Astroglial MicroRNA-219-5p in the Ventral Tegmental Area Regulates Nociception in Rats

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ABSTRACT

Background: The authors previously reported that noncoding microRNA miR-219-5p is down-regulated in the spinal cord in a nociceptive state. The ventral tegmental area also plays critical roles in modulating nociception, although the underlying mechanism remains unknown. The authors hypothesized that miR-219-5p in the ventral tegmental area also may modulate nociception.

Methods: The authors studied the bidirectional regulatory role of ventral tegmental area miR-219-5p in a rat complete Freund's adjuvant model of inflammatory nociception by measuring paw withdrawal latencies. Using molecular biology technologies, the authors measured the effects of astroglial coiled-coil and C2 domain containing 1A/nuclear factor κB cascade and dopamine neuron activity on the down-regulation of ventral tegmental area miR-219-5p–induced nociceptive responses.

Results: MiR-219-5p expression in the ventral tegmental area was reduced in rats with thermal hyperalgesia. Viral overexpression of ventral tegmental area miR-219-5p attenuated complete Freund's adjuvant–induced nociception from 7 days after complete Freund's adjuvant injection (paw withdrawal latencies: 6.09 ± 0.83 s *vs.* 3.96 ± 0.76 s; n = 6/group). Down-regulation of ventral tegmental area miR-219-5p in naïve rats was sufficient to induce thermal hyperalgesia from 7 days after lentivirus injection (paw withdrawal latencies: 7.09 ± 1.54 s *vs.* 11.75 ± 2.15 s; n = 8/group), which was accompanied by increased glial fibrillary acidic protein (fold change: 2.81 ± 0.38; n = 3/group) and reversed by intraventral tegmental area injection of the astroglial inhibitor fluorocitrate. The nociceptive responses induced by astroglial miR-219-5p down-regulation were inhibited by interfering with astroglial coiled-coil and C2 domain containing 1A/nuclear factor-κB signaling. Finally, pharmacologic inhibition of ventral tegmental area dopamine neurons alleviated this hyperalgesia.

Conclusions: Down-regulation of astroglial miR-219-5p in ventral tegmental area induced nociceptive responses are mediated by astroglial coiled-coil and C2 domain containing 1A/nuclear factor-κB signaling and elevated dopamine neuron activity. **(Anesthesiology 2017; 127:548-64)**

CHRONIC pain is an increasing public health problem, affecting approximately 30% of the general population worldwide and adversely reducing their quality of life.[1] However, the current therapeutic options, including opioids and nonsteroidal antiinflammatory drugs, are far from satisfactory. Therefore, unraveling the neurobiologic and molecular mechanisms underlying the persistent pain state will offer novel targets for developing effective therapeutic strategies.

MicroRNAs (miRNAs) are a class of endogenous small, noncoding RNAs that negatively regulate gene expression by recognizing the 3′-untranslated region (UTR) in a sequence-specific manner to induce the degradation or translational inhibition of target mRNAs. Many miRNAs are highly expressed in the adult nervous system in a spatially and temporally controlled manner under normal physiologic conditions, as well as under certain pathologic conditions.[2] These features make miRNAs ideal candidates for regulating complex processes, including neural development, neuronal plasticity, brain functions, and neurologic and neuropsychologic disorders.[3,4] Recent studies also have demonstrated that miRNAs are involved in neuronal plasticity in the peripheral and central nervous systems in multiple chronic pain states and play a critical role in the pathophysiology of chronic pain by regulating pain-related gene expression.[5] Because

What We Already Know about This Topic

- The down-regulation of microRNA miR-219-5p in the spinal cord supports nociceptive sensitization
- Brain centers such as the ventral tegmental area also are thought to control nociception, although the mechanisms have not been defined

What This Article Tells Us That Is New

- MiR-219-5p expression in the ventral tegmental area is reduced in the setting of hind paw inflammation and nociceptive sensitization
- Nociceptive sensitization related to reduced miR-219-5p expression was related to astrocytic activation

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endogenous expression patterns can be altered by the application of exogenous mimics or inhibitors, the manipulation of specific miRNAs has been shown to effectively alleviate inflammatory, neuropathic, and cancer pain in animal models.[6] Based on the advantages of miRNA-based therapeutics, including regulation of many components of the same pathway/cell, stability and durability, and effective *in vivo* regulation,[7] their potential for use in treating chronic pain appears promising.

MiR-219-5p is a central nervous system (CNS)-specific miRNA that is conserved between rodents and humans. Several studies have demonstrated that miR-219-5p is involved in neurologic and psychiatric disorders, including Alzheimer disease,[8] multiple sclerosis,[9] epilepsy,[10] schizophrenia,[11] and depression,[12] suggesting a potential functional role of miR-219-5p in dysfunctional CNS diseases. We recently reported that miR-219-5p mediates persistent inflammatory pain by targeting calcium/calmodulin-dependent protein kinase IIγ at the spinal level.[13] However, it still remains unclear whether and how it regulates nociception at the supraspinal level.

The ventral tegmental area (VTA), a well-studied midbrain region involved in reward and aversion, has been implicated in nociceptive modulation.[14,15] Dopamine neurons in this region exhibit altered activity in response to multiple acute noxious stimuli including findings in human functional magnetic resonance imaging studies.[16–18] Neural adaptation of the mesolimbic reward circuitry also occurs under chronic inflammatory and neuropathic pain states.[19–21] Furthermore, several studies have demonstrated that miRNAs are essential for dopamine neuron development, maintenance, survival, and functioning in adult midbrain areas.[22,23] Therefore, we hypothesize that miR-219-5p likely acts as a nociception-related gene regulator and is a key player in regulating nociceptive responses in the VTA.

In this study, by using lentivirus-mediated overexpression or down-regulation of miR-219-5p in the VTA, we found that astroglial miR-219-5p and its downstream target, coiled-coil and C2 domain containing 1A (CC2D1A), which is a putative signal transducer that positively regulates the nuclear factor-κB (NF-κB) cascade, regulate nociception by modulating dopamine neuron activity. Our findings provide novel insight into the underlying molecular and cellular mechanisms of nociceptive processing in the mesolimbic system.

Materials and Methods

Animals

Adult male Wistar rats (250 to 350 g) were purchased from the Experimental Animal Center of Xuzhou Medical University (Xuzhou, Jiangsu Province, China). Rats were housed with controlled relative humidity (20 to 30%) and temperature (23 ± 2°C) under a 12-h light-dark cycle (lights on, 8:00 AM to 8:00 PM) with free access to food and water. All experimental protocols were approved by the Animal Care and Use Committee of Xuzhou Medical University and in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication 85–23) to ensure minimal animal use and discomfort. All rats were randomized to different experimental groups. Blinding was attempted whenever possible for all experiments. The investigators who performed and analyzed the tests were unbiased and blinded to the experimental conditions of the animals.

Inflammatory Pain Model

Rats were anesthetized with sevoflurane (2%), and 100 μl complete Freund's adjuvant (CFA; Sigma-Aldrich, USA) was injected subcutaneously into the plantar surface of the left hind paw; control rats were injected with saline.

Measurement of Thermal Hyperalgesia

Paw withdrawal latencies (PWLs) in response to thermal stimuli were measured with an IITC plantar analgesia meter (IITC Life Science, Inc., USA) according to the method described by Hargreaves *et al.*[24] To summarize in brief, rats were placed in plastic cages on a glass platform and allowed to acclimatize to their environment for 30 min before testing. A radiant heat source was positioned directly beneath the glass and focused on the plantar surface of the left hind paw. The nociceptive endpoint in the radiant heat test was the lifting or licking of the hind paw. The time from onset of the radiant heat to the reaction was considered as the PWL. The radiant heat intensity was adjusted at the beginning of the experiment to obtain a basal PWL of 12 to 15 s. Rats with a baseline PWL of less than 6 s were excluded from the study to avoid false-positive results (approximately 10% of the animals were excluded during the screening). The cutoff time for radiant heat exposure was set at 25 s to prevent tissue damage. Each animal was tested five times at an interval of 5 min.

Two minutes before testing thermal hyperalgesia, motor functions of the rats were evaluated by observing the righting reflex. In this test, the rat was placed on its back, and attempts to reassume the prone position within 15 s were noted. This simple test often is used to assess an animal's motor function when it is not the primary outcome measure. Rats with signs of motor dysfunction were excluded from the experiments (none of the animals exhibited motor dysfunction before nociceptive testing).

Conditioned Place Aversion (CPA)

CPA test was carried out as described with a minor modification,[25] which combined von Frey stimulation and place conditioning. To summarize, the CPA apparatus (Clever Systems, Inc., USA) consists of two large Plexiglas chambers with different-colored walls (black or black and white stripes) connected by an internal door. Movements of the rats and the time spent in each chamber are monitored and automatically recorded with TopScan software (Clever Systems, Inc.). Pretests were performed 3 days after down-regulation of miR-219-5p by miR-219-5p-sponge injection or overexpression

miR-219-5p by miR-219-5p-O/E injection (miR-219-5p-O/E was injected 3 days after CFA injection). On the pretest day, rats were given free access to the entire conditioning apparatus for 15 min, and the amount of time spent in each compartment was recorded. Animals spending greater than 720 s or less than 180 s in any chamber were excluded from further testing (approximately 10% of animals). For conditioning sessions, each group of rats received four consecutive days of pairings in which they were confined to a chamber paired with one painful stimulus (15-g fiber) in the morning and then alternately confined to the opposite chamber paired with a nonpainful stimulus (1-g fiber) 4 h later in the afternoon. The left hind paw was stimulated every minute for 15 min during each session. Twenty-four hours after the last conditioning session (test day), all rats were allowed free access to each compartment, and CPA was determined. CPA is represented as the difference between the time (seconds) the rat spent in the pain-paired chamber on the test day and the time it spent in the same chamber during the pretest session.

Novelty-suppressed Feeding (NSF)

Six days after miR-219-5p-sponge or miR-219-5p-O/E injection (miR-219-5p-O/E was injected 3 days after injection), all groups of rats were deprived of food for 24 h with free access to water and were moved to the dimly lit testing room 1 h before testing. Rats were placed in the corner of an open field apparatus (50 × 50 × 20 cm) with an opaque white acrylic floor. A food pellet was placed in the center of the open field. The latency for the rat to approach the pellet and begin eating was recorded with a limit of 15 min. As soon as the rat was observed to eat or the 15-min time limit was reached, the rat was removed from the open field and placed in the home cage.

Locomotion Activities (LAs)

Three days after miR-219-5p-sponge or miR-219-5p-O/E injection (miR-219-5p-O/E was injected 3 days after CFA injection), rats were placed in an open field apparatus (50 × 50 × 20 cm) with an overhead camera. With the use of TopScan software (Clever Systems, Inc.), animal movements were tracked during a 20-min test. The total distance traveled was calculated.

