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The association between spinal cord trauma-sensitive miRNAs and pain sensitivity, and their regulation by morphine

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Abstract

Increased pain sensitivity is a common sequela to spinal cord injury (SCI). Moreover, drugs like morphine, though critical for pain management, elicit pro-inflammatory effects that exacerbate chronic pain symptoms. Previous reports showed that SCI results in the induction and suppression of several microRNAs (miRNAs), both at the site of injury, as well as in segments of the spinal cord distal to the injury site. We hypothesized that morphine would modulate the expression of these miRNAs, and that expression of these SCI-sensitive miRNAs may predict adaptation of distal nociceptive circuitry following SCI. To determine whether morphine treatment further dysregulates SCI-sensitive miRNAs, their expression was examined by qRT-PCR in sham controls and in response to vehicle and morphine treatment following contusion in rats, at either 2 or 15 days post-SCI. Our data indicated that expression of miR1, miR124, and miR129-2 at the injury site predicted the nociceptive response mediated by spinal regions distal to the lesion site, suggesting a molecular mechanism for the interaction of SCI with adaptation of functionally intact distal sensorimotor circuitry. Moreover, the SCI-induced miRNA, miR21 was induced by subsequent morphine administration, representing an alternate, and hitherto unidentified, maladaptive response to morphine exposure. Contrary to predictions, mRNA for the pro-inflammatory interleukin-6 receptor (IL6R), an identified target of SCI-sensitive miRNAs, was also induced following SCI, indicating dissociation between miRNA and target gene expression. Moreover, IL6R mRNA expression was inversely correlated with locomotor function suggesting that inflammation is a predictor of decreased spinal cord function. Collectively, our data indicate that miR21 and other SCI-sensitive miRNAs may constitute therapeutic targets, not only for improving functional recovery following SCI, but also for attenuating the effects of SCI on pain sensitivity.

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Keywords

spinal cord contusion; microRNA; dysregulation; neuronal plasticity; nociception; morphine

1. Introduction

The effects of spinal cord injury (SCI) can be severely debilitating, especially the chronic presence of neuropathic pain that affects close to two-thirds of all spinal cord-injured patients (Anderson, 2004; Siddall and Loeser, 2001). One of the primary treatments for chronic pain following SCI is administration of opiate analgesics such as morphine, which are also given during the acute phase of spinal injury for pain mitigation (Dworkin et al., 2003; McCarberg, 2004). However, administration of morphine in the acute phase of a spinal contusion injury in rats has been shown to significantly attenuate locomotor function and increase expression of chronic pain symptoms (Hook et al., 2007; Hook et al., 2009). The increased chronic pain associated with morphine administration has been attributed to the activation of astrocytes and microglia by morphine and inhibition of analgesic effects by increased expression of pro-inflammatory cytokines TNF, IL-1, and IL-6 (Cui et al., 2006; Johnston et al., 2004; Raghavendra et al., 2002; Song and Zhao, 2001; Tai et al., 2006). Accordingly, the application of an interleukin-1 receptor antagonist prior to intrathecal morphine prevents the maladaptive effects of morphine on neuropathic pain and functional recovery (Hook et al., 2011).

While these results are promising, there is limited knowledge of the remodeling of pain neuro-circuitry and the activation of potentially maladaptive gene regulatory networks following morphine treatment in SCI. MiRNAs are short, 18 to 25 nucleotide-long non-coding small RNAs that play an important regulatory role in gene expression by inhibiting protein translation or targeting mRNA transcripts for degradation (Alvarez-Garcia and Miska, 2005; Zamore and Haley, 2005). Individual miRNAs are able to coordinate gene networks to affect a specific cellular endpoint by simultaneously controlling expression of several hundred mRNAs. Furthermore, miRNAs have been implicated in physiological processes relevant to neuropathic pain, including innate immune responses involved in wound inflammation (Roy and Sen, 2011; Williams et al., 2008) and the regulation of inflammation in response to morphine treatment in human monocyte-derived macrophages (Dave and Khalili, 2010).

