Alteration of P2X1-6 receptor expression in retrograde Fluorogold-labeled DRG neurons from rat chronic neuropathic pain model

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Abstract. Accumulating evidence indicates that P2X receptors may serve an important role in pain and nociceptive sensations. However, recent studies of regulation of P2X receptor expression following nerve injury have produced variable or conflicting results. In the present study the alteration of expression of P2X1-6 receptor subunits in retrograde Flurorogold (FG)-labeled L4+L5 dorsal root ganglion (DRG) neurons were evaluated following unilateral chronic constriction injury (CCI) of the rat sciatic nerve using immunohistochemistry combined with a retrograde fluorescence-tracing method. It was demonstrated that there was no significant difference in the proportion of FG-labeled DRG neurons between the sham and CCI groups (P>0.5). The percentages of P2X1-immunoreactive (IR) and P2X2-IR FG-labeled DRG neurons were not significantly different between the sham and CCI groups (41.5±8.2 vs. 45.2±7.4% and 58.1±6.2 vs. 69.1±3.5%, P>0.05). The percentages of P2X3-IR and P2X6-IR FG-labeled DRG neurons significantly increased in the CCI group compared with the sham group (51.6±4.1 vs. 28.5±3.4% and 41.8±2.2 vs. 22.6±3.3%, P>0.01). By contrast, the percentage of P2X4-IR FG-labeled DRG neurons significantly decreased in the CCI group compared with the sham group (29.4±3.3 vs. 45.0±3.7%, P<0.01). The P2X5-IR positive FG-labeled neurons were not detected in the CCI and sham groups. The results of the present study provided the first evidence regarding the regulation of the expression of the P2X1-6 receptor in sensory neurons being directly associated with chronic nerve injury in rats and also suggest that compared with the P2X3 receptor, the P2X2/3 heteromeric receptor is not the major receptor involved in peripheral neuropathic pain sensation. In addition, the possible functional role of P2X6 receptors in peripheral neuropathic pain requires further investigation.

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Introduction

P2X receptors are ligand-gated ion channels that are activated by extracellular adenosine triphosphate. To date, seven functional mammalian P2X receptor subunits (P2X1-7) have been identified that assemble as either homo- or heterotrimeric receptors (1). Accumulating evidence indicates that P2X receptors serve an important role in the generation and transmission of pain and inflammation nociceptive signals (1,2). In particular, the P2X3 homomeric and P2X2/3 heteromeric receptors occur in a subset of putative nociceptive sensory neurons (1,2) and the expression of these receptors has been reported to increase in several peripheral nociceptive conditions (1,2). Furthermore, using selective antagonists, antisense oligonucleotides and gene knock-out mice, several studies confirmed that these receptors are closely associated with peripheral nociceptive mechanisms (1,2).

Previous studies suggested that in addition to P2X3 and P2X2/3 receptors, other P2X receptors may be also involved in the peripheral nociceptive mechanism. For instance, except for P2X7, all other P2X receptor subunits are expressed in various primary sensory neurons including the dorsal root ganglion (DRG) and trigeminal ganglion neurons (1,2). However, compared with P2X3 and P2X2/3 receptors, the functional role of other P2X receptors in the peripheral nociceptive mechanism remains largely unknown. Specifically, little information is available about the regulation of the receptor expression in peripheral nociceptive conditions.

The aim of the present study was to evaluate the alteration of expression of the P2X1-6 receptor subunits in retrograde Flurorogold (FG)-labeled L4+L5 DRG neurons following unilateral chronic constriction injury (CCI) of the rat sciatic nerve using immunohistochemistry combined with a retrograde fluorescence-tracing method. The results of the present study provide the first evidence regarding the regulation of P2X1-6 receptor expression in sensory neurons directly associated with chronic nerve injury in rats.

