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Research report

Changes in expression of P2X purinoceptors in rat cerebellum during postnatal development

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Abstract

Changes in expression of P2X receptors ($P2X_{1–7}$) during postnatal development of the rat cerebellum are described. At P3, immunoreactivity (ir) to all the P2X receptors, except for $P2X_3$ receptors, was found in Purkinje cells and deep cerebellar nuclei, $P2X_5$-ir being most prominent. Granular and microglial cells were labeled for $P2X_5$ (weakly) and $P2X_4$ receptors, respectively. At P7, expression of all the P2X receptors (with the exception of $P2X_3$) was up-regulated, $P2X_5$ and $P2X_6$ receptors being most prominent. Scattered P2X receptor-ir in unipolar brush cells in the granular cell layer and $P2X_1$- and $P2X_7$-ir of microglial cells was also present. At P14, the dendritic trees of Purkinje cells were intensely labeled by $P2X_{1–7}$ receptor antibodies, except for $P2X_3$, while $P2X_1$, $P2X_4$ and $P2X_7$ receptor immunostaining in microglial cells and $P2X_5$ receptor immunostaining in granular cells was up-regulated. At P21, expression of all P2X receptors (except $P2X_3$) was down-regulated in the Purkinje cells and deep cerebellar nuclei; $P2X_1$, $P2X_4$ and $P2X_7$ receptors-ir was present in microglial cells. In contrast, expression of $P2X_5$-ir in granular cells was up-regulated. At P60, expression levels of all the P2X receptors (except $P2X_3$) were similar with those at P21. In double-labeling experiments, almost all the P2X-ir Purkinje cells were immunoreactive for calbindin-D28k, while 60–80% of P2X-ir cells in the granular cell layer were immunoreactive for calretinin. The possible short- and long-term functional significance of the changes in expression of P2X receptors during postnatal development is discussed.

Theme: Development and regeneration
Topic: Neurotransmitter systems and channels

Keywords: Postnatal; Calbindin; Calretinin; Purkinje cells

1. Introduction

Extracellular ATP is now established as an excitatory neurotransmitter or neuromodulator in both the peripheral and central nervous systems that acts via nucleotide receptors [6,9,10,12]. Much evidence indicates that ATP is released as a co-transmitter with other neurotransmitters such as noradrenaline, acetylcholine, nitric oxide, glutamate, γ-amino butyric acid, dopamine and 5-hydroxytryptamine [3,7,8,11]. Purinergic neurotransmission may play an important role in the cerebellum, since high expression levels have been described for different P2X receptors, especially in Purkinje cells, and to a lesser extent, in the granular layer [14,15,25,32]. Seven different members of the P2X family of ligand-gated ion channels have been cloned [13]. Of the seven cloned P2X receptors, $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_6$ receptors have been reported as being expressed in the cerebellum of adult rat [4,14,32,40]. mRNA expression for $P2X_1$ and $P2X_2$ receptors in cerebellum seems to be relatively high in postnatal day 5 rats, although it is much lower in adult animals [25,26]. $P2X_4$ and $P2X_6$ receptors are highly expressed by Purkinje cells in adult rats [14]. Mateo et al. used single-cell fluorescence microscopy and a fura-2 method to study $[Ca^{2+}]_i$ signals elicited by extracellular ATP in cultured

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Purkinje cells from postnatal days 7–8 rat cerebellum [33]. Extracellularly applied ATP evoked fast $[Ca^{2+}]_i$ rises revealed by a rapid and transient increase in fura-2 F340/F380 ratio in all Purkinje cells tested. The receptor antagonists suramin and pyridoxalphosphate-6-azophenyl-2′, 4′disulphonic acid effectively blocked the responses elicited by ATP [33]. These results demonstrate that the functional ionotropic P2X purinoceptor in the cerebellar Purkinje cells is the $P2X_2$ receptor as $P2X_4$ and $P2X_6$ receptors cannot be effectively blocked by these antagonists in the rat [33,35]. These data imply that a switch from $P2X_2$ to $P2X_4$ and $P2X_6$ receptors may take place during the development of the cerebellum. In the present study, using single- and double-labeling immunofluorescence, we carried out a detailed analysis of changes in expression of all the P2X receptors during postnatal development in order to clarify the expression patterns of P2X receptors and confirm whether there is a switch from $P2X_2$ to $P2X_4$ and $P2X_6$ receptors on cerebellar Purkinje cells during the postnatal period.

2. Materials and methods

2.1. Animals and tissue preparation

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with UK Home Office regulations (Schedule One Procedures). Twenty-five Wistar rats were killed by asphyxiation with CO_2 at P3, P7, P14, P21 and P60 (n = 5 for each age group) and perfused through the aorta with 100 ml 0.9% NaCl solution and 250 ml 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. The brains were removed, postfixed overnight in the same fixative at 4 °C and cryoprotected in 20% sucrose solutions. Coronal and sagittal sections of the cerebellar hemispheres were cut using a Leica cryostat (25 μm), floated and then kept in 0.01 mol/L pH 7.2 phosphate-buffered saline (PBS).

2.2. Immunocytochemistry

The distribution and intensity of P2X immunoreactivity was systematically evaluated to assess developmental trends. The conclusions were based on evaluation of immunoreactivity in lobes VI–IX since these could be consistently identified in the different age groups studied. Each series of experiments was carried out on several sections from each animal and 5 animals were used per age group.

Polyclonal antibodies were raised in New Zealand rabbits by multiple injections of synthetic peptides corresponding to the carboxyl termini of the cloned rat P2X receptors, covalently linked to Keyhole Limpet Haemocyanin. The peptide sequences are as follows: $P2X_1$, ATSSTLGLQENMRTS; $P2X_2$, QQDSTSTDPKGLAQL; $P2X_3$, VEKQSTDSGAYSIGH; $P2X_4$, YVEDYEQGLSGEMNQ; $P2X_5$, RENAIVNVKQSQILH; $P2X_6$, EAGFYWRTKYEEARA; $P2X_7$, TWRFVSQDMADFAIL. The specificity of the antisera was verified by immunoblotting with membrane preparations from native tissue sources (rat vas deferens for $P2X_1$ and rat dorsal root ganglion for $P2X_3$) and/or CHO K1 cells expressing the cloned $P2X_1$ to $P2X_6$ receptors. Immunoglobulin G (IgG) fractions were isolated from the immune sera and the pre-immune controls using chromatography on DEAE Affi-Gel blue gel (Bio-Rad, Hemel Hempstead, UK). The specificity of the $P2X_1$ to $P2X_7$ polyclonal antibodies has been reported previously [38]. The immunocytochemical method was modified from our previous report [47]. Briefly, the preparations were washed 3 × 5 min in 0.01 mol/L pH 7.2 PBS, then incubated in 1.0% H_2O_2 for 30 min to block the endogenous peroxidase. The preparations were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with P2X antibodies, diluted 1:500–1:1000 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4 °C. Subsequently, the preparations were incubated with biotin-conjugated donkey-anti-rabbit IgG (Jackson Immunoresearch, PA, USA), diluted 1:500 in antibody dilution solution for 2 h at room temperature (RT), and then with streptavidin-HRP (Sigma Chemical Co., Poole, UK), diluted 1:1000 in PBS for 1 h at RT. Finally, a nickel-intensified diaminobenzidine (DAB) reaction in a solution containing 0.05% 3,3′-DAB, 0.04% nickel ammonium sulphate, 0.2% glucose, 0.004% ammonium nitrate, and 1.2 μl/ml glucose oxidase in 0.01 M PBS was used to visualize immunoreactivity. All the incubations and reactions were separated by 3 × 10 min washes in PBS. The preparations were mounted, dehydrated, cleared and covered.

