Purinergic signalling in a latent stem cell niche of the rat spinal cord

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Abstract The ependyma of the spinal cord harbours stem cells which are activated by traumatic spinal cord injury. Progenitor-like cells in the central canal (CC) are organized in spatial domains. The cells lining the lateral aspects combine characteristics of ependymocytes and radial glia (RG) whereas in the dorsal and ventral poles, CC-contacting cells have the morphological phenotype of RG and display complex electrophysiological phenotypes. The signals that may affect these progenitors are little understood. Because ATP is massively released after spinal cord injury, we hypothesized that purinergic signalling plays a part in this spinal stem cell niche. We combined immunohistochemistry, *in vitro* patch-clamp whole-cell recordings and Ca^{2+} imaging to explore the effects of purinergic agonists on ependymal progenitor-like cells in the neonatal (P1–P6) rat spinal cord. Prolonged focal application of a high concentration of ATP (1 mM) induced a slow inward current. Equimolar concentrations of BzATP generated larger currents that reversed close to 0 mV, had a linear current–voltage relationship and were blocked by Brilliant Blue G, suggesting the presence of functional P2X7 receptors. Immunohistochemistry showed that P2X7 receptors were expressed around the CC and the processes of RG. BzATP also generated

Ca^{2+} waves in RG that were triggered by Ca^{2+} influx and propagated via Ca^{2+} release from internal stores through activation of ryanodine receptors. We speculate that the intracellular Ca^{2+} signalling triggered by P2X7 receptor activation may be an epigenetic mechanism to modulate the behaviour of progenitors in response to ATP released after injury.

Keywords Stem cells · Purinergic signalling · P2X7 receptors · Ca^{2+} waves · Ependyma · Spinal cord

Introduction

The central canal (CC) of the mammalian spinal cord is believed to be a “latent neural stem cell niche” [1]. Within this niche, different kinds of progenitor-like cells are organized in functional domains [2]. The cells on the lateral domains combine morphological and molecular traits of ependymocytes and radial glia (RG) and are electrically coupled via connexin 43 (Cx43). The cells lining the ventral and dorsal poles express neural stem cell markers and have RG morphology with slender processes that extend from the CC lumen to the pia [2–4]. These cells appear uncoupled and display complex electrophysiological phenotypes [2]. In addition, other CC-contacting cells display molecular and functional features similar to immature neurons in adult neurogenic niches [5]. In response to spinal cord injury (SCI), some ependymal cells proliferate and migrate to the lesion site where they differentiate mostly into scar-forming astrocytes and a few myelinating oligodendrocytes [3, 6]. This reaction is required to restrict secondary enlargement of the lesion and further axonal loss [7]. However, the signals that may regulate the biology

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of CC-contacting progenitors in the normal and injured spinal cord remain poorly understood.

ATP signalling plays a key role both in the normal and pathological brain. During development, ATP acting on purinergic receptors regulates processes such as progenitor cell proliferation, migration, differentiation and synapse formation [8, 9]. On the other hand, a variety of CNS insults lead to massive release of ATP from different sources [10]. It has been proposed that ATP may act as a diffusible “danger signal” to alert about damage and to start repair [11–13]. Following spinal cord injury (SCI), ATP levels increase around the lesion epicentre [14] activating the ionotropic receptor P2X7 which contributes to the extension of the damage [15].

To test the possibility that ATP signals on CC-contacting cells, we combined patch-clamp recordings and Ca^{2+} imaging with immunohistochemistry and transmission electron microscopy (TEM). We found that ependymal cells in both the medial and lateral domains have functional ionotropic P2X7 receptors. Their activation generates a slow inward current and a Ca^{2+} wave propagated from the site of ATP application. It is tempting to speculate that P2X7 receptor activation may be a key component of the response of the ependymal stem cell niche to spinal cord injury.

Materials and methods

General

Neonatal rats (Sprague Dawley, P1–P6) of either sex were used in accordance with the ethical guidelines established by our local Committee for Animal Care and Research at the Instituto de Investigaciones Biológicas Clemente Estable. Every precaution was taken to minimize animal stress and the number of animals used.

Slice preparation and electrophysiology

Rats anesthetized with isoflurane (Forane, Abbott England) were decapitated, and the cervical enlargement was dissected out in chilled Ringer's solution of the following composition (in mM): NaCl, 101; KCl, 3.8; MgCl_2 , 18.7; CaCl_2 , 1; MgSO_4 , 1.3; HEPES, 10; KH_2PO_4 , 1.2 and glucose, 25; saturated with 5 % CO_2 and 95 % O_2 (pH 7.4). Transverse 300- μm -thick slices were cut, placed in a chamber (1 ml volume) and superfused (1 ml/min) with Ringer's solution (in mM): NaCl, 124; KCl, 2.4; NaHCO_3 , 26; CaCl_2 , 2.4; MgSO_4 , 1.3; HEPES, 1.25; KH_2PO_4 , 1.2 and glucose, 10; saturated with 5 % CO_2 and 95 % O_2 (pH 7.4). For low- Ca^{2+} Ringer's solution, CaCl_2 was lowered to 0.2 mM and MgSO_4 increased to 4 mM. All experiments were performed at room temperature (22–24 °C). Cells were visualized with

