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Research Paper

Activation of cGMP-PKG signaling pathway contributes to neuronal hyperexcitability and hyperalgesia after *in vivo* prolonged compression or *in vitro* acute dissociation of dorsal root ganglion in rats

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Abstract: Injury or inflammation affecting sensory neurons in the dorsal root ganglia (DRG) causes hyperexcitability of DRG neurons that can lead to spinal central sensitization and neuropathic pain. Recent studies have indicated that, following chronic compression of DRG (CCD) or acute dissociation of DRG (ADD) treatment, both hyperexcitability of neurons in intact DRG and behaviorally expressed hyperalgesia are maintained by activity in cGMP-PKG signaling pathway. Here, we provide evidence supporting the idea that CCD or ADD treatment activates cGMP-PKA signaling pathway in the DRG neurons. The results showed that CCD or ADD results in increase of levels of cGMP concentration and expression of PKG-I mRNA, as well as PKG-I protein in DRG. CCD or ADD treated-DRG neurons become hyperexcitable and exhibit increased responsiveness to the activators of cGMP-PKG pathway, 8-Br-cGMP and Sp-cGMP. Hyperexcitability of the injured neurons is inhibited by cGMP-PKG pathway inhibitors, ODQ and Rp-8-pCPT-cGMPS. *In vivo* delivery of Rp-8-pCPT-cGMPS into the compressed ganglion within the intervertebral foramen suppresses CCD-induced thermal hyperalgesia. These findings indicate that the *in vivo* CCD or *in vitro* ADD treatment can activate the cGMP-PKG signaling pathway, and that continuing activation of cGMP-PKG pathway is required to maintain DRG neuronal hyperexcitability and/or hyperalgesia after these two dissimilar forms of injury-related stress.

Key words: compression; dissociation; dorsal root ganglion; hyperexcitability; hyperalgesia; cGMP; PKG

大鼠背根节慢性压迫或急性分离引起的cGMP-PKG信号通路持续激活介导背根节神经元的异常兴奋性和痛觉过敏

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摘 要：背根节(dorsal root ganglion, DRG)损伤或炎症可导致DRG神经元兴奋性异常增强和痛觉过敏。我们近期研究显示，长期慢性在体压迫(chronic compression of DRG, CCD)或急性离体分离(acute dissociation of DRG, ADD)背根节导致的神经元兴奋性异常增强和痛觉过敏受环鸟苷酸(cGMP)-蛋白激酶G (PKG)信号通路活动的调控。本研究采用大鼠CCD模型和ADD模型，直接在DRG上检测cGMP浓度和PKG mRNA及其蛋白质的表达，进一步证明了cGMP-PKG信号通路活动在CCD和ADD DRG所致神经元兴奋性异常增强和痛觉过敏中的重要作用。酶联免疫吸附测定(ELISA)和逆转录聚合酶链反应(RT-PCR)的实验结果显示，CCD或ADD明显增高DRG内的cGMP浓度，上调I型PKG mRNA和PKG蛋白质表达。电生理膜片钳全细胞记录结果显示，CCD和ADD显著增强伤害特异性DRG细胞的兴奋性及其对cGMP-PKG信号通路激动剂的反应强度。增强的细胞兴奋性可以被cGMP-PKG通路阻断剂所抑制。在体压迫DRG的椎间孔内注射cGMP-PKG抑制剂显著减轻痛觉过敏。以上研究结果表明，CCD和ADD可以激活DRG细胞内的cGMP-PKG信号通路，而损伤的DRG细胞的超兴奋性和痛觉过敏的维持则需要cGMP-PKG信号通路处于持续的激活状态。

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关键词：慢性压迫；急性分离；背根节；超兴奋性；痛觉过敏；环鸟苷酸；蛋白激酶G
中图分类号：R338.1+1

Peripheral nerve injury produces long-lasting hyperexcitability of sensory neurons in diverse species. In mammals, injury affecting the axons or somata of sensory neurons having their somata in the dorsal root ganglia (DRG) often causes hyperexcitability that may lead to spinal central sensitization and neuropathic pain [1–4]. Electrophysiological mechanisms contributing to the expression of hyperexcitability in DRG neuronal somata following injury to the peripheral nervous system have been investigated intensively, but signals that induce and maintain this hyperexcitability remain elusive. Our previous studies showed that two signaling pathways, the cAMP-PKA and cGMP-PKG pathways, are important for maintaining both DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia in an *in vivo* animal model of neuropathic pain, chronic compression of rat dorsal root ganglia (CCD) treatment, and an *in vitro* model of acute dissociation of DRG (ADD) treatment [5,6]. During CCD treatment, the somata of DRG neurons are mechanically compressed and probably exposed to inflammatory mediators [7–9]. In ADD preparation, 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 [6,10,11].

We have continued to investigate the upstream molecules mediating the increased activity of cAMP-PKA pathway following these two dissimilar forms of injury-related stress. We found that the DRG neuronal protease-activated receptors (PARs) subtype PAR2 is activated during prolonged compression *in vivo* as well as acute dissociation *in vitro*. Activation of PAR2 is involved in mediating the cAMP-dependent, DRG neuronal hyperexcitability and behaviorally expressed hyperalgesia [12]. The cGMP-PKG signaling pathway found to maintain CCD effects [5,6] was interesting because, in acute experiments, the cGMP-PKG pathway has more often been associated with depressive effects on DRG neuronal excitability [13–16] than with sensitizing effects [17–20]. In addition, our previous study showed that cGMP-PKG and cAMP-PKA contribute to CCD-induced DRG neuronal hyperexcitability and hyperalgesia, but these pathways had little effect on behavior or soma excitability in the absence of CCD treatment and/or DRG somata dissociation [5,6]. These observations appeared to conflict with earlier reports of cAMP-induced DRG neuronal hyperexcitability, but our excitability tests were performed on sensory neuron somata that remained in place within excised but intact ganglia, whereas previous studies implicating the cAMP-PKA pathway in hyperexcitability had been performed either on dissociated DRG neuronal somata [21–30] or on neurons in excised ganglia that had previously been compressed [31]. This suggested that injury-related stress, caused in these cases by either ADD or CCD, induces an increase in electrophysiological responsiveness to both cAMP and cGMP, and this increased responsiveness is important for maintaining hyperexcitability. The possibility that ADD produces cAMP- and cGMP-dependent DRG neuronal hyperexcitability is also important because it would suggest that these sensitizing responses do not require the recruitment of inflammatory cells for their induction or early maintenance and might involve mechanisms intrinsic to injured neurons. This property has been demonstrated in an invertebrate nociceptor [32,33] and strongly implicated in dissociated DRG neurons [34]. Indeed, our study has shown that ADD produces acute cAMP- and cGMP-dependent DRG neuronal hyperexcitability that is remarkably similar to that produced by CCD [6]. In this study, we further investigated the alterations of cGMP-PKG pathway after CCD and ADD and roles cGMP-PKG pathway in the hyperexcitability as well as hyperalgesia.

