MicroRNA cluster miR-17-92 regulates multiple functionally related voltage-gated potassium channels in chronic neuropathic pain

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miR-17-92 is a microRNA cluster with six distinct members. Here, we show that the miR-17-92 cluster and its individual members modulate chronic neuropathic pain. All cluster members are persistently upregulated in primary sensory neurons after nerve injury. Overexpression of miR-18a, miR-19a, miR-19b and miR-92a cluster members elicits mechanical allodynia in rats, while their blockade alleviates mechanical allodynia in a rat model of neuropathic pain. Plausible targets for the miR-17-92 cluster include genes encoding numerous voltage-gated potassium channels and their modulatory subunits. Single-cell analysis reveals extensive co-expression of miR-17-92 cluster and its predicted targets in primary sensory neurons. miR-17-92 downregulates the expression of potassium channels, and reduced outward potassium currents, in particular A-type currents. Combined application of potassium channel modulators synergistically alleviates mechanical allodynia induced by nerve injury or miR-17-92 overexpression. miR-17-92 cluster appears to cooperatively regulate the function of multiple voltage-gated potassium channel subunits, perpetuating mechanical allodynia.

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A microRNA (miRNA) cluster is a polycistronic gene in which several miRNAs are encoded in a single primary, or nascent, transcript. Small non-coding mature miRNAs are produced by stepwise cleavage of primary miRNA transcribed from the genome, and inhibit expression of diverse genes through a sequence-dependent binding to a specific 3' untranslated region (UTR) sequence in miRNAs. About 40% of microRNAs are estimated to form clusters whose physiological importance is largely unknown, although the roles of individual miRNAs in a variety of physiological and pathological states are increasingly being recognized.

The miR-17-92 cluster encodes six distinct miRNAs in a single primary transcript (miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a) compared with common miRNA clusters that comprise only two or three miRNAs. The miR-17-92 null mutation is lethal because of lung hypoplasia and ventricular septal defects, and miR-17-92 dysregulation has been observed in diverse neurological diseases such as autism spectrum disorder and Alzheimer’s disease. Our previous microarray analysis showed that miR-17-92 cluster members were upregulated in the dorsal root ganglion (DRG) after nerve injury. The pathophysiological significance of miRNA cluster in neurological disorders is not yet understood.

Voltage-gated potassium channels are critical regulators of neuronal excitability, acting by modulating action potential generation, firing rate or neurotransmitter release. Voltage-gated potassium channels are encoded by ~40 genes: six encode the Kv1.4, Kv3.3, Kv3.4 and Kv4.1–4.3 channel subtypes that mediate fast-inactivating A-type currents, while the others are delayed rectifiers. Expression of a variety of voltage-gated potassium channels—and thus both currents—are consistently decreased in DRG neurons after nerve injury, making potassium channels attractive targets for the treatment of neuropathic pain.

Neuropathic pain is frequently caused by lesions or disease of primary sensory neurons; their inputs to the spinal cord are critical for the development and maintenance of chronic pain. miRNAs reportedly regulate gene translation in neuropathic pain states. Although the molecular mechanisms regulating the expression of several potassium channels have recently been illuminated, the pathophysiological mechanism by which potassium channel expression is coordinately downregulated in DRG neurons is incompletely understood.

Here, we show the combinatorial impact of miR-17-92 cluster members on chronic neuropathic pain through regulation of functionally related multiple voltage-gated potassium channels and their modulatory subunits, especially those responsible for the A-type potassium current.

Results

miR-17-92 cluster is upregulated in DRG neurons after injury.

Using quantitative PCR (qPCR), we first confirmed that expression of each miR-17-92 cluster member (Supplementary Fig. 1a) was significantly upregulated in the DRG 14 days after ligation of the fifth lumbar (L5) nerve, as observed in our previous microarray analysis (Fig. 1a). Uregulation was sustained from day 1 to day 28 after L5 spinal nerve ligation (SNL; Fig. 1a). Neuronal pain was also evident at the same time (Fig. 1b). Next, we examined the expression of pri-miR-17-92 (the primary transcript of miR-17-92 cluster), finding that it was also upregulated from days 1 to 28 after SNL (Fig. 1c), suggesting that the upregulation of mature miR-17-92 cluster miRNA expression reflected transcriptional upregulation of the miR-17-92 cluster. In contrast, expression of miR-17-363 (miR-106a-363 homologue in humans), a miR-17-92 paralog encoding similar mature miRNAs (Supplementary Fig. 1a), was not consistently elevated (Supplementary Fig. 1b). miR-17-92 was also upregulated in the DRG neurons in another neuropathic pain model, spared nerve injury (Supplementary Fig. 2a). In contrast, miR-17-92 expression was unchanged in the injury-spared L4 DRG and L5 dorsal spinal cord ipsilateral to the L5 SNL (Supplementary Fig. 2b,c). Expression of miR-17-92 cluster members was not elevated in the L5 DRG in rats with inflammatory pain of the hind paw provoked by complete Freund’s adjuvant (CFA) (Supplementary Fig. 2d), despite the partial overlap of the molecular mechanisms of inflammatory and neuropathic pain and despite CFA inducing comparable mechanical allodynia (Supplementary Fig. 2e). These results suggest nerve injury-specific involvement of miR-17-92 in pain behaviours.
Bioinformatic analysis of miR-17-92 cluster targets. To illuminate the mechanisms underlying miR-17-92-mediated mechanical allodynia, we searched target genes for miR-17-92 cluster members that could be responsible for pain behaviour using Ingenuity Pathway Analysis (IPA; Qiagen K.K., Tokyo, Japan). The number of putative target genes for miR-17a, miR-19a/b (miR-19a and miR-19b have the same seed sequence) and miR-92a were 695, 1,448 and 1,138, respectively (a total of 2,834 genes). We further restricted the potential target genes based on the miRNA expression profile 28 days after L5 SNL, as persistent miRNA-target mRNA complexing can lead to mRNA decay\(^1^9\).