differential interference contrast (DIC) optics (Leica DM LFS) with a $\times 40$ (0.8 NA) objective. Patch-clamp whole-cell recordings were obtained with electrodes filled with (in mM): K-gluconate, 122; $\text{Na}_2\text{-ATP}$, 5; MgCl_2 , 2.5; CaCl_2 , 0.003; EGTA, 1; Mg-gluconate, 5.6; K-HEPES, 5; H-HEPES, 5; biocytin, 10 and Alexa 488 hydrazide, 0.25–0.5 (Invitrogen); pH 7.4; 5–10 M Ω . Voltage-clamp recordings were performed with a Multiclamp 700B (Molecular Devices). Voltage steps were generated with pClamp10 (Molecular Devices) which was also used for further analysis. Seal resistances were between 4 and 18 G Ω . Series resistance and whole-cell capacitance were not compensated. In voltage-clamp mode, cells were held at -70 mV and the resting membrane potential was estimated from the current–voltage relationship (at $I=0$). Liquid junction potentials were determined and corrected off-line [16]. Values are expressed as the mean $\pm$ the standard error of the mean (S.E.M.). Statistical significance was evaluated using the Mann–Whitney U test.

Drugs

ATP (0.5–1 mM, Sigma-Aldrich), 3'-*O*-(4-benzoyl) benzoyl-ATP (BzATP, 0.5–1 mM, Tocris) and 2-methylthioadenosine diphosphate trisodium salt (2MesADP, 1 mM, Tocris) diluted in normal Ringer's solution were pressure ejected using a Picospritzer III (Parker Instrumentation) controlled by Clampex10. No responses were observed during puffs of normal Ringer alone (data not shown). Brilliant Blue G (BBG, 10 μM , Sigma-Aldrich) and dantrolene (40 μM , Tocris, diluted in dimethyl sulphoxide) were added to the bath solution.

Morphological identification of recorded cells

During whole-cell patch-clamp recordings, cells were filled with a fluorophore and/or biocytin. In most cases, the cells were first imaged in living slices with an EM CCD LucaR camera (Andor Technology) using Imaging Workbench 6.0 (INDEC Biosystems). Then, the slices were fixed by immersion in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB) for 12–24 h. After overnight PB rinsing, the slices were blocked with 0.5 % BSA in PB (1 h) and then incubated in a solution containing 0.3 % Triton-X100 with either the streptavidin-fluorophore complex (streptavidin-Alexa 488 or 633, 2 h) or diaminobenzidine. Slices incubated with fluorophores were mounted in glycerol and examined with a confocal microscope (Olympus FV 300). Some of the recorded cells were processed to be examined using TEM techniques (see below).

Immunohistochemistry

Animals were anesthetized (50 mg/kg pentobarbital; i.p.) and fixed by intracardiac perfusion with 4 %

paraformaldehyde in 0.1 M PB (pH 7.4). The following primary antibodies were used: (1) anti-P2X7 (rabbit polyclonal, 1:200 Millipore) and (2) anti-nestin (mouse monoclonal, 1:10; Developmental Studies Hybridoma Bank, Iowa City, IA). We used HOECHST (Molecular Probes) as a nuclear stain. Tissues were sectioned with a vibrating microtome (60–80 μm) and placed in PB with 0.5 % bovine serum albumin (BSA) for 30 min and then incubated with the primary antibodies diluted in PB with 0.3 % Triton X-100 (Sigma-Aldrich). Incubation times and antibody concentrations were optimized for each case. After washing in PB, the tissues were incubated in secondary antibodies conjugated with different fluorophores or horseradish peroxidase revealed with 3,3'-diaminobenzidine (DAB, Sigma-Aldrich). The P2X7 antibody specificity was tested by pre-absorption with a control antigen provided by the manufacturer (Millipore, 1 μg of peptide with 1 μg of primary antibody for 1 h at room temperature) followed by the usual immunohistochemical procedures. When performing co-labelling experiments, Alexa 488 and 633 (Invitrogen) conjugated secondary antibodies were used to avoid cross-talk. The histological material was visualized with confocal microscopy (Olympus VF300), and images were acquired with Fluoview 5 (Olympus) and then exported to Photoshop for image adjustment.

Ca²⁺ imaging

Cells were filled with the Ca²⁺-sensitive dye Fluo-4 (250 μM , pentapotassium salt, Invitrogen) added to the patch solution. Time-lapse imaging (2–4 Hz) was performed with Imaging Workbench 6.0. Intensity measurements for regions of interest (ROI) are expressed as the change of fluorescence relative to background fluorescence ($\Delta F/F_0$).

TEM

The spinal cords were fixed by transcardiac perfusion with an aldehyde mixture consisting of 4 % paraformaldehyde and 2 % glutaraldehyde dissolved in 0.1 M PB. The spinal cords were cut in 1-mm-thick portions, washed several times in the same buffer and post-fixed with OsO₄ (1 % in PB). Dehydration was carried out in graded alcohol series, and the pieces were finally embedded in epoxy resin. Ultrathin sections were mounted on Formvar-coated slot grids (2 $\times$ 1 mm) and contrasted with uranyl acetate and lead citrate. In two instances, vibrating microtome sections containing cells that were electrophysiologically recorded and biocytin filled were fixed by immersion in buffered 4 % paraformaldehyde and subsequently treated with DAB and nickel sulphate to reveal the injected cells. The sections were post-fixed

with OsO₄ (1 % in PB), dehydrated, embedded and treated as usual for TEM examination.

