Up-regulation of P2X7 receptors mediating proliferation of Schwann cells after sciatic nerve injury

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Abstract Peripheral nerve injury (PNI) is a common disease, which results in a partial or total loss of motor, sensory and autonomic functions, leading to a decrease in quality of life. Schwann cells play a vital role in maintaining the peripheral nervous system and in injury and repair. Using immunohistochemistry, Western blot, calcium assay and bromodeoxyuridine (BrdU) proliferation assay, the present study clearly demonstrated that P2X7 receptors (R) were expressed in myelinating and non-myelinating Schwann cells in longitudinal sections of sciatic nerves. After sciatic nerve injury (SNI), P2X7R expression in Schwann cells of injured sciatic nerves was significantly up-regulated during the early days of SNI. Double immunofluorescence of proliferating cell nuclear antigen (PCNA) and

P2X7R implied that P2X7R may be involved in proliferation of Schwann cells. Further experiments on primary cultures of Schwann cells showed that P2X7R are functionally expressed in Schwann cells of rat sciatic nerves; ATP via P2X7R can promote Schwann cell proliferation, possibly via the MAPK/ERK intracellular signalling pathway. Other possible roles of P2X7R on Schwann cells are discussed.

Keywords P2X7 receptor · Schwann cell · Proliferation · Sciatic nerve injury

Introduction

Peripheral nerve injury (PNI) can reduce one's quality of life, as a consequence of a partial or a total loss of motor, sensory and autonomic functions. Schwann cells play a vital role in maintaining the peripheral nervous system. PNI initiates a cascade of degenerative cellular and molecular changes at the site of injury and in distal segments of nerves. In the early stages of nerve injury, Schwann cell proliferation is an important event in the degeneration/regeneration process. The function of this injury-induced Schwann cell proliferation is likely to replace dead or dying cells, since Schwann cells are essential for the production of neurotrophic factors to support the survival of injured neurons, promote macrophage infiltration to the injured nerve and supply growing axons with a substrate to grow on [13, 14, 16, 27].

At the site of nerve injury, high concentrations of extracellular ATP are released from cells. As the ATP degradation system is inhibited in injury and at sites of inflammation [5, 10], high extracellular ATP would persist for a long time. High extracellular ATP acts on local cells, including Schwann cells, via P2 receptors (R).

P2R are divided into P2X and P2Y receptor families [1]. Currently, seven subtypes of ionotropic P2XR (non-selective

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cation channels) and eight subtypes of metabotropic P2YR (G protein-coupled receptors) have been identified [5].

Previous data has shown that the Schwann cells mainly express P2X7R [20, 21, 24]. Early electrophysiological and pharmacological data showed that P2X7R are functionally expressed in myelinating Schwann cells of peripheral nerves [15]. With methods of patch-clamp recording, fluorescent dye uptake and immunocytochemistry, P2X7R were detected in cultured mouse Schwann cells [8]. Recent data has shown that P2X7R control myelination in sciatic nerves and contribute to the death of Schwann cells transplanted into the spinal cord [12, 20].

In this study, we found that expression of P2X7R increased in a rat model of sciatic nerve injury (SNI), the cells with positive P2X7R immunoreactivity (ir) were proliferating Schwann cells. Further in vitro experiments demonstrated that up-regulation of P2X7R in injured nerves may be involved in Schwann cell proliferation.

Material and methods

Animals and surgical procedures

Male Sprague–Dawley rats weighing 250–300 g were provided by the Animal Center of the Second Military Medical University. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Second Military Medical University and conformed to the UK Animals (Scientific Procedures) Act 1986 and associated guidelines on the ethical use of animals.

SNI: rats were anaesthetized with 10 % (w/v) chloral hydrate (400 mg/kg) intraperitoneally. After the right thigh and buttock were shaved, a longitudinal incision was made along the median femur to expose ~2 cm of the sciatic nerve. The greater sciatic foramen was cut at the tendon level of the obturator internus muscle. The incision was sutured with 10/0 nylon monofilament under a surgical microscope.