1 MATERIALS AND METHODS

1.1 *Animals, anesthesia, drugs, and administration*

Adult, male Sprague-Dawley rats (150–250 g, n = 66, Charles River Laboratories, Wilmington, MA, USA) were used in the experiments. All investigations were conducted in conformity with the APS's "Guiding Principles in the Care and Use of Animals", and protocols were approved by the Institutional Committee on the Care and Use of Experimental Animals. All surgeries were done under anesthesia with sodium pentobarbital (50 mg/kg, i.p.). Contributions of cGMP-PKG pathways to hyperexcitability of dissociated and previously compressed DRG neurons were investigated by applying the following drugs in the bath to the excised DRG

or to dissociated DRG neurons (final concentration): a cGMP analog, 8-Br-cGMP (50 μmol/L); a PKG activator, Sp-cGMPS (50 μmol/L); a PKG inhibitor, Rp-8-pCPT-cGMPS (50 μmol/L); and a soluble guanylyl cyclase inhibitor, ODQ (5 μmol/L in 0.01% DMSO). We purchased all of these drugs from Sigma-Aldrich (St. Louis, MO, USA). DMSO at 0.01% produces no significant effects on RMP or excitability of either intact DRG neurons [5] or acutely dissociated DRG neurons (data not shown). Although we have not conducted systematic dose-response studies on these drugs, all were previously found to have electrophysiological effects at doses between 10 and 500 μmol/L in excised ganglia [5]. Each was diluted in the buffered solution just before application, or diluted initially with distilled water for a stock solution that was subsequently diluted with oxygenated buffered solution prior to application. Application of all drugs began 10–30 min prior to and continued during the 3–4 h of electrophysiological recording. *In vivo* drug administration is described in the next paragraph of CCD.

1.2 *CCD*

Hollow stainless-steel, L-shaped rods (4 mm in length and 0.6 mm in diameter) were surgically implanted unilaterally into the intervertebral foramen (ivf) at L_4 and L_5 to chronically compress the DRG (CCD treatment)[5,8,35]. Some of these rods were with one hole drilled on each side and one outlet on each end to permit delivery of drugs to the DRG during compression[5]. 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 drugs were injected through this tubing during CCD. After surgery, the muscle and skin layers were sutured. To deliver drugs or saline to the DRG in previously naïve rats, a sharp, stainless steel needle, 0.4 mm in diameter (with a right angle to limit penetration), was inserted ~4 mm into the ivf at L_5. Injections took 1.5–2 min, and the needle was withdrawn.

1.3 *Excised, intact DRG preparation*

Some DRG neurons were tested while still in place in excised ganglia prepared as described previously [5,35], using L_4 and/or L_5 ganglia in CCD and sham-operated rats. Briefly, each rat was anesthetized after its final behavioral test. The sciatic nerve was transected at the mid-thigh level, and its proximal portion traced to the ganglia. A laminectomy was performed, and the location of the rod in CCD rats checked. Ice-cold, oxygenated, buffered solution containing (in mmol/L) 140 NaCl, 3.5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 4.5 HEPES, 5.5 HEPES-Na and 10 glucose (pH 7.3, osmolarity 310–320 mOsmol/L) was dripped onto the surface of the ganglion during the procedure. The ganglia from the injured side (left) of the L_4 and L_5 segments were removed and placed in 35-mm petri dishes containing ice-cold, oxygenated, buffered solution. The perineurium and epineurium were peeled off and the attached sciatic nerve and dorsal roots transected adjacent to the ganglion. The otherwise intact ganglion was then treated with collagenase (type P, 1 mg/mL, Roche Diagnostics, Indianapolis, IN) for 30 min at 35 °C and then incubated at room temperature for patch-clamp recordings or other purposes. Naïve DRGs was used as a control for the intact DRGs in the ELISA, RT-PCR, 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 the rats that had previously received CCD treatment for 7–10 d. This intact, CCD DRGs are named "CCD DRGs" in this article. The intact DRGs from the naïve rats were also used as control for the intact CCD DRG preparation.

1.4 *ADD*

DRG neurons were dissociated from L_4 and/or L_5 ganglia taken from CCD and sham-operated rats using conventional methods[37,38]. In brief, the excised ganglion was minced using microdissection scissors, the DRG fragments transferred into 10 mL of the buffered solution containing collagenase (type IA, 1 mg/mL, Sigma) and trypsin (0.5 mg/mL, Sigma), and then incubated for 30 min at 35 °C. The DRG fragments were removed, rinsed 5–6 times in the buffered solution, and put into the buffered solution (5 mL) containing DNase (0.2 mg/mL, Sigma) to prevent possible toxicity from DNA leaking from ruptured cells. Individual neurons were dissociated by passing DRG fragments through a set of fire-polished glass pipettes with decreasing diameter. The dissociated cells were then transferred to acid-cleaned glass coverslips, and electrophysiological tests were performed at 22 °C within 2–6 h after dissociation.

