European Journal of Neuroscience, Vol. 25, pp. 3319–3331, 2007 doi:10.1111/j.1460-9568.2007.05562.x

Expression and localization of P2 nucleotide receptor subtypes during development of the lateral ventricular choroid plexus of the rat

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Keywords: ATP, brain, embryo, epithelium, neuroependyma, Sprague–Dawley rat

Abstract

The choroid plexuses secrete cerebrospinal fluid (CSF) and regulate the brain's internal environment via the blood–CSF barrier. The permeability properties of the blood–CSF interface have been studied previously in adult and immature brains, however, little is known about the development of CSF secretion and its modulation. ATP influences secretion in other epithelia via ionotropic P2X or metabotropic P2Y receptors. P2 receptors have frequently been found to be down-regulated in the postnatal period, suggesting a developmental role for purinergic and pyrimidine signalling. The present study investigated the expression of P2 receptors in lateral ventricular choroid plexus in relation to recent studies of aquaporin-1 expression and rapid expansion of the lateral ventricles in rat embryos. In the present study mRNAs for all known mammalian nucleotide receptor subtypes, except $P2X_7$, were identified from as early as E15. $P2X_7$ mRNA was detected from E18. Indications of differential expression patterns were observed for the different subtypes during development: an apparent increase in expression for $P2Y_2$ and $P2X_7$, a decline in $P2X_{1\text{-}2,4}$, no detectable difference in expression levels for $P2X_6$ and $P2Y_{12\text{-}13}$ and transient expression peaks for $P2X_{3,5}$ and $P2Y_{1,4,6,14}$. $P2X_{4,5,7}$ and $P2Y_{1,4}$ receptor proteins were detected immunohistochemically in the choroidal epithelium from early in development (E15 or E18). Their differing developmental profiles suggest specific roles in the development of CSF secretion that may have particular relevance for the rapid expansion of the ventricles that occurs in the embryo. $P2X_5$ and $P2Y_6$ were also detected in the developing neuropendyma from P0 and P9, respectively.

Introduction

The choroid plexuses are the site of the blood–cerebrospinal fluid (CSF) barrier, formed by the presence of continuous tight-junctions between adjacent epithelial cells (Brightman & Reese, 1969). The blood–CSF and the blood–brain barriers protect and regulate the internal environment of the brain, which is vital for its proper development and function (Saunders, 1992). Previous work has shown that both these barriers are tight and functional from very early stages in brain development (Tauc *et al*., 1984; Habgood *et al*., 1992; Ek *et al*., 2001, 2003, 2006; Johansson *et al*., 2006). Choroid plexuses are also largely responsible for production of CSF (Davson & Segal, 1996). CSF is produced by formation of ion gradients created by the actions of numerous ion transporters and pumps located in the choroidal epithelial cells. The ion gradients drive the subsequent movement of water across the polarized epithelia into the ventricles through the water-channel aquaporin 1 in the apical (Nielsen *et al*., 1993; Oshio *et al*., 2005; Brown *et al*., 2005) and basolateral (Johansson *et al*., 2005) membranes. Little is known about the mechanisms controlling ion and water transfer in the developing plexuses. In rodents, aquaporin 1 is present in the choroid plexus within one day of its appearance, indicating early onset of fluid secretion (Johansson *et al*., 2005). The limited information available on ion transport in the developing rat choroid plexus shows that gradients between blood and CSF for sodium, potassium and chloride have become established around the time of birth (Amtorp & Sørensen, 1974; Jones & Keep, 1987). However, nothing is known about ion gradients, or the mechanisms generating them, during embryonic brain development. We have therefore investigated the possibility that P2 receptors might be involved in embryonic CSF secretory mechanisms.