Immunohistochemistry

After 2 days (d), 4, 7, 14, 30 or 60 d of nerve injury, rats were anaesthetized and perfused intracardially with saline, followed by 4 % (w/v) paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS), pH 7.4. The sciatic nerves were removed and fixed overnight in 4 % (w/v) paraformaldehyde, then transferred to 25 % sucrose in PBS and kept in the solution for 2 days. Thereafter, the tissue blocks were rapidly frozen, and longitudinal sections (10 µm in thickness) were cut with a Leica cryostat and thawed on gelatine-coated slides.

The following protocol was used for immunofluorescence. The sections were washed 3–5 min in PBS, and then preincubated in a blocking solution (10 % normal bovine serum, 0.2 %

Triton X-100, 0.4 % sodium azide in 0.01 mol/L PBS, pH 7.2) for 30 min followed by incubation with the primary antibodies: P2X7R (1:1000), Alomone, rabbit polyclonal, APR-004; S100β (1:400), Tuj-1 (1:200), p75NTR, PCNA (1:200), MBP (1:200), mouse monoclonal antibodies from Boster, CASPR (1:400) mouse monoclonal antibodies from Santa Cruz, at room temperature overnight. Subsequently, the sections were incubated with Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch) diluted 1:400 for P2X7R, FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) 1:200 for S100β, Tuj-1, p75NTR and PCNA. All incubations were separated by 5–10 min washes in PBS.

Western blot

For Western blotting, the rats were killed after 2, 4, 7, 14, 30 or 60 d of nerve injury, and the sciatic nerves were removed immediately and lysed with 20 mM Tris–HCl buffer, pH 8.0, containing 1 % NP-40, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.1 % L-mercaptoethanol, 0.5 mM dithiothreitol and a mixture of proteinase and phosphatase inhibitors (Sigma). Schwann cells were also treated with the same method. Protein concentration was determined by the BCA protein assay method using bovine serum albumin (BSA) as standard. Fifty micrograms of protein samples was loaded per lane, separated by SDS-PAGE (10 % polyacrylamide gels) and then electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10 % non-fat milk in Tris-buffered saline for 1 h and incubated overnight at 4 °C with P2X7R (1:1000, Alomone), p-ERK(1:1000, CST), ERK (1:1000, CST) and GAPDH (1:1000, Beyotime) antibodies diluted in 2 % BSA in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Sigma) diluted 1:5000 in 2 % BSA in PBS for 1 h at room temperature. The colour development was performed with 400 µg/mL nitro-blue tetrazolium, 200 µg/mL 5-bromo-4-chloro-3-indolyl phosphate and 100 mg/mL levamisole in TSM2 (0.1 mol/L Tris–HCl₂ buffer, pH 9.5, 0.1 mol/L NaCl and 0.05 mol/L MgCl₂) in the dark. Bands were scanned using a densitometer (GS-700, Bio-Rad Laboratories).

Primary culture of Schwann cells

Schwann cells were isolated from sciatic nerves from P1 Sprague–Dawley rats. The rats were placed on ice to be anaesthetized; when the animals stopped moving and became numb, the animals were rinsed using 70 % alcohol for 3 min. Both sciatic nerves were dissected out from each pup. The sciatic nerves were placed into one 35 mm dish containing 2 mL Hank's Buffered Salt Solution (HBSS) which was refreshed leaving 1 mL HBSS. Non-nervous tissues, including the epineurium and perineurium, were gently stripped under a dissecting

microscope. The nerves were cut into 1–2 mm segment; then 1 mL 0.25 % trypsin–EDTA was added and incubated for 45 min at 37 °C. Dulbecco's Modified Eagle's medium (DMEM)/F-12 containing 10 % foetal bovine serum (FBS) was then added to the suspension at a ratio of 4:1 in order to terminate the activity of the trypsin. The mixture was centrifuged at 1000 rpm for 10 min. The supernatant was discarded, and the cells were resuspended in DMEM/F-12 containing 10 % FBS. Finally, the cells were plated onto a 60-mm dish at 37 °C, under 5 % CO₂ for 1 h, before the cells were plated onto a poly-L-lysine-

