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Changes in expression of sensory organ-specific microRNAs in rat dorsal root ganglia in association with mechanical hypersensitivity induced by spinal nerve ligation

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Abstract

Chronic neuropathic pain caused by peripheral nerve injury is associated with global changes in gene expression in damaged neurons. To understand the molecular mechanisms underlying neuropathic pain, it is essential to elucidate how nerve injury alters gene expression and how the change contributes to the development and maintenance of chronic pain. MicroRNAs are non-protein-coding RNA molecules that regulate gene expression in a wide variety of biological processes mainly at the level of translation. This study investigated the possible involvement of microRNAs in gene regulation relevant to neuropathic pain. The analyses focused on a sensory organ-specific cluster of microRNAs that includes miR-96, -182, and -183. RT-PCR analyses confirmed that these microRNAs were highly enriched in the dorsal root ganglion (DRG) of adult rats. Using the L5 spinal nerve ligation (SNL) model of chronic neuropathic pain, we observed a significant reduction in expression of these microRNAs in injured DRG neurons compared to controls. *In situ* hybridization and immunohistochemical analyses revealed that these microRNAs are expressed in both myelinated (N52 positive) and unmyelinated (IB4 positive) primary afferent neurons. They also revealed that the intracellular distributions of the microRNAs in DRG neurons were dramatically altered in animals with mechanical hypersensitivity. Whereas microRNAs were uniformly distributed within the DRG soma of non-allodynic animals, they were preferentially localized to the periphery of neurons in allodynic animals. The redistribution of microRNAs was associated with changes in the distribution of the stress granule protein TIA-1. These data demonstrate that SNL induces changes in expression levels and patterns of miR-96, -182, and -183, implying their possible contribution to chronic neuropathic pain through translational regulation of pain-relevant genes. Moreover, stress granules were suggested to be assembled and associated with microRNAs after SNL, which may play a role in modification of microRNA-mediated gene regulation in DRG neurons.

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Keywords

Real-time quantitative RT-PCR; *In situ* hybridization; Immunohistochemistry; Stress granules

Introduction

Neuropathic pain is a pathological chronic pain caused by disease or injury to the nervous system. Alterations in nerve function, responsiveness, activity, neurotransmitter and receptor expression, morphology, and synaptic connections contribute to the allodynia, hyperalgesia, and spontaneous pain that characterize neuropathic pain states (Woolf and Salter, 2000; Zimmermann, 2001; Campbell and Meyer, 2006; Scholz and Woolf, 2007). Long-lasting modifications in pain transmission pathways develop as a result of global changes in gene expression in specific neuronal and glial cells (Newton et al., 2000; Kim et al., 2001; Costigan et al., 2002; Wang et al., 2002; Xiao et al., 2002). However, it is largely unknown how nerve injury brings about such global changes in gene expression to induce chronic pain.

MicroRNAs are a class of non-protein-coding, small (21–23 nucleotides) RNA molecules that primarily promote translational suppression by binding to the 3' untranslated regions (3'-UTRs) of target mRNAs in a sequence specific manner (Bartel, 2004; Valencia-Sanchez et al., 2006). Many microRNAs are expressed either predominantly or exclusively in the nervous system. Several classes are implicated in the regulation of genes responsible for nervous system development and neural plasticity (Lagos-Quintana et al., 2002; Krichevsky et al., 2003; Miska et al., 2004; Sempere et al., 2004; Giraldez et al., 2005; Vo et al., 2005; Wienholds et al., 2005; Conaco et al., 2006; Schratt et al., 2006). Because the long-lasting changes in pain sensitivity induced by nerve injury are accompanied by altered gene regulation, the interesting possibility exists that microRNAs expressed in nociceptive pathways influence the development and maintenance of neuropathic pain conditions.

Among microRNAs expressed in the nervous system, the miR-183 family is unique in that they are highly enriched in sensory organs. In vertebrates, this family consists of three members; miR-96, -182, and -183. The corresponding genes are located within a 4 kb genomic segment and co-expressed in the eyes, ears, nose epithelium, and cranial ganglia of embryonic zebrafish (Wienholds et al., 2005), in photoreceptors and retinal cells in adult mouse (Xu et al., 2007), hair cells of the inner ear in neonatal mice (Weston et al., 2006), and dorsal root ganglia (DRG) of embryonic mice (Kloosterman et al., 2006). Among invertebrates, orthologs of the miR-183 family members (miR-263b (arthropods) and miR-228 (nematodes)) are also expressed in putative sensory tissues and organs (Pierce et al., 2008). Thus, the miR-183 family appears to be an evolutionarily conserved group of microRNAs specifically expressed in tissues/organs involved in sensory perception. The specific and restricted expression of the miR-183 family suggests these microRNAs are involved in sensory organ-specific development and/or function. In the DRG, it is possible that miR-183 family members influence translation of the genes important to the unique function of nociceptive and mechanosensitive primary afferent neurons. Therefore, any changes in the expression of miR-183 family members may contribute to alterations in gene expression and neuronal properties observed after peripheral nerve injury.

In addition to changes in their expression levels, activity of microRNAs may be influenced post-transcriptionally by the protein complexes they associate with (Dostie et al., 2003; Kim et al., 2004; Liu et al., 2005; Leung et al., 2006; Valadi et al., 2007). For example, when cultured cells are subjected to certain stress, microRNAs are shown to associate with newly assembled RNA-protein complexes known as stress granules (SGs) (Leung et al., 2006) and this change may have a significant impact on regulatory activity of microRNAs (Bhattacharyya et al.,

2006; Vasudevan and Steitz, 2007) (reviewed in (Leung and Sharp, 2007)). It is possible that nerve injury may also induce changes in the association between microRNAs and binding proteins thereby altering microRNA regulatory activity in injured neurons. Therefore, in order to determine how nerve injury influences regulatory activity of microRNAs, we need to examine not only changes in their expression levels but also changes in their interactions with other molecules.

To investigate the possible involvement of microRNAs in neuropathic pain, we examined expression levels and patterns of the miR-183 family in DRG using an established model of neuropathic pain in adult rats. We determined that expression of these microRNAs is significantly altered in a quantitative and qualitative manner in association with the development of mechanical hypersensitivity. Our findings strongly suggest that sensory organ-specific microRNAs play a role in gene regulation relevant to chronic pain induced by nerve damage.

Materials and Methods

Animals, surgical procedures and behavioral testing

Animal handling and experimental procedures were performed in accordance with the policies and recommendations of the Guide for Care and Use of Laboratory Animals, and with the approval of the University of Iowa Institutional Animal Care and Use Committee. The minimum number of rats was used and every effort was made to reduce their discomfort and stress.