Results

Progenitors in the CC have functional purinergic receptors

Immunohistochemical, electron microscopy and electrophysiological studies have reported the presence of ionotropic purinergic P2X2 receptors in CC-contacting neurons of the rat spinal cord [5, 17]. However, it is not known whether ATP is capable of generating responses in different types of progenitor cells around the CC. To test purinergic signalling in RG contacting the CC poles, we made patch-clamp recordings combined with focal applications of ATP. Identification of these cells was confirmed by including a fluorophore in the recording pipette (Fig. 1a). In line with our previous observations [2], cells in the ventral and dorsal CC poles displayed hyperpolarized resting membrane potentials (MP, -87 ± 0.2 mV, $n=20$ cells) and relatively low input resistances (248.8 ± 42.8 M Ω , $n=27$ cells). In contrast to what was previously reported on CC-contacting neurons [5], we did not find sizeable membrane currents in RG cells when we applied short pulses (50–500 ms) of ATP (1 mM, $n=8$ cells, Fig. 1b(1)). Small inward currents appeared when ATP was puffed for 1 s (4 of 7 cells, 13.3 ± 2.62 pA, $n=3$, MP = -80 mV, Fig. 1b(1)). The low sensitivity of CC-contacting RG to ATP suggested the presence of P2X7 receptors. Because homodimeric BzATP is 10–30 times more potent than ATP to activate P2X7 receptors [18], we focally applied BzATP to RG. BzATP (1 mM) generated inward currents in most RG (31 of 44 cells, Fig. 1b(2)). Inward currents were larger than those induced by equimolar concentrations of ATP (80.7 ± 25.61 pA, $n=8$, MP = -80 mV, $p < 0.05$, Fig. 1b(2)). The delay between current initiation and current peak amplitude was in average 2.35 ± 0.48 s ($n=8$ cells). The BzATP-induced currents reversed near 0 mV (-1.54 ± 5.35 mV, $n=6$ cells, Fig. 1c(1)) and had a linear current–voltage (I/V) relationship (Fig. 1c(2)). This value corresponds to the reversal potential of non-selective cationic currents. BzATP-induced currents were reduced by bath application of the P2X7 receptor antagonist BBG (10 μM , $n=3$ cells, Fig. 1d).

The cells in the lateral aspects of the CC also responded to BzATP (10 of 13 cells, Fig. 2). As in RG, we found that ependymocytes responded with larger currents when we applied BzATP rather than ATP (Fig. 2b). The current generated by BzATP in the cells lining the lateral aspects of the ependyma had a slow time course and reversed close to 0 mV (-2.56 ± 7.26 , $n=5$ cells, Fig. 2c). The time between current initiation and current peak amplitude was 1.67 ± 2.2 s ($n=6$ cells), similar to that of RG in the CC poles ($p > 0.05$).

Fig. 1 Responses of progenitor-like cells contacting the CC poles to purinergic agonists. **a** Representative radial glia (RG) contacting the ventral pole of the CC. The position of the puff pipette is indicated. **b** Currents induced by puff application of 1 mM ATP (*I*) with pulses of 50 ms (blue), 500 ms (green) and 1 s (red) in a CC-contacting RG. In the cell shown in **a**, BzATP (1 s, 1 mM) produced a larger inward current (2)

than those induced by equimolar concentrations of ATP. **c** Responses of a CC-contacting RG to BzATP at different membrane potentials (*I*). The current–voltage (*I/V*) relationship of BzATP-induced currents in RG (2) was linear ($n=6$). **d** BBG (10 μ M) antagonized BzATP-induced currents. **a** Confocal Z-stack projection

Immunohistochemical detection of P2X7 receptors

Our electrophysiological data suggested that CC-contacting progenitor cells display functional P2X7 receptors. We performed immunohistochemistry to examine the localization of P2X7 within the ependymal region of the neonatal rat spinal

cord. We observed P2X7 expression in most cells surrounding the CC (Fig. 3a). To test whether RG in midline domains expressed this receptor, we combined immunohistochemistry for P2X7, HOECHST to label nuclei and nestin to label some RG in the dorsal and ventral poles of the rat CC [2, 4, 19]. We found that some nestin+ fibres in the dorsal pole express the

Fig. 2 Functional P2X7 receptors in the lateral domains of the CC. **a** Ependymocyte recorded on the lateral aspect of the CC. **b** As in RG, puff application of BzATP (1 mM) generated inward currents larger than those induced by ATP. **c** Responses to BzATP at different membrane potentials (*I*) and *I/V* relationships (2) in cells lying in the lateral aspects of the CC ($n=5$). **a** Epifluorescence image in a living slice