The role of nucleotides as extracellular cell signalling molecules is well established (Burnstock, 1972, 1997, 2006). Seven P2X receptors (ionotropic, $P2X_{1-7}$) and eight P2Y receptors (metabotropic, $P2Y_{1,2,4,6,11-14}$) have been identified in mammals (North, 2002; Burnstock & Knight, 2004). In adult choroid plexus, expression of $P2X_{1,2,4-7}$ (Xiang & Burnstock, 2005) and $P2Y_4$ (Webb *et al*., 1998) receptors has previously been demonstrated and they have been suggested to be involved in CSF secretion. Further, chloride ion currents were not induced in the choroidal epithelium in response to GTPγS when ATP was omitted from the solution (Kotera & Brown, 1994). Both P2X and P2Y receptors are highly expressed in other secretory epithelia and have been found to be involved in the modulation of fluid secretion (Leipziger, 2003; Schwiebert & Zsembery, 2003). The P2 receptor subtypes have been found to be

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Received 11 December 2006, revised 12 March 2007, accepted 26 March 2007

down-regulated during postnatal development in most systems investigated, indicating a significant role for purinergic and pyrimidinergic transmission during development (Burnstock, 2001). Given the influence of nucleotides on ion gradients and secretion in other epithelia and their importance during development, purinergic and pyrimidinergic signalling could have a role in early CSF secretion. This possibility is supported by the findings of the present study of the ontogeny of P2 receptors in rat lateral ventricular choroid plexus.

Materials and methods

Animals

All animal experiments were conducted according to NHMRC guidelines and approved by the University of Melbourne Animal Experimentation Ethics Committee. Time-mated females at different gestational ages and postnatal Sprague–Dawley rats were used. All animals were terminally anaesthetized with an intraperitoneal injection of urethane (Sigma, 25% w/v, 1 mL/100 g bodyweight). The day of discovery of the overnight plug was counted as embryonic day (E) 0 and the day of birth as postnatal day (P) 0. The developmental stages of the embryos were confirmed by their weight and crown-rump length.

RT-PCR

Lateral ventricular choroid plexuses from E15, E18, P0 and adult rats were carefully dissected out in DEPC-treated saline and immediately submersed in RNase Later (Qiagen). Both lateral ventricular choroid plexuses from approximately seven embryos were pooled at E15, five at E18, two neonates at P0 and from one adult. Three pooled samples from each age were used ($n = 3$). Total RNA was extracted and eluted into 50 μL dH_20 using the RNeasy Mini Kit (Qiagen) with the inclusion of an on-column DN*ase* treatment step (RN*ase*-Free DN*ase* Set, Qiagen) in order to avoid false positives by amplification of residual genomic DNA. This resulted in an RNA yield between 50 and 100 ng/mL as measured on a spectrophotometer (SmartSpec 3000, Bio-Rad). The integrity of the RNA was assessed by using denaturing-agarose gel electrophoresis. Isolated RNA (0.1 μg) was converted to cDNA and RT-PCR was performed using a thermal cycler (Mastercycler EP gradient S, Eppendorf) in a one-step reaction using SuperScript™ One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen). Primer sequences used are shown in Table 1. The thermal cycling protocol was; 42 °C for 30 min followed by 94 °C for 2 min, then 35 cycles of 94 °C for 45 s, 60 °C for 30 s (see Table 1 for primer specific annealing temperatures) and 72 °C for 1 min and finally 72 °C for 10 min. The resulting PCR products were resolved in a 2% agarose gel containing ethidium bromide and observed under ultraviolet illumination. Negative controls were run using no RNA and always appeared blank. All primer pairs were first tested on a positive control tissue (kidney and/or brain). The relative intensity of the bands, corresponding to 1 μg of total RNA, at different developmental ages were visually compared to each other and scored (–, not detectable to +++ very strong band in gel).

Tissue preparation for morphological studies

Brains were dissected out from E15, E18, P0, P9 and adult terminally anaesthetized Sprague–Dawley rats and immediately immersed in Bouin's fixative ($n = 2$–3 per age). The specimens were dehydrated with graded alcohols, cleared in xylene and embedded in paraffin wax (Merck, melting point 52 °C). Serial sections, 5-μm thick, were cut in coronal orientation and placed on silanized slides. Representative sections of each series were stained with haematoxylin and eosin for routine histology.