Persistent mechanical hypersensitivity was induced using a modified version of the spinal nerve ligation (SNL) model (Kim and Chung, 1992). In this model, only the L5 spinal nerve was ligated in 6 week old male Sprague-Dawley rats (Sasco Charles River; Portage, IN) weighing 80–100 g at the time of surgery. We chose to use an L5 only ligation instead of the originally described L5 and L6 ligation because the former is a less invasive approach compared to the original model (Engle et al., 2006). Sham-operated rats were treated identically in all respects to the ligated rats except that the L5 spinal nerve was exposed but not ligated. Rats were housed two per cage on a 12-h reverse-light cycle with free access to water and a soy-free diet for two weeks after surgery (Engle et al., 2006). We chose the 2-week time point because differences in withdrawal thresholds between ligated and sham-operated rats are near their maximum level at this time (Engle et al., 2006). At the end of the two-weeks, paw withdrawal thresholds to mechanical stimuli were assessed using Von Frey filaments as previously described (Chaplan et al., 1994). At the completion of behavioral testing, rats were euthanized using carbon dioxide and ipsilateral and contralateral L4-6 DRG were harvested and frozen at -80°C . The following tissues were also collected from naïve rats for microRNA analysis: L4-6 DRG, spinal cord, brainstem, cortex, cerebellum, heart, intestine, kidney, liver, and lung.

Quantitative Real-time PCR Analysis of microRNAs

For each biological replicate, total RNA fractions were extracted from tissue samples using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA; #AM1560). For the comparative analysis of microRNA levels in the DRG, spinal cord, brainstem, cortex, cerebellum, heart, intestine, kidney, liver, and lung in naïve animals, tissue from a single animal was used for each biological replicate. In total, we analyzed three biological replicates for each tissue. For the analysis of naïve, sham-operated, and ligated DRG samples, DRGs from one to two rats were used for a single biological replicate. In total, we analyzed eight biological replicates each for sham ipsilateral, ligated ipsilateral, and ligated contralateral L5 DRG samples and five for naïve L5 DRG samples.

Quantitative real-time polymerase chain reaction (qPCR) was performed using the TaqMan MicroRNA Assay kit with primers specific to the small RNAs of interest (mmu-miR-96 #373372, hsa-miR-182 #4373271, hsa-miR-183 #4373114, hsa-miR-23b #4373073, and 4.5S RNA(H) #4386736 (Applied Biosystems Inc., Foster City, CA, USA)) following the manufacturer's instructions. Each qPCR assay was conducted in triplicate using cDNA derived from 50 ng total RNA from a biological replicate. To compare the expression of miR-183 family members across different rat tissues, samples were run together with a 10-fold dilution series of corresponding microRNA oligo to create a standard curve and allow for conversions of sample CT values into fg of microRNA per ng of total RNA. Next, the expression of microRNAs in each tissue sample relative to the DRG was estimated. To quantify the influence of SNL on the expression of microRNAs in DRG, changes in microRNA levels in sham ipsilateral, ligated contralateral, and ligated ipsilateral relative to naïve DRGs were calculated using the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001). Samples were normalized using the 4.5S RNA as an endogenous control. The ratios of microRNA amounts were compared among samples using a one-way analysis of variance (ANOVA) followed by a Student-Newman Keuls multiple comparison test.

In situ hybridization and immunohistochemistry

To characterize microRNA expression patterns in myelinated and unmyelinated DRG neurons, we combined *in situ* hybridization with immunohistochemistry. In brief, DRGs from naïve rats were freshly frozen in Tissue-Tek II O.C.T. Compound, sectioned at 16 μ m, fixed on slides in 4% paraformaldehyde/PBS for 20 min, dehydrated, acetylated, and pre-hybridized with pre-hybridization buffer (mRNA-locator-Hyb kit, Ambion) for 2 hrs. The tissue sections were incubated overnight with 7 pmol of anti-sense miRCURY™ probes for microRNAs modified with locked nucleic acid (Exiqon Inc., Woburn, MA, USA) in hybridization buffer at the probe-specific temperature (miR-96: 53°C, miR-182: 47°C, miR-183: 53°C, Let-7a: 48°C, miR-124a: 53°C, miR-23b: 55°C). Probes were digoxigenin-labeled using a DIG Oligonucleotide Tailing Kit (#3–353–583, Roche Applied Science, Indianapolis, IN, USA). Sections were subjected to four 30-min stringency washes at the probe-specific temperature (see above) incubated in antibody-blocking buffer, and then incubated 1–3 days at 4°C with alkaline phosphatase-conjugated polyclonal sheep anti-Digoxigenin Fab fragments (1:500; #11–093–274–910, Roche Applied Science, Indianapolis, IN, USA), monoclonal mouse anti-neurofilament 200 (N52) (1:4,000; #NO142, Sigma-Aldrich Inc., Saint Louis, MO, USA) and biotinylated *Griffonia simplicifolia* Lectin I (IB4) (1:1,000; #M1022, Vector Laboratories Inc., Burlingame, CA, USA). After washing, sections were processed for alkaline phosphatase staining using NBT/BCIP (#34042, Pierce/Thermo Fisher Scientific Inc., Rockford, IL, USA) monitoring occasionally under the microscope until signal was evident (~30 min). After *in situ* staining had reached adequate levels, sections were washed in PBS, incubated with Cy-2 conjugated streptavidin (1:200; #016–220–084) and Cy-3 conjugated goat anti-mouse IgG (1:200; #115–165–164) (Jackson Immuno Research Inc., West Grove, PA, USA) at room temperature for 2 hrs, washed, and cover-slipped with Aqua-Mount (Lerner Laboratories, Pittsburg, PA, USA). Sections were examined with a Nikon E800 epifluorescence microscope and appropriate filter sets including a narrow band excitation filter set for Cy-3 that precluded bleed-through from the Cy-2 label. To compare the expression pattern and intracellular distribution of microRNAs in naïve, sham-operated, and ligated animals, sections were processed as described above, but were not processed for N52 or IB4.

In situ hybridization for microRNAs was also combined with immunohistochemistry for the SG associated protein, T-Cell intracellular antigen 1 (TIA-1). The aforementioned costaining protocol was modified to enhance the TIA-1 fluorescence signal. In brief, *in situ* hybridization was carried out as described above. Following stringency washes and antibody blocking steps, sections were incubated 1–3 days at 4°C with polyclonal goat anti-TIA-1 (C20) (1:100;

#sc-1751, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The sections were washed, incubated with Alexa Fluor® 488 donkey anti-goat IgG (1:100; #A-11055, Invitrogen, Carlsbad, CA, USA), washed, and photographed. Following the image acquisition of the TIA-1 signal, slides were then incubated with alkaline phosphatase conjugated polyclonal sheep anti-Digoxigenin Fab fragments, washed, processed for alkaline phosphatase staining using NBT/BCIP, and re-photographed.

