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Original Article

Expression of purinergic receptor P2Y4 in Schwann cell following nerve regeneration

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Abstract: Objective: Emerging evidences suggested an important role of purinergic receptor P2Y4 in nerve system. The present study aims to investigate the localization and possible function of P2Y4 receptor in recurrent laryngeal nerve (RLN) following regeneration. Methods: Right RLN of fifty Sprague-Dawley rats was cut and immediately repaired with PLGA/chitosan nerve conduit. Immunofluorescence, real-time qPCR and Western blot were used to detect the expression alterations of P2Y4 receptor. Results: Weak immunostaining signals of P2Y4 receptor were located on the plasmalemma of Schwann cell (SC) with regular arrangement of axons in normal RLN. On the post-injury 4th day, the sprouting axons regrowed along the degenerated SCs intensively expressing P2Y4 receptor. On the post-injury 7th day, the regenerating axons existed in multicellular cords of P2Y4 receptor-positive SCs occupying the nerve gap. On the post-injury 14th day, the axons grew along the bands of P2Y4 receptor-positive SCs exhibiting the regularly parallel distribution. On the post-injury 30th day, mild immunostaining signals of P2Y4 receptor still existed on SC surface, and the regenerated axons were located inside the remodeled endoneurium tube. In accordance with immunofluorescence findings, the transcription and protein expression levels of P2Y4 were significantly increased after the injury and the peak value appeared on the post-injury 7th day, compared to control group ($P < 0.05$). Conclusion: Data from the present study suggested a potential role of P2Y4 receptors in functional modulation of SCs in the regeneration of RLN.

Keywords: Purinergic receptor, Schwann cell, nerve regeneration, recurrent laryngeal nerve

Introduction

Vocal fold paralysis is one common disease in otolaryngology clinic, and its causes varied, such as iatrogenic, traumatic, and tumor-derived, etc. Unsatisfactory functional recovery and synkinesis of laryngeal muscles are mainly due to poor or misdirectional regeneration of axons after the injury to recurrent laryngeal nerve (RLN). How to promote the regeneration and functional reconstruction of RLN is always a difficult clinical issue. It is well-known that Schwann cell (SC), as a specific type of glial cells, plays an important role in the development and regeneration process of peripheral nerves. SCs could secrete a variety of active substances such as nerve growth factor and neurotrophic factor, and could also regulate the axon regeneration and the myelin remodeling [1-3]. Wallerian's degeneration occurs in the damaged peripheral nerve, which mainly appeared as the necrosis, decomposition and absorption of axon and myelin, as well as the proliferations of SCs. The nerve-injuring signals could induce the dedifferentiated SCs to largely proliferate, migrate and differentiate. On one hand, SCs could provide the signals and matrixes for the axonal regeneration of nerve fibers. On the other hand, SCs would spirally extend and wrap the newborn axons, and then formed the specific myelin structure. This process was involved in complex molecular and cellular events, and fine regulation mechanism as well [3-5]. The integration of SC function ensures the sufficient number of regenerated axons and the correct direction of axonal regeneration, so

it was the important factor that could affect the degree of functional recovery of the target organ (skeletal muscle).

The nerve regeneration depends on the adaptive regulation of SC function and states, and the regulation mechanisms were extremely precise and complicated. Purins and their metabolic products, as activity-dependent signaling molecules and neurotransmitters, are widely engaged in injury and regeneration of central and peripheral neuronal system via regulating glia-glia and glia-neuron communications [6]. Their effects are mainly mediated by purinergic receptor families: P2X (ligand-gated cationic channels) and P2Y (G protein-coupled receptors) types [7]. Recent studies had indicated that the purinergic receptors might participate in the axonal regeneration, regulating the information exchange among the neurons and glial cells, among which the purinergic receptor P2Y subtype member might be involved in regulating the activation, migration, and differentiation of SCs [8] . As a member of P2Y family, P2Y4 receptor was reportedly expressed in rat dorsal root ganglion and the spinal cord [9]. However, the localization of P2Y4 receptor remains unclear in the peripheral nerve, as well as after nerve injury and regeneration. Thus, the present study aims to explore the expression alteration of P2Y4 receptor using an animal model of RLN injury, which is expected to highlight the role of P2Y4 receptor in peripheral nerve system.

