with a continuous independent variable (e.g., recovery period, rostral-caudal histological sections), mixed-design ANOVAs were used. In cases where significant between-subject differences were obtained (main effect of a single variable), group means were compared using the Duncan's New Multiple Range Test ($p < 0.05$). Group differences on dichotomous variables (e.g., mortality) were evaluated using chi-square probability tests.

3. Results

3.1. Experiment : A single dose of morphine increases expression levels of IL-1 β

Day 1 BBB scores were indicative of a moderate contusion injury: prior to morphine treatment, subjects assigned to the morphine and saline treatment groups, respectively, displayed mean ($\pm$ SEM) converted BBB scores of 2.0 ± 0.56 and 2.75 ± 0.58 (equivalent to untransformed BBB scores of 2.69 ± 0.76 and 3.56 ± 0.89 , respectively).

There was a significant main effect of systemic morphine treatment on levels of IL-1 β in spinal tissue taken from the injury site 24 hrs after drug treatment ($F(1, 14) = 23.50, p < 0.005$). As can be seen in Figure 1A, morphine significantly increased IL-1 β levels. Although morphine increased levels of IL-6 also, this effect was not significant ($F(1, 14) = 3.49, p = 0.08$, Figure 1B).

3.2. Experiment 2

Day 1 BBB scores were balanced across groups. An ANOVA confirmed that there were no differences across conditions prior to treatment (both F 's $< 1.49, p > 0.05$). Converted BBB scores ranged from of 2.67 ± 0.35 , for the morphine group assessed 24 hrs after administration, to 3.19 ± 0.37 , for the morphine group assessed 30 min post administration (equivalent to untransformed BBB scores of 3.67 ± 0.61 and 4.06 ± 0.56 , respectively).

As found for systemic morphine administration, there was a significant main effect of intrathecal morphine on levels of IL-1 β in spinal tissue taken from the injury site 30 min and 24 hrs after drug treatment ($F(1, 28) = 13.14, p < 0.005$). As can be seen in Figure 2A, morphine significantly increased IL- β levels at both time points. There was also a significant main effect of time on the concentration of IL-1 β ($F(1, 28) = 6.85, p < 0.05$). Morphine increased the levels of IL-1 β at both time points, relative to vehicle treated controls, but particularly at 30 minutes post administration (Figure 2A). Also commensurate with the findings for systemic administration, there was a tendency for increased levels of IL-6 in subjects treated with intrathecal morphine, but this effect was not quite significant ($F(1, 28) = 3.19, p = 0.08$, Figure 2B). There was a significant main effect of time for IL-6, however ($F(1, 28) = 5.05, p < 0.05$): levels of IL-6 were elevated 30 minutes post morphine administration relative to the 24 hour time point (Figure 2B). It should also be noted that the levels of pro-inflammatory cytokines observed with intrathecal morphine administration were substantially higher than those observed in the systemic treatment model. The presence of the intrathecal catheter (in both the morphine and vehicle-treated groups) may enhance the glial responses to injury.

3.3. Experiment 3

3.3.1. Intrathecal morphine attenuates pain reactivity in the acute stage of injury—To verify the effectiveness of drug treatment, both a spinal (tail withdrawal from radiant heat or shock) and supraspinal (vocalization to heat or shock) measure of nociceptive reactivity were recorded. The 90 μ g dose of morphine significantly increased the latency to tail-flick irrespective of the dose of IL-1ra treatment. Prior to drug treatment subjects in the

morphine and vehicle groups displayed similar tail-flick latencies of 5.70 ± 0.29 and 5.05 ± 0.25 sec, respectively. After drug treatment, subjects treated with morphine increased their average tail-flick latency to 7.47 ± 0.25 sec, while vehicle treated subjects displayed a latency similar to that recorded pre-treatment (4.78 ± 0.25 sec). An ANCOVA (with pre-treatment baseline scores as the covariate) verified that there was a significant effect of morphine on tail withdrawal from radiant heat test ($F(1, 54) = 20.62, p < 0.0001$). There was also a significant main effect of morphine on the latency to vocalize to the heat stimulus ($F(1, 54) = 16.52, p < 0.001$; Morphine, 5.53 ± 0.23 sec, Vehicle = 5.10 ± 0.29 sec). Again, there was no effect of the IL-1ra on this measure. For the incremented shock stimulus, morphine also significantly decreased the shock threshold for tail movement ($F(1, 54) = 10.76, p < 0.01$; Morphine, 0.07 ± 0.01 mV, Vehicle = 0.12 ± 0.05 mV) and to display a vocalization response ($F(1, 54) = 6.01, p < 0.05$; Morphine, 0.13 ± 0.03 mV, Vehicle = 0.19 ± 0.07 mV).