coated flask (25 cm) and maintained in DMEM/F-12, containing 10 % FBS and forskolin (2 µM) for 1 day. To eliminate fibroblasts, the cells were treated with cytosine arabinoside (10 mM, Sigma) twice for 24 h after the 2-day culture; the cells were washed twice with phosphate-buffered solution (PBS; pH 7.2), and 0.05 % trypsin–EDTA was added (2 mL enzymatic solution per flask). The flasks were continuously shaken vertically for 1 min and subsequently horizontally until most of the Schwann cells were detached from the flask. As soon as the majority of the Schwann cells were suspended, a

Fig. 1 Expression of P2X7R in longitudinal sections of normal sciatic nerves. **a–c** show co-localization of P2X7R ir (red) and S100Aβ ir (green). Note an *arrowhead* showing a fibre-like structure and an *arrow* showing a trapezoid structure in **a** and **b**; **c** is the merged image of **a** and **b**. Note an *arrow* indicating a trapezoid structure double labelled by both P2X7R and S100β antibodies (yellow). **d–f** show co-localization of P2X7R ir (red) and Tuj-1 ir (green). Note an *arrow* showing a trapezoid structure in **d** and an *arrow* showing an axon in **e**. **f** is the merged image of **d** and **e**; note an *arrow* indicating a green axon passing through the middle of five trapezoid structures. **g–i** show

co-localization of P2X7R ir (red) and p75NTR ir (green). Note an *arrow* showing a fibre-like structure in **g** and **h**. **i** is the merged image of **g** and **h**. An *arrow* indicates a double-labelled non-myelinating Schwann cell with P2X7R and p75NTR antibodies (yellow). **j–l** show co-localization of P2X7R ir (red) and MBP ir (green). Note an *arrow* showing a fibre-like structure with P2X7R ir, which was not labelled by MBP in **l**. **m–o** show co-localization of P2X7R ir (red) and CASPR ir (green). Note that no colocalization of P2X7R ir and CASPR ir was observed. Scale bars in **a–c** and **g–i**=100 µm; scale bars in **d–f**=50 µm

fourfold volume of DMEM/F-12 containing 10 % FBS was added to the cell suspension to terminate the activity of the trypsin. The suspended cells were subsequently collected into a conical tube and centrifuged at 1000 rpm for 10 min. After removal of the supernatant, the pellet was resuspended in DMEM/F-12 containing 10 % FBS and forskolin and plated onto a culture plate at a density of 5×10^5 cells/mL for 3 days. The cells were then collected for Western blots, immunocytochemistry, proliferation assays and calcium assays. The purity of the Schwann cells can reach about 95 % as shown by immunostaining with an antibody against the Schwann cell marker, S100.

Bromodeoxyuridine (BrdU) proliferation assays

In order to study the role of P2X7R in Schwann cell proliferation, primary cultured Schwann cells were treated with an agonist and an antagonist for P2X7R and BrdU. ATP and A740003 were supplied by Tocris Bioscience and BrdU (BrdU-B5002) by Sigma-Aldrich. The working concentrations of ATP, A740003, BrdU, AraC and SCH772984 were 100, 1/L, 30, 2 and 50 μ M, respectively. Schwann cell specimens were treated with BrdU, BrdU+ATP, BrdU+ATP+A740003, BrdU+ATP+AraC or BrdU+ATP+SCH772984 for 6 h.

Subsequently, the cells were collected for Western blots, immunocytochemistry or BrdU immunostaining.

BrdU immunostaining

Schwann cell specimens were fixed in 4 % (w/v) paraformaldehyde/PBS for 30 min at room temperature, treated with 0.2 N HCl (in distilled water) for 30 min at 37 °C, neutralized with 0.1 M Na borate (pH 8.5) for 10 min at room temperature, incubated with 1 % BSA in PBS at room temperature for 30 min, incubated with mouse anti-BrdU (1:500) in 1 % BSA/PBS at 4 °C overnight, incubated with FITC-conjugated donkey anti-mouse IgG for 1 h at room temperature, counterstained with DAPI and mounted with 50 % glycerol in PBS. All incubations were separated by 5–10 min washes in PBS.