MiRNAs are also dysregulated following SCI (Liu et al., 2009; Nakanishi et al., 2010; Strickland et al., 2011; Yunta et al., 2012). We previously reported that miR124, miR129, and miR1 were significantly down-regulated following spinal cord contusion, while both miR21 and miR146a were transiently induced, and changes in miR146a and miR129-2 expression significantly explained the variability in injury severity (Strickland et al., 2011). We hypothesized that the temporal expression of miR146a, miR21, and other SCI-sensitive miRNAs may be further dysregulated following administration of morphine in the acute phase of a spinal contusion injury. Such dysregulation could constitute an important role in both inflammation and the progression of neuropathic pain during the chronic phase of injury. Moreover, these miRNAs may contribute to the regulation of the nociceptive circuitry and chronic effects of morphine exposure. To assess these possibilities, the current

study investigated the relationship between SCI-sensitive miRNA expression and morphine administration, evaluated at 2 and 15 days post-SCI to explore the effects of acute morphine treatment in both the acute and chronic phases of SCI. In addition, we also investigated the relationship between miRNA expression and the variance in baseline and morphine-attenuated pain sensitivity following SCI.

2. Experimental Procedures

2.1. Subjects

All of the experiments were reviewed and approved by the institutional animal care committee at Texas A&M University and all NIH guidelines for the care and use of animal subjects were followed. Male Sprague–Dawley rats (*Rattus norvegicus*) obtained from Harlan (Houston, TX) at approximately 90–110 days old (300–350 g) 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.

2.2 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 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 and 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. Sham controls received a laminectomy, but the cord was not contused with the MASCIS device. Following surgery, the wound was closed with Michel clips. T12 level contusion models have been routinely used by members of our group to define spinal cord learning circuits and molecular mechanisms involved with recovery of function (Brown et al., 2011; Ferguson et al., 2008; Hook et al., 2011). Lesions at this level result in well-defined and replicable sensory-motor deficits, and we therefore chose to utilize contusion at this level to also examine changes in miRNA expression.

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-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 hind-paw pain reactivity. 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.

2.3. Drug Administration

After baseline assessments of locomotor and sensory function, rats were assigned to a morphine or vehicle treatment group. Twenty-four hours or 14 days after injury, the rats were given an intrathecal infusion of morphine sulfate (90 µg i.t., Mallinckrodt, Hazelwood, MI) dissolved in 2 µL of distilled water. This dose of morphine was chosen based on previous studies that demonstrated that this high dose is required to achieve strong anti-nociception 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; Hook et al., 2011). Control subjects were treated with 2 µL of vehicle. These drug injections were followed by a 10 µL injection of saline, to flush the catheter. Locomotor ability was assessed with the BBB scale prior to and after drug treatment. This behavioral index was used to ensure that injury severity was balanced across groups prior to drug treatment.

2.4. Assessment of locomotor function

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 forelimb-hindlimb 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.

2.5. Sensory Reactivity

For the assessment of morphine efficacy, nociceptive reactivity was assessed immediately before, and 30 minutes after intrathecal morphine administration. Thermal reactivity was

assessed using radiant heat in the tail-flick test. Subjects were placed in the restraining tubes with their tail positioned in a 0.5 cm deep groove, cut into an aluminum block, and allowed to acclimate to the apparatus (IITC Inc., Life Science, CA) and testing room for 15 min. The testing room was maintained at 26.5°C. Thermal thresholds were then assessed. Thermal reactivity was tested using a halogen light that was focused onto the rat's tail. Prior to testing, the temperature of the light, focused on the tail, was set to elicit a baseline tail-flick response in 3–4 sec (average). This pre-set temperature was maintained across all subjects. In testing, the latency to flick the tail away from the radiant heat source (light) was recorded. If a subject failed to respond, the test trial was automatically terminated after 8 s of heat exposure. Two tests occurred at 2-minute intervals, and the second test's tail-flick latencies were recorded. 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.

2.6. Isolation of RNA for qRT-PCR

Subjects were euthanized (100 mg/kg of pentobarbital, i.p.) 24 hrs after drug treatment. A 5 mm segment of spinal cord from the injury site, hearts, and carotid arteries were collected, snap frozen in liquid nitrogen, and stored at –80 °C until further analysis. Specimens were crushed and RNA was isolated using the TRIzol (Invitrogen; Carlsbad, CA) protocol. Total RNA was then quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific; Wilmington, DE) and stored at –80 °C.