The following protocol was used for double-staining of $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$, calbindin D-28k (a marker for Purkinje cells), calretinin (a marker for unipolar brush cells (UBCs)), gliofibrillar acid protein (GFAP; a marker for astrocytes) and ED1 (a microglia marker). Floating sections were washed 3 × 5 min in PBS, then pre-incubated in antibody dilution solution for 30 min, followed by incubation with P2X antibodies diluted 1:500–1:1000, calbindin (mouse-anti-rat, SWANT, Bellinzone, Switzerland) diluted 1:5000, calretinin (mouse-anti-rat, SWANT) diluted 1:2000, GFAP (mouse-anti-rat, Sigma) diluted 1:400, and ED1 (mouse-anti-rat, Chemicon) diluted 1:600 in antibody dilution solution overnight at 4 °C. Subsequently the sections were incubated with FITC or Cy3-conjugated donkey anti-rabbit IgG diluted 1:200 for P2X antibodies and Cy3- or FITC-conjugated donkey anti-mouse IgG (Jackson) diluted 1:200 for calbindin, calretinin, GFAP and ED1 in antibody dilution solution for 1 h at RT. All the incubations and reactions were separated by 3 × 10 min washes in PBS.

2.3. Photomicroscopy

Images of immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged using Adobe-Photoshop 5.0.

2.4. Controls

The negative control sections from P3, P7, P14, P21 and P60 were processed as the methods described above, but with the omission of the primary antibody or preabsorption of the primary antibody with the relative P2X peptides as follows: seven tubes contained 20 μl different P2X peptides (1 μg/1 μl) ($P2X_1$ to $P2X_7$) respectively, 478 μl antibody dilution solution and relative rabbit anti-P2X antibodies 2.5 μl (1 μg/μl) were added in each tube, respectively; after incubations at 4 °C overnight tubes were centrifuged at 4 °C and 10 000 rmp. The supernatant was used as the primary antibody incubation solution for negative control experiments.

Table 1
The staining intensity of Purkinje cells (PC), deep nuclei (DN), granular cells (GC) and microglial cells (MGC) by P2X antibodies in developing postnatal cerebellum

Stage	Cell	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
P3	PC	+	+	–	+	++	+/–	+
	DN	+	+	–	–	++	+	+
	GC	–	–	–	–	+/–	–	–
	MGC	–	–	–	++	–	–	–
P7	PC	++	++	–	++	++	++	++
	DN	+	+	–	+	+++	++	+
	GC	–	–	–	–	+/–	–	–
	MGC	+	–	–	++	+	–	+
P14	PC	+++	+++	–	+++	+++	+++	+++
	DN	++	++	–	+	+++	++	++
	GC	–	–	–	–	+	–	–
	MGC	++	–	–	+++	–	–	++
P21	PC	++	++	–	++	++	++	++
	DN	+	+	–	+	+++	++	+
	GC	–	–	–	–	++	–	–
	MGC	+	–	–	++	–	–	+
P60	PC	++	++	–	++	++	++	++
	DN	+	+	–	+	+++	++	+
	GC	–	–	–	–	++	–	–
	MGC	–	–	–	++	–	–	–

Intensity of P2X immunoreactivity was ranked on a 5-point scale: +++, most intense; ++, moderate; +, weak; +/–, barely detectable; and –, undetectable.

3. Quantitative analysis

The sections stained by P2X receptor, calbindin D-28k and calretinin antibodies were also used to perform a quantitative analysis. Positively-stained neuronal cell bodies in the cerebellar Purkinje cell layer and granular cell layer were counted per visual field. Ten randomly chosen fields in each section and 5 sections for each rat were analyzed. The percentage of neurons immunoreactive for a P2X receptor that were also immunoreactive for calbindin D-28k or calretinin and the percentage of neurons immunoreactive for calbindin D-28k or calretinin that were also immunoreactive for a P2X receptor were calculated and expressed as mean ± standard error of the mean (n = number of rats used).

4. Results

A summary of the expression of P2X receptors at postnatal stages P3 to P60 is shown in Table 1 and selected examples of P2X receptor-ir are illustrated in Figs. 1 and 2.

At P3, all P2X receptors immunoreactivities, except for $P2X_3$, were found in Purkinje cells and deep cerebellar nuclei, although the immunostaining intensity of each receptor was different (Figs. 1A–H). The highest level of expression among the P2X receptors was for the $P2X_5$ receptor at this stage (Fig. 1D). Staining was localized to the cell bodies with no positive staining of dendrites. In the granular cell layer there were many cells labeled by the $P2X_5$ receptor antibody (Fig. 1D) and $P2X_4$ receptor-immunoreactive microglial cells were also found in some areas of the cerebellar white matter (Figs. 1C, F). Some of these cells were oval and some were polygons with a few short branches.

At P7, expression of all the P2X receptors (with the exception of $P2X_3$) was up-regulated. The primary dendrites of Purkinje cells were positively stained and the immunostaining intensity of each receptor was similar at this stage (Figs. 2A–E). Scattered P2X-ir cells were also found at the granular cell layer; these cells were larger than the granular cells (Figs. 2A–D). The granular cells with $P2X_5$-ir were weakly stained at this stage (Fig. 2D). The number and staining intensity of microglial cells with $P2X_4$-ir was increased (Fig. 2C). $P2X_1$- and $P2X_7$-ir microglial cells were also found at this stage (Figs. 2A, E).

At P14, expression of all the P2X receptors (except $P2X_3$) continued to be up-regulated. The dendritic trees of Purkinje cells were positively stained and branched extensively and reached the border of the germ layer of the cerebellum. The staining intensity of all the P2X receptors was strong in neurones (Figs. 3A, B, D–F). Some areas of Purkinje cell primary branches and cell bodies were much more heavily stained with the $P2X_5$ antibody than other areas of the cell (Fig. 3E). In deep cerebellar nuclei, the staining intensity of neurons with P2X-ir was moderate to strong (Figs. 3G, H). The number and intensity of microglial cells with $P2X_4$-ir reached a peak at P14 (Fig. 3C). The number of $P2X_1$- and $P2X_7$-ir microglial cells also increased at this stage.