Calcium assays

Schwann cells grown on coverslips in dishes were washed with PBS (NaCl 8 g/L, KCl 0.4 g/L, glucose 1 g/L, KH_2PO_4 60 mg/L, Na_2HPO_4 47.5 mg/L, pH 7.2) and incubated with 5 μ M fura-2/AM (Sigma) for 30 min at 37 °C (5 % CO_2 balanced air). The cells were then washed and kept in 200 μ L PBS in each

Fig. 2 Expression of P2X7R in teased sciatic nerve specimens. **a–c** show co-localization of P2X7R ir (red) and CASPR ir (green). Note an arrow showing a typical Ranvier node in **a** and **b**; **c** is the merged image of **a** and **b**. Note that the trapezoid structures with P2X7R ir were not labelled by CASPR ir. **d–f** show co-localization of P2X7R ir (red) and MBP ir (green). Note an arrow showing a Ranvier node with P2X7R ir in **d** and **f**. **f** is the merged image of **d**, **e** and an image of the same nerve fibre under phase-contrast microscopy; the arrow shows a typical Ranvier node with P2X7R ir. **g–i** show co-localization of P2X7R ir (red) and S100 β ir (green). Note that the fibre-like structure with P2X7R ir was also labelled by S100 β ir. **j–l** show co-localization of P2X7R ir and BMP ir. No colocalization of P2X7R ir and BMP ir was observed. All scale bars=50 μ m

Fig. 3 Expression of P2X7R (red) and S100 β (green) in longitudinal sections after SNI. **a** is the merged image of **a1** and **a2**, showing colocalization of P2X7R ir and S100 β ir 2 days after SNI. Note that almost all the P2X7R ir cells are also labelled by S100 β . **b** is the merged image of **b1** and **b2**, showing colocalization of P2X7R ir and S100 β ir 4 days after SNI. **c** is the merged image of **c1** and **c2**, showing colocalization of P2X7R ir and S100 β ir 7 days after SNI. **d** is the merged image of **d1** and **d2**, showing colocalization of P2X7R ir and S100 β ir 14 days after SNI. Note that almost all the P2X7R ir cells are also labelled by the S100 β antibody in **b**, **c** and **d**. **e** is the merged image of **e1** and **e2**, showing colocalization of P2X7R ir and S100 β ir 30 days after SNI. Note that most of the P2X7R ir cells are also labelled by the S100 β antibody, but some round cells with P2X7R ir were not labelled by the S100 β antibody, as indicated by an arrow. **f** is the merged image of **f1** and **f2**, showing colocalization of P2X7R ir and S100 β ir 60 days after SNI. Scale bars in **a–f**=100 μ m; scale bars in all inserted small figures=250 μ m

dish. The coverslips were mounted on an inverted microscope (Olympus) and visualized with a 40 $\times$ objective. To obtain Ca^{2+} measurements, the field was alternatively excited at 340 and 380 nm with a scanning monochromator, and the fluorescence emitted >520 nm from a region of interest surrounding individual Schwann cells was imaged with a charge-coupled device (CCD) camera and analysed (MetaFluor). All Ca^{2+} measurements were performed at room temperature. To determine dye extrusion, a control sample was run in parallel. Dye extrusion was subtracted from the sample data to obtain the corrected fura-2 ratio. After a 5-min interval at 37 $^{\circ}\text{C}$, 100 μM ATP was added; 1 μM A740003 was added after the ratio reached a maximum.

Statistical analysis

All data are presented as mean $\pm$ SD ($n=5$). Statistical analysis was performed using one-way ANOVA followed by Dunnett's post hoc test. Statistical significance was calculated with a p value of <0.05 .

