

# Downregulation of miR-219 enhances brain-derived neurotrophic factor production in mouse dorsal root ganglia to mediate morphine analgesic tolerance by upregulating CaMKII $\gamma$

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## Abstract

**Background:** Increasing evidence suggests that microRNAs are functionally involved in the initiation and maintenance of pain hypersensitivity, including chronic morphine analgesic tolerance, through the posttranscriptional regulation of pain-related genes. We have previously demonstrated that miR-219 regulates inflammatory pain in the spinal cord by targeting calcium/calmodulin-dependent protein kinase II gamma (CaMKII $\gamma$ ). However, whether miR-219 regulates CaMKII $\gamma$  expression in the dorsal root ganglia to mediate morphine tolerance remains unclear.

**Results:** MiR-219 expression was downregulated and CaMKII $\gamma$  expression was upregulated in mouse dorsal root ganglia following chronic morphine treatment. The changes in miR-219 and CaMKII $\gamma$  expression closely correlated with the development of morphine tolerance, which was measured using the reduction of percentage of maximum potential efficiency to thermal stimuli. Morphine tolerance was markedly delayed by upregulating miR-219 expression using miR-219 mimics or downregulating CaMKII $\gamma$  expression using CaMKII $\gamma$  small interfering RNA. The protein and mRNA expression of brain-derived neurotrophic factor were also induced in dorsal root ganglia by prolonged morphine exposure in a time-dependent manner, which were transcriptionally regulated by miR-219 and CaMKII $\gamma$ . Scavenging brain-derived neurotrophic factor via tyrosine receptor kinase B-Fc partially attenuated morphine tolerance. Moreover, functional inhibition of miR-219 via miR-219-sponge in naive mice elicited thermal hyperalgesia and spinal neuronal sensitization, which were both suppressed by CaMKII $\gamma$  small interfering RNA or tyrosine receptor kinase B-Fc.

**Conclusions:** These results demonstrate that miR-219 contributes to the development of chronic tolerance to morphine analgesia in mouse dorsal root ganglia by targeting CaMKII $\gamma$  and enhancing CaMKII $\gamma$ -dependent brain-derived neurotrophic factor expression.

## Keywords

Morphine tolerance, hyperalgesia, miR-219, CaMKII $\gamma$ , brain-derived neurotrophic factor, dorsal root ganglia

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## Background

Morphine is one of the most commonly used drugs for the treatment of moderate to severe pain. However, the clinical administration of morphine for pain management is restricted by the development of analgesic tolerance following prolonged morphine usage, which manifests as a progressive loss of anti-nociceptive potency. In this situation, pain relief can be achieved by increasing the morphine dose, but this also augments the negative side effects of morphine.<sup>1</sup> The desensitization and trafficking of the  $\mu$ -opioid receptor (MOR), and altered expression and function of neurochemical signals in the dorsal root ganglia (DRG) neurons are known to cause tolerance to morphine analgesia.<sup>2,3</sup> However, the molecular and genetic mechanisms underlying this phenomenon in DRG have not been fully elucidated.

MicroRNAs (miRNAs) are non-coding RNAs, 18–25 nucleotides in length, that regulate gene expression at the posttranscriptional level. They degrade mRNA and inhibit translation by binding to the 3'-untranslated region (UTR) of targeted mRNAs, both of which inhibit expression of the target proteins.<sup>4,5</sup> Accumulating evidence suggests that neuroinflammation and nerve injury can alter the expression of miRNAs in the DRG.<sup>6</sup> Affected miRNAs may regulate processes such as inflammation- or neuropathy-induced pain hypersensitivity, as their target genes are involved in pain-associated peripheral and central sensitization.<sup>7,8</sup> Here, we investigated the role of miR-219 in the development of morphine tolerance in the DRG for the following reasons. (1) Morphine tolerance is a type of hyperalgesia that has both similar and distinct mechanisms to inflammatory or neuropathic pain, especially in the peripheral nervous system.<sup>9,10</sup> (2) We have previously demonstrated that miR-219 mediates inflammatory pain by negatively regulating calcium/calmodulin-dependent protein kinase II gamma (CaMKII $\gamma$ ) expression in the dorsal horn of the mouse spinal cord,<sup>11</sup> but whether this occurs in the DRG is unknown. (3) CaMKII is located in small- and medium-diameter DRG neurons and plays important roles in nociceptive signal transmission.<sup>12</sup>

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family and is mainly synthesized within DRG neurons. BDNF is anterogradely transported to the central terminals of the spinal dorsal horn, where the transduction of pain signaling by different pain stimuli is modulated. Numerous studies have shown that BDNF expression increases in the primary sensory neurons following peripheral inflammation and nerve injury and acts as a neuromodulator between DRG neurons during inflammatory and neuropathic pain.<sup>13,14</sup> In addition, many factors have been reported to promote BDNF production in certain neuropsychiatric disorders. Evidence suggests that CaMKII directly stimulates BDNF activity and contributes to synaptic plasticity

and learning and memory.<sup>15,16</sup> However, the importance of CaMKII $\gamma$  and DRG-derived BDNF in the development of morphine tolerance is poorly understood.

In the present study, using a recognized mouse model of morphine analgesic tolerance, we provide the first evidence that repetitive subcutaneous (s.c.) injection of morphine produced rapid and lasting analgesic tolerance, associated with decreased miR-219 expression and increased protein expression of its target CaMKII $\gamma$  in the DRG. The upregulation of miR-219 or downregulation of CaMKII $\gamma$  expression significantly delayed the development of morphine tolerance via miR-219 mimics or CaMKII $\gamma$  small interfering RNA (siRNA), respectively. Furthermore, altered miR-219 or CaMKII $\gamma$  expression in the DRG affected BDNF production in both naive and morphine-tolerant mice.

## Methods

### Animals

Male CD-1 mice weighing 20–25 g at 8–10 weeks of age were supplied from Experimental Animal Center of Xuzhou Medical College. Mice were housed under a constant 12-h light-dark cycle at 22–24°C with food and water ad libitum. All animal-handling procedures were approved by the Institutional Animal Care and Use Committee of Xuzhou Medical University, and all experimental protocols were consistent with the NIH Guide for the Care and Use of Laboratory Animals and the IASP Ethical Issues for pain research.<sup>17</sup> Best efforts were exerted to minimize the amount of animals use and their suffering.

### Models of morphine analgesic tolerance

According to previous reports in our lab,<sup>18,19</sup> for testing acute tolerance, a single morphine (10 mg/kg, s.c.) was injected 24 h after a single morphine (100 mg/kg, s.c.) injection (protocol identified as morphine acute administration in primed mouse) to elicit acute morphine tolerance after 2 h. In addition, for testing chronic tolerance, mice were treated with morphine (10 mg/kg, s.c.) twice a day at 12 h intervals (08:00 and 20:00 for seven days) to establish systemic chronic tolerance to morphine anti-nociception.

