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Chronic compression or acute dissociation of dorsal root ganglion induces cAMP-dependent neuronal hyperexcitability through activation of PAR2

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ABSTRACT

Chronic compression (CCD) or dissociation of dorsal root ganglion (DRG) can induce cyclic adenosine monophosphate (cAMP)-dependent DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia. Here, we report that protease-activated receptor 2 (PAR2) activation after CCD or dissociation mediates the increase of cAMP activity and protein kinase A (PKA) and cAMP-dependent hyperexcitability and hyperalgesia in rats. CCD and dissociation, as well as trypsin (a PAR2 activator) treatment, increased level of cAMP concentration, mRNA, and protein expression for PKA subunits PKA-RII and PKA-c and protein expression of PAR2, in addition to producing neuronal hyperexcitability and, in CCD rats, thermal hyperalgesia. The increased expression of PAR2 was colocalized with PKA-c subunit. A PAR2 antagonistic peptide applied before and/or during the treatment, prevented or largely diminished the increased activity of cAMP and PKA, neuronal hyperexcitability, and thermal hyperalgesia. However, posttreatment with the PAR2 antagonistic peptide failed to alter either hyperexcitability or hyperalgesia. In contrast, an adenylyl cyclase inhibitor, SQ22536, administrated after dissociation or CCD, successfully suppressed hyperexcitability and hyperalgesia, in vitro and/or in vivo. Trypsin-induced increase of the intracellular calcium $[Ca^{2+}]_i$ was prevented in CCD or dissociation DRG neurons. These alterations were further confirmed by knockdown of PAR2 with siRNA. In addition, trypsin and PAR2 agonistic peptide-induced increase of cAMP was prevented by inhibition of PKC, but not Gαs. These findings suggest that PAR2 activation is critical to induction of nerve injury-induced neuronal hyperexcitability and cAMP-PKA activation. Inhibiting PAR2 activation may be a potential target for preventing/suppressing development of neuropathic pain.

1. Introduction

Injury or inflammation involving axon or somata of dorsal root ganglion (DRG) neurons produces long-lasting neuronal hyperexcitability in diverse species and often causes neuropathic pain [21,32,34,39]. Although electrophysiological mechanisms contributing to the expression of hyperexcitability in DRG neuronal somata following injury to the peripheral nervous system have been investigated intensively, not much is known about the signals that induce and maintain this hyperexcitability. We have recently shown that the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway is important for maintaining both DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia in an animal model of neuropathic pain: chronic compression of DRG (CCD treatment) [22], and further, dissociation of DRG neurons produces acute cAMP-dependent hyperexcitability that is remarkably similar to that produced by CCD [24,36]. These findings suggest that injury-related stress, caused in these cases by either dissociation or CCD, induces an increase in electrophysiological responsiveness to cAMP, and this increased responsiveness is important for maintaining neuronal hyperexcitability and behaviorally expressed hyperalgesia. The upstream molecules mediating such an increased activity of cAMP-PKA pathway following these 2 dissimilar forms of injury-related stress remain unknown.

During CCD treatment, somata of DRG neurons are mechanically compressed and probably exposed to inflammatory mediators [14,22,23,25]. In DRG dissociation, trypsin was used to conduct proteolysis of tissue extracellular matrix and primary cell isolation in addition to the mechanical stress and the accompanied inevitable injury during dissociating process [4,5,36]. Trypsin excites neurons by cleaving protease-activated receptors (PARs) subtype PAR2 [27]. PARs, currently 4 subunits PAR1-4, are a family of G-protein-coupled receptors activated by endogenous serine

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proteases that cleave the N-terminal domain of the receptor, unmasking a "tethered ligand" sequence [13]. PAR2 is broadly distributed in neurons, including a large part of DRG neurons, fibroblasts, and inflammatory cells [37], and has been shown to be involved in neurogenic inflammation, nociceptive pathway, and hyperalgesia, probably partly through releasing calcitonin gene-related peptide and substance P [16,29,31]. It is also suggested that PAR2 activation can increase cAMP level [3,13]. Recent studies indicate that trypsin and PAR2 activating peptide can excite the DRG neurons [2,12]. Here, we provide the first evidence that the DRG neuronal PAR2 may be activated during prolonged compression in vivo as well as acute dissociation in vitro and involved in mediating the cAMP-dependent DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia. A preliminary report of some of these data has appeared in abstract form [9].

2. Methods

2.1. Experimental animals, anesthesia, drugs, and administration

Adult male Sprague-Dawley rats (180–250 g wt) (Charles River Laboratories, Wilmington, MA, USA) were used. All protocols were approved by the Institutional Animal Care and Use Committees. Surgeries were done under anesthesia with pentobarbital (50 mg/kg intraperitoneally). We purchased trypsin, soy bean trypsin inhibitor (SBI), adenylyl cyclase inhibitor SQ22536 (SQ), and PKC inhibitor GF109203X (GFX) from Sigma (St. Louis, MO, USA); PAR2 activating peptide 2-(2-Furoyl)-LIGRLO-NH_2 (PAP), PAR2 antagonistic peptide FSLLRY-NH_2 (PIP), and related control peptide LRGILS-NH_2 (PNP) from Bachem Inc (Torrance, CA, USA); and Gαs-subunit-selective G-protein antagonist NF449 from Calbiochem Inc. (Gibbstown, NJ, USA). Trypsin (0.5 mg/mL), SBI (1 mg/mL), PAP (50 μM), PNP (50 μM), PIP (100 μM), and SQ (0.1 mM) were applied onto the DRG neurons during patch-clamp whole-cell recordings in vitro. To determine the level of cAMP concentration and PKA activity, the intact or dissociated DRG cells were incubated for 30 minutes in bath solution containing trypsin, PAP, PNP, PIP, NF449 (1 μM), or GFX (2 μM) or their combination, as indicated. PIP (1 mM), PNP (1 mM), and SQ (1 mM) (each in 10 μL) were injected, respectively, in vivo into the intervertebral foramen (ivf) through a silicon tube connected to the previously implanted hollow stainless steel rod (see Section 2.3).

2.2. Small-interference RNA (siRNA) knockdown of PAR2

Stealth RNAi siRNA (set of 3 oligos) that targets rat PAR2 (F2rl1, GenBank Accession No. NM_053897.2) and Stealth RNAi Negative Control High GC (catalog no. 12935-400) were purchased from Invitrogen Inc (Carlsbad, CA, USA). Sequences were as follows. The siRNA1: ID RSS300559, 5′-GCUCUGCAAGGUGCUCAUUGGCUUU-3′ and 5′-AAAGCCAAUGAGCACCUUGCAGAGC-3′; siRNA2: ID RSS300560, 5′-CCAGCUCUUACUCCUCCAGCUCAA-3′ and 5′-UUGAGCUGGAGGAGUAAGAGCUGGA-3′; and siRNA3: ID RSS300558, 5′-CAUGGACGAGCACUCGGAGAAGAAA-3′ and 5′-UUUCUUCUCCGAGUGCUCGUCCAUG-3′. Sequences were subjected to a BLAST search (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov) to ensure that there was no significant homology with other genes. Stealth RNAi used in our experiments was 25-bp blunt-end dsRNA chemically modified to eliminate nonspecific stress response of interferon.

The siRNA (siRNA1, siRNA2, or siRNA3 described in the paragraph above) was dissolved in RNase-free water at the concentration of 1 μg/μL as stock solution. The siRNA was mixed with branched polyethyleneimine (PEI; Sigma) 10 minutes at room temperature before in vivo intrathecal (i.t.) injection, to increase cell membrane penetration. PEI was dissolved in 5% glucose, and 1 μg of siRNA was mixed with 0.18 μL of PEI [11,28]. In procedures of the intact DRG neuron preparations from naïve rats, the PAR2 siRNA was administered daily (each at 2 μg, i.t.) for 3 consecutive days. The L_4 and L_5 DRGs were taken from the rats 24 hours after the last siRNA injection. In the rats that received CCD treatment, pretreatment of siRNA was administered prior to surgery (daily for 3 consecutive days, each at 2 μg). After surgery, siRNA was continuously administered in the same dose daily for another 5 consecutive days during CCD treatment. Then the compressed DRGs at L_4 and L_5 were removed from the CCD rats 24 hours after the last siRNA injection. PAR2 knockdown was analyzed by immunoblotting with antibody to PAR2.

2.3. CCD

Hollow stainless steel rods were surgically implanted unilaterally into the ivf at L_4 and L_5 to chronically compress the DRG (CCD treatment [22]). The hollow stainless steel rods were made from 25-gauge needles. Some of these rods had one hole drilled on each side and one outlet on each end to permit delivery of drugs to the DRG during compression [24]. The other end of the tubing was sealed, except when injecting drugs. In some experiments, the rod was implanted into the ivf and connected to silicon tubing filled with saline. The PIP, PNP, and SQ were injected, respectively, through this tubing during CCD treatment and expected to prevent or inhibit PAR2 activation.