TABLE 1. Primer sequences for P2 receptors

Gene	Sequence 5′–3′	Amplicon	T*
P2X1[§]	F GCTGACTATGTCTTCCCAGC R GACTCTCGCACCACATAGC	454 bp	56 °C
P2X2*	F GAATCAGAGTGCAACCCCAA R TCACAGGCCATCTACTTGAG	357 bp	60 °C
P2X3*	F TGGCGTTCTGGGTATTAAGATCGG R CAGTGGCCTGGTCACTGGCGA	440 bp	60 °C
P2X4*	F GAGGCATCATGGGTATCCAGATCAAG R GAGCGGGGTGGAAATGTAACTTTAG	447 bp	62 °C
P2X5[§]	F CCTCAACTCCACAAAGTTAGG R GCTGACTATGATGTCTCTGG	332 bp	58 °C
P2X6*	F AAAGACTGGTCAGTGTGTGGCGTTC R TGCCTGCCCAGTGACAAGAATGTCAA	520 bp	60 °C
P2X7*	F GTGCCATTCTGACCAGGGTTGTATAAA R GCCACCTCTGTAAAGTTCTCTCCGATT	354 bp	62 °C
P2Y1[‡]	F CCTGCGAAGTTATTTCATCTA R GTTGAGACTTGCTAGACCTCT	318 bp	56 °C
P2Y2[†]	F CTGCCAGGCACCCGTGCTCTACTT R CTGAGGTCAAGTGATCGGAAGGAG	339 bp	66 °C
P2Y4[†]	F CACCGATACCTGGGTATCTGCCAC R CAGACAGCAAAGACAGTCAGCACC	377 bp	66 °C
P2Y6[†]	F GACCTTGCCTGCCGCCTGGTA R TACCACGACAGCCATACGGGCCGC	481 bp	66 °C
P2Y12[‡]	F TCCCATTGCTCTACACTGTC R TGTCCTTTCTTCTTATTTGC	895 bp	56 °C
P2Y13[‡]	F CAGGGACACTCGGATGACA R TGTTCGGCAGGGAGATGA	425 bp	56 °C
P2Y14[§]	F CCCTGCACCCGGAACA R CGAAGAACGCGATGCTGAC	300 bp	58 °C

F, forward primer; R, reverse primer. Primers from *Shibuya *et al.*, 1999; [†]Fries *et al.*, 2004; [‡]Fumagalli *et al.*, 2004; [§]designed using Primer Express software (PE Applied Biosystems). All primers were assessed for sequence homology with other genes by using BLAST searches (http://www.ncbi.nlm.nih.gov/blast).

Immunohistochemistry

Paraffin embedded sections were dewaxed by heating (60 °C for 20 min) followed by histolene treatment and then rehydrated by a series of graded ethanols. Antigen retrieval was performed by heating the sections in citrate solution in a microwave oven (10 mM, 2 × 3 min). Sections were incubated in Peroxidase Blocker (Dako-Cytomation) to remove any endogenous peroxidase activity, followed by incubation in Protein Blocker (DakoCytomation). The primary antibody was left on the sections overnight at 4 °C. P2X antibodies were obtained from previous studies (Xiang & Burnstock, 2005) and P2Y antibodies were obtained from Alamone Laboratories. The P2X antibodies have been used previously in immunohistochemical procedures and have been tested for their specificity using both immunoblots and immunohistochemistry (Oglesby *et al.*, 1999; Cheung & Burnstock, 2002; Xiang & Burnstock, 2005). The P2Y receptors have been used in previous immunohistochemical studies and have been tested in rat tissues by the manufacturer using

immunoblots, which revealed specific reactivity that could be removed by preincubation with the control peptide antigen (e.g. Cheung *et al*., 2003; Fries *et al*., 2004; Giaroni *et al*., 2006). All antibodies were used in a 1 : 50 dilution. The overnight incubation was followed by 3 × 5-min washes in PBS containing 0.1% Tween-20 (Sigma). Subsequently, two incubations in swine anti-rabbit immunoglobulins (DakoCytomation, diluted 1 : 200) and rabbit peroxidase-anti peroxidase (DakoCytomation, diluted 1 : 200) were performed at room temperature for 2 h each. Each incubation was followed by three washes in PBS/Tween-20 buffer. The peroxidase reaction was developed in DAKO 3,3′-diaminobenzidine tetrahydrochloride (DAB) liquid substrate solution for 5 min and then stopped by immersing the slides in distilled water. The sections were counterstained with toluidine blue, followed by dehydration in a series of graded ethanols, cleared in histolene and mounted in Ultramount 4 (Fronine) mounting medium. As a negative control, the primary antibody was omitted and these sections always appeared blank. All antibodies were first tested on positive control tissue (kidney and adult choroid plexus).