Quantitative analysis of microRNA redistribution

Using DRG sections stained for miR-96, we quantitatively analyzed the staining pattern of naïve, sham-operated ipsilateral, and ligated ipsilateral L5 DRGs. For each DRG/animal we obtained three 20X images, each from three different tissue sections. The images were opened in ImageJ (Abramoff et al., 2004) and the freehand tool was used to trace the outline of each large diameter neuron (800–2,800 μm^2 ; (Hammond et al., 2004)). The average pixel intensity, standard deviation of pixel intensities, and area within each tracing were obtained using the measure tool. The area within each tracing was then used to estimate the average diameter of each neuron in pixels. Next, the enlarge tool was used to create a second set of tracings identical in shape and position to the first set but shrunk by 15% of the pixel diameter of the original. That is, the new tracings have the same shape and center as the original tracing but have an average diameter 70% of the original (15% reduced from each side). The measure tool was again used to obtain the average pixel intensities, standard deviation of pixel intensities, and area within the new “smaller” set of tracings. The measurements from corresponding larger and smaller tracing were then used to deduce the average pixel intensity in the area between the larger and smaller tracings representing the periphery of the neuron cell body. The staining intensity in the outer, peripheral area and area within the inner, smaller tracing area were then compared by calculating a ratio of the average pixel intensities in the outer area to inner area. These ratio values were compared among the naïve, sham, and ligated groups using a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by a Dunn’s multiple comparison test. Next, we quantified the absolute value of the differences in pixel intensity between the outer and inner portions of naïve neurons. The difference was then divided by the standard deviation in pixel intensity for the inner portion of the neuron to estimate the number of standard deviations accounting for the difference in pixel intensity between the outer and inner areas. Finally, we quantified the percentage of neurons for each treatment group which show a redistribution of microRNA signal to the cell periphery as: any neuron with an average pixel intensity in the peripheral area greater than the average pixel intensity of the inner area plus one standard deviation. The percentage of neurons in each image frame with redistribution to the periphery was compared across treatments using a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by a Dunn’s multiple comparison test.

RESULTS

Paw withdrawal thresholds were significantly different among treatment groups (Figure 1; Kruskal-Wallis ANOVA, $H = 32.78$, $P < 0.001$). The withdrawal thresholds of the ipsilateral hindpaws of SNL-operated rats were significantly lower compared to the contralateral paws of ligated rats and ipsilateral paws of sham-operated and naïve rats (Dunn’s pairwise test, $P < 0.05$). The remaining comparisons were not significantly different (Dunn’s pairwise test, $P > 0.05$). These results were consistent with our previous observations (Engle et al., 2006). However, not all rats showed the expected level of mechanical sensitivity after behavioral testing (Fig. 1 data-points with asterisk). Specifically, two of the 16 rats that received SNL did not develop mechanical hypersensitivity, while one of the 15 sham-operated rats developed mechanical hypersensitivity. Because of the uncertainties surrounding the causes for the “abnormal” behavioral phenotype in these animals, they were removed from further testing. Thus, only rats displaying the expected paw withdrawal thresholds were used for qPCR analysis

to examine the effects of SNL on expression levels of microRNAs (naïve: n = 5; sham: n = 11; ligated: n = 11) and for *in situ* hybridization experiments to determine the expression patterns of microRNAs after SNL (naïve: n = 4; sham: n = 3; ligated: n = 3).

MiR-183/96/182 cluster is highly expressed in the adult rat DRG

Using qPCR we examined the expression level of miR-183 family members among various tissues in adult rats. All three members of the miR-183 family were highly enriched in the adult rat DRG compared to any other tissue examined in this analysis (Fig. 2A). The second highest level of expression was observed in the intestine for miR-182, where its expression was six fold less than that in the DRG. Among nervous system tissues outside the DRG, the spinal cord had the highest expression of a miR-183 family member (miR-182). However, the level of expression in the spinal cord was 40 times lower than that estimated for the DRG. Thus, enrichment of miR-183 family members in the DRG during embryonic development (Kloosterman et al., 2006) appears to be maintained in adults. The expression levels of the three different members of the miR-183 family did not differ significantly within the DRG (ANOVA, $P = 0.34$; Fig. 2B).

Spinal nerve ligation causes down-regulation of miR-183/96/182

Enriched expression of miR-183 family members in the adult DRG prompted us to examine if their expression was changed by SNL. We compared expression levels of miR-96/182/183 in injured and uninjured DRG using qPCR. In injured DRG, the expression levels were down-regulated approximately 40–68% for miR-183 family members compared to naïve DRGs (Fig. 3). For miR-96 and miR-183, a one-way ANOVA indicated a significant difference among samples (miR-96, $P = 0.002$; miR-183, $P = 0.01$). For both miR-96 and miR-183, ligated ipsilateral samples were significantly different from naïve, sham ipsilateral, and ligated contralateral samples ($P < 0.05$; Fig. 3) and the remaining comparisons were not significantly different. MiR-182 levels were also significantly different among samples (ANOVA, $P = 0.03$). However, only the ligated ipsilateral and naïve samples were significantly different ($P < 0.05$; Fig. 3) and the remaining comparisons were not.

To explore the possibility that there may be a global, non-specific depression in microRNA in injured DRG, we also measured expression levels of miR-23b. MiR-23b was selected because it is expressed in neurons (Dostie et al., 2003), but is not a member of the miR-183 family. Unlike the decrease observed for miR-183 microRNAs, miR-23b in injured DRGs was not significantly different compared to the uninjured controls (ANOVA, $P = 0.48$; Fig. 3).

MiR-183/96/182 are expressed in unmyelinated and myelinated neurons

To determine whether miR-183 family members are expressed by a specific population of primary afferent neurons, we used *in situ* hybridization and immunohistochemical staining to simultaneously detect miR-183 family members and cell type-specific markers in naïve DRG. *In situ* hybridization label for miR-183 family members co-localized with markers for both large myelinated fibers (N52, large arrow) as well as small unmyelinated fibers (IB4, small arrow) in the DRG (Fig. 4). We did not observe any neurons that were IB4 or N52 positive but negative for the microRNAs examined. We observed several small diameter neurons that were positive for microRNA signal but negative for IB4 and N52 (Fig. 4, arrow head). These neurons presumably express calcitonin-gene related peptide (CGRP). However, as we were unable to obtain suitable labeling for CGRP in sections that underwent *in situ* hybridization, it was not possible to test this hypothesis.