Sucrose Preference (SP)

Rats were singly housed and initially habituated to two 50-ml tubes with stoppers fitted with ball-point sipper tubes filled with drinking water 2 days before the SP measurements. Three days after miR-219-5p-sponge or miR-219-5p-O/E injection (miR-219-5p-O/E was injected 3 days after CFA injection), rats were given access to a two-bottle choice of water or 1% sucrose solution. Rats were allowed to drink freely for 12 h, and the total volume consumed from each tube was recorded. The preference for sucrose was calculated as ([sucrose ml consumed/total ml consumed] × 100).

Stereotaxic Surgery and Microinjections

The lentivirus and small interfering RNA (siRNA) reagents were purchased from GenePharma (China). Fluorocitrate, minocycline, baclofen, and DK-AH269 were purchased from Sigma-Aldrich.

Rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally), and their scalps were shaved and sterilized with povidone-iodine. Each rat was fixed in a stereotaxic apparatus (David Kopf Instruments, USA), and a 1-cm longitudinal skin incision was made with a number 10 scalpel to expose the skull surface. The periosteum was cleared with 3% hydrogen peroxide and washed with normal saline. For microinjections, 33-gauge needles were placed into the VTA (anterior/posterior, −5.04 mm; lateral/medial, +0.7 mm; dorsal/ventral, −8.3 mm) or the substantia nigra pars compacta (SNc; anterior/posterior, −5.04 mm; lateral/medial, +2.1 mm; dorsal/ventral, −7.8 mm) and a volume of 0.5 to 1.0 μl was injected at a rate of 0.1 μl/min. Needles were slowly removed 5 min after injection to prevent backflow. After microinjection, the skin was sutured with 5-0 silk threads and covered with an antibiotic ointment. The rats receiving siRNAs or a glial inhibitor received a second surgery for injection. Three animals exhibited postoperative neurologic deficits after intracranial injection and were excluded from the experiments.

Histology

At the end of the experiments, rats were anesthetized with chloral hydrate (350 mg/kg, intraperitoneally) and intracardially perfused with 200 ml saline and 300 ml 4% ice-cold paraformaldehyde solution. Then, the brains were dissected rapidly and postfixed in 4% paraformaldehyde solution for 6 to 8 h and subsequently allowed to equilibrate in 30% sucrose overnight at 4°C. Coronal midbrain slices (30 μm) were prepared.

For cresyl violet staining, frozen sections were mounted on glass slides, air dried overnight, placed directly into 1:1 alcohol/chloroform overnight, and rehydrated through 100 and 95% alcohol to distilled water. Then, sections were stained in 0.1% cresyl violet solution (Millipore, USA) for 5 to 10 min, rinsed quickly in distilled water, differentiated in 95% ethyl alcohol for 2 to 30 min, dehydrated in 100% alcohol 2 × 5 min, cleared in xylene 2 × 5 min, and mounted with permanent mounting medium. The sites of intracerebral infusion were examined under light microscopy (Olympus, Japan). Only successful penetrations located in the VTA were used for data analyses (the data from five rats were excluded after such validation because of off-target injections).

Immunofluorescence Staining

For immunofluorescence staining, brain sections were blocked with 3% donkey serum for 1 h at room temperature and incubated overnight at 4°C with the following primary antibodies: rabbit anti-green fluorescent protein (anti-GFP,

1:600, #183734; Abcam, USA), rabbit anti-p-NF-κBp65 (1:200, #3033; Cell Signaling, USA), mouse antityrosine hydroxylase (anti-TH, 1:600, #318; Millipore), rabbit anti-c-Fos (1:200, #209794; Abcam), mouse antineuronal nuclei (1:500, #104224; Abcam), mouse antiglial fibrillary acidic protein (anti-GFAP, 1:400, #3670; Cell Signaling), and goat anti-ionized calcium binding adaptor molecule 1 (1:200, #5076; Abcam). The sections were then incubated for 1 h at room temperature with secondary antibodies: Alexa Fluor 488 antirabbit, AlexaFluor 594 antimouse, and AlexaFluor 546 antigoat (Molecular Probes, USA). Sections were rinsed 3 × 10 min with phosphate-buffered saline after each step. The sections were examined with the use of laser scanning confocal microscopy (FluoView FV1000; Olympus).

Western Blotting

VTA tissue was harvested from coronal brain slices with 12-gauge punches. To obtain enough protein, tissues from two rats per time point were pooled. Tissues were then sonicated in 30 μl homogenization buffer containing 320 mM sucrose, 5 nM HEPES, 1% sodium dodecyl sulfate (v/v), phosphatase inhibitor cocktails I and II (Sigma-Aldrich), and protease inhibitors (Roche Diagnostics, USA). Protein concentrations were determined with a DC protein assay (Bio-Rad, USA). Proteins (20 to 40 μg) were electrophoresed in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and transferred onto polyvinylidene difluoride membranes. The membranes were incubated at 4°C overnight with the primary rabbit anti-GFAP (1:400, #12389; Cell Signaling), rabbit anti-CC2D1A (1:1,000, #16816-1-AP; Proteintech, China), rabbit anti-p-NF-κBp65 (1:200, #3033; Cell Signaling), and mouse anti-beta-Actin (1:1,000, #021020-02; EarthOx Life Sciences, USA). The membranes were washed and then incubated for 2 h at room temperature with the secondary antibodies conjugated to alkaline phosphatase (1:500; Santa Cruz Biotechnology, USA). The immune complexes were detected with a nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate assay kit (Beyotime, China). Blots were analyzed with Adobe Photoshop software (Adobe Systems, Inc., USA), and the grayscale values of protein bands were normalized to those of β-actin.

Quantitative Real-time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from VTA tissues with Trizol reagent (Invitrogen, USA). The cDNA was synthesized from 400 to 800 ng total RNA with M-MLV reverse transcriptase (Invitrogen) and oligo(dT) primers or specific stem-loop primers. Glyceraldehyde 3-phosphate dehydrogenase and U6 RNAs were used as internal controls. qRT-PCR was carried out with a Light Cycler 480II (Roche Diagnostics) with SYBR Premix (Takara, China). Each cDNA was run in triplicate 20-μl reaction mixtures. The data were evaluated with the comparative C_T method ($2^{-\Delta\Delta CT}$). The detailed primer sequences (Sangon Biotech, China) are as follows: miR-219-5p, 5′-TCAGTGATTGTCCAAACGCAA-3′ (forward) and 5′-GTGCAGGGTCCGAGGTAT-3′ (reverse); U6, 5′-TTATGGGTCCTAGCCTGAC-3′ (forward) and 5′-CACTATTGCGGGTCTGC-3′ (reverse); NF-κB, 5′-GCAGAAAGAGGACATTGAGGTG-3′ (forward) and 5′-ACGTTTCTCCTCAATCCGGTGA-3′ (reverse); CC2D1A, 5′-GTGGATGTCGCTGAATTGC-3′ (forward) and 5′-CAAGCGATCCTCCCATCTT-3′ (reverse); glyceraldehyde 3-phosphate dehydrogenase, 5′-TCGGTGTGAACGGATTTGGC-3′ (forward) and 5′-CCTTCAGGTGAGCCCCAGC-3′ (reverse).

Enzyme-linked Immunosorbent Assay (ELISA)

Rat interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) ELISA kits were purchased from Neobioscience Technology (China). Protein samples were prepared as described for Western blotting. For each reaction in a 96-well plate, 100 μg of protein was used, and ELISAs were performed according to the manufacturer's protocols. The standard curves were included in each experiment.

Construction of Plasmids

The oligonucleotides for the 3′-UTR segment of CC2D1A were subcloned into a dual-reporter psi-CHECK2 plasmid (Promega, USA) digested with *Xho*I and *Not*I (NEB, China). The predicted binding sites that interact with miR-219-5p (Sangon Biotech) are 5′-TCGACAGACAATCAGTGGACAATCA-3′ (forward) and 5′-GGCCTGATTGTCCACTGATTGTCTG-3′ (reverse) for the wild type (pCHK-wt-CC2D1A). The mutated sequences are 5′-TCGACAGACAATCAGTaGtCcAgCA-3′ (forward) and 5′-GGCCTGcTgGaCcACTGATTGTCTG-3′ (reverse) for the mutant (pCHK-mut-CC2D1A).

Cell Culture and Luciferase Assay

All transfections were performed in serum-free Dulbecco's Modified Eagle's Medium with GenEscortI (Wisegen, China) according to the manufacturer's protocol. HEK293T cells and C6 glioma cells were plated in 24-well plates and cotransfected with 500 ng miR-219-5p mimics (GenePharma) and 50 ng pCHK-wt-CC2D1A, pCHK-mut-CC2D1A, or an empty vector. Luciferase assays were performed 48 to 72 h after transfection with the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample.

Statistical Analysis

Data are presented as mean ± SD. Statistical analyses were performed with GraphPad Prism 5.0 software (Graph Pad Software, Inc., USA). The sample sizes were estimated based on previous experience, and a formal statistical power analysis was not used to guide sample size estimation in this study. Differences among three or more groups were compared with a one-way ANOVA followed by Tukey's *post hoc* test. An unpaired Student's *t* test was used if only two groups were compared. A Mann–Whitney *U* test was used when the variances of the

two populations were not equal. The significance of any difference in thermal PWLs in the behavioral tests was assessed with a two-way ANOVA with repeated measures and Bonferroni's *post hoc* test. All *P* values given are based on two-tailed tests. A $P < 0.05$ was considered to indicate statistical significance.