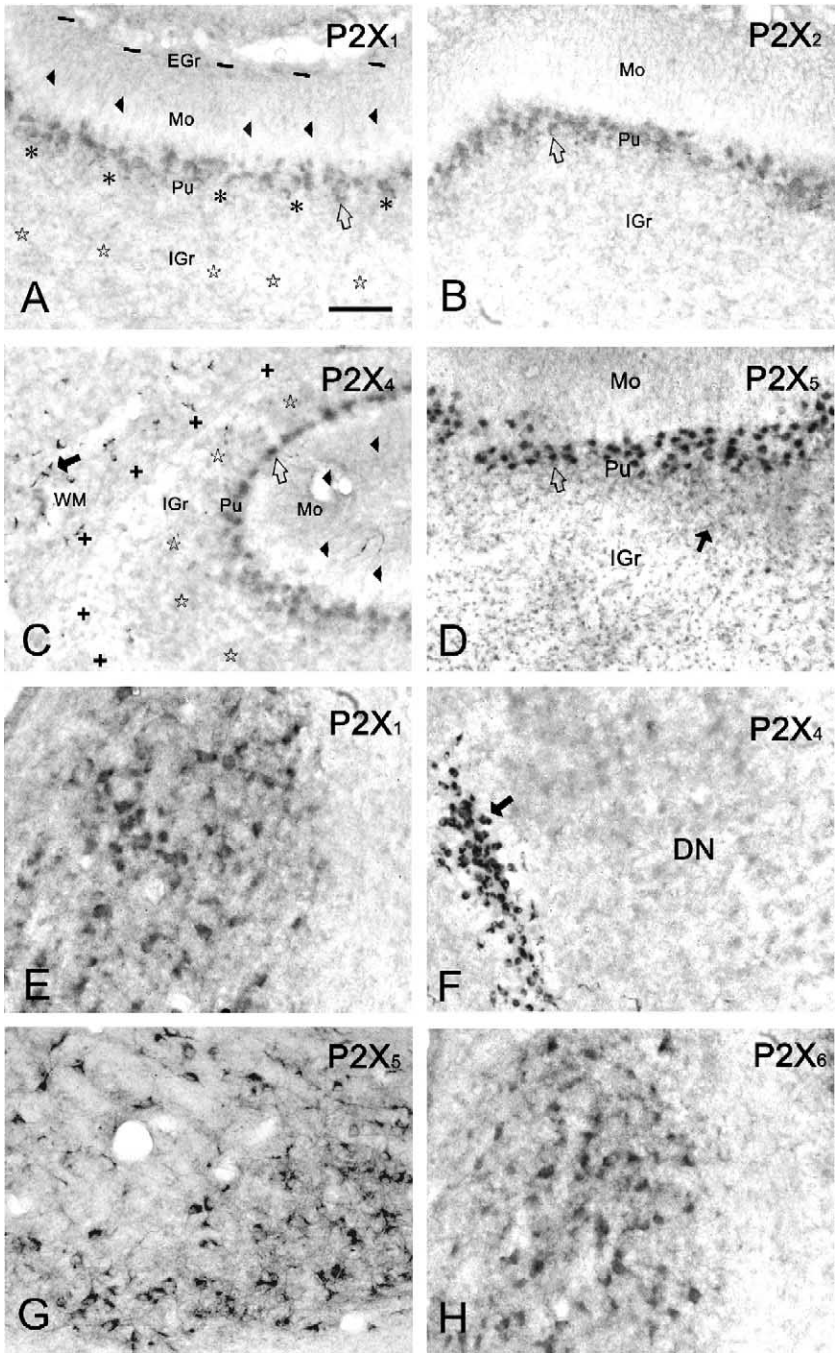

Fig. 1. P2X receptor-ir in rat cerebellar cortex and deep cerebellar nuclei at P3. (A) Cerebellar cortex $P2X_1$-ir in Purkinje cells (open arrow) in the Purkinje cell layer (Pu, asterisks). (B) Cerebellar cortex $P2X_2$-ir of Purkinje cells (open arrow). (C) Cerebellar cortex $P2X_4$-ir of Purkinje cells (open arrow), microglial cells (solid arrow) in the white matter (WM, crosses). (D) Cerebellar cortex $P2X_5$-ir of Purkinje cells (open arrow) and weakly-stained granular cells (solid arrow) in the internal granular cell layer (IGr, open stars)). (E) $P2X_1$-ir cells in deep cerebellar nuclei. (F) Cerebellar cortex $P2X_4$-ir of microglial cells (solid arrow); note absence of staining in the area of deep cerebellar nuclei (DN). (G) $P2X_5$-ir cells in deep cerebellar nuclei. (H) $P2X_6$-ir cells in deep cerebellar nuclei. Note absence of P2X receptor-ir in external granular layer (EGr; black bars) and the molecular layer (Mo, black arrowheads). Scale bar = 100 μm.

At P21, expression of all the P2X receptors (except $P2X_3$) was down-regulated in the Purkinje cells and deep cerebellar nuclei. $P2X_1$, $P2X_4$ and $P2X_7$ receptor expression was also decreased in microglial cells when compared to P14, but the expression of the $P2X_5$ receptor in neurones in deep cerebellar nuclei was similar to that at P14 (Figs. 4A–G). In contrast, expression of $P2X_5$-ir in granular cells was up-regulated (Fig. 4D).

At P60, expression levels of all the P2X receptors (except $P2X_3$) were similar to that at P21 in Purkinje cells and granular cells of the cerebellar cortex and neurons in