Fig. 3 Immunohistochemistry for P2X7 receptors in cells contacting the CC. **a** Immunoreactivity of P2X7 receptors in cells contacting the CC (arrowheads). **b** Expression of P2X7 receptors in nestin⁺ cells in the dorsal pole of the CC (1, arrowheads). Orthogonal images of the region marked with a dotted circle in panel 1 are shown in panel 2. Note the presence of P2X7 receptor immunoreactivity surrounded by nestin⁺ fibres (arrows). **c** Nestin and nuclear staining reveals nestin⁺ cells in the dorsal raphe (box in 1) that express P2X7 receptors (ellipse in 2–4). P2X7⁺ punctate staining (arrows) were also observed in the processes that are not immunoreactive for nestin. The asterisk indicates a process from the cell in the box running ventrally towards the CC (2–4). **d** P2X7 receptor immunoreactivity in cells lining the lateral aspects of the CC. **e** Negative control of P2X7 immunohistochemistry by pre-incubation of a control antigen (1 μ g control antigen plus 1 μ g P2X7 antibody) before addition of the secondary antibody. Notice the absence of P2X7 punctate staining. **a–e** Single confocal optical planes. **b** Confocal Z-stack projection

P2X7 receptor (Fig. 3b(1–2)). As shown previously [2], nuclear staining revealed that nestin⁺ processes originated from cell bodies located at different distances from the CC (Fig. 3c(1)). Some nestin⁺ processes belonging to the CC-contacting cells lying in the raphe were strongly immunoreactive for P2X7 (Fig. 3c(2–4), ellipses). Notice that in the same region, there were processes immunoreactive for P2X7 that did not express nestin (Fig. 3c(2 and 4), arrows). Figure 3d

shows abundant P2X7 punctate staining (arrows) on the cells lining the lateral aspects of the CC. No P2X7 staining was observed when negative controls were performed by pre-absorption with a control antigen (Fig. 3e). These data indicated that P2X7 receptors occur in CC-contacting cells both in the midline and lateral domains of the endyma.

Activation of P2X7 induces Ca²⁺ waves in CC-contacting progenitor cells

P2X7 receptors are highly permeable to Ca²⁺ [11], so their activation may generate intracellular Ca²⁺ signalling in RG contacting the CC poles. To test this possibility, we loaded cells with the Ca²⁺ indicator Fluo-4 during whole-cell recordings and puff applied BzATP. We found that BzATP induced Ca²⁺ waves in RG progenitors contacting the poles of the CC (24 of 36 cells, Fig. 4). Time-lapse imaging showed that Ca²⁺ waves travelled along the entire cell, starting at the site where the agonist was applied (Fig. 4a, b). When BzATP was applied on the endfoot of RG, the Ca²⁺ wave spread from the CC towards the distal process (Fig. 4a(1–6)) whereas application of BzATP on the cell body of the same cell generated a wave that spread both towards the CC and the pia, as shown in the time-lapse images (Fig. 4b(1–6)) and in the $\Delta F/F_0$ in different ROI (Fig. 4c, d). An inward current with a faster time course preceded the Ca²⁺ wave onset (1.36 ± 0.45 s, $n=6$) (Fig. 4c, d). Ca²⁺ waves propagated at a mean speed of 27.64 ± 5.06 μ m/s ($n=4$ waves).

To determine if the Ca²⁺ wave generated by P2X7 receptor activation needs a triggering influx of Ca²⁺, we lowered Ca²⁺ in the extracellular medium. Under this condition, Ca²⁺ waves induced by BzATP were reduced ($n=4$ cells, Fig. 5a, b), showing that extracellular Ca²⁺ influx is required for Ca²⁺ wave initiation. This data suggests that Ca²⁺ wave propagation may result from the activation of ryanodine receptors in the endoplasmic reticulum and mobilization of Ca²⁺ from internal stores. In line with this interpretation, dantrolene (40 μ M, $n=3$ cells)—an inhibitor of ryanodine receptors—slightly reduced the Ca²⁺ increase at the site of BzATP application but completely blocked the propagation of the Ca²⁺ wave (Fig. 5c, d).

Ca²⁺ waves induced in RG by activation of metabotropic purinergic receptor P2Y1 have been also reported in the developing cerebral cortex [8]. We tested whether activation of P2Y1 receptors may contribute to the Ca²⁺ wave in CC-contacting RG by applying 2MesADP, a potent purinergic agonist for P2Y1. In contrast to BzATP (Fig. 6a(4)), no inward currents were observed following 2MesADP application ($n=13$ cells, Fig. 6b(4)) and concomitant Ca²⁺ responses were not observed in most RG tested (12 of 13 cells, Fig. 6b(1–4)), except for a cell that

Fig. 4 Ca^{2+} waves in RG contacting the poles of the CC. **a** RG in the dorsal pole of the CC filled with Fluo-4 before (1), during (2) and after (3–6) application of BzATP (1 s, 1 mM) on the apical process that contacts the CC. The cell morphology is shown in the *inset* in 6. The *arrowheads* indicate the sequence of Ca^{2+} increase along the cell. **b** The same cell shown in **a** before (1), during (2) and after (3–6) application of BzATP on the soma. Note that the direction of Ca^{2+} wave propagation (*arrowheads*) is reversed compared to that shown in **a**. **c** $\Delta F/F_0$ analysis of ROIs in **a1** (*open circles*) showed the time course of $[\text{Ca}^{2+}]_i$ increase after BzATP application. An inward current (*black*) preceded the increment in $[\text{Ca}^{2+}]_i$. **d** $\Delta F/F_0$ of ROIs shown in **b1** (*open circles*) and the associated inward current (*black*) generated by BzATP application at the soma. **a**, **b** Pseudocolor images