Materials and methods

RLN injury and regeneration models

All animal work was reviewed and approved by the ethics committee of Second Military Medical University. Fifty adult New-Zealand rabbit were used in this study, and randomly divided into 5 groups: the post-injury 4th-, 7th-, 14th- and 30th- day group, and sham operation in normal rabbits were set as the control group (n = 10). All animals were anesthetized by intraperitoneal injection with sodium pentobarbital (40 mg/kg). RLN on the right side was transected at the level of about 3-4 tracheal rings, and 1 mm length of both nerve stumps was introduce into the prepared PLGA/chitosan nerve conduit (3.5 mm in length and 1.2 mm in diameter). The conduit and the epineurium were fixed using 11-0 prolene suture. All animals were intraperitoneally injected penicillin sodium (80000 units) one time per day for postoperative infection prevention, with free feeding and water daily, as well as the alternating cycle of 12-h light and 12-h darkness. The rabbits of each subgroup were sacrificed by the intraperitoneal injection of overdose phenobarbital sodium on the designed postoperative time points. The RLN was cut from the level of cricothyroid joint to the distal end of nerve conduit, and about 1 cm nerve specimen was removed. Some samples were fixed in 4% paraformaldehyde-phosphate buffer solution overnight, followed by dehydration in a graded sucrose series and cryoprotected in 30% sucrose phosphate-buffered saline at -20°C until further immunofluorescence staining. The other samples were stored directly in liquid nitrogen for later use in real-time qPCR and Western blot.

Immunofluorescence

Eight μm serial longitudinal frozen sections were labeled using indirect immunofluorescence techniques. The primary rabbit polyclonal P2Y4 antibody (APR-006, 1:100) was purchased from Alomone Labs Ltd. Mouse monoclonal S100 (sc-58837, 1:50), monoclonal neurofilament (sc-20014, 1:100) and rabbit polyclonal $p75^{NGFR}$ (sc-8317, 1:100) antibodies were purchased from Santa Cruz Biotechnology, Inc. In order to block any nonspecific reactions, all sections were incubated in 0.1 mol/L PBS containing 20% horse serum and 0.5% Triton X-100 for 10 min, and then were incubated with the primary antibodies at 4°C overnight, followed by incubation with the secondary antibodies for 1 h at 37°C. The FITC-conjugated donkey anti-mouse and Cy3-conjugated donkey anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:1000 were used as the secondary antibodies. Nuclei were counterstained with 25 μg/mL 4',6'-diamidino-2'-phenylindole (DAPI; Sigma Aldrich, Inc., St. Louis, MO). The primary antibody was substituted by PBS in the same slides as the negative control. Immunofluorescence staining was examined with a Nikon Eclipse 300 fluorescence microscope (Nikon, Tokyo, Japan).

Real-time qPCR

The frozen tissues were thawed and immediately homogenized in 1 mL of Trizol® reagent (Invitrogen Co., Carlsbad, CA). Total RNA was