Results

CFA-induced Nociception Down-regulates Expression of MiR-219-5p in the VTA

Intraplantar injection of CFA produced persistent thermal hyperalgesia, which lasted at least 7 days and returned to the baseline level 14 days after injections (fig. 1A). Consistent with the time course of behavioral changes, qRT-PCR showed that CFA also decreased the expression of miR-219-5p in the VTA contralateral to the CFA injection at day 3 and day 7, with expression levels recovering at day 14 after CFA injections (fig. 1B). To assess whether manipulating miR-219-5p expression in the contralateral VTA alters CFA-induced thermal hyperalgesia in rats, we used a lentivirus vector to overexpress miR-219-5p (miR-219-5p-O/E) in the VTA 3 days after CFA injection, which was confirmed by qRT-PCR (fig. 1C). The behavioral results showed that overexpression of miR-219-5p in the contralateral VTA

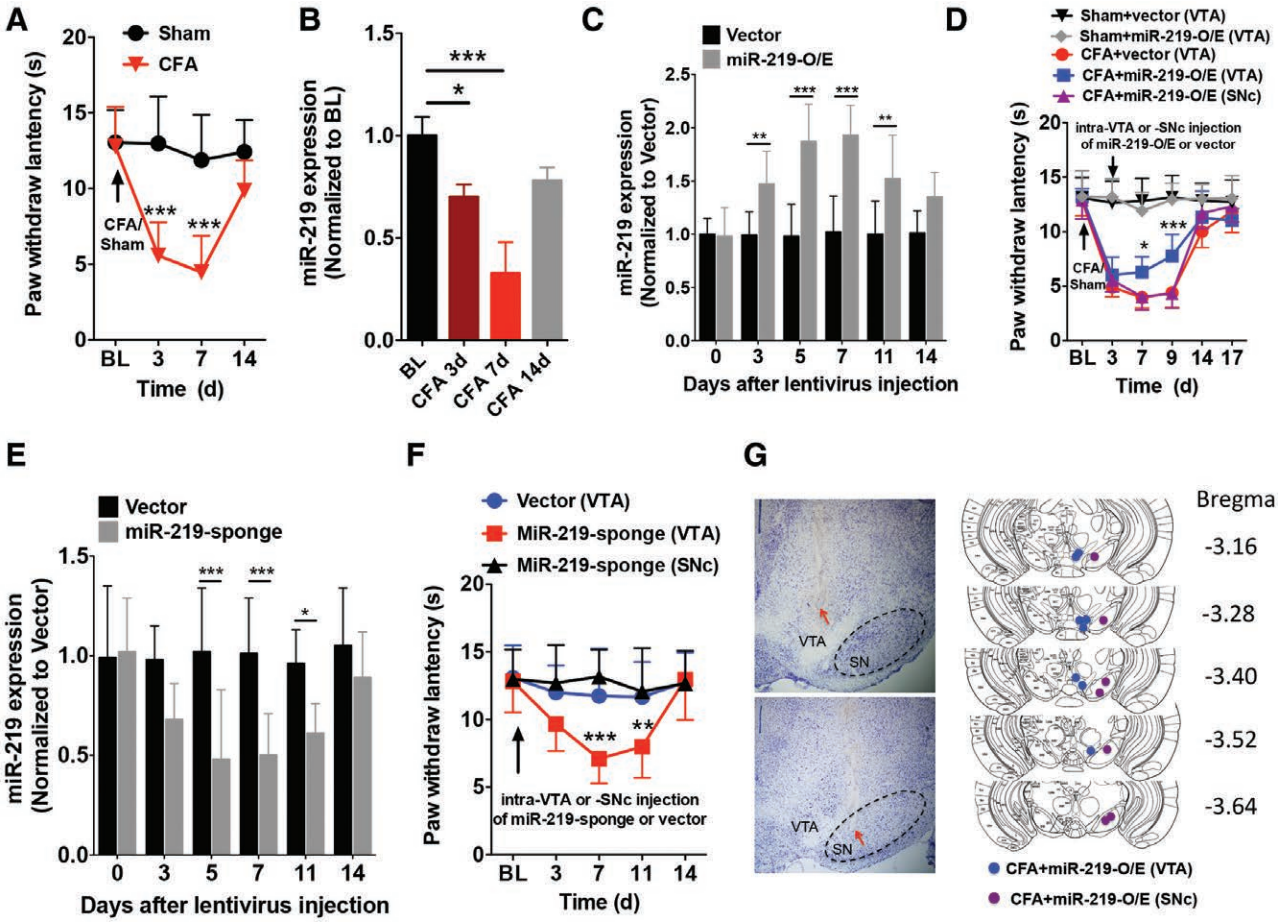

Fig. 1. Ventral tegmental area (VTA) miR-219-5p (miR-219) regulates thermal nociception. (*A*) Intraplantar injections of complete Freund's adjuvant (CFA) induced persistent thermal hyperalgesia (two-way repeated-measures ANOVA, treatment effect: $F_{1, 40} = 38.17$; *post hoc* test, ***$P < 0.001$ *vs.* sham group; n = 6 rats/group). (*B*) Time course of VTA miR-219-5p expression after intraplanter injections of CFA. Samples were collected from the contralateral VTA (one-way ANOVA, $F_{3, 20} = 37.16$; *post hoc* test, *$P < 0.05$, ***$P < 0.001$ *vs.* baseline level; n = 6). (*C*) Time course of VTA miR-219-5p expression after intra-VTA miR-219-5p-O/E injection (two-way repeated-measures ANOVA, treatment effect: $F_{1, 96} = 86.95$; *post hoc* test, **$P < 0.01$, ***$P < 0.001$ *vs.* vector group; n = 9). (*D*) Microinjection of miR-219-5p-O/E in the VTA significantly attenuated CFA-induced thermal hyperalgesia (two-way repeated-measures ANOVA, treatment effect: $F_{4, 145} = 102.50$; *post hoc* test, *$P < 0.05$, ***$P < 0.001$ *vs.* CFA + vector group; n = 8 rats in CFA + vector and VTA CFA + miR-219-5p-O/E groups and n = 6 rats in other groups). (*E*) Time course of VTA miR-219-5p expression after intra-VTA injection of miR-219-5p-sponge (two-way repeated-measures ANOVA, treatment effect: $F_{1, 96} = 37.69$; *post hoc* test, *$P < 0.05$, ***$P < 0.001$ *vs.* vector group; n = 9). (*F*) Microinjection of miR-219-5p-sponge in the VTA induced thermal hyperalgesia in naïve rats (two-way repeated-measures ANOVA, treatment effect: $F_{2, 105} = 12.49$; *post hoc* test, **$P < 0.01$, ***$P < 0.001$ *vs.* vector group; n = 8 rats/group). (*G*) Schematic representation of microinjection sites for the experiments shown in (*D*) and histologic plates illustrating the intra-VTA and -substantia nigra pars compacta (SN) injection sites adopted from the rat brain atlas. *Error bars* show SD. BL = baseline.

significantly reversed the established thermal hyperalgesia induced by CFA, as indicated by the increased PWLs in CFA rats at day 7 and day 9 after CFA injection (fig. 1D). This antinociceptive effect was not observed in rats receiving an injection of the vector in the contralateral VTA or an injection of miR-219-5p-O/E into the SNc, another dopamine neuron–enriched brain region in close proximity to the VTA.

To test whether down-regulation of miR-219-5p is sufficient to induce thermal hyperalgesia in naïve rats, we injected miR-219-5p-sponge, a "miRNA-loss-of-function" strategy, into the VTA to knockdown endogenous miR-219-5p expression, as evidenced by the decrease of miR-219-5p expression in the VTA from 5 to 11 days after miR-219-5p-sponge injection (fig. 1E). The results showed that the PWLs were decreased dramatically at day 7 after intra-VTA miR-219-5p-sponge injection and recovered at day 14 in comparison with those from vector or SNc injection control rats (fig. 1F). Figure 1G depicts the microinjection sites in the VTA and SNc with cresyl violet staining. Collectively, these data indicate that VTA miR-219-5p is both necessary and sufficient for thermal hyperalgesia induced by CFA.

Because previous studies identified an important role for the VTA in the processing of the affective dimension of nociception,[15,16] we tested whether miR-219-5p modulation had effects on nociception-related negative emotions and normal physical functions. All groups were put through the CPA test as a measurement of nociception-related negative emotional component, the NSF test as a measurement of rat's spontaneous aversion to eating in a novel environment, as well as the SP to assess anhedonia and the LA test for general physical activity. As shown in figure 2, CFA-treated rats (fig. 2A) and rats with miR-219-5p down-regulation in the VTA (fig. 2E) spent significantly less time in the nociception-paired conditioning chamber than the sham and vector control animals, respectively. However, overexpression of miR-219-5p in the contralateral VTA was not sufficient to block this aversion. None of the manipulations affected the responses of rats toward SP (fig. 2, B and F), NSF (fig. 2, C and G), or LA (fig. 2, D and H) tests. Taken together, these results suggest that miR-219-5p manipulation in the VTA mainly affects the sensory discriminative component of nociceptive information and has minimal impact on the affective-motivational aspects of nociception, with no effect on physical function. Therefore, subsequent experiments were focused mainly on the role and mechanism of VTA miR-219-5p in nociceptive sensory regulation.

Down-regulation of VTA MiR-219-5p Induces Thermal Hyperalgesia via *Astroglial Activation in Naïve Rats*

To explore the cellular mechanism of thermal hyperalgesia induced by miR-219-5p down-regulation, miR-219-5p-sponge or a viral vector that fused with GFP was injected into the VTA of naïve rats. We first validated the GFP expression using immunofluorescent staining 7 days after virus injection and found that GFP was expressed predominantly in GFAP-labeled astroglia but not in neuronal nuclei–labeled neurons or ionized calcium binding adaptor molecule 1–labeled microglia (fig. 3A).

Activation of astroglia is characterized by cellular hypertrophy, hyperplasia, and increased GFAP expression.[26] Here, we investigated whether VTA astroglia were activated by down-regulation of miR-219-5p. As shown in figure 3B, miR-219-5p-sponge, but not vector, dramatically increased GFAP expression and induced a hypertrophic morphology of astroglia in the VTA. The increase in GFAP expression by miR-219-5p down-regulation in the VTA also was confirmed by Western blotting (fig. 3C). To further determine whether thermal hyperalgesia induced by down-regulation of miR-219-5p depends on the activation of astroglia, we injected fluorocitrate (1 μl at 1 nmol/10 μl), an inhibitor of astroglia, or a microglial inhibitor minocycline (1 μl at 50 ng/10 μl) into the VTA 7 days after miR-219-5p-sponge injection. The behavioral results showed that after 2 h, injection of fluorocitrate, but not minocycline, significantly reversed the thermal hyperalgesia induced by VTA miR-219-5p down-regulation (fig. 3, D and E). Taken together, these results suggest that down-regulation of miR-219-5p in VTA-induced nociception is mediated by the activation of astroglia.

NF-κB Is Required for Thermal Hyperalgesia Induced by Down-regulation of VTA MiR-219-5p

Astroglia are involved in the complex regulation of CNS inflammation, and their activation can lead to abnormal function of astroglia and can trigger proinflammatory responses.[27] NF-κB, a protein complex that controls DNA transcription and cytokine production, mediates astroglial activation–induced proinflammatory responses. Previous studies have shown that NF-κB impacts nociceptive transmission and processing.[28] Therefore, we examined the effect of miR-219-5p down-regulation on NF-κB expression. Immunofluorescence staining and Western blotting showed that down-regulation of miR-219-5p induced a significant increase in p-NF-κBp65 expression in astroglia, but not in dopamine neurons or microglia, 7 days after miR-219-5p-sponge injection in the VTA (fig. 4A–C). These data indicate that down-regulation of miR-219-5p activates astroglial NF-κB signaling in the VTA.