1.5 *Determination of cGMP level*

The cGMP concentration in DRG was measured by ELISA using cGMP Enzyme Immunoassay kit (R&D systems, Minneapolis, MN, USA) according to the

manufacturer's instructions.

1.6 *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 Platium Tag (Invitrogen, Carlsbad, CA, USA). The relative mRNA level of PKG subunit gene expression was analyzed using Quantity One (Bio-Rad, Hercules, CA, USA). The statistical results were obtained from three repeats of the experiment. The primer sets synthesized by Integrated DNA Technologies (Coralville, IA, USA): PKG-I forward 5'-CCATGACATTTCAGCCGACT-3'; reverse 5'-TCCCATCCTGAGTTGTCATC-3'; PKG II forward 5'-CCGAGGGTAGACTGGAGGTGTT-3'; reverse 5'-GAATGGGGAGGTTGAGGAGAAT-3'; β-actin forward 5'-TCTACAATGAGC TGCGTGTG-3'; reverse 5'-AATGTCACGCACGATTTCCC-3'.

1.7 *Immunofluorescent staining and histological analysis*

DRG sections (10 μm) were first blocked with 10% goat serum, then incubated overnight with primary antibodies (anti-PKG, 1:200, Novus Biologicals, Inc Littleton, CO) or rabbit IgG, as an isotype control (1:200, Vector Laboratories, Burlingame, CA). The secondary antibody was Fluorescent labeled anti-rabbit-IgG (1:200, Vector Laboratories). The sections were then incubated with PI counterstaining solution.

The morphologic details of the immunofluorencent staining on the large- and medium-sized and small cells were studied under a fluorescence microscope (Olympus BX51WI, Olympus America Inc., Melville, NY, USA). Ten random fields at 400× magnification were selected for each slide among the different groups. To distinguish cell size-specific changes, DRG neurons were divided into small (<600 μm^2), medium (600–1 200 μm^2), and large (>1 200 μm^2) subpopulations. Levels of immunoreactivity in different cell types were scored as negative (−), weak (+), moderate (++), and strong (+++). To obtain quantitative measurements of immunofluorescence, the multiple microphotographs were also taken at 400× magnification with an Olympus U-CMAD3 camera. All image analysis was performed by using MicroSuite image analysis software (Olympus America Inc.). The cells were randomly selected and the images captured. On average, 15–20 fields (5–20 cells each) for PKG, secondary only and isotype control staining of each group were evaluated and photographed at the same exposure time to generate the raw data. Fluorescence intensities of the different cells in these fields of view, were Analyzed by using MicroSuite image analysis software (Olympus America Inc.). The intensity of fluorescence within the cells was measured with background subtraction. We measured the average green fluorescence intensity of pixels which normalized with the background. The control and experimental tissue was processed in identical manners, and only background levels of fluorescence were detected in the control groups (Isotype group). These studies were performed in four identical experiments.

1.8 *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) in the small cells (soma diameter: 15–30 μm; membrane capacitance: <45 pF) from the intact or dissociated DRGs. These small cells largely correspond to neurons with C-fiber conduction velocities [39]. The protocols were similar to that we have previously described [6]. Glass electrodes were fabricated with a Flaming/Brown micropipette puller (P-97, Sutter instruments). Electrode impedance was 3–5 MΩ when filled with saline containing (in mmol/L) 120 K^+-gluconate, 20 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 ethyleneglycol-bis-(β-aminoethyl-ether) N,N,N',N',-tetraacetic acid (EGTA), 2 Mg-ATP and 10 HEPES-K (pH 7.2, osmolarity 290–300 mOsmol/L). 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 a RMP of −45 mV or more negative. To compare the excitability of the intact naïve, intact CCD and ADD DRG neurons, we focused on evaluating the action potential (AP) threshold current (APTC) and repetitive discharges evoked by the standard depolarizing current. The RMP was taken 2–3 min after a stable recording was first obtained. APTC was defined as the minimum cur-

rent required evoking an AP 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}) 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-s, intracellular pulse of depolarizing current normalized to 2.5 times the APTC.

1.9 *Thermal withdrawal*

Thermal hyperalgesia was indicated by a decrease in the latency of foot withdrawal evoked by a radiant heat stimulus, as described previously [5,35,36]. In brief, each rat was placed in a box containing a smooth glass floor maintained at (26 ± 0.5) °C. Radiant heat was focused on part of the hindpaw that was flush against the glass, and delivered until the hindpaw moved (or up to 20 s, to prevent tissue damage). The latency of foot withdrawal in naïve, control rats is 9–12 s [5,35]. Thermal stimuli were delivered 4 times to each hind paw at 5–6 min intervals. Withdrawal latencies were normalized by subtracting each value on the treated side from the corresponding value on the contralateral side, and the results were expressed as difference scores. The rats were tested on each of 2 successive days prior to surgery. Postoperative tests were conducted 1, 3, 7 and 9 d after surgery and/or on the day of electrophysiological recording (days 3–10). Thermal hyperalgesia for a given rat was defined as a postoperative decrease of foot withdrawal latency from the mean preoperative value, with a difference score ≥3 s [5,35]. Only rats that exhibited thermal hyperalgesia after CCD treatment were used for the electrophysiological studies.

1.10 *Statistical analysis*

Comparisons among the groups of intact, intact CCD, ADD were performed with one-way ANOVA followed by Newman-Keuls tests. Specific hypotheses about differences between each drug-treated group and its corresponding control group were tested with individual *t*-tests. All data are presented as means ± SEM. Statistical results are considered significant if $P < 0.05$.

2 RESULTS

2.1 CCD or ADD treatment increases level of cGMP concentration in DRG

To provide direct evidence supporting the hypothesis that the cGMP-PKG signaling pathway contributes to chronically compressed or acutely dissociated DRG neuronal hyperexcitability [5,6], we first directly measured level of cGMP concentration in the DRG. ELISA measurement showed that level of cGMP 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 cGMP in this study. However, the level of cGMP concentration in the CCD or ADD DRG was significantly increased (Fig. 1). The samples were collected on the 7th day after CCD and within 2–4 h after acute dissociation, respectively.

