RE1-silencing transcription factor controls the acute-to-chronic neuropathic pain transition and *Chrm2* receptor gene expression in primary sensory neurons

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Neuropathic pain is associated with persistent changes in gene expression in primary sensory neurons, but the underlying epigenetic mechanisms that cause these changes remain unclear. The muscarinic cholinergic receptors (mAChRs), particularly the M2 subtype (encoded by the *cholinergic receptor muscarinic 2* (*Chrm2*) gene), are critically involved in the regulation of spinal nociceptive transmission. However, little is known about how *Chrm2* expression is transcriptionally regulated. Here we show that nerve injury persistently increased the expression of RE1-silencing transcription factor (REST, also known as neuron-restrictive silencing factor [NRSF]), a gene-silencing transcription factor, in the dorsal root ganglion (DRG). Remarkably, nerve injury–induced chronic but not acute pain hypersensitivity was attenuated in mice with *Rest* knockout in DRG neurons. Also, siRNA-mediated *Rest* knockdown reversed nerve injury–induced chronic pain hypersensitivity in rats. Nerve injury persistently reduced *Chrm2* expression in the DRG and diminished the analgesic effect of muscarine. The RE1 binding site on the *Chrm2* promoter is required for REST-mediated *Chrm2* repression, and nerve injury increased the enrichment of REST in the *Chrm2* promoter in the DRG. Furthermore, *Rest* knockdown or genetic ablation in DRG neurons normalized *Chrm2* expression and augmented muscarine's analgesic effect on neuropathic pain and fully reversed the nerve injury–induced reduction in the inhibitory effect of muscarine on glutamatergic input to spinal dorsal horn neurons. Our findings indicate that nerve injury–induced REST up-regulation in DRG neurons plays an important role in the acute-to-chronic pain transition and is essential for the transcriptional repression of *Chrm2* in neuropathic pain.

Chronic neuropathic pain is a debilitating condition caused by damage to the somatosensory system. Persistent up-regulation of pronociceptive genes and down-regulation of antinociceptive genes account for the long-lasting aberrant excitability of dorsal root ganglion (DRG)[2] neurons in chronic neuropathic pain (1–3). For example, nerve injury causes up-regulation of $\alpha2\delta$-1 in the DRG (4, 5), which leads to increased synaptic *N*-methyl-D-aspartate receptor activity and nociceptive input to spinal dorsal horn neurons. On the other hand, nerve injury reduces the expression of voltage-gated K^+ channels (2, 6), contributing to increased excitability of DRG neurons. However, the mechanisms underlying the transition from acute to chronic pain after nerve injury remain unclear. Thus, identifying the epigenetic and transcriptional factors responsible for altered gene expression in injured DRG neurons is important for understanding the mechanisms of neuropathic pain development and for improving the treatment of chronic neuropathic pain.

The repressor element-1 (RE1)-silencing transcription factor (REST; also known as neuron-restrictive silencer factor (NRSF)) is a zinc-finger DNA-binding protein and a master regulator of gene expression during neuronal development (7). REST binds to the RE1 site, which is composed of specific consensus DNA sequences, and inhibits transcription of its target genes by recruiting the cosuppressors mSin3 and coREST. These co-repressor complexes epigenetically modify target genomic regions through several chromatin-modifying enzymes (8–10). In nerve injury–induced neuropathic pain, REST is involved in silencing the expression of certain K^+ channels and μ-opioid receptors (*Oprm1*) in the DRG (6, 11). Nevertheless, the function of REST and its target genes in the injured DRG remain to be determined.

The muscarinic acetylcholine receptors (mAChRs) are G protein–coupled receptors that produce powerful analgesic effects upon activation. Spinally administered mAChR agonists or acetylcholinesterase inhibitors reduce pain in both animal models and humans (12–14). Furthermore, blocking mAChRs at the spinal cord level causes pain hypersensitivity, indicating that mAChRs tonically control nociceptive transmission (15). Among the five mAChRs, encoded by *Chrm1–5*, the odd-number group (M1, M3, and M5) and the even-number group (M2

[2] The abbreviations used are: DRG, dorsal root ganglion/ganglia; mAChR, muscarinic cholinergic receptor; SNL, spinal nerve ligation; SNI, spared nerve injury; cKO, conditional knockout; HEK, human embryonic kidney; EPSC, excitatory postsynaptic current; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; REST, RE1-silencing transcription factor; ANOVA, analysis of variance.

and M4) preferentially bind to $G_{q/11}$ and $G_{i/o}$ proteins, respectively (16, 17). In the DRG, about 75% of mAChR transcripts are *Chrm2* (2). The M2 subtype is also abundantly expressed at primary afferent terminals in the spinal dorsal horn (12, 18) and predominantly mediates the analgesic effect of mAChR agonists (12, 14, 19–21). However, little is known about how the expression of *Chrm2* in the DRG is regulated in neuropathic pain.

In this study, we provide new evidence that REST in DRG neurons not only contributes to the transition from acute to chronic pain after nerve injury but is also responsible for nerve injury–induced *Chrm2* repression and diminished muscarinic analgesia in neuropathic pain. Thus, REST-dependent epigenetic remodeling is a central mechanism involved in regulating *Chrm2* expression in primary sensory neurons and in neuropathic pain development.

Results

REST expression is increased in the injured DRG and maintains chronic pain induced by nerve injury

REST is a key transcriptional repressor that is normally expressed at a low level in differentiated neurons (22). To determine how nerve injury affects *Rest* expression in the DRG, we performed L5 and L6 spinal nerve ligation (SNL) in rats, a commonly used rodent model of neuropathic pain (23, 24). Quantitative PCR analysis showed that the mRNA level of *Rest* in the DRG was significantly higher in SNL rats than in sham-operated control rats on days 5, 10, and 21 after surgery ($p < 0.0001$, F(1,30) = 120.5, $n = 6$ rats/group; Fig. 1A). In contrast, SNL had no significant effect on the mRNA level of *Rest* in the dorsal spinal cord (Fig. 1B).

To determine whether REST plays a role in nerve injury–induced pain hypersensitivity, we first used an siRNA to knock down *Rest* in rats subjected to SNL. Three weeks after SNL, intrathecal injection of 2 μg/day of *Rest*-specific siRNA, but not control siRNA, for 5 days fully restored the mRNA level of *Rest* in the injured DRG to that observed in the DRG of rats that underwent sham surgery ($p < 0.0001$, F(2,15) = 56.64, $n = 6$ rats/group; Fig. 1C). However, treatment with *Rest*-specific siRNA had no effect on the mRNA level of REST-corepressor 1 (*Rcor1*) in the DRG (Fig. 1C). Western blotting showed that treatment with *Rest*-specific siRNA restored the protein level of REST in the injured DRG to that found in sham control rats ($p < 0.0001$, F(2,15) = 23.24, $n = 6$ rats/group; Fig. 1D).

SNL rats treated with control siRNA displayed a sustained reduction in the withdrawal threshold of the ipsilateral hind paw in response to tactile and noxious pressure stimuli (Fig. 1, E and F). By comparison, treatment with *Rest*-specific siRNA (2 μg/day) significantly attenuated tactile allodynia and mechanical hyperalgesia caused by SNL ($n = 9$ rats/group; Fig. 1, E and F). These results suggest that nerve injury increases REST expression in the DRG and that REST up-regulation is involved in maintaining chronic neuropathic pain.

REST in DRG neurons contributes to chronic but not acute pain induced by nerve injury

To further elucidate the role of REST in DRG neurons in the development of chronic pain after nerve injury, we generated

Rest conditional knockout mice (*Rest*-cKO) by crossing *Rest*-floxed mice (25) with a primary sensory neuron-specific Cre mouse line, *Advillin*-Cre (26, 27), such that *Rest* was selectively ablated from DRG neurons. The ambulatory behavior and motor coordination, measured with the rotarod test, were similar in *Rest*-cKO mice and WT littermate controls (Fig. 2A). The protein and mRNA levels of REST in the DRG were diminished in *Rest*-cKO mice compared with those in WT controls ($n = 8$ mice/group; Fig. 2, B and C). Furthermore, spared nerve injury (SNI) significantly increased the mRNA level of *Rest* in the DRG in WT ($p = 0.0004$, F(1.84,9.20) = 179, $n = 6$) but not in *Rest*-cKO mice (Fig. 2D).

In WT mice, SNI induced a persistent reduction in the tactile and pressure withdrawal thresholds of the ipsilateral hind paw that lasted for at least 4 weeks ($n = 8$ mice; Fig. 2, E and F). The withdrawal thresholds did not differ significantly between WT and *Rest*-cKO mice during the first week after SNI. Remarkably, the nerve injury–induced reduction in the withdrawal thresholds was significantly attenuated in *Rest*-cKO mice 8 to 29 days after SNI (Fig. 2, E and F). These data indicate that REST-dependent epigenetic remodeling in DRG neurons contributes to the transition from acute to chronic pain after nerve injury.