Fig. 4 Expression of P2X7R detected by Western blotting in the sciatic nerves of normal and 2, 4, 7, 14, 30 and 60 d after SNI. **a** shows Western blots of P2X7 receptor protein (top panel) from the control group and 2, 4, 7, 14, 30 and 60 d after SNI. In the lower panel, blots were normalized to GAPDH to control for unequal protein loading between lanes. Data are representative of five rats/group. **b** The ratio of P2X7 receptor protein/GAPDH was analysed by one-way ANOVA followed by Dunnett's post hoc test (* $p<0.05$, ** $p<0.01$ vs. control group)

Fig. 5 Colocalization of P2X7R ir (red) and PCNA ir (green) in longitudinal sections of the sciatic nerve 2 days after SNI. **a** and **b** are P2X7R ir and PCNA ir cells, respectively. **c** is the merged image of **a** and **b**. **d** is the merged image of **c** and DAPI counterstaining (blue). An arrow indicates a typical cell with P2X7R ir, PCNA ir and DAPI counterstaining in **a**, **b**, **c** and **d**. All scale bars=100 μ m

Results

In longitudinal sections of sciatic nerves of normal adult rats, P2X7R ir was mainly detected in two tissue structures: one, a thin and long fibre-like structure and the other a trapezoid or square structure (Fig. 1a). In order to confirm which type of cell expressed P2X7R ir, double immunofluorescence of P2X7R (red) and S100 β (green) or p75NTR (p75 neurotrophin receptor) (green) was carried out. Almost all the trapezoid or square structures were also labelled by the S100 β antibody, but the fibre-like structures were also labelled by the S100 β antibody, although the S100 β immunostaining was much weaker in this structure (Fig. 1c). In order to confirm whether the fibre-like structures were nerve fibres, double immunofluorescence of P2X7R (red) and Tuj-1 (green) was carried out. The results showed that the fibre-like structures were not labelled by the Tuj-1 antibody (Fig. 1f). Interestingly, the nerve fibres stained with Tuj-1 ir always passed through the middle of the trapezoid structures with P2X7R ir, although these two structures were not labelled by both P2X7R and Tuj-1 (Fig. 1f). In order to determine whether the fibre-like structures were non-myelinating Schwann cells, double immunofluorescence of P2X7R and p75NTR or MBP was carried out. The results showed that the fibre-like structures were all labelled by the p75NTR antibody (Fig. 1g–i), but not labelled by MBP (Fig. 1j–l). In order to further identify the location of P2X7R ir trapezoid or square structures, double immunofluorescence of P2X7R and CASPR (a Ranvier node marker) was carried out. The results showed that the trapezoid or square structures with

P2X7R ir were not located in the regions of Ranvier node as shown in Fig. 1m–o.

In order to clearly identify P2X7R ir in the substructures of Schwann cells, teased sciatic nerves were used. In teased

Fig. 6 Purity analysis of primary cultured Schwann cells. **a** shows Schwann cells with S100 β ir (red). **b** shows the merged image of **a** and DAPI counterstaining (blue). Note that almost all cells are labelled with S100 β ir. An arrow shows an S100 β ir-negative cell. Scale bars=40 μ m

sciatic nerves, the distribution pattern of P2X7R ir was similar with that in the longitudinal sections of sciatic nerves. Two tissue structures with P2X7R ir were also detected (Fig. 2). In addition, P2X7R ir was detected in the region of Ranvier nodes as shown in Fig. 2a, d. The fibre-like structures with P2X7R ir were labelled by S100 β , but not MBP (Fig. 2g–i).