2.4. Excised, intact DRG neuron preparation

This preparation allows us to test DRG neurons while still in place in excised ganglia. The protocol was the same as that we have described previously [24,36]. The intact, L_4 and/or L_5 DRG was treated with collagenase (type P, 1 mg/mL, Roche Diagnostics, Indianapolis, IN, USA) for 30 minutes at 35°C and then incubated at room temperature for patch-clamp recordings or other purposes. Naïve DRGs were used as a control for the intact DRGs in the enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR), Western blot, immunofluorescent staining, and histological analysis. Compared with the intact DRG (group of Intact), the naïve DRGs (group of Naïve) were taken from naïve rats and did not receive any further treatments. The intact DRGs were also taken from rats that had previously received CCD treatment for 7–10 days. These intact, CCD DRGs are named "CCD DRGs" in this article. The intact DRGs from naïve rats were also used as control for the intact CCD DRG preparation.

2.5. Dissociation of DRG neurons (dissociation treatment)

The protocol was the same as that we used for preparing dissociated neurons for patch-clamp recordings [36].The excised L_4 and/or L_5 DRGs from naïve rats were minced and then the fragments were transferred into the buffered solution containing collagenase (type IA, 1 mg/mL; Sigma) and trypsin (0.5 mg/mL), incubated for 30 minutes at 35°C, then removed, rinsed, and put into the buffered solution containing DNase (0.2 mg/mL; Sigma). Individual neurons were dissociated by passing DRG fragments through a set of fire-polished glass pipettes with decreasing diameter.

2.6. Level of cAMP and PKA activity determination

cAMP concentration and PKA activity were measured by ELISA using cAMP Enzyme Immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) and PKA Enzyme Immunoassay kit (R&D systems, Minneapolis, MN, USA), respectively, according to the manufacturer's instructions.

2.7. mRNA isolation and RT-PCR

The mRNA was isolated using Oligotex Direct mRNA Mini Kit (QIAGEN Inc, Valencia, CA, USA). The samples were treated with DNase I (0.2 U/μl; Ambion, Austin, TX, USA). The RT-PCR was carried out by using SuperScript One-Step RT-PCR with Platinum Tag (Invitrogen). The relative mRNA level of PKA subunit gene expression was analyzed using Quantity One (Bio-Rad Laboratories, Hercules, CA, USA). The statistical results were obtained from 3 repeats of the experiment. The primer sets [19] synthesized by Integrated DNA Technologies (Coralville, IA, USA): PKA-RI forward 5′-CAGCTACCGGAGAATCCTCATGGG-3′; reverse 5′-ATCTGAGCATGGGCCAAGGACG-3′ PKA-RII forward 5′-ACCTCAGACGGCTCCCTTTG-3′; reverse 5′-CGTCTCCAACCGCATAAGCAG-3′ PKA-C forward 5′-ACCTTGGGAACGGGTTCCTTCG-3′, reverse 5′-TACACCCAATG CCCACCAGTCC-3′; β-actin forward 5′-TCTACAATGAGCTGCGTGTG-3′, reverse 5′-AATGTCACGCACGATTTCCC-3′.

2.8. Western blot analysis

Expression of PAR2 protein was examined by Western blot analysis. The membranes were incubated with the primary antibodies anti-PAR2 (SAM11, 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-glyceraldehyde 3-phosphate dehydrogenase (1:2000, Sigma). The blots were developed using a SuperSignal WestPico Kit (Thermo Scientific, Rockford, IL, USA) with horseradish peroxidase-conjugated secondary antibodies (R&D Systems). Data were analyzed with the Molecular Imager (ChemiDoc XRS, Bio-Rad Laboratories) and the associated software Quantity One-4.6.5 (Bio-Rad Laboratories).

2.9. Immunofluorescent staining and histological analysis

DRG sections (10 μm) were first blocked with 10% goat serum then incubated overnight with primary antibodies (anti-PKA RII, 1:100, Epitomics, Burlingame, CA, USA; anti-PKA-c, 1:50, Cell Signaling Technology, Danvers, MA, USA) or rabbit immunoglobulin G (IgG), as an isotype control (1:200, Vector Laboratories, Burlingame, CA, USA). The secondary antibody was fluorescent-labeled anti-rabbit IgG, (1:200, Vector Laboratories). The sections were then either incubated with propidium iodide counterstaining solution or stained with Alexa-488-labeled anti-PAR2 antibody (SAM11, Santa Cruz Biotechnology). The morphologic details of PKA immunofluorescent staining on DRG neurons were examined using a fluorescence microscope. Ten random fields at 400× magnification were selected from each section for counting the positive stained cells.

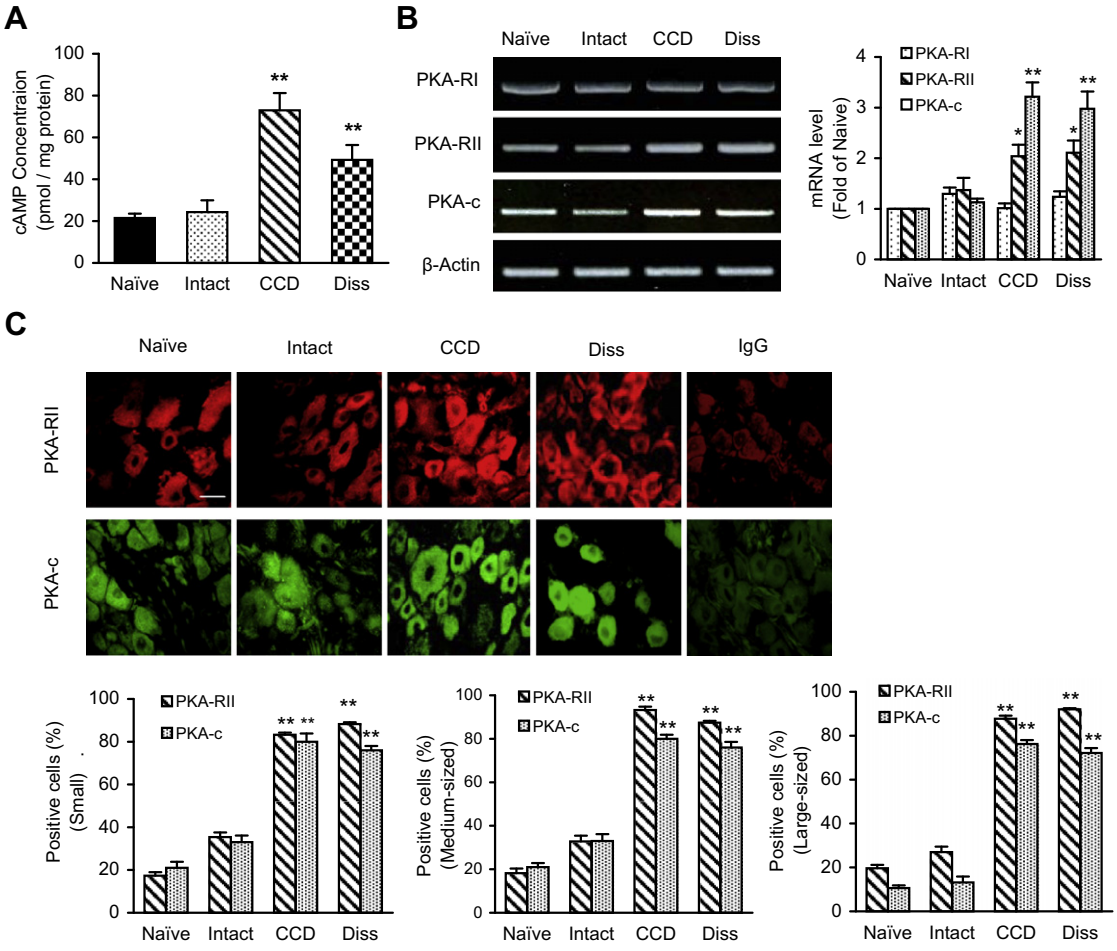

Fig. 1. Alteration of level of cyclic adenosine monophosphate (cAMP) concentration and mRNA level and protein expression of protein kinase A (PKA) subunits in dorsal root ganglion (DRG) after chronic compression of DRG (CCD) and dissociation (Diss) treatment, respectively. (A) Level of cAMP concentration was measured by enzyme-linked immunosorbent assay using cAMP Enzyme Immunoassay kit. (B) mRNA level of PKA subunits, PKA-RI, PKA-RII, and PKA-c analyzed by reverse transcription polymerase chain reaction (RT-PCR). Left: representative bands. Right: data summary. Three samples were included in each group and 4 ganglia in each sample. The samples were collected 7 days after CCD or 2 hours after Diss. (C) Protein expression of PKA subunits PKA-RII and PKA-c by immunostaining. Each group included in the small, medium-, and large-sized cell categories included 500 cells counted from 5 ganglia (100 cells from each ganglion). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate the significant difference compared with the corresponding control group in Naïve. Bar in (C): 30 μm.