then extracted with chloroform, precipitated with isopropanol, and resuspended in 100 μL of RNAse-free water. The concentration of isolated total RNA was determined by measuring the optical density at 230 nm (OD_{230}) with an ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA). The reverse transcription of 1 μg of total RNA was performed to synthesize complementary DNA (cDNA) using a PrimerScript™ RT reagent kit (TaKaRa Biotechnology Co. Ltd., Dalian, China) with the GeneAmp PCR System 9700 (Applied Biosystems, Inc., Foster City, CA). Nucleotide sequence of Rabbit P2Y4 receptor (XM_002720078.2) was obtained from GenBank, and the housekeeping gene β-actin (NM_001101683.1) was used as the internal control. Primers for target gene messenger RNA (mRNA) were designed using the online software Primer 3 Plus (Wageningen University and Research Centre, Gelderland, the Netherlands). The primer sequences for P2Y4 gene was as follows: (1) P2Y4 receptor: upstream primer 5'-AGCTATGCGGTCGTCTTTGT-3', and downstream primer 5'-GGACGCTGCAG-TAGAGGTTC-3', yielding a fragment of 250 bp. (2) β-actin: upstream primer 5'-TTTATTTTGA-ATGGTCAGCCATC-3', and downstream primer 5'-TGTTCAGGTGTGCACTTTTATTG-3', yielding a fragment of 162 bp. Real-time qPCR was performed using the reagents and protocol supplied with a SYBR® Premix Ex Tag™ II kit (TaKaRa Biotechnology). Reactions were performed in a volume of 20 μL containing 0.5 μL of cDNA. The amplification program consisted of an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 10 s, and elongation at 72°C for 10 s, with a final elongation step at 72°C for 10 min. Data were collected and analyzed on a Rotor-Gene Q real-time PCR cycler (Qiagen Pty Ltd., Doncaster, Victoria, Australia). The specificity of the PCR product was examined based on the melting curves of the reactions. Relative expression levels of target genes were analyzed using the $2^{-\Delta\Delta CT}$ quantitative method.

Western blot

Approximately 100 mg of nerve tissue from each sample was homogenized in lysis buffer (Applied Biosystems). After incubated for 1 hour in an ice bath, the homogenates were centrifuged at 12000 rpm for 15 min, and the supernatant was harvested. For immunoblotting assay, aliquots of the supernatant containing 100 μg of protein were loaded into individual lanes of 12% sodium dodecyl sulfate-polyacrylamide gels, separated by electrophoresis under standard conditions (200 mA, 180 min), and electroblotted onto PVDF membranes (Millipore Co., Billerica, MA). To block the non-specific binding sites, the membranes were incubated with 5% nonfat milk in PBS for 2 h at room temperature. Subsequently, the membranes were incubated overnight with the rabbit polyclonal anti-P2Y4 antibody diluted 1:400. The membranes were then washed three times in PBS containing 0.05% Tween-20 (PBST) before being incubated with horseradish peroxide-conjugated anti-rabbit IgG (1:2000; Sigma-Aldrich) for 2 h at room temperature. After washing three times in PBST, the membranes were reacted with Pierce enhanced chemiluminescent substrate (Thermo Fisher Scientific) for 1 min, according to the manufacturer's instructions. The membranes were exposed and photographed using the Flourchem® FC2 system (Alpha Innotech Co., San Leandro, CA) equipped with imaging software that automatically analyzes the density of each band. After imaging, the membranes were stripped, and glyceraldehyde phosphate dehydrogenase (GAPDH) was used as internal control. The relative protein levels were deduced from the actual density divided by the density of the corresponding GAPDH.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Student's t-test for multiple comparisons and were presented as the means ± standard deviation. For all comparisons, $P < 0.05$ was considered as the statistical significance.

Results

The present study used S100 and p75NGFR (P75) to label the SCs, and neurofilament (NF) to label the axons. Double immunofluorescence staining was adopted to detect the localization of P2Y4 receptor in RLN. As shown in **Figure 1A**, the expression of P2Y4 receptor in normal RLN was very weak, and located on the plas malemma of S100-positive SCs (green) with regular arrangement of axons. On the post-injury 4th day (see **Figure 1B**, **1C**), the obvious gap was generated by retraction of transected