Materials and methods

Animals and neuropathic pain model. All animal experiments in the present study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the

Animal Care and Use Committee of Jianghan University (Wuhan, China). A total of 24 male Sprague-Dawley rats (250-270 g), 7 weeks old, which were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China) were individually housed in cages in a temperature and humidity (23±1˚C and 50-55%) controlled room under a reversed 12-h light-dark cycle with food and water freely available. The CCI model was produced as previously described (3). Briefly, twelve rats were anesthetized by injection of pentobarbital sodium (25 mg/kg, i.p), following induction in sample bottles containing cotton balls dipped in ether used as anesthetic jars for 2 min. After the right common sciatic nerve was exposed, ~7 mm of the nerve was freed from adhering tissue and four ligatures (4.0 chromic gut) were tied loosely with ~1-mm spacing proximal to the sciatica's trifurcation. Twelve rats with the right sciatic nerve exposed without a ligature served as sham controls.

Mechanical and thermal sensitivity measurements. Mechanical allodynia and heat hyperalgesia were determined as previously described (4,5). An automated Dynamic Plantar Aesthesiometer (UGO Basile, Camerio, Italy) was used to detect the paw mechanical withdrawal threshold (MWT). Briefly, rats were placed on a wire mesh floor in clear cylindrical plastic enclosures. Following 20 min of acclimation, a von Frey filament was placed on the plantar surface of the right hind paw and the force was increased gradually until a withdrawal response was evoked, and the amount of force needed to cause the withdrawal response was recorded. A maximum cut-off value of 50 g was used. Each trial was repeated 3 times at ~5-min intervals and the mean force producing withdrawal response was determined. Thermal nociceptive responses were determined using a plantar test instrument (Ugo Basile). The rats were acclimatized to the apparatus that consisted of three individual perspex boxes on a glass table. A mobile radiant heat source was located under the table and focused onto the desired paw. The paw withdrawal latency was recorded three times for the right hind paw and the average was taken as the value. In order to prevent tissue damage, an automatic cut-off at 30 sec was set.

Retrograde Flurorogold (FG)-tracing of DRG neurons. A total of 15 days following CCI, the rats were anesthetized by injection of pentobarbital sodium (25 mg/kg, i.p). The right common sciatic nerve was exposed and bisected completely. Then, 2 μl 2% FG (Fluorochrome, LLC, Denver, CO, USA) was smeared on the distal cuff of the ligature on the sciatic nerve. The fascia and skin were then closed.

Tissue preparation and immunohistochemistry staining. A total of 3 days after FG retrograde, rats were anesthetized and then were systemically perfused intracardially with 250 ml ice-cold normal saline followed by 250 ml 4% paraformaldehyde in 0.01 M PBS (pH 7.4). The corresponding segments (L4+L5) of DRG were carefully separated following fixation. After paraffin embedding, DRG paraffin tissue blocks were cut into 4-μm-thick slices. The 4-μm serial sections were deparaffinized in xylene, rehydrating in graded ethanol, rinsed in distilled water and then pre-incubated with 3% hydrogen peroxide for 15 min to inactivate endogenous peroxidase. Antigen retrieval slides were incubated at 95˚C in 10 mM citric acid buffer (pH=6.0) in a microwave oven (750 W) for 15 min. Following washing with PBS three times, the preparations were preincubated with 10% normal goat serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 40 min in a moisture chamber at 37˚C. The sections were then incubated with rabbit anti-P2X1-6 (1:200; cat. nos. APR-022, APR-025, APR-026, APR-024, APR-027 and APR-028; Alomone Labs, Inc., Jerusalem, Israel) overnight at 4˚C. After 3 rinses in PBS, the sections were then incubated with fluorescent secondary antibody (1:200; cat. no. ab150079; Abcam, Cambridge, UK) in the dark at 37˚C for 40 min. The prepared sections were given three times washes again in PBS before mounted in mounting medium and then cover slipped. After these steps, the sections were observed with fluorescence microscopy. The negative controls were processed in the same manner except that PBS was used instead of the primary antibody.