Two days after SNI, the trapezoid structures with P2X7R ir disappeared completely in the distal segments of the sciatic nerves (Fig. 3a, a2). The fibre-like structures with P2X7R ir increased in number and in thickness at the diameter. These P2X7R ir structures were all labelled by the S100 β antibody (Fig. 3a). Between 4 and 14 days following SNI, almost all the

Fig. 7 Expression of P2X7R in primary cultured Schwann cells (red). **a** shows the expression of P2X7R ir in normal Schwann cells. **b** shows the expression of P2X7R ir in Schwann cells stimulated with 100 μ M/L ATP for 6 hours. Note that the expression of P2X7R was increased significantly. **c** shows the expression of P2X7R ir in Schwann cells stimulated with 100 μ M/L ATP+1 μ M/L A740003 for 6 h. Note that the expression level of P2X7R is similar to that of the control group. **d** shows the expression of P2X7R ir in Schwann cells stimulated with 100 μ M/L ATP+2 μ M/L AraC. Note that the expression level of P2X7R is similar to that of the control group. **e** is the result of Western blots: *top panel* is P2X7R protein from the control group, the ATP-

stimulated group and the ATP+A740003 group. Blots were normalized to GAPDH (*bottom panel*) to control for unequal protein loading between lanes. Data are representative of five Schwann cell specimens/group. **f** is the ratio of P2X7R protein/GAPDH analysed by one-way ANOVA followed by Dunnett's post hoc test (* p <0.05, ** p <0.01). **g** is the result of Western blots: *top panel* is P2X7R protein from the control group, the ATP-stimulated group and the ATP+AraC group. Blots were normalized to GAPDH (*bottom panel*) to control for unequal protein loading between lanes. Data are representative of five Schwann cell specimens/group. **h** is the ratio of P2X7R protein/GAPDH analysed by one-way ANOVA followed by Dunnett's post hoc test (* p <0.05, ** p <0.01)

P2X7R ir cells also displayed positive labelling for the S100 β antibody in the distal segments of sciatic nerves (Fig. 3b–d). After 30 days of SNI, expression of P2X7R ir in the distal segments decreased dramatically (Fig. 3e, e2). After 60 days of SNI, the pattern of P2X7R ir was similar to that in the normal group (Fig. 1a, d).

The up-regulation of P2X7R after SNI was further confirmed by Western blots. The results showed that the P2X7R was significantly up-regulated in distal segments of injured sciatic nerves from 2 to 30 days after SNI. Expression levels of P2X7R were not significantly up-regulated by 60 days after SNI (Fig. 4).

In examining the possible roles of P2X7R up-regulation in the early days after SNI, double immunofluorescence of P2X7R (red) and PCNA (green) was carried out. During 1 week after SNI, in particular at 2 days, most of the P2X7R ir cells were also labelled by PCNA (a cell proliferation marker) as shown in Fig. 5, which implied that up-regulation of P2X7R after SNI might be involved in the proliferation of Schwann cells. In order to clarify this, the effects of ATP via P2X7R on the proliferation of Schwann cells were further studied in primary cultured Schwann cells.

Schwann cells were isolated and purified from sciatic nerves of postnatal day 1 rats. The purity of the Schwann cells was assayed using S100 β antibody immunostaining. About 95 % of primary cultured cells were labelled by the S100 β antibody (Fig. 6).

Primary cultured Schwann cells expressed P2X7R at a low level, as detected by immunocytochemistry (Fig. 7a) and Western blot (Fig. 7d). Six hours after stimulation with ATP, the expression of P2X7R had increased significantly, as shown by both immunocytochemistry and Western blot analysis (Fig. 7b, e, f). An antagonist of the P2X7R, A740003, and an anti-mitotic reagent, AraC, inhibited the up-regulation of P2X7R induced by ATP (Fig. 7c–h).

In order to determine whether the P2X7R in primary cultured Schwann cells was functional, the effect of ATP and A740003 on intracellular Ca^{2+} concentrations was examined in Schwann cells in fura-2/AM-loaded slices. Single-cell calcium measurements showed that the P2X7R agonist ATP (100 μM) led to a rapid increase in intracellular Ca^{2+} , and the ATP-induced $[\text{Ca}^{2+}]_i$ increases were abolished after pre-application with A740003 (Fig. 8).

In normal conditions, the proliferation rate of primary cultured Schwann cells was about 29 ± 5 %. Following ATP stimulation, the proliferation rate increased significantly, as shown in Fig. 7, and the P2X7R antagonist A740003 and the ERK1/2 inhibitor, SCH772984, inhibited significantly the proliferation of primary cultured Schwann cells (Fig. 9).