2.7. Quantitative RT-PCR

MiRNA expression data was measured with quantitative reverse transcription (qRT)-PCR for miRNAs, based on the protocol of the miRCURY™ LNA microRNA Universal RT-PCR system (EXIQON; Woburn, MA). RNA samples were converted to cDNA, and qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Forward and reverse primers (EXIQON; Woburn, MA) for hsa-miR124, hsa-miR1, hsa-miR21, hsa-miR129-2, and hsa-miR146a were used for PCR amplification, and real time data was normalized using U6 RNA. Similarly, mRNA expression of IL6R was measured using qRT-PCR for mRNAs, based on the protocol for PerfeCTa® SYBR® Green SuperMix with ROX™ (Quanta Biosciences; Gaithersburg, MD). RNA samples were converted to cDNA using qScript™ cDNA SuperMix (Quanta Biosciences; Gaithersburg, MD), and qRT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Forward and reverse primers (Integrated DNA Technologies; Coralville, IA) for IL-6R were used for PCR amplification, and real time data was normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative miRNA and mRNA expression was determined by calculating the mean difference between cycle threshold of either the miRNA from the U6 small nuclear RNA (U6_{SNB}) normalized control, or the IL6R mRNA from the GAPDH normalized control for each sample [cycle threshold (CT)] within each sample group (samples with same miRNA ID, time, and condition parameters) and expressed as - CT for relative change in expression. Sample means that were greater than ± 2 standard deviations from the mean CT, or ± 3 standard deviations from the mean CT after exclusion, were considered outliers and removed from the analysis.

Of the 218 data points in 36 groups used in the analysis, only 14 data points were excluded according to this criteria, and no more than one data point in any individual experimental group was excluded. Fold change in miRNA/mRNA expression was determined by calculating the difference between the mean CTs of sham, morphine, and vehicle sample groups at the same time point ($-CT$), and expressed as a baseline-corrected percentage of fold-change ($[2^{-CT-1}] * 100$).

2.8. Data analysis

All data were analyzed using SPSS software version 18 (SPSS; Chicago, IL). MicroRNA expression, verified by qRT-PCR, was analyzed by multivariate analysis of variance (ANOVA) using Pillai's trace statistic, and further analyzed using *post hoc* univariate ANOVA and Fisher's least significant difference (LSD) test. Other data were analyzed using ANOVAs followed by *post hoc* LSD using planned comparisons. In all cases, the *a priori* value was set at 0.05. Data were expressed as mean $\pm$ SEM, as indicated in the figure legends.

Correlations between initial injury severity and either miRNA or IL6R mRNA expression, and between tail-flick latency and either miRNA or IL6R mRNA expression, were determined by Pearson's product-moment correlation using $-CT$ values of either the miRNAs or IL6R, initial BBB scores, day 13 BBB scores, and tail-flick latency as separate independent variables. All partial correlations were corrected for time only. The *a priori* value was set at 0.05, and data were expressed as the mean difference in cycle threshold change of either each miRNA relative to the cycle threshold of U6 controls ($-CT = CT_{U6} - CT_{miRNA}$), as indicated in the legend of Figures 2, 3, and 4, or IL6R expression relative to the cycle threshold of GAPDH controls ($-CT = CT_{GAPDH} - CT_{IL6R}$), as indicated in the legend of Figure 7.

3. Results

3.1. Quantification of morphine-induced changes in spinal cord miRNA expression

We previously reported that miR1, miR21, miR124, miR129-2, and miR146a were significantly dysregulated following spinal cord contusion (Strickland et al., 2011). To determine whether morphine treatment further dysregulates SCI-sensitive miRNAs, their expression was examined by qRT-PCR in sham controls and in response to vehicle and morphine treatment following contusion, at either 2 or 15 days post-SCI (Figure 1). Multivariate ANOVA of qRT-PCR data indicated a significant main effect of time (Pillai's Trace Statistic, $F_{(5,19)} = 21.094$; $p < 0.005$) and treatment ($F_{(10,40)} = 3.742$; $p < 0.005$), and a significant interaction effect between time and treatment ($F_{(10,40)} = 6.478$; $p < 0.005$). *Post hoc* univariate ANOVAs indicated a main effect of treatment on miR21, miR129-2, and miR146a ($F_{(2,23)} = 65.038$; $p < 0.005$, $F_{(2,23)} = 17.265$; $p < 0.005$, and $F_{(2,23)} = 16.163$; $p < 0.005$, respectively), and a significant interaction effect of time and treatment on miR21 and miR146a expression ($F_{(2,23)} = 8.229$; $p < 0.005$ and $F_{(2,23)} = 10.055$; $p < 0.005$, respectively). In addition, *post hoc* LSD *t*-tests indicated that both miR21 and miR146a expression is increased following spinal cord trauma, irrespective of morphine treatment ($p_{miR21} < 0.001$ and $p_{miR146a} < 0.001$; Fig. 2a, b). In contrast, miR129-2 expression was down-regulated

following both morphine and vehicle administration ($p_{\text{miR129-2}} < 0.001$ and $p_{\text{miR129-2}} < 0.001$, respectively), and there was no change in the expression of either miR1 or miR124 (Figure 2a, b). *Post hoc* planned comparisons indicated that there was a significant increase in expression of miR21 in response to morphine treatment relative to vehicle at 15 days post-SCI (Student's two-tailed *t*-test, $p < 0.05$; Figure 2c, d). In addition, Pearson's product-moment correlations indicated a statistically significant correlation between miR21 expression and time (Pearson's $r = 0.61$, $p < 0.005$). Controlling for time, partial correlation analysis showed that miR21 expression was significantly correlated with the expression of miR1, miR124, and miR146a (Pearson's $r = 0.51$, $p < 0.03$, Pearson's $r = 0.58$, $p < 0.01$, and Pearson's $r = 0.83$, $p < 0.001$, respectively).