Intracellular distributions of microRNAs are dramatically changed in cell bodies of DRG neurons following SNL

As described earlier, expression levels of the miR-183 family members were significantly reduced in DRG in response to SNL. To determine whether the reduction occurred in a particular class of primary afferent neurons, *in situ* hybridization analysis for miR-96 was carried out. We observed miR-96 signal in both large and small diameter neurons of sham-operated and ligated rats. There appeared to be a reduction in the intensity of *in situ* hybridization signal in both large and small diameter DRG neurons after SNL (Fig. 5), which is consistent with the decreased quantity of miR-183 family members detected by qPCR. Unexpectedly, it was found that there was a significant qualitative difference in the intracellular distribution of miR-96 following SNL (Fig. 5). The *in situ* hybridization signals in DRG of naïve animals (Fig. 4A, C, E) as well as the ipsilateral DRG of sham-operated rats (Fig. 5A, C) were uniformly distributed across the cytoplasm of the cell body with some weak nuclear staining (Fig 5C, arrow). In contrast, the miR-96 staining in the ipsilateral L5 DRG of SNL rats was concentrated around the cell periphery and was relatively weak within the cytoplasm near the center of the cell (Fig. 5B, D). This pattern is particularly evident in large diameter neurons where it can clearly be differentiated from weaker staining across the nucleus.

Each member of the miR-183 family we examined showed the same redistribution of labeling to the periphery of DRG neurons in rats that exhibited mechanical hypersensitivity after SNL (Figs. 5, 6A–D). To determine if this phenomenon was restricted to the miR-183 cluster of microRNAs or observed for other microRNAs, we examined the staining patterns of the ubiquitously expressed let-7a (Sempere et al., 2004) and neuron-specific miR-124a (Lagos-Quintana et al., 2002; Sempere et al., 2004). In addition, we examined the neuronally expressed miR-23b (Dostie et al., 2003), whose expression levels were not altered following SNL. All three microRNAs exhibited a redistribution of label to the periphery of DRG neurons in rats with mechanical hypersensitivity similar to what was observed for the miR-183 cluster (Fig. 6E–J). These observations indicate that not only the miR-183 family members but many, if not all, microRNAs change their intracellular distributions in DRG neurons after peripheral nerve injury.

There was the possibility that the very dense labeling of the cytoplasm by some of the microRNAs could have obscured the prior existence of a preferential localization of microRNAs to the periphery of the neurons, something that would become apparent upon a reduction in the intensity of cytoplasmic labeling after injury. However, this possibility was ruled out because we observed little or no evidence of a preferential localization of microRNAs to the periphery of DRG neurons in uninjured DRGs even for those microRNAs whose labeling was not intense (e.g., Fig. 6E–F and I–J). Furthermore, the ring pattern was present for miR-23b staining which did not display altered expression levels by qPCR analysis following SNL. These observations suggest that the appearance of microRNA accumulation near the cell periphery is not secondary to a generalized reduction in the intensity of intracellular labeling.

Quantification of microRNA redistribution in injured neurons

The pixel intensity ratios between the outer and inner areas were determined for miR-96 *in situ* hybridization staining using the method described in Materials and Methods. They were significantly different among naïve, ligated, and sham operated animals (Table 1; ANOVA, $P < 0.001$). Ligated animals had larger ratios and were significantly different from sham and naïve animals ($P < 0.05$). The ratios for sham and naïve animals were not significantly different from each other ($P > 0.05$) and approximated one. For naïve animals, the absolute difference in pixel intensity between the inner and outer portions of the neurons was ~ 0.8 standard deviations of the pixel intensity in the inner portion with a 95% confidence interval of 0.70 to 0.90 S.D.. In other words, in approximately 95% of the large diameter neurons of naïve animals,

the average pixel intensity in the outer portion of the neuron is less than the average pixel intensity of the inner portion of the neuron plus one S.D. Based on this observation, we considered any neuron with an average pixel intensity in the outer portion of the neuron greater than the average pixel intensity of the inner portion plus one S.D. to have a redistribution of signal to the cell periphery. Using this criterion, there was a significant difference among treatment groups in the percentage of neurons with redistribution to the periphery (Table 1; ANOVA, $P < 0.001$). Ligated animals had a redistribution to the cell periphery in 55% of all large diameter neurons, which was significantly different ($P < 0.05$) from both naïve and sham operated animals that had redistribution in 2% and 4% of all large diameter neurons, respectively. The percentages for sham and naïve animals were not significantly different from each other ($P > 0.05$).

Possible association of microRNAs with stress granules following SNL

A final set of experiments examined the distribution of the stress granule (SG) associated protein T-Cell intracellular antigen 1 (TIA-1) in DRG neurons after SNL. Similar to the microRNA redistribution, we observed a corresponding redistribution of TIA-1 to the periphery of DRG neurons in rats that exhibited mechanical hypersensitivity after SNL (Fig 7). This redistribution of microRNAs and TIA-1 is most evident in large diameter neurons. In sham-operated animals, both the TIA-1 and miR-96 staining was evenly distributed in the cytoplasm of large diameter neurons (Fig 7A–B, large arrow). However, in injured large diameter DRG neurons, TIA-1 and miR-96 displayed a clear, almost identical accumulation near the edge of the cell, implying possible association of the microRNAs with SGs (Fig. 7C–D, large arrow). For small diameter neurons, the smaller cytoplasmic area made it difficult to determine if the microRNAs or the TIA-1 protein are accumulating near the cell periphery. However, in uninjured small diameter neurons TIA-1 is present in the nucleus while in injured small diameter neurons TIA-1 staining in the nucleus is largely absent, suggesting a change in the intracellular distribution of this SG protein following nerve injury in small diameter neurons as well (Figure 7A, C, small arrow). This also highlights a qualitative difference between the large and small diameter neurons; TIA-1 is present in the nucleus of injured large diameter neurons (Figure 7C, large arrow) but absent in the nucleus of injured small diameter neurons (Figure 7C, small arrow).

DISCUSSION

The principal finding of this study was that the levels of three different members of the sensory organ-specific miR-183 family (miR-96, miR-182, and miR-183) were significantly reduced in the injured ipsilateral L5 DRG two weeks after L5 SNL. This change appears to be the result of specific regulation of miR-183 family members rather than a global change in microRNA or small RNA levels because the expression level of miR-23b, which is not a member of this family, was not significantly altered after SNL. Although the SNL-induced reduction in expression levels of miR-183 members was not large, this change may have important functional consequences for injured DRG neurons. Little is known about the relationship between endogenous microRNA expression levels and the extent of target mRNA suppression. However, experiments using cell culture systems with a reporter gene suggest that a certain threshold microRNA level is needed to achieve significant target gene suppression (Brown et al., 2007). That is, microRNA suppression of target genes is minimal below a specific expression level but, even with small changes, it becomes significant once the threshold level is reached. Thus, it is possible that in DRG neurons 40–68% reductions of miR-183 family microRNAs are sufficient to achieve significant changes in gene expression. Moreover, a recent report suggested that even small changes in microRNA expression may be biologically relevant due to the cumulative effect of the simultaneous regulation of multiple genes (Calin and Croce,

2006). Given the restricted expression of miR-183 family members in sensory tissues it is expected that any change will have a significant influence on sensory neuron function.