Fig. 5 Ca^{2+} influx and release from internal stores is required for Ca^{2+} wave generation and propagation. **a** RG in the ventral pole of the CC filled with Fluo-4. The position of the puff pipette is indicated. **b** Ionic current (1, *black trace*) and $\Delta F/F_0$ (1, *colours* #1–4) corresponding to ROIs shown in **a** (#1–4, *open circles*). In low Ca^{2+} (0.2 mM), the Ca^{2+} wave did not propagate (2). BzATP still evoked an inward current in low Ca^{2+} (2, *black trace*). **c** RG recorded in a CC pole in which BzATP (1 mM) was applied at the soma. **d** $\Delta F/F_0$ (1) in the ROIs indicated in **c** (*open circles*) shows a Ca^{2+} wave starting in the soma (#1) and propagating towards the distal process (#2, 3, 4). Dantrolene (40 μM) blocked Ca^{2+} wave propagation (2). **a**, **c** Epifluorescence images in a living slice

Fig. 6 BzATP but not 2MesADP generates Ca^{2+} waves in RG. **a** Time-lapse images showing the response of a RG to BzATP (1 mM) application on the soma (1–3). BzATP generated an inward current (4, black trace) and a propagated Ca^{2+} wave (ROI #1 and 2, colour traces). **b** Application

of 2MesADP (1 mM) in the same cell as in **a** (1–3). This agonist of metabotropic purinergic receptors did not generate a membrane current (4, black trace) nor increased Fluo-4 fluorescence (4, colour traces) in the ROIs analysed (white circles, #1 and 2 in **b**)

responded to 2MesADP generating a Ca^{2+} wave (1 of 13 cells, data not shown).

A marked increase in Fluo-4 fluorescence and an inward current were also observed when we applied BzATP to ependymal cells in the lateral domains (5 of 7 cells; Fig. 7a, b). The Ca^{2+} waves propagated along the basal process of those cells after BzATP puff application at the soma (Fig. 7c(1–2)). Collectively, these data suggest the presence of functional P2X7 receptors in most cells lining the CC of the rat spinal cord.

Medial RG have an extended system of endoplasmic sacs and vesicles

Our data suggests that for a Ca^{2+} wave to propagate there should be an intracellular system to store Ca^{2+} distributed over the whole extension of CC-contacting cells. To identify potential intracellular Ca^{2+} stores, we studied the fine structure of progenitor cells lining the dorsal and ventral poles of the CC. We used TEM to analyse RG that had been recorded and filled with biocytin (Fig. 8a). Their unambiguous light and TEM

Fig. 7 Ca^{2+} signalling on the lateral domains of the CC. **a** Time-lapse imaging of an ependymocyte filled with Fluo-4 in the lateral domain of the CC (inset in 1) before (1), during (2) and after (3, 4) application of BzATP (1 mM). **b** $\Delta F/F_0$ in the ROI (circle in **a**1) and the simultaneous inward current (black trace) generated by BzATP. **c** Lateral

ependymocyte with a thin distal process in which BzATP was applied on the soma (1). $\Delta F/F_0$ in ROIs selected on the distal process (white open circles in 1) shows that the Ca^{2+} wave propagated from the soma to the distal process. **a** Pseudocolour images in a living slice. **c**1 Confocal Z-stack projection

Fig. 8 Endoplasmic sacs and vesicles in CC-contacting progenitors. **a** RG recorded in one of the poles of the CC filled with Alexa 488 (*inset*) and biocytin revealed with the DAB–Ni complex (*main panel*). **b** The electron-dense precipitate allowed the identification of the different RG compartments with TEM (*1* to *4* in **a**). Note the presence of enlarged sacs and irregular cavities delimited by endoplasmic membranes at different RG levels (*asterisks* in *1*, *2* and *4* and *arrowheads* in *3*). **c–e** TEM images obtained from better-preserved tissue that was not exposed to detergents also showed the presence of similar membrane compartments at both the CC level (*asterisks* in **c**) and in the processes travelling along the dorsal raphe (*asterisks*, **d** and **e**). The *inset* in **c** shows the insertion site of a single cilium in the apical pole of the cell. **a** Epifluorescence image in a living slice (*inset*) and corresponding light microscope image of the same cell in fixed and processed tissue (*main panel*)

identification was possible because after adequate processing with the DAB–Ni complex, they exhibited a conspicuous electron-dense labelling (Fig. 8b). The cell shown in Fig. 8a was ultrathin sectioned perpendicularly to its main radial axis. The sections corresponding to different levels (dotted lines 1 to 4 in Fig. 8a) were examined with TEM. At all levels, we found the occurrence of enlarged sacs and irregular cavities, free of the electron-dense precipitate (Fig. 8b(1–4), asterisks in 1, 2 and 4 and arrowheads in 3). TEM images from spinal cords that were fixed and treated with procedures allowing a better cytological preservation also showed the same kind of endoplasmic sacs (Fig. 8c–e). Interestingly, large endoplasmic sacs were observed close to the primary cilium (Fig. 8c) whereas nearby the nucleus many small vesicles predominated (Fig. 8b(3)).