Nerve injury causes a persistent reduction in Chrm2 expression and increases REST occupancy at the Chrm2 promoter in the DRG

Chrm2 is the most dominant mAChR subtype expressed in the DRG (2, 19). Quantitative PCR analysis showed that 5 days after surgery, the mRNA level of *Chrm2* in the DRG was significantly lower in SNL rats than in control rats subjected to sham surgery ($p < 0.001$, F(1,10) = 619.4, $n = 6$ rats/group, Fig. 3A). This nerve injury-induced *Chrm2* down-regulation lasted for at least 3 weeks after surgery (Fig. 3A). However, the mRNA level of *Chrm2* in the dorsal spinal cord was not significantly different between SNL and sham control rats on days 5, 10, and 21 after surgery (Fig. 3B). Also, the mRNA level of *Chrm4* in the DRG did not differ significantly between the SNL and sham control groups (Fig. 3C).

We next used ChIP followed by quantitative PCR to determine whether nerve injury affects the amount of REST in the promoter region of *Chrm2* in the DRG. In DRG tissues from sham control rats, the REST occupancy at the *Chrm2* promoter was very low and similar to that precipitated by the IgG-negative control (Fig. 3D). However, SNL caused a large increase in the enrichment of REST in the promoter region of *Chrm2* in the DRG ($p = 0.0002$, t(10) = 5.65, $n = 6$ rats/group; Fig. 3D). In contrast, the level of REST in the promoter region of *Gapdh*, a highly expressed gene in the DRG, did not differ significantly between the SNL and control groups (Fig. 3E). These results indicate that nerve injury increases REST enrichment at the *Chrm2* promoter in the DRG.

The RE1 site is essential for transcriptional repression of Chrm2 by REST

DNA sequences containing the RE1 site typically recruit the binding of REST and are associated with repression of gene expression (7). We initially used Matinspector, a software tool (28), to search for transcription factor binding sites to the pro-
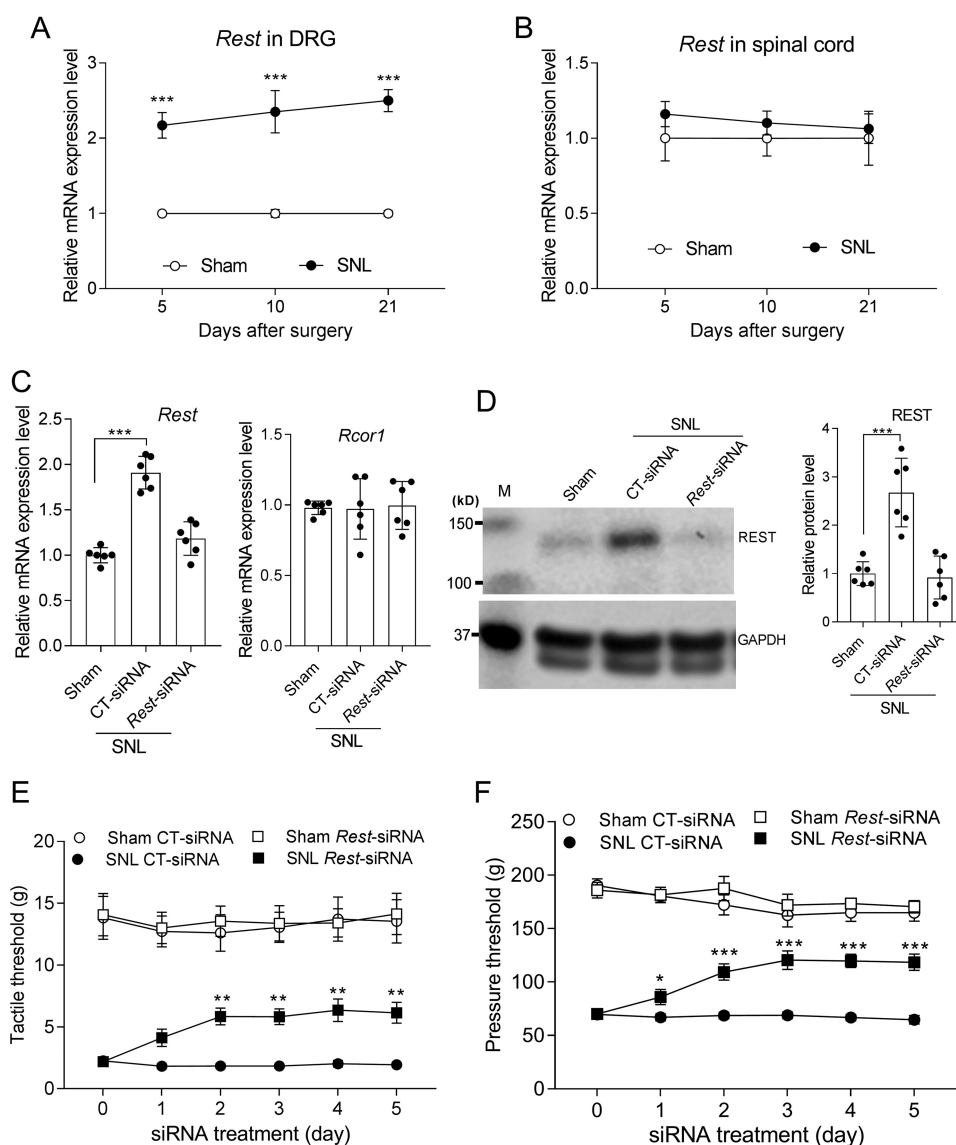
Figure 1. Nerve injury-induced REST up-regulation in the DRG contributes to chronic pain. *A* and *B*, real-time PCR data showing the mRNA level of *Rest* in the rat DRG (*A*) and dorsal spinal cord (*B*) on days 5, 10, and 21 after SNL or sham surgery. *Gapdh* was used as an endogenous control, and the mean value in the sham group was set to 1 (*n* = 6 rats/group). *C*, real-time PCR data showing the mRNA levels of *Rest* and *Rcor1* in the DRG of sham control and SNL rats treated intrathecally with control (*CT*) siRNA or *Rest*-specific siRNA for 5 days (*n* = 6 rats/group). ***, *p* < 0.001 (one-way ANOVA). *D*, original immunoblotting images and quantification of the REST protein level in the DRG in sham control and SNL rats treated with control siRNA or *Rest*-specific siRNA for 5 days (*n* = 6 rats/group). GAPDH was used as an internal control. *M*, molecular weight marker. *E* and *F*, time course of changes in the tactile (*E*) and noxious pressure (*F*) withdrawal thresholds in sham control rats and SNL rats treated with control siRNA or *Rest*-specific siRNA for 5 days (*n* = 9 rats/group). Data are shown as means ± S.D. (*A*–*D*) or means ± S.E. (*E* and *F*). *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001 compared with the sham group at the same time point (two-way ANOVA).

moter region of rat *Chrm2*. We found an RE1-binding site (taTTCAGgAtacagaagaaGGACtGggaagt; uppercase letters indicate conserved sequences) near the transcription start site (between nucleotides −150 and −119) of *Chrm2* (Fig. 4*A*). We then used a luciferase reporter assay to determine directly whether the RE1 site is involved in suppression of *Chrm2* expression by REST. In HEK293 cells transduced with *Chrm2* promoter/luciferase expression vectors, luciferase activity was significantly lower in cells with the *Chrm2* promoter containing the RE1 sequence than in cells without the RE1 sequence (*p* = 0.0286, *n* = 4; Fig. 4*B*).

We repeated the luciferase reporter assay using the F11 cell line, which is a hybrid of a rat embryonic DRG neurons with mouse neuroblastoma cells (29). Luciferase activity was

also significantly lower in cells transduced with the *Chrm2* promoter containing the RE1 sequence than in F11 cells with the RE1 sequence–deficient *Chrm2* promoter (*p* = 0.0286, *n* = 4; Fig. 4*B*). These data indicate that the *Chrm2* RE1 site is necessary and sufficient to repress *Chrm2* promoter activity.

REST is critically involved in nerve injury–induced repression of Chrm2 in the DRG and the diminished analgesic effect of muscarine