Image analysis and quantification. Fluorescence images of DRG sections were acquired with an OLYMPUS BX51 fluorescence microscope outfitted with the relevant filter blocks, a Hamamatsu C5810 color CCD camera and its proprietary Image Processor software v1.7 (Hamamatsu Photonic System, Bridgewater, NJ, USA). Cell sizes were determined by the previously described method (6). Cell diameters <30 μm were classified as small-diameter neurons, cell diameters from 30 to 50 μm were medium-diameter neurons and cell diameters >50 μm were large-diameter neurons (6). The numbers of FG-labeled neurons and FG /P2X1-6 double-tagged neurons for each animal were counted. This procedure was performed in a blinded manner.

Statistical analysis. Mechanical and thermal sensitivity measurements were repeated three times. All results were expressed as the mean ± standard error of the mean. GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses. Statistical significance of results was analyzed with Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rat neuropathic pain model assessment. A total of 3 days following the sciatic nerve CCI operation, the rats gradually exhibited the typical signs of hyperalgesic responses including toe closing, foot eversion and paw-licking. By contrast, the behavior of the sham-operated rats did not obviously alter. The changes in ipsilateral MWT and thermal withdrawal latency (TWL) are demonstrated in Fig. 1. The MWT and TWL values for rats in CCI group significantly decreased ($P<0.001$) on day 3 following CCI operation and further reduced on day 5 ($P<0.001$) compared with the sham-operated rats, indicating that the mechanical allodynia and thermal hyperalgesia were established on the third day following CCI operation.

Retrograde FG-tracing of DRG neurons. As presented in Fig. 2A, neurotracer FG-labeled neurons were identified in L4+L5 DRG neurons in the sham and CCI groups. The average proportions of the FG-labeled neurons were 44±7.6 and 55±6.2% of total L4+L5 DRG neurons in the sham

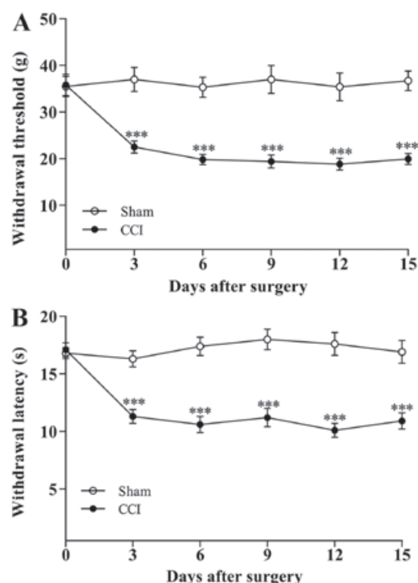

Figure 1. Mechanical and thermal sensitivity assessments. The CCI rats (n=8) demonstrated a significantly reduced threshold levels to (A) mechanical stimuli and (B) thermal stimuli following surgery compared with sham-operated rats (n=8), indicating the development of allodynia. ***P<0.001 vs. the sham group. CCI, chronic constriction injury.