2.2 CCD or ADD treatment increases level of expression of PKG-I mRNA and protein in DRG

We examined the relative mRNA level of PKG subunit gene expression in the DRG by RT-PCR in the same conditions. Level of mRNA for PKG-I was significantly ($P < 0.01$) increased after CCD or ADD treatment, while level of mRNA for PKG-II was not changed or slightly decreased ($P > 0.05$). In contrast, none of these mRNA expressions was significantly changed in the intact compared with the naïve control DRGs ($P > 0.05$) (Fig. 2).

Immunofluorescence staining showed that protein expression of PKG broadly distributed and significantly increased in all the three categories of the small and the medium- and large-sized DRG neurons after CCD or ADD. Examples of staining of PKG-I and its negative control IgG in the naïve DRG are shown in Fig. 3. The expression of PKG-I protein was greatly increased in

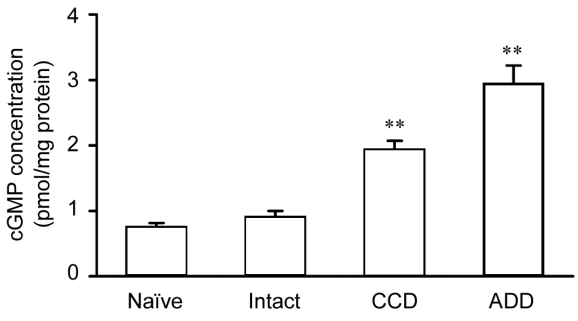

Fig. 1. Alteration of level of cGMP concentration in DRG after ADD and CCD treatment, respectively. cGMP concentration was measured by enzyme-linked immunosorbent assay using cGMP enzyme immunoassay kit. The samples were collected 7 d after CCD or 2 h after ADD. Three samples were included in each group and 4 ganglia in each sample. $^{**}P < 0.01$ *vs* control group, naïve.

CCD- and ADD-DRGs (Fig. 4*A* and *B*). Expression of PKG-I was also increased in the medium-sized cells in intact-DRGs although it was significantly ($P < 0.05$) less than that in the CCD and ADD DRGs (Fig. 4*A* and *B*). The PKG-I immunoreactivity is significantly stronger in the nociceptive small and medium-sized cells than in the large-sized cells ($P < 0.05$, Fig. 4*C*). Taken together, the results presented in the last two paragraphs indicate that CCD and ADD treatment can activate the cGMP-PKG signaling pathway in the DRG neurons.

2.3 cGMP-PKG pathway agonists and antagonists, respectively, enhanced and reduced CCD or ADD treatment-induced DRG neuronal hyperexcitability

Our previous studies hypothesized that cGMP-PKG contributes to injury-related hyperexcitability of DRG neurons proposes that injury increases the electrophysiological responsiveness of DRG neurons to activation of the pathway [5,6]. To further test this proposition, we compared effects of membrane-permeant agonists and antagonists of cGMP-PKG pathway on the nociceptive, small DRG neurons in the three groups described

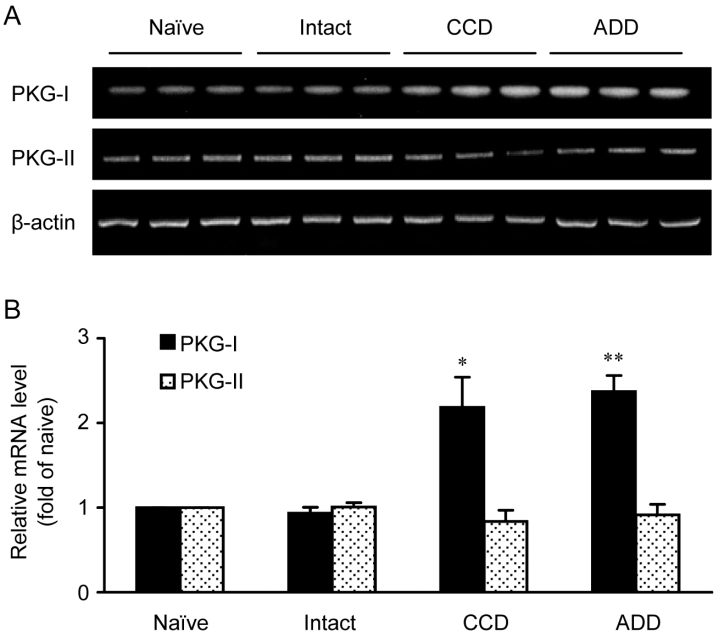

Fig. 2. Alteration of level of mRNA of PKG-I and PKG-II in DRG after ADD and CCD treatment, respectively. The mRNA level of PKG-I and PKG-II was analyzed by RT-PCR. *A*: representative bands. *B*: Data summary. The samples were collected 7 d after CCD or 2 h after ADD. Three samples were included in each group and 4 ganglia in each sample. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* control group, naïve.

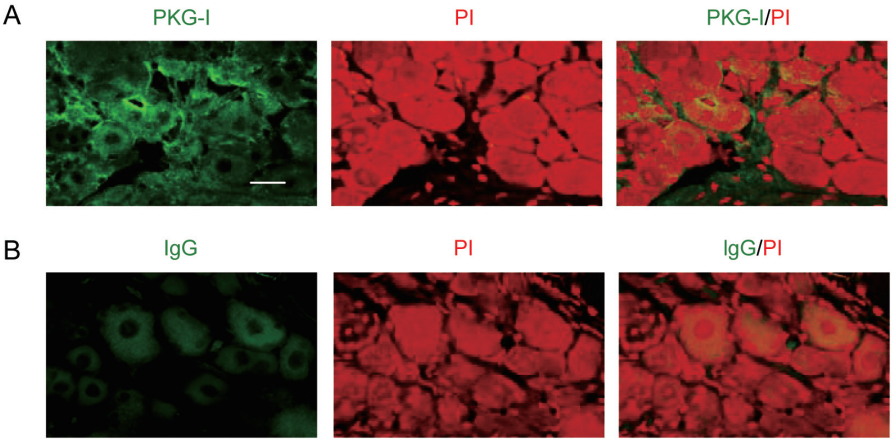

Fig. 3. Examples of immunofluorescence showing the staining of PKG-I (*A*) and its isotype control IgG (*B*) in the Naïve DRG. Scale bar, 30 μm.