We used a *Rest*-specific siRNA in SNL rats to determine whether REST is responsible for nerve injury–induced transcriptional repression of *Chrm2* in the DRG. Intrathecal treatment with the *Rest*-specific siRNA fully restored the mRNA
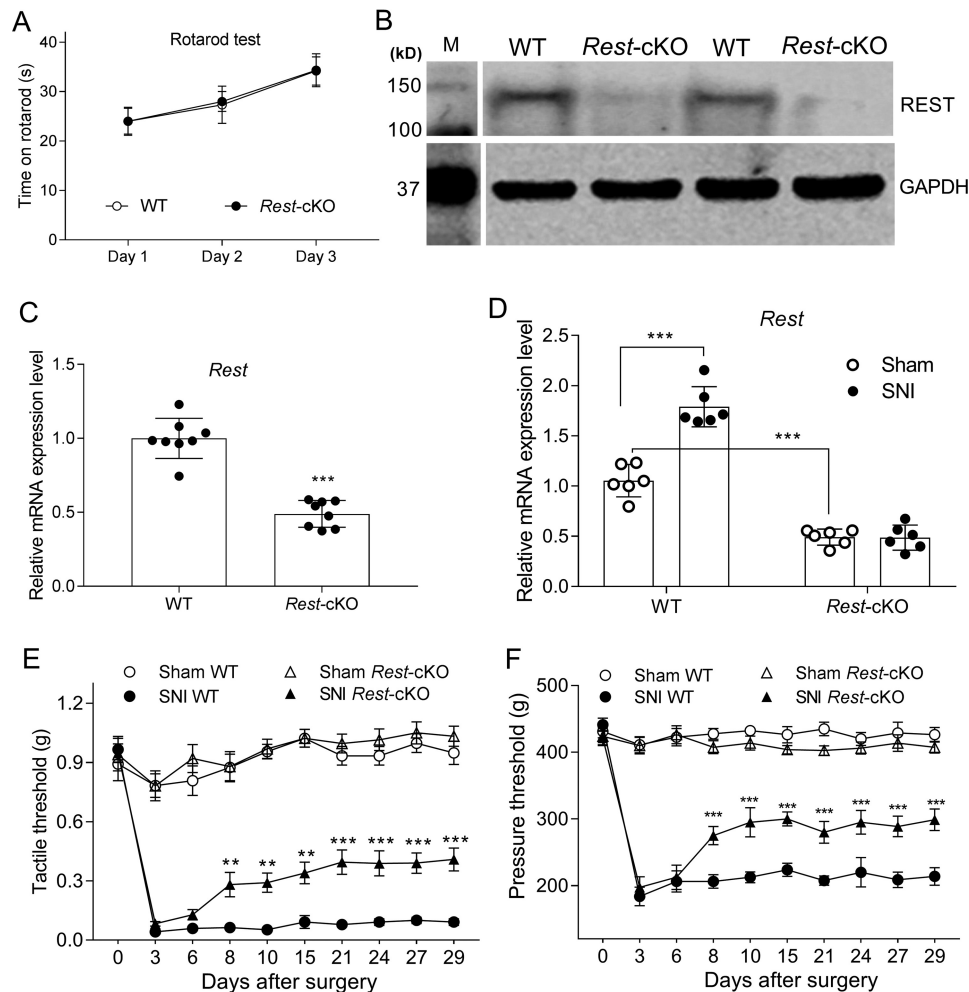
Figure 2. REST in DRG neurons contributes to chronic pain hypersensitivity induced by nerve injury. *A*, motor coordination function, measured with the rotarod test, in WT and *Rest*-cKO mice (*n* = 6 mice/group). *B*, Western blot images showing the REST protein band in the DRG of two WT and two *Rest*-cKO mice. GAPDH was used as an internal control. *M*, molecular weight marker. *C*, real-time PCR data showing the mRNA level of *Rest* in the DRG of WT and *Rest*-cKO mice. ***, $p < 0.001$ (*n* = 8 mice/group, paired Student's *t* test). *D*, real-time PCR data showing the mRNA level of *Rest* in the DRG from WT and *Rest*-cKO mice subjected to sham or SNI surgery. *Tuba1a* was used as an endogenous control, and the mean value in the WT group was set to be 1. ***, $p < 0.001$ (*n* = 6 mice/group, one-way ANOVA). *E* and *F*, time course of changes in the tactile (*E*) and noxious pressure (*F*) withdrawal thresholds in WT and *Rest*-cKO mice subjected to sham or SNI surgery. Data are shown as means ± S.D. (*A–D*) or means ± S.E. (*E* and *F*). **, $p < 0.01$; ***, $p < 0.001$ *versus* the SNI-WT group at the same time point (*n* = 8 mice/group, two-way ANOVA).

level of *Chrm2* in the DRG that was decreased by SNL ($p < 0.0001$, F(2,15) = 21.56, *n* = 6 rats/group, Fig. 5*A*). Neither *Rest* knockdown nor SNL had any effect on the mRNA level of *Chrm4* in the DRG (Fig. 5*B*). Furthermore, treatment with *Rest*-specific siRNA normalized the M2 protein level in the DRG that was reduced by SNL ($p = 0.0012$, F(2,15) = 10.92, *n* = 6 rats/group; Fig. 5*C*).

Because M2 plays a major role in mediating mAChR agonist–induced analgesia (19, 21), we examined whether nerve injury–induced *Chrm2* repression in DRG neurons influences the analgesic effect of muscarine in neuropathic pain. Treatment with *Rest*-specific siRNA only slightly augmented the analgesic effect of intrathecal injection of muscarine in sham control rats (Fig. 5, *D* and *E*). Compared with the sham control group, intrathecal injection of 2 μg of muscarine in SNL rats produced a minimal analgesic effect on tactile allodynia and mechanical hyperalgesia (*n* = 6 rats/group; Fig. 5, *D* and *E*). Strikingly, the effect produced by intrathecal injection of 2 μg of muscarine on pain hypersensitivity was much larger in SNL rats treated with

Rest-specific siRNA than in SNL rats treated with control siRNA (Fig. 5, *D* and *E*).

REST in DRG neurons is required for the nerve injury–induced repression of Chrm2 and the diminished analgesic effect of muscarine

We then used *Rest*-cKO mice to confirm the role of REST in DRG neurons in the diminished analgesic effect of muscarine in nerve injury–induced neuropathic pain. In WT mice, SNI significantly reduced the mRNA ($p = 0.0003$, F(3,20) = 9.93, *n* = 6 mice/group) and protein ($p = 0.0025$, F(3,20) = 6.77, *n* = 6 mice/group) levels of M2 in the DRG (Fig. 6, *A* and *B*). In contrast, in *Rest*-cKO mice, SNI failed to significantly reduce the mRNA and protein levels of M2 in the DRG (Fig. 6, *A* and *B*). The mRNA and protein levels of M2 in the injured DRG of *Rest*-cKO mice were comparable with those in the DRG of sham-operated WT mice.

In WT mice subjected to SNI, intrathecal injection of 0.2 μg and 0.5 μg of muscarine produced only a minimal effect on pain
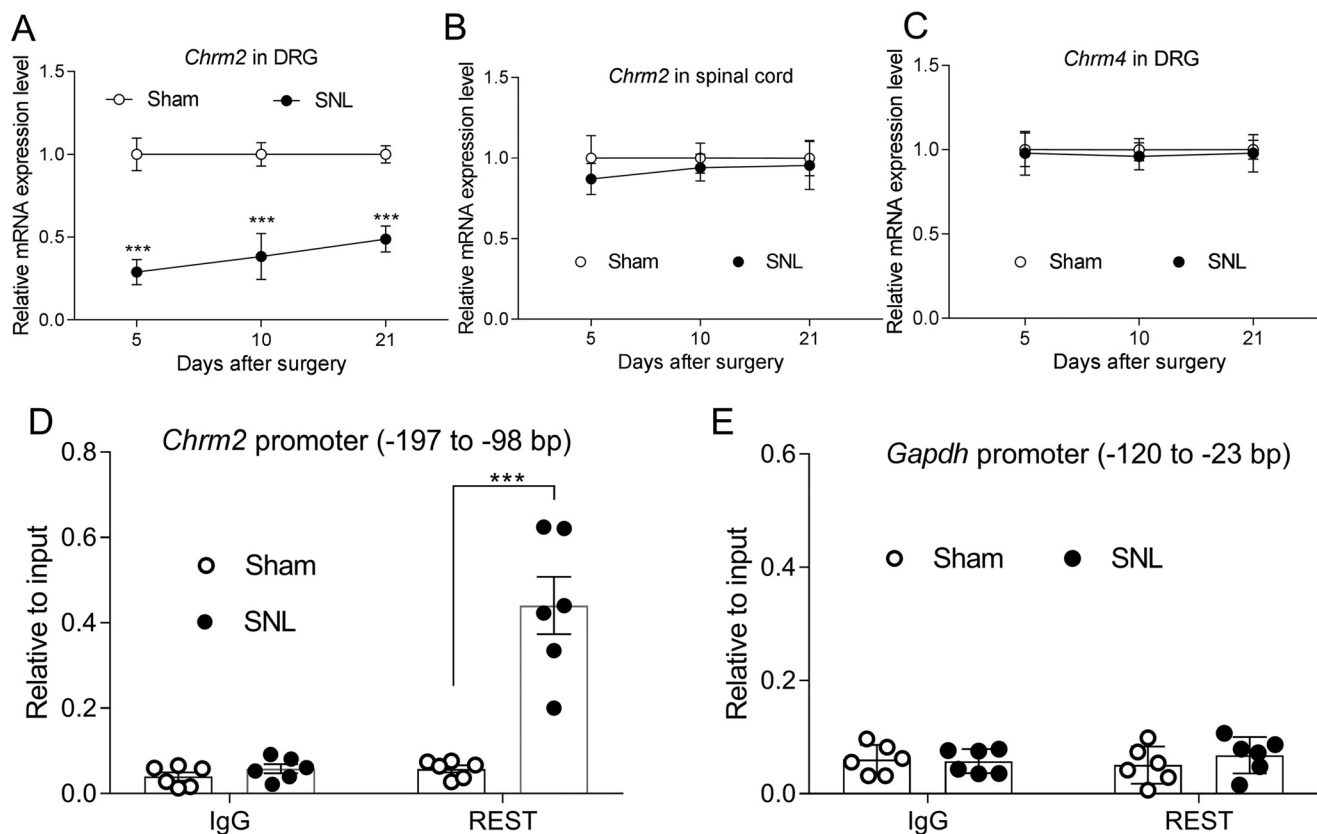
Figure 3. Nerve injury decreases *Chrm2* expression and increases the enrichment of REST at the *Chrm2* promoter in the DRG. *A* and *B*, real-time PCR data showing the mRNA level of *Chrm2* in the rat DRG (*A*) and dorsal spinal cord (*B*) on days 5, 10, and 21 after SNL or sham surgery. *Gapdh* was used as an endogenous control, and the mean value in the sham group was set to be 1 (*n* = 6 rats/group). ***, *p* < 0.001 compared with the sham group at the same time point (two-way ANOVA). *C*, real-time PCR data showing the mRNA level of *Chrm4* in the rat DRG on days 5, 10, and 21 after SNL or sham surgery (*n* = 6 rats/group). *D* and *E*, ChIP-PCR quantification data showing the enrichment of REST at the *Chrm2* promoter (*D*, −197 to −98 bp upstream of ATG) and *Gapdh* promoter (*E*, −120 to −23 bp upstream of ATG) in DRG tissues from sham and SNL rats 21 days after surgery. IgG was used as a negative control for the REST antibody. Data are shown as means ± S.D., and ChIP-PCR values were calculated relative to the input. **, *p* < 0.01 (*n* = 6 rats/group, paired Student's *t* test).