The expression levels of miR-183 was reported to be reduced in rat trigeminal ganglion neurons after induction of inflammatory muscle pain by injection of complete Freund's adjuvant (CFA) into the masseter muscle of rats (Bai et al., 2007). This finding is significant because miR-183 changes in the same direction in both inflammatory and neuropathic pain models that produce mechanical hypersensitivity despite fundamental differences in their causes, development, molecular mediators, changes in gene expression, and associated physical manifestations (Honore et al., 2000; Malmberg and Zeitz, 2004; Rodriguez Parkitna et al., 2006). Thus, shared changes between the two distinct chronic pain models are of interest because they support a possible common function of miR-183 in contributing to changes in nociceptive sensitivity. It should be noted that the magnitude of miR-183 expression change is different between the two studies. In the trigeminal ganglion neurons an 85–90% reduction in miR-183 expression was observed within 4 hrs after CFA injection (Bai et al., 2007). This might be explained by the different experimental time points. We only assayed microRNA expression levels at two weeks post surgery. It is possible that the expression levels of the miR-183 cluster microRNAs are changed more dramatically at other time points following the initial insult to the neurons in the SNL model. Future studies of temporal changes in miR-183 expression after SNL will provide important insights into this issue.

One underlying assumption of this study is that the miR-183 family may regulate nociceptive sensitivity by regulating the expression of pro- or anti-nociceptive proteins. To this end, the results of *in silico* studies (Lewis et al., 2003; Krek et al., 2005) predict that the miR-183 family members target several important pain-related genes (selected examples provided in Table 2). Furthermore, the likelihood of predictions for pain related target genes (i.e., PicTar and TargetScan score) are comparable to those for confirmed miR-183 family targets ((Xu and Wong, 2008); Table 2). This suggests that some of the modifications in the signaling properties and excitability of sensory neurons observed after SNL may be attributed to the down-regulation and/or changes in cellular distribution of miR-183 family members. For example, a voltage-gated sodium channel Nav 1.3, which is present at abnormally high levels in injured sensory neurons, is suggested to be involved in neuropathic pain (Waxman et al., 1994). It is possible that down-regulation of miR-96 may relieve suppression of Nav_v 1.3 translation and play a role in the up-regulation of Nav 1.3. Other potential targets for miR-183 family members includes: neurotrophic factors involved in nerve regeneration following injury (Song et al., 2008), kinases involved in intracellular signaling cascades involved in the sensitization of primary afferent nociceptors with chronic pain (Song et al., 2006; Velazquez et al., 2007), and neuropeptides involved in nociception (Marchand et al., 1994). This may parallel the activity of other microRNAs influencing neuronal function (Cheng et al., 2007), morphology (Vo et al., 2005; Schratt et al., 2006), and development (Visvanathan et al., 2007). Thus, miR-183 family members may coordinately regulate multiple diverse nociceptive genes significantly influencing the distinct neuronal changes associated with the development and maintenance of chronic pain conditions. Furthermore, due to sequence conservation of miR-183 family members, several genes are predicted to be targeted by more than one member of the family (Table 2). It is expected that the combined effect of down regulating of all three family members will have a significant influence on the expression of genes targeted by multiple members of the miR-183 family. Therefore, experimental confirmation of the endogenous targets of miR-183 family members should provide valuable insights into gene expression changes associated with chronic pain development.

In addition to quantitative changes in microRNA expression, we unexpectedly observed a striking alteration in intracellular distribution of microRNA labeling, from uniform distribution in the cytoplasm to compartmentalization near the cell periphery, in DRG neurons when

mechanical hypersensitivity was induced by SNL (see Fig. 5–Fig. 7). This phenomenon appears to be general for most, if not all, microRNAs. Our observation that microRNAs and the SG-associated protein TIA-1 colocalize to the periphery of injured DRG neurons indicates that the redistribution of microRNAs is likely due to the recruitment of microRNAs into SGs that are assembled as a result of the nerve injury. SG formation is microtubule-dependent process (Ivanov et al., 2003) involving dynein motor proteins (Tsai et al., 2009). Therefore, it is likely that the differential distribution of TIA-1 in ligated- and sham-operated DRG neurons involve actual movement of stress granule structures to the cell periphery. Although we do not fully know what may cause the differential distribution of microRNAs it is likely that they are shuttled along with SG components to the cell periphery ((Leung et al., 2006))

MicroRNAs and their associated binding proteins have been previously shown to localize to SGs in culture cells following exposure to stress stimuli (e.g. chemical insult, heat shock, oxidative stress) (Leung et al., 2006; Vasudevan and Steitz, 2007). Notably, the SG associated protein, Fragile X Mental Retardation Protein (Kim et al., 2006) that is involved in the microRNA pathway has been recently shown to play an important role in the development of mechanical allodynia following nerve injury (Price et al., 2007), implying a possible connection between microRNAs, SGs, and neuropathic pain. At present it is not known how microRNA and SG accumulation near the cell periphery affects regulatory functions of miR-183 family microRNAs in injured DRG neurons. However, it might contribute to relief of miR-183 family-mediated translational suppression by two potential mechanisms. First, although microRNAs typically suppress expression of target transcripts, several recent reports have indicated that microRNAs may become activators of translation when they are compartmentalized with SGs (Bhattacharyya et al., 2006; Vasudevan and Steitz, 2007) (reviewed in (Leung and Sharp, 2007)). Second, compartmentalization of microRNAs appears to create a large area in the cytoplasm where mRNAs may be free of translational inhibition by microRNAs.

In conclusion, our data indicate that SNL causes a specific down-regulation of the miR-183 family of microRNAs in the ipsilateral L5 DRG. Furthermore, injury that results in mechanical hypersensitivity is accompanied by a generalized redistribution of microRNAs to the periphery of both unmyelinated and myelinated DRG neurons, quite possibly in association with SGs. These findings strongly suggest that microRNA activity is altered in DRG neurons in response to nerve injury, which likely have significant effects on development and maintenance of neuropathic pain. Future functional studies will be needed to elucidate exactly how miR-183 family of microRNAs and their interactions with SGs are involved in development and maintenance of a neuropathic pain state.

Abbreviations

DRG, Dorsal Root Ganglion; SNL, Spinal Nerve Ligation; 3'-UTRs, 3' Untranslated Regions; ANOVA, Analysis of Variance; CGRP, Calcitonin-Gene Related Peptide; qPCR, Quantitative Real-time Polymerase Chain Reaction; TIA-1, T-Cell Intracellular Antigen 1; SG, Stress Granule; CFA, Complete Freund's adjuvant.