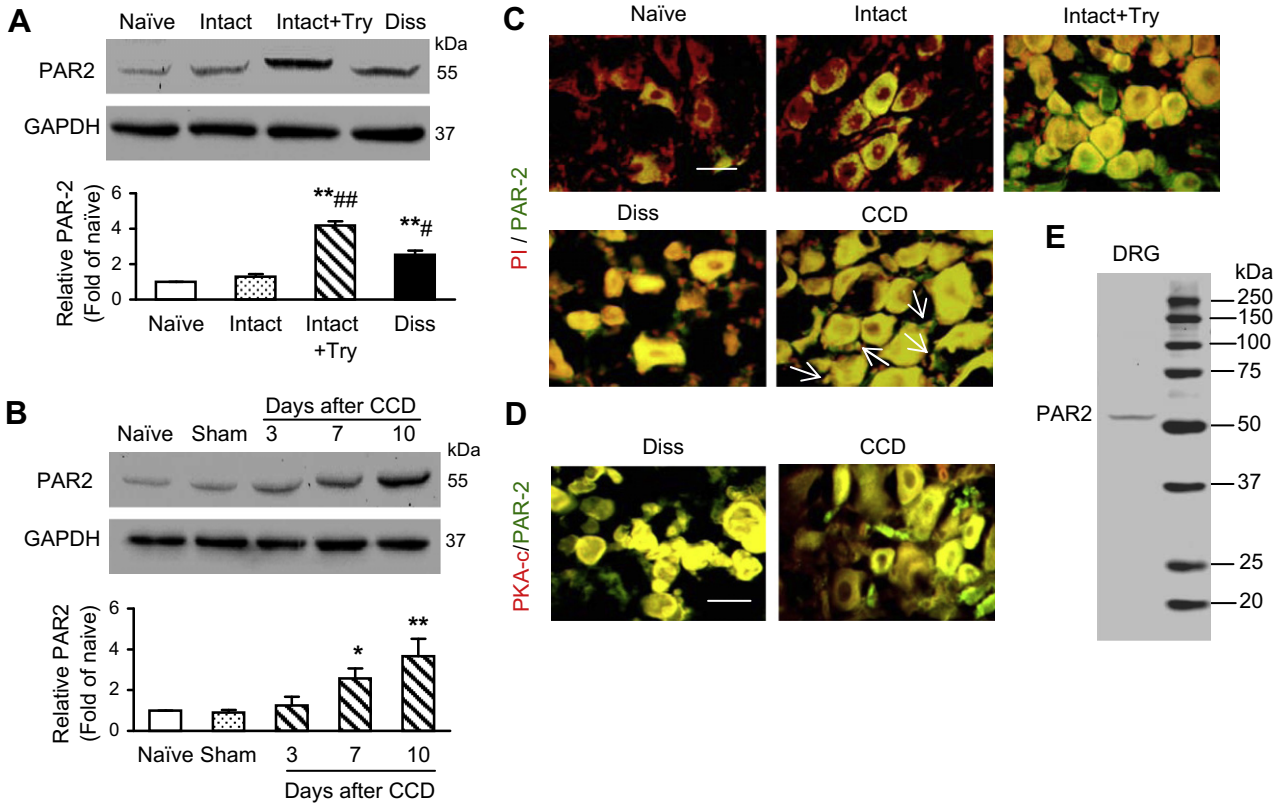

Fig. 2. Trypsin (Try), dissociation (Diss), or chronic compression of dorsal root ganglion (DRG) (CCD) treatment activates protease-activated receptor 2 (PAR2). (A, B) Western blot analysis for protein expression of PAR2 following treatment of Try, Diss (A), or CCD (B). Four samples were included in each group and 4 ganglia in each sample. $^{*}P < 0.05$, $^{**}P < 0.01$ indicates significant difference compared with the corresponding control group Naïve (A), or Sham (B). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicates significant difference compared with the group of intact (A).(C) Co-localization (yellow) of PAR2 (green) with DRG cells (PI, red) in DRGs that previously received treatment of Try, Diss, or CCD. Note that all the yellow-stained cells are neurons (larger-sized with diameter > ~10 μm), but not the very small glial cells (<~5 μm) in groups of naïve, intact, intact + Try, and Diss. In contrast, the DRG neurons and some glial cells (arrow) in CCD ganglia were shown in yellow. (D) Co-localization (yellow) of PAR2 (green) with protein kinase A (PKA)-c (red). Bars in (C) and (D): 30 μm. (E) Examination of specificity of the PAR2 antibody used for detecting PAR2 expression in the Western blot analysis and immunofluorescent staining.

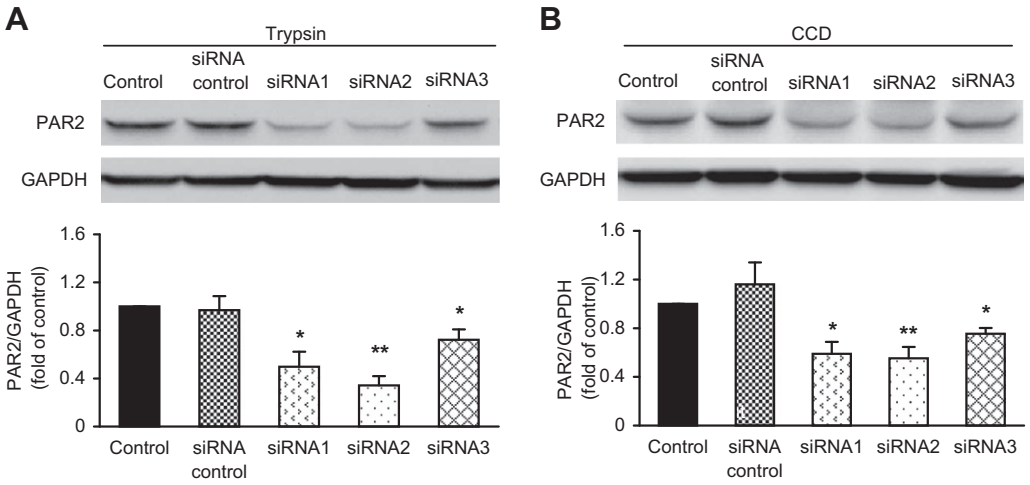

Fig. 3. In vivo pretreatment with protease-activated receptor 2 (PAR2) siRNAs inhibits trypsin- or chronic compression of dorsal root ganglion (DRG) (CCD)-induced PAR2 expression in intact ganglia. (A) Trypsin-induced PAR2 expression. siRNA1, siRNA2 or siRNA3 was repetitively administrated (intrathecally [i.t.], each at 2 μg, daily) for 3 consecutive days prior to trypsin treatment in naïve rats. (B) CCD-induced PAR2 expression. siRNA1, siRNA2, or siRNA3 was repetitively administrated (i.t., each at 2 μg, daily) for 3 consecutive days prior to and 5 consecutive days during CCD treatment., The L_4 and L_5 DRGs in both (A) and (B) were taken from the rats 24 hours after the last siRNA injection. Four samples were included in each group and 4 ganglia in each sample. $^{*}P < 0.05$, $^{**}P < 0.01$ indicates significant difference compared with the corresponding control group.

2.10. Measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Intact DRGs taken from naïve or CCD rats and dissociated DRGs from naïve rats, respectively, were incubated in artificial cerebrospinal fluid (ACSF) containing Fura-2/AM (5 μM) and Pluronic F-127 (0.5 mg/mL) (Invitrogen Inc). Fluorescence in the small and medium-sized DRG neurons (diameter, 15–45 μm), but not the glia cells surrounded the neurons, was measured at 340 nm