hypersensitivity. By comparison, the analgesic effect of intrathecally administered muscarine at both 0.2 µg and 0.5 µg was markedly enhanced in *Rest*-cKO mice subjected to SNI (*n* = 8 mice/group; Fig. 6, *C–F*). Collectively, these findings indicate that REST in DRG neurons is responsible for nerve injury–induced transcriptional repression of *Chrm2* and the diminished analgesic effect of muscarine in neuropathic pain.

REST in DRG neurons mediates the reduced muscarinic inhibition of glutamatergic input to spinal dorsal horn neurons in neuropathic pain

At the spinal cord level, M2 is predominately expressed at the central terminals of primary afferent nerves and plays a critical role in regulating glutamate release to spinal dorsal horn neurons (14, 18, 30). We performed electrophysiological recordings in spinal cord slices to determine the role of REST-dependent expression of presynaptic M2 in the control of glutamate release from primary afferent nerve terminals after nerve injury. We recorded excitatory postsynaptic currents (EPSCs) monosynaptically evoked by electrical stimulation of the dorsal root, which reflect glutamate release from central terminals of primary afferent nerves (18, 30). Bath application of muscarine at concentrations of 0.1 to 10 µM reduced the amplitude of evoked EPSCs of lamina II neurons of sham control rats treated with control siRNA or *Rest*-specific siRNA in a concentration-

dependent manner (*n* = 12 neurons per group; Fig. 7, *A* and *B*). The baseline amplitude of evoked EPSCs in lamina II neurons was significantly higher in SNL than in sham control rats, as reported previously (4, 31). The inhibitory effect of muscarine (at concentrations of 1–10 µM) on the amplitude of evoked monosynaptic EPSCs was significantly lower in neurons of SNL rats treated with control siRNA (*n* = 11 neurons) than in neurons of sham rats treated with control siRNA (Fig. 7, *A* and *B*). Remarkably, treatment with *Rest*-specific siRNA in SNL rats completely restored the inhibitory effect of muscarine on the amplitude of evoked EPSCs (*n* = 12 neurons; Fig. 7, *A–C*) to the level observed in sham control rats treated with control siRNA.

In addition, we conducted electrophysiological recording in spinal cord slices from WT and *Rest*-cKO mice subjected to SNI or sham surgery. In both WT and *Rest*-cKO mice subjected to sham surgery, bath application of 0.1 to 10 µM muscarine similarly reduced the amplitude of monosynaptically evoked EPSCs of lamina II neurons in a concentration-dependent manner (*n* = 15 neurons/group; Fig. 8, *A* and *B*). Muscarine produced only a small inhibitory effect on the amplitude of evoked monosynaptic EPSCs in WT mice subjected to SNI (*n* = 16 neurons/group; Fig. 8, *A* and *B*). In contrast, in *Rest*-cKO mice subjected to SNI, muscarine potently reduced the amplitude of evoked EPSCs of lamina II neurons (*n* = 15 neurons;
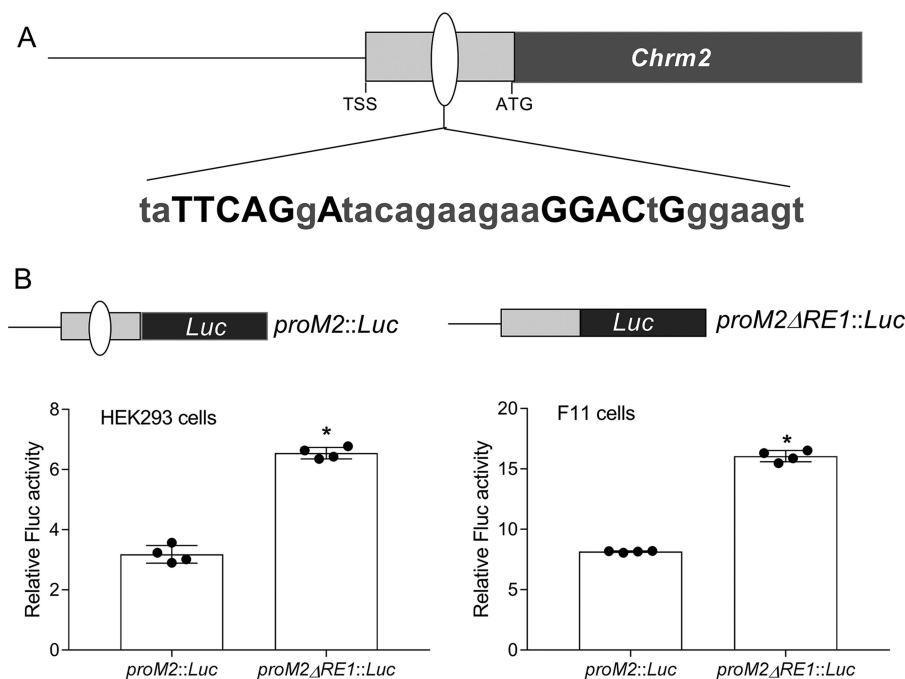
Figure 4. The RE1 site in the *Chrm2* promoter region is required for repressing *Chrm2* expression. *A*, schematic showing that the RE1 site (*white ellipse*) is located in the 5′ UTR (*gray box*) of the *Chrm2* gene promoter (-150 and -119 bp upstream of ATG). The conserved RE1 sequences are indicated by *uppercase letters*. *TSS*, transcription start site. *B*, luciferase (*Luc*) assay analysis of the promoter activity of *Chrm2* with and without the RE1 sequence in HEK293 cells and F11 cells. Although the promoter of *Chrm2* (*proM2::Luc*) can drive luciferase gene expression, the version without the RE1 sequence (*proM2ΔRE1::Luc*) has relatively greater gene promotion activity. Data are shown as means ± S.D. *, $p < 0.05$; $n = 4$ independent experiments/group (Mann–Whitney test).

Fig. 8, *A–C*). These data indicate that REST in DRG neurons plays an essential role in the nerve injury–induced reduction in the ability of mAChR activation to inhibit glutamatergic transmission from primary afferent nerves to spinal dorsal horn neurons.

Discussion

Our study provides new evidence that REST-dependent transcriptional repression in injured DRG neurons plays an important role in the transition from acute to chronic pain after nerve injury. Long-term changes in gene expression profiles in DRG neurons are mediated by epigenetic mechanisms that include DNA methylation, histone modifications, and transcriptional activation and repression (1, 2, 6, 32, 33). By binding to RE1 sites, REST serves as a molecular scaffold that recruits modulatory and epigenetic cofactors to itself. The REST macromolecular repressor complex has both transient and long-term effects on gene transcription in neurons. REST is expressed at higher levels in embryonic and adult neural progenitor cells in neuronal tissues than in nonneuronal tissues (25, 34). In nonneuronal mammalian cells and tissues, REST silences neuron-specific genes (34, 35). REST is expressed at low levels in mature neurons in several brain regions (22, 36). We found in this study that peripheral nerve injury caused a large and sustained increase in REST expression in the DRG. Although previous studies have reported that REST expression in the DRG is increased after sciatic nerve ligation (6, 11, 37), the functional significance of REST up-regulation in neuropathic pain development is still unclear. Interestingly, we found that, although the acute pain phase after nerve injury was similar in *Rest*-cKO and WT mice, genetic ablation of *Rest* in DRG

neurons significantly impaired the development of chronic pain 1 week after nerve injury. Furthermore, we showed that knockdown of REST with siRNA attenuated the chronic pain hypersensitivity induced by nerve injury. These findings suggest that nerve injury–induced up-regulation of REST in DRG neurons predominantly contributes to the maintenance of chronic pain. It has been reported that knockdown of *Rest* with antisense oligonucleotides does not reduce nerve injury–induced pain hypersensitivity (11). However, the antisense treatment was conducted only for the first 6 days (*i.e.* the acute pain phase) after nerve injury (11), which could explain why no effect of *Rest* knockdown on neuropathic pain was observed. As a transcriptional regulator, REST may maintain the chronic phase of neuropathic pain by targeting a select group of genes in the DRG, which may include various voltage-gated K^+ channels and *Oprim1* (2, 6, 11, 38). Nevertheless, the exact REST target genes in the DRG that are involved in the acute-to-chronic pain transition remain to be identified.