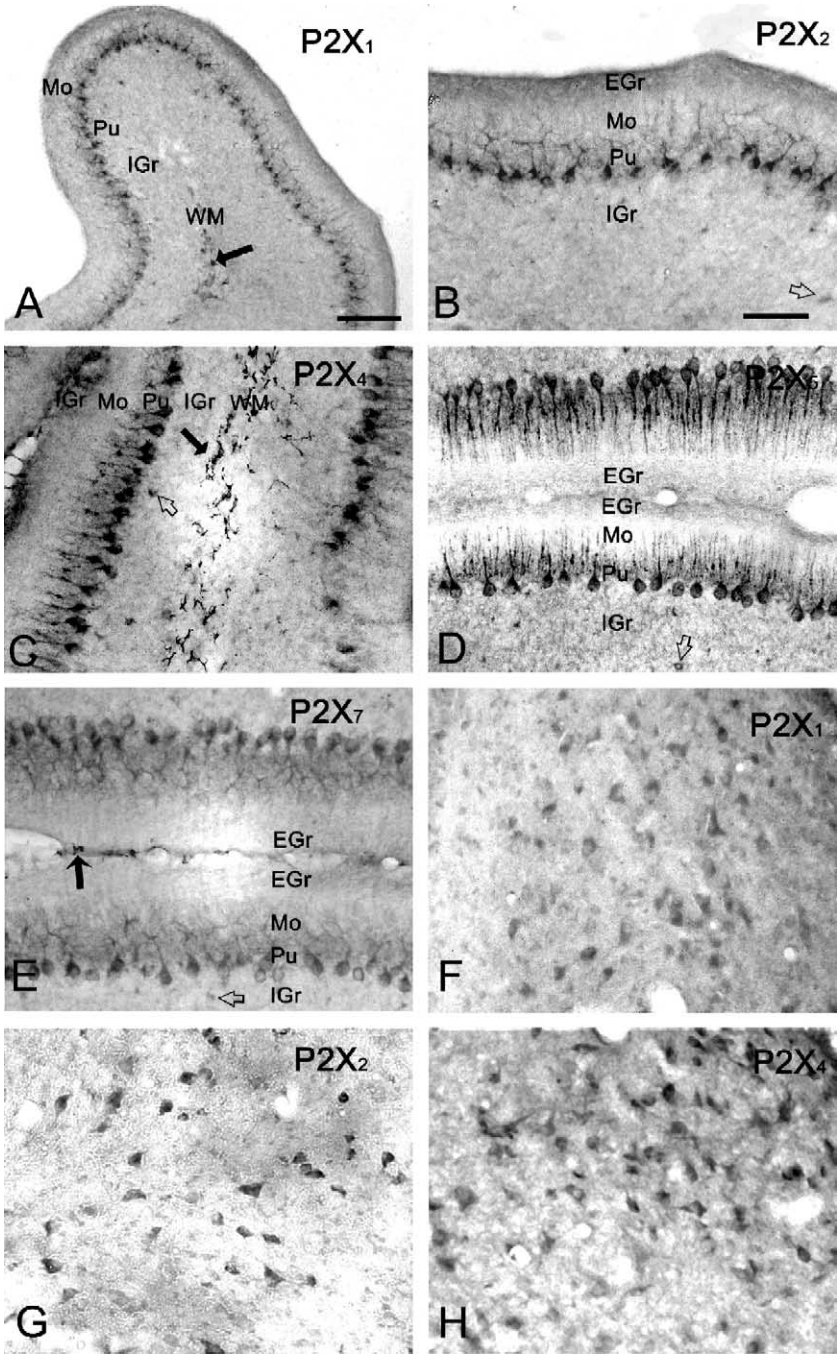

Fig. 2. P2X receptor-ir in rat cerebellar cortex and deep cerebellar nuclei at P7. (A) Cerebellar cortex $P2X_1$-ir of Purkinje cells in the Purkinje cell layer (Pu) and microglial cells (solid arrow) in the white matter (WM). (B) Cerebellar cortex $P2X_2$-ir of Purkinje cells in the Purkinje cell layer (Pu) and a unipolar brush cell (UBC, open arrow). (C) Cerebellar cortex $P2X_4$-ir cells of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo), microglial cells (solid arrow) in the white matter (WM) and a UBC (open arrow) in the internal granular cell layer (IGr). (D) Cerebellar cortex $P2X_5$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a UBC (open arrow) in the internal granular cell layer (IGr). (E) Cerebellar cortex $P2X_7$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo), a UBC (open arrow) in the internal granular cell layer (IGr) and a macrophage (solid arrow) in the pia mater. (F) $P2X_1$-ir cells in deep cerebellar nuclei. (G) $P2X_2$-ir cells in deep cerebellar nuclei. (H) $P2X_4$-ir cells in deep cerebellar nuclei. Note absence of P2X receptor-ir in external granular layer (EGr) and internal granular cell layer (IGr). Scale bar in A = 200μm and in B–H = 100μm.

deep cerebellar nuclei of white matter. The developmental trends were summarized and the intensity of P2X immunoreactivity was ranked on a 5-point scale (see Table 1).

In control experiments, preabsorption with P2X receptor peptides, or omission of the primary antibody resulted in no positive immunostaining (see for example $P2X_7$ receptors, Fig. 4H).

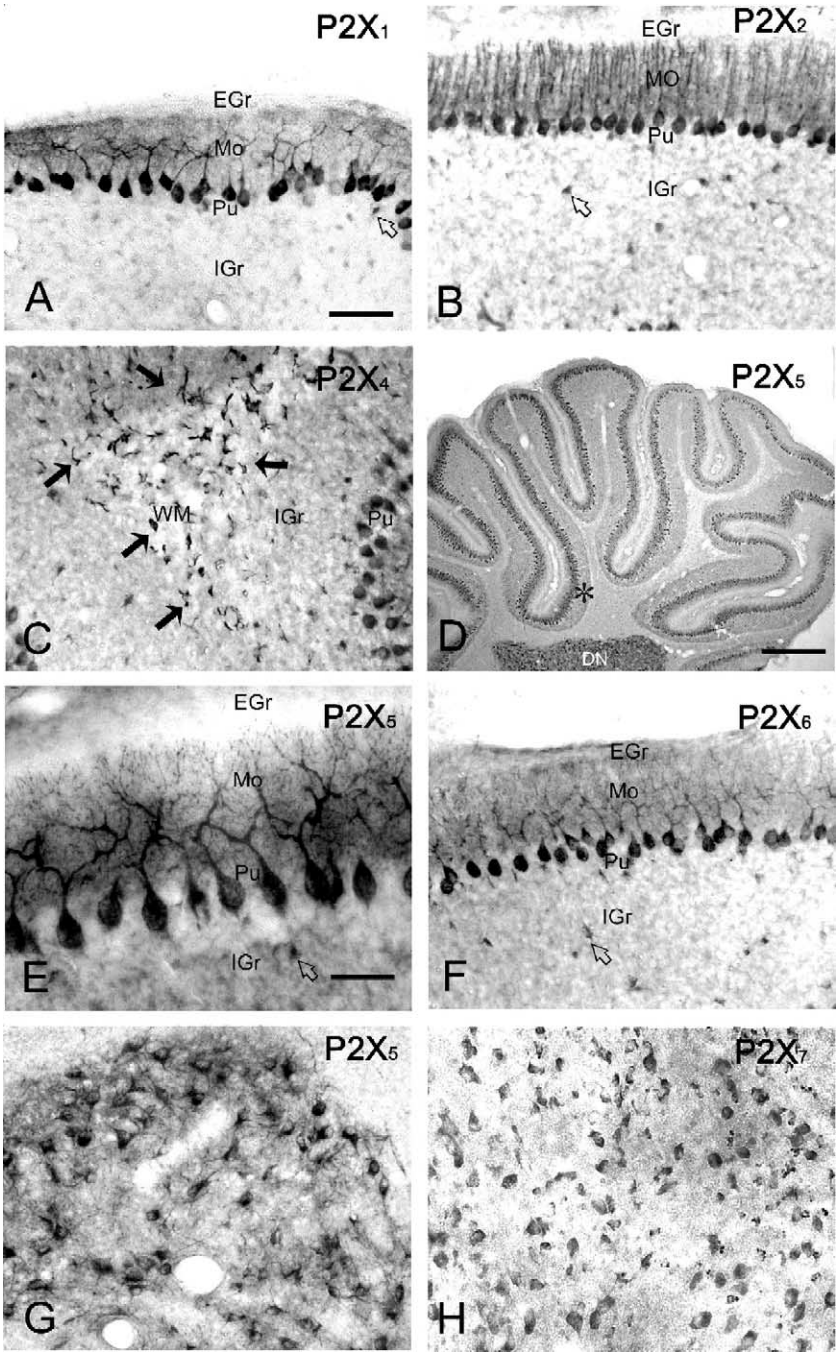

Fig. 3. P2X receptor-ir in rat cerebellar cortex and deep cerebellar nuclei at P14. (A) Cerebellar cortex $P2X_1$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a UBC (open arrow) in the internal granular cell layer (IGr). (B) Cerebellar cortex $P2X_2$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a UBC (open arrow) in the internal granular cell layer (IGr). (C) Cerebellar cortex $P2X_4$-ir of Purkinje cells in the Purkinje cell layer (Pu) and microglial cells (solid arrows) in the white matter (WM). (D) Low magnification cerebellar $P2X_5$-ir of deep cerebellar nuclei (DN). (E) High magnification of the area in D indicated by an asterisk showing Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a UBC (open arrow) in the internal granular cell layer (IGr). (F) Cerebellar cortex $P2X_6$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a UBC (open arrow) in the internal granular cell layer (IGr). (G) $P2X_5$-ir cells in deep cerebellar nuclei. (H) $P2X_7$-ir cells in deep cerebellar nuclei. Note absence of P2X receptor-ir in external granular layer (EGr) and internal granular cell layer (IGr). Scale bar in A–C, F–H = 100μm, in D = 500μm and in E = 50 μm.

The $P2X_3$ antibody, while giving no positive staining in this series of experiments, has been used to successfully label dorsal root ganglion cells and gut ganglion cells in studies in our laboratory [45,46].