3.3.2. IL-1ra blocks the effects of morphine on locomotor recovery—BBB

scores, collected on Day 1 and prior to drug treatment, did not differ across treatment groups. There was no main effect of morphine or IL-1ra treatment, and no interaction effect (all F s $< 1.0, p > 0.05$). Mean BBB scores on Day 1 ranged from 2.15 ± 0.30 (prior to transformation BBB score = 2.85 ± 0.51) for the group to be treated with $3 \mu\text{g}$ of IL-1ra and then vehicle, to 2.40 ± 0.41 (prior to transformation BBB score = 2.90 ± 0.53) for subjects treated with the $1 \mu\text{g}$ IL-1ra prior to vehicle.

Day 1 BBB scores were, however, a significant covariate ($F(1, 53) = 70.12, p < 0.0001$), accounting for 56% of the variance in recovery seen across days. Subsequent analyses, using Day 1 scores as a covariate (ANCOVA), revealed a significant interaction between treatment with morphine and the dose of IL-1ra across the recovery period ($F(22, 583) = 1.88, p < 0.01$). As can be seen in Figure 3, treatment with $90 \mu\text{g}$ of morphine impaired recovery across the 21 days post injury, compared with vehicle controls. Administration the IL-1ra, prior to morphine treatment, blocked the adverse consequences of morphine on long-term recovery. Comparisons of the average locomotor scores of the groups over Days 15–21, when performances had stabilized, confirmed the interaction between morphine and the IL-1ra treatments (ANCOVA, $F(2, 53) = 4.19, p < 0.05$). Post hoc analyses comparing the group means confirmed that subjects treated with the $90 \mu\text{g}$ dose of morphine alone recovered significantly less motor function than vehicle treated controls ($p < 0.05$, Figure 3). Additionally, trend analysis revealed a significant linear effect of IL-1ra dose on terminal (Days 15–21) BBB scores ($F(1, 53) = 7.20, p < 0.01$). The level of motor recovery significantly decreased, in subjects that were not treated with morphine, as the dose of IL-1ra increased.

3.3.3. IL-1ra decreases symptoms of at-level pain—There was no effect of morphine or IL-1ra treatment on either motor or vocal reactivity to heat or shock stimuli in the chronic phase of recovery (21–28 days post injury). Similarly, neither morphine ($F(1, 52) < 1.0, p > 0.05$) nor the IL-1ra ($F(2, 52) = 1.73, p > 0.05$) affected motor or vocal reactivity to mechanical stimuli 21–28 days after injury. However, there was a significant main effect of IL-1ra dose on girdle reactivity on day 21 ($F(2, 53) = 3.85, p < 0.05$). Treatment with $3 \mu\text{g}$ of the IL-1ra prior to morphine, and alone, significantly decreased the expression of at-level neuropathic pain symptoms compared with vehicle treated controls ($p < 0.05$). As can be seen in Figure 4, treatment with the IL-1ra prior to morphine reduced vocal responses to a level commensurate with vehicle-treated (no morphine) controls. Independent analyses for the vehicle and morphine-treated subjects separately, also revealed a significant linear trend for the morphine groups ($F(1, 26) = 5.62, p < 0.05$) indicative of a decrease in vocal reactivity as the dose of IL-1ra increased (Figure 4).