Another important finding of our study is that nerve injury causes a sustained reduction in *Chrm2* expression in the DRG and diminishes the analgesic effect of muscarine at the spinal cord level in chronic neuropathic pain. M2 is the most abundant mAChR subtype in the DRG and spinal cord; it mainly mediates mAChR agonist–produced analgesia (12, 19, 20, 39). It has been shown that M2 is down-regulated in facial motoneurons after axotomy (facial nerve transection) (40) and in the hippocampus after traumatic brain injury (41). Glutamate is the major excitatory neurotransmitter that conveys nociceptive information from primary sensory nerves to spinal dorsal horn neurons (42, 43). The M2 sub-
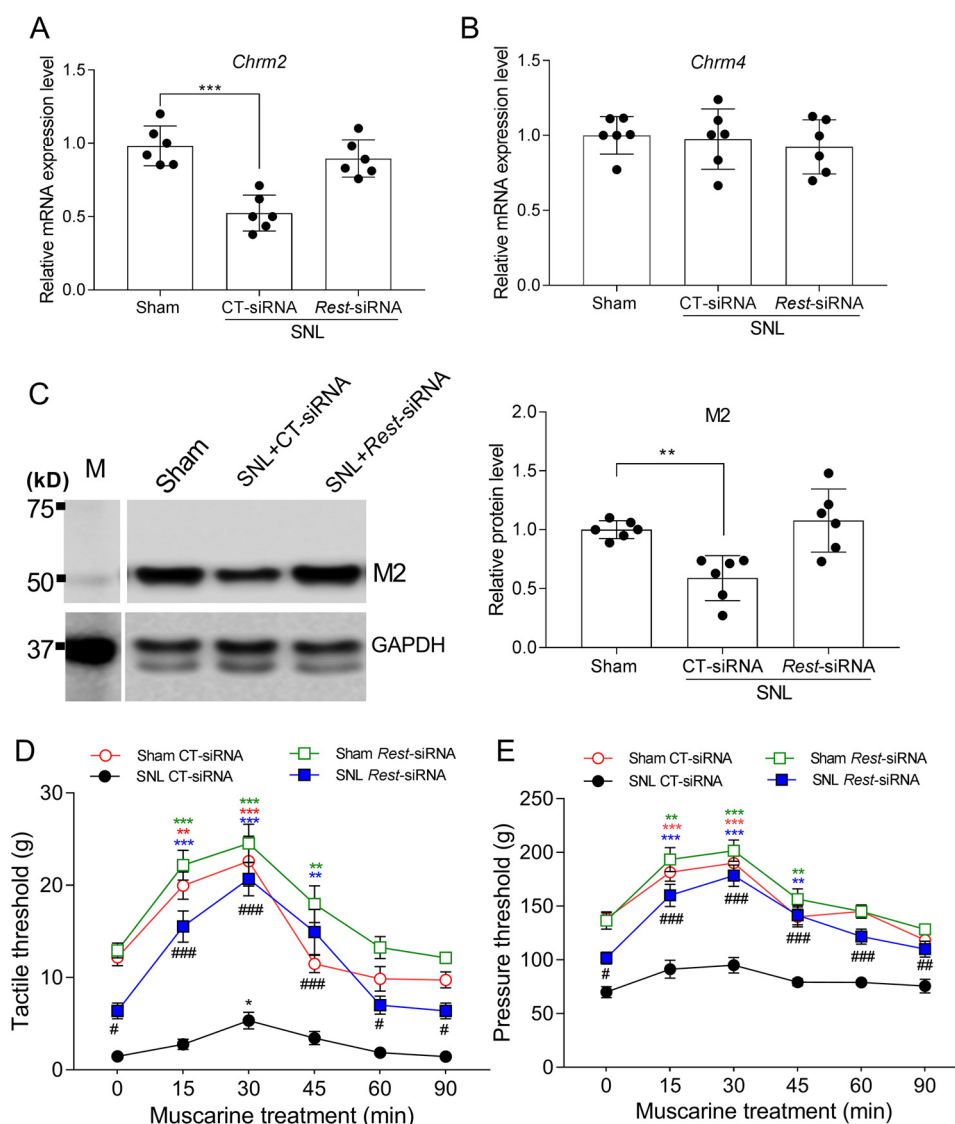
Figure 5. Nerve injury represses *Chrm2* expression in the DRG and diminishes the analgesic effect of muscarine at the spinal cord level through REST. *A* and *B*, real-time PCR data showing the mRNA level of *Chrm2* (*A*) and *Chrm4* (*B*) in the DRG of rats that underwent sham surgery or SNL and were treated intrathecally with control siRNA or *Rest*-specific siRNA (2 μg/day, 5 days) 3 weeks after surgery. ***, $p < 0.001$ ($n = 6$ rats/group, one-way ANOVA). *C*, original blotting images and quantification data showing the protein level of M2 in the DRG of sham and SNL rats treated with control (*CT*) siRNA or *Rest*-specific siRNA. GAPDH was used as an internal control. *M*, molecular weight marker. **, $p < 0.01$ ($n = 6$ rats/group, one-way ANOVA). *D* and *E*, time course of the effects of intrathecal injection of muscarine (2 μg) on the tactile (*D*) and pressure (*E*) withdrawal thresholds in sham and SNL rats treated with control siRNA or *Rest*-specific siRNA 3 weeks after sham or SNL surgery. Data are shown as means ± S.D. (*A–C*) or means ± S.E. (*D* and *E*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the respective baseline (time 0). #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with SNL rats treated with control siRNA ($n = 6$ rats/group, two-way ANOVA).

type expressed at central terminals of primary sensory nerves in the spinal dorsal horn plays a critical role in regulating nociceptive glutamatergic input to dorsal horn neurons (14, 18, 19, 21, 39). Thus, activation of presynaptic M2 in the spinal dorsal horn produces analgesia by inhibiting glutamatergic transmission from primary sensory nerves to dorsal horn neurons (14, 18, 39). Repression of *Chrm2* expression in the DRG after nerve injury can lead to a reduction in the amount of M2 expressed at presynaptic terminals, which accounts for the attenuation of muscarine-mediated inhibition of nociceptive transmission at the spinal cord level.

Another salient finding of our study is that REST plays an obligatory role in nerve injury–induced repression of *Chrm2*

expression in the DRG. Our *in vitro* study showed that the RE1 binding site in the *Chrm2* promoter region is required for silencing of *Chrm2*. Also, nerve injury caused a large increase in enrichment of REST at the *Chrm2* promoter in the DRG. Importantly, we showed that knockdown of *Rest* using siRNA or ablation of *Rest* in DRG neurons completely normalized the expression level of *Chrm2*, which had been diminished by nerve injury. In addition, *Rest* knockdown or *Rest* ablation in DRG neurons fully restored the reduced muscarinic inhibition of glutamate release from primary afferent nerves to spinal dorsal horn neurons, restoring the muscarinic analgesic effect. These results strongly support the notion that REST is essential for nerve injury–induced *Chrm2* silencing and the diminished analgesic effect of muscarine in neuro-
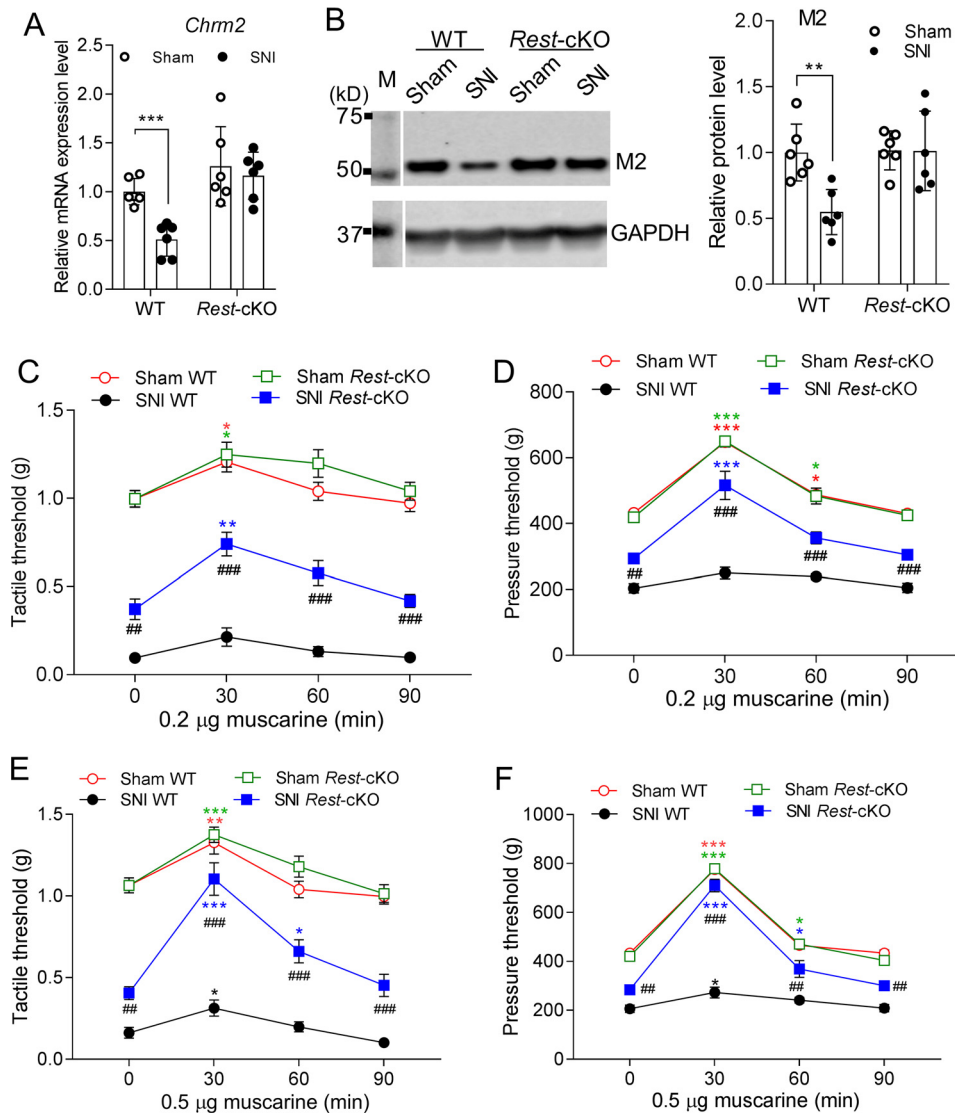
Figure 6. REST in DRG neurons is required for the nerve injury–induced repression of *Chrm2* expression and the diminished analgesic effect of muscarine. *A* and *B*, quantitative PCR (*A*) and Western immunoblotting analyses (*B*) show the effect of SNI on the levels of *Chrm2* mRNA and M2 protein in the DRG of WT control and *Rest*-cKO mice (*n* = 6 mice/group). *M*, molecular weight marker. For the mRNA data, *Tuba1a* was used as an endogenous control, and the value in the WT group was considered to be 1. The amount of M2 protein was normalized to the level of GAPDH in the same samples, and the mean value of M2 levels in sham control WT mice was considered to be 1. **, $p < 0.01$; ***, $p < 0.001$ (*n* = 6 mice/group, one-way ANOVA). *C–F*, time course of the effects of intrathecal injection of 0.2 μg (*C* and *D*) or 0.5 μg (*E* and *F*) muscarine on the tactile and pressure withdrawal thresholds in WT and *Rest*-cKO mice subjected to sham or SNI surgeries. Data are shown as means ± S.D. (*A* and *B*) or means ± S.E. (*C–F*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the respective baseline (time 0). ##, $p < 0.01$; ###, $p < 0.001$ compared with WT mice subjected to SNI (*n* = 8 mice/group, two-way ANOVA).