Image analysis

Agarose gels were captured under ultraviolet illumination using the Kodak Image Station. Tissue sections were viewed under an Olympus BX50 light microscope and photographed using and attached Olympus DP70 digital camera connected to Olympus DP controller software. Captured images were transferred into Adobe Photoshop 5.5. In some images the contrast and brightness were adjusted to obtain a light (sections) or black (gels) background. No other image manipulations were performed.

Results

P2 receptor mRNA expression in the developing choroid plexus

The expression of P2 receptor RNA was assessed using RT-PCR. Although the form of RT-PCR used is only semiquantitative the three different pooled samples from each age showed consistent age-related differences in the magnitudes of the RT-PCR signals. An indication of these apparent differences in expression levels is shown in Table 2 (– not detectable to +++ very strong band in gel). All P2Y and P2X receptor mRNAs investigated, except $P2X_7$, were expressed in the lateral ventricular choroid plexus from the earliest day examined (E15, Fig. 1, Table 2) which is only one day after the differentiation of the first epithelial cells in this plexus (Johansson *et al*., 2005). $P2X_6$, $P2Y_{12}$ and $P2Y_{13}$ mRNAs were detected at similar levels at all ages examined. $P2X_7$ mRNA, the only receptor subtype that was not expressed at E15 (Fig. 1), was detected from E18 and showed a stronger signal after birth. The intensity of the $P2Y_2$ amplification product appeared to increase continuously, with the highest mRNA levels detected in adult choroid plexus. The relative expression of $P2X_1$, $P2X_2$ and $P2X_4$ decreased during development. During development, but not in the adult, two bands were detected using the $P2X_2$ primer (Fig. 1B). This has been described previously for $P2X_2$ using RT-PCR (e.g. Fumagalli *et al*., 2004; Doctor *et al*., 2005) and is likely due to alternative splicing (Brändle *et al*., 1997). Finally, $P2X_3$, $P2X_5$, $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ appeared to have a different developmental pattern showing a transient increase in levels of mRNA at an intermediate age (E18 for $P2X_3$, $P2Y_1$ and $P2Y_6$ and at P0 for $P2X_5$, $P2Y_4$ and $P2Y_{14}$, Table 2).

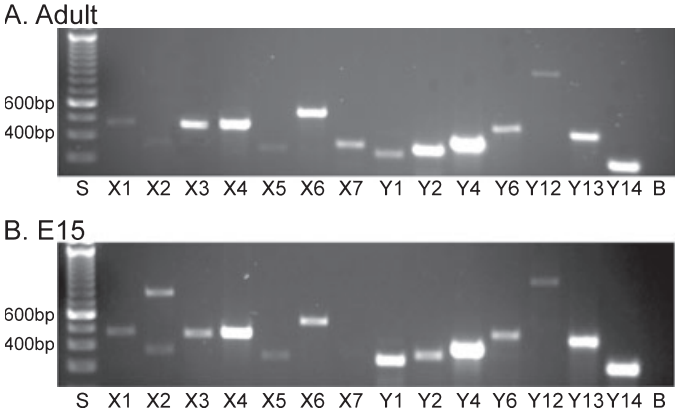

FIG. 1. RT-PCR analysis of P2 receptor mRNA in rat lateral ventricular choroid plexus. (A) In the adult choroid plexus mRNA for all P2 receptor subtypes was detected. (B) In the embryonic day (E) 15 choroid plexus, one day after its appearance, all subtypes were expressed except $P2X_7$. The two bands for $P2X_2$ are probably due to alternative splicing (Brändle *et al*., 1997). S, molecular standards, B, blank (no RNA).

P2X receptor immunohistochemistry

The presence of $P2X_4$, $P2X_5$ and $P2X_7$ in the choroid plexus was investigated in sections from brains of E15, E18, P0 and adult rats and is summarized in Table 3.