### Behavioral tests

**Hot-plate test.** For evaluating morphine tolerance generation, the thermal anti-nociceptive behavior was evaluated using a hot-plate test.<sup>18,19</sup> In brief, the hot-plate apparatus was maintained at 55°C, and mouse was placed on the plate to observe its behavioral response. Then the mouse was removed from the plate immediately

after performing hindpaw-lick or four-paw-jump behavior. A cutoff time of 30 s was set to avoid potential tissue injury. Mice were tested at 30 min after morphine injection each afternoon. The percentage of maximum potential efficiency (MPE%) was calculated as following formulation:  $MPE\% = (\text{drug response latency} - \text{basal latency}) / (30\text{ s} - \text{basal latency}) \times 100\%$ .

**Hargreaves test.** The algogenic effect of miR-219-sponge on thermal hyperalgesia was assessed by paw withdrawal latency (PWL) according to a classical protocol.<sup>20</sup> A Plantar Analgesia Meter (IITC Life Science Inc., Victory Blvd Woodland Hills, CA, USA) was applied to provide a radiant heat source, and each mouse was placed in a plastic chamber with a clear glass floor. The heat source was placed underneath the glass and focused on the plantar surface of the hindpaw. The time-course from onset of radiant heat to endpoint of characteristic hindpaw-lift or -lick was recorded as the PWL. The basal PWL was adjusted to 12–15 s at the beginning of the experiment with an automatic 25 s cutoff to prevent potential tissue injury. Each mouse was tested three times on each hindpaw at intervals of 5 min.

**miRNA mimics, siRNA, and miRNA-219 sponge.** miR-219 mimics, scrambled mimics (served as control), CaMKII $\gamma$  siRNA, scrambled siRNA (served as control), miR-219-sponge, and lentivirus-empty vector (served as control) were purchased from GenePharma (Suzhou, China). CaMKII $\gamma$  siRNA (sense, 5'-GUAGAGUGCU UACGCAAUUTT-3'; antisense, 5'-AUUUGCGUAA GCACUCUACTT-3') or scrambled siRNA (sense, 5'-UUCUCCGAACGUGUCACGUAUdTdT-3'; antisense, 5'-ACGUGACACGUUCGGAGAAAdTdT-3') was designed and validated in vitro and in vivo as described previously.<sup>11</sup> miRNA-219-sponge forward and reverse sequence were synthesized respectively (219F, 5'-P-CGCGAGAATTGCGAACGACA ATCAGCTAAGA ATGCGAACGACAATCAGCTAAGAATTGCGAAC GACAATAGCTAAGAATTGCGAACGACAATCA-3'; 219R, 5'-P-CGTGATTGTCGTTTCGCAATTCTTA GCTGATTGTCGTTTCGCAATTCTTAGCTGATTGT CGTTGCAATTCTTAGCTGATTGTCGT TCGCAAT TCT-3'). 219F and 219R were annealed and inserted into PLVTHM plasmid digested by MluI and ClaI and named as PLV-219.

**Lentivirus package and infection.** According to our previous report,<sup>21</sup> PLV-219 (16  $\mu$ g) and two envelope plasmids, PSPAX2 (12  $\mu$ g) and PMD2G (4.8  $\mu$ g), were cotransfected into HEK293T cells in a six-well plate according to manufacturer instructions of Lipofectamine 2000 (11668–027, Invitrogen). After 48 h, the supernatant was collected and concentrated by using a Centricon Plus-70 filter unit (UFC910096, Millipore). Lentivirus

with titers 108 TU/ml was used in the experiment. The assays of lentivirus in vitro and in vivo infection were performed according to the previous description. Briefly, 20  $\mu$ l lentivirus and 1.5  $\mu$ l polybrene (1.4  $\mu$ g/ $\mu$ l; H9268, Sigma–Aldrich) were added in a 24-well plate containing  $1 \times 10^5$  HEK293T cells and 400  $\mu$ l DMEM without FBS; after 24 h, the transfection medium was replaced with 500  $\mu$ l fresh complete medium containing 10% FBS; cells were collected at 48 h after culture. Each time, 1  $\mu$ l lentivirus was injected in vivo.

**Drug administration.** Each time, miR-219 mimics (20  $\mu$ M, 5  $\mu$ l), scrambled mimics (20  $\mu$ M, 5  $\mu$ l), CaMKII $\gamma$  siRNA (5  $\mu$ M, 5  $\mu$ l), scrambled siRNA (5  $\mu$ M, 5  $\mu$ l), Tyrosine receptor kinase B-Fc (TrkB-Fc) (2  $\mu$ g, 5  $\mu$ l, T8694, Sigma, St Louis, MI, USA), or phosphate buffer saline (PBS, 5  $\mu$ l) was delivered using a method of intrathecal (i.t.) injection. miR-219-sponge (1  $\mu$ l) or lentivirus-empty vector (1  $\mu$ l) was delivered using a method of intragan-gliotic (i.g.) injection into the right L4-L5 DRGs. The administration time and dosage were determined by preliminary experiments. For i.t. injection, intraspinal puncture was performed with a 30-gauge syringe needle between the L5–6 interspace to deliver the drug. The correct subarachnoid positioning of the needle tip was verified by a tail- or paw-flick response. Then the needle was left for a further 15 s. For i.g. injection, the paraspinal muscles and tissues were removed in order to identify the intervertebral foramen. The right L4 and L5 DRGs were exposed, and 1  $\mu$ l of miR-219-sponge (1  $\mu$ l) or lentivirus-empty vector were gently injected into each DRG. The wound was then closed. Motor function was evaluated by the observation of placing or stepping reflexes and righting reflexes at 2 min before behavioral test. Mice with motor dysfunction signs were excluded from experiments.

**miRNA quantification by qRT-PCR.** According to our previous report,<sup>11</sup> total RNA (including miRNA) was isolated from DRG or spinal cord using Trizol-based method (Invitrogen, Carlsbad, CA, USA). RNA concentrations were determined by NanoDrop 2000 (Wilmington, DE, USA). U6 snRNA (U6F, 5'-CTCGCTTCGGCAG CACATATACT-3'; U6R, 5'-ACGCTTCA CGAATTT GCGTGTC-3'; U6 Taqman probe, 5'-FAM-CTTGC GCAGGGGCCATGCTAA-MGB-3') was used as an internal control of miRNA-219. Specific reverse transcription primer of miRNA-219 (000522, Applied Biosystems) and U6R primer were respectively used for reverse transcription with TaqMan<sup>®</sup> MicroRNA Reverse Transcription Kit (4366596, Applied Biosystems) in a total volume of 10  $\mu$ l. Each reaction contained 1  $\mu$ g RNA as template. Real-time PCR was performed respectively using specific PCR primers (000522, Applied Biosystems) for miRNA-219, primer

pair U6F, and U6R for U6 RNA in TaqMan Universal PCR Master Mix (4324018, Applied Biosystems) according to the manufacture instruction with LightCycler<sup>®</sup> 480 System (Roche Applied Science, Mannheim, Germany). Each reaction was run in triplicate. The miRNA-219 abundance in each sample was normalized to U6 RNA reference. The expression of miRNA-219 was calculated with the  $\Delta\Delta C_t$  method.<sup>22,23</sup>