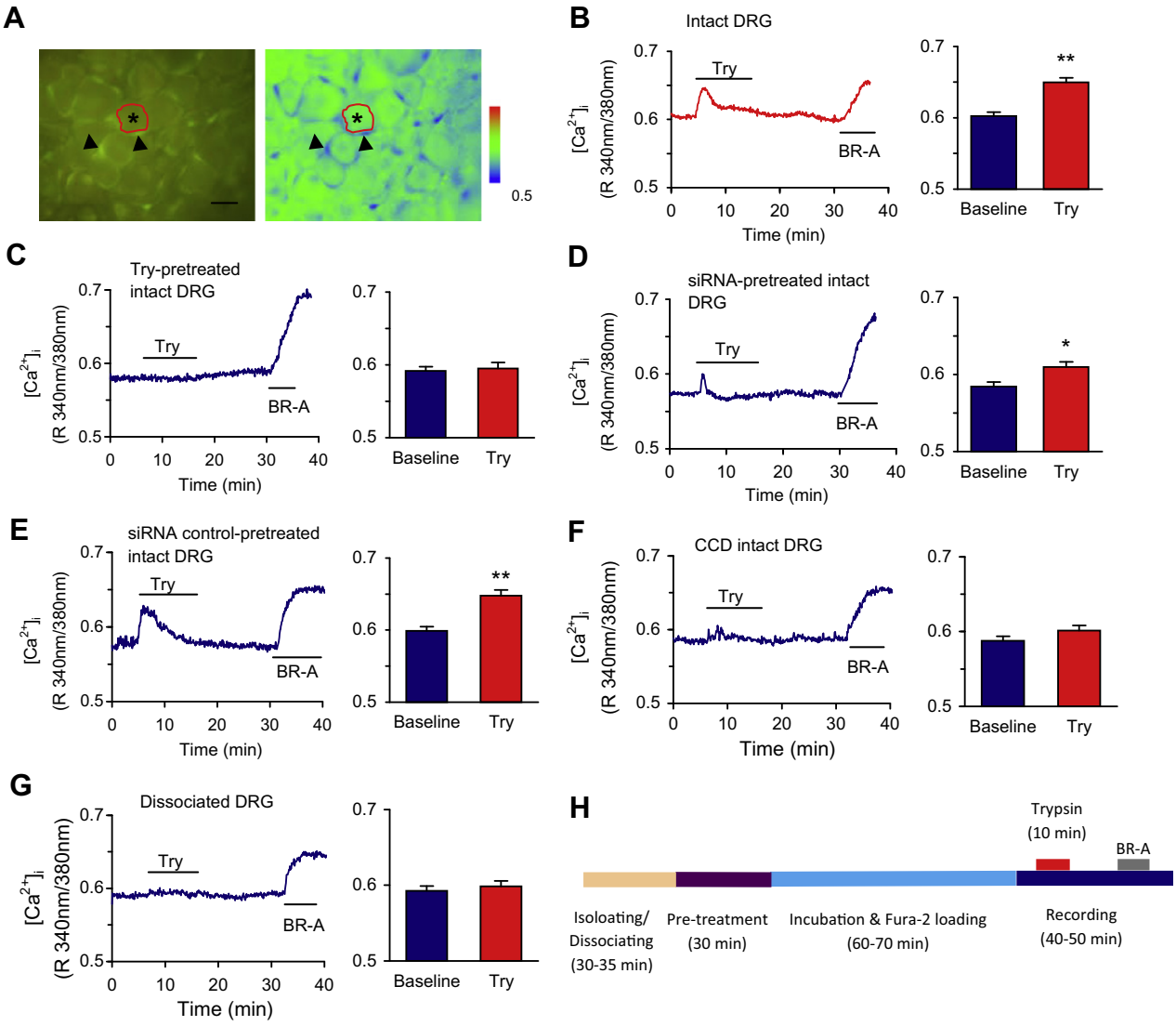

Fig. 4. Trypsin (Try), Dissociation (Diss), or chronic compression of dorsal root ganglion (DRG) (CCD) treatment activates protease-activated receptor 2 (PAR2). (A) Representative 340/380 nm fluorescence (left) and ratiometric (right) pseudocolor images showing measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in intact DRGs from naïve rats. Fluorescence in DRG neurons (∗), but not the glial cells (▲) surrounding the neurons, was measured. (B) $[Ca^{2+}]_i$ in intact DRG neurons (n = 56) from naïve rats was significantly, transiently increased by Try treatment. (C–G) Try-induced increase of $[Ca^{2+}]_i$ in the DRG neurons that had previously received treatment of Try in vitro (n = 45, C), siRNA in vivo (n = 49, D), siRNA control in vivo (n = 43, E), CCD (n = 56, F), or Diss (n = 44, G) was significantly reduced or completely abolished. IB-A was used to check cellular viability. (H) Protocols of preparing the intact, CCD intact, and dissociated DRG neurons and the drug treatment and recordings. Scale bar in A: 30 μm. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference compared with the baseline. Data in baseline groups were copied from the recordings at the first 5 minutes before administration of Try.

and 380 nm excitation and 520 nm emission (Olympus IX51 with ORCA-R2 digital camera, Hamamatsu Inc, Japan). The 340/380-nm emission ratio was used to determine $[Ca^{2+}]_i$ [18,27]. After each recording, 4-bromo A-23187 (BR-A; 10 μM, Sigma) was used to check the viability of the cells.

2.11. Whole cell current-clamp recordings

To test excitability of the nociceptive DRG neurons, Whole-cell patch-clamp recordings were made with an Axopatch-200B amplifier (Molecular Devices, Union City, CA, USA) in the small cells (soma diameter 15–30 μm; membrane input capacitance < 45 pF) from intact or dissociated DRGs. These small cells largely correspond to neurons with C-fiber conduction velocities [15]. Conduction velocity was not measured in the present study. The protocols were similar to that we have previously described [36]. Glass electrodes were fabricated with a Flaming/Brown micropipette puller (P-97, Sutter Instruments, Novato, CA, USA). Electrode impedance was 3–5 MΩ when filled with saline containing (in mM) 120 K^+-gluconate, 20 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 ethylene glycol-bis-(β-aminoethyl-ether) N,N,N′,N′,-tetraacetic acid, 2 Mg-ATP, and 10 HEPES-K (pH 7.2, osmolarity 290–300 mOsm). Electrode position was controlled by a 3-D hydraulic micromanipulator (MHW-3; Narishige, Japan).When the electrode tip touched the cell membrane, gentle suction was applied to form a tight seal (serial resistance >2 GΩ). Under −70 mV command voltage, additional suction was applied to rupture the cell membrane. After obtaining the whole cell mode, the recording was switched to bridge mode (I = 0) and the resting membrane potential (RMP) was recorded.

All the DRG cells accepted for analysis had an RMP of −40 mV or more negative. To compare the excitability of the intact, intact CCD, and dissociated DRG neurons, we examined the RMP, action potential threshold current (APTC), and repetitive discharges evoked by the standard depolarizing current. The RMP was taken 2–3 minutes after a stable recording was first obtained. APTC was defined as the minimum current required evoking an action potential by delivering intracellular currents from −0.1 to 0.5 nA (50-ms pulses) in increments of 0.02–0.1 nA. The whole cell input capacitance (C_{in})