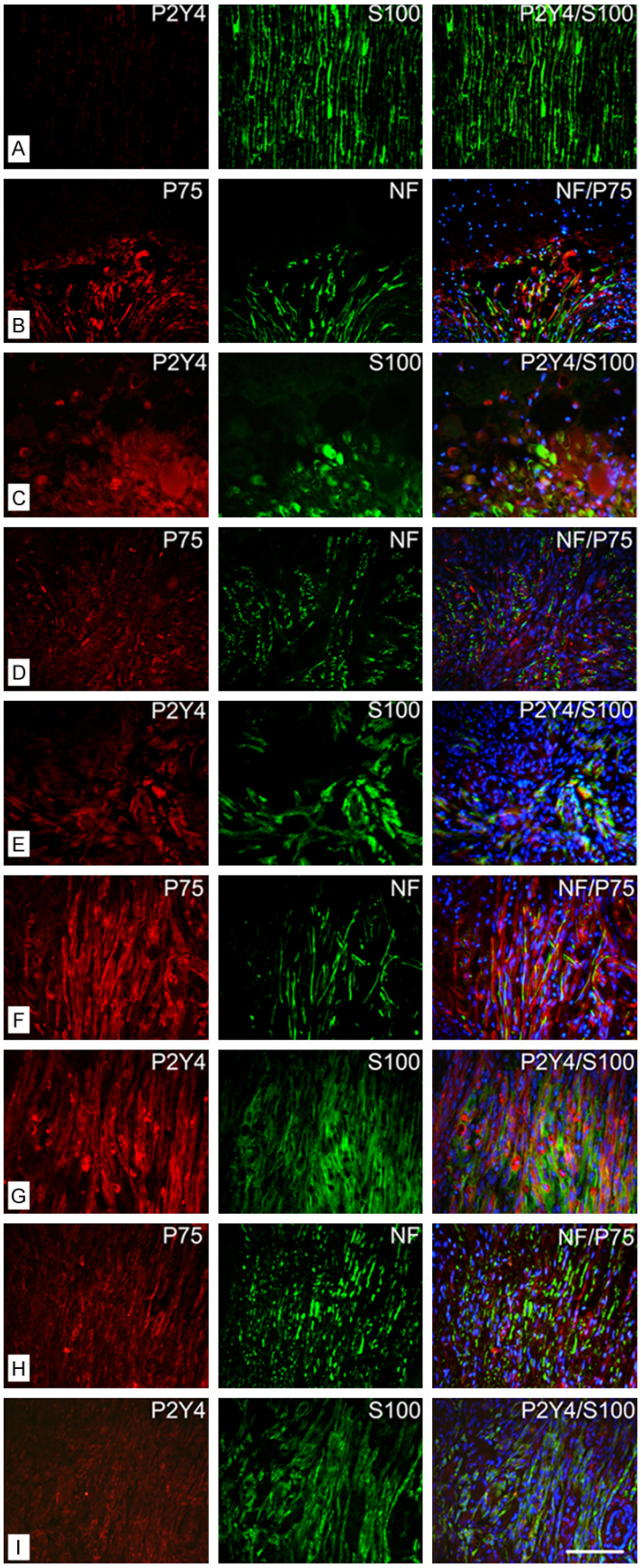

Figure 1. Expression of P2Y4 receptors in recurrent laryngeal nerve. Immunofluorescence staining for Schwann cell maker $P75^{NGFR}$ (P75) or S100, and axonal neurofilament (NF). Nuclei were counterstained with DAPI. A. Weak immunopositive signal of P2Y4 (red) was located on the plasmalemma of S100-positive SCs (green) with regular arrangement of axons in normal nerve. B, C. On the post-injury 4th day, an obvious gap was seen between both sides of transected nerve stumps, and sprouting axons (NF-positive, green) regrowed towards the lesion site along the SCs (P75-positive, red). P2Y4 receptor-positive SCs displayed promiscuous distribution. D, E. On the post-injury 7th day, the regrowing axons traveled through the gap, and formed newly "nerve bridge". Discrete cell cords formed by SCs were found in the nerve gap, which intensively co-expressed P2Y4 receptor. F, G. On the post-injury 14th day, the regenerating axons were located in the bands of SCs distributed regularly. Intensive immunostaining signals for P2Y4 receptor were detectable on the plasmalemma of SCs. H, I. On the post-injury 30th day, the axons and SCs showed regular alignment similar to normal nerve, while positive immunostaining signals for P2Y4 receptor were mild on the SC surface. The scale bar represents 100 μm.