Discussion

Here, we show for the first time that the progenitor-like cells lining the CC of the rat spinal cord have functional P2X7 receptors. In these cells, P2X7 receptors are activated by high

concentrations of ATP, generating inward currents and Ca^{2+} waves that propagated over the entire length of the cell. The intracellular Ca^{2+} signalling triggered by P2X7 receptor activation could be a key mechanism regulating the biology of the CC-contacting progenitors, with implications for the endogenous response to spinal cord injury.

Purinergic signalling in the CC: P2X7 receptors rule in CC-contacting progenitors

The CC-contacting progenitor-like cells in midline and lateral domains responded rather reluctantly to ATP as they needed prolonged applications (>1 s) of a high concentration (1 mM). This is in sharp contrast to CC-contacting neurons which are strongly excited by ATP via P2X2 receptors [5]. BzATP was more potent than ATP in CC-contacting progenitors, a pharmacological hallmark of P2X7 receptors [20–23]. In line with this interpretation, the P2X7 receptor antagonist BBG [21, 24] reduced BzATP-mediated currents. However, BBG at the concentrations used in our study can also partly antagonize P2X2 and P2X4 receptors [22, 25]. A major involvement of P2X2 receptors is unlikely as they are highly sensitive to ATP and

P2X2-mediated currents show a strong inward rectification [5, 18] whereas the BzATP-induced current had a linear I/V relationship with a reversal at 0 mV. P2X4 receptors are also unlikely to mediate the responses observed in CC-contacting RG because ATP is tenfold more potent to activate P2X4 over P2X7 receptors [25] and P2X4 currents show a slow inactivation [18]. Finally, P2X7 immunoreactivity was found in all aspects of the ependyma and the processes of CC-contacting RG. This is in line with previous studies showing the expression of P2X7 mRNA in ependymal cells of the adult rat [26]. Functional P2X7 receptors seem to be a general trait of ependymal cells as they are found in the microvilli of cells lining the lateral ventricles [27]. Collectively, our results suggest that purinergic signalling in CC-contacting RG and ependymocytes is dominated by P2X7 receptors.

Ca²⁺ waves in CC-contacting progenitors

Activation of P2X7 receptors in the RG contacting the poles of the CC generated Ca²⁺ waves that propagated bi-directionally from the stimulated site at a speed similar to that of Ca²⁺ waves in other glial cells (e.g., astrocytes, [28]). Ca²⁺ waves play an important role during cortical development by synchronizing the cell cycle of a cohort of RG [8]. Spontaneous Ca²⁺ waves in cortical progenitors are generated by activation of P2Y1 receptors leading to IP3-mediated Ca²⁺ release from internal stores [8]. Our data suggests that the generating mechanism of Ca²⁺ waves in progenitor-like cells in the CC of neonatal animals is different, as it relies on Ca²⁺ entry via P2X7 receptors as a trigger. Dantrolene prevented the spread of the Ca²⁺ signal, indicating that the initial rise in cytosolic Ca²⁺ activates ryanodine receptors evoking Ca²⁺ release from internal stores. As in other cell types [29, 30], the Ca²⁺-induced Ca²⁺ release would provide a regenerative mechanism for propagation analogous to “toppling dominos” [31]. Indeed, our TEM data demonstrated an extensive cytoplasmic system of cisternae spanning from the very tip of the endfoot to the distal process of CC-contacting cells, providing a framework for the propagation of Ca²⁺ signals. The fact that in a minority of CC-contacting progenitors the P2Y1 agonist 2MesADP could induce a Ca²⁺ wave suggests that the IP3 pathway may be involved in a small subset of RG, adding to the functional heterogeneity of CC-contacting progenitors reported before [2]. Because the ependymal cells in the lateral domains are coupled via Cx43 [2], it is possible that Ca²⁺ signals spread to neighbouring cells as their counterparts in the developing cortex [8]. Loading a large group of cells with Fluo-4 AM around the CC will be necessary to explore this possibility.