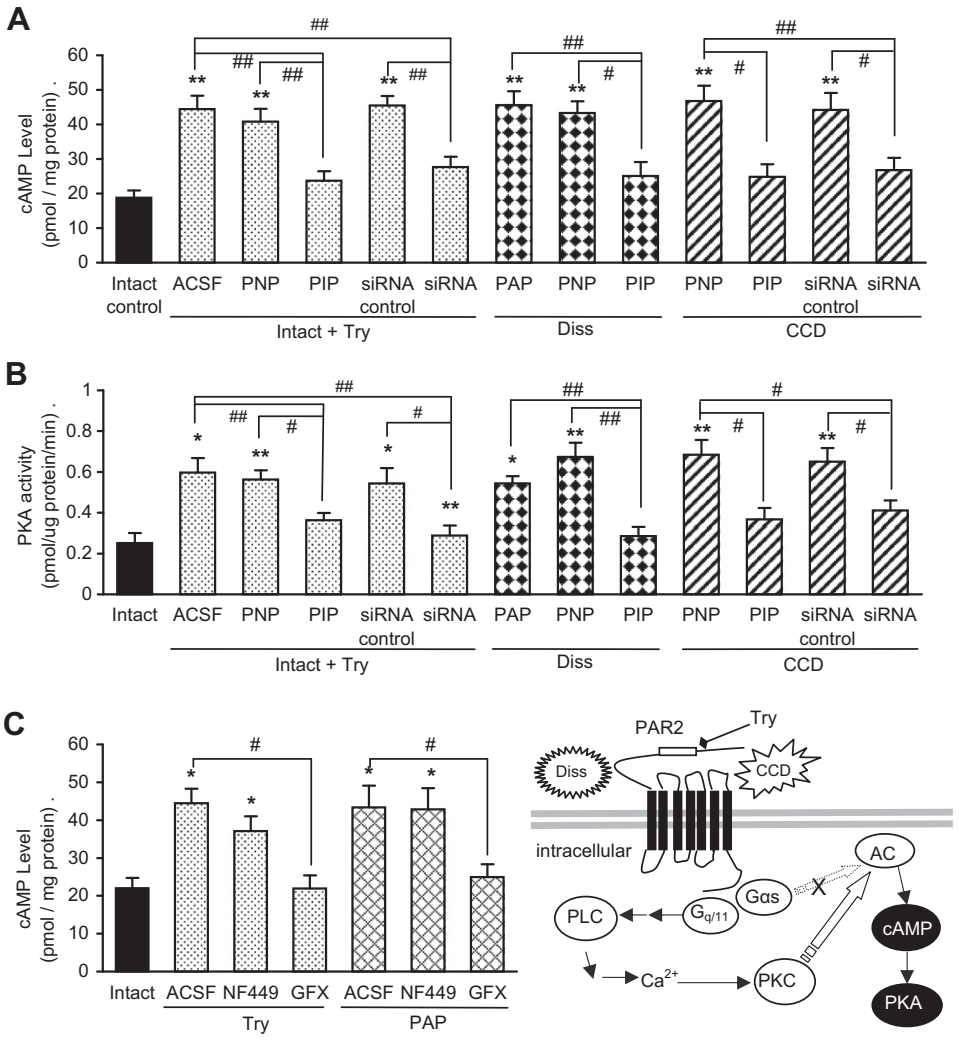

Fig. 5. Antagonistic peptide and siRNA of protease-activated receptor 2 (PAR2) prevent trypsin (Try)-, dissociation (Diss)-, or chronic compression of dorsal root ganglion (DRG) (CCD)-induced increase of cyclic adenosine monophosphate (cAMP) level and protein kinase (PK) A activity. (A) Level of cAMP concentration. (B) PKA activity. (C) The PKC inhibitor GFX, but not Gαs antagonist NF449, inhibits Try- or PAR2 agonistic peptide (PAP)-induced increase of cAMP. Right: schematic of PKC modulation of PAR2 activation-induced increase of cAMP. (A–C) Three samples were included in each group and 4 ganglia in each sample. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference compared with the corresponding control group of intact. $^{\#}P < 0.05$ indicates significant difference compared with the corresponding controls.

was calculated by integration of the capacity transient evoked by a 10-mV pulse in voltage clamp mode. Repetitive discharge was measured by counting the spikes evoked by 1-second, intracellular pulses of depolarizing current normalized to 2.5 times the APTC. All electrophysiological recordings and data analyses were conducted by experimenters blind to previous pharmacological or compression treatment of the cells.

2.12. Thermal withdrawal

Hind paw thermal sensitivity of the rats was determined by measuring foot withdrawal latency during heat stimulation [24,33]. In brief, each rat was placed in a box containing a smooth glass floor maintained at 26–27°C. Radiant heat was focused on part of the hind paw that was flush against the glass, and delivered until the hind paw moved (or up to 20 seconds, to prevent tissue damage). The latency of foot withdrawal in naïve, control rats is 9–12 seconds [24,33]. Thermal stimuli were delivered 4 times to each hind paw at 5–6-minute intervals. The rats were tested on each of 2 successive days prior to surgery. Postoperative tests were conducted after 3, 5, 6, and 7 days, with additional tests conducted 2, 4, and 8 hours after injection of drugs in some experiments. Thermal hyperalgesia for a given rat was defined as a postoperative decrease of foot withdrawal latency from the mean preoperative value $\geqslant$3 seconds [24,33]. All the CCD DRGs in this study were taken from rats that previously received CCD treatment for 7–10 days and exhibited thermal hyperalgesia measured before and on the day.

2.13. Statistical analysis

SPSS Rel 15 (SPSS Inc., Chicago, IL, USA) was used to conduct all the statistical analyses. Alteration of expression of the proteins detected and the behavioral responses to thermal and mechanical stimuli over time among groups were tested with 1-way and 2-way analyses of variance with repeated measures followed by Bonferroni post hoc tests, respectively. All data are presented as means ± SEM. Statistical results are considered significant if $P < 0.05$.

3. Results

3.1. CCD or dissociation treatment increases level of cAMP concentration and mRNA expression of PKA subunits

To provide direct evidence supporting the hypothesis that the cAMP-PKA signaling pathway contributes to chronically

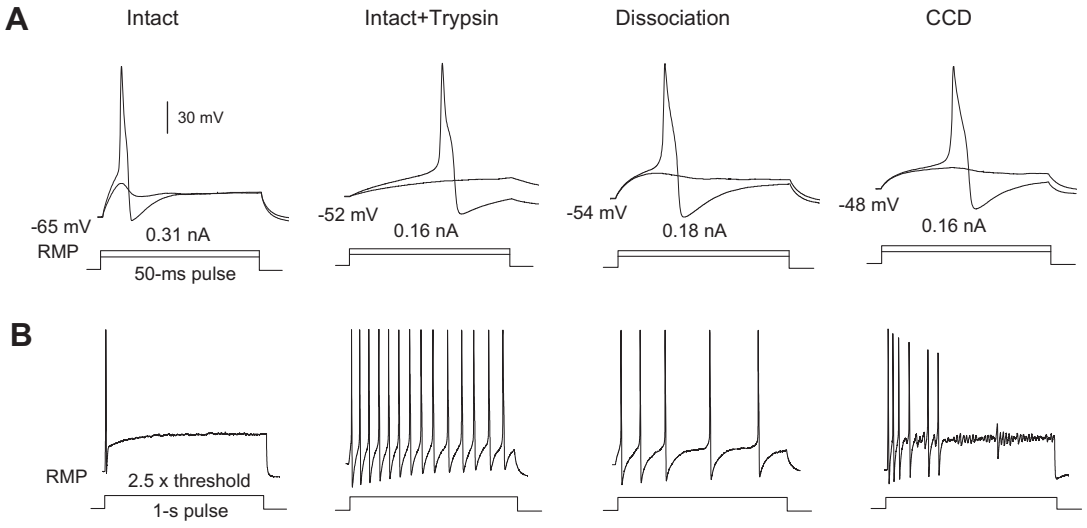

Fig. 6. Electrophysiological alterations of excitability of dorsal root ganglion (DRG) neuronal somata after treatment of trypsin, dissociation, and chronic compression of DRG (CCD), respectively. (A) Examples of neural responses recorded with whole-cell patch-clamp electrodes under current clamp during the test sequence used to determine action potential threshold. Only 2 of the depolarizing 50-ms pulses (bottom) and corresponding responses (top) are illustrated in each case. (B) Examples of neural discharge patterns evoked by depolarizing current with strength of 2.5× threshold at 1-s. The cell in each of the categories in (A) and (B) was the same cell. The threshold current used in (B) was that firstly determined in (A).

compressed or acutely dissociated DRG neuronal hyperexcitability [24,36], we first directly measured level of cAMP concentration in the DRG. ELISA measurement showed that level of cAMP concentration in the intact DRGs taken from naïve animals was not significantly altered compared to its naïve control, suggesting that the protocol used for making the intact DRG preparation produced the least injury to the ganglia, and such inevitably minimal injury was not enough to alter the cAMP in this study. However, the level of cAMP concentration in the CCD or dissociated DRGs was significantly increased (Fig. 1A). The samples were collected on the 7th day after CCD and within 2–4 hours after acute dissociation, respectively. We further examined the relative mRNA level of PKA subunit gene expression in the DRG by RT-PCR in the same conditions. The level of mRNA for PKA-RII and PKA-c, but not PKA-RI, was significantly increased after CCD or dissociation. In contrast, none of these mRNA expressions was significantly changed in the intact compared with the naïve control DRGs (Fig. 1B). Immunofluorescence staining showed that protein expression of PKA-RII and PKA-c broadly distributed and significantly increased in all 3 categories of DRG neurons, the small and the medium- and large-sized neurons, after CCD or dissociation (Fig. 1C). These results indicate that CCD and dissociation treatment can activate the cAMP-PKA signaling pathway.

3.2. CCD and dissociation treatment increases expression of PAR2 protein

Activation of PAR2 plays an important role in responses to injury and inflammation [6]. We examined whether CCD and dissociation treatment could excite PAR2 in the DRG. Protein expression of PAR2 was detected from preparations of naïve, intact, dissociation, and CCD, respectively. In the intact and naïve control groups, expression of PAR2 was shown at a similar level ($P > 0.05$), although it was slightly higher in the intact group. In contrast, PAR2 expression was greatly increased in the intact DRGs treated with trypsin (0.5 mg/mL) and the dissociated DRGs compared with either Naïve or intact group (Fig. 2A). Trypsin induced the greatest increase of expression of PAR2 among these 4 groups. Trypsin has been shown to excite neurons by cleaving PAR2 [27]. CCD treatment produced a time-related increased expression of PAR2, which started between 3 and 7 days and maintained a high level at 10 (the last examination) postoperative days (Fig. 2B). The increased expression of PAR2 after trypsin and dissociation, as well as CCD treatment, was distributed in the small, medium- and large-sized DRG neurons (Fig. 2C). In some immunostaining slices from the intact DRG, expression of PAR2 was slightly increased (Fig. 2C), which corresponded to the slightly increased expression detected by Western blot (Fig. 2A). We assumed that this slight alteration was induced by the protocol used for intact DRG preparation that produced the least, but inevitable, injury. Further, the increased expression of PAR2 in CCD and dissociated DRG neurons was shown co-localized with the increased PKA-c subunit (Fig. 2D). Specificity of the mouse monoclonal anti-PAR-2 antibody from Santa Cruz Biotechnology used in this study to detect PAR2 expression has been well confirmed in previous reports [3,17,35]. A clean band of PAR2 is also shown in DRG in this study (Fig. 2E).