Comprehensive microarray analysis of mRNA expression changes\(^2^0\) available in Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo/; GEO accession GSE42198) identified 1,470 significantly downregulated mRNAs in the DRG 28 days after L5 SNL. Intriguingly, 20.3% of the downregulated genes (299 genes) were predicted as targets of pain-relevant miR-17-92 cluster members (Supplementary Fig. 7a and Supplementary Data 1), suggesting a broad modulatory role for miR-17-92 cluster following nerve injury. These plausible target genes were then analysed using IPA to explore their associated functions. Downstream effects analysis, which predicts the likelihood of involvement in several downstream biological processes, identified 'neurological disease' as the most significant (Supplementary Fig. 7b), supporting a functional role for miR-17-92 cluster in the modulation of gene expression after SNL. Network analysis also showed that plausible target genes comprised a relatively high proportion of genes associated with the network function of cell-to-cell signalling and interaction, nervous system development and function, and neurological disease (Supplementary Fig. 7c,d).
miR-17-92 downregulates voltage-gated potassium channels. Many voltage-gated potassium channel α subunits were among the plausible target genes downregulated following nerve injury and predicted as targets of pain-relevant miR-17-92 cluster members (Fig. 4a), including Kv1.1, Kv1.4 and Kv4.3 (refs 21–25). In addition, positive modulators of the voltage-gated potassium channels DPP10 and Navβ1 (refs 26–28) were also predicted miRNA targets. Therefore, we examined whether voltage-gated potassium channel subunits are targeted by miR-17-92 cluster miRNAs using a luciferase assay. As an miRNA generally recognizes the 3′-UTR of mRNAs19, each 3′-UTR sequence of a candidate target gene was inserted downstream of the firefly luciferase gene in a plasmid vector. Activities of luciferases with putative target 3′-UTR sequences were generally decreased by their corresponding miRNAs (Fig. 4b), although Kcna1 and Dpp10 3′-UTRs were not targeted by miR-92a or miR-18a, respectively. Prior transfection of TuD miRNA antisense RNAs inhibited the decrease in luciferase activity by corresponding miRNAs (Supplementary Fig. 5), validating the effectiveness of the antisense RNAs. To confirm that the predicted seed sequences were responsible for miRNA-mediated luciferase suppression, these seed sequences were mutated to mismatch the miRNA sequences (Supplementary Fig. 8). As miR-19a/b and miR-92a have two predicted binding sites for Kcna4 3′-UTR and Kcnc4 3′-UTRs, respectively, both sites were mutated. Activities of luciferases with mutated 3′-UTRs were no longer suppressed by corresponding miRNAs (Fig. 4b), indicating that the predicted seed sequences were directly targeted in a sequence-specific manner by corresponding miRNAs. Importantly, all seed sequences are conserved among mammals (Supplementary Fig. 8), highlighting the potential importance of potassium channel modulation by miR-17-92.