In double-labeling experiments, almost all of the P2X-ir Purkinje cells at different stages were labeled by the calbindin D-28k antibody (a marker for Purkinje cells) and almost all of the cells with calbindin-ir in the Purkinje

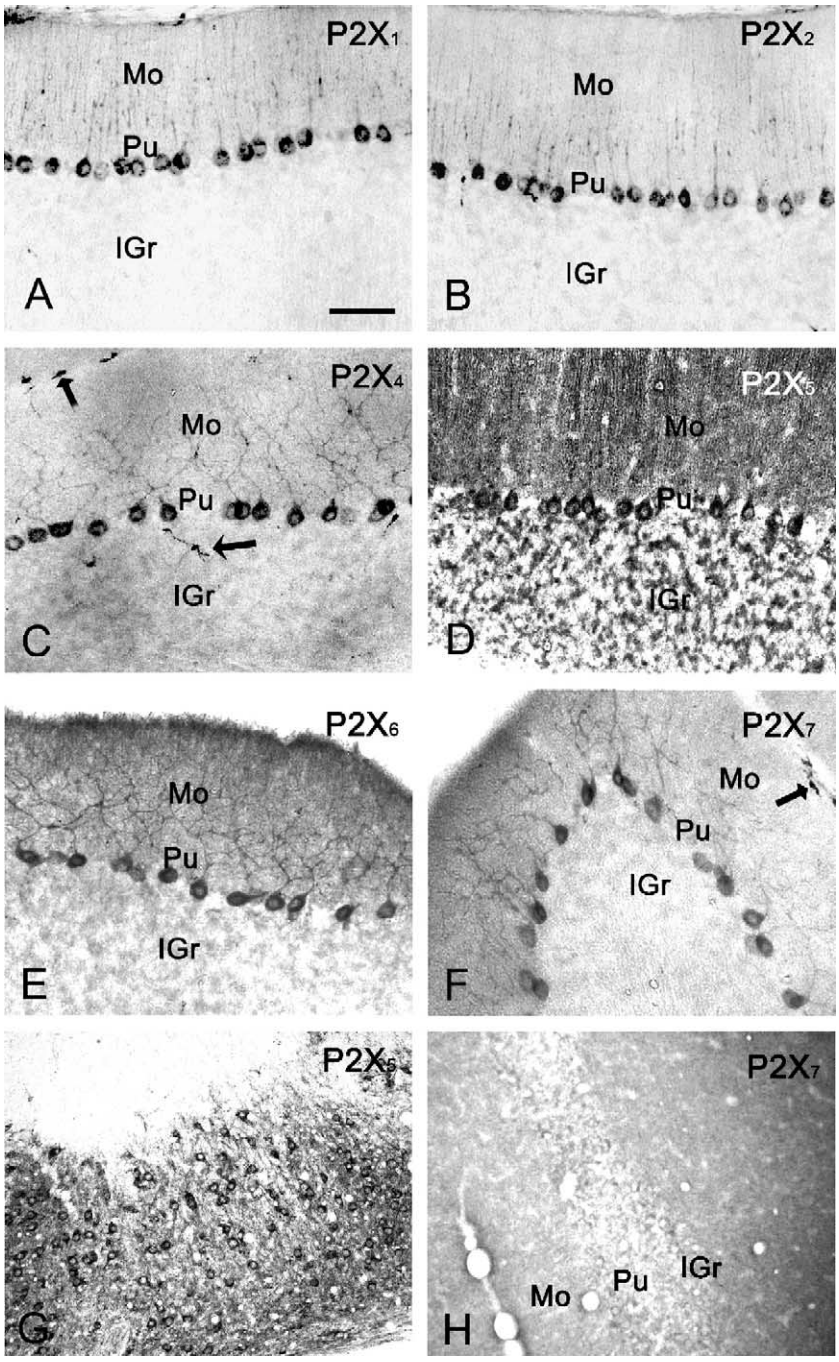

Fig. 4. P2X receptor-ir in rat cerebellar cortex and deep cerebellar nuclei at P21. (A) Cerebellar cortex $P2X_1$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo). (B) Cerebellar cortex $P2X_2$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo). (C) Cerebellar cortex $P2X_4$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and microglial cells (sold arrows). (D) Cerebellar cortex $P2X_5$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and granular cells in the internal granular cell layer (IGr). (E) Cerebellar cortex $P2X_6$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo). (F) Cerebellar cortex $P2X_7$-ir of Purkinje cells in the Purkinje cell layer (Pu) with processes into the molecular layer (Mo) and a macrophage (solid arrow) in the pia mater. (G) $P2X_5$-ir cells in deep cerebellar nuclei. (H) Preabsorption with $P2X_7$ peptides; no positive cells are shown in cerebellar cortex of P21 rat. Scale bar in A–H = 100μm.

cell layer were also labeled by the P2X antibodies, except for $P2X_3$ (Figs. 5A–H). Some of the P2X-ir cells in the granular cell layer were bigger in size than the granular cells and were labeled by the calretinin antibody (a marker for UBCs) (Figs. 5I–N). Since the percentage of P2X-ir cells that were also immunoreactive for calbindin D-28k or calretinin were almost the same at the five developmental stages, the data from P14 rats was selected for illustration in Table 2. $P2X_1$, $P2X_4$ and $P2X_7$-ir cells that look like microglial cells in the white matter at different stages were all labeled by the ED1 antibody but not labeled by GFAP, calbindin and calretinin antibodies (Fig. 5O).

5. Discussion

This paper is the first to study systematically the changes in expression of P2X receptor in rat cerebellum during postnatal development, using immunocytochemistry. We found that all P2X receptors, with the exception of $P2X_3$ receptors, were expressed in the cerebellum. This is consistent with the lack of cerebellar $P2X_3$ receptors described by Kidd et al. [27], although Garcia-Lecea et al. [20] claimed that $P2X_3$ receptors were expressed in Purkinje cells, cultured from 7-day newborn rats. Among the P2X receptors found in the rat cerebellum in the present study, $P2X_5$ and $P2X_7$ receptors were identified for the first time.

There have been a number of pharmacological and physiological studies focused on P2X receptor-mediated effects on Purkinje cells. P2X receptors found on Purkinje neurons for example were shown to be sensitive to suramin and PPADS blockade at low concentrations [20,21,33]. $P2X_4$ and $P2X_6$ homomeric receptors studied in expression systems have been shown to be resistant to blockade by suramin and PPADS [4,5,14]. Thus, the properties of P2X receptors in neonatal Purkinje cells appear to be dominated by $P2X_2$ receptor expression [33]. The responses to ATP of all functional homomeric P2X receptors are inhibited by acidification, except for $P2X_2$ receptors, where ATP responses are potentiated by acidification and inhibited by alkalinization [28,44]. This property applies to some extent