Purinergic signalling and its possible functional role in the CC stem cell niche

Besides the myriad functions executed in health, ATP signalling plays a pivotal role in different pathological conditions [11, 32]. It has been proposed that ATP may act as a diffusible “danger signal” to alert about damage and start repair mechanisms [13]. The unique functional properties of the P2X7 receptor (e.g. low affinity [0.1 to 3 mM], high Ca²⁺ permeability and increased permeability to sustained agonist application) make it a suitable candidate to act as a detector of tissue damage. Ependymal cells react to spinal cord injury by proliferating and migrating towards the lesion site, where they differentiate mostly in astrocytes [3]. This reaction is fundamental for the formation of the glial scar delimiting the extent of the injury [7]. Because after spinal cord injury ATP levels increase around the lesion epicentre [14], it is possible that the massive increase of ATP induced by traumatic spinal cord injury may activate P2X7 receptors in CC-contacting progenitors generating Ca²⁺ waves. Nestin⁺ and vimentin⁺ cells in midline and lateral domains of the CC have long distal processes ideally suited to detect ATP released by injury of the dorsal, lateral or ventral aspects of the spinal cord. The activation of P2X7 receptors in the distal processes lying within injured tissue would generate a Ca²⁺ wave propagating towards the CC, generating a local Ca²⁺ increase in key cellular compartments such as the nucleus and the apical process of CC-contacting progenitors. Ca²⁺ transients may modulate nuclear gene expression, activating or repressing function-specific transcription factors that may affect events such as proliferation, differentiation and migration [33, 34] of ependymal cells. For example, interference of Ca²⁺ signalling by blockade or genetic knockdown of purinergic receptors impairs the migration of intermediate neuronal progenitors to the subventricular zone [35]. The apical pole of neural progenitors is a peculiar cellular compartment with structural features such as a primary cilium and a distinctive apical protein complex [36], which are main determinants of the progenitor cell fate [37, 38]. Indeed, nestin⁺ CC-contacting cells in the CC poles share some of these basic features, like a single primary cilium [2]. The conspicuous Ca²⁺ waves propagated to or generated at the apical pole of CC-contacting RG may generate changes (e.g. resorption of cilia [39]) in this cellular compartment with major functional consequences. Interestingly, it has been recently demonstrated that signalling via nicotinic acetylcholine receptors modulates cell proliferation of ependymal cells in the spinal cord [40], highlighting the importance of neurotransmitter regulation of progenitor cell behaviour.

Taken together, our findings suggest P2X7 receptors and downstream cellular events (e.g. Ca^{2+} waves) as possible targets to manipulate the response of the ependymal stem cell niche to ATP released by spinal cord injury. In line with this idea, recent work showed increased P2X7 expression in the spinal cord after injury [41]. Whether the tuning of purinergic signalling in the latent CC stem cell niche may modulate the healing induced by ependymal cells [7, 34] will require the combination of in vivo and in vitro animal models to target selectively P2X7 receptors within the niche.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in this study involving animals were in accordance with the ethical standards of the local Committee for Animal Care and Research at the Instituto de Investigaciones Biológicas Clemente Estable. Every precaution was taken to minimize animal stress and the number of animals used.

References

- Göritz C, Frisén J (2012) Neural stem cells and neurogenesis in the adult. *Cell Stem Cell* 10:657–659. doi:10.1016/j.stem.2012.04.005
- Marichal N, García G, Radmilovich M et al (2012) Spatial domains of progenitor-like cells and functional complexity of a stem cell niche in the neonatal rat spinal cord. *Stem Cells* 30:2020–2031. doi:10.1002/stem.1175
- Meletis K, Barnabé-Heider F, Carlén M et al (2008) Spinal cord injury reveals multilineage differentiation of ependymal cells. *PLoS Biol* 6:1494–1507. doi:10.1371/journal.pbio.0060182
- Petit A, Sanders AD, Kennedy TE et al (2011) Adult spinal cord radial glia display a unique progenitor phenotype. *PLoS One* 6, e24538. doi:10.1371/journal.pone.0024538
- Marichal N, Garcia G, Radmilovich M et al (2009) Enigmatic central canal contacting cells: immature neurons in “standby mode”? *J Neurosci* 29:10010–10024. doi:10.1523/JNEUROSCI.6183-08.2009
- Barnabé-Heider F, Göritz C, Sabelström H et al (2010) Origin of new glial cells in intact and injured adult spinal cord. *Cell Stem Cell* 7:470–482. doi:10.1016/j.stem.2010.07.014
- Sabelström H, Stenudd M, Réu P et al (2013) Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice. *Science* 342:637–640. doi:10.1126/science.1242576
- Weissman TA, Riquelme PA, Ivic L et al (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. *Neuron* 43:647–661
- Zimmermann H (2006) Nucleotide signalling in nervous system development. *Pflügers Arch* 452:573–588
- Di Virgilio F, Boeynaems JM, Robson SC (2009) Extracellular nucleotides as negative modulators of immunity. *Curr Opin Pharmacol* 9:507–513. doi:10.1016/j.coph.2009.06.02
- Khakh BS, North RA (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442:527–532
- Surprenant A, North RA (2009) Signaling at purinergic P2X receptors. *Annu Rev Physiol* 71:333–359. doi:10.1146/annurev.physiol.70.113006.100630
- Abbracchio MP, Burnstock G, Verkhratsky A et al (2009) Purinergic signalling in the nervous system: an overview. *Trends Neurosci* 32:19–29. doi:10.1016/j.tins.2008.10.001
- Wang X, Arcuino G, Takano T et al (2004) P2X7 receptor inhibition improves recovery after spinal cord injury. *Nat Med* 10:821–827
- Peng W, Cotrina ML, Han X et al (2009) Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. *Proc Natl Acad Sci U S A* 106:12489–12493. doi:10.1073/pnas.0902531106
- Barry PH, Diamond JM (1970) Junction potentials, electrode standard potentials, and other problems in interpreting electrical properties in membranes. *J Membr Biol* 3:93–122. doi:10.1007/BF01868010
- Stoeckel ME, Uhl-Bronner S, Hugel S et al (2003) Cerebrospinal fluid-contacting neurons in the rat spinal cord, a gamma-aminobutyric acidergic system expressing the P2X2 subunit of purinergic receptors, PSA-NCAM, and GAP-43 immunoreactivities: light and electron microscopic study. *J Comp Neurol* 457: 159–174
- North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067
- Hamilton LK, Truong MK, Bednarczyk MR et al (2009) Cellular organization of the central canal ependymal zone, a niche of latent neural stem cells in the adult mammalian spinal cord. *Neuroscience* 164:1044–1056. doi:10.1016/j.neuroscience.2009.09.006
- Bianchi BR, Lynch KJ, Touma E et al (1999) Pharmacological characterization of recombinant human and rat P2X receptor subtypes. *Eur J Pharmacol* 376:127–138
- North RA, Surprenant A (2000) Pharmacology of cloned P2X receptors. *Annu Rev Pharmacol Toxicol* 40:563–80
- Anderson CM, Nedergaard M (2006) Emerging challenges of assigning P2X7 receptor function and immunoreactivity in neurons. *Trends Neurosci* 29:257–262
- Jiang LH, Baldwin JM, Roger S et al (2013) Insights into the molecular mechanisms underlying mammalian P2X7 receptor functions and contributions in diseases, revealed by structural modeling and single nucleotide polymorphisms. *Front Pharmacol* 4:55. doi:10.3389/fphar.2013.00055
- Evans RJ, Lewis C, Buell G et al (1995) Pharmacological characterization of heterologously expressed ATP-gated cation channels (P2X purinoceptors). *Mol Pharmacol* 48:178–183
- Jiang LH, Mackenzie AB, North RA et al (2000) Brilliant blue G selectively blocks ATP-gated rat P2X7 receptors. *Mol Pharmacol* 58:82–88
- Yu Y, Ugawa S, Ueda T et al (2008) Cellular localization of P2X7 receptor mRNA in the rat brain. *Brain Res* 1194:45–55. doi:10.1016/j.brainres.2007.11.064
- Genzen JR, Platel JC, Rubio ME et al (2009) Ependymal cells along the lateral ventricle express functional P2X(7) receptors. *Purinergic Signal* 5:299–307. doi:10.1007/s11302-009-9143-5
- Cornell-Bell AH, Finkbeiner SM, Cooper MS et al (1990) Glutamate induces calcium waves in cultured astrocytes: long-range glial signalling. *Science* 247:470–473