pathic pain. G9a, a histone methyltransferase, may function as a REST partner (44) in the injured DRG; knockdown of REST or G9a can restore the nerve injury–induced reduction in *Oprm1* expression in the DRG and opioid-induced analgesia in neuropathic pain (11, 38). However, RNA sequencing data show that the reduced mRNA level of *Chrm2* in the DRG caused by nerve injury is not restored by G9a inhibition (2). Thus, other REST-interacting epigenetic enzymes and cofactors, such as histone demethylases and mSin3 (45), may cooperate with REST to create a relatively condensed chromatin feature and thus silence the expression of *Chrm2* in the injured DRG.

Although it has been reported that the RE1 site is required for the promoter activity of *Chrm4* in several nonneuronal cell lines (46–48), we found in this study that neither nerve injury nor *Rest*-specific siRNA knockdown had any effect on the expression level of *Chrm4* in the DRG. It is not fully known what determines the specificity of interaction between REST and its targets as well as the enrichment of REST at the promoter of a given target gene. It should be noted that experiments performed in cell lines do not reflect the cellular environment and epigenetic landscape, which vary with developmental stages, cell types, and disease conditions. For instance, the REST target genes in the hippocampal CA1 altered by ischemia are distinct from those in the hippocampal CA3 related to seizure activity (49, 50). Other transcription factors and epigenetic marks may also influence the REST binding affinity of target genes. Thus, REST likely controls the expression of different ensembles of target genes in different cell types and disease conditions.
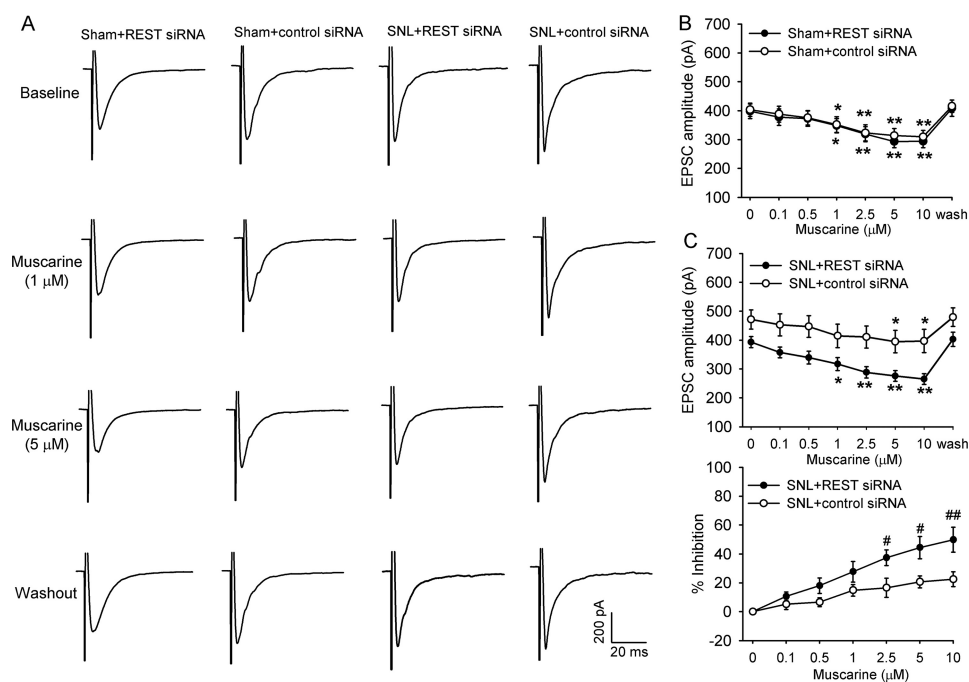
Figure 7. REST knockdown normalizes the inhibitory effect of muscarine on glutamate release from primary afferent terminals in SNL rats. *A–C*, original recording traces (*A*) and mean data (*B* and *C*) showing the effect of bath application of 0.1–10 μM muscarine on the amplitude of evoked monosynaptic EPSCs of dorsal horn neurons in sham control rats treated with *Rest*-specific siRNA ($n = 12$ neurons) or control siRNA ($n = 12$ neurons) and SNL rats treated with *Rest*-specific siRNA ($n = 12$ neurons) or control-siRNA ($n = 11$ neurons). Because the baseline amplitude of evoked EPSCs was different in SNL rats treated with control siRNA and those treated with *Rest*-specific siRNA, the amplitude of evoked EPSCs was normalized to the baseline for each recorded neuron in *C*. Data are shown as means $\pm$ S.E. *, $p < 0.05$; **, $p < 0.01$ compared with the respective baseline. #, $p < 0.05$; ##, $p < 0.01$ compared with SNL rats treated with control siRNA (two-way ANOVA).

In summary, our study provides important new evidence that REST up-regulation in the injured DRG contributes to the transition from acute to chronic pain after nerve injury. Furthermore, *Chrm2* expression in the DRG and the muscarinic analgesic effect are diminished in neuropathic pain through a transcriptional mechanism involving REST. This information suggests new strategies for treating patients with chronic neuropathic pain. The potentiated analgesic effects of muscarine induced by *Rest* knockdown or ablation in neuropathic pain suggest that a combination of REST inhibitors and mAChR agonists may be effective for the treatment of chronic neuropathic pain. REST inhibitors may increase the analgesic efficacy of mAChR agonists, thereby reducing the dose of mAChR agonists and improving their therapeutic windows. In addition, reduced cholinergic activity/M2 levels and increased REST expression have been detected in the brain tissues of patients with Alzheimer and Huntington diseases (51–55). Thus, our findings regarding the transcriptional control of *Chrm2* by REST may also be relevant for understanding the molecular mechanisms underlying the development of these neurodegenerative diseases.

Experimental procedures

Animal models

The surgical procedures and experimental protocols were approved by the Animal Care and Use Committee at the University of Texas MD Anderson Cancer Center. All animals were housed (2–3 rats/cage) on a standard 12:12 h light-dark cycle and received food and water *ad libitum*. Male Sprague-Dawley rats (7–8 weeks old, Harlan, Indianapolis, IN) were subjected to

SNL as described previously (23, 24). Briefly, rats were anesthetized with 2–3% isoflurane, and the left L5 and L6 spinal nerves were exposed and ligated separately with 6-0 silk sutures under a surgical microscope. Sham surgery (without nerve ligation) was performed as the control.