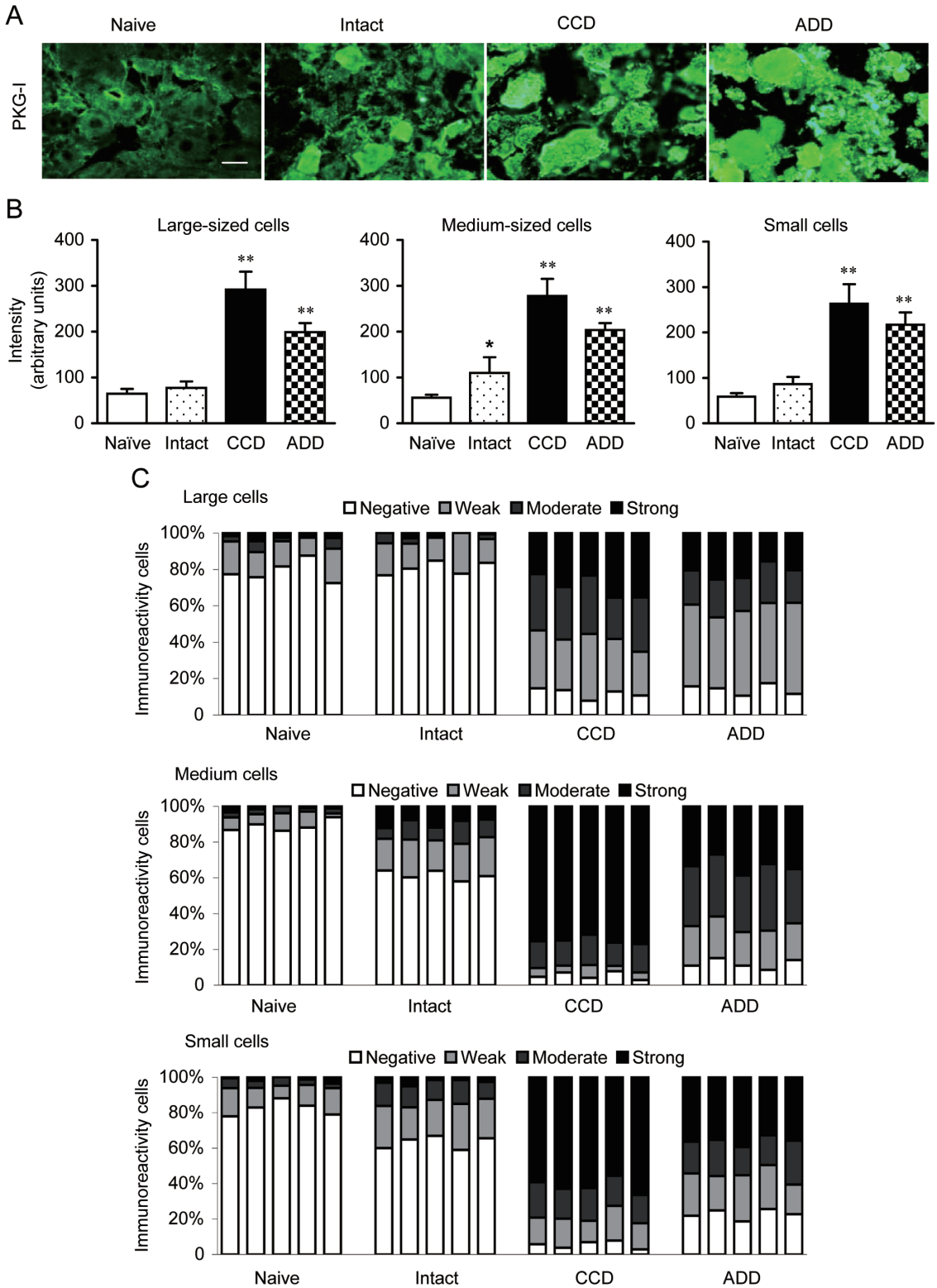

Fig. 4. Alteration of immunofluorescence staining of PKG-I protein in DRG after ADD and CCD treatment, respectively. *A*: Examples of immunostaining of PKG-I after different treatment. Scale bar, 30 μm. *B*: Immunofluorescence intensity of cells in different categories. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* corresponding control group, naïve. *C*: Proportion of the cells in categories of the negative, weak, moderate and strong immunoreactivity. Each group included in the large- and medium-sized and small cell categories included 500 cells counted from 5 ganglia (100 cells from each ganglion).

above: a) minimally stressed neurons in intact DRG from naive animals (Naïve intact), b) neurons in intact DRG from CCD-treated animals (CCD), and (c) dissociated neurons from naive animals (ADD). Fig. 5 illustrates these comparisons graphically across two electrophysiological properties, the action potential threshold current (APTC) and the repetitive discharges of DRG neurons, and shows the similarity in pattern of