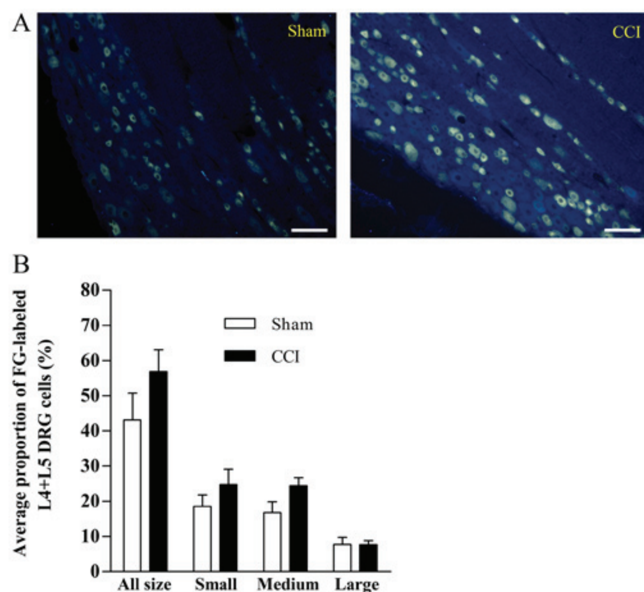

Figure 2. Detection of retrograde FG-labeled L4+L5 DRG neurons. (A) Photomicrographs demonstrating fluorescence of FG-labeled neurons in L4+L5 DRG from sham and CCI groups. (B) Graph demonstrating the average proportions of the FG-labeled L4+L5 DRG neurons vs. cell size between sham (n=6) and CCI (n=7) groups. Scale bars, 100 μm. CCI, chronic constriction injury; DRG, dorsal root ganglion; FG, Fluorogold.

and CCI groups, respectively, and no significant difference was detected in different sizes of neurons between these two groups (Fig. 2B). The diameter of cells varied from 17 to 70 μm.

P2X1-6 receptor expression in FG-labeled neurons of L4+L5 DRG. The protein expression of P2X1-6 receptor subtypes in FG-labeled L4+L5 DRG neurons following sciatic nerve CCI were compared. It was demonstrated that all P2X receptor proteins were expressed in FG-labeled DRG neurons of the sham and CCI groups, except the signal of P2X5 receptors was not detected just like a previous study (Fig. 3) (7).

In retrograde FG-labeled L4+L5 DRG neurons, the percentages of P2X1-immunoreactive (IR) neurons were 41.5±8.2 and 45.2±7.4% between the sham and CCI groups, and these values were not significantly different (P<0.05). In the small-, medium- and large-diameter FG-labeled L4+L5 DRG neurons, the percentages of P2X1-IR neurons were 10.5±2.9 and 13.0±4.5% (P<0.05), 20.7±5.5 and 20.4±5.7% (P<0.05), 10.4±4.1 and 11.9±2.9% (P<0.05) between the sham and CCI groups (Fig. 3; Table I).

In retrograde FG-labeled L4+L5 DRG neurons, the percentages of P2X2-IR neurons were 58.1±6.2 and 69.1±3.5% between the sham and CCI groups, and these values were not significantly different (P>0.05). In the small-, medium- and large-diameter FG-labeled L4+L5 DRG neurons, the percentages of P2X2-IR neurons were 21.5±3.5 and 29.3±5.8% (P<0.05), 20.5±2.3 and 26.8±2.7% (P<0.05), 15.5±5.1 and 13.9±4.2% (P<0.05), which were significantly different between the sham and CCI groups. (Fig. 3; Table I).

In retrograde FG labeled L4+L5 DRG neurons, the percentage of P2X3-IR neurons in the CCI group significantly increased compared with the in sham group (51.6±4.1 vs. 28.5±3.4%, P<0.01). In small-, medium-, large-diameter FG-labeled L4+L5 DRG neurons, the percentages of P2X3-IR neurons in CCI group significantly increased compared with the sham group (19.3±3.6 vs. 9.6±2.3%, P<0.05; 25.7±3.3 vs. 15.5±2.3%, P<0.05; 6.6±1.1 vs. 3.5±0.9%, P<0.05, respectively; Fig. 3; Table I).

In retrograde FG-labeled L4+L5 DRG neurons, the percentage of P2X4-IR neurons in CCI group significantly decreased compared with the sham group (29.4±3.3 vs. 45.0±3.7%, P<0.05). In small- and large-diameter FG-labeled L4+L5 DRG neurons, the percentages of P2X4-IR neurons in CCI group were not significantly different compared with the sham group (12.0±3.4 vs. 18.7±4.1%, P<0.05; 5.3±1.5 vs. 6.2±1.7%, P<0.05). However, in medium-diameter FG-labeled L4+L5 DRG neurons, the percentage of P2X4-IR neurons in CCI group significantly decreased compared with the sham group (12.1±2.6 vs. 20.1±2.4%, P<0.05; Fig. 3; Table I).