Next, we investigated the role of miR-17-92 in the modulation of potassium channel subunit expression in vivo. miR-17-92-expressing cell types and potassium channel expression patterns were examined using laser microdissection followed by reverse transcription PCR (RT-PCR). Pri-miR-17-92 expression in laser-captured single DRG neurons (Fig. 5a,b) was positively detected.
miR-17-92 decreases potassium currents in DRG neurons. Potassium channel α subunits targeted by miR-17-92 include three of six known potassium channels constituting rapidly inactivating potassium currents, or A-type currents (Fig. 4a). In addition, DPP10 and NaV1 subunits are shown to positively modulate Kv4 channels, which are major contributors to A-type currents, further suggesting the importance of miR-17-92 in the modulation of A-type potassium currents. Therefore, we performed whole-cell patch clamping of primary sensory neurons to measure A-type and non-A-type potassium currents. The non-A-type potassium current was recorded in the presence of the A-type potassium channel blocker, 3,4-diaminopyridine (DAP)12. The DAP-sensitive A-type potassium current was obtained by subtracting the non-A-type current from the total potassium currents. In L5 DRG neurons prepared from rats injected with AAV vector encoding the whole miR-17-92 cluster 7 days beforehand, total potassium currents were diminished in small DRG neurons of cell size <600 μm² and medium/large neurons of cell size >600 μm² (Fig. 5c). Small and large DRG neurons represent putative C-fibre and A-fibre neurons, respectively. RT-PCR also detected a massive overlap of potassium channel subunits targeted by miR-17-92 (Fig. 5c). Importantly, voltage-gated potassium channel subunits were extensively detected in DRG neurons positive for the expression of miR-17-92. To further address the cell types expressing miR-17-92, TAC1 and P2X3 expressions were examined as peptidergic DRG neurons, although it was also expressed in the TAC1-positive peptidergic DRG neurons (Fig. 5c). Then, we analysed the expression of potassium channels in the L5 DRG of rats 7 days after AAV vector injection. Microarray analysis revealed that injection of an AAV vector encoding the whole miR-17-92 cluster decreased the expression of all voltage-gated potassium channel subunits targeted by miR-17-92 (n = 4; Supplementary Table 1). miR-17-92 overexpression also modulated the expression of other potassium channels (Supplementary Table 1) and non-potassium channel genes (Supplementary Data 2). Many of these genes that were not predicted as direct targets of miR-17-92 may also be indirectly modulated through the downregulation of miR-17-92 targets. qPCR confirmed the increased expression of all voltage-gated potassium channel subunits targeted by miR-17-92 (Fig. 5d). Similarly, overexpression of each miR-17-92 cluster member decreased expression of corresponding voltage-gated potassium channel subunits (Fig. 5d), although NaVβ1 expression was mostly unaffected by miR-19a. Notably, the whole miR-17-92 cluster more robustly downregulated expression of potassium channel subunits targeted by several miRNAs than each cluster member alone. In contrast, other pain-related potassium channels (Kr1.2, Kr6.1 and BA_Ca) that were not predicted as miR-17-92 targets did not show significant expression changes (Supplementary Fig. 9). Because Kv1.1, Kv3.4 and Kv4.3 are reportedly involved in mechanical allodynia, but not thermal hyperalgesia, these potassium channels appear to be particularly important for miR-17-92-mediated mechanical allodynia.
SNL significantly reduced it (Supplementary Fig. 10). The slight reduction brought about by miR-17-92 cluster can likely be explained by its predominant targeting of the \( K_v7.5 \) channel among non-A-type channels in small DRG neurons. The \( V_{\text{half}} \) and \( k \) of total, A-type and non-A-type potassium currents were unaffected by miR-17-92 (Supplementary Fig. 11a), indicating that miR-17-92 did not affect the voltage-dependent activation of potassium channels in small DRG neurons. In medium/large DRG neurons of cell sizes >600 \( \mu m^2 \), miR-17-92 did not significantly reduce total, non-A-type or A-type potassium currents (Fig. 6b,d). \( V_{\text{half}} \) and \( k \) in medium/large DRG neurons were also unaffected by miR-17-92 (Supplementary Fig. 11b). These results indicate that miR-17-92 has a critical role in the function of voltage-gated potassium channels, particularly those mediating A-type currents.

**Blockade of miR-17-92 members rescues potassium currents.** To address the causal involvement of miR-17-92 in reduced potassium currents associated with neuropathic pain, the protein expressions of pore-forming potassium channel subunits targeted by miR-17-92 were examined in the L5 DRG 28 days after SNL. L5 DRGs were obtained 28 days after SNL from rats injected with mixture of AAV vectors expressing antisense RNAs against miR-18a, miR-19a, miR-19b and miR-92a 7 days after SNL. Blockade of these miR-17-92 members significantly restored the

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**Table 1. List of voltage-gated potassium channel subunits predicted as miR-17-92 target genes; these subunits were reportedly downregulated in a previous microarray study of SNL.** Parenthesized targeting miRNAs represent predicted miRNAs not validated by a luciferase assay.

| Gene symbol | Fold change | Targeting miRNA       | Function                  |
|-------------|-------------|-----------------------|---------------------------|
| Kcna1       | 1.650       | 18a (92a)             | Delayed rectifier         |
| Kcn4        | 3.184       | 19a/b \( x2 \)        | Fast-inactivating (A-type current) |
| Kcnc4       | 1.933       | 19a/b                 | Fast-inactivating (A-type current) |
| Kcnd3       | 8.028       | 18a                    | Fast-inactivating (A-type current) |
| Kcnd3       | 1.605       | 19a/b                 | Delayed rectifier (M current) |
| Dpp10       | -52.375     | (18a) 92a             | Positive regulator        |
| Scn1b       | -2.904      | 19a/b                 | Positive regulator        |