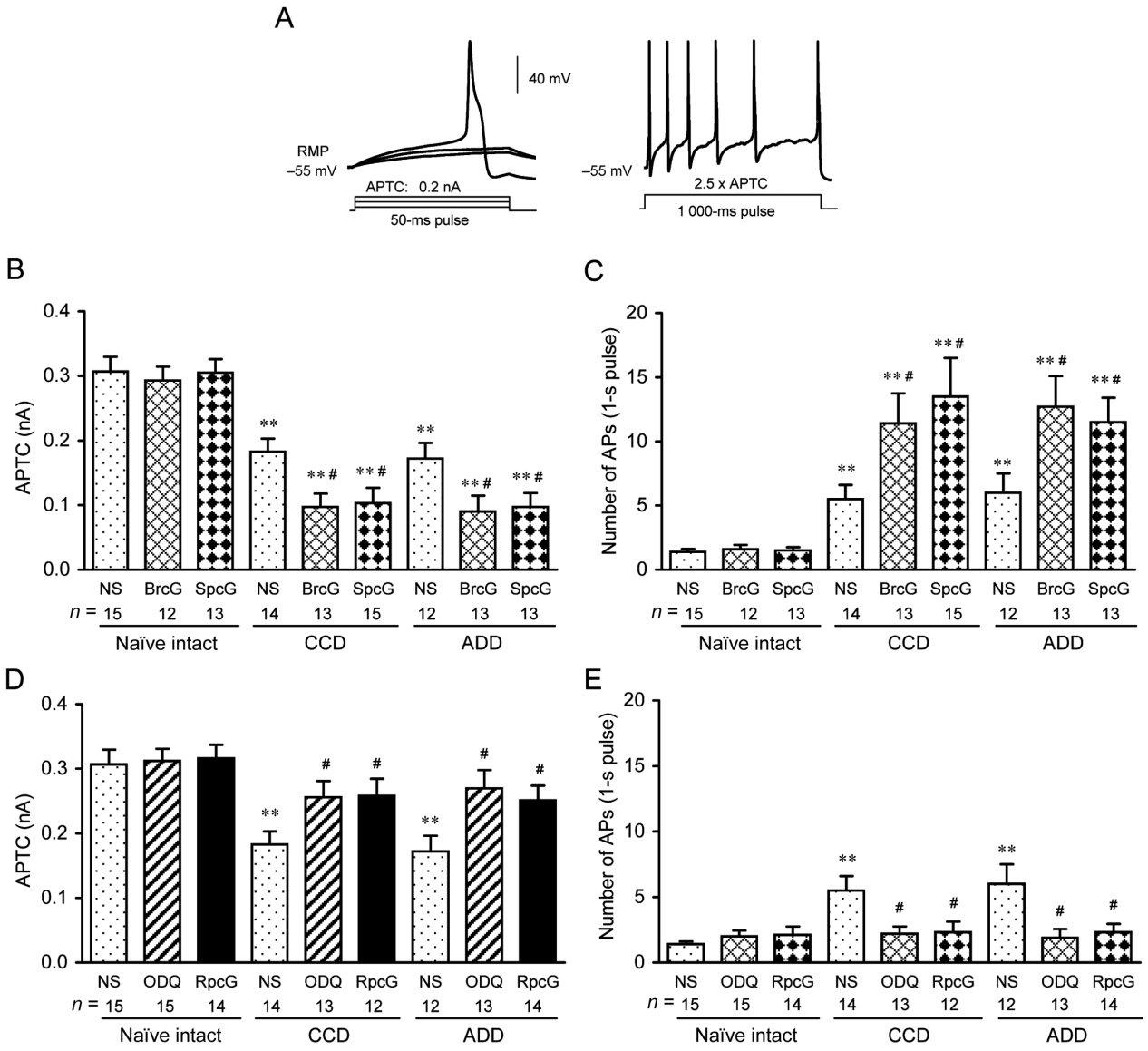

Fig. 5. Effects of agonists and antagonists of the cGMP-PKG pathway on excitability in DRG neuron somata after CCD and ADD treatment, respectively. *A*: Responses of a small DRG neurons recorded with whole-cell patch electrodes under current clamp during the test sequence used to determine action potential (AP) threshold current (APTC). Only some of the hyperpolarizing and depolarizing 50-ms pulses (below) and responses (above) are illustrated. Right, Discharge patterns of DRG neurons tested with 1-s pulses at 2.5 × the 50-ms APTC. Responses shown in the left and the right are from the same neuron (diameter: 24 μm, Cin: 35 pF). *B* and *C*: Data summary of effects of agonists of cGMP-PKG pathway on excitability in neuronal somata in naïve, CCD and ADD DRGs, respectively. *D* and *E*: Data summary of effects of antagonists of cGMP-PKG pathway on excitability in neuronal somata in naïve, CCD and ADD DRGs, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* corresponding control group, naïve Intact. $^{\#}P < 0.05$ indicates significant difference between corresponding groups in the presence and absence of the indicated agonist. NS: Saline ; BrcG: 8-Br-cGMP (50 μmol/L); SpcG: Sp-cGMPS (50 μmol/L); ODQ (5 μmol/L); RpcG: Rp-8-pCPT-cGMP (50 μmol/L). Numbers of cells in each group are indicated in the figures (*B–E*). Note that data of NS group in *B-E* were from the same cells in the corresponding groups of the categories of naïve intact, CCD and ADD, respectively.

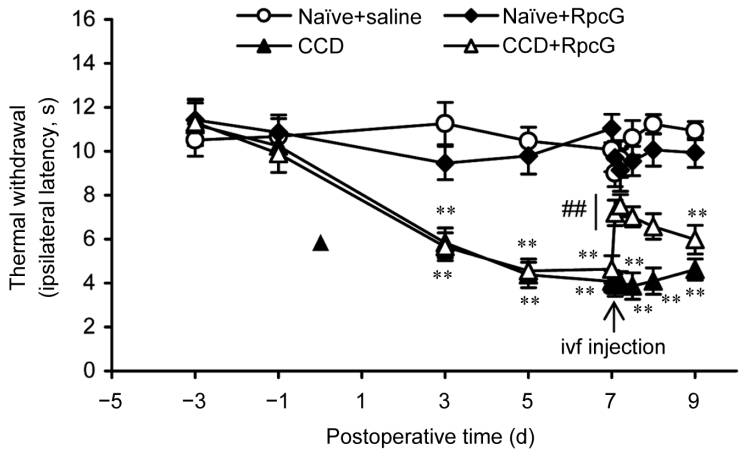

Fig. 6. Effects of antagonist of cGMP-PKG pathway, Rp-8-PCPT-cGMP (RpcG), on CCD-induced thermal hyperalgesia. A single injection of RpcG (intervertebral foramen, ivf, indicated by the arrow, 0.5 mmol/L, 20 μL) administrated on the 7th postoperative day (▲) suppressed CCD-induced thermal hyperalgesia. Eight rats were included in each group. $^{**}P < 0.01$ *vs* corresponding control groups, naïve+saline and naïve+RpcG. $^{\#\#}P < 0.01$ *vs* corresponding group of CCD or the baseline before the single injection of RpcG.

