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Full-length Article

Protraction of neuropathic pain by morphine is mediated by spinal damage associated molecular patterns (DAMPs) in male rats

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ABSTRACT

We have recently reported that a short course of morphine, starting 10 days after sciatic chronic constriction injury (CCI), prolonged the duration of mechanical allodynia for months after morphine ceased. Maintenance of this morphine-induced persistent sensitization was dependent on spinal NOD-like receptor protein 3 (NLRP3) inflammasomes—protein complexes that proteolytically activate interleukin-1β (IL-1β) via caspase-1. However, it is still unclear how NLRP3 inflammasome signaling is maintained long after morphine is cleared. Here, we demonstrate that spinal levels of the damage associated molecular patterns (DAMPs) high mobility group box 1 (HMGB1) and biglycan are elevated during morphine-induced persistent sensitization in male rats; that is, 5 weeks after cessation of morphine dosing. We also show that HMGB1 and biglycan levels are at least partly dependent on the initial activation of caspase-1, as well as Toll like receptor 4 (TLR4) and the purinergic receptor P2X7R—receptors responsible for priming and activation of NLRP3 inflammasomes. Finally, pharmacological attenuation of the DAMPs HMGB1, biglycan, heat shock protein 90 and fibronectin persistently reversed morphine-prolonged allodynia. We conclude that after peripheral nerve injury, morphine treatment results in persistent DAMP release via TLR4, P2X7R and caspase-1, which are involved in formation/activation of NLRP3 inflammasomes. These DAMPs are responsible for maintaining persistent allodynia, which may be due to engagement of a positive feedback loop, in which NLRP3 inflammasomes are persistently activated by DAMPs signaling at TLR4 and P2X7R.

1. Introduction

Opioids remain a gold-standard analgesic for moderate-to-severe chronic pain. Despite the recent escalation in opioid prescriptions for chronic pain, there are no clinical studies that have rigorously evaluated the long-term consequences of opioid use (Chou et al., 2015). Beyond simply an absence of benefit, there is growing evidence of harm among adults prescribed long-term opioid therapy (Chou et al., 2015; Frank et al., 2017; Hoffman et al., 2017; Sommer, 2017). We have discovered an additional negative consequence for pain in rats: a 5-day course of a moderate morphine dose, starting 10 days after sciatic chronic constriction injury (CCI), prolonged the duration of CCI-allodynia for months after morphine ceased (Grace et al., 2016). Maintenance of this morphine-induced persistent sensitization was dependent on spinal NOD-like receptor protein 3 (NLRP3) inflammasomes—protein complexes that proteolytically activate interleukin-1β (IL-1β) via caspase-1 (Grace et al., 2016). However, an unresolved question from our study is how NLRP3 inflammasome signaling is maintained long after morphine is cleared (Grace et al., 2016).

NLRP3 inflammasomes can be primed and activated by signaling through Toll like receptor 4 (TLR4) and the purinergic receptor P2X7, and both receptors are essential for morphine-induced persistent sensitization (Grace et al., 2016). TLR4 and P2X7R are activated after peripheral nerve injury by endogenous damage associated molecular patterns (DAMPs) that are released by stressed cells in the spinal cord (Grace et al., 2014). As a consequence of inflammasome activation, IL-1β may exacerbate cellular DAMP release (e.g. by disrupting glutamate homeostasis). As morphine-induced persistent sensitization was associated with persistent downregulation of the spinal astrocyte glutamate transporter GLT-1 (Grace et al., 2016), exacerbated DAMP release could be a consequence that leads to continued activation of NLRP3 inflammasomes in a positive feedback loop.

The first of three goals of this study was to determine whether the spinal DAMPs high mobility group box 1 (HMGB1) and

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biglycan were elevated in the lumbar dorsal spinal cord in our model of morphine-induced persistent sensitization. HMGB1 is a nuclear protein that is expressed by most cells. HMGB1 is released principally by neurons upon peripheral nerve injury, where it can induce an inflammatory response in surrounding cells (Agalave and Svensson, 2015). Biglycan is also expressed in the nucleus of spinal neurons, and in cultured astrocytes, but its function in these cells is not understood (Koops et al., 1996; Liang et al., 1997). Biglycan is upregulated after CNS injury, where it can be expressed by macrophages/microglia, and neurons (Stichel et al., 1995). A potential role for biglycan in pain has not been previously investigated. These representative DAMPs were selected due to their potential to activate NLRP3 inflammasomes: HMGB1 is a TLR4 agonist, while biglycan is an agonist of both TLR4 and P2X7R (Babelova et al., 2009; Frank et al., 2015; Grace et al., 2014). The second goal was to determine whether levels of HMGB1 and biglycan were dependent on TLR4, P2X7R or caspase-1. Finally, we aimed to identify a causal role for spinal DAMPS in the maintenance of morphine-induced persistent sensitization.