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References

- Abramoff MD, Magelhaes PJ, Ram SJ. Image Processing with ImageJ. *Biophotonics International* 2004;11:36–42.
- Alloui A, Zimmermann K, Mamet J, Duprat F, Noel J, Chemin J, Guy N, Blondeau N, Voilley N, Rubat-Coudert C, Borsotto M, Romey G, Heurteaux C, Reeh P, Eschaliere A, Lazdunski M. TREK-1, a K⁺ channel involved in polymodal pain perception. *Embo J* 2006;25:2368–2376. [PubMed: 16675954]
- Bai G, Ambalavanar R, Wei D, Dessem D. Downregulation of selective microRNAs in trigeminal ganglion neurons following inflammatory muscle pain. *Mol Pain* 2007;3:15. [PubMed: 17559665]
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;116:281–297. [PubMed: 14744438]
- Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006;125:1111–1124. [PubMed: 16777601]
- Black JA, Liu S, Tanaka M, Cummins TR, Waxman SG. Changes in the expression of tetrodotoxin-sensitive sodium channels within dorsal root ganglia neurons in inflammatory pain. *Pain* 2004;108:237–247. [PubMed: 15030943]
- Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G, Galli C, Naldini L. Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 2007;25:1457–1467. [PubMed: 18026085]
- Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866. [PubMed: 17060945]
- Campbell JN, Meyer RA. Mechanisms of neuropathic pain. *Neuron* 2006;52:77–92. [PubMed: 17015228]
- Chaplan SR, Bach FW, Pogrel JW, Chung JM, Yaksh TL. Quantitative assessment of tactile allodynia in the rat paw. *J Neurosci Methods* 1994;53:55–63. [PubMed: 7990513]
- Cheng HY, Papp JW, Varlamova O, Dziema H, Russell B, Curfman JP, Nakazawa T, Shimizu K, Okamura H, Impey S, Obrietan K. microRNA modulation of circadian-clock period and entrainment. *Neuron* 2007;54:813–829. [PubMed: 17553428]
- Conaco C, Otto S, Han JJ, Mandel G. Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc Natl Acad Sci U S A* 2006;103:2422–2427. [PubMed: 16461918]
- Costigan M, Belfort K, Karchewski L, Griffin RS, D'Urso D, Allchorne A, Sitarski J, Mannion JW, Pratt RE, Woolf CJ. Replicate high-density rat genome oligonucleotide microarrays reveal hundreds of regulated genes in the dorsal root ganglion after peripheral nerve injury. *BMC Neurosci* 2002;3:16. [PubMed: 12401135]
- Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, Karbani G, Jafri H, Mannan J, Raashid Y, Al-Gazali L, Hamamy H, Valente EM, Gorman S, Williams R, McHale DP, Wood JN, Gribble FM, Woods CG. An SCN9A channelopathy causes congenital inability to experience pain. *Nature* 2006;444:894–898. [PubMed: 17167479]
- Dostie J, Mourelatos Z, Yang M, Sharma A, Dreyfuss G. Numerous microRNPs in neuronal cells containing novel microRNAs. *Rna* 2003;9:180–186. [PubMed: 12554860]
- Engle MP, Gassman M, Sykes KT, Bettler B, Hammond DL. Spinal nerve ligation does not alter the expression or function of GABA(B) receptors in spinal cord and dorsal root ganglia of the rat. *Neuroscience* 2006;138:1277–1287. [PubMed: 16427742]
- Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, Baskerville S, Hammond SM, Bartel DP, Schier AF. MicroRNAs regulate brain morphogenesis in zebrafish. *Science* 2005;308:833–838. [PubMed: 15774722]
- Hammond DL, Ackerman L, Holdsworth R, Elzey B. Effects of spinal nerve ligation on immunohistochemically identified neurons in the L4 and L5 dorsal root ganglia of the rat. *J Comp Neurol* 2004;475:575–589. [PubMed: 15236238]
- Honore P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, Clohisy DR, Mantyh PW. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of

neurochemical changes in the spinal cord and sensory neurons. *Neuroscience* 2000;98:585–598. [PubMed: 10869852]

Ivanov PA, Chudinova EM, Nadezhdina ES. Disruption of microtubules inhibits cytoplasmic ribonucleoprotein stress granule formation. *Exp Cell Res* 2003;290:227–233. [PubMed: 14567982]

Kim DS, Lee SJ, Park SY, Yoo HJ, Kim SH, Kim KJ, Cho HJ. Differentially expressed genes in rat dorsal root ganglia following peripheral nerve injury. *Neuroreport* 2001;12:3401–3405. [PubMed: 11711894]

Kim J, Krichevsky A, Grad Y, Hayes GD, Kosik KS, Church GM, Ruvkun G. Identification of many microRNAs that copurify with polyribosomes in mammalian neurons. *Proc Natl Acad Sci U S A* 2004;101:360–365. [PubMed: 14691248]

Kim SH, Chung JM. An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain* 1992;50:355–363. [PubMed: 1333581]

Kim SH, Dong WK, Weiler IJ, Greenough WT. Fragile X mental retardation protein shifts between polyribosomes and stress granules after neuronal injury by arsenite stress or in vivo hippocampal electrode insertion. *J Neurosci* 2006;26:2413–2418. [PubMed: 16510718]

Kloosterman WP, Wienholds E, de Bruijn E, Kauppinen S, Plasterk RH. In situ detection of miRNAs in animal embryos using LNA-modified oligonucleotide probes. *Nat Methods* 2006;3:27–29. [PubMed: 16369549]

Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. *Nat Genet* 2005;37:495–500. [PubMed: 15806104]

Krichevsky AM, King KS, Donahue CP, Khrapko K, Kosik KS. A microRNA array reveals extensive regulation of microRNAs during brain development. *Rna* 2003;9:1274–1281. [PubMed: 13130141]

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol* 2002;12:735–739. [PubMed: 12007417]

Leung AK, Sharp PA. microRNAs: a safeguard against turmoil? *Cell* 2007;130:581–585. [PubMed: 17719533]

Leung AK, Calabrese JM, Sharp PA. Quantitative analysis of Argonaute protein reveals microRNA-dependent localization to stress granules. *Proc Natl Acad Sci U S A* 2006;103:18125–18130. [PubMed: 17116888]

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell* 2003;115:787–798. [PubMed: 14697198]

Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* 2005;7:719–723. [PubMed: 15937477]

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001;25:402–408. [PubMed: 11846609]

Luo ZD, Chaplan SR, Higuera ES, Sorkin LS, Stauderman KA, Williams ME, Yaksh TL. Upregulation of dorsal root ganglion (alpha)2(delta) calcium channel subunit and its correlation with allodynia in spinal nerve-injured rats. *J Neurosci* 2001;21:1868–1875. [PubMed: 11245671]

Malmberg, AB.; Zeitz, KP. Studies of pain mechanisms in genetically manipulated mice. In: Mogil, JS., editor. *The Genetics of Pain. Progress in Pain Research and Management*. Seattle, WA: IASP Press; 2004. p. 21-48.

