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MicroRNA-124 and microRNA-146a both attenuate persistent neuropathic pain induced by morphine 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 microglial reactivity and Toll-like receptor 4 signaling. Given that microRNAs (miRNAs) such as miR-124 and miR-146a possess the ability to modulate such signaling, we directly compared their function in this model. We found that both miRNAs reversed established allodynia in our model of morphine-induced persistent sensitization. The efficacy of miR-124 and miR-146a were comparable, and in both cases allodynia returned within hours to days of miRNA dosing conclusion. Our findings demonstrate that miRNAs targeting Toll-like receptor signaling are effective in reversing neuropathic pain, which underscores the clinical potential of these non-coding RNAs.

Keywords

TLR4; P2×7R; danger signals; priming; opioid-induced hyperalgesia

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that, by and large, negatively regulate gene expression by binding the 3'-UTR of mRNA, to post-transcriptionally inhibit protein translation [1]. The expression levels of many miRNAs are altered by noxious stimuli, suggestive that they may be involved in the response to peripheral nerve injury [2]. For example, miR-124 was decreased in spinal dorsal horn neurons after hindpaw formalin, and

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systemic administration (which is peripherally restricted) of a miR-124 mimic attenuated nociceptive hypersensitivity induced by formalin and spared nerve injury [3,4]. MiR-146a was upregulated in the spinal cord after spinal nerve ligation, and supplementation by intrathecal miR-146a mimic further attenuated allodynia [5]. Among other functions, these miRNAs negatively regulate immune signaling, such as Toll like receptor signaling, and pro-inflammatory M1 phenotype polarization [4,6]—key mechanisms of neuropathic pain [7–10].

We have recently demonstrated that a 5-day course of morphine, starting 10 days after sciatic chronic constriction injury (CCI), prolonged the duration of CCI-allodynia for months after morphine had ceased [11]. This morphine-induced persistent sensitization was dependent on spinal immune signaling, as sensitization was reversed when such proinflammatory signaling was inhibited [11,12]. Since miR-124 and miR-146a negatively regulate immune signaling, the goal of this study was to test whether intrathecal supplementation of either miRNA could reverse allodynia in our model of persistent sensitization.

Results

We sought to determine whether immune modulating miRNAs could reverse established morphine-induced persistent sensitization when administered intrathecally. CCI surgery was performed, followed ten days later by a 5-day course of morphine (5 mg/kg b.i.d., s.c). We have previously shown that CCI-allodynia in F344 male rats/resolves 5 weeks after saline treatment, while CCI-allodynia persists for 11 weeks after morphine treatment [11]. Consistently, CCI-allodynia was evident at 5 weeks post morphine treatment (Fig. 1). MiRNAs were then intrathecally administered daily, for 4 days, at this timepoint. MiR-124 and miR-146a reversed established persistent sensitization in F344 rats, compared to missense control (Fig 1; Time $\times$ Treatment: $F_{12,84} = 2.04$, $P < 0.05$; Time: $F_{6,84} = 33.86$, $P < 0.001$; Treatment: $F_{2,14} = 7.52$, $P < 0.01$). MiR-146a significantly reversed persistent sensitization for 48 h after the last dose ($P < 0.05$), before allodynia returned at 72 h. On contrast, miR-124 significantly reversed persistent sensitization for 6 h following the last dose ($P < 0.05$), and completely returned to control levels by 72 h.

Discussion

Here, we show that miR-124 and miR-146a reverse morphine-induced persistent sensitization. In addition, we report for the first time that miR-146a has efficacy when injected intrathecally, indicating that the spinal cord and/or DRGs are a site of action. These data support prior reports that miR-124 and miR-146a attenuate nociceptive hypersensitivity in models of neuropathic pain [3–5].

MiR-124 and miR-146a have a myriad of regulatory functions in the innate immune system, including repression of key TLR4 signaling molecules like MyD88, TRAF6, IRAK and NF κ B [6,13,14]. In addition, miR-124 increased markers of anti-inflammatory M2 polarization by microglia [4], and attenuated microglia activation in experimental autoimmune encephalomyelitis by downregulating C/EBP- α and PU.1 [14]. Notably, these

are key processes in morphine-induced persistent sensitization, as we found that activation of microglia, and signaling through TLR4 were causal to maintenance of allodynia [11]. As miR-124 and miR-146a interact with this pathway, we do not predict that the protective effects are specific to morphine, but will generalize to any TLR4 signal (other TLR4 ligands besides morphine are sufficient to induce persistent sensitization [11]). These miRNAs likely have direct effects on neurons in the pain neuraxis, as miR-124 was recently found to repress CREB1 gene expression [15], a key mediator of central sensitization [16]. Subsequent studies will be aimed at investigating whether miR-124 and miR-146a modulate these pathways in this model of neuropathic pain enhancement by morphine.