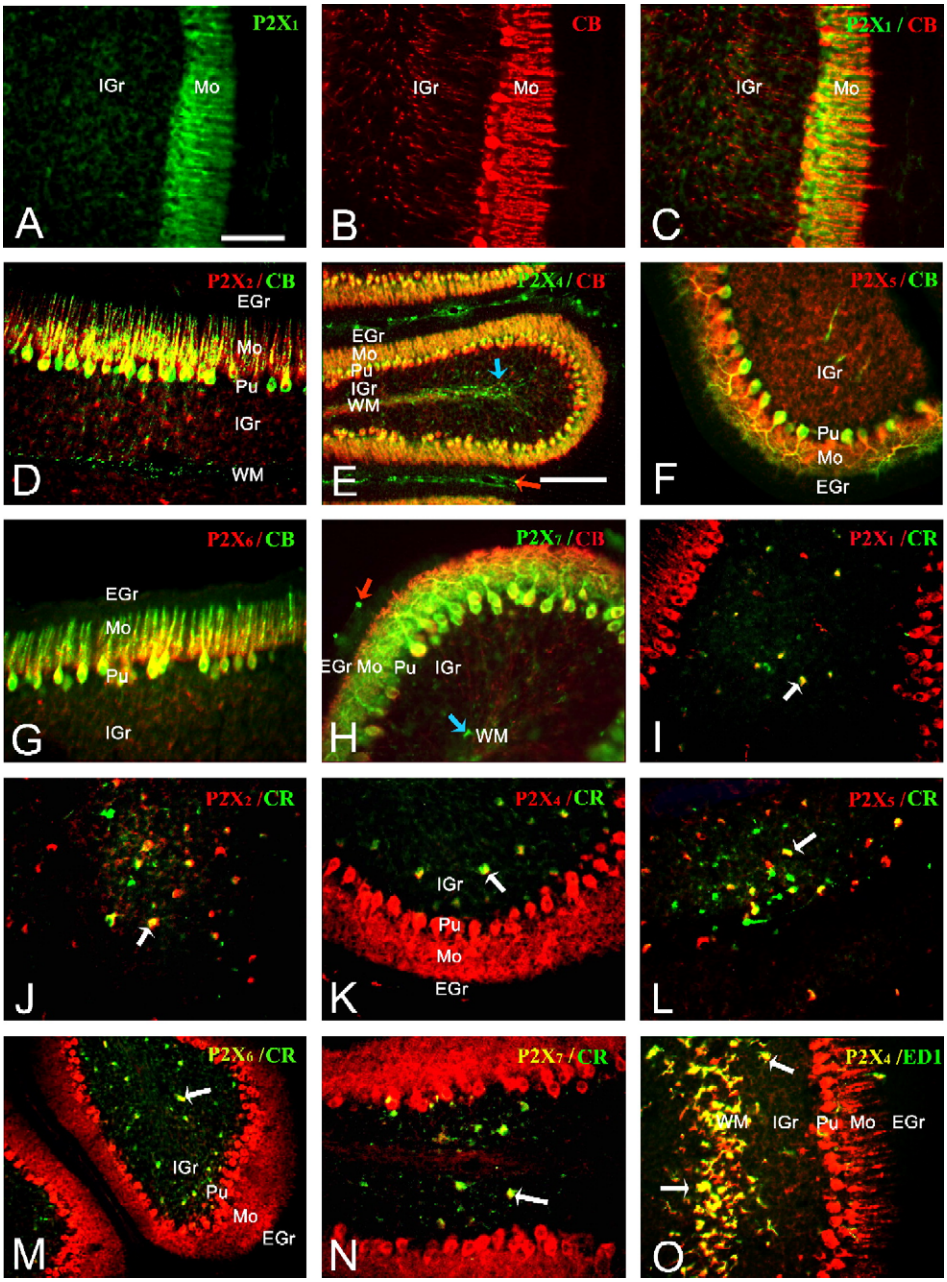

Table 2
The percentage of P2X-ir neurons which were also immunoreactive for calbindin D-28k or calretinin and the percentage of calbindin D-28k-ir or calretinin-ir neurons which were also immunoreactive for P2X receptor subunits in the Purkinje cell layer (PCL) or granular cell layer (GCL) at P14

P2X receptor subunits	Calbindin D-28k			Calretinin		
	PCL		GCL (%)	PCL (%)	GCL	
	A (%)	B (%)			C (%)	D (%)
$P2X_1$	97 ± 3	96 ± 4	0	0	67 ± 7	76 ± 8
$P2X_2$	98 ± 2	99 ± 1	0	0	63 ± 6	74 ± 7
$P2X_4$	98 ± 2	97 ± 3	0	0	78 ± 9	68 ± 6
$P2X_5$	99 ± 1	95 ± 5	0	0	79 ± 8	69 ± 7
$P2X_6$	96 ± 4	97 ± 3	0	0	70 ± 5	76 ± 8
$P2X_7$	98 ± 2	95 ± 6	0	0	78 ± 10	68 ± 9

(A) The number of P2X-ir neurons with calbindin D-28k-ir/the total number of P2X-ir neurons × 100%. (B) The number of calbindin D-28k-ir neurons with P2X-ir/the total number of calbindin D-28k-ir neurons × 100%. (C) The number of P2X-ir neurons with calretinin-ir/the total number of P2X-ir neurons × 100%. (D) The number of calretinin-ir neurons with P2X-ir/the total number of calretinin-ir neurons × 100%.

to heteromeric receptors likely to be expressed in Purkinje cells [21].

It is possible that replacement of $P2X_2$ for $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors may take place during development of cerebellum, in a comparable way to developmental changes in GABA or NMDA receptors [1,18,48]. It seems likely that $P2X_{2/6}$ or $P2X_{2/4}$ heteromultimers may be present in the cerebellum.

In the present study, we have shown that there are differences in the expression of P2X-ir on Purkinje cells during postnatal development. There was an increase in expression levels of P2X receptors from P3, peaking at P14 and showing some decline at P21 and P60. $P2X_5$ receptor expression was high in deep nuclei at P3 followed by a further increase in immunoreactivity at P7 and is sustained through to P60. Only the $P2X_5$ receptor was present in granular cells from P14 and showed increased expression at P21 and P60, but was barely present at P3 and P7. $P2X_4$-ir was expressed in cerebellar microglial cells at P3, showed increased expression up to P14 and later reverted to the earlier level; $P2X_7$ receptor expression appeared transiently between P7 and P21 in the white matter of cerebellum.

mRNA for all the P2X receptors, measured by RT-PCR, except for $P2X_6$, was expressed in cultured cerebellar granular cells from newborn rats (8–10 days) [22]. The $P2X_7$ antagonist, oxidized ATP, was reported to be highly toxic to the cultured granular cells of the cerebellum from newborn rats and this toxicity was inhibited by co-incubation with BzATP, a $P2X_7$ agonist [17]. However, in our study, only the $P2X_5$ receptor was found in the granular cells of the cerebellum. Maybe the levels of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$ receptor proteins are too low to be detected by our immunocytochemical method or maybe these P2X receptors are expressed in granular cells in culture, but not in vivo.

Calbindin D-28k is often used as a selective marker for Purkinje cells in the cerebellum [16,33,42]. In this study, almost all of the Purkinje cells with calbindin D-28k immunoreactivity at all the developmental stages examined were also labeled by $P2X_1$, $P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ antibodies. This means that most, if not all, Purkinje cells express these six P2X receptors at the same time. So it seems likely that P2X receptors in Purkinje cells co-assemble into heterooligomeric receptors such as those reported previously: $P2X_{4/6}$ [30], $P2X_{1/5}$ [31] and $P2X_{2/6}$ [29]. Therefore, Purkinje cells in the cerebellum, especially those at P7 to P14, would be a good model for the study of native heteromultimer P2X receptors.