Next, we investigated whether the activation of NF-κB is involved in astroglial activation and nociceptive responses induced by miR-219-5p down-regulation in the VTA. To address this question, we used an siRNA strategy to knockdown endogenous NF-κB (small interfering NF-κB [siNF-κB]) 5 days after miR-219-5p-sponge injection (fig. 5A); astroglial activation was detected 2 days after siNF-κB injection. The results showed that knockdown of NF-κB reversed the increased GFAP expression, cellular hypertrophy, and hyperplasia of astroglia induced by down-regulation of miR-219-5p in the VTA (fig. 5, B and C), which

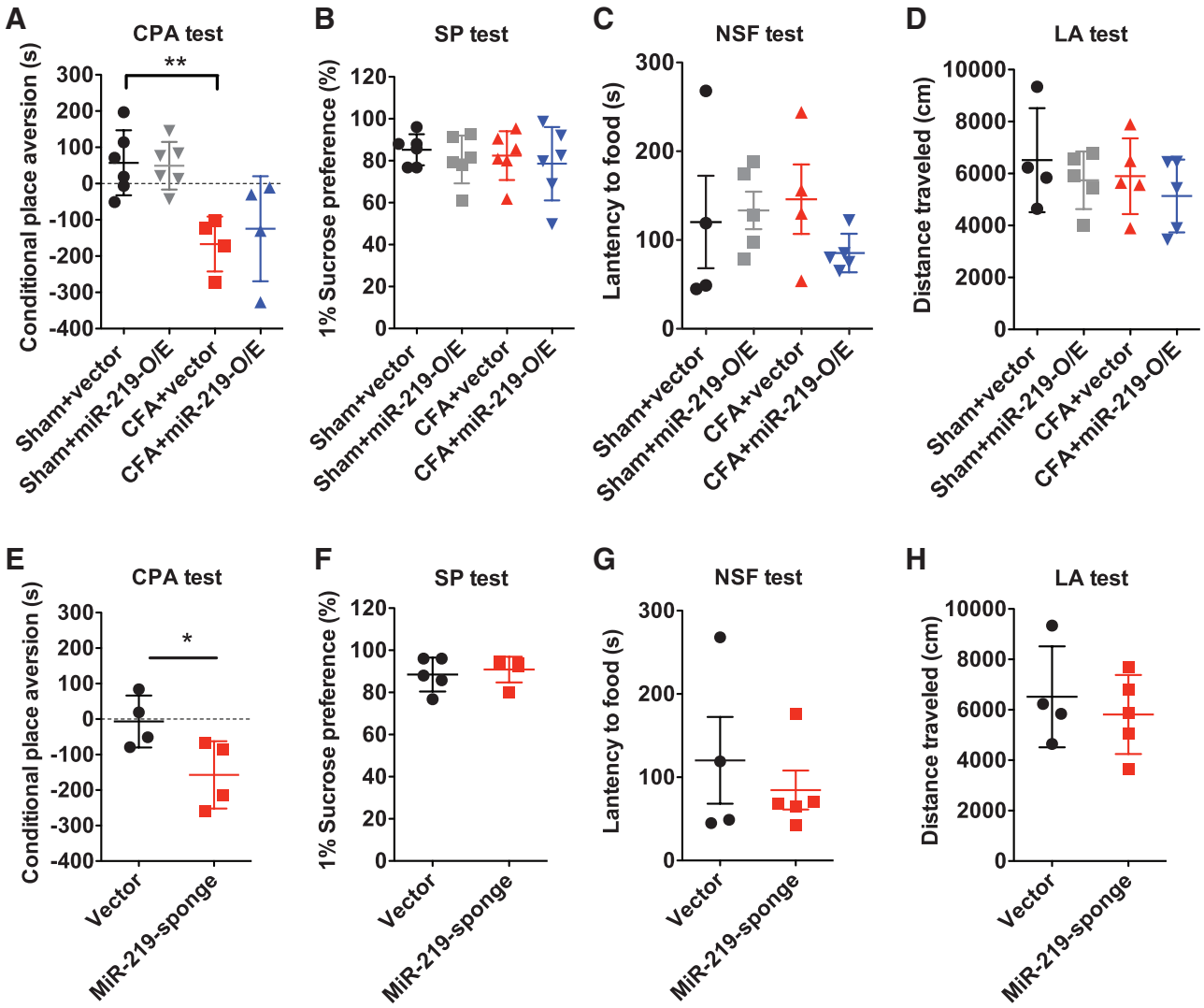

Fig. 2. Effects of the ventral tegmental area (VTA) miR-219-5p manipulation on affective aspects of nociception. (*A*) Rats receiving intraplantar injections of complete Freund's adjuvant (CFA) spent less time in the pain-paired chamber (one-way ANOVA, $F_{3, 16} = 7.29$; *post hoc* test, $^{**}P < 0.01$ *vs.* sham + vector group; n = 4 rats in sham + vector and sham + miR-219-5p-O/E groups and n = 6 rats in CFA + vector and CFA + miR-219-5p-O/E groups). MiR-219-5p-O/E treatment did not attenuate this negative affective state ($P > 0.05$ *vs.* CFA + vector group). (*B–D*) There were no differences among the groups in (*B*) the sucrose preference (SP) test (one-way ANOVA, $F_{3, 20} = 0.31$; n = 6 rats/group), (*C*) novelty suppressed feeding (NSF) test (one-way ANOVA, $F_{3, 14} = 0.72$; n = 4 rats in sham + vector and CFA + vector groups and n = 5 rats in sham + miR-219-5p-O/E and CFA + miR-219-5p-O/E groups), or (*D*) locomotion activity (LA) test (one-way ANOVA, $F_{3, 15} = 0.65$; n = 4 rats in sham + vector group and n = 5 rats in other groups). (*E*) Rats receiving intra-VTA injections of miR-219-5p-sponge spent less time in the pain-paired chamber (unpaired *t* test, $t_6 = 2.51$, $^{*}P < 0.05$ *vs.* vector group; n = 4 rats/group). (*F–H*) There were no differences among the groups in (*F*) the SP test (unpaired *t* test, $t_8 = 0.52$; n = 5 rats/group), (*G*) NSF test (unpaired *t* test, $t_7 = 0.67$; n = 4 rats in vector group and n = 5 rats in miR-219-5p-sponge group), or (*H*) LA test (unpaired *t* test, $t_7 = 0.59$; n = 4 rats in vector group and n = 5 rats in miR-219-5p-sponge group). CPA = conditioned place aversion.

were accompanied with the reversal of thermal hyperalgesia, as evidenced by the increased PWLs (fig. 5D). These findings suggest that NF-κB–mediated astroglial activation participates in nociception induced by down-regulation of miR-219-5p in the VTA.

The activation of the NF-κB family of transcription factors is a key step in the regulation of proinflammatory cytokine expression. Activated astroglia can increase the synthesis and release of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, which contribute to chronic pain persistence and central sensitization.[29–33] Therefore, we examined the changes in TNF-α, IL-1β, and IL-6 expression in the VTA after treatment with miR-219-5p-sponge or miR-219-5p-sponge combined with siNF-κB. The results of ELISA showed that down-regulation of miR-219-5p significantly increased the expression of these cytokines in the VTA, which was reversed by knockdown of NF-κB (fig. 5E–G). Together, these findings suggest that down-regulation of