3.2. Impact of injury severity and functional recovery on miRNA expression in spinal cord

We previously observed a significant relationship between the expression of both miR129-2 and miR146a and injury severity, as indicated by initial BBB scores following surgery. Accordingly, we assessed whether this relationship would be replicated by the new data set. Indeed, there was a statistically significant correlation between BBB scores at 24 hours post-SCI and the expression of both miR129-2 and miR146a across both time points (Pearson's $r = 0.66$, $p < 0.001$ and Pearson's $r = -0.55$, $p < 0.001$, respectively; Figure 3a,b). Contrary to our previous findings, we also found a statistically significant correlation between the expression of miR21 and initial BBB scores (Pearson's $r = -0.88$, $p < 0.001$; Figure 3c). Additionally, we analyzed the relationship between recovery of function, as indicated by Day 13 BBB scores, and miRNA expression at 15 days post-SCI. Similar to initial injury severity, there were statistically significant correlations between BBB scores at 13 days post-SCI and expression of miR129-2, miR146a, and miR21 (Pearson's $r = 0.50$, $p < 0.05$, Pearson's $r = -0.70$, $p < 0.001$, and Pearson's $r = -0.80$, $p < 0.001$, respectively; Figure 4a-c).

3.3. Relationship between SCI-sensitive miRNA expression and sensory reactivity

In order to assess the possibility that pain sensitivity is associated with the expression of SCI-sensitive miRNAs, thermal thresholds were measured by the tail flick-latency test, following SCI, both before and after treatment of morphine. Accordingly, there were statistically significant correlations between baseline tail-flick latency and expression of miR1, miR124, and miR129-2 regardless of time point (Pearson's $r = -0.41$, $p < 0.05$, Pearson's $r = -0.43$, $p < 0.05$, and Pearson's $r = -0.41$, $p < 0.05$, respectively; Figure 5a-c), and as predicted these correlations were abolished following morphine administration.

3.4. Expression of miR-1 in vascular tissues following SCI

We previously observed a significant decrease in miR1 expression following spinal cord contusion (Strickland et al., 2011), but did not find a significant change in expression following SCI and administration of the tail-flick test. Given the positive correlation of miR1 expression with sensory reactivity and the capability of miR1 to inhibit angiogenesis (Stahlhut et al., 2012), added to the fact that miR1 is heavily expressed in the cardiovascular system (Fichtlscherer et al., 2011; Jakob and Landmesser, 2012), it is possible that the change in expression of miR1 at the injury site may represent a broader response of vascular and cardiac tissues to SCI. We therefore assessed miR-1 expression in heart and carotid

arterial tissue. Quantitative RT-PCR indicated that there was not a significant change in miR1 expression in either of these tissues following SCI (Figure 6), suggesting that the changes in miR-1 expression previously observed (Strickland et al., 2011) are localized within the injury site rather than due to adaptive changes in the vascular system.