TABLE 2. P2 receptor mRNA in rat lateral ventricular choroid plexus

	X1	X2	X3	X4	X5	X6	X7
E15	++	+*/++†	+	+++	+	+++	–
E18	+	++/+	+++	+++	(+)	+++	+
P0	+	++/+	++	+++	++	+++	+
Adult	+	+/–	++	++	+	+++	++
	Y1	Y2	Y4	Y6	Y12	Y13	Y14
E15	++	+	++	+	+	++	++
E18	+++	+	++	+++	+	++	++
P0	++	++	+++	++	+	++	+++
Adult	+	+++	++	++	+	++	++

E, embryonic day; *P*, postnatal day; +++, very strong band on the agarose gel; ++, strong and clearly visible band; +, weak band; (+), barely visible band and –, band not visible see Fig. 1. $n = 3$ pooled samples (for the number of choroid plexuses in each pooled sample see Materials and methods). *Smaller molecular weight band, †larger molecular weight band of $P2X_2$ probably due to alternative splicing (Brändle *et al*., 1997).

TABLE 3. P2 receptor immunoreactivity in the developing lateral ventricular choroid plexus epithelial cells

	P2X4	P2X5	P2X7	P2Y1	P2Y4	P2Y6
E15	++	+	+	–	+	–
E18	+++	+	–	+++	+++	(+)
P0	++	+++	(+)	++	++++	–
Adult	–	+++++	+	++	++	–

The number of positive epithelial cells assessed in a sliding scale from – to +++++, where – is no detectable immunoreactivity in any cells, +++++ is many positive epithelial cells but not all and (+) is slight staining of a few cells. E, embryonic day; *P*, postnatal day; $n = 2–3$.

P2X4

The presence of $P2X_4$ was detected at all developmental ages examined; however, the distribution patterns changed with age (Fig. 2). At E15 the immunohistochemical reaction product appeared to be localized within the cytoplasm of clusters of epithelial cells (Fig. 2B). At E18, the immunostaining was limited to the apical surface of some epithelial cells, which were distributed along the length of choroid plexus, with an apparently higher number of positive cells seen at the tip of the plexus (Fig. 2D). At P0 immunoreactivity was present in many epithelial cells but appeared, at this age, to be cytoplasmic and more diffuse (Fig. 2E), resembling the distribution pattern previously reported in the adult choroid plexus (Xiang & Burnstock, 2005).

P2X5

The presence of $P2X_5$ was detected at all ages examined with a larger number of positive cells observed at older ages (Fig. 3 and Table 3). At all ages examined the immunostaining appeared to be cytoplasmic, however, the appearance of the staining was different at different ages corresponding to the changing pattern of the presence of glycogen in the epithelial cells. Glycogen is lost from the cells during tissue processing, leaving characteristic empty spaces in the epithelial cells. Thus at E15 and E18, both glycogen rich stages, only sparse cytoplasm can be seen around the glycogen deposits and the nucleus, possibly reducing the number of positive cells that are clearly visible (Fig. 3B and Table 3). After the disappearance of glycogen, the immunohistochemical reaction product was diffusely distributed throughout the cell cytoplasm as illustrated for adult choroid plexus in Fig. 3D.

P2X7

The presence of $P2X_7$ was detected in epithelial cells at the very root of the E15 choroid plexus (Fig. 4B). In all other ages there was only marginal staining of the choroidal epithelium (Table 3). However, in the adult there was detectable staining in endothelial cells of the choroidal blood vessels (Fig. 4D). Weak endothelial staining was also present in some earlier ages.

P2Y receptor immunohistochemistry

The presence of $P2Y_1$, $P2Y_4$ and $P2Y_6$ was also investigated in the lateral ventricular choroid plexus of E15, E18, P0 and adult rat brains and is summarized in Table 3.

P2Y1

Immunoreactivity for $P2Y_1$ was detected in the cytoplasm of the choroid plexus epithelial cells (Fig. 5). At the earliest day examined (E15) no reaction product was detected in the choroid plexus (not illustrated), however, three days later at E18, many epithelial cells were immunopositive in the cytoplasm in the apical (CSF facing) end of the cell above the abundant glycogen deposits (Fig. 5A). At P0 a similar localization was seen but fewer cells appeared positive (Fig. 5C, Table 3). In the adult some epithelial cells remained positive with immunoreactivity distributed uniformly in their cytoplasm (Fig. 5E). Additionally, in the adult some staining of endothelial cells in the plexus stroma was also observed (Fig. 5E).