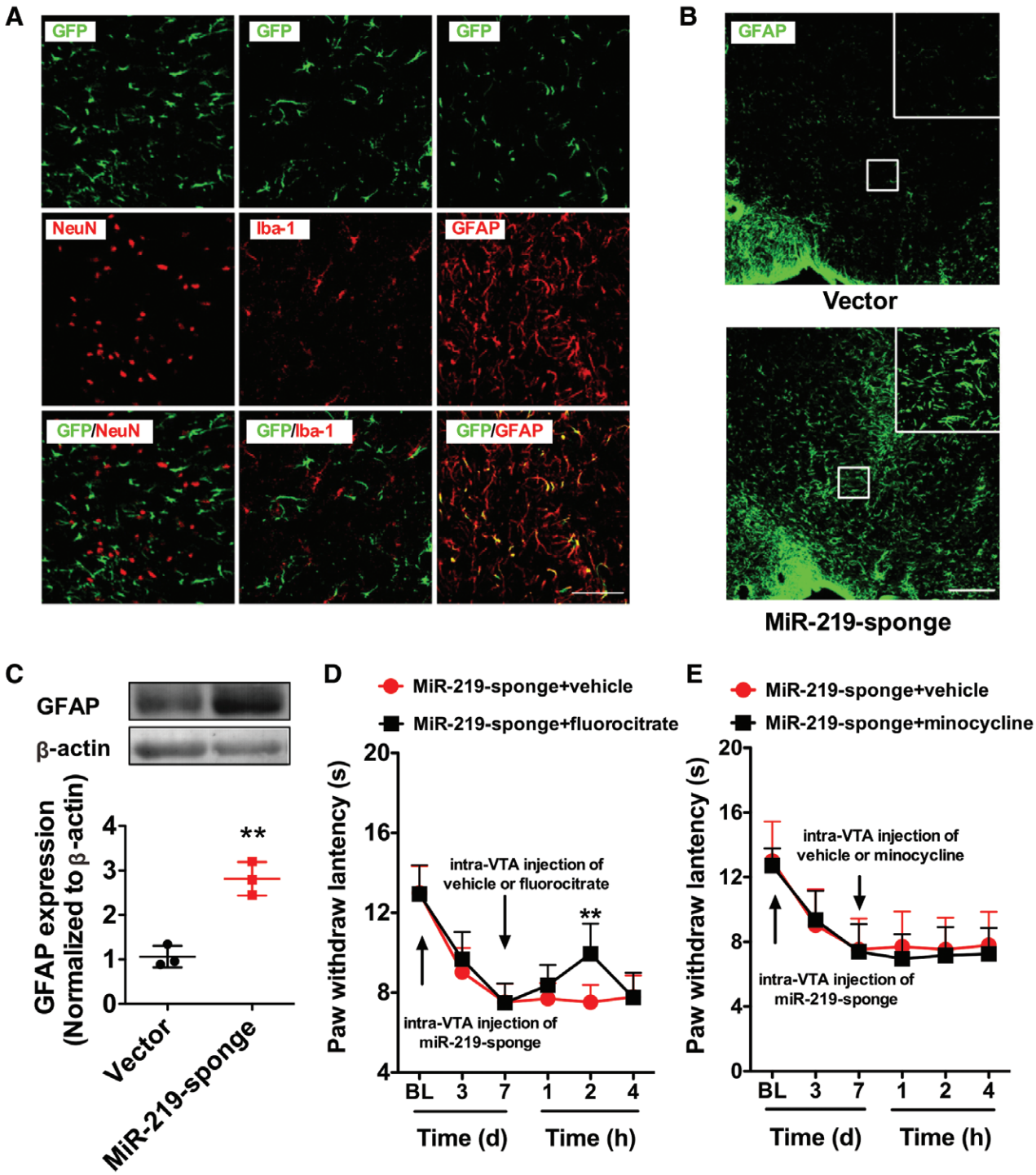

Fig. 3. Down-regulation of miR-219-5p activates astroglia in the ventral tegmental area (VTA) in naïve rats. (*A*) Double immunostaining shows that green fluorescent protein (GFP)-tagged miR-219-5p colocalized with glial fibrillary acidic protein (GFAP) but not with neuronal nuclei (NeuN) or ionized calcium binding adaptor molecule 1 (Iba-1). *Scale bar* = 125 μm. (*B*) Down-regulation of miR-219-5p increased GFAP immunoreactivity and hypertrophic morphology of astroglia in the VTA compared with that of vector treatment at 7 days after miR-219-5p-sponge injection. *Scale bar* = 500 μm. (*C*) Western blotting result validating the effect depicted in (*B*) (unpaired *t* test, $t_4 = 6.78$, $^{**}P < 0.01$ *vs.* vector group; n = 3). (*D* and *E*) Fluorocitrate (1 μl at 1 nmol/10 μl) partially reversed the down-regulation of miR-219-5p–induced thermal hyperalgesia 2 h after injection (two-way repeated-measures ANOVA, treatment effect: $F_{1, 40} = 6.06$; *post hoc* test, $^{**}P < 0.01$ *vs.* vector group; n = 6 rats/group), whereas minocycline (1 μl at 50 ng/10 μl) did not affect the paw withdrawal latencies (two-way ANOVA, treatment effect: $F_{1, 40} = 0.68$; n = 6 rats/group). *Error bars* show SD. BL = baseline.

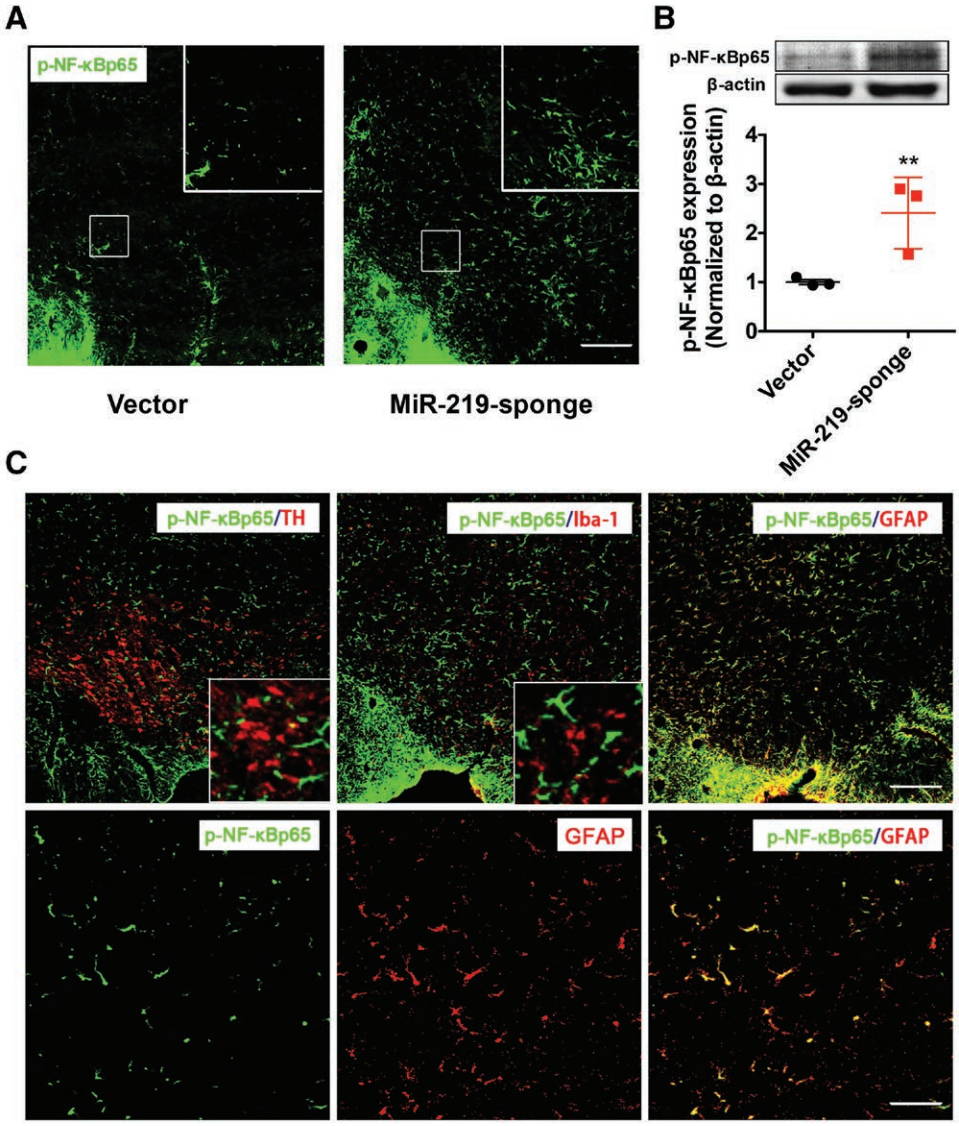

Fig. 4. Nuclear factor-κB (NF-κB) is activated in the ventral tegmental area (VTA) astroglia after down-regulation of miR-219-5p. (*A*) Immunofluorescence and (*B*) Western blotting results show that down-regulation of miR-219-5p induced a significant increase of p-NF-κBp65 expression in the VTA 7 days after miR-219-5p-sponge injection. *Scale bar* = 500 μm (Mann–Whitney *U* test, $^{**}P < 0.01$ *vs.* vector group; n = 3). (*C*) The majority of p-NF-κBp65^{+} cells expressed glial fibrillary acidic protein (GFAP), but not neuronal nuclei or ionized calcium binding adaptor molecule 1, in the VTA. *Scale bar* = 500 μm (*upper*) and 125 μm (*lower*). *Error bars* show SD.

miR-219-5p in the VTA induces nociception by modulating the release of proinflammatory cytokines in an NF-κB–dependent manner from activated astroglia.

MiR-219-5p Regulates Nociception by Targeting CC2D1A

Next, we used a bioinformatic analysis to identify a potential nociception-related gene target of miR-219-5p and found that the 3′-UTR of CC2D1A mRNA has strong hybridization sites for miR-219-5p. We then cloned the wild type CC2D1A 3′-UTR and the mutated CC2D1A 3′-UTR sequences into luciferase reporter plasmids, psi-CHECK2. This reporter vector was cotransfected with miR-219-5p mimics into HEK293T or C6 glioma cells. MiR-219-5p mimics significantly decreased the luciferase activity from pCHK-wt-CC2D1A–transfected cells compared with those from the miR-219-5p target site mutant pCHK-mut-CC2D1A– and empty vector–transfected cells (fig. 6, A and B). Furthermore, we found that CC2D1A protein expression was increased after knockdown of miR-219-5p and decreased by overexpression of miR-219-5p compared with that from naïve rats (fig. 6C). These *in vitro* and *in vivo* findings suggest that CC2D1A is a direct target of miR-219-5p.

To test the functional role of CC2D1A in astroglial activation, proinflammatory cytokines expression, and nociceptive responses induced by down-regulation of miR-219-5p,