3.3. siRNA of PAR2

We bought a set of 3 PAR2 siRNAs: siRNA1, siRNA2, and siRNA3. Their sequences are described in Methods. PAR2 knockdown by each of these 3 siRNAs was analyzed by immunoblotting with antibody to PAR2. Our results showed that trypsin-induced increased expression of PAR2 was significantly inhibited by in vivo administration of siRNA1, siRNA2, or siRNA3 (each at 2 μg, i.t., daily for 3 consecutive days) (Fig. 3A). Similarly, CCD-induced expression of PAR2 was also significantly inhibited by each of these 3 siRNAs (each at 2 μg, i.t., daily for 3 consecutive days before and 5 consecutive days during CCD treatment) (Fig. 3B). These results confirmed that these siRNAs may be used to knock down PAR2 in vivo. Among the 3 siRNAs, siRNA3 produced the least knockdown, while siRNA1 and siRNA2 produced the most knockdown of PAR2. We thus choose siRNA2, which seems to be even better than siRNA1 ($P > 0.05$) for our studies for the purpose of PAR2 knockdown in vivo in the same protocol. This siRNA2 is referred to as siRNA in descriptions in the following paragraphs.

3.4. CCD and dissociation treatment as well as PAR2 knockdown diminish trypsin-induced increase of PAR2-dependent intracellular Ca^{2+} ($[Ca^{2+}]_i$)

Increase of $[Ca^{2+}]_i$ is a sign for the PAR2 activation [27]. Trypsin excites neurons by cleaving PAR2 [27]. Our results showed that

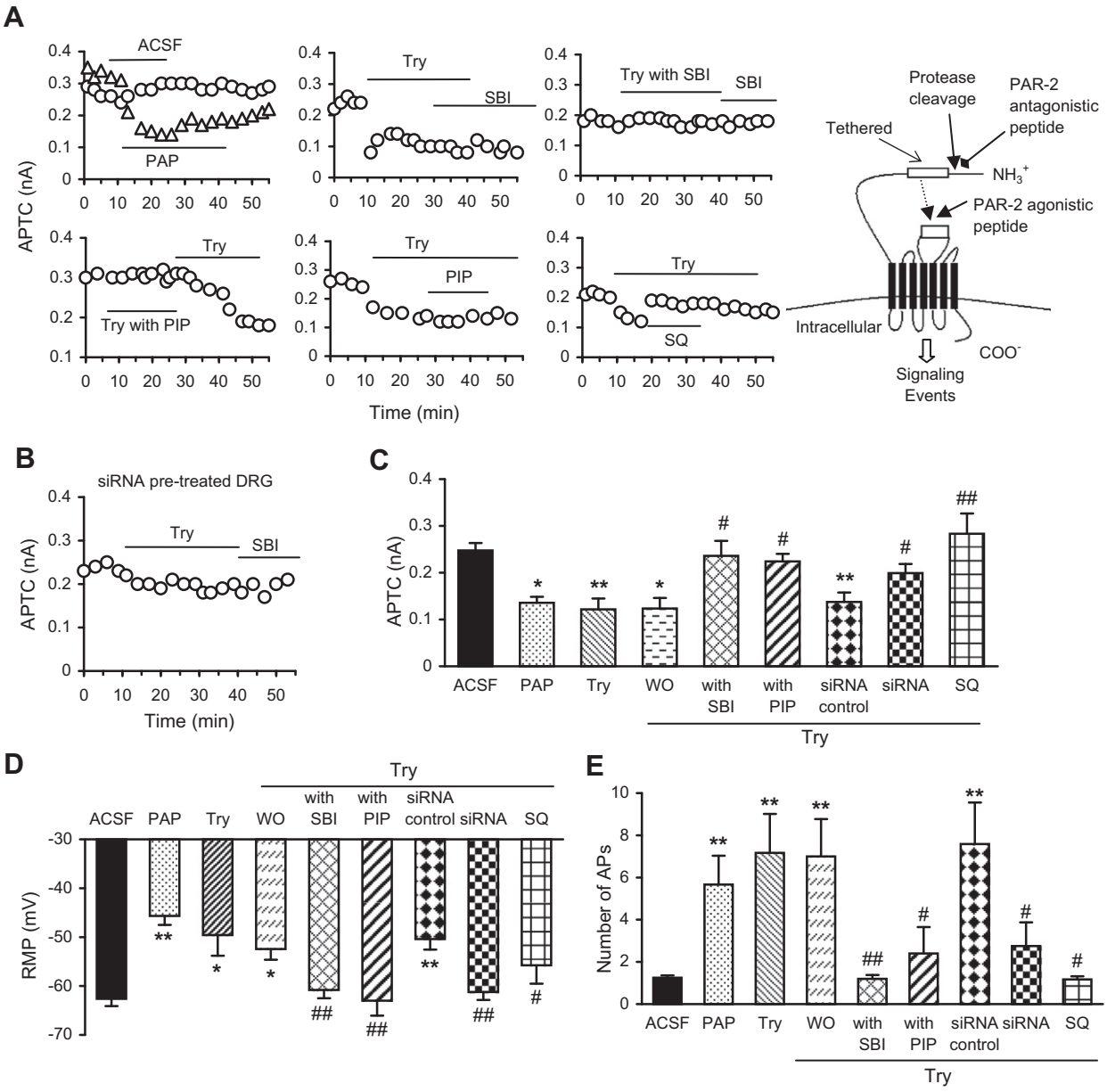

Fig. 7. Trypsin (Try)-induced hyperexcitability of small dorsal root ganglion (DRG) neurons are mediated by protease-activated receptor 2 (PAR2) and maintained by cyclic adenosine monophosphate (cAMP). (A) Examples of neuronal responses (alteration of APTC) to treatment of PAR2 agonist peptide (PAP) or Try with or without PAR2 inhibitors and adenylyl cyclase inhibitor SQ. Right: schematic of PAR2 structure and activation. (B) Pretreatment with siRNA inhibited Try-induced increased hyperexcitability (decreased action potential current threshold was reversed). (C–E) Data summary. Ten cells were tested in group of artificial cerebrospinal fluid (ACSF), 8 in group of siRNA and 6 in other groups. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference compared with the corresponding controls ACSF. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate significant difference compared with the corresponding controls, the group of Try. WO = washout.

transient perfusion of trypsin (0.5 mg/mL, same dose as we used previously in intact DRG preparation for electrophysiological recordings in the present and our previous studies [24,36]) resulted in a rapid, transient increase of $[Ca^{2+}]_i$, followed by a sustained elevation of $[Ca^{2+}]_I$ in the small and medium-sized neurons (n = 56) in the intact DRGs from naïve animals as Control (Fig. 4A, B). However, the same treatment of trypsin failed to induce a significant increase of $[Ca^{2+}]_i$ in the intact DRG neurons (n = 45) that had previously received trypsin treatment (0.5 mg/mL, 30 minutes) (Fig. 4C). BR-A was used to check cellular viability in all the cells tested, and examples are shown in Fig. 4B–E, in which all the cells tested were responsive to BR-A. The unresponsiveness to the second treatment of trypsin (Fig. 4C) may result from the irreversible activation character of PAR2 [2] after the first trypsin treatment (30 minutes, given 60–70 minutes before recordings). Further, repetitive in vivo treatment (i.t.) with PAR2 siRNA (n = 49, Fig. 4D), but not the control siRNA (n = 43, Fig. 4E), significantly reduced trypsin-induced increase of $[Ca^{2+}]_i$ (comparison between Fig. 4B and Fig. 4D), supporting that trypsin-induced increase of $[Ca^{2+}]_i$ is mediated, at least partly, through PAR2 activation. We used this pattern of $[Ca^{2+}]_i$ and PAR2 activation to evaluate status of PAR2 activity in CCD and dissociated DRG neurons. In CCD (n = 62, Fig. 4F) and dissociated (n = 44, Fig. 4G) DRG neurons, trypsin treatment induced slight, but not significant, increase in $[Ca^{2+}]_i$. This unresponsiveness to the trypsin in CCD and dissociated DRG neurons was similar to that in the trypsin-pretreated, or PAR2 knockdown intact DRGs (Fig. 4C, D). These results suggest that CCD or dissociation treatment may cause PAR2 activation by trypsin