P2Y4

Immunoreactivity for $P2Y_4$ was detected in the developing choroid plexus epithelial cells at all ages investigated; it showed a punctate pattern (Fig. 5). At E15, only very few cells were immunopositive for $P2Y_4$ and were found at the very root of the plexus (Fig. 5B). At E18 the number of positive epithelial cells was greatly increased (not illustrated), the positive cells were dispersed throughout the choroid plexus epithelium but somewhat concentrated at the root (Table 3). At P0, a significant increase in the number of positive epithelial cells as well as the number of punctate immunoreactivity foci was seen at the root of the lateral ventricular choroid plexus, with only few cells being positive along its length (Fig. 5D). In the adult plexus, the proportion of positive epithelial cells was much less compared to E18 and P0 (Fig. 5F).

P2Y6

At E18, the reaction product for $P2Y_6$ was present in some glycogen rich epithelial cells, in one out of three animals (not illustrated). Immunoreactivity for $P2Y_6$ was detected in endothelial cells in adult choroid plexus blood vessels (Fig. 6B).

P2 receptor immunoreactivity in developing neuroependyma lining the lateral ventricles

The choroid plexus epithelial cells arise from the neuroependymal cells lining the cerebral ventricles at the level of the interventricular foramen. The presence of P2 receptors in these cells during development was also investigated in the present study. The $P2X_5$ and $P2Y_6$ receptors, but not $P2X_4$, $P2X_7$, $P2Y_1$ or $P2Y_4$, were found in the neuroependymal lining (Fig. 7). $P2X_5$ immunoreactivity was identified in the neuroependymal cells from P0 (Fig. 7B). At this age the immunoreactivity appeared to be localized to the CSF-surface cell membrane, which is in contrast to cytoplasmic staining seen in at P9 (Fig. 7E) and in the adult (Fig. 7H). $P2Y_6$ immunoreactivity was detected in the neuroependyma from P9 (Fig. 7F), but not at P9 (Fig. 7C). The immunoreactivity had a punctate appearance localized to the lateral cell membranes (Fig. 7F), perhaps associated with intercellular junctions (see Discussion). The distribution pattern at P9 (Fig. 7F) and adult (Fig. 7I) was similar.

Discussion

CSF secretory mechanisms in the embryonic choroid plexus

It is clear from the rapid expansion of the ventricular system during embryonic stages of brain development (Johansson *et al.*, 2006) that effective CSF secretory mechanisms are already present in the early embryo. Actual measurements of CSF secretion have been obtained as early as 60 days gestation in the sheep fetus (term 150 days, Evans *et al.*, 1974) and postnatal day 3 in rats (Bass & Lundborg, 1973; Woodbury *et al.*, 1974). Extrapolation of estimated secretion rates in rats into the fetal period suggests that secretion starts around 18 days gestation. The lateral ventricles in the rat embryo increase rapidly