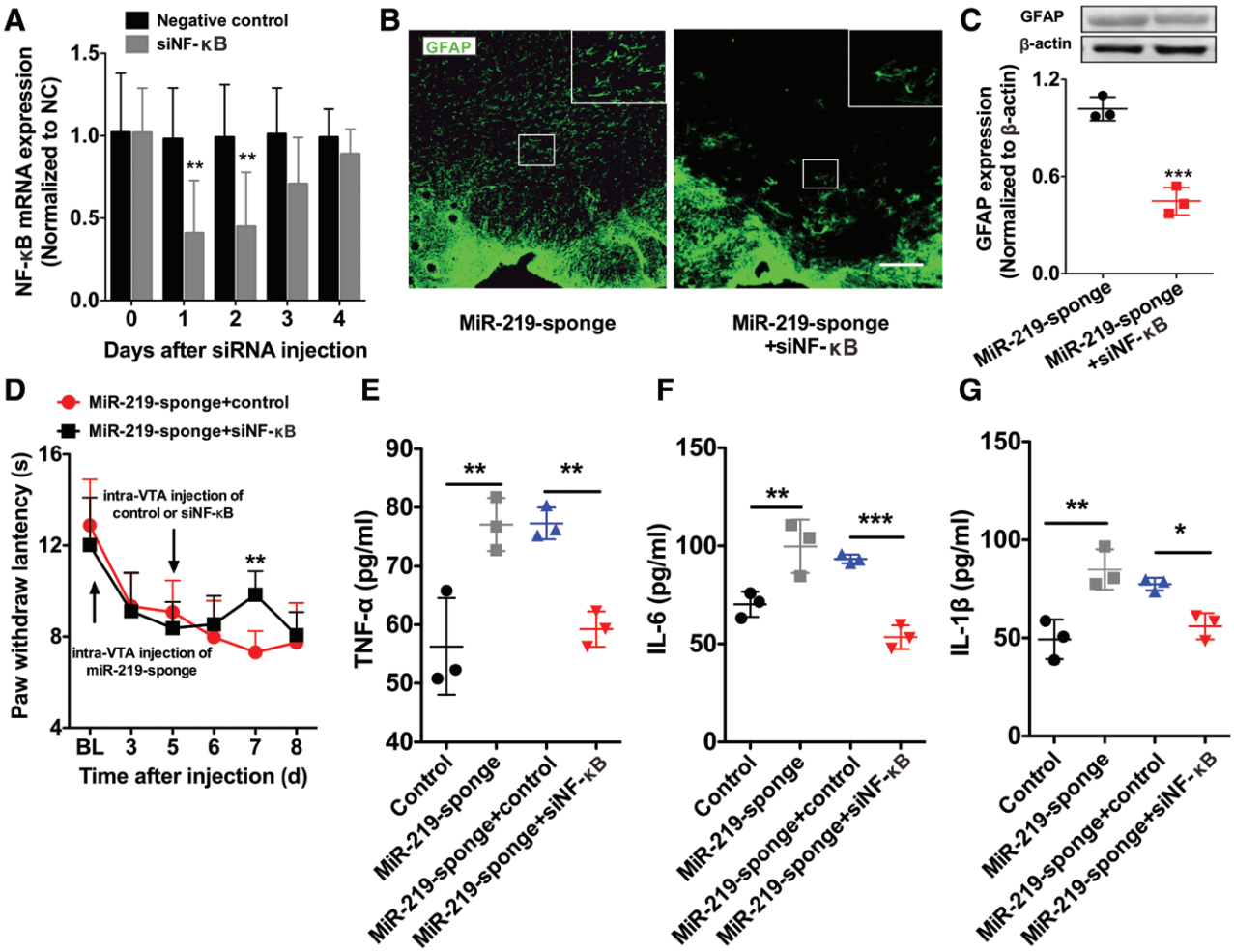

Fig. 5. Functional role of nuclear factor-κB (NF-κB) in down-regulation of miR-219-5p–induced nociception. (*A*) Time course of NF-κB mRNA expression after intraventral tegmental area (intra-VTA) small interfering NF-κB (siNF-κB) injection (two-way repeated-measures ANOVA, treatment effect: $F_{1, 80} = 25.01$; *post hoc* test, $^{**}P < 0.01$ *vs.* negative control group; n = 9). (*B*) SiNF-κB reversed the down-regulation of miR-219-5p–induced astroglial activation in the VTA. Glial fibrillary acidic protein (GFAP) was detected 2 days after siNF-κB injection (7 days after miR-219-5p-sponge injection). *Scale bar* = 500 μm. (*C*) Western blotting result validating the effect depicted in (*B*) (unpaired *t* test, $t_4 = 8.77$, $^{***}P < 0.001$ *vs.* miR-9-sponge group; n = 3). (*D*) SiNF-κB (1 μl at 20 μM) reversed the down-regulation of miR-219-5p–induced thermal hyperalgesia (two-way repeated-measures ANOVA, treatment effect: $F_{1, 56} = 4.43$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group; n = 8 rats/group). (*E–G*) Down-regulation of miR-219-5p elevated the levels of (*E*) tumor necrosis factor-α (TNF-α) (one-way ANOVA, $F_{3, 8} = 14.57$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group), (*F*) interleukin-IL-1β (one-way ANOVA, $F_{3, 8} = 13.06$; *post hoc* test, $^{**}P < 0.01$ *vs*. control group), (*G*) and interleukin-6 (one-way ANOVA, $F_{3, 8} = 20.52$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group), whereas siNF-κB reversed these effects. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* miR-219-5p-sponge + control group). Proinflammatory cytokines were measured 2 days after siNF-κB injection (7 days after miR-219-5p-sponge injection). Three samples were tested in each group. *Error bars* show SD.

we knocked down CC2D1A expression using an siRNA (small interfering coiled-coil and C2 domain containing 1A [siCC2D1A]) in the VTA of rats 5 days after miR-219-5p-sponge injection (fig. 6D). The results showed that knockdown of CC2D1A reversed astroglial activation and GFAP expression induced by down-regulation of miR-219-5p in the VTA (fig. 6, E and F), which was accompanied by decreased p-NF-κBp65 (fig. 6G) expression and an alleviation of nociceptive behavior (fig. 6H). This siCC2D1A injection also reversed the elevated TNF-α, IL-1β, and IL-6 expression induced by down-regulation of miR-219-5p in the VTA (fig. 6I–K), suggesting that down-regulation of miR-219-5p in the VTA induces nociceptive responses by targeting the CC2D1A/NF-κB pathway.

Down-regulation of MiR-219-5p Activates VTA Dopamine Neurons

Astroglial activation potentially can have profound effects on neuronal activity and promote a constant interaction between glial cells and neurons.[34] Therefore, we asked whether down-regulation of miR-219-5p changes dopamine neuron excitability in the VTA. To address this question, we performed double immunofluorescence staining for TH (regularly used as a marker for dopamine neurons) and c-Fos (a molecular

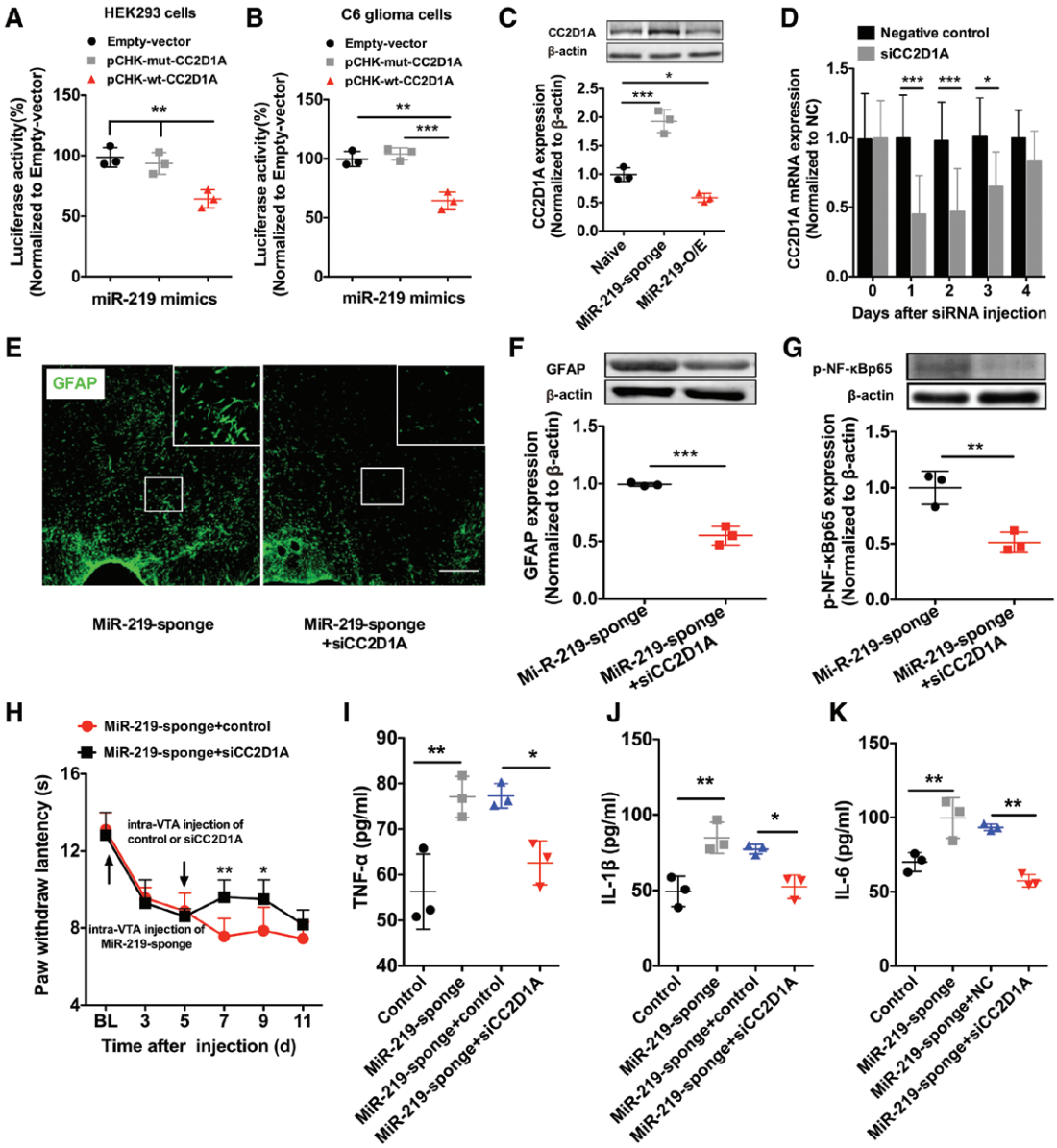

Fig. 6. Coiled-coil and C2 domain containing 1A (CC2D1A) is a direct target of miR-219-5p. (*A* and *B*) Luciferase activity from pCHK-wt-CC2D1A–transfected cells was decreased significantly by the administration of miR-219-5p mimics when tested in HEK293T (one-way ANOVA, $F_{2,6} = 15.18$; *post hoc* test, $^{**}P < 0.01$ *vs.* pCHK-wt-CC2D1A group; n = 3 cultures/group) and C6 glioma (one-way ANOVA, $F_{2,6} = 33.91$; *post hoc* test, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* pCHK-wt-CC2D1A group; n = 3 cultures/group) cells. (*C*) CC2D1A expression was increased after miR-219-5p-sponge injection and was decreased after miR-219-5p-O/E injection (one-way ANOVA, $F_{2,6} = 69.08$, $P < 0.0001$; *post hoc* test, $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* naïve group; n = 3). (*D*) Time course of CC2D1A mRNA expression after intraventral tegmental area (intra-VTA) small interfering coiled-coil and C2 domain containing 1A (siCC2D1A) injection (two-way repeated-measures ANOVA, treatment effect: $F_{1,80} = 29.56$; *post hoc* test, $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* negative control group; n = 9). (*E*) SiCC2D1A reversed down-regulation of miR-219-5p–induced astroglial activation in the ventral tegmental area 2 days after injection (7 days after miR-219-5p-sponge injection). *Scale bar* = 500 μm. (*F*) Western blotting result validating the effect depicted in (*E*) (unpaired *t* test, $t_4 = 9.43$, $^{***}P < 0.001$ *vs.* miR-219-5p-sponge group; n = 3). (*G*) SiCC2D1A reversed the overexpression of p-NF-κBp65 induced by miR-219-5p-sponge injection (unpaired *t* test, $t_4 = 4.85$, $^{**}P < 0.01$ *vs.* miR-219-5p-sponge group; n = 3). p-NF-κBp65 was measured 2 days after siCC2D1A injection (7 days after miR-219-5p-sponge injection). (*H*) SiCC2D1A (1 μl at 20 μM) reversed the down-regulation of miR-219-5p–induced thermal hyperalgesia (two-way repeated measures ANOVA, treatment effect: $F_{1,40} = 15.86$; *post hoc* test, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* miR-219-5p-sponge + control group; n = 6 rats/group). (*I–K*) Down-regulation of miR-219-5p elevated the levels of (*I*) tumor necrosis factor-α (TNF-α) (one-way ANOVA, $F_{3,8} = 11.25$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group), (*J*) interleukin-1β (IL-1β) (one-way ANOVA, $F_{3,8} = 13.49$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group), and (*K*) interleukin-6 (IL-6) (one-way ANOVA, $F_{3,8} = 18.91$; *post hoc* test, $^{**}P < 0.01$ *vs.* control group). SiCC2D1A reversed these effects ($^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* miR-219-5p-sponge + control group). Proinflammatory cytokines were measured 2 days after siCC2D1A injection (7 days after miR-219-5p-sponge injection). Three samples were tested in each group. *Error bars* show SD. BL = baseline; GFAP = glial fibrillary acidic protein; NC = negative control.