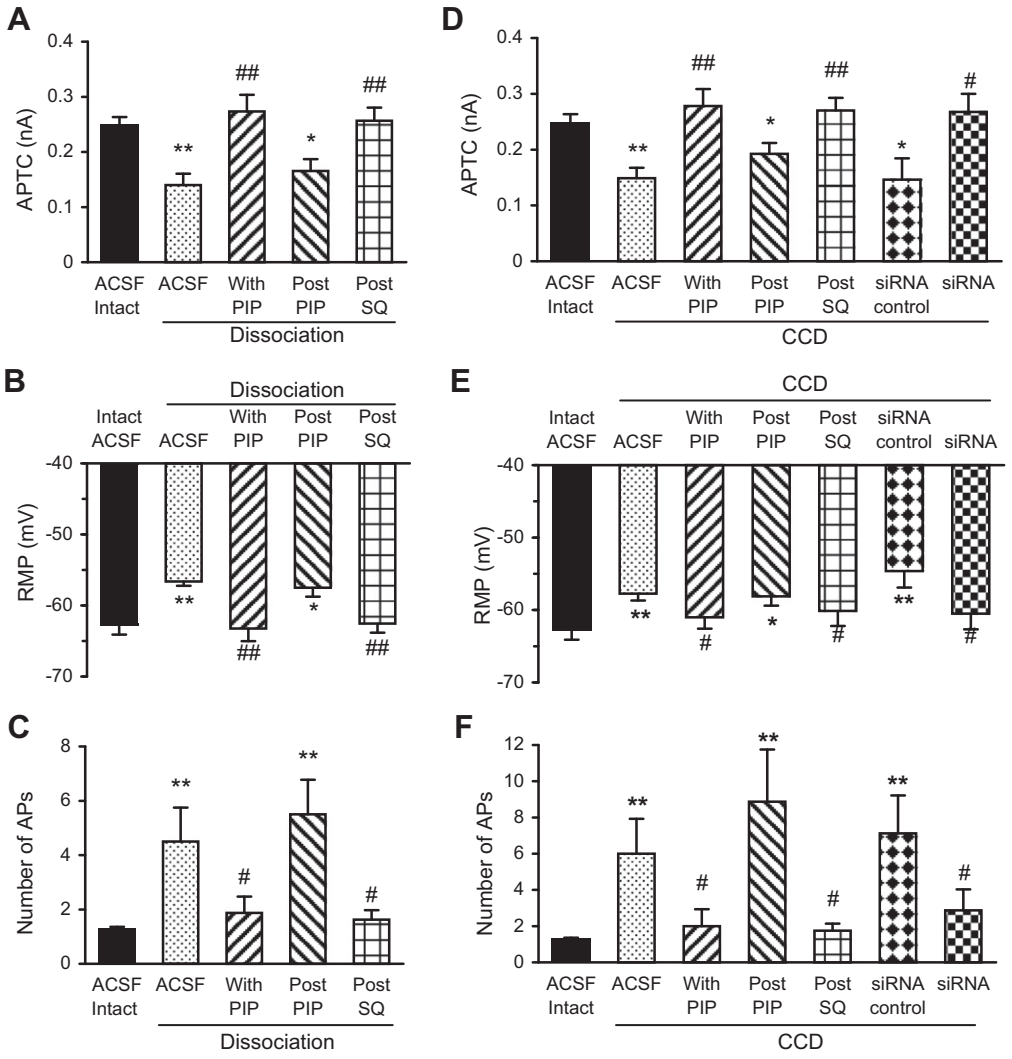

Fig. 8. Dissociation- and chronic compression of dorsal root ganglion (DRG) (CCD)-induced hyperexcitability of small DRG neurons are mediated by protease-activated receptor 2 (PAR2) and maintained by cyclic adenosine monophosphate (cAMP). (A–C) Effects of PAR2 antagonistic peptide (PIP) and adenylyl cyclase inhibitor SQ22536 (SQ) on dissociation-induced neural hyperexcitability. Numbers of cells tested in each group: 10 (intact), 8 (artificial cerebrospinal fluid [ACSF]), and 10 for each group of dissociation with PIP, post PIP, and Post SQ.(D–F) Effects of PIP, SQ, and siRNA of PAR2 on CCD-induced hyperexcitability. Numbers of cells: 10 (intact), 8 (ACSF), 11 (with PIP), 11 (post PIP), 10 (Post SQ), 9 (siRNA control), and 12 (siRNA). The group of "intact" used as normal control was the same data as the ACSF group in 7. $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference compared with the corresponding control ACSF/intact. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate significant difference compared with the corresponding control ACSF/dissociation (A–C) or ACSF/CCD (D–F).

endogenously released during compression in CCD DRGs or exogenously applied during dissociating in dissociated DRG neurons.

3.5. CCD, dissociation, and trypsin treatment increases cAMP level and PKA activity through PAR2 activation

Given that CCD and dissociation treatment increases level of cAMP concentration and expression of PKA subunits and activates PAR2, we examined whether PAR2 activation might mediate CCD- and/or dissociation-induced increase of cAMP and PKA activity. Our ELISA analysis showed that level of cAMP concentration (Fig. 5A, also see Fig. 1A) and PKA activity (Fig. 5B) was significantly increased after trypsin, dissociation, and CCD treatment, respectively (Fig. 5A, B). The increased cAMP concentration and PKA activity were greatly inhibited by PAR2 antagonistic peptide PIP when it was co-applied with trypsin (trypsin treatment of the intact DRG), dissociation preparation (during dissociating procedure), or CCD (during CCD treatment, PIP or PNP was injected into the targeted ivf in vivo). The control peptide PNP did not alter the cAMP and PKA activity. PAR2 agonistic peptide PAP applied onto the ganglion during dissociating did not further increase the cAMP and PKA activity, suggesting that the increased cAMP and PKA activity was due to the PAR2 activation caused by the dissociating procedure. Further, spinal administration of siRNA of PAR2 (PAR2 knockdown) during CCD treatment greatly suppressed CCD-induced increase of cAMP concentration and PKA activity (Fig. 5A, B). The siRNA was administered prior to surgery (each at 2 μg i.t. daily for 3 consecutive days) and during CCD treatment, in the same doses and protocol, for another 5 consecutive days. These results indicate that CCD- or dissociation-induced activation of cAMP-PKA signaling may be mediated, in the upstream, by PAR2 activation. Further, in the intact DRGs taken from naïve rats, PKC inhibitor GFX blocked trypsin- or PAP-induced increase of cAMP, while the Gαs-subunit-selective G-protein antagonist NF449 failed to do so (Fig. 5C). This suggests that the PAR2 activation-dependent cAMP-PKA activity may be modulated by and/or mediated through PKC.

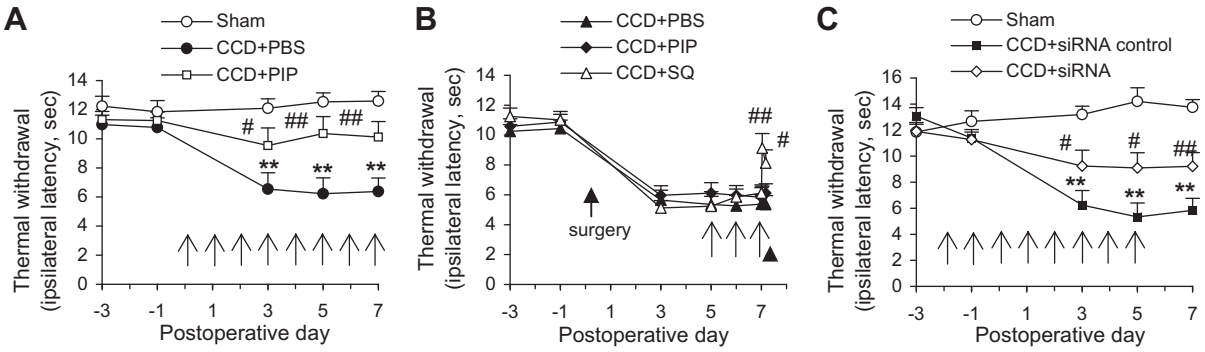

Fig. 9. Effects of protease-activated receptor 2 (PAR2) antagonistic peptide PIP, adenylyl cyclase inhibitor SQ and PAR2 siRNA on chronic compression of dorsal root ganglion (DRG) (CCD)-induced thermal hyperalgesia. (A) Repetitive administration of PIP (intervertebral foramen [ivf], each at 1 mM, 10 μL, starting at 30 minutes before surgery, daily for total 8 doses) suppressed the hyperalgesia. (B) Post-administration of PIP (ivf, each at 1 mM) on 5, 6, and 7 postoperative days failed to reduce hyperalgesia. A single dose of SQ (1 mM) administered on the 7th postoperative day (▲) produced an immediate analgesic effect. (C) Repetitive administration of siRNA, but not the siRNA control (intrathecally, each 2 μg, daily for 3 consecutive days before surgery; and another 5 consecutive days during CCD treatment) suppressed the hyperalgesia. Eight rats were included in each group in (A–C). $^{*}P < 0.05$, $^{**}P < 0.01$ indicate significant difference compared with the corresponding control Sham (A, C). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate significant difference compared with the corresponding control groups of CCD + PBS (phosphate-buffered saline) (A), CCD + siRNA control (C) or the baseline before the single injection of SQ (B).