2. Methods

2.1. Subjects

Pathogen-free adult male Fischer 344 (F344) rats (n = 5–7 rats/-group for each experiment; 10–12 wks old on arrival; Harlan Labs, Indianapolis, IN, USA) were used. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder.

2.2. Drugs

Morphine was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC and Bethesda, MD, USA). (+)-Naloxone was synthesized by Dr. Kenner Rice. BoxA (HMGBiotech, Milan, Italy), fibronectin tetrapeptide (Santa Cruz Biotechnology, Dallas, TX) and 17-dimethylaminoethylamino-17-desmethoxygeldanamycin (17-DMAG; Calbiochem, San Diego, CA) were obtained commercially. Where applicable, drugs were prepared and are reported as free base concentrations.

2.3. RNA interference

Small interfering RNAs (siRNAs) against biglycan (sense: GAUCUCACCUGAUACCACAtt; antisense: UGUGGUAUCAGGUGA GAUCtc) were purchased from Life Technologies (Carlsbad, CA). siRNA or missense control (0.24 μg/μl in DNase-free water) was mixed at a 1:1 ratio with RNAiMAX transfection reagent (6% v/v in PBS; Life Technologies), according to manufacturer instructions.

2.4. Chronic constriction injury (CCI)

Neuropathic pain was induced using the CCI model of sciatic nerve injury (Bennett and Xie, 1988), as described previously (Grace et al., 2016). Four sterile chromic gut sutures (cuticular 4–0 chromic gut, Ethicon, Somerville, NJ) were loosely tied around the gently isolated sciatic nerve. For sham surgery, the sciatic nerve was isolated, but no chromic gut sutures were tied around the nerve.

2.5. Acute and chronic intrathecal catheter implantation

The method of acute intrathecal drug administration and the construction and implantation of the indwelling intrathecal catheters was based on that described previously (Grace et al., 2016). In brief, intrathecal operations were conducted under isoflurane anesthesia by threading sterile polyethylene-10 tubing (PE-10 Intramedic Tubing; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA) guided by an 18-gauge needle between the L5 and L6 vertebrae. The catheters were attached to a pre-loaded osmotic minipump where appropriate (Alzet, 2001, Cupertino, CA).

2.6. Subcutaneous and intrathecal drug administration

Morphine was administered subcutaneously (s.c.) at 5 mg/kg, twice daily. Intrathecal osmotic minipump infusions were as follows, (+)-naloxone: 60 μg/h; A438079: 30 ng/h; ac-YVAD-cmk: 1 μg/h, based on our previous report (Grace et al., 2016). The DAMP inhibitor cocktail was composed of *Bgn* siRNA (1.2 μg in 10 μl), BoxA (5 μg in 5 μl), 17-DMAG (10 μg in 1 μl) and fibronectin tetrapeptide (10 μg in 10 μl). The cocktail was administered daily for 7 days, beginning 5 weeks after morphine dosing conclusion. Respective equivolume vehicles were used as controls.

2.7. Western blotting

Western blot analyses were performed on L4/5 spinal ipsilateral dorsal quadrants obtained from rats used in our previous study (Grace et al., 2016), that had been transcardially perfused with saline under sodium pentobarbital anesthesia (100 mg/kg; intraperitoneal). Protein extraction, gel electrophoresis, membrane transfer and antibody incubation was performed as previously described (Grace et al., 2016). Primary antibodies and dilution ratios used were: rabbit HMGB1 1:4000 (Abcam, Cambridge, MA), rabbit biglycan 1:200 (Santa Cruz). Mouse β actin 1:100,000 (Sigma, St. Louis, MO) was used against loading control protein. Secondary antibodies used were: Goat anti-mouse IRDye 680RD 1:15,000 (LI-COR Biosciences), and goat anti-rabbit IRDye 800CW 1:15,000 (LI-COR Biosciences). Bands were quantified using Image Studio (LI-COR Biosciences).

2.8. Mechanical allodynia

Testing was conducted blind with respect to group assignment. The von Frey test (Chaplan et al., 1994) was performed at the distal region of the heel in the hind paws, within the region of sciatic innervation as previously described (Grace et al., 2016). The behavioral responses were used to calculate absolute threshold (the 50% probability of response) as described previously (Grace et al., 2016).

2.9. Statistics

Mechanical allodynia was analyzed as the interpolated 50% thresholds (absolute threshold). One-way ANOVAs followed by Tukey's post hoc test was used to confirm that there were no baseline differences in absolute thresholds between treatment groups. Differences between treatment groups were determined using repeated measures two-way ANOVA, followed by Sidak's post hoc test. Integrated Densities for Western blots were analyzed by two-way ANOVA and Sidak's post hoc test, or unpaired *t*-test, as appropriate. All data are presented as mean ± SEM, and $P < 0.05$ was considered significant.

3. Results

3.1. DAMPs are elevated with morphine-induced persistent sensitization

We first tested whether levels of the spinal DAMPs HMGB1 and biglycan were elevated by the CCI and morphine treatment. As

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