**Western blot.** The protein samples of bilateral L4-L6 DRG and L4-6 spinal dorsal horn were collected 2 h after morphine/drugs injection. Protein samples (40  $\mu$ g total protein per lane) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were probed with rabbit anti-CaMKII gamma antibody (1:400, Abcam, Cambridge, MA, USA), rabbit anti-BDNF antibody (1:800, Abcam), rabbit anti-phosphorylated extracellular signal-regulated kinase (p-ERK) antibody (1:800, Cell Signaling Technology, Boston, MA, USA), rabbit anti-phosphorylated cAMP response element binding protein (p-CREB) antibody (1:1000, Cell Signaling Technology), rabbit anti-c-Fos antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:50000, Sigma), respectively. Band intensity was quantified using Quantity One Analysis Software (Version 4.6.5, Bio-Rad Laboratories, Hercules, CA, USA). The protein band density of CaMKII $\gamma$  and BDNF was measured and normalized against the density of GAPDH. The fold change of the control group was set as 1 for quantifications.<sup>24</sup>

**Statistical analysis.** All data are expressed as mean  $\pm$  standard error of the mean (SEM), and all statistical analysis were conducted by GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Alterations of miRNA and protein expression were detected using one-way analysis of variance (ANOVA) with repeated measures followed by the Dunnett multiple comparison test; changes of behavioral response to radiant heat stimulation over time among groups were detected using two-way ANOVA with repeated measures followed by Bonferroni post hoc test. Pearson correlation was used for the linear correlation analysis. Statistical differences were considered significant if  $p < 0.05$ .

## Results

### *Downregulation of miR-219 correlated negatively with CaMKII $\gamma$ expression in DRG after chronic morphine treatment*

In agreement with our previous results from a complete Freund's adjuvant (CFA)-induced inflammatory pain

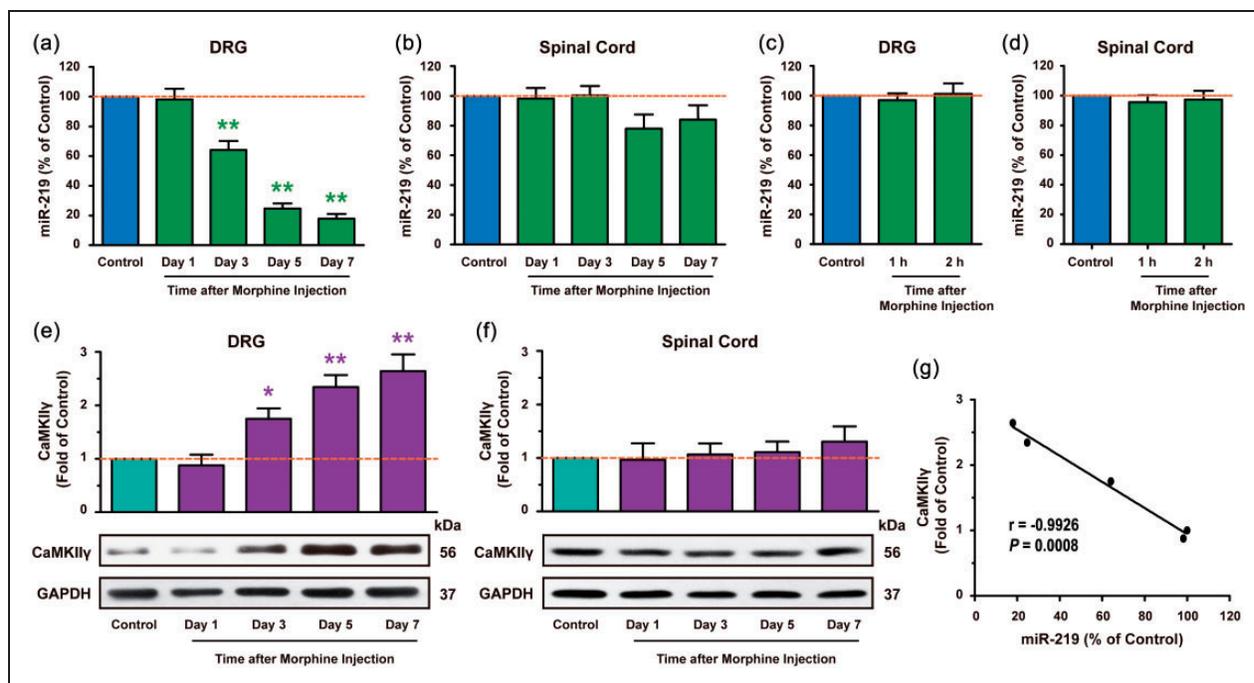
mouse model,<sup>11</sup> quantitative reverse-transcription polymerase chain reaction (qRT-PCR) showed that miR-219 expression was downregulated in a time-dependent manner in the bilateral DRG following repeated morphine injections (10 mg/kg, s.c.). Expression began to decrease on day 3 and remained at a low level until the last test day (Figure 1(a)). However, the expression of miR-219 in the dorsal horn of the spinal cord was not affected (Figure 1(b)). Additionally, a single 100 mg/kg (s.c.) morphine injection followed by a single 10 mg/kg (s.c.) morphine injection 24 h later (which has been shown to elicit acute morphine tolerance after 2 h<sup>18</sup>) did not alter miR-219 expression in the DRG or the dorsal spinal cord (Figure 1(c) and (d)).

CaMKII $\gamma$  was identified as a potential target of miR-219 by three independent prediction programs (PicTar, miRanda and TargetScan) and has been experimentally validated in our previous study.<sup>11</sup> Therefore, we investigated whether CaMKII $\gamma$  expression changed in the DRG after the downregulation of miR-219 expression in response to repeated morphine exposure. Similar to the rapid decrease in miR-219 expression after morphine treatment, CaMKII $\gamma$  expression significantly increased from days 3 to 7 (Figure 1(e)). The expression of CaMKII $\gamma$  protein remained low in the spinal cord during all tested time points (Figure 1(f)). We also found a linear correlation between miR-219 and CaMKII $\gamma$ . As shown in Figure 1(g), the protein expression of CaMKII $\gamma$  correlated negatively with miR-219 expression in the DRG following repetitive morphine injections.

### *Altered miR-219 and CaMKII $\gamma$ expression is associated with chronic morphine tolerance*

Repeated morphine administration produced robust analgesia to thermal stimuli from day 1. Compared with control groups (naive or saline-injected mice), morphine had an anti-nociceptive effect (evaluated by MPE%) until day 7 (see experimental protocol in Figure 2(a)). However, the elevated MPE% of morphine gradually declined from days 3 to 7, indicating the development of tolerance to morphine anti-nociception. The MPE% was not significantly altered in control groups during the observed period (Figure 2(b)). Changes in behavior occurred parallel to changes in the expression of miR-219 and CaMKII $\gamma$  following morphine treatment. Linear correlation analysis showed that the expression of miR-219 correlated positively with the MPE% of morphine (Figure 2(c)). In contrast, the expression of CaMKII $\gamma$  correlated negatively with the MPE% (Figure 2(d)).