3.5. MiRNA regulation of pain neuro-circuitry associated cytokines in spinal cord

As SCI-induced miRNA is further dysregulated following morphine administration, we assessed the extent to which changes in miR21 expression were associated with alterations in known cytokine mRNA targets. MiR21 has previously been shown to regulate the interleukin-6 receptor, IL6R (Frankel et al., 2008). To determine if IL6R was additionally dysregulated by alteration of pain neuro-circuitry, the expression of mRNA transcripts for IL6R was examined by qRT-PCR in sham controls and in response to vehicle and morphine treatment following contusion injury. An ANOVA revealed a main effect of both time and treatment on IL6R mRNA ($F_{(1,28)}=4.533$; $p<0.05$, and $F_{(2,28)}=10.881$; $p<0.01$, respectively), and *post hoc* LSD *t*-tests indicated that IL6R expression is increased following spinal cord trauma, irrespective of morphine treatment ($p_{\text{Contused Vehicle vs. Sham}} < 0.0001$ and $p_{\text{Contused Morphine vs. Sham}} < 0.001$; Figure 7a, b). In addition, there were significant correlations between IL6R mRNA expression and miR21, miR124, miR129-2, and miR146a (Pearson's $r=0.63$, $p<0.001$, Pearson's $r=-0.38$, $p<0.05$, Pearson's $r=-0.59$, $p<0.001$, and Pearson's $r=0.57$, $p<0.001$, respectively). Partial correlations controlling for the effects of time also indicated that the expression of IL6R mRNA was significantly correlated with expression of both miR124 and miR146a (Pearson's $r=-0.54$, $p<0.05$, and Pearson's $r=0.47$, $p<0.05$, respectively). Furthermore, IL6R expression was predictive of nociceptive sensitivity, as there was a statistically significant correlation between baseline tail-flick latency and IL6R expression across both time points (Pearson's $r=0.59$, $p<0.01$; Figure 8a), indicating that pain sensitivity decreased as IL6R expression increased, and this correlation was abolished following morphine administration. Finally, there was a statistically significant correlation between BBB scores at 24 hours post-SCI and IL6R expression, indicating that variation in initial injury severity is predictive of variation in IL6R expression (Pearson's $r=-0.66$, $p<0.01$; Figure 8b), but there was not a significant correlation between day 13 BBB scores and IL6R mRNA expression ($p=0.49$).

4. Discussion

Morphine treatment plays an important role in pain mitigation in both the acute and chronic phase of spinal cord trauma. However, its deleterious effects on functional recovery and exacerbation of chronic pain systems, through activation of innate immune responses, provide a substantial challenge towards clinical efforts to minimize the short and long term effects of SCI (Dworkin et al., 2003; Hook et al., 2007; Hook et al., 2009; Hook et al., 2011; McCarberg, 2004; Scholz and Woolf, 2007; Watkins et al., 2005; Watkins et al., 2007). Given their dysregulation following SCI and ability to coordinate the expression of large gene networks, it is likely that miRNAs are involved in SCI-induced remodeling of nociceptive circuitry, and consequently, the effects of opiates like morphine on this circuitry. Therapeutic manipulation of these miRNAs could attenuate the maladaptive effects of acute morphine administration on both the acute and chronic phases of SCI by suppressing

activation of inflammation and inhibiting the synaptic remodeling that increases both neuropathic pain and morphine tolerance.

In the current study, we investigated the regulation of SCI-sensitive miRNAs following morphine treatment. Specifically, we replicated our previous observation that miR129-2 is decreased following spinal cord contusion, while miR21 and miR146a were induced, irrespective of morphine treatment (Strickland et al., 2011). Interestingly, in this study, neither miR1 nor miR124 were significantly altered in SCI animals. One possible explanation that merits further investigation is that the measurement of pain sensitivity itself exposed SCI animals to nociceptive stimuli, which may have normalized miR1 and miR124 expression. This possibility is supported by reports showing that peripheral inflammation, which is also known to trigger nociception, results in persistent elevation in miR1 in the dorsal horn of the spinal cord (Kusuda et al., 2011). Additionally, both miR1 and miR124 are predicted to play a central role in the late phase gene repression associated with long-term potentiation-dependent neuronal plasticity (Ryan et al., 2012), and therefore, the normalization of these miRNAs may be important for activation of neural circuitry associated with pain as well.

We found that morphine exposure significantly induced miR21 expression and resulted in a near-significant increase in miR146a expression 15 days after SCI. This is the first evidence for the potential involvement of miRNAs in a post-trauma response to morphine. Though it remains to be determined, it is intriguing to hypothesize that miRNA-regulated gene networks may mediate morphine's effects on plasticity in the spinal cord following SCI. In addition, changes in expression of miR21 were significantly explained by the variance in expression of miR1, miR124, and miR146a, suggesting that these miRNAs may be co-regulated. Moreover, while the expression of miR21 and miR146a was significantly correlated with locomotor behavioral performance following SCI, their expression did not predict pain sensitivity. Consequently, the induction of these miRNAs by morphine is unlikely to be related to the analgesic actions of morphine, but rather, to its pro-inflammatory actions. Several lines of evidence support this hypothesis. Firstly, the expression of both miR146a and miR21 is induced by pro-inflammatory cytokines (Loffler et al., 2007; Nakasa et al., 2008; O'Connell et al., 2007; Taganov et al., 2006; Tili et al., 2007), and reciprocally, both miRNAs target mRNA transcripts of pro-inflammatory cytokines or their receptors (Frankel et al., 2008; Li et al., 2010a; Nahid et al., 2009). Secondly, both miR21 and miR146a play an important role in the innate immune response (Li et al., 2010a; Li et al., 2010b; Moschos et al., 2007; Nahid et al., 2009; Taganov et al., 2006), because increased expression of both miR21 and miR146a negatively regulates innate immune signaling. Finally, miR21 and miR146a have been found to be highly expressed in activated astrocytes, and their overexpression results in attenuation of astrocytic hypertrophy and suppression of the astrocyte-mediated inflammation response, respectively (Bhalala et al., 2012; Iyer et al., 2012). As a result, it is more likely that these miRNAs serve as a component of morphine-induced inflammation rather than being involved in the morphine-triggered signaling cascade driving analgesia.