29. Clapham DE (1995) Calcium signalling. *Cell* 80:259–268
30. Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 1:11–21
31. Iino M (2010) Spatiotemporal dynamics of Ca^{2+} signalling and its physiological roles. *Proc Jpn Acad Ser B Phys Biol Sci* 86:244–256
32. Franke H, Krügel U, Illes P (2006) P2 receptors and neuronal injury. *Pflugers Arch* 452:622–644
33. Glaser T, Resende RR, Ulrich H (2013) Implications of purinergic receptor-mediated intracellular calcium transients in neural differentiation. *Cell Commun Signal* 11:12. doi:[10.1186/1478-811X-11-12](https://doi.org/10.1186/1478-811X-11-12)
34. Miras-Portugal MT, Gomez-Villafuertes R, Gualix J, Diaz-Hernandez JJ, Artalejo AR, Ortega F, Delicado EG, Perez-Sen R (2015) Nucleotides in neuroregeneration and neuroprotection. *Neuropharmacology*. doi:[10.1016/j.neuropharm.2015.09.002](https://doi.org/10.1016/j.neuropharm.2015.09.002)
35. Liu X, Hashimoto-Torii K, Torii M et al (2008) The role of ATP signalling in the migration of intermediate neuronal progenitors to the neocortical subventricular zone. *Proc Natl Acad Sci U S A* 105: 11802–11807. doi:[10.1073/pnas.0805180105](https://doi.org/10.1073/pnas.0805180105)
36. Fietz SA, Huttner WB (2011) Cortical progenitor expansion, self-renewal and neurogenesis—a polarized perspective. *Curr Opin Neurobiol* 21:23–35. doi:[10.1016/j.conb.2010.10.002](https://doi.org/10.1016/j.conb.2010.10.002)
37. Alvarez-Buylla A, García-Verdugo JM, Tramontin AD (2001) A unified hypothesis on the lineage of neural stem cells. *Nat Rev Neurosci* 2:287–293
38. Götz M, Huttner WB (2005) The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* 6:777–788
39. Goto H, Inoko A, Inagaki M (2013) Cell cycle progression by the repression of primary cilia formation in proliferating cells. *Cell Mol Life Sci* 70:3893–3905. doi:[10.1007/s00018-013-1302-8](https://doi.org/10.1007/s00018-013-1302-8)
40. Corns LF, Atkinson L, Daniel J et al (2015) Cholinergic enhancement of cell proliferation in the postnatal neurogenic niche of the mammalian spinal cord. *Stem Cells* 33:2864–76. doi:[10.1002/stem.2077](https://doi.org/10.1002/stem.2077)
41. Gómez-Villafuertes R, Rodríguez-Jiménez FJ, Alastrue-Agudo A et al (2015) Purinergic receptors in spinal cord-derived ependymal stem/progenitor cells and its potential role in cell-based therapy for spinal cord injury. *Cell Transplant* 24:1493–509. doi:[10.3727/096368914X682828](https://doi.org/10.3727/096368914X682828)