Marchand JE, Wurm WH, Kato T, Kream RM. Altered tachykinin expression by dorsal root ganglion neurons in a rat model of neuropathic pain. *Pain* 1994;58:219–231. [PubMed: 7816489]

Miska EA, Alvarez-Saavedra E, Townsend M, Yoshii A, Sestan N, Rakic P, Constantine-Paton M, Horvitz HR. Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol* 2004;5:R68. [PubMed: 15345052]

Newton RA, Bingham S, Davey PD, Medhurst AD, Piercy V, Raval P, Parsons AA, Sanger GJ, Case CP, Lawson SN. Identification of differentially expressed genes in dorsal root ganglia following partial sciatic nerve injury. *Neuroscience* 2000;95:1111–1120. [PubMed: 10682718]

Noguchi K, Kawai Y, Fukuoka T, Senba E, Miki K. Substance P induced by peripheral nerve injury in primary afferent sensory neurons and its effect on dorsal column nucleus neurons. *J Neurosci* 1995;15:7633–7643. [PubMed: 7472514]

- Obata K, Noguchi K. BDNF in sensory neurons and chronic pain. *Neurosci Res* 2006;55:1–10. [PubMed: 16516994]
- Pierce ML, Weston MD, Fritzsche B, Gabel HW, Ruvkun G, Soukup GA. MicroRNA-183 family conservation and ciliated neurosensory organ expression. *Evol Dev* 2008;10:106–113. [PubMed: 18184361]
- Price TJ, Rashid MH, Millecamps M, Sanoja R, Entrena JM, Cervero F. Decreased nociceptive sensitization in mice lacking the fragile X mental retardation protein: role of mGluR1/5 and mTOR. *J Neurosci* 2007;27:13958–13967. [PubMed: 18094233]
- Rodriguez Parkitna J, Korostynski M, Kaminska-Chowanec D, Obara I, Mika J, Przewlocka B, Przewlocki R. Comparison of gene expression profiles in neuropathic and inflammatory pain. *J Physiol Pharmacol* 2006;57:401–414. [PubMed: 17033093]
- Scholz J, Woolf CJ. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci* 2007;10:1361–1368. [PubMed: 17965656]
- Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. *Nature* 2006;439:283–289. [PubMed: 16421561]
- Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brainexpressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* 2004;5:R13. [PubMed: 15003116]
- Song XJ, Wang ZB, Gan Q, Walters ET. cAMP and cGMP contribute to sensory neuron hyperexcitability and hyperalgesia in rats with dorsal root ganglia compression. *J Neurophysiol* 2006;95:479–492. [PubMed: 16120663]
- Song XY, Li F, Zhang FH, Zhong JH, Zhou XF. Peripherally-derived BDNF promotes regeneration of ascending sensory neurons after spinal cord injury. *PLoS ONE* 2008;3:e1707. [PubMed: 18320028]
- Tsai NP, Tsui YC, Wei LN. Dynein motor contributes to stress granule dynamics in primary neurons. *Neuroscience* 2009;159:647–656. [PubMed: 19171178]
- Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;9:654–659. [PubMed: 17486113]
- Valencia-Sanchez MA, Liu J, Hannon GJ, Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 2006;20:515–524. [PubMed: 16510870]
- Vasudevan S, Steitz JA. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. *Cell* 2007;128:1105–1118. [PubMed: 17382880]
- Velazquez KT, Mohammad H, Sweitzer SM. Protein kinase C in pain: involvement of multiple isoforms. *Pharmacol Res* 2007;55:578–589. [PubMed: 17548207]
- Visvanathan J, Lee S, Lee B, Lee JW, Lee SK. The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev* 2007;21:744–749. [PubMed: 17403776]
- Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH, Impey S. A cAMP-response element binding protein-induced microRNA regulates neuronal morphogenesis. *Proc Natl Acad Sci U S A* 2005;102:16426–16431. [PubMed: 16260724]
- Wang H, Sun H, Della Penna K, Benz RJ, Xu J, Gerhold DL, Holder DJ, Koblan KS. Chronic neuropathic pain is accompanied by global changes in gene expression and shares pathobiology with neurodegenerative diseases. *Neuroscience* 2002;114:529–546. [PubMed: 12220557]
- Waxman SG, Kocsis JD, Black JA. Type III sodium channel mRNA is expressed in embryonic but not adult spinal sensory neurons, and is reexpressed following axotomy. *J Neurophysiol* 1994;72:466–470. [PubMed: 7965028]
- Weston MD, Pierce ML, Rocha-Sanchez S, Beisel KW, Soukup GA. MicroRNA gene expression in the mouse inner ear. *Brain Res* 2006;1111:95–104. [PubMed: 16904081]
- Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. MicroRNA expression in zebrafish embryonic development. *Science* 2005;309:310–311. [PubMed: 15919954]
- Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 2000;288:1765–1769. [PubMed: 10846153]

- Xiao HS, Huang QH, Zhang FX, Bao L, Lu YJ, Guo C, Yang L, Huang WJ, Fu G, Xu SH, Cheng XP, Yan Q, Zhu ZD, Zhang X, Chen Z, Han ZG, Zhang X. Identification of gene expression profile of dorsal root ganglion in the rat peripheral axotomy model of neuropathic pain. *Proc Natl Acad Sci U S A* 2002;99:8360–8365. [PubMed: 12060780]
- Xu J, Wong C. A computational screen for mouse signaling pathways targeted by microRNA clusters. *Rna* 2008;14:1276–1283. [PubMed: 18511500]
- Xu S, Witmer PD, Lumayag S, Kovacs B, Valle D. MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster. *J Biol Chem* 2007;282:25053–25066. [PubMed: 17597072]
- Yamanaka H, Obata K, Fukuoka T, Dai Y, Kobayashi K, Tokunaga A, Noguchi K. Induction of plasminogen activator inhibitor-1 and -2 in dorsal root ganglion neurons after peripheral nerve injury. *Neuroscience* 2005;132:183–191. [PubMed: 15780477]
- Zimmer A, Zimmer AM, Baffi J, Usdin T, Reynolds K, Konig M, Palkovits M, Mezey E. Hypoalgesia in mice with a targeted deletion of the tachykinin 1 gene. *Proc Natl Acad Sci U S A* 1998;95:2630–2635. [PubMed: 9482938]
- Zimmermann M. Pathobiology of neuropathic pain. *Eur J Pharmacol* 2001;429:23–37. [PubMed: 11698024]

Figure 1.

The paw withdrawal thresholds for naïve, sham-operated, and ligated rats used in the qPCR, *in situ* hybridization, and immunohistochemistry analyses. Squares depict the threshold for the indicated hindpaw of each rat. Circles with error bars are the mean $\pm$ S.E.M. for each treatment group. Symbols with an asterisk indicate rats that did not show the expected pain behavior. n = the number of animals in each group.

Figure 2.