Rest conditional knockout mice (*Rest* cKO) were generated by crossing female mice carrying loxP sites flanking the first coding exon 4 of the *Rest* gene (*Rest*-loxP$^{+/+}$, stock no. 024549, The Jackson Laboratory, Bar Harbor, ME) (25) and male mice with primary sensory neuron–specific *Advillin*-Cre (*Advillin*-Cre$^{+/-}$) (26, 27). *Advillin*-Cre$^{+/-}$: *Rest*-loxP$^{+/-}$ mice obtained from the first generation were crossed with female *Rest*-loxP$^{+/+}$ mice to get *Advillin*-Cre$^{+/-}$: *Rest*-loxP$^{+/+}$ mice (*Rest*-cKO mice). Littermates without Cre (*Advillin*-Cre$^{-/-}$: *Rest*-loxP$^{+/+}$) were used as controls. Mice were ear-marked at the time of weaning (3 weeks after birth), and tail biopsies were used for PCR genotyping. Both male and female adult mice (8–10 weeks of age) were used for electrophysiological and behavioral studies.

SNI was performed as described previously (2, 56). Mice were anesthetized with 2–3% isoflurane, and the sciatic nerve and its three terminal branches (the sural, common peroneal, and tibial nerves) of the left leg were exposed under a surgical microscope. The tibial and common peroneal nerves were tightly ligated with a 6-0 silk suture and sectioned distal to the ligation sites, leaving the sural nerve intact.

Intrathecal catheter placement and treatment

Rats were anesthetized with 2–3% isoflurane. A small incision was made at the back of the neck, and a PE-10 catheter (8
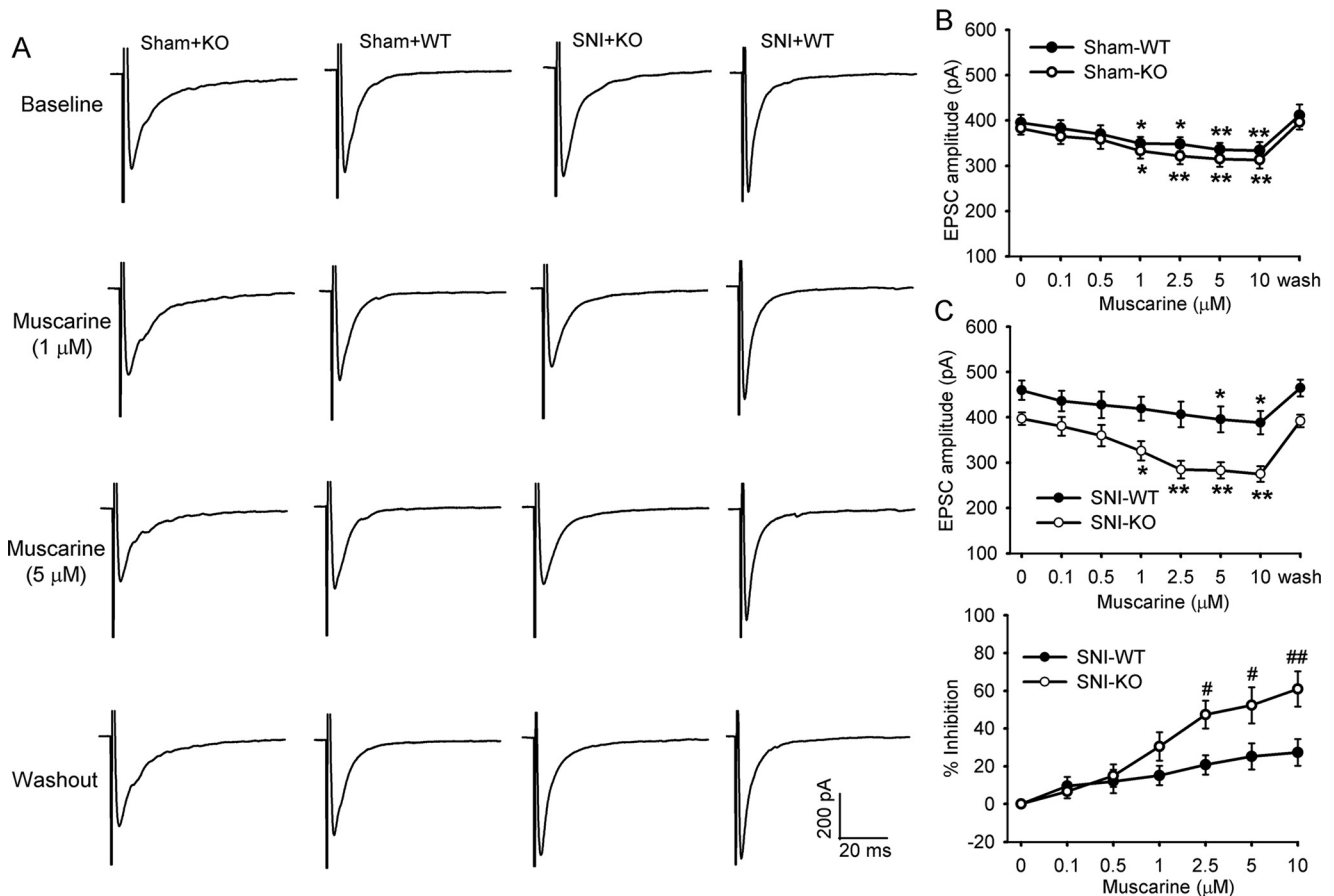
Figure 8. Ablation of REST in DRG neurons restores the inhibitory effect of muscarine on glutamate input from primary afferent terminals to dorsal horn neurons in mice with nerve injury. *A–C*, representative recording traces (*A*) and mean data (*B* and *C*) showing the inhibitory effect of bath application of 0.1–10 μM muscarine on the amplitude of EPSCs of dorsal horn neurons evoked monosynaptically from dorsal root stimulation in WT mice subjected to SNI (n = 16 neurons) or a sham surgery (n = 15 neurons) and *Rest*-cKO mice subjected to SNI (n = 15 neurons) or a sham surgery (n = 15 neurons) 3 weeks after surgery. Because the baseline amplitude of evoked EPSCs was different in WT and *Rest*-cKO mice subjected to SNI, the amplitude of evoked EPSCs was normalized to the baseline for each recorded neuron in *C*. Data are shown as means ± S.E. *, $p < 0.05$; **, $p < 0.01$ compared with the respective baseline. #, $p < 0.05$; ##, $p < 0.01$ compared with WT mice subjected to SNI (two-way ANOVA).

cm) was inserted via a small opening made in the atlanto-occipital membrane of the cisterna magna so that the catheter tip reached the lumbar spinal enlargement (57). The rostral end of the catheter was exteriorized, and the wound was closed with sutures. The animals were allowed to recover for 5–7 days before intrathecal injections.

Rest siRNA (SASI-RN01-00040248) and control siRNA (SIC001, MISSION siRNA Universal Negative Control #1) were obtained from Sigma-Aldrich (St. Louis, MO). The sequence for *Rest* was GCAAUUAUGUGGCCUCUAA[dT][dT]. The transfection reagent iFect (NI35130, Neuromics, Edina, MN) was used to dissolve the siRNA for intrathecal delivery (2, 58). A mixture of 2 μg of siRNA with 10 μl of iFect was intrathecally injected daily for 5 days beginning 3 weeks after surgery. Muscarine chloride (M6532) was obtained from Sigma-Aldrich.

Nociceptive behavioral tests

For tactile sensitivity measurements, rats or mice were habituated on a wire grid panel for 30 min before testing. The plantar surface of the hind paw was subjected to a series of von Frey filaments (Stoelting, Wood Dale, IL). Brisk withdrawal or flinching of the paw was considered a positive response. In the absence of a response, we applied the filament of the next

greater force. We calculated the tactile threshold that produced a 50% likelihood of a withdrawal response using the "up–down" method (24).

To measure the mechanical nociceptive threshold, the paw pressure test was performed using an analgesiometer (Ugo Basile, Varese, Italy). A foot pedal was pressed to activate a motor that applied a linearly increasing force to the hind paw (19). The pedal was immediately released when the animal displayed pain by either withdrawing the paw or vocalizing. Each trial was repeated two or three times at 2-min intervals, and the mean value was used as the force needed to produce a withdrawal response.

Rotarod test

The rotarod test was carried out on a rotarod apparatus (LE8205, Harvard Apparatus, Holliston, MA) programmed to accelerate from 4 to 40 rpm during a 120-s window. Mice were placed on a moving rotarod (4 rpm) and habituated for 5 min. After accelerating, the time on the rotarod was recorded, and the mean value from three repeated trials was obtained. The test was conducted on three consecutive days.

Transcriptional regulation in neuropathic pain

Western immunoblotting

Total protein was extracted from the lumbar DRG and spinal cord tissues (at L5/L6 levels in rats and at L3/L4 levels in mice) using an extraction buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and 1 mM NaF in the presence of a proteinase inhibitor mixture (Sigma-Aldrich). The protein concentration was determined using the DC protein assay kit (Bio-Rad). 30 μg of protein from each sample was loaded and separated on 4–12% BisTris SDS-PAGE gel (Invitrogen). The following primary antibodies were used: rabbit anti-REST (07-579, 1:5,000, Millipore) [59], rabbit anti-M2 (ab109226, 1:5,000, Abcam) [60], and rabbit anti-GAPDH (5174, 1:5,000, Cell Signaling Technology, Danvers, MA). Horseradish peroxidase–conjugated anti-rabbit IgG (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary antibody. The protein bands were detected with an enhanced chemiluminescence kit (Thermo Fisher Scientific), and the protein band density was visualized and quantified using an Odyssey Fc Imager (LI-COR Biosciences, Lincoln, NE).

