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Prior exposure to repeated morphine potentiates mechanical allodynia induced by peripheral inflammation and neuropathy

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ABSTRACT

Opioids, such as morphine, induce potent analgesia and are the gold standard for the treatment of acute pain. However, opioids also activate glia, inducing pro-inflammatory cytokine and chemokine production, which counter-regulates the analgesic properties of classical opioid receptor activation. It is not known how long these adverse pro-inflammatory effects last or whether prior morphine could sensitize the central nervous system (CNS) such that responses to a subsequent injury/inflammation would be exacerbated. Here, multiple models of inflammation or injury were induced two days after morphine (5 mg/kg b.i.d., five days , s.c.) to test the generality of morphine sensitization of later pain. Prior repeated morphine potentiated the duration of allodynia from peripheral inflammatory challenges (complete Freund's adjuvant (CFA) into either hind paw skin or masseter muscle) and from peripheral neuropathy (mild chronic constriction injury (CCI) of the sciatic nerve). Spinal cord and trigeminal nucleus caudalis mRNAs were analyzed to identify whether repeated morphine was sufficient to alter CNS expression of pro-inflammatory response genes, measured two days after cessation of treatment. Prior morphine elevated IL-1β mRNA at both sites, MHC-II and TLR4 in the trigeminal nucleus caudalis but not spinal cord, but not glial activation markers at either site. Finally, in order to identify whether morphine sensitized pro-inflammatory cytokine release, spinal cord was isolated two days after morphine dosing for five days , and slices stimulated *ex vivo* with lipopolysaccharide. The morphine significantly induced TNFα protein release. Therefore, repeated morphine is able to sensitize subsequent CNS responses to immune challenges.

1. Introduction

Opioids are the gold standard for treatment of severe acute and chronic pain. Opioids exert their potent analgesic properties via classical opioid receptors. However, opioids also induce a pro-inflammatory response within the central nervous system (CNS). The primary cell type initiating such pro-inflammatory responses is likely microglia, the predominant immunocompetent cell within the CNS. These cells have been implicated in chronic pain and dysregulating the effects of opioids (Hutchinson et al., 2007, 2008a, 2011; Watkins et al., 2007).

Activation of microglia by inflammation or injury can lead to the production of pro-inflammatory mediators (Watkins et al., 2007). Evidence is now emerging that activated microglia can either return to a homeostatic surveying state or become "primed" (sensitized) following the initial immune challenge. If these "primed" microglia are challenged within a certain period with a second immune challenge, the pro-inflammatory response is exacerbated. Most studies that have investigated glial priming have focused on the hippocampus (Frank et al., 2010a,b, 2012; Jurgens and Johnson, 2012). However, microglial priming can also occur in the spinal cord resulting in heightened pain responses to later challenge (Alexander et al., 2009; Hains et al., 2010). Heightened pain responses following later inflammatory challenge from systemic or intrathecal lipopolysaccharide (LPS) are associated with enhanced pro-inflammatory cytokine levels in spinal cord and are blocked by intrathecal interleukin-1 receptor antagonist (Hains et al., 2011; Loram et al., 2011). Such examples are suggestive that sensitization, likely of microglial origin, can occur in the spinal cord, resulting in exaggerated pain following a subsequent challenge.

Morphine can also induce pro-inflammatory responses within the CNS, likely via the activation of microglia (Hutchinson et al., 2011). To date, most studies that have explored the pro-inflammatory responses to morphine have focused on acute effects soon after morphine administration. The blockade of IL-1 with IL-1 receptor antagonist in the spinal cord increased the

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intensity and duration of morphine-induced analgesia (Hutchinson et al., 2008a). In addition, repeated morphine enhances the pro-inflammatory response when measured within 2 h of the last dose of morphine (Hutchinson et al., 2008a). However, it is not known how long the morphine-induced pro-inflammatory response lasts or whether morphine exposure sensitizes CNS responses to subsequent peripheral injury/inflammation. If morphine induces, or sensitizes, long duration pro-inflammation, even after termination of morphine administration, this may potentially impact the development of pain to subsequent inflammatory/traumatic events.

Therefore, the aim of this study was to discover whether a multi-day morphine regimen is able to sensitize neuroinflammatory and/or pain responses to a later challenge. Here we explored whether such sensitized responses may occur in response to a variety of later challenges, including peripheral inflammation of both the hind paw and orofacial muscle and peripheral nerve injury.

2. Materials and methods

2.1. Animals

Pathogen-free male Sprague–Dawley rats (300–350 g, Harlan Laboratories, Madison, WI) were housed two per cage with temperature (23 ± 0.3 °C) and light (12:12 light:dark cycle; lights on at 07:00) controls. Rats had free access to water and standard chow and acclimated 1 wk before experimentation. All behavioral testing occurred during lights on. The Institutional Animal Care and Use Committee of the University of Colorado at Boulder approved all procedures. In all experiments, behavior and tissue were analyzed using blinded procedures.