nerve stumps. And NF-positive sprouting axons (green) regrowed towards the lesion site along the p75-positive SCs (red). P2Y4 receptor-positive SCs displayed promiscuous distribution. On the post-injury 7th day (see **Figure 1D**, **1E**), the regenerating axons sprout and travel through the gap. SCs in the form of multicellular cords migrated across the lesion site, and intensively co-expressed P2Y4 receptor. On the post-injury 14th day (see **Figure 1E**, **1F**), the sprouting axons were mainly located in the bands of SCs with the regular distribution. Positive immunostaining signals for P2Y4 receptor were detectable on the plasmalemma of SCs. On the post-injury 30th day (see **Figure 1H, 1I**), the distribution of SCs inside the nerve trunk was regular. The regenerated axons were located

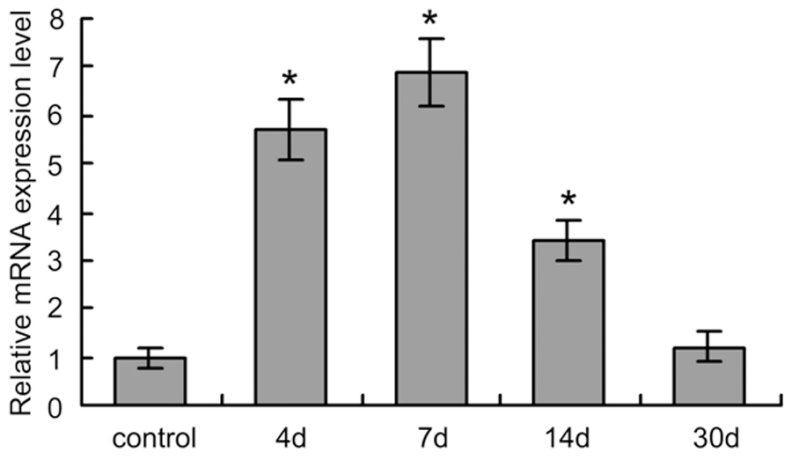

Figure 2. Real-time qPCR for detecting mRNA expression level of P2Y4 receptor in recurrent laryngeal nerve. The quantitative assessment showed that transcription expression level of P2Y4 receptors was significantly up-regulated after injury, and peaked on the post-injury 7th day, compared to control ($P < 0.05$). However, relative transcription level of P2Y4 receptor on the post-injury 7th day was down-regulated to the baseline level of the control ($P < 0.05$).

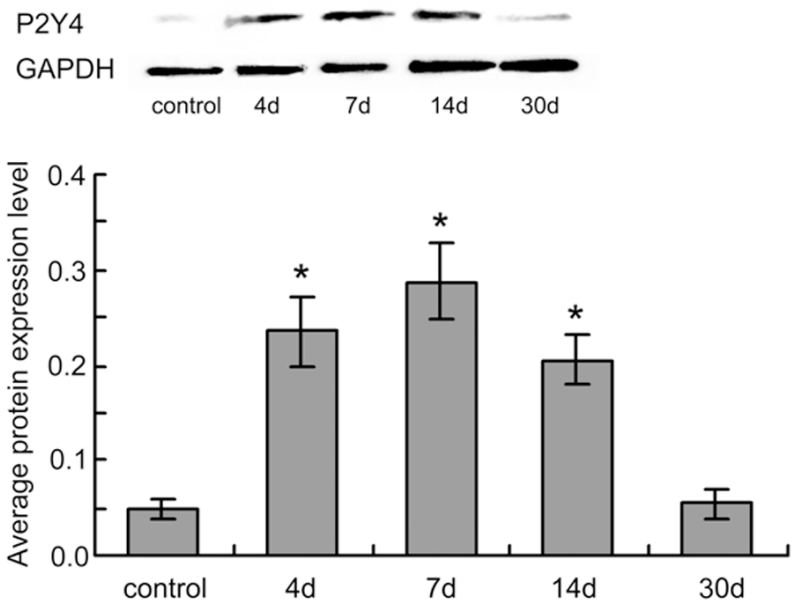

Figure 3. Western blot for detecting protein expression level of P2Y4 receptor in recurrent laryngeal nerve. The measurement was repeated at least 3 times. Control, 4 d, 7 d, 14 d and 30 d lanes represent the expression of P2Y4 receptor and GAPDH in the control and on the 4th-, 7th-, 14th- and 30th- day post-injury. A representative image showed that the protein of P2Y4 receptors was detected in all lanes. Further optical density analyses showed that the highest relative levels of P2Y4 receptor protein presented on the post-injury 7th- day group. The post-injury 4th-, 7th- and 14th- day groups showed significantly statistical difference compared to the control group, respectively ($P < 0.05$).