In order to identify the possible intracellular signalling pathway induced by the activity of ATP on P2X7R, the expression of p-ERK was assayed. The results showed that expression levels of p-ERK in normal Schwann cell specimens were low, but increased significantly after ATP stimulation (Fig. 10). The P2X7R antagonist inhibited significantly the

Fig. 8 Calcium imaging of ATP-evoked responses from primary cultured Schwann cells. **a** is a representative single-cell calcium response to ATP in the absence and presence of A740003. ATP was applied at the beginning of each experiment. Calcium responses are shown as F340/F380. The time between each agonist application was 3 min. **b** shows the areas under the curves after ATP and ATP+A740003 stimuli. **c** shows calcium response peak values after ATP and ATP+A740003 stimuli. Note that A740003 significantly decreases the area and peak values (** $p < 0.01$)

Fig. 9 Assay of cell proliferation rates in primary cultured Schwann cells. **a, d, g** and **j** show BrdU-positive cells (green) in control, ATP, ATP+A740003 and ATP+ SCH772984 groups, respectively. **b, e, h** and **k** show counterstaining with DAPI of **a, d, g** and **j**. **c, f, i** and **l** are the merged images of **a** and **b**, **d** and **e**, **g** and **h** and **j** and **k**, respectively. **m** shows the statistical analysis of the Schwann cell proliferation rate. The results show that ATP increased significantly the Schwann cell proliferation rate, which was inhibited by the P2X7R antagonist A740003 and SCH772984. Scale bars=40 μ m (** p <0.01)

expression levels of p-ERK induced by ATP stimulation (Fig. 10).

Discussion

In this study, we have clearly demonstrated P2X7R expression in myelinating and non-myelinating Schwann cells. Up-regulation of P2X7R expression in Schwann cells of injured sciatic nerves was associated with proliferation of Schwann cells. The role of ATP via P2X7R promoting Schwann cell proliferation was further confirmed using primary cultured Schwann cells.

Previous studies have presented evidence that P2X7R are expressed in the paranodal membrane of rat Schwann cells *in vivo* [15], in cultured mouse Schwann cells [8, 21, 24], in

longitudinal sciatic nerve sections of rat and mouse [20] and in cross and longitudinal sciatic nerve sections of mouse [12]. In this study, we clearly demonstrate localization of P2X7R in two types of Schwann cells in longitudinal sciatic nerve sections. In myelinating Schwann cells, P2X7R are preferentially localized in Schmidt–Lanterman incisures and Ranvier node regions. In non-myelinating Schwann cells, P2X7R are localized throughout the cytoplasm of the Schwann cells. The trapezoid or square structures with P2X7R were not located in the region of Ranvier nodes as shown by the double-labelling of P2X7 and CASPR. As such, these structures may be Schmidt–Lanterman incisures because there is cytoplasm only in these regions of the myelin [29].

Previous data has shown that both myelinating and non-myelinating Schwann cells express S100 protein, but p75NTR is only expressed in non-myelinating Schwann cells [2, 3].

Fig. 10 Phosphorylation levels of ERK detected by Western blotting in primary cultured Schwann cells. **a** is the result of Western blots of p-ERK (*top panel*) from the control group, the ATP group and the ATP+ A740003 group. Blots were normalized to total ERK (*bottom panel*) to control for unequal protein loading between lanes. Data are representative of five Schwann cell specimens/group. **b** is the ratio of p-ERK protein/ total ERK analysed by one-way ANOVA followed by Dunnett's post hoc test (* $p < 0.05$, vs. control group)

Schwann cells show a considerable amount of plasticity. Both myelinating and non-myelinating Schwann cells can dedifferentiate into immature Schwann cells after axonal amputation. Once axons regenerate, these cells differentiate to myelinating or non-myelinating Schwann cells [2, 16, 18]. It is important to determine whether P2X7R are involved in this process of differentiation and dedifferentiation.