3.3.4. IL-1ra increases weight loss after injury—A day after injury, prior to drug treatment, mean weights ranged from 321.20 ± 5.94 to 355.88 ± 9.97 . There was a significant effect of group for this measure ($F(2, 54) = 5.24, p > 0.01$). Post hoc analyses showed that subjects in the 3 μg IL-1ra group were significantly heavier than vehicle treated controls at this time-point. To control for the variability observed in starting weights, we used a change from baseline (Day 1) weight in subsequent analyses. An ANOVA revealed a significant IL-1ra dose X Days interaction for weight ($F(22, 594) = 2.14, p < 0.005$). Subjects treated with 3 μg IL-1ra lost much more weight than vehicle treated controls and subjects treated with 1 μg of IL-1ra (Figure 5). A similar effect of dose was found for the vehicle-treated subjects when comparisons were made on the average terminal weight loss for 15–21 days ($F(2, 27) = 4.47, p < 0.05$). Again, subjects treated with 3 μg IL-1ra lost much more weight than subjects treated with 1 μg of IL-1ra and vehicle-treated controls ($p < 0.05$; Figure 5). Also, as can be seen in Figure 5, subjects treated with morphine alone lost substantially more weight than vehicle-treated controls, although this effect was not quite significant in this study ($F(1, 18) = 4.17, p = 0.056$).

3.3.5. Effects of morphine on autophagia, mortality, spasticity and bladder function—Neither morphine nor the IL-1ra affected mortality, autophagia or spasticity ($p > 0.05$ for all chi-squared tests). Similarly, neither drug affected the recovery of bladder function (Morphine, $F(1, 52) < 1.0, p > 0.05$; IL-1ra, $F(2, 52) = 1.92, p > 0.05$).

3.3.6. The IL-1ra has a dose-dependent effect on lesion size—Rather than effects of morphine *per se* on lesion size and tissue sparing, there were significant interaction effects between the dose of IL-1ra and morphine. For % relative lesion (damage + missing), there was no effect of morphine or the IL-1ra at the injury center but a main effect of morphine emerged for the sections caudal to the center of the lesion ($F(1, 44) = 5.48, p < 0.05$). As can be seen in Figure 6, subjects treated with vehicle had higher % relative lesion caudal to the injury and this effect was primarily driven by the data from subjects treated with 3 μg of IL-1ra. There was also a significant interaction effect for the IL-1ra dose X Morphine rostral to the injury center ($F(2, 44) = 4.64, p < 0.05$). Again this interaction was due to a dose-dependent effect of IL-1ra administered alone; subjects treated with 3 μg of IL-1ra had increased lesion size whereas a decreased area of lesion was derived for subjects treated with 1 μg of IL-1ra.

The effects of the IL-1ra were largely due to increased missing tissue in the 3 μg IL-1ra group. As found for the overall relative lesion indice, there was a main effect of morphine caudal to the injury center ($F(1, 44) = 4.91, p < 0.05$). As seen in Figure 6, subjects treated with 3 μg of IL-1ra alone were missing significantly more tissue, caudal to the lesion center, relative to those treated with morphine and the IL-1ra. Similarly there was a nonsignificant tendency for a main effect of IL-1ra dose at the center of the lesion ($F(2, 48) = 2.67, p < 0.08$); there was more missing tissue for the 3 μg IL-1ra group compared with the 1 μg IL-1ra group.

The increased lesion and missing tissue indices found for the 3 μg IL-1ra group were due to loss of both grey and white matter. There was a significant main effect of IL-1ra dose on gray matter sparing at the center of the lesion ($F(2, 48) = 3.73, p < 0.05$). Post hoc analyses confirmed that subjects treated with 3 μg of IL-1ra had significantly less gray matter than those treated with 1 μg of IL-1ra ($p < 0.05$; Figure 6). While there was a tendency for similar effects of the high dose of IL-1ra rostral and caudal to the lesion center, decreasing gray matter sparing, no other effects were significant. For white matter, commensurate with the missing tissue effects, there was a main effect of morphine caudal to the center of the injury ($F(1, 44) = 4.58, p < 0.05$). Morphine-treated subjects had more spared white matter. Also, rostral to the injury, there was a significant IL-1ra dose X Morphine interaction ($F(2, 44) =$

5.17, $p < 0.01$). Vehicle subjects treated with 3 μg of IL-1ra displayed less white matter sparing rostral to the injury site.