We further investigated the anti-nociceptive effect of miR-219 upregulation or CaMKII $\gamma$  downregulation on morphine-tolerant behaviors. The technique of



**Figure 1** Expression changes of miR-219 and CaMKII $\gamma$  in the DRG and spinal cord following morphine treatment in mice. qRT-PCR data show time course of miR-219 expression in the DRG and spinal cord of chronic morphine-injected mice (a, b) or acute morphine-injected mice (c, d). Western blot data show time course of CaMKII $\gamma$  protein expression in the DRG (e) and spinal cord (f) of chronic morphine-injected mice. Samples were collected at days 1, 3, 5, and 7 following chronic morphine injection; or at 1 h or 2 h after acute morphine injection. \* $p < 0.05$ , \*\* $p < 0.01$  versus naive group,  $n = 5$  in each group. (g) The correlation analysis between the CaMKII $\gamma$  protein expression and the miR-219 expression in the time-line following morphine treatment. The CaMKII $\gamma$  shows a negative correlation with miR-219 in the DRG of chronic morphine-injected mice ( $r = -0.9926$ ,  $p = 0.0008$ ).

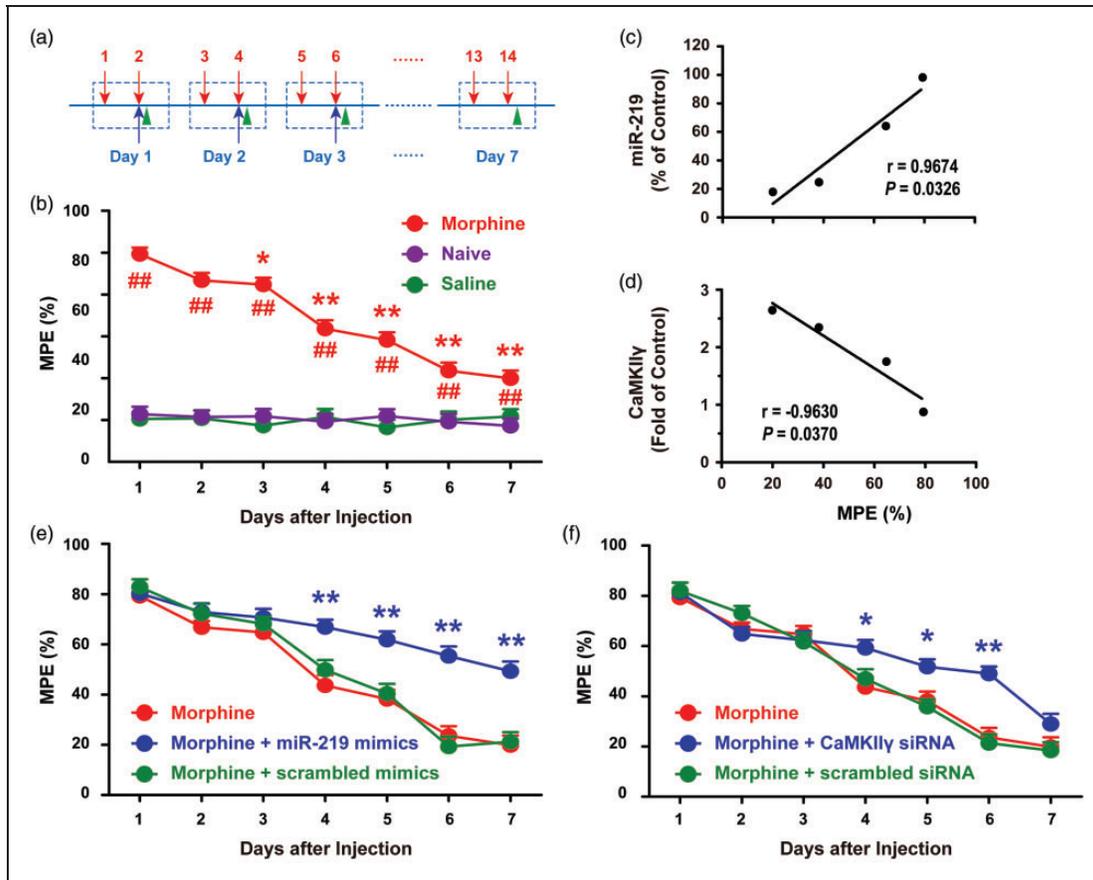
intrathecal (i.t.) administration allows agents to act on DRG directly,<sup>25,26</sup> and the administration time and dosage were determined by preliminary experiments. To detect this, miR-219-mimics for miR-219 upregulation (20  $\mu$ M, 5  $\mu$ l) or CaMKII $\gamma$  siRNA for CaMKII $\gamma$  knockdown (5  $\mu$ M, 5  $\mu$ l) was i.t. delivered once daily along with the second, fourth, and sixth morphine injection (see experimental protocol in Figure 2(a)). The behavioral results showed that repetitive i.t. injection of miR-219-mimics significantly delayed the initiation of MPE% reduction from days 4 to 7 after morphine treatment, however, scrambled mimics (for control) did not alter the declined MPE% of morphine-injected mice (Figure 3(e)). Furthermore, compared to mice exposed to morphine and control siRNA, i.t. injection of CaMKII $\gamma$  siRNA also significantly blocked the morphine tolerance from days 4 to 6 after sustained morphine injection (Figure 3(f)).

### *BDNF is highly expressed in DRG following sustained morphine exposure and is involved in the development of morphine tolerance*

Accumulating evidence suggests that BDNF can transmit and modulate nociceptive signals and plays an important

role in the development of inflammatory, neuropathic, bone cancer pain, and morphine-induced hyperalgesia.<sup>27–30</sup> Here, we explored the time-course of BDNF expression during the development of morphine analgesia tolerance in the DRG. The Western blot and qRT-PCR analysis showed that the protein (Figure 3(a)) and mRNA expression (Figure 3(b)) of BDNF were upregulated from day 5 and day 3, respectively, following morphine administration.

Previous reports have shown that administration of the BDNF scavenger TrkB-Fc (Tyrosine receptor kinase B-Fc) attenuates thermal hyperalgesia and mechanical allodynia in rodent models of neuropathic pain.<sup>31,32</sup> Here, we report the effects of TrkB-Fc on the chronic tolerance to morphine anti-nociception. Repetitive i.t. administration of TrkB-Fc (2  $\mu$ g in 5  $\mu$ l PBS, once daily along with the second, fourth, and sixth morphine injection) did not significantly delay the initiation of analgesic tolerance at the early phase (days 1, 2, and 3 of morphine treatment, see experimental protocol in Figure 3(c)) of morphine tolerance (Figure 3(d)). However, established morphine tolerance, characterized by decreased MPE%, were dramatically reversed by repetitive i.t. administration of the same dose of TrkB-Fc (once daily along with the 10th, 12th, 14th morphine injection) at the late phase (days 5, 6, and 7 of morphine treatment, see