The P2XR is considered to be a ligand-gated Ca^{2+} channel with a higher Ca^{2+} flux compared to other neurotransmitter channels, such as acetylcholine or glutamate [11, 25]. In this study, calcium assay showed that ATP via the Schwann cell P2X7R induced Ca^{2+} entry, which was blocked significantly by the P2X7R antagonist A740003. Ca^{2+} is an important second messenger in living cells. Ca^{2+} entry through P2X7R is involved in Schwann cell functions. The MAPK/ERK pathway is involved in the regulation of a variety of growth and differentiation pathways through several phosphorylation cascades. The signalling cascade is activated by a number of receptors, including receptor tyrosine kinase, integrins and ion channels, which then transduce the signal to adaptors that eventually activate Raf, MEK1/2 and ERK [6, 18]. Previous data have shown that the ERK signalling pathway is very important in Schwann cells after PNI [17, 18, 23]. The study by Napoli et al. [23] showed that ectopic ERK1/2 activation was sufficient to trigger myelin breakdown, dedifferentiation and proliferation of Schwann cells in the absence of nerve

injury. Furthermore, the study also showed that Schwann cell-specific ERK1/2 activation was sufficient to induce other responses associated with nerve injury and repair [23]. The present study shows that ATP acting via P2X7R on Schwann cells promotes proliferation. A possible mechanism for this might be that ATP, acting via P2X7R, induces intracellular Ca^{2+} increase, which activates in turn the MAPK/ERK pathway, since p-ERK and the proliferation rate increased significantly after ATP stimulation, both of which were inhibited by the P2X7R antagonist A740003 in primary cultured Schwann cells.

One of the most important functions of Schwann cells is to myelinate axons. In addition, Schwann cells also assist in the survival of neurons [4, 22, 26]. After an injury occurs in the peripheral nervous system, there is disruption in the conductance of nerve signals due to axonal damage. Such an injury is accompanied by an inflammatory response by Schwann cells. The Schwann cell helps to phagocytize the damaged axons and then forms the regeneration tube of the axon that is connected to the cell body. While forming this tube, Schwann cells proliferate rapidly and provide the axon with a path to grow along. This trail is formed by the Schwann cells (Büngner's Band) that are still present in the distal stump [14].

At the site of nerve injury, high concentrations of extracellular ATP are released from injured cells. As the ATP degradation system is inhibited in injured and at sites of inflammation [5, 10], the concentration of local ATP would be high for a time, to then act on Schwann cells via P2X7R. Accordingly, ATP could promote Schwann cells to proliferate, form Büngner's Bands in the injured nerves, and then promote axon growth and elongation.

The present study has shown that a high level of P2X7R was present in the Ranvier node region of myelinating Schwann cells of normal sciatic nerves. P2X7R in Ranvier node regions disappeared completely after SNI, which implies that P2X7R have an important role. Interestingly, axons seem to receive various mRNA packed in shedding microvesicles from Schwann cells at nodes of Ranvier. By shedding microvesicles, gene expression of these glial cells seems to locally sustain protein synthesis in axons independently, at least in part [7, 9, 19]. Shedding of microvesicles from microglia, astrocytes, oligodendrocytes and neurons is triggered by ATP via P2X7R [28]. Increasing evidence shows that shedding of vesicles participates in important biological processes, such as surface-membrane trafficking and horizontal transfer of protein and RNA among neighbouring cells, which are necessary for rapid phenotype adjustments in a variety of conditions [7, 28]. It would be important to study the effects of ATP via P2X7R on local axonal functions at Ranvier node regions.

In summary, we have clearly demonstrated that P2X7R are expressed on myelinating and non-myelinating Schwann cells in longitudinal sections of sciatic nerves. After SNI, P2X7R

expression in Schwann cells of injured sciatic nerves is up-regulated, which appears to be involved in proliferation of Schwann cells. Experiments on primary cultured Schwann cells showed that ATP, via P2X7R, can promote Schwann cell proliferation via the MAPK/ERK intracellular signalling pathway.

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