Calretinin is usually used as a selective marker for unipolar brush cells (UBCs) in the cerebellum [2,19,37]. In this study we showed that 60–80% of $P2X_1$-, $P2X_2$-, $P2X_4$-, $P2X_5$-, $P2X_6$- and $P2X_7$-immunopositive neurons in the

Fig. 5. Co-existence among P2X receptor-ir, calbindin D-28K-ir (Purkinje cells), calretinin-ir (unipolar brush cells) and ED-1-ir (microglia) in rat cerebellum at P14. (A) Immunostaining for $P2X_1$ receptors (green). (B) Immunostaining for calbindin D-28k (CB; red) a marker for Purkinje cells. (C) Double-immunostaining (yellow) with $P2X_1$ receptors (green) and CB (red); note that most CB-ir cells (Purkinje cells) are also immunopositive for $P2X_1$ receptors. (D) Double-immunostaining (yellow) for $P2X_2$ receptors (red) and CB (green); note that most CB-ir cells are also immunopositive for $P2X_2$ receptors. (E) Double-immunostaining (yellow) for $P2X_4$ receptors (green) and CB (red); note that most cells that are immunopositive for CB also express $P2X_4$ receptors. A blue arrow indicates $P2X_4$-ir microglial cells in the white matter (WM) and a red arrow indicates $P2X_4$-ir cells in pia matter. (F) Double immunostaining (yellow) for $P2X_5$ receptors (red) and CB (green); note that most cells that are immunopositive for CB also express $P2X_5$ receptors. (G) Double immunostaining (yellow) for $P2X_6$ receptors (red) and CB (green); note that most cells that are immunopositive for CB also express $P2X_6$ receptors. (H) Double immunostaining (yellow) for $P2X_7$ receptors (green) and CB (red); note that most cells that are immunopositive for CB also express $P2X_7$ receptors. A blue arrow indicates $P2X_7$-ir microglial cells in the white matter (WM) and a red arrow indicates $P2X_7$-ir cells in pia matter. (I) Double-immunostaining (yellow) for $P2X_1$ receptors (red) and calretinin (CR; green) a marker for unipolar brush cells. (UBCs); note that some CR-ir UBCs express $P2X_1$ receptors in the granular cell layer, a white arrow indicates a double-labeled neuron. (J) Double-immunostaining (yellow) for $P2X_2$ receptors (red) and CR (green); note that some CR-ir UBCs express $P2X_2$ receptors in the granular cell layer, a white arrow indicates a double-labeled neuron. (K) Double- immunostaining (yellow) for $P2X_4$ receptors (red) and CR (green); note some CR-ir UBCs express $P2X_4$ receptors in the granular cell layer (IGr), a white arrow indicates a double-labeled neuron. (L) Double-immunostaining (yellow) for $P2X_5$ receptors (red) and CR (green); note that some CR-ir UBCs express $P2X_5$ receptors in the granular cell layer, a white arrow indicates a double-labeled neuron. (M) Double-immunostaining (yellow) for $P2X_6$ receptors (red) and CR (green); note that some CR-ir UBCs express $P2X_6$ receptors in the granular cell layer, a white arrow indicates a double-labeled neuron. (N) Double-immunostaining (yellow) for $P2X_7$ receptors (red) and CR (green); note that some CR-ir UBCs express $P2X_7$ receptors in the granular cell layer, a white arrow indicates a double-labeled neuron. (O) Double-immunostaining (yellow) for $P2X_4$ receptors (red) and ED1-ir (green; a marker for microglia) in white matter (WM) not in Purkinje cells; white arrows indicate double-labeled cells in the white matter. Mo: molecular layer, Pu: Purkinje cell layer, IGr: internal granular cell layer, EGr: external granular cell layer. Scale bar in A–D, F–O = 100μm and in E = 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cerebellar granular layer are immunoreactive for calretinin, identifying the majority of these cells as UBCs. UBCs are excitatory interneurons associated with granular cells in the granular layer of the cerebellar cortex [24,34,43]. In rat, UBCs usually have a single, thick and short dendrite provided with a tuft of dendrioles resembling a paint brush [34], a thin axon that forms several branches within the granular layer and synapses with numerous granule cells and other UBCs [36,39]. Excitatory input to the UBCs is usually provided by a single mossy fiber rosette with which the dendrioles form extensive synaptic contacts estimated to measure 20 μm^2 or more [23,41]. Since so many P2X receptor subtypes are present in the UBCs, extracellular ATP, as an excitatory neurotransmitter or neuromodulator, may regulate the activities of the giant synapses in the granular cell layer. However, no pharmacological studies of the properties of P2X receptors in the UBCs have been carried out to date.

In summary, we have presented details of the expression changes of P2X receptors that occur in rat postnatal developing cerebellum. Extracellular ATP as a neurotransmitter or neuromodulator may mediate or modulate rapid excitatory signaling by P2X receptors in Purkinje cells, granular cells, UBCs and microglia cells of the developing cerebellum. Since some of the changes in P2X receptor expression are transient, the possibility that they are involved in longer term trophic events such as cell proliferation, differentiation, movement and death during development cannot be excluded.

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References

[1] C. Akazawa, R. Shigemoto, Y. Bessho, S. Nakanishi, N. Mizuno, Differential expression of five *N*-methyl-D-aspartate receptor subunit mRNAs in the cerebellum of developing and adult rats, J. Comp. Neurol. 347 (1994) 150–160.

[2] R. Anelli, E. Mugnaini, Enrichment of unipolar brush cell-like neurons in primary rat cerebellar cultures, Anat. Embryol. 203 (2001) 283–292.

[3] M.R. Bennett, Non-adrenergic non-cholinergic (NANC) transmission to smooth muscle: 35 years on, Prog. Neurobiol. 52 (1997) 159–195.

[4] X. Bo, Y. Zhang, M. Nassar, G. Burnstock, R. Schoepfer, A P2X purinoceptor cDNA conferring a novel pharmacological profile, FEBS Lett. 375 (1995) 129–133.

[5] G. Buell, C. Lewis, G. Collo, R.A. North, A. Suprenant, An antagonist-insensitive P2X receptor expressed in epithelia and brain, EMBO J. 15 (1996) 55–62.

[6] G. Burnstock, Purinergic nerves, Pharmacol. Rev. 24 (1972) 509–581.

[7] G. Burnstock, Do some nerve cells release more than one transmitter? Neuroscience 1 (1976) 239–248.

[8] G. Burnstock, Purinergic mechanisms, Ann. N. Y. Acad. Sci. 603 (1990) 1–17.

[9] G. Burnstock (Guest Editor), Purinergic neurotransmission, Semin. Neurosci. 8 (1996) 171–257.

[10] G. Burnstock, The past, present and future of purine nucleotides as signalling molecules, Neuropharmacology 36 (1997) 1127–1139.

[11] G. Burnstock, Purinergic cotransmission, Brain Res. Bull. 50 (1999) 355–357.

[12] G. Burnstock, Purinergic receptors in the nervous system, in: E.M. Schwiebert (Ed.), Current Topics in Membranes: Purinergic Receptors and Signalling, vol. 54, Academic Press, San Diego, 2003, pp. 307–368.

[13] G. Burnstock, Introduction: P2 receptors, Curr. Top. Med. Chem. 4 (2004) 793–803.

[14] G. Collo, R.A. North, E. Kawashima, E. Merlo-Pich, S. Neidhart, A. Surprenant, G. Buell, Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels, J. Neurosci. 16 (1996) 2495–2507.

[15] G. Collo, S. Neidhart, E. Kawashima, M. Kosco-Vilbois, R.A. North, G. Buell, Tissue distribution of the P2X7 receptor, Neuropharmacology 36 (1997) 1277–1283.

[16] R.M. Cowell, F.S. Silverstein, Developmental changes in the expression of the chemokine receptor CCR1 in the rat cerebellum, J. Comp. Neurol. 457 (2003) 7–23.

[17] M.W. Craighead, K.M. Middlehurst, R. LeFeuvre, I. Kimber, N.J. Rothwell, Oxidised adenosine 5′-triphosphate, a $P2X_7$ antagonist, is toxic to rat cerebellar granule neurones in vitro, Neurosci. Lett. 311 (2001) 77–80.