inside the remodeled endoneurial tubes. Mild immunostaining signals for P2Y4 receptor were still detectable on the SC surface.

As shown in **Figure 2**, real-time qPCR assays showed that the expression level of P2Y4 mRNA was significantly up-regulated after RLN injury, and was about 5.8 times to the control level ($P < 0.05$), which reached the highest level on the post-injury 7th day with about 6.9 times to the control level ($P < 0.05$). The high level was maintained until 14 days after injury. However, the expression level was decreased on the post-injury 30th day, with no statistical significance to the control ($P > 0.05$).

The positive bands of P2Y4 receptor were detected in all the groups (see **Figure 3**). The ratio of P2Y4 to GAPDH was determined by optical density analyses. The relative expression level of P2Y4 receptor protein was increased on the post-injury 4th day and peak on the post-injury 7th day. The level on the post-injury 4th, 7th and 14th day was about 4.6, 5.5 and 4.1 times than the control group, respectively ($P < 0.05$). However, the relative expression level of P2Y4 receptor on the post-injury 30th day showed no significantly statistical difference compared with the control group ($P > 0.05$).

Discussion

It is well known that functional recovery is dependent on the type and extent of nerve lesion. In case of nerve crush injury, regeneration is particularly successful because the integrity of the basal lamina surrounding the axon/SC nerve unit allows highly efficient and accurate reinnerva-

tion, while poor regeneration occurs in case of nerve transection which significantly disrupt nerve structure [10]. Although this severe lesion of nerve transection was adopted in this study, immunostaining findings showed that successful regeneration of RLN occurred 30 days after nerve cut, suggesting that immediate repair using PLGA/chitosan nerve conduit contributed to good outcome. The obvious gap was generated due to the retraction of either side of transected nerve stumps on post-injury 4th day. Many mononuclear cells existing in this gap are possibly infiltrated inflammatory cells or fibroblasts, which has proven to be essential for nerve regeneration [11]. These cells formed newly tissues, referred to as the "nerve bridge" [12], to reconnect the proximal and distal stumps of RLN from 7th to 14th day post-injury. During above period, the regenerating axons were found to sprout and pass through the newborn "nerve bridge" to reach the distal stump. On post-injury 30th day, the regenerated nerve displayed histomorphological characteristics close to normal nerve, in which the axons aligned regularly with detectable continuity.

The present study focused on the morphological change of SC in the process of RLN regeneration. It was found that SC began to proliferate on the post-injury 4th day, and then formed discrete cell cords in the proximal stump and collectively migrated towards the lesion site on the post-injury 7th day. Compared to normal nerve, SCs exhibited disorderly arrangement upon injury, and ultimately regained the relatively regular alignment on the post-injury 30th day. During the above period, sprouting axons changed from promiscuous regrowth to regular parallel distribution, and positive staining signals for neurofilament were always located in SCs and remodeled endoneurial tubes, suggesting the morphological and function regulation of SCs may be involved in the directional regrowth of axons. As a kind of glial cell of peripheral nerves, SCs arise from trunk neural crest cells that find their way to the emerging axons of motor neurons during development, in which SCs interpret different extracellular cues to regulate their migration, proliferation, and the remarkable morphological changes associated with the sorting, ensheathment, and myelination of axons [1]. SCs are important for nerve regeneration through replenishing lost or damaged tissue by proliferation, and producing a favorable environment for axonal regrowth both by helping to clear myelin debris and by forming cellular conduits or corridors, known as bands of Bungner, that guide axons through the degenerated nerve stump and back to their targets. After injury, SCs can proliferate and migrate along the outgrowing axons and wrap axons, ultimately accomplish the remyelination, which is believed to be regulated by axon-derived survival signals. Each SC typically first envelops multiple axonal segments but ultimately surrounds a segment of a single axon [2, 13]. In case of peripheral nerve transection injury, continuity of axons as well as endoneurial tubes is disconnected. It is speculated that the migrating SCs may be the only axonal guidance at an early stage of regeneration. Directed migrating SCs exhibited a range of distinct motile morphologies, characterized by their symmetry and number of extensions [4]. Loss of guidance from SCs would result in aberrant reinnervation of renew axons [5]. In addition, SCs could synthesize dozens of neurotrophic cytokines and remodel the extracellular matrix, which are crucial in regulating the regeneration of axons and the formation of myelin sheath [3]. The formation and function of the neuromuscular junction during development and regeneration also depends on SCs, which actively modulate synaptic activity [14]. Although many investigations have explored the functional regulation of SCs, the underlying mechanism is not well known yet.