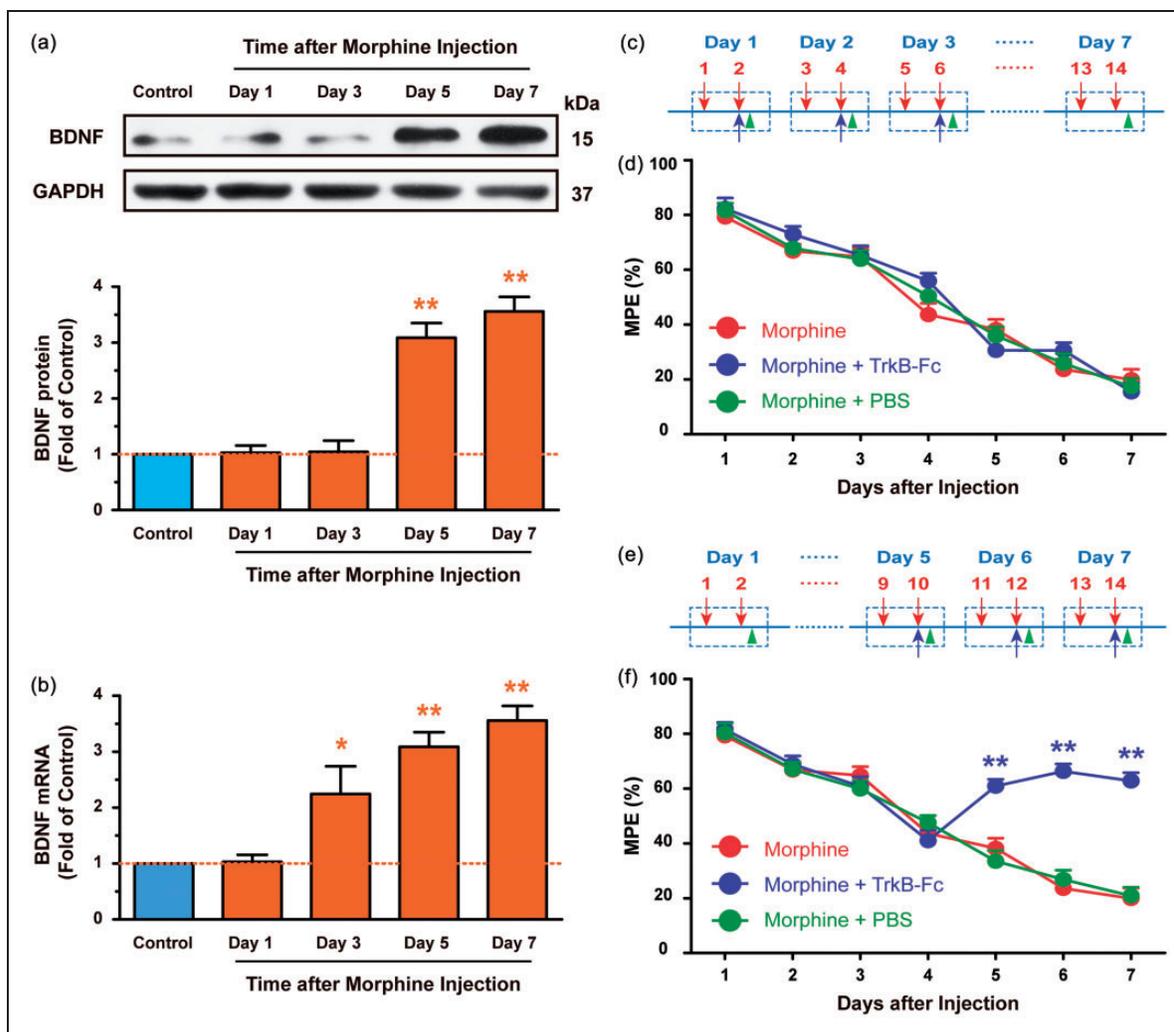
**Figure 2.** Effects of miR-219 and CaMKII $\gamma$  in the DRG on chronic tolerance to morphine anti-nociception. (a) Schematic illustration of morphine injection, drugs application, and behavioral test for (b), (e), and (f). Red arrows indicate morphine injection; blue arrows indicate drugs application; and green arrowheads indicate behavioral test. (b) Repetitive morphine injection (10 mg/kg, s.c., twice a day) elicits a progressive reduction in MPE% during seven days. \* $p$  < 0.05, \*\* $p$  < 0.01 versus morphine group at day 1; # $p$  < 0.05, ## $p$  < 0.01 versus naive group,  $n$  = 8 in each group. (c) The miR-219 expression in the DRG is positively correlated to the MPE% of chronic morphine-injected mice in the time-line of morphine treatment ( $r$  = 0.9674,  $p$  = 0.0326). (d) The CaMKII $\gamma$  expression in the DRG is negatively correlated to the MPE% of chronic morphine-injected mice in the time-line of morphine treatment ( $r$  = -0.9630,  $p$  = 0.0370). Morphine-induced induction of analgesic tolerance is delayed by i.t. injection of both miR-219 mimics (e) or CaMKII $\gamma$  siRNA (f) once a day along with the second, fourth, and sixth morphine injection. \* $p$  < 0.05, \*\* $p$  < 0.01 versus morphine + vehicle,  $n$  = 8 in each group.

experimental protocol in Figure 3(e)) of morphine tolerance (Figure 3(f)). The basal pain threshold was not altered by TrkB-Fc or PBS treatment in naive mice (data not shown). Together, these results indicated that DRG-derived BDNF is involved in the development of morphine tolerance, particularly after tolerance has been established.

#### Upregulation of miR-219 attenuates BDNF over-expression by downregulating CaMKII $\gamma$ in the DRG of morphine-tolerant mice

It has previously been reported that CaMKII stimulation in the central nervous system (CNS) regulates BDNF expression and neuronal plasticity.<sup>15,16</sup> We further investigated the correlation between BDNF and the CaMKII isoform CaMKII $\gamma$  in the development of

morphine tolerance. As shown above, the protein and mRNA expression of BDNF were increased in the DRG of morphine-tolerant mice after five days of morphine exposure. Here, we observed that the increased BDNF expression was reduced by the i.t. administration of CaMKII $\gamma$  siRNA (5  $\mu$ M, once daily along with the second, fourth, and sixth morphine injections), but not by scrambled miRNA (Figure 4(a) and (b)). In addition, the increased protein expression of CaMKII $\gamma$  and BDNF were both inhibited by i.t. injection of miR-219 mimics (20  $\mu$ M, once daily along with the second, fourth, and sixth morphine injection), but not scrambled mimics (Figure 5(c) and (d)). Taken together, these results indicated that CaMKII $\gamma$  could regulate BDNF production at the transcription level. Furthermore, both direct knockdown of CaMKII $\gamma$  via CaMKII $\gamma$  siRNA or indirect inhibition of CaMKII $\gamma$