**Figure 4.** miR-17-92 cluster members differentially target the 3'-UTR sequences of voltage-gated potassium channels and modulatory subunits. (a) List of voltage-gated potassium channel subunits predicted as miR-17-92 target genes; these subunits were reportedly downregulated in a previous microarray study of SNL. Parenthesized targeting miRNAs represent predicted miRNAs not validated by a luciferase assay. (b) Activities of luciferase with voltage-gated potassium channel subunit 3'-UTRs or mutated 3'-UTRs in HEK293 cells co-transfected with control or miR-17-92 cluster member-expressing plasmid vector. Numbers of cell cultures are indicated at the base of each bar. Error bars are s.e.m. *\( P<0.05 \), **\( P<0.01 \) and ***\( P<0.001 \) (Kcna1, \( P<0.001 \) for miR-18a and \( P=0.004 \) for miR-92a; Kcna4, \( P<0.001 \) for miR-19a and \( P<0.001 \) for miR-19b; Kcnc4, \( P<0.001 \) for miR-19a and \( P<0.001 \) for miR-19b and \( P<0.001 \) for miR-92a; Kcnd3, \( P=0.021 \) for miR-18a; Kcnd3, \( P=0.008 \) for miR-19a and \( P=0.008 \) for miR-19b; Dpp10, \( P=0.006 \) for miR-92a; Scn1b, \( P<0.001 \) for miR-19a and \( P<0.001 \) for miR-19b) compared with control plasmid, unpaired \( t \)-test or Dunnett’s test.
channel protein expressions (Fig. 7a,b). In line with the protein expression changes, injection of the AAV vector mixture 7 days before SNL significantly blocked the reduction in total, A-type and non-A-type potassium currents at day 7 following SNL (Fig. 7c,d).

Potassium channel modulators suppress mechanical allodynia. As miR-17-92 cluster suppressed the activity of multiple voltage-gated potassium channels, we examined the effects of available potassium channel modulators on neuropathic pain. Seven days after SNL, systemic administration of flupirtine, a Kv7 potassium channel activator, partially attenuated mechanical allodynia (Fig. 8a), consistent with a previous report that flupirtine compensated for potassium channel downregulation30. Furthermore, NS5806, another potassium channel modulator that increases potassium current through the Kv4.3 channel, also attenuated mechanical allodynia to a similar extent (Fig. 8a). Intriguingly,
co-administration of flupirtine and NS5806 showed more potent suppression of mechanical allodynia than each potassium channel modulator alone (Fig. 8a), suggesting the importance of concurrent enhancement of multiple potassium channels. Then, to address the correlation of voltage-gated potassium channels in mechanical allodynia mediated by miR-17-92, we administered these potassium channel modulators to rats overexpressing miR-17-92. Both flupirtine and NS5806 were partially effective (Fig. 8b), as was the case in SNL-induced allodynia. Furthermore, combination therapy with both drugs showed more potent alleviation of mechanical allodynia induced by miR-17-92 overexpression (Fig. 8b).

**Discussion**

We have shown the combinatorial impact of miR-17-92 cluster members on mechanical allodynia through concurrent regulation of many functionally related voltage-gated potassium channel subunits. Furthermore, bioinformatic analysis indicated that many genes predicted as targets of miR-17-92 were downregulated by nerve injury and were associated with neurological disease. Thus, these genes other than potassium channel subunits may also be involved in the miR-17-92-mediated mechanical allodynia. On the other hand, miR-17-92 is reported to enhance axonal growth in embryonic cortical neurons31, which is consistently enhanced in DRG neurons affected by nerve injury. Thus, miR-17-92 may also orchestrate nerve regeneration following nerve injury. Furthermore, miR-17-92 dysregulation is reportedly observed in neurological diseases including autism spectrum disorder3–5,32–34. Taken together, miR-17-92 as a cluster can effectively and cooperatively regulate many aspects of physiological and/or pathological processes. Therefore, thorough investigation of the influence of multifunctional miR-17-92 cluster on neural function will likely advance our understanding of the pathophysiology of diverse neurological disorders as well as neuropathic pain.

miR-17-92 cluster members concurrently suppressed multiple voltage-gated potassium channel α subunits and modulatory
subunits in DRG neurons. Expression of these subunits detected using RT-PCR, immunohistochemistry or microarray analysis were substantially decreased in DRG neurons after nerve injury. KV1.1, KV1.4, KV3.4 and KV4.3 are reportedly downregulated in small and medium/large neurons after nerve injury21–25. In this study, we detected co-expression of multiple potassium genes and miR-17-92 cluster in single cells. The cell profile expressing the potassium channel subunits was comparable with previous histological studies: KV1.1 was preferentially detected in medium/large DRG neurons 24,35 and KV3.4 was broadly expressed in various sized DRG neurons22, while KV1.4, KV4.3, KV7.5 and DPP10 were preferentially detected in small DRG neurons22–24,36,37. Notably, DPP10 expression was detected only in small DRG neurons that expressed KV4.3, exactly consistent with a previous report38. In contrast, NaVb1 distribution, which was mainly detected in medium/large DRG neurons in this study, is poorly understood, although NaVb1 null mutant mice exhibited hyperexcitability of small DRG neurons37. Given that miR-17-92