Interestingly, we observed that although the interleukin receptor IL6R was dysregulated following SCI, it was not additionally dysregulated by morphine administration. Moreover,

contrary to our prediction, the transcript for IL6R, the identified miR21 target, was persistently up-regulated following SCI, suggesting a dissociation between miRNA-target gene networks following SCI, as has been observed in other models of neural damage (Pappalardo et al., 2013). *In silico* analyses (MirWalk, (Dweep et al., 2011)) indicate that aside from miR21, other SCI miRNAs including miR124, miR129-2 and miR146a are predicted to target IL6R. While changes in expression of IL6R were significantly explained by the variance in all four SCI-sensitive miRNAs, the expression of miR124 and miR129-2 were negatively correlated with IL6R expression. It is therefore likely that while IL6R regulation involves a network of miRNAs, miR124 and miR129-2 may serve a more conventional role as negative regulators of gene expression.

We also observed that variations in IL6R mRNA expression explained a statistically significant portion of the variance in initial injury severity, suggesting that decreased expression of IL6R, and presumably lower levels of inflammation, was associated with a less severe initial injury. Indeed, this relationship is likely to be causal in nature, because application of an anti-IL6R antibody immediately after injury has been shown to significantly decrease tissue damage, increase axonal regeneration, and improve functional recovery (Mukaino et al., 2010). Finally, since variability in IL6R mRNA expression also explained a statistically significant proportion of the variability in pain sensitivity (58.5%), it is possible that IL6R also plays a significant role in remodeling inflammation-driven pain neuro-circuitry following SCI and opiate exposure. While this initial analysis focused specifically on IL6R, *in silico* analyses (DIANA-mirPath/microT-CDS, (Vlachos et al., 2012)) predict that 3 out of 4 SCI miRNAs, miR21, miR-129-2 and miR-146a, are likely to collectively target cytokine-cytokine receptor pathways (KEGG pathway hsa04060, FDR-corrected $p < 0.02$) that mediate inflammation and pain-sensitivity. It will be important to determine the extent to which inflammatory pathways are generally susceptible to miRNA regulation following SCI.

We were able to reaffirm our previous finding that expression changes of both miR129-2 and miR146a were significantly explained by the variance of initial injury severity (Strickland et al., 2011). In addition, we found that fluctuations in miR21 expression were significantly explained by the variance in BBB scores 24 hours post-SCI, and that changes in miR129-2, miR146a, and miR21 were also significantly explained by the variance in functional recovery at 13 days post-SCI. Similar to miR146a, miR21 is a negative regulator of inflammation, but it also has anti-apoptotic activity (Roy and Sen, 2011; Sathyan et al., 2007). As such, its up-regulated expression may result in increased neuronal survival following SCI by inhibiting inflammation and cell death, factors which are likely to result in better locomotor function and higher initial BBB scores. One therapeutically relevant prediction from these data that merits further investigation is that application of miR21 or miR146a mimetics prior to or concurrent with morphine administration in the acute phase of SCI may attenuate opioid mediated inflammation, and could also reduce apoptosis (protecting the potential for recovery) in this early phase of injury.

Finally, we observed that miRNA expression at the injury site explained a significant proportion of the sensitivity to nociceptive stimuli, i.e., the response of sensory-motor circuitry distal to the injury site. Not surprisingly, these correlations between pain sensitivity

and miRNA expression were abrogated by morphine administration, due to the analgesic effects of morphine. While these correlations do not necessarily imply causality, they do advance the possibility that there is a degree of molecular integration between the injury site and more caudal, but anatomically intact neuro-circuitry. A mechanism for cross-talk between miRNAs at the injury site and caudal neuro-circuitry is unclear at this time, but the possibility that these interactions serve an adaptive function is intriguing, and needs further investigation.