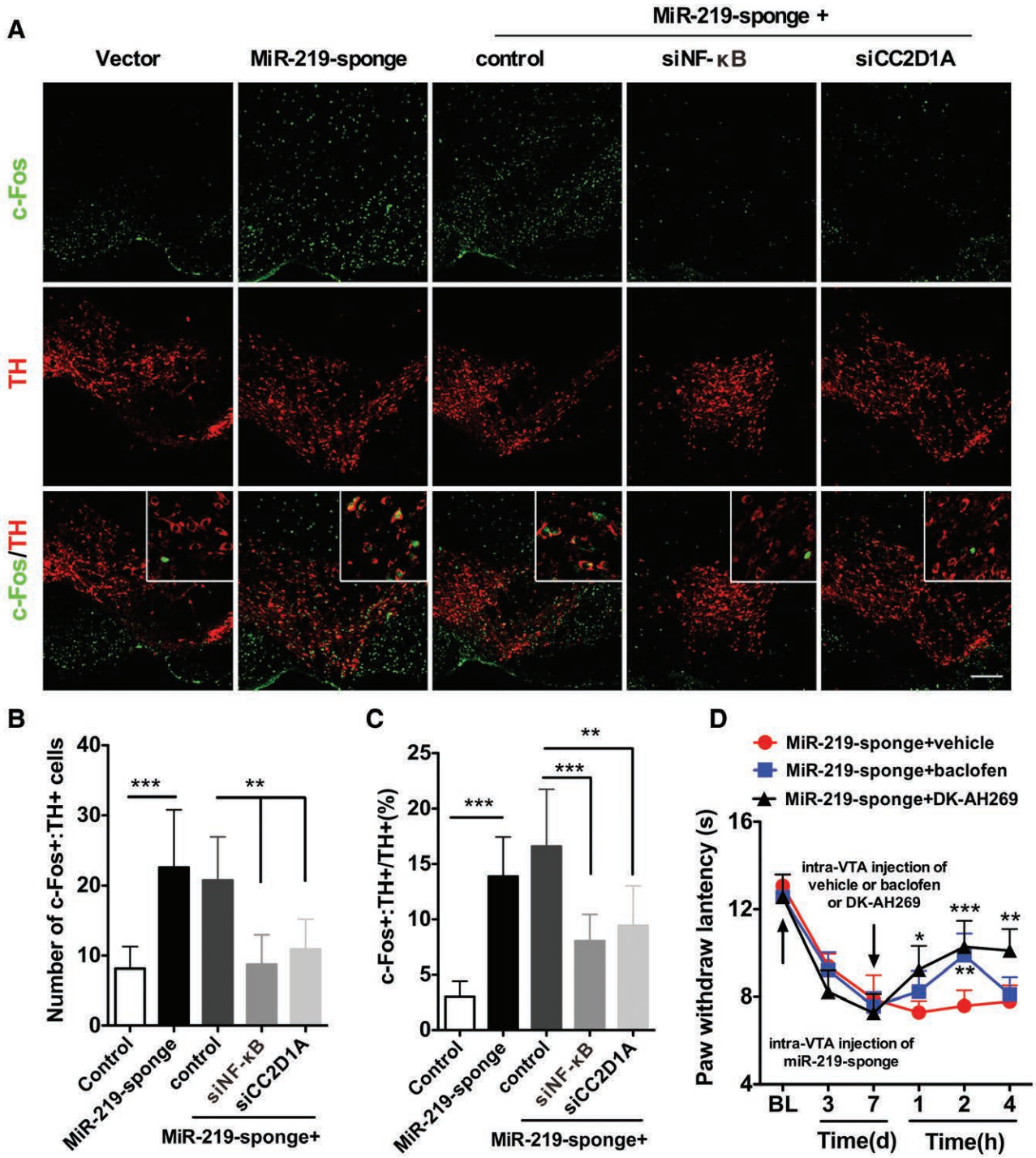

Fig. 7. Down-regulation of miR-219-5p activates ventral tegmental area (VTA) dopamine neurons. (*A–C*) MiR-219-5p-sponge injection significantly increased the number of TH⁺:c-Fos⁺ neurons (one-way ANOVA, $F_{4, 34} = 12.05$; *post hoc* test, ***$P < 0.001$ *vs.* control group) and the proportion of c-Fos⁺ neurons among TH⁺ VTA cells (one-way ANOVA, $F_{4,34} = 17.81$; *post hoc* test, ***$P < 0.001$ *vs.* control group), which was blocked by small interfering coiled-coil and C2 domain containing 1A (siCC2D1A) and siNF-κB (**$P < 0.01$, ***$P < 0.001$ *vs.* miR-219-5p-sponge + control group). Immunostaining was performed 2 days after siCC2D1A or siNF-κB injection (7 days after miR-219-5p-sponge injection) (n = 8 in control group, n = 10 in miR-219-5p-sponge + seCC2D1A group, or n = 7 in other groups). Two or three nonadjacent sections were selected randomly from three to five rats in each group. *Scale bar* = 500 μm. (*D*) High-dose baclofen (0.2 μg/0.5 μl) or DK-AH269 (1.2 μg/0.5 μl) reversed the hyperalgesia induced by miR-219-5p-sponge injection (two-way repeated-measures ANOVA, treatment effect: $F_{2,60} = 18.59$; *post hoc* test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* miR-219-5p-sponge + vehicle group; n = 6 rats/group). *Error bars* show SD. TH = tyrosine hydroxylase.

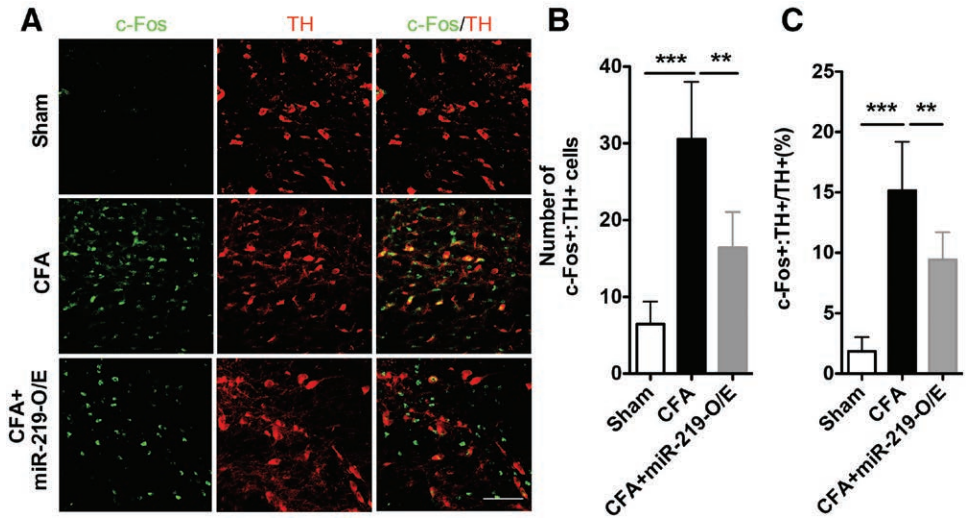

Fig. 8. Overexpression of miR-219-5p reduces complete Freund's adjuvant (CFA)-induced hyperalgesia by inhibiting dopamine neuron activity. (*A–C*) Overexpression of miR-219-5p reduced the increased number of TH⁺:c-Fos⁺ neurons (one-way ANOVA, $F_{2, 29}$ = 48.40; *post hoc* test, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* CFA group) and the increased proportion of c-Fos⁺ neurons among the TH⁺ ventral tegmental area cells (one-way ANOVA, $F_{2, 29}$ = 55.63; *post hoc* test, $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* CFA group) induced by CFA injection. Immunostaining was performed 6 days after miR-219-5p-O/E injection (9 days after CFA injection) (n = 14 in CFA + miR-219-5p-O/E group or n = 9 in other groups). Two or three nonadjacent sections were selected randomly from three to five rats in each group. *Scale bar* = 50 μm. *Error bars* show SD. TH = tyrosine hydroxylase.

marker for neuronal activation) in the VTA 7 days after miR-219-5p-sponge injection. As shown in figure 7A–C, miR-219-5p-sponge, but not vector, significantly increased the number of TH⁺:c-Fos⁺ neurons and the proportion of c-Fos⁺ neurons among the TH⁺ VTA cells, which was reversed by knockdown of CC2D1A or NF-κB in the VTA.

To further investigate whether the activation of VTA dopamine neurons is involved in nociceptive responses induced by down-regulation of VTA miR-219-5p, we pharmacologically inhibited VTA dopamine neuron activity by microinjecting the selective $GABA_B$ receptor agonist, baclofen (0.2 μg/0.5 μl), or I_h blocker DK-AH269 (1.2 μg/0.5 μl), which are reagents known to reliably decrease VTA dopamine neuron firing,[35,36] into the VTA at day 7 after miR-219-5p-sponge injection. PWLs were measured 1 h after the last injection. The behavioral results showed that baclofen increased PWLs, an effect that lasted 2 h, whereas the antinociceptive effect of DK-AH269 lasted for at least 4 h (fig. 7D). These findings suggest that the increased activity of dopamine neurons contributes to nociceptive responses induced by down-regulation of miR-219-5p in the VTA.

Finally, we found that intraplantar CFA robustly increased the number of TH⁺:c-Fos⁺ neurons and the proportion of c-Fos⁺ neurons among the TH⁺ cells in the contralateral VTA, which was reversed partially by an intra-VTA infusion of miR-219-5p-O/E (fig. 8A–C), suggesting that the antinociceptive effect of miR-219-5p overexpression in CFA-induced inflammatory nociception also might be mediated by inhibiting VTA dopamine neuron activity.

Discussion

MiRNAs have attracted increasing interest as research tools, biomarkers, and potential new drug targets for modulating pain processing and analgesia. Recently, it has been demonstrated that miRNA expression is altered drastically in the reward system in chronic pain states. For example, miR-132 and miR-125b were shown to be changed in the hippocampus under neuropathic and inflammatory pain conditions.[37,38] In the nucleus accumbens (NAc), which is highly involved in both reward and chronic pain processing, miR-200b and miR-429 are decreased in a neuropathic pain model.[39] In the prefrontal cortex, miR-155 and miR-223 are increased after facial carrageenan injection.[40] However, despite the central role of the VTA in the reward system, no study has shown the miRNA expression profile or a functional role of specific miRNAs in the VTA in a state of chronic nociceptive sensitization. Our recent study demonstrated that miR-219-5p is down-regulated in the spinal cord under a CFA-induced nociceptive state, and the manipulation of spinal miR-219-5p could regulate central sensitization and chronic pain behaviors by targeting calcium/calmodulin-dependent protein kinase IIγ.[13] In this study, we showed that intraplantar CFA injection decreased miR-219-5p expression in the VTA. Viral-mediated up-regulation of miR-219-5p in the VTA reversed CFA-induced thermal hyperalgesia, and down-regulation of miR-219-5p in the VTA was sufficient to induce thermal hyperalgesia in naïve rats. These results indicate a critical role of miR-219-5p in nociceptive regulation in the CNS.