Figure 7 | miR-17-92 inhibition rescues potassium channel expressions and potassium currents in DRG neurons in the neuropathic pain state. (a) Representative images of immunoblotting for voltage-gated potassium channel α subunits in the L5 DRG obtained from SNL rats at day 28. Full blots can be found in the Supplementary Fig. 12. AAV vectors expressing either a control AAV vector or mixture of AAV vectors encoding TuD antisense RNAs against miR-18a, miR-19a, miR-19b and miR-92a were administered 7 days after SNL. GAPDH was used as a loading standard. (b) Expression levels of voltage-gated potassium channel protein, as a percentage of expression level in intact L5 DRG. Numbers of samples are indicated at the base of each bar. *P<0.05 and **P<0.01 (P=0.004 for KV1.1; P=0.038 for KV1.4; P=0.015 for KV3.4; P=0.028 for KV4.3; P=0.003 for KV7.5), Welch’s test. (c,d) Potassium currents were recorded in acutely-dissociated DRG neurons 7 days after SNL. Control AAV vector or AAV vector mixture was injected 7 days before SNL. Only EGFP-fluorescent DRG neurons were recorded. (c) Representative traces of total, non-A-type and A-type potassium currents elicited by stepwise depolarization from −70 to 60 mV at a holding potential of −80 mV in small DRG neurons. (d) Current density of each potassium current component plotted against voltage in small DRG neurons obtained from three rats. Error bars are s.e.m. *P<0.05 (P=0.045 for total current; P=0.046 for non-A-type current; P=0.015 for A-type current), two-way repeated-measures ANOVA.
cluster members targeted 3'-UTR sequences to repress gene expression, miR-17-92 may directly decrease multiple potassium channel expression in a cell-autonomous fashion. Additionally, euchromatic histone-lysine N-methyltransferase-2 (G9a) mediates downregulation of most potassium channel z subunits in the DRG after nerve injury. Although potassium channels modulated by G9a partially overlap with the miR-17-92 target channels, miR-17-92 and G9a modulate potassium channel expressions at distinct steps of gene expression; miR-17-92 blocks translational steps, leading to mRNA degradation, whereas G9a inhibits transcription through histone methylation. Thus, expression of potassium channels may be modulated in the injured DRG neurons through both transcriptional and post-transcriptional regulations.

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Kv subtypes (Kv1.4, Kv3.3, Kv3.4 and Kv4.1–4.3), three of which (Kv1.4, Kv3.4 and Kv4.3) are miR-17-92 targets, mediate fast-inactivating A-type currents. However, Kv4.2 is reportedly expressed at very low levels in the DRG, further indicating the importance of miR-17-92 for fast-inactivating voltage-gated potassium channels in DRG neurons. In addition, DPP10 and Nav1.1, positive modulators of Kv4 channels, were also downregulated by miR-17-92. Dipeptidyl peptidase-like proteins (DPP6 and DPP10) are necessary for Kv4 to fully constitute the A-type current. The transient outward potassium current is reduced in Nav1.1 null mutant mice with hyperexcitable DRG neurons, although the reported influences of Nav1.1 on Kv4 are contradictory. In line with these results, miR-17-92 significantly reduced A-type potassium currents in small, putative nociceptive DRG neurons, consistent with the reduced current in injured DRG neurons observed in this study and previous reports. In contrast, non-A-type potassium currents were not significantly affected by miR-17-92 in small DRG neurons, although antisense RNAs restored the non-A-type potassium currents in the neuropathic pain condition. Among the voltage-gated potassium channels targeted by miR-17-92, Kv1.1 and Kv7.5 are non-A-type delayed rectifiers, but we only detected Kv7.5 in small DRG neurons. Similarly, medium/large DRG neurons did not show significant reduction in total, A-type and non-A-type currents after nerve injury. Consistent with this, other investigators have reported slight reduction in potassium currents in injured medium/large DRG neurons. In fact, among the miR-17-92 targets only Kv3.4 and Kv1.1, which mediate A-type and non-A-type currents respectively, are expressed in medium/large DRG neurons. Overall, miR-17-92 appears particularly critical for the modulation of fast-inactivating A-type current in the nociceptive DRG neurons.