In retrograde FG-labeled L4+L5 DRG neurons, the percentage of P2X6-IR neurons in CCI group significantly increased compared with the sham group (41.8±2.2 vs. 22.6±3.3%, P<0.01). In small- and medium-diameter FG labeled L4+L5 DRG neurons, the percentages of P2X6-IR neurons were not significantly different from the sham group (13.9±3.3 vs. 7.1±1.9%, P>0.05; 18.1±3.2 vs. 11.8±2.6% P<0.05). However, in large-diameter FG-labeled L4+L5 DRG neurons, the percentage of P2X4-IR neurons in CCI group significantly increased compared to that in the sham group (9.8±2.5 vs. 3.7±0.6%, P<0.01; Fig. 3; Table I).

Discussion

Out of the seven cloned functional mammalian P2X receptor subunits, a growing body of evidence suggests that P2X3 and P2X2/3 receptors serve important roles in the generation and transduction of sensory nociceptive signals. For instance, it has been reported that antagonist A-317491 selective for P2X3

Table I. The percentage (%) of Fluorogold-labeled L4+5 dorsal root ganglion neurons with P2X 1, 2, 3, 4, 6-immunoreactive positive staining between sham (n=6) and CCI (n=7) groups.

		Cell size		
Receptor	All size	Small	Medium	Large
P2X1				
Sham	41.5±8.2	10.5±2.9	20.7±5.5	10.4±4.1
CCI	45.2±7.4	13.0±4.5	20.4±5.7	11.9±2.9
P2X2				
Sham	58.1±6.2	21.5±3.5	20.5±2.3	15.5±5.1
CCI	69.1±3.5	29.3±5.8	26.8±2.7	13.9±4.2
P2X3				
Sham	28.5±3.4	9.6±2.3	15.5±2.3	3.5±0.9
CCI	51.6±4.1[b]	19.3±3.6[a]	25.7±3.3[a]	6.6±1.1[a]
P2X4				
Sham	45.0±3.7	18.7±4.1	20.1±2.4	6.2±1.7
CCI	29.4±3.3[a]	12.0±3.4	12.1±2.6[a]	5.3±1.5
P2X6				
Sham	22.6±3.3	7.1±1.9	11.8±2.6	3.7±0.6
CCI	41.8±2.2[b]	13.9±3.3	18.1±3.2	9.8±2.5

[a]P<0.05, [b]P<0.01 vs. the sham group. CCI, chronic constriction injury.