effects produced by each agonist on each of the three stressed groups. The lowered APTC and increased repetitive discharges indicate increased DRG neuronal excitability. Fig. 5*A* gives examples of measurement of APTC and the repetitive discharges evoked by the standardized, depolarizing current. Agonists of the cGMP-PKG pathway, 8-Br-cGMP and Sp-cGMPS (each 50 μmol/L), produced no significant effects on excitability in the minimally stressed, sham intact group. In contrast, excitability of the previously compressed (CCD) or dissociated (ADD) neurons was significantly increased by these agonists (Fig. 5*B* and *C*). Meanwhile, CCD- or ADD-induced hyperexcitability was significantly suppressed by the antagonists of the cGMP-PKG pathway, ODQ (5 μmol/L) and Rp-8-cPCT-cGMPS (50 μmol/L)(Fig. 5*D* and *E*). These results showed that CCD and ADD treatment enhanced electrophysiological responsiveness of the cells to the cGMP-PKG signaling pathways. These results were consistent to our previous findings [5,6]. The largely reduced electrophysiological effects of CCD and ADD treatment by inhibiting cGMP-PKG pathway provided strong evidence for an important contribution of this pathway to the maintenance of hyperexcitability after CCD and ADD treatment.

2.4 *In vivo* delivery of PKG inhibitor into suppressed CCD-induced thermal hyperalgesia

Our findings indicate that the cGMP-PKG pathway is important for maintaining hyperexcitability of DRG neurons during DRG compression, and this hyperexcitability likely contributes to hyperalgesia. Thus, we further tested our hypothesis that application of a blocker of cGMP-PKG pathway can alter the hyperalgesia produced by DRG compression *in vivo*. The cGMP-PKG pathway inhibitor, Rp-8-pCPT-cGMPS (0.5 mmol/L, $n = 8$), was administered into the intervertebral foramen (ivf) of L_5 on the third day after CCD when thermal hyperalgesia was well developed. This inhibitor significantly interrupted the ongoing hyperalgesia, as evidenced by transient recovery of the CCD-induced shortened latencies of foot withdrawal. The inhibition of hyperalgesia started within 2 h, peaked at 6–12 h and lasted for ~24 h. In contrast, in naive rats Rp-8-pCPT-cGMPS (0.5 mmol/L, $n = 8$) failed to altered the latencies of foot withdrawal (Fig. 6), which is consistent to that we report previously[5]. These results suggest that application of PKG inhibitor to the DRG is not normally analgesic, but instead acts to reduce the sensitization induced by CCD treatment.

3 DISCUSSION

This study demonstrates that the prolonged *in vivo* compression or acute dissociation of DRG neurons can activate cGMP-PKA signaling pathway, leading to neuronal hyperexcitability and behaviorally expressed hyperalgesia. CCD or ADD treatment increases levels of cGMP concentration and expression of PKG-I mRNA and protein in DRG. The injured DRG neurons exhibit increased responsiveness to cGMP, and the DRG neu-

ronal hyperexcitability is inhibited by inhibitors of cGMP-PKG pathway. *In vivo* delivery of inhibitors of cGMP-PKG pathway onto the compressed ganglion suppresses CCD-induced hyperalgesia. These findings indicate that CCD or ADD treatment activates the cGMP-PKA signaling pathway and continuing activation of cGMP-PKG pathway is required to maintain hyperexcitability and/or hyperalgesia after CCD and ADD treatment.

CCD or ADD treatment can induce an increase in electrophysiological responsiveness to cGMP and this increased responsiveness is important for maintaining DRG neuronal hyperexcitability. These dissimilar forms of injury-related stress greatly enhance the electrophysiological responsiveness of the stressed neurons to activity in the cGMP-PKG pathway, suggesting interesting possibilities. One possibility is that the enhanced excitatory effects of inflammatory mediators on DRG neurons by prior nerve injury [9,40] or CCD treatment [7] involve an increased responsiveness within the neuron to the cGMP-PKG pathway because excitatory effects of some inflammatory mediators are mediated by this pathway [17]. Another implication of our findings in dissociated neurons [6] is that previous demonstrations of cGMP-PKG contributions to hyperexcitability of DRG somata may have depended at least partly on dissociation increasing the electrophysiological responsiveness of the DRG neurons to cGMP. In this study, we have provided direct evidence that the level of cGMP concentration and PKG activity are increased by CCD or ADD. These findings strongly support the hypothesis that the cGMP-PKG signaling plays an important role in the injury-related stress-induced neuronal hyperexcitability, leading to behaviorally expressed hyperalgesia.