5. Conclusion

Our evidence shows that acute morphine administration results in a very specific and limited profile of changes in SCI-sensitive miRNA expression during the chronic phase of injury. Interestingly, expression changes are confined to miRNAs, like miR21, that are both induced by and inhibitors of pro-inflammatory cytokines, suggesting that morphine may potentially interfere with miRNA-mediated negative feedback pathways. Like miR21, IL6R mRNA was persistently up-regulated after SCI, and its expression negatively correlated with initial injury severity, suggesting that IL6R and miR21 may be a coordinately regulated, adaptive response to SCI. Furthermore, miR1, miR124, and miR129-2 expression at the injury site appears to play a substantial role in the regulation of distal nociceptive signaling pathways. Collectively, these data suggest that SCI-sensitive miRNAs constitute an important component of emerging pain-sensitivity and inflammation, and consequently, these miRNAs could provide therapeutic targets following SCI.

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Abbreviations

ANOVA	analysis of variance
BBB	Basso, Beattie, and Bresnahan
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
IL6R	interleukin-6 receptor
LSD	least significant difference
miRNA	microRNA
qRT	quantitative reverse transcription
SCI	spinal cord injury

Highlights

- MiRNAs exhibit changes in the chronic phase of injury following morphine administration
- Anti-inflammatory miRNAs are induced
- MiR21 and IL6R are both induced and indicate a dissociation between miRNA and target mRNA expression
- Changes in trauma-sensitive miRNAs appear to play a role in the regulation of distal nociceptive signaling pathways

Figure 1.

Timeline for experimental procedures. A contusion or sham injury was administered on Day 0. Assessment of motor function and testing of sensory reactivity before and after morphine or vehicle administration occurred on either Day 1 (sham, n=6; contused with vehicle, n=6; contused with morphine, n=6) or Day 14 (sham, n=5; contused with vehicle, n=8; contused with morphine, n=5), followed by the harvesting of tissue for miRNA analysis one day later.

Figure 2.

Bar graphs depicting qRT-PCR analysis of miRNA expression of miR1, miR21, miR124, miR129-2, and miR146a at the lesion site for sham animals and after administration of either morphine or vehicle in contused animals at either 2 (a, c) or 15 (b, d) days post-SCI. (a-b) The x-axis denotes miRNA of interest, and the y-axis denotes the mean change in cycle threshold, where CT represents the mean difference of miRNA and U6 control CT values. Animals received either a laminectomy only (sham) or a T12-T13 spinal contusion followed by administration of vehicle or morphine 24 hrs later, and sacrificed at 2 or 15 days post-surgery for harvesting of the cords. Data are expressed relative to U6 controls (a value of zero equates to no difference in expression between the miRNA and U6, positive values equate to higher miRNA expression, and negative values vice versa), with asterisks indicating significance (LSD) compared with sham controls; *p* values are as indicated in the text. (c-d) The x-axis denotes miRNA of interest, and the y-axis denotes the baseline-corrected percentage of fold expression change of miRNA using the formula $(2^{-CT} - 1) \times 100$, where CT represents the mean difference of contused vehicle and sham, contused morphine and sham, or contused morphine and contused vehicle CT values. Data are expressed as a percentage of change (a value of zero equates to no change between surgery conditions), with asterisks indicating significance (LSD) compared with sham controls and hash tags indicating significance (LSD) compared with contused vehicle controls; *p* values are as indicated in the text.

Figure 3.

Correlation analysis to assess the relationship between miRNA expression and initial injury severity. (a-c) The x-axis represents the BBB score for baseline locomotor function, 24 hours following spinal cord contusion, and the y-axis depicts the mean difference in cycle threshold change (-CT) between miRNA expression at both 2 and 15 days post-SCI and U6 controls. Pearson's product-moment correlations indicated a significant correlation between miR129-2 (a), miR146a (b), and miR21 (c), and initial BBB score (Pearson's $r=0.66$, $P<0.001$, Pearson's $r=-0.55$, $P<0.001$, and Pearson's $r=-0.88$, $P<0.001$, respectively). Data are represented as the mean change in cycle threshold of miRNA expression relative to U6 controls (-CT = $CT_{U6} - CT_{miRNA}$).

Figure 4.

Correlation analysis to assess the relationship between miRNA expression and functional recovery. (a-c) The x-axis represents the BBB scores recorded 13 days following spinal cord contusion, and the y-axis depicts the mean difference in cycle threshold change (-CT) between miRNA expression at 15 days post-SCI and U6 controls. Pearson's product-moment correlations indicated a significant correlation between miR129-2 (a), miR146a (b), and miR21 (c), and Day 13 BBB score (Pearson's $r=0.50$, $P<0.05$, Pearson's $r=-0.70$, $P<0.001$, and Pearson's $r=-0.80$, $P<0.001$, respectively). Data are represented as the mean change in cycle threshold of miRNA expression relative to U6 controls (-CT = $CT_{U6} - CT_{miRNA}$).