[18] M. Farrant, D. Feldmeyer, T. Takahashi, S.G. Cull-Candy, NMDA-receptor channel diversity in the developing cerebellum, Nature 368 (1994) 335–339.

[19] A. Floris, M.R. Diflo, D.M. Jacobowitz, E. Mugnaini, The unipolar brush cells of the rat cerebellar cortex and the cochlear nucleus are calretinin-positive: a study by light and electron microscopic immunocytochemistry, Anat. Embryol. 189 (1994) 495–520.

[20] M. Garcia-Lecea, E.G. Delicado, M.T. Miras-Portugal, E. Castro, $P2X_2$ characteristics of the ATP receptor coupled to $[Ca^{2+}]_i$ increases in cultured Purkinje neurons from neonatal rat cerebellum, Neuropharmacology 38 (1999) 699–706.

[21] M. Garcia-Lecea, R.P. Sen, F. Soto, M.T. Miras-Portugal, E. Castro, P2 receptors in cerebellar neurons: molecular diversity of ionotropic ATP receptors in Purkinje cells, Drug Dev. Res. 52 (2001) 104–113.

[22] C. Hervas, R. Perez-Sen, M.T. Miras-Portugal, Coexpression of functional P2X and P2Y nucleotide receptors in single cerebellar granule cells, J. Neurosci. Res. 73 (2003) 384–399.

[23] D. Jaarsma, R.J. Wenthold, E. Mugnaini, Glutamate receptor subunits at mossy fiber-unipolar brush cell synapses: light and electron microscopic immunocytochemical study in cerebellar cortex of rat and cat, J. Comp. Neurol. 357 (1995) 145–160.

[24] D. Jaarsma, M.R. Diño, H. Ohishi, R. Shigemoto, E. Mugnaini, Metabotropic glutamate receptors are associated with non-synaptic appendages of unipolar brush cells in rat cerebellar cortex and cochlear nuclear complex, J. Neurocytol. 27 (1998) 303–327.

[25] R. Kanjhan, G.D. Housley, P.R. Thorne, D.L. Christie, D.J. Palmer, L. Luo, A.F. Ryan, Localization of ATP-gated ion channels in cerebellum using P2X2R subunit-specific antisera, NeuroReport 7 (1996) 2665–2669.

[26] E.J. Kidd, C.B. Grahames, J. Simon, A.D. Michel, E.A. Barnard, P.P. Humphrey, Localization of P2X purinoceptor transcripts in the rat nervous system, Mol. Pharmacol. 48 (1995) 569–573.

[27] E.J. Kidd, K.J. Miller, A.J. Sansum, P.P. Humphrey, Evidence for $P2X_3$ receptors in the developing rat brain, Neuroscience 87 (1998) 533–539.

[28] B.F. King, L.E. Ziganshina, J. Pintor, G. Burnstock, Full sensitivity of $P2X_2$ purinoceptors to ATP revealed by changing extracellular pH, Br. J. Pharmacol. 117 (1996) 1371–1373.

[29] B.F. King, A. Townsend-Nicholson, S.S. Wildman, T. Thomas, K.M. Spyer, G. Burnstock, Coexpression of rat $P2X_2$ and $P2X_6$ subunits in *Xenopus* oocytes, J. Neurosci. 20 (2000) 4871–4877.

[30] K.T. Lê, K. Babinski, P. Seguela, Central $P2X_4$ and $P2X_6$ channel subunits coassemble into a novel heteromeric ATP receptor, J. Neurosci. 18 (1998) 7152–7159.

[31] K.T. Lê, E. Boue-Grabot, V. Achambault, P. Seguela, Functional and biochemical evidence for heteromeric ATP-gated channels composed of $P2X_1$ and $P2X_5$ subunits, J. Biol. Chem. 274 (1999) 15415–15450.

[32] A. Loesch, G. Burnstock, Electron-immunocytochemical localization of $P2X_1$ receptors in the rat cerebellum, Cell Tissue Res. 294 (1998) 253–260.

[33] J. Mateo, M. Garcia-Lecea, M.T. Miras-Portugal, E. Castro, Ca^{2+} signals mediated by P2X-type purinoceptors in cultured cerebellar Purkinje cells, J. Neurosci. 18 (1998) 1704–1712.

[34] E. Mugnaini, A. Floris, The unipolar brush cell: a neglected neuron of the mammalian cerebellar cortex, J. Comp. Neurol. 339 (1994) 174–180.

[35] R.A. North, Molecular physiology of P2X receptors, Physiol. Rev. 82 (2002) 1013–1067.

[36] M.-G. Nunzi, E. Mugnaini, Unipolar brush cell axons form a large system of intrinsic mossy fibers in the postnatal vestibulocerebellum, J. Comp. Neurol. 422 (2000) 55–65.

[37] M.G. Nunzi, R. Shigenoto, E. Mugnaini, Differential expression of calretinin and metabotropic glutamate receptor mGluR1α defines subsets of unipolar brush cells in mouse cerebellum, J. Comp. Neurol. 451 (2002) 189–199.

[38] I.B. Oglesby, W.G. Lachnit, G. Burnstock, A.P.D.W. Ford, Subunit specificity of polyconal antisera to the carboxy terminal regions of P2X receptors $P2X_1$ through $P2X_7$, Drug Dev. Res. 47 (1999) 189–195.

[39] D.J. Rossi, S. Alford, E. Mugnaini, N.T. Slater, Properties of transmission at a giant glutamatergic synapse in cerebellum: the mossy fiber-unipolar brush cell synapse, J. Neurophysiol. 74 (1995) 24–42.

[40] M.E. Rubio, F. Soto, Distinct localization of P2X receptors at excitatory postsynaptic specializations, J. Neurosci. 21 (2001) 641–653.

[41] N.T. Slater, D.J. Rossi, G.A. Kinney, Physiology of transmission at a giant glutamatergic synapse in cerebellum, Prog. Brain Res. 114 (1997) 151–163.

[42] F. Soto, M.E. Rubio, Cloned P2X receptor subunits in cerebellum and hippocampus, Drug Dev. Res. 52 (2001) 133–139.

[43] J. Takács, L. Markova, Z. Borostyánköi, T.J. Görcs, J. Hámori, Metabotropic glutamate receptor type 1 expressing unipolar brush cells in the cerebellar cortex of different species: a comparative quantitative study, J. Neurosci. Res. 55 (1999) 733–748.

[44] S.S. Wildman, B.F. King, G. Burnstock, Zn^{2+} and pH modulation of ATP-responses at recombinant $P2X_4$ receptors, Br. J. Pharmacol. 126 (1999) 762–768.

[45] G. Wynn, W. Rong, Z. Xiang, G. Burnstock, Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum, Gastroenterology 125 (2003) 1398–1409.

[46] Z. Xiang, G. Burnstock, Development of nerves expressing $P2X_3$ receptors in the myenteric plexus of rat stomach, Histochem. Cell Biol. 122 (2004) 111–119.

[47] Z. Xiang, X. Bo, I.B. Oglesby, A.P. Ford, G. Burnstock, Localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamus, Brain Res. 813 (1998) 390–397.

[48] T.M. Zheng, W.J. Zhu, G. Puia, S. Vicini, D.R. Grayson, E. Costa, H.J. Caruncho, Changes in gamma-amino butyrate type A receptor subunit mRNAs, translation product expression, and receptor function during neuronal maturation in vitro, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 10952–10956.