Expression of miR-183 family microRNAs in tissues from the adult rat. (A) Amounts of miR-96, miR-182, and miR-183 in various adult rat tissues relative to that in the dorsal root ganglia. Data are the mean $\pm$ S.E.M. of determinations in 3 rats. (DRG = dorsal root ganglion; SC = spinal cord; Br St = brain stem; Cer = cerebellum; Cor = cortex; Hrt = heart; Int = intestine; Kid = kidney; Liv = liver; Lun = lung). (B) Data represented as fg of miR-96, miR-182, and miR-183 in 1 ng of total RNA extracted from adult rat dorsal root ganglia.

Figure 3.

Relative expression of microRNAs in the L5 DRG of ligated and sham-operated rats determined by qPCR for miR-96, miR-182, miR-183, and miR-23b. Data are the mean $\pm$ S.E.M. of eight determinations. Data for the ipsilateral L5 DRG of sham rats and ipsilateral and contralateral L5 DRG of ligated rats are expressed relative to average microRNA amount (fg/ng total RNA) in L5 DRG of naïve rats. Symbols above error bars indicate significant difference for ligated ipsilateral L5 DRG compared to naïve ipsilateral L5 DRG (^; ANOVA, $P \leq 0.05$) or ligated ipsilateral L5 DRG compared to naïve and sham operated ipsilateral L5 DRG and ligated contralateral L5 DRG (*; ANOVA, $P \leq 0.05$). The remaining comparisons were not significantly different (ANOVA, $P > 0.05$).

Figure 4.

Expression patterns of miR-96, -182, and 183 in L5 DRGs from naïve rats. *In situ* hybridization staining for microRNAs (A, C, E) was performed in combination with immunohistochemical labeling (B, D, F) for myelinated (N52-positive, red) and unmyelinated (IB4-positive, greens) neurons. Large and small arrows indicate microRNA/N52 co-labeling and microRNA/IB4 co-labeling, respectively. Arrow heads indicate microRNA-positive/N52 and IB4-negative neurons. (A, B) miR-96, (C, D) miR-182, (E, F) miR-183.

Figure 5.

Distribution of miR-96 *in situ* hybridization signal in ipsilateral L5 DRG from rats that underwent sham surgery (A, C) or L5 spinal nerve ligation (B, D). Spinal nerve ligation resulted in an overall reduction in the intensity of labeling and a redistribution of label within cytoplasm to the periphery of the neuron. Arrow in panel C indicates a neuron with weakly staining nucleus but uniform staining across cytoplasm, which can be differentiated from ligated neurons with strong staining on the edge of the cell with a weakly staining inner cytoplasmic area.

Figure 6. Representative examples of the distribution of *in situ* hybridization signal for miR-182 (A, B), miR-183 (C, D), let-7a (E, F), miR-23b (G, H), and miR-124a (I, J) in the ipsilateral L5 DRG two weeks after either sham surgery (A, C, E, G, I) or ligation of the L5 spinal nerve (B, D, F, H, J) in the rat.

Figure 7.

Co- *in situ* hybridization and immunohistochemistry for TIA-1 (A, C) and miR-96 (B, D) in ipsilateral L5 DRG two weeks after sham surgery (A, B) or ligation of the L5 spinal nerve (C, D) in the rat. TIA-1 and miR-96 costaining in large diameter and small diameter neurons indicated by large and small arrows, respectively. Large arrows in panel C and D indicate a large diameter neuron with TIA-1 and miR-96 staining colocalizing to edge of cell. In sham operated DRGs, TIA-1 is present in the nucleus of both large and small diameter neurons (A; large and small arrows, respectively). In ligated DRGs, TIA-1 is present in the nucleus of large diameter neurons and absent in the nucleus small diameter neurons (C; large and small arrows, respectively).

Table 1

Quantification of miR-96 staining in outer and inner regions of large diameter neurons from 4 naïve, 3 ligated, and 3 sham operated animals. Total neurons counted for each treatment group provided. The neurons were counted from 3 different tissue sections per animal/DRG.

		Naïve	Ligated	Sham
Neurons Counted		171	152	121
(A.) Pixel intensity ratio of outer to inner portion of neuron	Median:	0.91 (0.84–0.99)	*1.31 (1.14–1.62)	0.93 (0.85–1.05)
	Average:	0.92 ± 0.01	*1.70 ± 0.15	0.97 ± 0.02
(B.) % neurons with redistribution		1.66 ± 1.12	*55.10 ± 10.52	4.27 ± 4.27

(A.) The median with 25%–75% quartiles and average ± standard error ratio of the average pixel intensities in the outer to inner portions of neurons for each treatment group.

(B.) The percentage of neurons averaged across animals within a treatment group ± standard error with miR-96 redistribution to the cell periphery. Neurons were considered to show redistribution if the pixel intensity in the outer portion of the neuron was greater than the average pixel intensity plus 1 S.D. for the inner portion of the neuron. Asterisks indicate significant differences from naïve.

Asterisks indicate significant differences from naïve.

Table 2
Predicted targets of miR-183 family members from *in silico* studies of microRNA-mRNA complementary binding. Predictions taken from PicTar (Krek et al., 2005) (internet available at <http://pictar.bio.nyu.edu/>) and TargetScan (Lewis et al., 2003) (internet available at <http://www.targetscan.org/>).

Gene	NCBI reference	microRNA	PicTar score	TargetScan score	Reference
A.					
N _a , 1.3	NM 006922	96,182,183	5.9,6.2,3.9	-0.07, -0.06,-0.23	(Waxman et al., 1994; Black et al., 2004)
N _a , 1.7	NM 002977	96,182	3.5,3.5	-0.14,-0.15	(Cox et al., 2006)
BDNF	NM 170734	182	2.1	-0.18	(Obata and Noguchi, 2006)
Tac 1	NM 013998	96	3.9	-0.31	(Noguchi et al., 1995; Zimmer et al.,1998)
CaCN α 2 δ 1	NM 000722	96,182	3.6,3.6	-0.12,-0.13	(Luo et al., 2001)
PAI-2	NM 002575	96,182	3.9,3.3	-0.46,-0.23	(Yamanaka et al., 2005)
TREK-1	NM 014217	183	5.9	-0.36	(Alloui et al., 2006)
B.					
Irs1	NM 005544	96*,183*	3.0,2.3	-0.21,-0.42	(Xu and Wong, 2008)
Rasa1	NM 022650	96,182*	8.4,6.6	-0.33,-0.81	(Xu and Wong, 2008)
Grb2	NM 203506	96*,182*	5.1,2.9	-0.18,-0.20	(Xu and Wong, 2008)

(A) List of genes with nociceptive function predicted to be targeted by miR-183 family members.

(B) List of target predictions for genes confirmed to be targeted by miR-183 family members.

Asterisk designates the microRNAs confirmed to target the gene's 3' UTR. For each predicted gene the NCBI reference, predicted targeting microRNA, PicTar score, TargetScan Score, pain study reference (A.), and target confirmation reference (B.) are provided. Larger PicTar scores and more negative TargetScan scores indicate a higher likelihood.