The potassium channels affected by miR-17-92 are reportedly implicated in mechanical sensation, but not heat hyperalgesia, consistent with the effects of miR-17-92 on mechanical allodynia that we observed. Chien et al. reported that Kv3.4 or Kv4.3 knockdown with antisense oligodeoxynucleotide induced mechanical allodynia, but not thermal hyperalgesia. Kv1.1 is reportedly a mechanosensitive channel, inhibition of which causes mechanical allodynia, but not thermal hyperalgesia. Mechanical allodynia, but not heat hyperalgesia, was reported to be mediated by small non-peptidergic C fibres. Consistent with this, miR-17-92 was colocalized with Kv3.4 and Kv4.3 in non-peptidergic DRG neurons. On the other hand, ectopic discharge in large myelinated Aβ afferents is considered a source of neuropathic pain, while NaV1.8-expressing neurons (putative small DRG neurons) were previously shown not to be essential for neuropathic pain. The involvement of miR-17-92 in cold allodynia, another frequent symptom in neuropathic pain, remains unknown. Cold temperatures strongly and preferentially inhibit A-type currents but have fewer inhibitory effects on tetrodotoxin-resistant Na+ channels and non-inactivating K+ currents in small DRG neurons, suggesting a contribution of A-type K+ currents to cold pain. Therefore, miR-17-92 may also have a modulatory role in nociceptive cold sensation.

Despite concerns about adverse effects on the cardiovascular and nervous systems, potassium channels are attractive therapeutic targets for neuropathic pain. Flupirtine (a Kv7 activator) suppressed mechanical allodynia in the SNL model, findings consistent with other investigators, and a report of the successful use of flupirtine in a patient with refractory neuropathic pain due to small fibre neuropathy. Importantly, in our study combined application of flupirtine and NS-5806 (a Kv4.3 activator) induced substantial relief of mechanical allodynia compared with the analgesic effect of each modulator alone. Given the downregulation of diverse potassium channel subunits in neuropathic pain, concurrent modulation of various relevant potassium channel subunits may provide superior therapeutic efficacy with fewer adverse effects. In this context, therapeutic manipulation of miR-17-92 cluster would be advantageous, as its components miR-18a, miR-19a/b and miR-92a collectively modulate multiple potassium channel z subunits and auxiliary subunits in DRG neurons. In this study, miR-17-92-mediated mechanical allodynia was alleviated by potassium channel modulators, suggesting miR-17-92 as a therapeutic target for potassium channel modulation.
In conclusion, we have shown the orchestrated effects of miR-17-92 cluster miRNAs on multiple voltage-gated potassium channels, especially those mediating A-type currents, in DRG neurons after nerve injury. Combined application of potassium channel-activating drugs in vivo exerted potent relief of mechanical allodynia. These findings underline the growing importance of comprehensive analysis of cluster miRNAs in the nervous system, and may provide the opportunity to develop a novel analgesic strategy based on concurrent regulation of multiple functionally related proteins.

Methods

Animal models. Conduct of all experimental procedures was approved by Animal Experimental Ethical Review Committee of Nippon Medical School (Approval number, 27-037) and performed in accordance with the guidelines of the International Association for the Study of Pain46. Male Sprague–Dawley rats (5–6 weeks of age; Sankyo Labo Service Corporation, Tokyo, Japan) were used for all experiments. The animals were singly housed in a temperature and humidity-controlled vivarium with a 14-h light/dark cycle and allowed food and water ad libitum. All surgery was performed on rats under general anaesthesia induced by intraperitoneal sodium pentobarbital (50 mg kg−1) and inhaled isoflurane (2–3%). Rats were randomized to AAV or drug injection. The L5 SNL model of neuropathic pain was performed on the left side25. Briefly, the L5 nerve was exposed and tightly ligated with 2-0 suture thread in two regions including both main branches by about 1 mm from the dorsal root entry zone. A small spared nerve injury model of neuropathic pain, the left tibial and common peroneal nerves were tightly ligated with 4-0 silk thread and cut distal to the ligation to remove 2–3 mm of the nerve46. The right side was left intact as a control. As an inflammatory pain model, CFA solution (100 μl; Sigma-Aldrich Japan, Tokyo, Japan) was placed into the left hind paw intradermally and Dr. DW Russell)51 and helper (pHelper; Agilent Technologies) free triple transfection with AAV vector, AAV packaging (pRepCap 6as; kindly provided by Dr. Andrew McGeary, University of California, San Francisco). The titre of the AAV vector was determined by qPCR. For use, each AAV vector was diluted with PBS to 1 × 1011 vp/ml and was kept at −80°C. The AAV vector was further concentrated with Amicon Ultra-4 30 K (Merck Millipore, Darmstadt, Germany). The titre of the AAV vector was determined by qPCR. RT-PCR of microdissection samples. A single cell was obtained by laser capture microdissection. The L5 DRG was excised and then rapidly frozen in OCT compound (Sakura Finetek, Tokyo, Japan) using dry ice/acetone. The DRG was sectioned in 10μm thickness using a cryostat (Leica Microsystems Wetzlar, Germany) and then by CsCl density gradient centrifugation. After purification by dialysis, DRG neurons were stained with toluidine blue (0.05%, pH 4.1) for 15 min at 4°C to visualize the DRG neurons. After washing with RNase-free water for 1 min on ice, the section was dried with a hair dryer. A single DRG neuron was obtained using a laser microdissection system (LMD7000; Leica Microsystems).