Neuronal dissociation necessarily involves close axotomy. Therefore it would not be surprising for it to trigger some of the same neuronal responses that are evoked by other forms of injury. This was first demonstrated in *Aplysia* sensory neurons [32,33,41], and then in the mammalian small DRG neurons, which correspond to sensory neurons that are often nociceptive and have C-fiber conduction velocities [5,6,34,39,42]. Most of the effects produced by dissociation, such as decreased AP threshold and increased repetitive firing, increase excitability. Hyperexcitability in DRG neuronal somata are also produced by peripheral nerve injury or inflammation [5,6,8,9,35,43–49], raising the question of how closely related these various hyperexcitability states are. Interactions or occlusion between dissociation-induced hyperexcitability and hyperexcitability produced by other manipulations may result in experimental underestimates of some forms of hyperexcitability examined in dissociated neurons, which might confound mechanistic analyses (e.g., see [50] for unexpected mechanistic questions encountered in acutely dissociated DRG neurons). The dissociated DRG neurons respond to agonists of the cGMP-PKG pathway with a further increase in excitability, expressed as a dramatic decrease in AP threshold and a large increase in repetitive firing. However, in both studies, agonists of the cGMP-PKG pathway had only weak, statistically insignificant effects on the excitability of minimally stressed somata in intact DRG from naïve animals confirming our previous demonstrations [5,6]. It is unlikely that these differences reflect increased access of the agonists to the DRG neurons following CCD or ADD treatment because the soma membranes of all the tested neurons, including neurons recorded on the surface of minimally stressed, intact DRG, were directly exposed to the bath after preparation for whole cell patch recording by enzyme treatment and surgical removal of the perineurium and epineurium. These results indicate that ADD greatly enhances the electrophysiological responsiveness of the stressed neurons to activity in the cGMP-PKG pathways. Our studies indicate that the cGMP-PKG pathway also contributes to hyperexcitability in compressed ganglion. A cGMP analog, 8-Br-cGMP, and a PKG agonist, Sp-cGMPS, enhanced hyperexcitability in ADD and CCD DRG neurons, while the PKG inhibitor Rp-8-pCPT-cGMPS reduced the hyperexcitability produced by ADD or CCD treatment. The similar inhibitory effects of the antagonists after ADD or CCD treatment indicate that these injury-related stresses cause a similar hyperexcitable state that requires continuing activity in the cGMP-PKG pathway for at least several hours after dissociation or the end of compression. In principle, the enhanced excitability produced by PKG agonists might be explained by cross-activation of PKA (just as effects of PKA agonists might involve cross-activation of PKG). We have recently discussed roles of the cAMP-PKA pathway in DRG hyperexcitability and hyperalgesia [5,6,12]. However, the reduction of hyperexcitability by inhibitors that are highly selective for each pathway strongly indicates that, in the compressed DRG, the cGMP-PKG pathway, in addition to the cAMP-PKA pathway, helps to maintain DRG neuronal hyperexcitability and behaviorally

expressed hyperalgesia.

cGMP and PKG in the spinal cord are important for hyperalgesia (e.g.,[19,51–54]). We have provided evidence that this pathway operates also in the DRG to produce hyperalgesia. In fact, activation of the cGMP-PKG pathway in somatic sensory neurons has often been reported to have depressive rather than sensitizing effects (e.g.,[13–16]). Different results might be accounted for by different effects of the cGMP-PKG pathway in different subsets of DRG neurons [20]. Evidence consistent with sensitizing effects of the cGMP-PKG pathway are observations that cGMP can increase the excitability of dissociated DRG neurons [55,56] and that type 1 PKG is expressed in both developing and mature DRG neurons [57]. In addition, cGMP-PKG signaling is important for guidance and connectivity of sensory axons during development (e.g., [58,59]). Peripheral processes of adult DRG neurons are involved in some forms of hyperalgesia that depend upon peripheral nitric oxide (NO) release and local cGMP synthesis [22]. NO causes cGMP synthesis in cultured DRG neurons, but in ganglia NO-induced cGMP synthesis is suggested to occur primarily in glial cells rather than DRG neurons [60–62]. An interesting possibility is that compression leads to cGMP synthesis and activation of PKG in stressed sensory axons and subsequent retrograde transport of the active PKG to neuronal somata in the DRG. This occurs in *Aplysia* sensory neurons after nerve injury and results in hyperexcitability of the sensory neuron soma [41]. Potential sources of NO for stimulating cGMP synthesis in the compressed DRG and adjacent nerves include cytokines and inflammatory mediators such as bradykinin [10,63]. Here, we provide further evidence that the level of cGMP concentration and expression of PKG-I mRNA and protein are broadly distributed and significantly increased in the small as well as the medium- and large-sized cells, in addition to the glial cells in DRG, after CCD treatment. These results support the hypothesis that cGMP-PKG pathway contributes to the neuronal hyperexcitability and behavioral hyperalgesia after ADD and CCD treatment.

The close similarity between the hyperexcitable effects produced by the cGMP-PKG and cAMP-PKA pathways after injury we have demonstrated previously [5,6,12] and in this study is interesting, given the opposing effects that can be produced by these pathways in DRG neurons [64,65]. Where these pathways converge to modulate DRG neuronal excitability and how they interact are important questions that need further investigation. Nonetheless, the potent blockade of the effects of the cAMP-PKA and cGMP-PKG agonists by pretreatment with the corresponding antagonists, and the similar degrees of enhancement produced by the same concentrations of each of the different agonists suggest that the effects we have attributed to either the cGMP-PKG pathway or cAMP-PKA pathway are not explained by pharmacological cross-activation of the other pathway. The increased electrophysiological responsiveness to the cGMP-PKG and cAMP-PKA pathways following ADD or CCD may be part of a larger pattern of altered cellular signaling following injury and inflammation. Such findings suggest that complex interactions among the numerous cellular signals that induce and maintain hyperexcitability of DRG neurons may be complicated further by injury- or inflammation-dependent plasticity within some of the signaling pathways. As you may notice that some important points demonstrated in our previous publications [5,6] are again discussed and emphasized here with new supporting data. We have recently identified the upstream molecule that mediates the increased activity of cAMP-PKA pathway following these two dissimilar forms of injury-related stress [12]. The upstream molecules that mediate the increased activity of cGMP-PKG pathway after nerve injury needs to be examined.

In conclusion, this study, based on our previous demonstrations [5,6], provides further evidence supporting the idea that the cGMP-PKG pathway is activated following *in vivo* prolonged compression or *in vitro* acute dissociation of DRG and that continuing activation of cGMP-PKG pathway is required to maintain the injury-induced neuronal hyperexcitability and hyperalgesia.

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