Figure 5.

Correlation analyses to assess the relationship between miRNA expression and sensory reactivity following SCI. (a-c) The x-axis represents the mean difference in cycle threshold change ($-CT$) between miRNA expression at both 2 and 15 days post-SCI and U6 controls, and the y-axis depicts the baseline tail-flick latency for pain sensitivity assessed 24 hours following spinal cord contusion and prior to morphine or vehicle administration. Pearson's correlations indicated a significant correlation between miR1 (a), miR124 (b), and miR129-2 (c) and baseline tail-flick latency (Pearson's $r = -0.41$, $P < 0.05$, Pearson's $r = -0.43$, $P < 0.05$, and Pearson's $r = -0.41$, $P < 0.05$, respectively), but no significant correlations were found following vehicle or morphine administration. Data are represented as the mean change in cycle threshold of miRNA expression relative to U6 controls ($-CT = CT_{U6} - CT_{miRNA}$).

Figure 6.

Bar graph depicting qRT-PCR analysis of miR1 expression in hearts and carotid arteries following SCI. The x-axis denotes tissue of interest, and the y-axis denotes the mean change in cycle threshold, where CT represents the mean difference of miR1 and U6 control CT values. Animals received either a laminectomy only (sham) or a T12 spinal contusion, and were sacrificed 24 hrs post-surgery for harvesting of the cords. Data are expressed as measure of expression relative to U6 controls (a value of zero equates to no difference in expression between miR1 and U6, positive values equate to a higher expression of miR1, and negative values vice versa), and indicate that there was no significant change in miR1 expression between contused and sham groups, both in heart and carotid artery tissue ($p < 0.17$ and $p < 0.14$, respectively).

Figure 7.

Bar graphs depicting qRT-PCR analysis of mRNA expression of IL6R at the lesion site for sham animals and after administration of either morphine or vehicle in contused animals at 2 and 15 days post-SCI. (a) The x-axis denotes time following surgery, and the y-axis denotes the mean change in cycle threshold, where CT represents the mean difference of mRNA and GAPDH control CT values. Animals received either a laminectomy only (sham) or a T12 spinal contusion followed by administration of vehicle or morphine 24 hrs later, and sacrificed at 2 or 15 days post-surgery for harvesting of the cords. Data are expressed relative to GAPDH controls (a value of zero equates to no difference in expression between the miRNA and GAPDH, positive values equate to higher miRNA expression, and negative values vice versa), with asterisks indicating significance (LSD) compared with sham controls; *p* values are as indicated in the text. (b) The x-axis denotes time after surgery, and the y-axis denotes the baseline-corrected percentage of fold expression change of IL6R mRNA using the formula $(2^{-CT} - 1) * 100$, where CT represents the mean difference of contused vehicle and sham, contused morphine and sham, or contused morphine and contused vehicle CT values. Data are expressed as a percentage of change (a value of zero equates to no change between surgery conditions), with asterisks indicating significance (LSD) compared with sham controls and hash tags indicating significance (LSD) compared with contused vehicle controls; *p* values are as indicated in the text.

Figure 8.

Correlation analyses to assess the relationship between IL6R mRNA expression and sensory reactivity following SCI. (a) The x-axis represents the mean difference in cycle threshold change (-CT) between mRNA expression at both 2 and 15 days post-SCI and GAPDH controls, and the y-axis depicts the baseline tail-flick latency for pain sensitivity at 24 hours following spinal cord contusion and prior to morphine or vehicle administration. Pearson's product-moment correlations indicated a significant correlation between IL6R and baseline tail-flick latency (Pearson's $r=0.59$, $P<0.01$), but no significant correlations were found following vehicle or morphine administration. Data are represented as the mean change in cycle threshold of mRNA expression relative to GAPDH controls (-CT = $CT_{GAPDH} - CT_{IL6R}$). (b) The x-axis represents the BBB score for baseline locomotor function, 24 hours following spinal cord contusion, and the y-axis depicts the mean difference in cycle threshold change (-CT) between IL6R mRNA expression at both 2 and 15 days post-SCI and GAPDH controls. Pearson's product-moment correlations indicated a significant correlation between IL6R expression and initial BBB score (Pearson's $r=-0.66$, $P<0.01$). Data are represented as the mean change in cycle threshold of miR21 expression relative to GAPDH controls (-CT = $CT_{GAPDH} - CT_{IL6R}$).