AAV vector production. Serotype 6 AAV vectors were produced by adenovirus-free triple transfection with AAV vector, AAV packaging (pRepCap 6as; kindly provided by Dr. DW Russell) and helper (pHelper; Agilent Technologies) plasmids at a ratio of 1:1:1. For miRNA-expressing AAV vector and its control, plasmids were co-transfected into HEK293 cells (ATCC, Manassas, VA) using calcium phosphate precipitation as previously described52. Six hours after transfection, the culture medium was refreshed and cells were cultured for 3 days at 37°C in a humidified atmosphere of air and 5% CO2. Cells were suspended in PBS (phosphate buffered saline) and freeze-thawed three times. Cell debris was pelleted by centrifugation at 6,000 rpm, for 30 min at 4°C and AAV vectors were purified by ammonium sulfate precipitation and iodixanol continuous gradient centrifugation. AAV vectors expressing TuD antisense RNAs or its control were produced with ultracentrifugation-free chromatography-mediated purification, as previously described24,53. Plasmids were co-transfected into HEK293 cells using polyethylenimine (PEI) solution (Polyplus transfection, Illkirch, France) at an E:PEI ratio of 1:3 at 37°C for 4 h. The RNA of the AAV vector was determined by qPCR. For use, each AAV vector was diluted with PBS to ~5 × 105 vp/ml.

Quantitative PCR. Reagents and kits were provided by Life Technologies (Carlsbad, CA) unless otherwise stated. All procedures were performed according to the manufacturers’ protocols. Total RNA was extracted from the L4 and L5 DRGs and L5 dorsal spinal cord using RNAiso plus (Takara Bio, Shiga, Japan) or mirVana PARIS kit according to the manufacturers’ protocols. For miRNA quantification, total RNA (10 ng) was reverse-transcribed with a stem-loop primer specific for each mature miRNA using a TaqMan MicroRNA Reverse Transcription Kit. PCR mixture was prepared using TaqMan Universal PCR Master Mix and premixed TaqMan probe and primer pairs specific for each miRNA included in the TaqMan MicroRNA Assays (Supplementary Table 2). For quantification of miRNAs and pri-miRNAs, total RNA (500 ng) was reverse-transcribed using Script select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA) with a random primer. PCR amplification was performed with TaqMan Gene Expression Master Mix of gene-specific TaqMan probe and reporter (TaqMan Gene Expression Assays, Supplementary Table 2). In the case of Taq amplification, the forward primer (5'-CGCAATGCAAGACTACGAA-3'), reverse primer (5'-CGCGCGAGACA-3') and probe (5'-CGTAAA-CAAACCTGTAACACGATCTATCT-3') were used. The amplification efficiency per single PCR cycle was obtained by assaying serially-diluted samples (four points at 1:5 dilution) and the relative expression was calculated.

Plasmdos. To express miRNAs as a cluster or individually, sequences encoding the miRNAs were amplified from rat genome using primer pairs with EcoRI restriction sites attached at the 5' ends (Supplementary Table 3). The amplified sequences were subcloned into the EcoRI site of the AAV vector plasmid pAAV-EFPG49, which contains the CAG promoter upstream of the transgene and the EF1G gene driven by the B19 promoter. pAAV-EFPG50, which contains the EF1G gene driven by the CAG promoter, was used as a control. TuD was RNA was used to inhibit miR-19 family function17. A TuD sequence was inserted at the 3'UTR of the primed potassium channels. PCR amplification of the diluted preamplified product was conducted as described in the Quantitative PCR section above. More than ten-fold signal intensity compared with negative control was considered as positively detected.

RT-PCR of microdissection samples. A single cell was obtained by laser capture microdissection. The L5 DRG was excised and then rapidly frozen in OCT compound (Sakura Finetek, Tokyo, Japan) using dry ice/acetone. The DRG was sectioned in 10μm thickness using a cryostat (Leica Microsystems Wetzlar, Germany) and then by CsCl density gradient centrifugation. After purification by dialysis, AAV vectors were further concentrated with Amicon Ultra-4 30 K (Merck Millipore, Darmstadt, Germany). The RNA of the AAV vector was determined by qPCR. For use, each AAV vector was diluted with PBS to ~5 × 105 vp/ml.
To inject an AAV vector, rats were laid prone under deep anaesthesia. Then, paraspinal muscles were separated from the vertebrae and a small part of vertebrae overlying the L5 DRG was removed to expose the ganglion. AAV vector (5 μl) was slowly (> 5 min) injected into the left L5 DRG using a microsyringe with a curved-tip 27-gauge needle. Rats exhibiting motor disturbance were excluded from analysis. This procedure specifically introduces transgene into L5 DRG neurons of all cell sizes, as previously described6. In vivo injection into the L5 DRG neurons was confirmed for all AAV vectors by immunofluorescence for EGFP. A lack of apparent motor dysfunction assessed by the open field and rotated tests (Supplementary Fig. 6b,c) and no change in thermal withdrawal latency (Supplementary Fig. 4) by miR-17-92 overexpression or inhibition indicated that the AAV injection itself and miR-17-92 in the DRG neurons did not obviously affect motor function.