4. Discussion

As found in our previous studies, intrathecal morphine applied in the acute phase of SCI significantly undermined recovery of locomotor function, and increased the expression of symptoms of paradoxical pain in the chronic phase of injury. These adverse effects of morphine appear to depend on activation of the IL-1 receptor. Expression levels of IL-1 β in the injured spinal cord were significantly elevated 24 hrs after systemic morphine administration compared with vehicle controls. Similarly, intrathecal morphine significantly increased IL-1 β levels in the spinal tissue 30 min and 24 hrs post-administration. Functionally, there was a dose-dependent effect of an IL-1ra on recovery of locomotor function and girdle reactivity after morphine administration. Antagonizing the IL-1 receptor with the 3 μg dose of IL-1ra protected recovery and reduced symptoms of at-level neuropathic pain in morphine-treated rats. In the acute phase of a contusion injury, administration of morphine may potentiate the inflammatory response, moving it beyond an adaptive reaction to maladaptive levels.

The data suggest that the morphine-induced attenuation of function depends, at least in part, on the increased expression of IL-1 β . As noted previously, increases in IL-1 β expression is inherent to the acute phase of a spinal contusion injury, and is likely adaptive in this early stage (Nesic et al., 2001; Nakamura et al., 2003; Yang et al., 2004; Wang et al., 2005; Yang et al., 2005; Pineau & Lacroix, 2007). Both systemic and intrathecal morphine administration, however, further increased IL-1 β expression levels relative to vehicle-treated controls. Moreover, the effects of morphine on IL-1 β levels emerged within 30 minutes of morphine administration; decreasing, but maintaining a significantly higher level of expression 24 hrs post-administration (48 hrs post injury). It is now well-established that in addition to the classic opioid receptors, morphine binds to *non-classic* opioid receptors, such as the toll-like receptor 4 (TLR4), inducing glial activation and subsequently the release of pro-inflammatory cytokines (Watkins et al. 2007; Hutchinson et al., 2007; Hutchinson et al. 2010). Interestingly, TLR4 mRNA levels are significantly increased by 72 hrs following a spinal contusion injury (Kigerl et al. 2007). In the absence of pathogens TLR4 appears to be important for regulating inflammation and gliosis after SCI (Kigerl et al. 2007), but increased expression may also leave the injured spinal system more vulnerable to opioid-induced proinflammatory glial activation. Morphine has also been shown to regulate microglial activation and migration within minutes, by binding with μ -opioid receptors on the microglia, inducing activation of the P13K/Akt pathway and enhancing P2X₄ receptor expression (Horvarth & DeLeo, 2009). The P2X₄ receptor has been implicated in the development of neuropathic pain symptoms (mechanical allodynia) via BDNF release (Ulmann et al. 2008; Trang et al., 2009; Beggs & Salter, 2010). The direct effects of morphine on glial activation appears to disturb a critical balance in the early immune response to SCI, disrupting the natural physiological sequelae by potentiating proinflammatory glial activation.

Indeed, Liu et al. (2008) have also shown that the intrathecal administration of IL-1 β , for three days following a contusion injury, significantly undermines recovery of locomotor function. The administration of morphine may indirectly mimic this effect. Normally, the acute inflammatory response inherent to a spinal contusion subsides around 48 hours post injury (Nesic et al., 2001; Liu et al., 2008). At this time point, Liu et al. (2008) have shown that the decrease in IL-1 β is accompanied by an increase in an endogenous IL-1 receptor antagonist. They suggest that after SCI there is a reciprocal interaction between IL-1 β and IL-1ra levels; as IL-1 β expression increases, IL-1ra levels decrease and vice versa.

Unfortunately, by applying morphine to the vulnerable contusion site, we appear to have triggered a detrimental dysregulation of pro-inflammatory cytokines, and perhaps endogenous IL-1ra, and thereby attenuated functional plasticity. Based on this hypothesis, pre-administration of the IL-1ra, in the current study, may protect recovery by producing a reciprocal decrease in endogenous IL-1 β levels. Alternatively, blocking the IL-1 receptor may prevent the induction of molecular cascades that undermine functional recovery.

Elevations in IL-1 β expression levels could affect locomotor recovery through multiple mechanisms. Liu et al. (2008) suggest that the motor deficits they observed with IL-1 β administration may have resulted from the induction of glutamate toxicity and increased secondary tissue damage. This does not, however, appear to be the case in the present study. We did not find an effect of morphine on tissue loss at the site of injury. Rather, we suggest that the adverse effects of pro-inflammatory cytokines in these studies may depend on the modulation of molecular cascades mediating plasticity in the early phase of recovery. Although endogenous IL-1 β appears to be required for the induction and maintenance of hippocampal LTP (Schneider et al., 1998; Coogan et al., 1999), the increased expression of this pro-inflammatory cytokine is associated with an inhibition of the induction of LTP (Murray & Lynch, 1998; Curran et al., 2003). Yang et al. (2005) have also shown that high levels of IL-1 β inhibit functional transmission in cultured hippocampal neurons. Overstimulation, with elevated levels of IL-1 β , may saturate the neural system and disrupt LTP induction (McNaughton, 1993). In the present study, the morphine-induced elevation in IL-1 β expression levels may directly undermine the functional plasticity of the spinal system.