Quantitative PCR

Total RNAs were extracted from the lumbar DRG and spinal cord tissues using TRIsure (BIO-38032, Bioline, Taunton, MA). After treatment with RNase-free DNase (79254, Qiagen, Hilden, Germany), 1 μg of RNA was used for reverse transcription with the RevertAid RT reverse transcription kit (K1619, Thermo Fisher Scientific, Waltham, MA). 2 μl of complementary DNA diluted five times was added to a 20-μl reaction volume with SYBR Green real-time PCR mixture (A25780, Thermo Fisher Scientific). Real-time PCR was performed using the QuantStudio 7 Flex real-time PCR system (Applied Biosystems, Waltham, MA). The thermal cycling conditions were as follows: 1 cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and at 60 °C for 45 s. The following primers were used: rat *Chrm2* forward, CAC CCC AAC GAG TAC CAC TGT AGA; rat *Chrm2* reverse, CTG GTC ACT TTC TTT TCC CGG GA; rat *Rest* forward, GTT CAA CAC GTT CGA ACT CAC ACA G; rat *Rest* reverse, GGG TCA CTT CGT GCT GAT TAG AGG; rat *Chrm4* forward, TGC CAG TAT CGG AAC ATC GG; rat *Chrm4* reverse, TGG TAC CTC ACG GTG TCT GG; rat *Rcor1* forward, CGT ACC GAG TGG TGT GTG AT; rat *Rcor1* reverse, TTC CAC ATG GTT AGT GCG GT; rat *Gapdh* forward, CAT CCC AGA GCT GAA CGG GAA G; rat *Gapdh* reverse, GTC CTC AGT GTA GCC CAG GAT GC; mouse *Chrm2* forward, AGT GTG GAC AAT TGG CTA CTG G; mouse *Chrm2* reverse, ACC TTG TAG CGC CTA TGT TCT; mouse *Rest* forward, ATC GGA CGC GGG TAG CGA G; mouse *Rest* reverse, GGC TGC AGT TCA GCT TTT CG; mouse *Tuba1a* forward, CCA CTA CAC CAT GGC AAC GGA GA; and mouse *Tuba1a* reverse, GGA GGT GAA GCC AGA GCC AGT.

Vector constructs

A fragment (~770 bp) upstream of ATG in the *Chrm2* gene that contains RE1 binding sites was amplified using paired primers: GCG *AAG CTT* GGC CAG AGC TAC CTC TTC TGC AA and GCG *CCA TGG* TTT GTG TTC AGT AGT CAA GTG GCC A. After digestion with HindIII and NcoI, the sequence was ligated to pGL3 luciferase reporter vectors (E1751, Promega, Madison, WI) to generate a *proM2::Luc* (WT version) vector. The vector for proM2-del-Luc (RE1-deleted version) was obtained by deleting the RE1 binding site from vector *proM2ΔRE1::Luc* using the In-Fusion HD Cloning Plus kit (638916, Clontech Laboratories, Inc., Mountain View, CA) and the paired primers TAT ACT GTT CCC TGT CTG ACC AGG C and A GGG AAC AGT ATA ta TTC TCA AAA GGA AGA AAT CTG ATG TGT T. All constructs were confirmed by DNA sequencing.

Luciferase reporter assay

A luciferase reporter assay was carried out using the Luc-Pair Duo-Luciferase Assay Kit 2.0 (LF001, GeneCopoeia, Rockville, MD). F11 and HEK293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (10-013-CV, Corning, Corning, NY) supplemented with 10% fetal bovine serum (F4135, Sigma-Aldrich) and 1× penicillin–streptomycin solution (30-002-CI, Corning) in an incubator at 37 °C in 5% CO_2. Cells were grown in a 24-well cell culture plate (662160, Greiner Bio-One, Monroe, NC) to $4 – 8 \times 10^4$ cells/well and transfected with a two-plasmid mixture (0.5 μg of pRL-TK with 0.5 μg of *proM2::Luc* or 0.5 μg of pRL-TK with 0.5 μg of *proM2ΔRE1::Luc*) using Lipofectamine 3000 reagent (L3000-075, Invitrogen). Following 24 h of incubation, cells were washed with PBS and lysed with 100 μl of 1× lysis buffer at 25 °C for 10 min. FLuc assay working solution was then added to measure firefly luciferase, and RLuc working solution was added to measure *Renilla* luciferase using the 20/20ⁿ luminometer (Turner Biosystems, Sunnyvale, CA). The ratio of firefly luciferase to *Renilla* luciferase was used to indicate the promoter activity.

ChIP

ChIP assays were performed as described previously [2, 61]. Briefly, lumbar DRG (L5/L6) tissues were isolated and cross-linked with 2 mM disuccinimidyl glutarate for 35 min, followed by 2% formaldehyde for another 20 min at 25 °C. The DRG tissues were lysed with lysis buffer and sonicated to fragments of around 200 to 1000 bp using a water bath sonicator (Qsonica, Newtown, CT) at 4 °C (40 cycles of 30 s on and 30 s off). Chromatin was pulled down using Dynabeads Protein G magnetic beads (10003D, Thermo Fisher Scientific) conjugated with an anti-IgG antibody (ab124055, Abcam, Cambridge, MA) or anti-REST antibody (07-579, Millipore, Burlington, MA). After de-cross-linking, DNA was recovered using the QIAquick PCR purification kit (58106, Qiagen). Data were analyzed and corrected by input (10% of the amount used for ChIP). Primers used for the ChIP assay were as follows: rat *Chrm2* forward, TT CCA TCT AGA GTT TTT GCC TCT TGC C; rat *Chrm2* reverse, CAG ATT TCT TCC TTT TGA GAA ACT CCC; rat *Gapdh* forward, CAT TGC AGA GGA TGG TAG AGG A; and rat *Gapdh* reverse, GGA GAA GAA AGT CAG ATT AGC GTG G.

Electrophysiological recordings in spinal cord slices

The lumbar spinal cord was obtained through laminectomy from rats or mice anesthetized with isoflurane. Spinal cords were placed in ice-cold sucrose artificial cerebrospinal fluid (234 mM sucrose, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 12 mM glucose, and 25 mM $NaHCO_3$, presaturated with 95% O_2 and 5% CO_2). Transverse slices (400 μm) were cut in ice-cold sucrose artificial cerebrospinal fluid using a vibratome. The slices were preincubated in Krebs solution (117 mM NaCl, 3.6 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 11 mM glucose, and 25 mM $NaHCO_3$, oxygenated with 95% O_2 and 5% CO_2) at 34 °C for at least 1 h. The slices were then transferred into a glass-bottomed chamber (Warner Instruments, Hamden, CT) and perfused continuously with Krebs solution at 3 ml/min at 34 °C.

Neurons from the lamina II outer region were selected for whole-cell patch clamp recordings because they are involved in processing nociceptive input from primary afferent nerves (43, 62). A glass pipette (5–10 megaohm) was filled with an internal solution containing 135 mM potassium gluconate, 5 mM tetraethylammonium chloride, 2 mM $MgCl_2$, 0.5 mM $CaCl_2$, 5 mM HEPES, 5 mM EGTA, 5 mM magnesium ATP, 0.5 mM sodium GTP, 1 mM guanosine 5′-O-(2-thiodiphosphate) trilithium salt, and 10 mM lidocaine N-ethyl bromide (pH adjusted to 7.2–7.4 with 1 M KOH and osmotic pressure to 290–300 mosmol). EPSCs were elicited by electrical stimulation (0.2 ms, 0.6 mA, and 0.1 Hz) of the dorsal root and recorded at a holding potential of −60 mV. Monosynaptic EPSCs were identified on the basis of the constant latency and absence of conduction failure of evoked EPSCs in response to a 20-Hz electrical stimulation (18, 30). The input resistance was continuously monitored, and the recording was terminated when the input resistance changed by more than 15%. Signals were recorded using an amplifier (MultiClamp700B, Axon Instruments Inc., Union City, CA), filtered at 1 to 2 kHz, digitized at 10 kHz, and stored for off-line analysis. All drugs were freshly prepared in artificial cerebrospinal fluid before the experiments and delivered via syringe pumps to reach their final concentrations.

Statistical analysis

Data are presented as means ± S.E. or means ± S.D., as specified in the figure legends. Although no statistical methods were used to predetermine sample sizes, our sample sizes were similar to those generally employed in the field. Data collection was randomized, and the investigators performing behavioral tests and electrophysiological recordings were blinded to the treatment. In electrophysiological experiments, only one neuron was recorded from each spinal cord slice, and at least six animals were used for each group. The amplitude of the evoked EPSCs was quantified by averaging 10 consecutive EPSCs with Clampfit 10.0 software (Axon Instruments). We used Student's *t* test to compare the mRNA and protein levels in the two groups. Mann–Whitney nonparametric test was used for luciferase reporter assay data analysis. To determine the effects of treatment on the withdrawal thresholds, we used repeated measures analysis of variance followed by Dunnett's post hoc test. We used one-way and two-way analysis of variance followed by Tukey's post hoc test to compare values in more than two groups. Statistical analysis was performed using Prism version 7 (GraphPad Software Inc., La Jolla, CA). A *p* value of less than 0.05 was considered to be statistically significant.

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