Immunofluorescence. Rats were perfused transcardially with PBS (pH 7.4) followed by fresh 4% paraformaldehyde in PBS. DRGs were removed and post-fixed in the same fixative overnight, and then immersed in 20% sucrose in PBS. On the next day, DRGs were rapidly frozen in dry ice/acetone and sectioned (10 μm) using a cryostat (Leica Microsystems). The DRG sections were pre-incubated in PBS containing 5% normal donkey serum and 0.2% Triton X-100 for 30 min, and then incubated with a rabbit anti-green fluorescent protein antibody (1:1000; A11122, Life Technologies) at 4 °C overnight. After washing with PBS, sections were incubated with a secondary antibody labelled with Alexa Fluor 488 at room temperature for 1 h. Images were captured using a high-resolution digital camera equipped with a computer (Olympus, Tokyo, Japan). To measure the size of DRG neurons, two DRG sections (minimum separation, 60 μm) obtained from individual rats were analysed using ImageJ software (National Institutes of Health, Bethesda, MD).

Luciferase assay. Activities of firefly and Renilla luciferases were measured using the Dual-Glo Luciferase Assay System (Promega, Fitchburg, WI). HEK293 cells (System Biosciences, Palo Alto, CA) were cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin and streptomycin). The cells were seeded onto a white 96-well plate (2 × 10^4 cells per well). To assess TUd antisense RNA efficiency, HEK293 cells were transfected with pAAV-EGFP plasmids, prior to transfection for luciferase assays. The next day, cells were co-transfected with pMIR vector with 3'-UTR, pGL4.74[luc2r/TK] vector (Promega) and pAAV vector using Lipofectamine2000. Two days after transfection, Dual-Glo luciferase reagent was added to each well and firefly luminescence was measured using Wallac 1420 ARVOXSO (PerkinElmer, Waltham, MA). Dual-Glo Stop & Glo reagent was then added to each well and Renilla luminescence was measured using the same luminescence hardware. Firefly lucinescence was divided by Renilla luminescence and luminescence of control pMIR vector for normalization.

Microarray. Total RNA was isolated from L5 DRG using RNAiso Plus. Cy3-labelled cRNA was prepared from total RNA (200 ng) using the Low Input Quick Amp Labeling Kit according to the manufacturer’s protocol (Agilent Technologies). After purification, cRNA was hybridized overnight to a rat microarray slide containing 44,000 probes (Agilent). The fluorescent intensity of each spot was quantified with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin and streptomycin). The solution was replaced with F12 medium and the DRGs were incubated with anti-KV1.1 (1:300; K20/78, NeuroMab, Davis, CA), anti-KV1.4 (1:300; K31/31, NeuroMab), anti-KV1.3 (1:100; APOC-19, Alomone Labs, Jerusalem, Israel), anti-Kv-4.3 (1:100; K75/41, NeuroMab), anti-Kv-7.5 (1:200; APC-155, Alomone Labs) or anti-GAPDH (1:1,000; 14C10, Cell Signaling Technology, Danvers, MA) antibody at 4 °C overnight and then detected using HRP-conjugated secondary antibody (1:2,000; Cell Signaling Technology) and chemiluminescence (ECL Prime Western Blotting Detection Reagents; GE Healthcare). The luminescence was detected with a C-DiGit Blot Scanner (LI-COR Biotechnology, Lincoln, NE). GAPDH was used as a loading standard. Optical densities of bands were quantified using Scion Image Beta 4.03. Full images of the blots are shown in Supplementary Fig. 12.

Statistics. Values are expressed as mean ± standard error. SPSS (version 18, IBM, Armonk, NY) and KxPlot (KyenceLab, Tokyo, Japan) were used for statistical analyses. Sample sizes were not statistically estimated but were adopted to minimize the number of rats used. Normality of data was assessed by the Shapiro-Wilk test. Equality of variance was assessed by Levene’s test. The paired t-test, unpaired t-test and one-way factorial ANOVA followed by Dunnett’s test for multiple comparisons were used for normally distributed data sets with equal variance. Welch’s t-test was used for normally distributed data sets when equality of variance was rejected. When normality was rejected, the Mann–Whitney U test or the Steel test for multiple comparisons was used. Differences between groups were assessed using two-way repeated-measures ANOVA. All tests were two-tailed and P values <0.05 were considered statistically significant.

Data availability. Microarray data have been deposited in Gene Expression Omnibus (GSE98636). All other data are available on request from the authors.

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Author contributions
A.S. and H.S. designed and analysed the experiments and wrote the manuscript. A.S. and M.M. performed the animal experiments and microdissection. A.S. performed the qPCR, immunofluorescence, luciferase assay and immunoblotting. N.M., K.M., T.S. and T.O. produced the AAV vectors. F.S. performed and analysed the electrophysiological experiments.

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