The behavioral studies also showed that the manipulation of VTA miR-219-5p in rats mainly affects the sensory

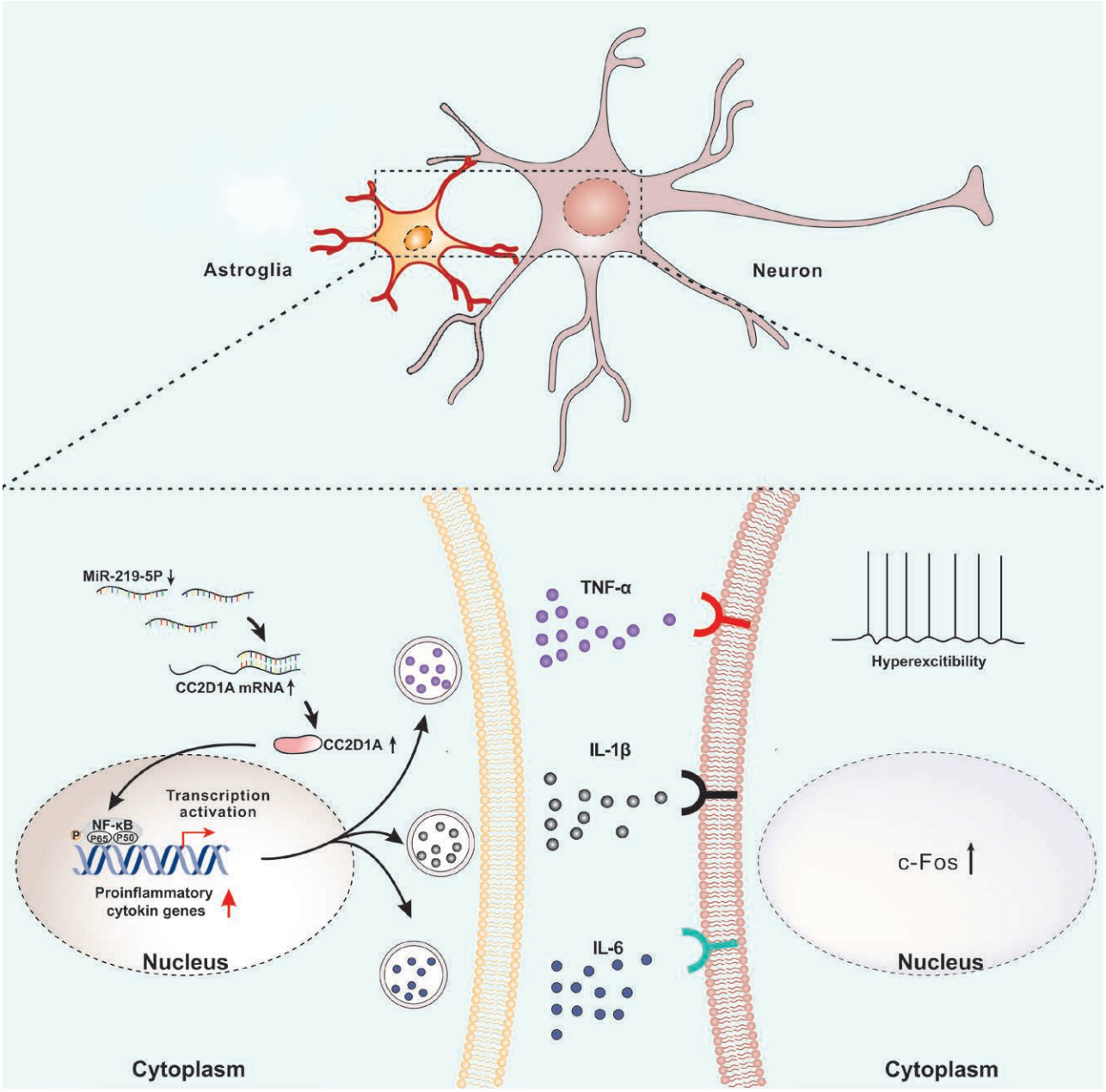

Fig. 9. Schematic shows how astroglial miR-219-5p regulates nociception in the ventral tegmental area. CC2D1A = coiled-coil and C2 domain containing 1A; IL-1β = interleukin-1β; IL-6 = interleukin-6; NF-κB = nuclear factor-κB; TNF-α = tumor necrosis factor-α.

discriminative component of nociceptive information and has little impact on the affective–motivational aspects of nociception, with no effect on physical function. These data suggest that VTA, the brain's reward center, plays a critical role in nociceptive sensory regulation. This nociception–modulatory role for VTA is not surprising, as it has reciprocal connections with several limbic brain regions, including NAc, anterior cingulate cortex, medial prefrontal cortex, amygdala, hippocampus, and insular cortex,[41,42] which are also important for pain regulation. The axons of the spinal nociceptive neurons carrying peripheral nociceptive information cross within the spinal cord and then ascend *via* the spinothalamic, spinoreticular (spinoparabrachial), and spinomesencephalic tracts to the thalamus, medulla, and brainstem. On the bases of these important intermediaries, the nociceptive information is relayed to the primary somatosensory cortex, as well as other cortical structures and limbic structures. Then, VTA primarily receives and processes the affective–motivational and cognitive aspects of nociceptive information. In addition, VTA has substantial projection to NAc, anterior cingulate cortex, medial prefrontal cortex, amygdala, and insular cortex, which are all involved in the pain matrix and project directly to periaqueductal gray and rostral ventromedial medulla.[43] Thus, VTA can affect the descending pain control

systems indirectly and modulate the sensory discriminative component of nociceptive information.

Accumulating evidence suggests that robust astroglial reaction in the CNS is general and evident in chronic pain states, including chronic neuropathic pain,[44] chronic inflammatory pain,[45] and bone cancer pain.[46] The astroglial toxin fluorocitrate and L-alpha-aminoadipate inhibit nerve injury– and nerve inflammation–induced pain behaviors.[47–49] In this study, we found that the lentivirus was expressed predominantly in the VTA astroglia. In addition, a dramatic activation of VTA astroglia, characterized by cellular hypertrophy, hyperplasia, and increased GFAP expression, was induced by down-regulation of miR-219-5p. Consistently, we found a fluorocitrate-induced transient antinociceptive effect in rats with viral down-regulation of miR-219-5p, suggesting the involvement of VTA astroglial activation in the nociceptive sensitization induced by this down-regulation. Interestingly, unlike our previous finding that miR-219-5p is expressed mainly in neurons in the spinal dorsal horn,[13] miR-219-5p expression was found mainly among astroglia in the VTA in this study. We speculate that miR-219-5p may function in different cell types in a location-dependent manner.

NF-κB is a transcription factor and has been implicated in the activation of astroglia.[27] In addition, it also is involved in regulating pain-related gene expression (*e.g.*, of cytokines and chemokines) in multiple pathologic states.[28] Here, we showed that phospho-NF-κBp65 (the activated form of NF-κB) was increased significantly by down-regulation of miR-219-5p in the VTA astroglia. Furthermore, knockdown of NF-κB in the VTA inhibited miR-219-5p down-regulation–induced astroglial activation, thermal hyperalgesia, and the release of the proinflammatory cytokines TNF-α, IL-1β, and IL-6. These results indicate that the NF-κB pathway may constitute the molecular mechanism underlying down-regulation of miR-219-5p–mediated nociceptive cellular and behavioral alterations.

Bioinformatic analysis revealed that the 3′-UTR of CC2D1A mRNA has strong hybridization sites for miR-219-5p, which makes CC2D1A a potential target gene of miR-219-5p. CC2D1A has been reported to play a crucial role in neurodevelopmental and psychiatric disorders,[50,51] and CC2D1A knockdown in neurons reduces dendritic complexity in autism spectrum disorder and intellectual disability.[51] In addition, CC2D1A has been shown to be required for mouse survival, to perform essential functions in controlling the maturation of synapses,[52] and is implicated in major depression.[53] In this study, for the first time, we reported that CC2D1A was involved in the nociceptive cellular (VTA astroglia) and behavioral alternations induced under conditions of miR-219-5p down-regulation in the VTA. Furthermore, we found that siCC2D1A effectively regulated the levels of p-NF-κBp65 and downstream cytokine release in the VTA, suggesting the involvement of the CC2D1A/NF-κB pathway in nociceptive information processing induced by down-regulation of miR-219-5p. Consistently, a previous study also demonstrated that CC2D1A regulates human intellectual and social functions by interfering with the NF-κB cascade.[50]

Activated astroglia produce multiple glial mediators, such as growth factors, proinflammatory cytokines, and chemokines, which contribute to maintaining chronic pain by modulating neuronal and synaptic activity.[54] The proinflammatory cytokines TNF-α, IL-1β, and IL-6 are among the most widely studied glial mediators. Incubation of spinal cord slices with TNF-α and IL-1β rapidly (within minutes) increases the frequency of spontaneous excitatory postsynaptic currents. IL-1β and IL-6 also inhibit the frequency of spontaneous inhibitory postsynaptic currents in spinal lamina II neurons.[32] Here, we showed that down-regulation of miR-219-5p not only increased TNF-α, IL-1β, and IL-6 expression in VTA tissues but also increased the activity of VTA dopamine neurons, indicating an enhanced interaction between astroglia and dopamine neurons in such nociceptive state.

The VTA is one of the most heterogeneous nuclei in the brain. Ambiguous roles of VTA dopamine neurons have been reported in studies of both humans and animals in acute and chronic pain states.[14–22] In animal studies, noxious stimuli, such as electric foot shock, formalin injection, and chronic nerve injury, were reported to increase VTA dopamine neuron firing activity,[17,19] whereas other studies suggested that these neurons are inhibited in acute and chronic pain states.[21,55] Here, we found that VTA dopamine neurons were activated with miR-219-5p down-regulation and in a CFA-induced nociceptive state. In addition, inhibiting the activity of dopamine neurons with high-dose baclofen, DK-AH269, or miR-219-5p-O/E showed markedly antinociceptive effects in rats treated with CFA or with down-regulated miR-219-5p expression. These discrepancies may be attributed to the heterogeneity of VTA dopamine neurons and highlight the importance of further exploring the roles of regional- and circuit-specific VTA dopamine neurons in different nociceptive states.

Conclusions

Our findings demonstrate that down-regulation of miR-219-5p induces activation of VTA astroglia and release of proinflammatory cytokines *via* the CC2D1A/NF-κB pathway to elicit nociceptive responses. In addition, we provide new insight into the involvement of VTA dopamine neuron activity in nociceptive modulation (fig. 9). Collectively, the results from this study show a novel local circuit mechanism underlying nociceptive processing in the VTA and provide more detailed molecular and gene targets for future analgesic development.

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Competing Interests

The authors declare no competing interests.

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