In our previous work, morphine-induced persistent sensitization was enduringly reversed when TLR4, the purinergic receptor P2 $\times$ 7, caspase-1, or danger associated molecular patterns (DAMPs) were inhibited for seven days, beginning 5 wk after morphine cessation [11,12]. In the present study, allodynia returned within hours to days of the final miRNA dose. This could be due to the shorter dosing duration, or that these miRNAs do not completely normalize the mechanisms underpinning persistent nociceptive hypersensitivity.

In conclusion, we have demonstrated that miR-124 and miR-146a both attenuate persistent sensitization of neuropathic pain by a short course of morphine early in neuropathic pain expression. Future studies will explore the underlying mechanisms, and determine whether the results translate to other models of morphine-enhanced pain [17–19]. As therapeutic miRNA modulators are in the clinical trials for glioblastoma [20], similar strategies may be employed to manage neuropathic pain.

Methods

Subjects

Pathogen-free adult male Fischer 344 (F344) rats (n = 6 rats/group for each experiment; 10-12 wks old on arrival; Harlan/Envigo Labs, Indianapolis, IN, USA) were used in all experiments, based on our prior studies of morphine-induced persistent sensitization [11]. Rats were pair-housed in temperature-controlled (23 $\pm$ 3°C) and light-controlled (12 h light:dark cycle; lights on at 07:00 h) rooms with standard rodent chow and water available *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder.

Chronic constriction injury (CCI)

Neuropathic pain was induced using the CCI model of sciatic nerve injury [21]. In brief, animals were anesthetized with isoflurane. The shaved skin was treated with Nolvasan and the surgery was aseptically performed. Four sterile chromic gut sutures (cuticular 4-0 chromic gut, Ethicon, Somerville, NJ) were loosely tied around the gently isolated sciatic nerve. Animals were monitored post-operatively until fully ambulatory prior to return to their home cage.

Morphine dosing

Morphine was obtained from the National Institute on Drug Abuse (Research Triangle Park, NC and Bethesda, MD, USA), and was prepared and reported as a free base concentration. Morphine was administered subcutaneously (s.c.) at 5 mg/kg/ml, twice daily for 5 days, beginning 10 days after CCI surgery.

Intrathecal catheterization and microRNA mimic dosing

The method of intrathecal drug administration, and the construction and implantation of the indwelling intrathecal catheters was based on that described previously [22]. 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 17 cm catheter was inserted such that the proximal catheter tip lay over the lumbosacral enlargement. The needle was removed and the catheter was sutured to the superficial musculature of the lower back, and then led subcutaneously to the nape of the neck. The catheters were heat sealed with approximately 3 cm of the catheter protruding to allow easy access for repeated dosing.

MicroRNA mimics for miR-124 (Sequence: CGUGUUCACAGCGGACCUUGAU; accession number: MIMAT0004728) and miR-146a (Sequence: CCUGUGAAGUUCAGUUCUUU; accession number: MIMAT0017132), as well as mirVana negative control were purchased from Life Technologies (Carlsbad, CA). miRNA or control (40 µg/ml in DNase-free water) was mixed at a 1:1 ratio with lipofectamine transfection reagent (6% v/v in PBS; Life Technologies), according to manufacturer instructions. MiR-124 mimic, miR-146a mimic, or missense control were administered daily at 0.3 µg in 15 µl for 4 successive days. MicroRNA mimic dosing began 5 weeks after morphine had concluded, the timepoint at which persistent sensitization is fully manifested [11].

Mechanical allodynia

Testing was conducted blind with respect to group assignment. Rats received at least three 60-minute habituations to the test environment prior to behavioral testing. The von Frey test [23] was performed at the distal region of the heel in the hind paws, within the region of sciatic innervation as previously described in detail [24,25]. Assessments were made prior to surgery (baseline), prior to morphine administration (day 10 post surgery), at weekly intervals following the conclusion of morphine administration, and over a 72 h timecourse after the conclusion of miRNA mimic administration. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL) were applied randomly to the left versus right hind paws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs ranged from manufacturer-designated 3.61 (0.40 g) to 5.18 (15.14 g) filaments. The behavioral responses were used to calculate absolute threshold (the 50% probability of response) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method [26,27].

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. Data are presented as mean $\pm$ SEM, and $P < 0.05$ was considered significant.

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- Morphine administered shortly after peripheral nerve injury can prolong neuropathic pain for weeks to months
- We show that an anti-inflammatory microRNAs miR-146a and miR-124 can reverse prolonged pain
- We further highlight a spinal site of action for these microRNAs in pain

Figure 1. MiR-124 and miR-146a attenuate morphine-induced persistent sensitization
 MiR mimics were administered intrathecally for 4 successive days beginning 5 weeks after morphine (5 days, administered 10 days after CCI; shaded panel) and absolute thresholds for mechanical allodynia quantified. CCI+saline treatment is included from our previous publication [11], for comparison. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, miR-146a vs. negative control. # $P < 0.05$, ## $P < 0.01$, miR-146a vs. negative control. $N = 6$ /group.