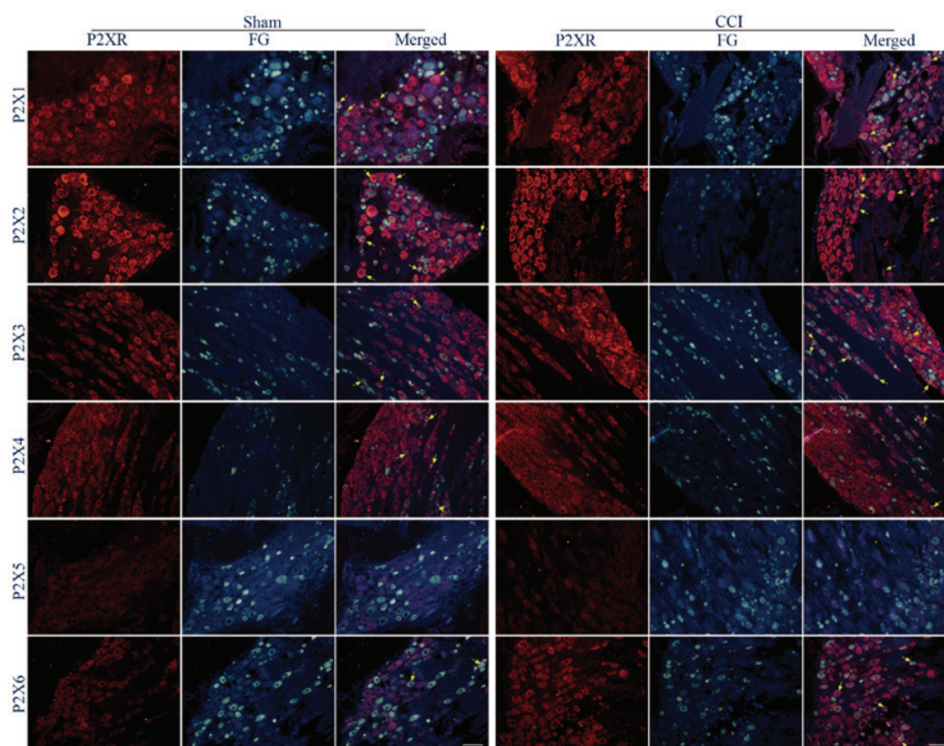

Figure 3. Detection of P2X1-6 receptor expression in FG-labeled neurons of L4+L5 DRG. Photomicrographs presenting fluorescence of FG-labeled and P2XR1-6 IR neurons in L4+L5 DRG from the sham and the CCI groups. Yellow arrows indicate the P2XR+/FG+ double-labeled neurons. Scale bars, 100 μm. IR, immunoreactive; FG, Fluorogold; DRG, dorsal root ganglion; CCI, chronic constriction injury.

and P2X2/3 subunit-containing channels could reduce persistent, chronic neuropathic and inflammatory pain in rats (8-10). In addition, studies using P2X3-selective antisense (11-13) or small interfering RNA (14), as well as P2X3-deficient mice (15,16) or P2X2/3 double knockout mice (17) revealed comparable results. However, the underlying cellular and

molecular mechanism of the involvement of P2X3 and P2X2/3 receptors in the generation and transduction of nociceptive signals has not been established.

Previous studies revealed that the P2X3 and P2X2/3 receptors are widely expressed in peripheral sensory neurons, especially in a subset of putative nociceptive sensory neurons (1,2). Notably, variable or conflicting experimental results have been reported regarding the alteration of expression of P2X3 receptors in different nociceptive conditions. For example, immunohistochemical studies indicate that the P2X3 receptor expression is markedly increased in DRG neurons following sciatic nerve CCI in rats (18,19). Similarly, P2X3 receptor upregulation has been reported in rat trigeminal primary sensory neurons following inferior alveolar nerve injury (20). By contrast, a significant reduction in P2X3 immunoreactivity was observed in DRG neurons following peripheral axotomy (21) and spinal nerve ligation (22) in rats. In addition, it has been reported that P2X3 receptor expression was not altered in rat DRG neurons following spinal nerve ligation (23) and in trigeminal ganglion neurons by lingual nerve injury in ferrets (22). Although the reason for these discrepancies remains unknown, several factors could be involved including animal species (7), animal models used to produce nerve injury and dynamic regulation of P2X receptor expression (22).

Similar to P2X3 and P2X2/3 receptors, *in situ* hybridization and immunohistochemical studies revealed that other P2X receptor subunits were widely expressed in sensory neurons (1,2), therefore raising the possibility that these P2X receptors may be also involved in nociceptive sensation. However, compared to P2X3 and P2X2/3 receptors little information is available about the alteration of expression of these P2X receptors in nociceptive conditions. Based on the limited information, variable or conflicting experimental results have also been reported regarding the expression of these P2X receptors in different experimental nociceptive conditions. For example, the gene expression of P2X6 receptors has been reported to decrease in the rat spinal nerve ligation experiment (24). By contrast, it has recently been demonstrated that the gene and protein expression of P2X6 receptors markedly increased following sciatic nerve CCI in rats (19).