2.2. Drugs

Morphine sulfate (gifted by Mallinckrodt, St. Louis, MO, USA) was prepared and reported as free base concentrations. Morphine was dissolved in endotoxin-free sterile saline at 5 mg/ml/kg. Morphine or saline was administered subcutaneously twice daily for five days at 08:00–10:00 and at16:00–18:00. Five days of morphine administration was selected as it has been shown previously to enhance the pro-inflammatory response over acute morphine administration (Hutchinson et al., 2008a; Johnston et al., 2004). A two day recovery period was chosen to ensure morphine had ample time to clear from the circulation and allow for the return of behavioral responses back to pre-morphine values. Complete Freund's adjuvant (CFA) was purchased from Sigma (F5881; Sigma–Aldrich, St. Louis, MO, USA) and 50 μl of heat killed *Mycobacterium tuberculosis* suspended in 50 μl of sterile saline to a 1:1 oil:saline emulsion was administered.

2.3. von Frey testing for mechanical allodynia

2.3.1. Hind paw

The von Frey test was performed on the plantar surface of each hind paw within the region of sciatic nerve innervation, as described previously (Milligan et al., 2000). Before testing, rats were habituated to the testing environment for 4 days, 40 min/day. A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (0.407–15.136 g, Stoelting, Wood Dale, IL, USA) was applied randomly to the hind paws, each for 8 s at constant pressure.

2.3.2. Orofacial

The von Frey test was performed on the face within the region of trigeminal nerve innervation, for both the V_1 and V_3 branches (Ren, 1999; Sugiyo et al., 2005). Before testing, rats were handled and habituated to remain still in a leather glove, unrestrained, while their nose rests between the tester's thumb and index finger. A logarithmic series of 10 calibrated Semmes–Weinstein monofilaments (2–65 g, Stoelting, Wood Dale, IL, USA) was applied for ~1 s, randomly to the cheek and above the eye. Each rat was tested three times upwards until three consecutive positive responses were identified.

The stimulus intensity threshold to elicit withdrawal responses (face or hind paw) 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) and is described as allodynia or mechanical sensitivity throughout the text.

2.4. RNA isolation and cDNA synthesis

RNA was extracted using phenol:chloroform with TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Total RNA was reverse transcribed using Superscript II First-Strand Synthesis System (Invitrogen, Carlsbad, CA) (Loram et al., 2011). cDNA was diluted 2-fold in nuclease-free water and stored at −80 °C until PCR was performed.

2.5. Real-time polymerase chain reaction (PCR)

Primer sequences (Genbank, National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) are displayed in Table 1. cDNA amplification was performed using Quantitect SYBR Green PCR kit (Qiagen, Valenica, CA) in iCycler iQ 96-well PCR plates (Bio-Rad, Hercules, CA) on a MyiQ single Color Real-Time PCR Detection System (Bio-Rad). Each sample was measured in duplicate using the MyiQ single Color Real-Time PCR Detection System (Bio-Rad) (Loram et al., 2011). Threshold for detection of PCR product was set in the log-linear phase of amplification and the threshold cycle (C_T) was determined for each reaction. The level of the target mRNA was quantified relative to the housekeeping gene (GAPDH) using the $\Delta\Delta C_T$ method. GAPDH was not significantly different between treatments.

2.6. Enzyme linked immunoassay (ELISA)

Supernatants were collected and assayed for TNFα using a rat multiplex ELISA (Aushon, CA, USA), according to manufacturer's instructions. Chemiluminescence was quantified on a SignaturePLUS CCD (Aushon, CA, USA) and analyzed using PROarray Analyst Software (Aushon).

Table 1
Primer sequences.

Gene	Primer sequence (5′–3′)	GenBank Accession No.
GAPDH	TCTTCCAGGAGCGAGATCCC (forward) TTCAGGTGAGCCCCAGCCTT (reverse)	NC_005103.2
TLR4	TCCCTGCATAGAGGTACTTC (forward) CACACCTGGATAAATCCAGC (reverse)	NM_019178.1
IL-1β	CCTTGTGCAAGTGTCTGAAG (forward) GGGCTTGGAAGCAATCCTTA (reverse)	NM_0315122.2
MHC-II	AGCACTGGGAGTTTGAAGAG (forward) AAGCCATCACCTCCTGGTAT (reverse)	NM_019111.4
CD11b	CTGGTACATCGAGACTTCTC (forward) TTGGTCTCTGTCTGAGCCTT (reverse)	NM_012711.1
GFAP	AGATCCGAGAAACCAGCCTG (forward) CCTTAATGACCTCGCCATCC (reverse)	NM_017009.2
CCL2	GTCTCAGCCAGATGCAGTTA (forward) CACAGATCTCTCTCTTGAGC (reverse)	NM_031530