Overexcitation of spinal neurons, with a morphine-induced elevation of IL-1 β , may also lead to central sensitization and symptoms of paradoxical pain in the chronic phase of SCI. The acute effects of pro-inflammatory cytokines on pain facilitation are well established. Intrathecal administration of IL-1 β produces mechanical and thermal hyperalgesia (Reeve et al., 2000; Sung et al., 2005), and treatment with an IL-1ra has been shown to reduce inflammatory pain (Zhang et al., 2008; Samad et al., 2001). Similarly, IL-1 β has been shown to oppose opioid-induced analgesia (Shavit et al., 2005; Hutchinson et al., 2008), decreasing morphine efficacy and contributing to the development of morphine tolerance. Conversely, an IL-1ra enhanced the magnitude and duration of morphine-induced analgesia and inhibited the development of hyperalgesia and allodynia with repeated intrathecal morphine administration (Johnston et al., 2004). The results of the current study support these findings. Early morphine treatment increased girdle reactivity, a symptom of at-level neuropathic pain, in the chronic phase of injury. Blocking IL-1 receptor activity, reduced the symptoms of paradoxical pain associated with morphine-treatment; the 3 μ g dose of IL-1ra significantly decreased girdle reactivity to levels commensurate with vehicle-treated controls. In subjects treated with 3 μ g dose of IL-1ra alone, the loss of white matter rostral to the injury may have attenuated supraspinal signaling, and consequently reduced vocal reactivity to stimuli applied around and caudal to the lesion site. However, subjects treated with morphine and the 3 μ g dose of IL-1ra did not show increased lesion size relative to vehicle controls, and also displayed significantly lower levels of girdle reactivity relative to vehicle-treated controls. These data indicate that blocking IL-1 receptor activation is sufficient to reduce the development of neuropathic pain symptoms, and is independent of reduced supraspinal signaling with increased cell death.

While increasing levels of pro-inflammatory cytokines appears to have adverse effects, significantly down-regulating IL-1 receptor activity may also be problematic. Despite the beneficial effects of the IL-1ra treatment for some indices of recovery, antagonizing the IL-1 receptor, independent of morphine administration, increased tissue loss across the rostral-caudal extent of the lesion, potentiated weight loss after injury and undermined recovery of

locomotor function. Treatment with 3 µg of the IL-1ra increased relative lesion size, and decreased gray and white matter sparing, when it was given *without* morphine. These detrimental effects of the IL-1ra were non-monotonic; they were not expressed when the lower 1 µg dose of IL-1ra was used, and were reduced when the 3 µg dose of IL-1ra was applied *with* morphine. It seems as though the significant reduction of IL-1 receptor activation undermines general health and recovery after SCI. Further, the alternative increase in pro-inflammatory cytokine levels with morphine administration had similar adverse effects. Commensurate with our previous studies, morphine alone decreased weight gain after injury relative to vehicle-treated controls. This effect has been remarkably consistent across studies and, given that morphine is applied intrathecally and acutely, is unlikely to be due to a simple decrease in food intake. We have, in fact, looked at food intake in a morphine self-administration model, and found no effect of this variable despite significant weight loss with morphine administration (Woller et al., in preparation). The mechanisms mediating weight loss after morphine require further examination. Nonetheless, these data again underscore the effects of disrupting a critical balance between adaptive repair and destructive pathological effects that depend on pro-inflammatory cytokine expression levels.