In the present study, in order to evaluate the regulation of expression of P2X receptors in the chronic neuropathic pain condition, the expression of P2X1-6 receptor subunits were analyzed in retrograde FG-labeled sensory neurons in L4+L5 DRG following unilateral CCI of the rat sciatic nerve using immunohistochemistry combined with retrograde fluorescence-tracing method. It was demonstrated that the average proportions of the FG-labeled neurons were 44 and 55% in the sham and CCI groups, and there were no significant differences detected in different sizes of neurons between these two groups. It was also demonstrated that all P2X receptor proteins were expressed in DRG neurons of CCI and sham groups, except the signal of P2X5 receptors was not detected just like a previous study reported (7).

The authors' previous study demonstrated that the expression of P2X1 receptors in rat DRG neurons increased following sciatic nerve CCI (18). The present study, however, revealed that in similar experimental conditions the expression of P2X1 receptors did not change significantly. The reason for this discrepancy is most likely due to the cells used for analysis between these two studies being different: In the previous study the cells were not labelled using retrograde fluorescence-tracing method and the cells used for analysis may not be directly associated with the nerve injury. Similarly, previous studies demonstrated that the expression of P2X2 receptors in rat DRG neurons increased following spinal nerve ligation (23) and sciatic nerve CCI (18). The experimental results of the present study demonstrated that the expression of P2X2 receptors slightly increased following CCI compared with the sham group, but the difference between these two groups was not significant. Again, the reason for this discrepancy is most likely due to different cells being used for analysis in different studies. Consistent with previous studies (17,18), the results of the present study demonstrated that the expression of P2X3 receptors in rat DRG neurons significantly increased following sciatic nerve CCI, supporting the functional role of this receptor involved in neuropathic pain sensation. It has been observed that the expression of P2X4 receptors in rat DRG neurons did not significantly alter following sciatic nerve CCI in the authors' previous study (18). In the present study, however, it was demonstrated that the expression of P2X4 receptors decreased compared with in the sham group. As mentioned above, the reason for this discrepancy is most likely due to different cells used for analysis between these two studies. The expression of P2X6 receptor in rat DRG neurons following sciatic nerve CCI has been demonstrated to increase in the authors' previous study (18) and similar results were demonstrated in the present study: In FG-labeled neurons (including small-, medium- and large-diameter cells), the percentage of P2X6-IR neurons in CCI group increased compared with in the sham group.

Present study to the best of our knowledge, provides the first evidence regarding the regulation of P2X1-6 receptors in retrograde FG-labeled sensory neurons directly associated with sciatic nerve injury in rats and it was demonstrated that among P2X1-6 receptors only the expression of P2X3 and P2X6 receptors increased. These results consistent with the previous studies regarding the role of P2X3 receptors in peripheral neuropathic pain sensation. Interestingly, the present study demonstrated that the expression of P2X2 receptors did not significantly increase, suggesting that compared with the P2X3 receptor, the P2X2/3 heteromeric receptor is not the major receptor involved in peripheral neuropathic pain sensation. It is noteworthy that in P2X2/3 double knockout mice the pain-associated behavior reduced in response to intraplantar injection of formalin, suggesting that heteromeric P2X2/3 receptors make an important contribution to nociceptive responses (11). However, the functional role of heteromeric P2X2/3 receptors in neuropathic pain sensation has not been clearly established. In addition, the present study revealed that the expression of P2X6 receptors significantly increased, which is similar to the authors' previous study (18). Based on the current information, however, P2X6 receptors seem unable to form functional homomultimers (1,2) and these receptors also do not appear to form heteromultimers with P2X3 receptors which was observed to significantly increase in the present study (24). Therefore, determining the functional role of P2X6 receptors in peripheral neuropathic pain sensation will be an interesting subject for future studies.

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Availability of data and materials

All data used and/or analyzed during this study are available from the corresponding author on reasonable request.

Authors' contributions

LC and CLi made substantial contributions to the conception and design of the study. CLeng and LC performed the experiments and analyzed the data. CLeng drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animals used in the experiments in the present study were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of Animal Care and Use Committee of Jianghan University (Wuhan, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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