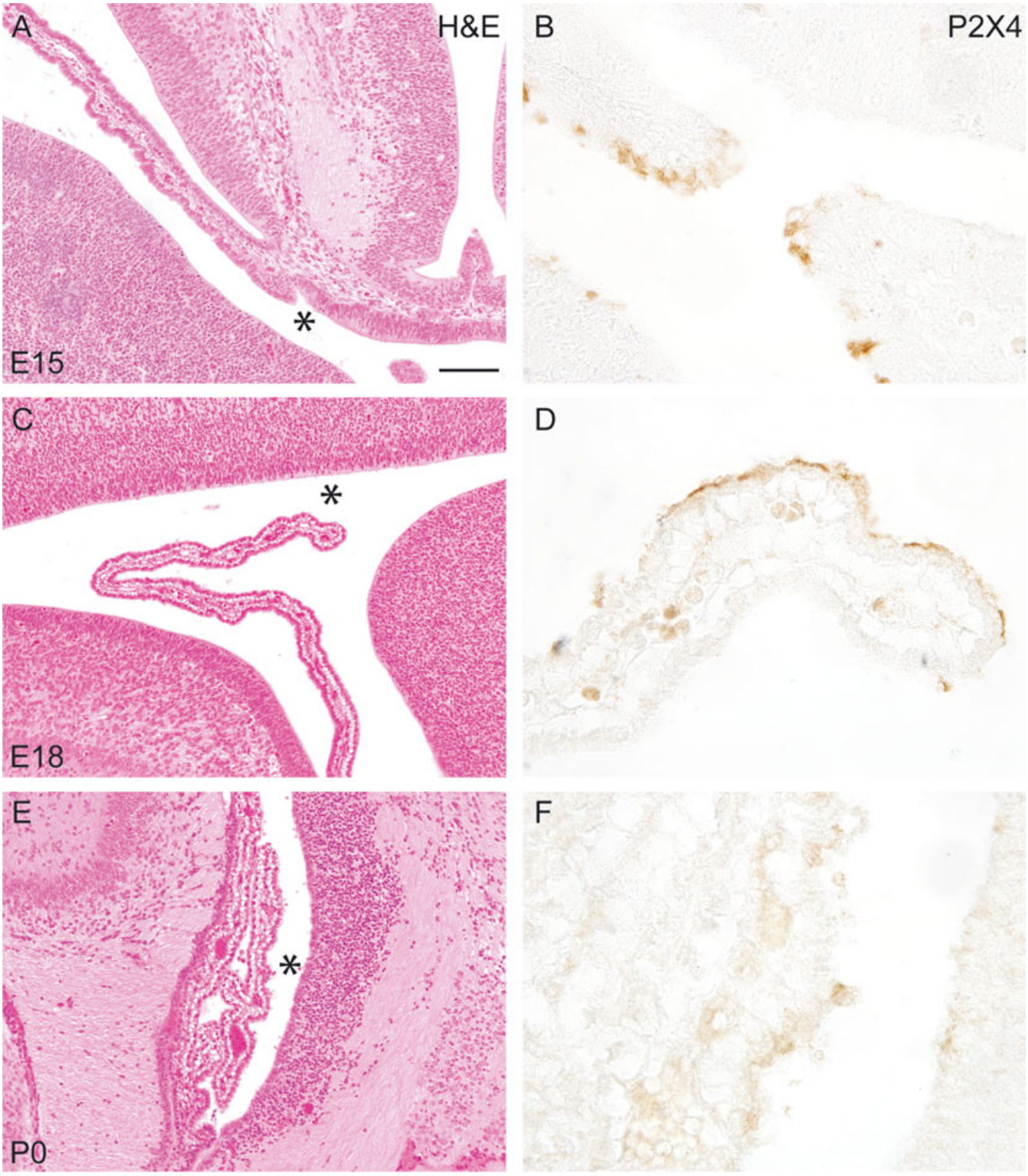

FIG. 2. $P2X_4$ immunoreactivity in the lateral ventricular choroid plexus of the developing rat. (A, C and E) Haematoxylin and eosin (H&E) and (B, D and F) $P2X_4$ immunoreactivity sections counterstained with toluidine blue. $P2X_4$ immunoreactivity on choroidal epithelial cells at E15 (A and B), E18 (C and D) and P0 (E and F). The immunoreaction product was distributed in the apical (CSF) surface of the cells at E15 and E18, but more generally throughout the cytoplasm at P0. Asterisk on H&E sections denotes the approximate location of the choroid plexus in a nearby section shown in the $P2X_4$ panel at higher magnification. Scale bar, 100 μm (A, C and E) and 20 μm (B, D and F).

between E14 and E16, more than doubling in volume; however, the actual volume increase is only approximately 1 μL, which would not be measurable by currently available dilution techniques, even if feasible in such small embryos. It has been suggested that an important part of the mechanism driving water entry into the lateral ventricles at this early stage of brain development is the high concentration of