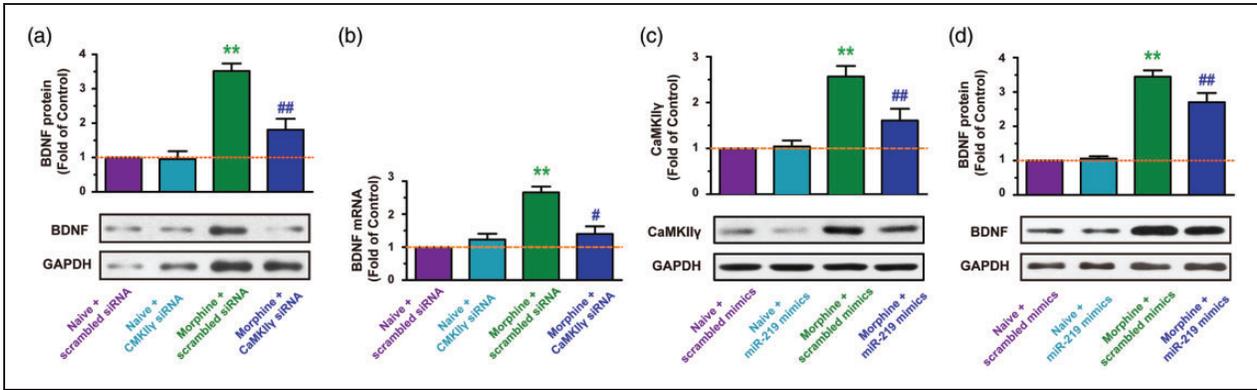
**Figure 3.** Expression and function of BDNF in morphine analgesic tolerance following chronic morphine injection. (a, b) Western blot and RT-PCR data show time course of BDNF expression (protein and mRNA) in the DRG of naive mice and morphine-injected mice. Samples were collected at days 1, 3, 5, and 7 following chronic morphine injection. \* $p < 0.05$ , \*\* $p < 0.01$  versus naive group,  $n = 5$  in each group. (c, e) Schematic illustration of morphine injection, drugs application and behavioral test for (d) and (e), respectively. Red arrows indicate morphine injection; blue arrows indicate drugs application; and green arrowheads indicate behavioral test. (d) Intrathecal administration of TrkB-Fc (once a day along with the second, fourth, and sixth morphine injection) does not delay the onset of morphine tolerance. (f) Intrathecal administration of TrkB-Fc (once a day along with the 10th, 12th, 14th morphine injection) significantly reverse the ongoing morphine tolerance. \*\* $p < 0.01$  versus morphine + PBS,  $n = 8$  in each group.

via miR-219 mimics was sufficient to suppress morphine-induced over-expression of BDNF in the DRG of morphine-tolerant mice.

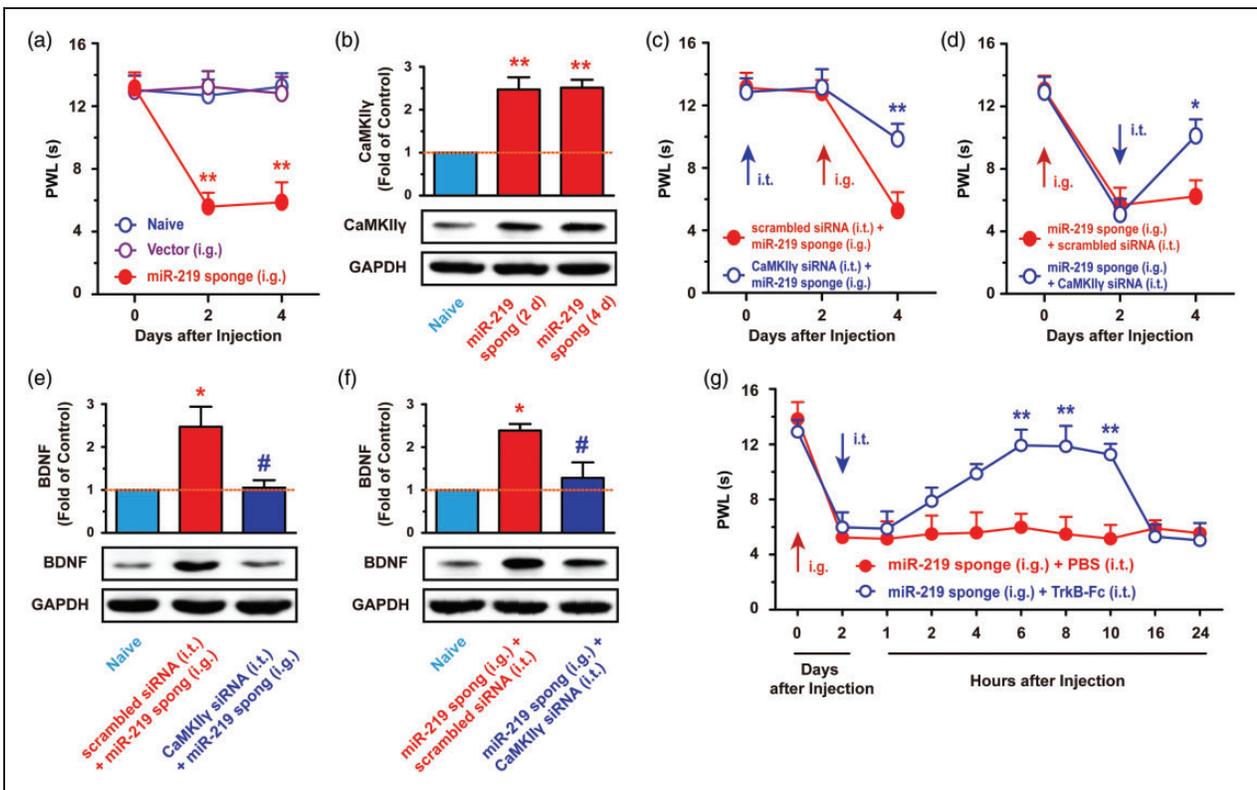
#### Downregulation of miR-219 elicits pain-like behavior and spinal neuronal sensitization in naive mice by inducing CaMKII $\gamma$ and BDNF expression

We have shown that miR-219 is expressed in the DRG and regulates pain-related behaviors. We then wanted to investigate whether miR-219 is sufficient to trigger hyperalgesic behaviors in the peripheral nociceptive system by intraganglionic (i.g.) injection of miR-219-sponge

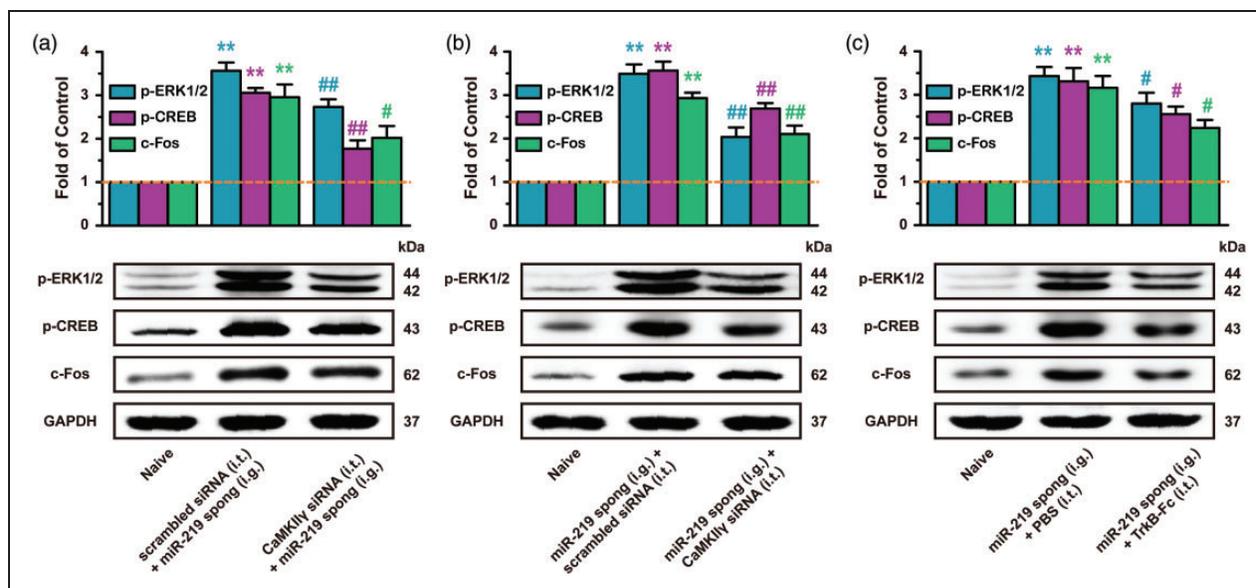
(a loss-of-function strategy of miRNA) into the naive mouse right L4 and L5 DRGs. The behavioral tests showed that miR-219-sponge decreased the paw thermal withdrawal latency (PWL) within two days after i.g. injection and continued to reduce the PWL for four days (Figure 5(a)). Western blot analysis demonstrated an increased expression of CaMKII $\gamma$  at days 2 and 4 after i.g. miR-219-sponge injection (Figure 5(b)). Moreover, the reduced PWL caused by miR-219-sponge was attenuated by CaMKII siRNA (i.e., 5  $\mu$ M, 5  $\mu$ l) two days either before or after miR-219-sponge injection (Figure 5(c) and (d)). We went on to investigate the importance of BDNF in miR-219-sponge-induced