3.6. CCD- and dissociation- as well as trypsin treatment-induced neuronal hyperexcitability is mediated by PAR2 and maintained by cAMP

Given that CCD and dissociation treatment may increase cAMP level and PKA activity through PAR2 activation, we continued to examine contributions of PAR2 to neuronal hyperexcitability after CCD or dissociation by means of whole-cell patch-clamp recordings from the nociceptive, small DRG neurons. The increased neuronal excitability manifested as depolarization of RMP, decreased APTC, and increased repetitive discharges evoked by a standard intracellular depolarizing current. Examples of neuronal excitability recorded from preparations of intact, intact plus trypsin, dissociation, and CCD DRGs are shown in Fig. 6. The intact DRG neurons from naïve animals were excited by PAR2 agonistic peptide PAP and trypsin. PAP- or trypsin-induced neuronal hyperexcitability lasted for up to 8 hours (recordings were made at 0.5–8 hours) after termination of perfusion of PAP or trypsin. Trypsin-induced hyperexcitability was prevented by coapplication with trypsin inhibitor SBI or PAR2 antagonistic peptide PIP. Posttreatment of SBI or PIP did not alter trypsin-induced neural hyperexcitability. However, posttreatment of an adenylyl cyclase inhibitor SQ successfully reversed trypsin-induced hyperexcitability. Examples of alterations of APTC following the treatments are given in Fig. 7A. However, trypsin failed to excite the DRG neurons that received pretreatment with PAR2 siRNA (siRNA, 2 μg, i.t., daily for 3 consecutive days) (Fig. 7B). Data are summarized in Fig. 7C–E. Posttreatment of SBI and PIP did not significantly change the neural excitability (examples are given in Fig. 7A and data not shown).

Further, we tested effects of PAR2 knockdown by PAR2 antagonistic peptide PIP and siRNA on DRG neuronal hyperexcitability induced by dissociation and CCD, respectively. The results showed that dissociation- or CCD-induced hyperexcitability was greatly suppressed by PIP applied during dissociating procedure in vitro or CCD treatment in vivo. However, posttreatment of PIP, that is, PIP was applied onto the neurons or intact ganglion after dissociation or CCD treatment in vitro, did not affect dissociation- or CCD-induced hyperexcitability. In contrast, posttreatment with adenyl cyclase inhibitor SQ significantly reversed dissociation- or CCD-induced hyperexcitability. Data are summarized in Fig. 8A–C (dissociation) and Fig. 8D–F (CCD), respectively. CCD-induced hyperexcitability was also greatly suppressed following spinal knockdown of PAR2 by repetitive i.t. PAR2 siRNA, but not the control siRNA (Fig. 8D–F). Consistently, CCD-induced thermal hyperalgesia was attenuated by pretreatment of PIP (Fig. 9A), but not by the posttreatment of PIP (Fig. 9B). However, a single dose of SQ (injected on the 7th postoperative day) significantly inhibited the ongoing thermal hyperalgesia in CCD rats (Fig. 9B). Spinal knockdown before and during CCD treatment with PAR2 siRNA, but not the control siRNA, significantly attenuated CCD-induced thermal hyperalgesia. The inhibitory effects of the siRNAs lasted for at least 2 more days after termination of the last injection of siRNA on the 5th day after CCD (Fig. 9C). These results further support that DRG neuronal hyperexcitability induced by CCD or dissociation may be mediated, at least partly, by PAR2 activation, and maintained by cAMP.

4. Discussion

This study demonstrates that the prolonged compression and acute dissociation of DRG neurons can activate PAR2 and cAMP-PKA signaling; PAR2 activation may mediate the increased cAMP and PKA activity and cAMP-dependent neuronal hyperexcitability. These findings provide a new mechanism that underlies cAMP-PKA activation and DRG neuronal hyperexcitability after CCD as well as dissociation treatment. Thus, PAR2 may be a potential target for preventing/suppressing induction of sensory neuron hyperexcitability following these dissimilar forms of injury-related stress. Further, inhibiting PAR2 activation may result in inhibition of PAR2-mediated activation of cAMP-PKA pathway, which contributes to maintenance of neural hyperexcitability and hyperalgesia.

CCD or dissociation treatment can induce an increase in electrophysiological responsiveness to cAMP, and this increased responsiveness is important for maintaining DRG neuronal hyperexcitability [24,36]. These dissimilar forms of injury-related stress greatly enhancing the electrophysiological responsiveness of the stressed neurons to activity in the cAMP-PKA pathway suggest interesting possibilities. One possibility is that the enhanced excitatory effects of inflammatory mediators on DRG neurons by prior nerve injury [25,26] or CCD treatment [14] involves an increased responsiveness within the neuron to the cAMP-PKA pathway because excitatory effects of some inflammatory mediators are mediated by this pathway [1]. Another implication of our findings in dissociated neurons [36] is that previous demonstrations of cAMP-PKA contributions to hyperexcitability of DRG somata may have depended at least partly on dissociation increasing the electrophysiological responsiveness of the DRG neurons to cAMP. Here, we provide direct evidence that the cAMP level and PKA activity are significantly increased by CCD or dissociation. These findings support the hypothesis that the cAMP-PKA signaling contributes

greatly to the injury-related stress-induced neuronal hyperexcitability and behaviorally expressed hyperalgesia.

PARs play important roles in responses to injury and inflammation [6]. In particular, agonists of PAR2 have widespread proinflammatory effects. Trypsin and activating peptides cause nitric oxide-dependent vasodilation [7,20], induce extravasation of plasma proteins and infiltration of neutrophils [10,30], and stimulate secretion of proinflammatory cytokines [8]. A large proportion of primary spinal afferent neurons, which express PAR2, also contain the proinflammatory neuropeptides calcitonin gene-related peptide and substance P. Trypsin and the mast cell tryptase can directly signal to neurons to stimulate release of these neuropeptides, which mediate inflammatory edema induced by agonists of PAR2 [27]. Here, we demonstrate that, in DRG neurons, PAR2 is activated by DRG compression and dissociation treatment. Knockdown of PAR2 by spinal administration of a PAR2 antagonistic peptide PIP or PAR2 siRNA can prevent CCD-induced DRG neuronal hyperexcitability and hyperalgesia or dissociation-induced hyperexcitability. However, posttreatment with PIP fails to reverse the developed hyperexcitability and hyperalgesia. These results suggest that PAR2 may contribute primarily to production of the hyperexcitability and hyperalgesia and demonstrate a crucial role for serine proteases in generating DRG neuronal hyperexcitability via a PAR2-dependent mechanism.

Further, PAR2 activation may mediate the increase of cAMP-PKA activity in these 2 forms of injury-related stress. CCD or dissociation treatment increases expression of PAR2 protein and PKA subunits. PAR2 and PKA-c are also colocalized in the DRG neurons. PAR2 agonistic peptide increases cAMP level and PKA activity, while PAR2 antagonistic peptide inhibits trypsin-, dissociation-, or CCD-induced increase of cAMP level and PKA activity. PAR2 activation increasing cAMP concentration is also supported by recent reports [3,13]. Moreover, we here provide further evidence that supports our hypothesis that chronic compression or acute dissociation-induced DRG neuronal hyperexcitability is maintained by increased responsiveness to cAMP [24,36]. Thus, PAR2 activation may greatly contribute to production of the hyperexcitability and hyperalgesia and mediate enhancement of cAMP-PKA activity, which involves maintaining the hyperexcitability and hyperalgesia. Additionally, we have found that trypsin- or PAR2 agonistic peptide-induced increase of cAMP level is suppressed by an inhibitor for PKC, but not the Gαs, suggesting that the PAR2-dependent cAMP activity may be mediated via the PKC pathway. PKC regulation of adenylyl activity and PKC modulation of ability of the adenylyl cyclase isoforms to respond to different G protein subunits have been discussed [38]. Further studies are required to elucidate the possible pathways that may mediate PAR2 activation, cAMP activity, and DRG neuronal hyperexcitability during and after injury-related stress.

This study may suggest a new strategy for treating chronic pain induced by nerve injury/compression or similar disorders in clinic. In the early phase of development of chronic pain, inhibiting PAR2 activation may prevent or greatly suppress induction of neuropathic pain and the associated sensory neuron hyperexcitability. Further, inhibition of PAR2 activation may result in inhibition of the PAR2-mediated activation of cAMP-PKA pathway, which then contributes to maintenance of neural hyperexcitability and hyperalgesia. In the late phase of chronic pain, targeting the activated cAMP-PKA pathway, which is mediated at least partly by PAR2 activation and PKC pathway, may be an effective therapeutic approach for suppressing the cAMP-dependent neural hyperexcitability and behaviorally expressed hyperalgesia.

Conflict of interest statement

There are no conflicts of interest to declare associated with this study.

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