Overall, the current studies implicate the IL-1 receptor, and IL-1β, in the morphine-induced attenuation of locomotor function and the development of paradoxical pain. Importantly, these data suggest that pre-treatment with an IL-1ra, in a clinical setting, may effectively block the induction of the morphine-induced attenuation of function seen after SCI. Future studies should now focus on deriving the mechanisms mediating the protective effects of IL-1ra treatment. While the present study demonstrates that an IL-1ra attenuates the negative effect of morphine, it does not give congruent information on the molecular mechanisms underlying the effect. Decreased pro-inflammatory cytokine concentrations, reduced glial activation, or altering the concentration of pro versus anti-inflammatory cytokine concentrations, with IL-1ra pre-treatment, may account for the altered impact of morphine after SCI. At present, these data implicate the immune system in the adverse effects, and suggest that further examination of critical changes in this system will increase our understanding of the cellular processes underlying the morphine-induced attenuation of function, as well as the development of neuropathic pain.

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Figure 1.

A single dose of systemic morphine (20 mg/kg, i.p.) significantly increased expression levels of IL-1 β in spinal tissue from the contusion injury site (A). The tissue was assayed 24 hrs after morphine administration, and 48 hrs after injury. Morphine did not significantly affect IL-6 levels (B). n = 8 for both groups, and ** $p < 0.05$.

Figure 2.

As found for systemic morphine, there was a significant main effect of intrathecal morphine on expression levels of IL-1 β at the contusion injury site (A). Increased expression levels were observed at both 30 min and 24 hrs following morphine administration. However, expression levels determined at 30 min were also significantly higher than those observed at 24 hrs. Similarly, there was a significant main effect of time on IL-6 expression levels (B). Although there was a strong tendency for morphine to increase levels of this pro-inflammatory cytokine also ($p = 0.08$), this effect was not significant. $n = 8$ for all groups** $p < 0.05$.

Figure 3.

Intrathecal morphine (90 μg , i.t.) administered 24 hrs after a moderate contusion significantly attenuated recovery of locomotor function, relative to vehicle-treated controls (0 μg IL-1ra). Pretreatment with an IL-1ra (1 and 3 μg) blocked the adverse effects of morphine on recovery. Further analyses of the average BBB scores when performances stabilized (days 15–21 post-injury) also revealed a significant linear trend for decreased recovery as the dose of IL-1ra was increased for vehicle-treated (no morphine) controls. $n = 10$ for all groups, ** $p < 0.05$.

Figure 4.

The number of vocal responses made to the application of an innocuous von Frey filament in the girdle region is depicted. Pre-treatment with 3 μ g of the IL-1ra prior to morphine, and alone, significantly decreased vocal reactivity compared with vehicle-treated subjects. For the morphine-treated groups, there was also a significant linear trend for a dose-dependent decrease in vocal reactivity to von Frey stimulation as the dose of IL-1ra increased.

Figure 5.

The mean weight loss for each group ($\pm$ SEM) over the 21 days recovery period is depicted as a change from baseline (Day 1) score. In the 0 µg IL-1ra groups, there was a tendency for morphine alone to increase weight loss, compared with vehicle-treated subjects, but this effect was not significant ($p = 0.056$). There was, however, a significant effect of the dose of IL-1ra on weight loss. Independent of morphine treatment, there was a concomitant increase in weight loss as the IL-1ra dose increased. Analyses of the average weight loss from Days 15–21 confirmed that vehicle controls (no morphine) treated with 3 µg IL-1ra lost significantly more weight than vehicle control subjects treated with 1 µg of IL-1ra or vehicle only. ** $p < 0.05$.

Figure 6.

Morphine treatment appeared to protect the contused cord against effects of the high dose IL-1ra (3 μ g) treatment on lesion size and the amount of gray and white tissue sparing. Subjects treated with morphine displayed less lesion caudal to the injury center (where the morphine and IL-1ra was applied), with less missing tissue and increased areas of gray matter sparing, compared with vehicle-treated controls (A, main effect of morphine treatment, $p < 0.05$). The increased tissue loss observed in the vehicle-treated subjects was driven primarily by treatment with the 3 μ g of IL-1ra. Pre-treatment with 3 μ g of IL-1ra significantly increased relative lesion size rostral to the injury site and decreased white matter sparing in the vehicle treated controls, but the same dose did not effect these indices

in morphine-treated subjects (B , interaction between morphine and IL-1ra dose, $p < 0.05$). There was also a significant effect ($**p < 0.05$) of the 3 μg IL-1ra dose on gray matter sparing at the center of the lesion, compared with the 1 μg IL-1ra dose.