**Figure 4.** Intrathecal administration of miR-219 mimics or CaMKII $\gamma$  siRNA inhibits morphine-induced over-expression of CaMKII $\gamma$  and/or BDNF in the DRG of morphine-tolerant mice. (a, b) Western blot and RT-PCR data show that i.t. administration of CaMKII $\gamma$  siRNA (once a day along with the second, fourth, and sixth morphine injection) suppresses the increased protein and mRNA expression of BDNF at day 5 following morphine injection. Additionally, i.t. administration of miR-219 mimics (once a day along with the second, fourth, and sixth morphine injection) suppressed the increased protein expression of CaMKII $\gamma$  (c) and BDNF (d) at day 5 following morphine injection. The bilateral L4–6 DRG tissues were collected at day 5 after the last morphine injection.  $**p < 0.01$  versus naive + vehicle.  $\#p < 0.05$ ,  $\#\#p < 0.01$  versus morphine + vehicle,  $n = 5$  in each group.



**Figure 5.** Intraganglionic injection of miR-219-sponge induces thermal hyperalgesia through CaMKII $\gamma$ -dependent activation of BDNF in the DRG of naive rats. (a) Intraganglionic (i.g.) administration of miR-219-sponge (1  $\mu$ l) markedly decrease paw withdrawal latency (PWL) at day 2 and 4 after injection.  $**p < 0.01$  versus naive group,  $n = 6$  in each group. (b) miR-219-sponge also increased CaMKII $\gamma$  expression in the DRG of naive mice. Samples were collected two and four days after miR-219-sponge injection.  $**p < 0.01$  versus naive group,  $n = 5$  in each group; however, i.t. pre- or posttreatment of CaMKII $\gamma$  siRNA two days before or after miR-219-sponge administration prevents or alleviates miR-219-induced decline of PWL (c, d) and increase of BDNF expression (e, f).  $*p < 0.05$  versus naive group.  $\#p < 0.05$  versus scrambled siRNA + miR-219-sponge or miR-219-sponge + scrambled siRNA,  $n = 5$  or 6 in each group. (g) i.t. delivery of TrkB-Fc, two days after miR-219-sponge injection, alleviates miR-219-induced PWL reduction in a time-dependent manner.  $**p < 0.01$  versus miR-219-sponge + PBS,  $n = 6$  in each group.



**Figure 6.** Involvement of CaMKII $\gamma$  and BDNF in miR-219-induced activation of ERK/CREB signaling and c-Fos in the spinal cord of naive mice. (a, b) Western blot data show that miR-219-sponge-induced upregulation of p-ERK, p-CREB, and c-Fos are inhibited by i.t. pre- or post-injection of CaMKII $\gamma$  siRNA, two days before or after miR-219-sponge administration. Samples were collected at day 4 after behavioral test.  $**p < 0.01$  versus naive group.  $\#p < 0.05$ ,  $###p < 0.01$  versus scrambled siRNA + miR-219-sponge or miR-219-sponge + scrambled siRNA,  $n = 5$  in each group. (c) miR-219-sponge-induced upregulation of p-ERK, p-CREB, and c-Fos are inhibited by i.t. administration of TrkB-Fc, two days after miR-219-sponge injection. Samples were collected 6 hours after TrkB-Fc administration.  $**p < 0.01$  versus naive group.  $\#p < 0.05$ ,  $###p < 0.01$  versus miR-219-sponge + PBS,  $n = 5$  in each group.

pain behavior. BDNF protein expression was significantly increased in the DRG at days 2 and 4 after i.g. miR-219-sponge injection, and this was markedly inhibited by CaMKII siRNA (Figure 4(e) and (f)). The miR-219-sponge-induced hyperalgesia was also reversed by TrkB-Fc (i.t., 2  $\mu$ g, 5  $\mu$ l) in a time-dependent manner (Figure 5(g)).

Enhanced activation of peripheral sensory afferents during nociceptive processing contributes to the initiation and maintenance of central sensitization in the spinal cord and supraspinal regions. ERK/CREB signaling and c-Fos protein are biomarkers for neuronal activation in the spinal cord level and are rapidly activated after exposure to noxious stimuli.<sup>33,34</sup> We examined the expression of phosphorylated ERK (p-ERK), phosphorylated CREB (p-CREB), and c-Fos in the spinal dorsal horn after injection of miR-219-sponge. As shown by Western blot, miR-219-sponge increased the expression of p-ERK, p-CREB, and c-Fos in the spinal cord after two or four days. However, i.t. injection of CaMKII $\gamma$  siRNA (5  $\mu$ g, 5  $\mu$ l) two days before (Figure 6(a)) or after (Figure 6(b)) miR-219-sponge injection significantly prevented or blocked miR-219-induced phosphorylation of ERK and CREB and expression of c-Fos. Additionally, these induced expression of neuronal activation markers were also inhibited by i.t. TrkB-Fc (2  $\mu$ g, 5  $\mu$ l) injection (Figure 6(c)).

## Discussion

The present study is a continuation of our previous research, which showed that miR-219 is downregulated after CFA injection and participates in CFA-induced inflammatory pain by targeting CaMKII $\gamma$  in the spinal dorsal horn.<sup>11</sup> Here, we demonstrate that miR-219 promotes anti-nociceptive tolerance in the mouse DRG following continuous morphine treatment and enhances BDNF production by upregulating CaMKII $\gamma$ . These findings may help to uncover a specific posttranscriptional mechanism for the development of chronic tolerance to morphine analgesia.