Intriguingly, co-localization of P2Y4 receptor with S100 (a maker for SCs) was found in the present study. Immunostaining intensity of P2Y4 receptor was significantly increased on the post-injury 4th day, compared to normal RLN. The intensive immunostaining signal for P2Y4 receptor continuously were localized on the plasmalemma of S100-positve SCs until on the post-injury 14th day. Quantitative assay using real-time qPCR and Western blot both showed that the transcription and protein expression level of P2Y4 receptor peaked on the post-injury 7th day. In accordance with immunofluorescence findings, no statistical significance was found between post-injury 30th day group and control group.

P2Y4 receptor belongs to purinergic P2Y receptor family, which are metabotropic G-protein-coupled receptors including eight subtypes [7]. In response to peripheral nerve injury, extrac-

ellular purine nucleotides and nucleosides are released and activate through purinergic receptors expressed on the SC surface, which regulating neurotransmission, neuromodulation, chemoattraction and acute inflammation [15]. Therefore, purinergic receptors might act as a kind of "damage-susceptible receptors" or "immunomodulatory receptors", thus participating the regulation of neural axon regeneration and regulating the information exchange between the neurons and the glial cells [16]. It was reported that no in situ hybridization signals for P2Y4 were seen in any cellular components of rat dorsal root ganglion, whereas P2Y4 mRNAs were expressed by some of the dorsal horn neurons and the motor neurons in the ventral horn in the spinal cord [9]. We also found that immunostaining signals for P2Y4 receptor were hardly detectable in normal RLN, while immunostaining intensity was significantly enhanced and located on the SC surface after injury, indicating that activation of P2Y4 receptor may participate in functional regulation of SCs.

Effect of P2Y4 receptor distinguished from other P2Y subtypes was proven to be crucial for specific ligand activation, membrane partitioning and consequent functional regulation [17]. The specific role of P2Y4 receptors was found in the modulation of inner retinal signaling [18], and possibly mobilize intracellular Ca^{2+} concentration in central nerve system [19]. The G-protein-coupled receptor signaling was involved in myelination during development and remyelination during regeneration [8]. Other P2Y subtypes, such as P2Y1 and P2Y2, were reportedly involved in regulating SCs [20, 21]. Recent studies had shown that cross-talk between growth factor and purinergic signaling via ERK/MAPK signaling pathway may positively and negatively regulate SC proliferation before the onset of myelination [22]. Emerging evidence has implicated that activation of P2Y receptor family may initiate and regulate a large number of biological processes, especially in cytoskeleton rearrangement [23]. The present study demonstrated that specific spatial-and-temporal expressional alteration of P2Y4 receptor in RLN, suggesting its potential role in regulating the function of SCs for peripheral nerve regeneration. An increasing number of studies regarding specific purinergic receptor agonists and antagonists provide a promising therapeutic strategy for peripheral nerve regeneration. Further researches would be needed to clarify the exact role of P2Y4 receptor in SCs, and reveal the underlying mechanism.

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Disclosure of conflict of interest

None.

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