### miR-219 in primary afferent neurons evokes pain sensitivity by targeting CaMKII $\gamma$ in naive mice

MiRNAs function as posttranscriptional repressors to negatively regulate target gene expression by mRNA degradation, translation inhibition, or other silencing mechanisms. As crucial micro-modulators, miRNAs influence a wide variety of neurophysiological processes in the central and peripheral nervous systems, including neural development, axonal regeneration, and synaptic plasticity.<sup>35,36</sup> But meanwhile, the dysregulation of miRNAs are associated with an extensive range of neuropathological progression, such as stroke,

Alzheimer's disease, and Parkinson's disease.<sup>37,38</sup> Recently, increasing evidence suggests that miRNA dysregulation in pain-signaling pathway regions (including primary afferent neurons, spinal dorsal neurons, brain stem, and brain) participates in the development and maintenance of pain processes by modulating pain-relevant protein expression. These miRNAs represent potential therapeutic targets for pain prevention and relief.<sup>39</sup> Among them, we have previously identified neuronal miR-219 as a regulator of chronic pain development in the spinal cord and have shown that CaMKII $\gamma$  is a target of miR-219 and is involved in this spinal miR-219-mediated pain hypersensitivity.<sup>11</sup>

Here, we specifically focused on the expression and function of miR-219 and CaMKII $\gamma$  in the DRG, as nociceptive signaling is principally modulated in the DRG and elucidating the function of miR-219 in the DRG would further our understanding of the role of miR-219 in pain modulation. Similar to our previous report that downregulation of spinal miR-219 induced spinal neuronal sensitization and pain behaviors, we observed that the functional inhibition of miR-219 in DRG neurons promoted thermal hyperalgesia and enhanced the activation of ERK/CREB and c-Fos signaling in the spinal cord of naive mice. Inhibition of miR-219 also increased CaMKII $\gamma$  protein expression in the DRG. CaMKII $\gamma$  knockdown significantly attenuated miR-219-induced thermal hyperalgesia and neuronal sensitization. Taken together, our findings (plus our previous study) suggest that miR-219 mediates the modulation of nociceptive signaling in both primary and secondary sensory neurons through a CaMKII $\gamma$ -relevant mechanism.

### *MiR-219/CaMKII $\gamma$ in the DRG facilitates the development of morphine analgesic tolerance*

Chronic morphine application induces tolerance via repetitive activation of  $\mu$ -opioid receptor (MOR) but does not change MOR expression at the transcriptional level.<sup>40</sup> Recent findings have shown that morphine modifies the expression of certain genes in the central nervous system (CNS), which serve as negative feedback mediators of MOR expression at the post-transcriptional level.<sup>41</sup> The regulation of MOR expression by miRNAs plays an important role in the development of morphine tolerance. For example, let-7 and miR-103/miR-107 contribute to morphine tolerance by inhibiting MOR expression.<sup>42,43</sup> Chronic exposure to morphine also changes the expression, distribution, and function of neurotransmitters, receptors, and signaling molecules, which are associated with morphine analgesic tolerance.<sup>44,45</sup> Thus, we hypothesized that miRNA-mediated expression of pain-related genes may represent a principal mechanism underlying these aberrant alterations. However, to the best of our knowledge, the functional significance of opioid

receptor-independent miRNAs in the development of chronic morphine tolerance has not been studied.

In the present research, we focused on the potential link between miR-219 expression and chronic morphine treatment in the DRG, as morphine tolerance may be initiated by primary sensory neurons. MiR-219 expression was downregulated and CaMKII $\gamma$  was upregulated in the DRG after chronic morphine exposure. Tolerance to morphine anti-nociception was significantly delayed by enhancing miR-219 expression or inhibiting CaMKII $\gamma$  expression. We have previously shown that miR-219 and CaMKII $\gamma$  regulate chronic pain processes in spinal dorsal horn neurons after CFA injection or chronic sciatic nerve constriction.<sup>11</sup> These parallel findings on the expression and function changes of miR-219 in the context of different types of pain could be viewed as one of the shared mechanisms between morphine tolerance and inflammatory/neuropathic pain, as a long-held view.<sup>46,47</sup> Interestingly, similar to our previous findings that the spinal expression of miR-219 was downregulated in chronic CFA pain models rather than acute formalin pain model, we did not observe any expression change of miR-219 in the DRG following acute morphine tolerance establishment. These findings partially reveal that there might exist inconsistent mechanisms between chronic morphine tolerance and acute morphine tolerance.

### *BDNF in the DRG is responsible for miR-219/CaMKII $\gamma$ -mediated morphine analgesic tolerance and pain hypersensitivity*

BDNF is widely expressed in regions of the pain-signaling pathway (including the DRG and spinal dorsal horn) and is involved in the development of pathological pain (including inflammatory pain and neuropathic pain). For example, in the DRG, BDNF is synthesized and produced increasingly in small- and medium-sized DRG neurons of rodent models of inflammatory pain.<sup>48</sup> Enhanced BDNF expression may function as an autocrine or paracrine signal between DRG neurons by activating pre-synaptic TrkB receptors and participate in pain modulation by activating the phosphorylation of extracellular signal-regulated kinases (ERK1/2), or stimulating the secretion of substance P and calcitonin gene-related peptide (CGRP).<sup>27</sup> In the spinal cord, BDNF is a modulator of synaptic transmission and neuronal sensitization,<sup>14,28</sup> and blocking BDNF reduces hyperalgesia caused by nerve injury.<sup>31,49,50</sup> Here, we have shown that BDNF expression in the DRG was elevated following chronic morphine exposure and the onset of morphine tolerance. In addition, spinal application of the BDNF scavenger TrkB-Fc significantly reversed chronic morphine tolerance.

We further demonstrated an upstream and downstream relationship between CaMKII $\gamma$  and BDNF,

which is consistent with previous findings that CaMKII mediates BDNF-dependent neural plasticity in the CNS for long-term memory formation.<sup>51</sup> Furthermore, BDNF is transported from primary afferent neurons to the spinal dorsal horn, where it binds to TrkB receptors on second-order sensory neurons to activate pain-signaling cascades.<sup>13</sup> Thus, we herein investigated the effect of miR-219 on spinal neuronal sensitization by examining the activation of ERK, CREB, and c-Fos. As expected, inhibition of miR-219 altered the expression of p-ERK, p-CREB, and c-Fos in the spinal dorsal horn, which was inhibited by CaMKII $\gamma$  siRNA or TrkB-Fc. Above all, our results, at least partially, suggest that DRG-derived BDNF is a functional downstream of CaMKII $\gamma$  in miR-219-mediated morphine tolerance and pain hypersensitivity.

## Conclusions

Repetitive morphine injection reduced miR-219 expression in the DRG, which may contribute to the chronic tolerance to morphine analgesia by upregulating CaMKII $\gamma$  protein expression. BDNF is a critical regulator of pain signaling and could be a promising downstream target for miR-219/CaMKII $\gamma$ -mediated pain sensitization in naive mice and morphine-tolerant mice. Our findings have uncovered a novel pain-related biomarker and a potential therapeutic strategy for morphine anti-nociceptive tolerance.

## Authors contributions

WS and ZQP conceived and designed this study. XMH analyzed the data and drafted the manuscript. SBC carried out the qRT-PCR and Western blot experiments. HLZ performed the animal surgery and behavioral tests. DML coordinated and supervised the experiments. LPC and HX participated in part of the Western blot experiments and behavioral tests. All authors read and approved the final manuscript.

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