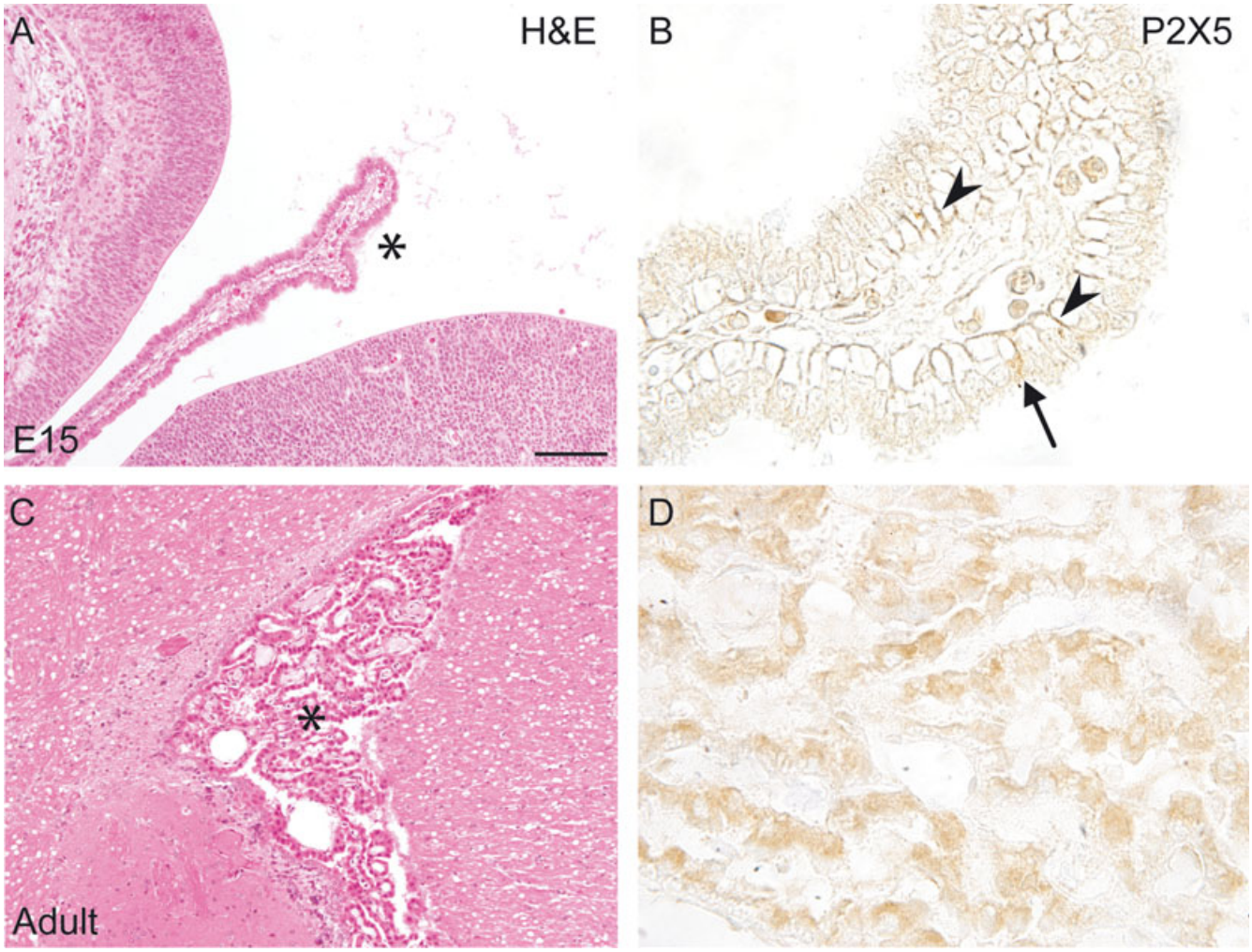

FIG. 3. $P2X_5$ immunoreactivity in the lateral ventricular choroid plexus of the developing rat. (A and C) Haematoxylin and eosin (H&E) and (B and D) $P2X_5$ immunoreactivity sections counterstained with toluidine blue. $P2X_5$ immunoreactivity in choroidal epithelial cells at E15 (B, arrow) and in adult (D). It appeared to be distributed in the cytoplasm at both ages, but at E15, because of the presence of a large amount of glycogen within the cells, the immunoreactivity was confined to a rim of strong staining around outer borders of the cell (arrowheads). Asterisk on A and C sections denotes the approximate location of the choroid plexus in a nearby section shown in B and D at higher magnification. Scale bar, 100 μm (A and C) and 20 μm (B and D).

proteins in CSF in the embryonic brain, with water entry through abundant aquaporin-1 channels in the apical membrane of the choroid plexus epithelial cells (Johansson *et al.*, 2005, 2006). Although aquaporin-1 was originally thought to be a pure water channel recent evidence show that aquaporin-1 also appear to mediate a cGMP-gated cationic current (Na^+, K^+ and Cs^+, Boassa & Yool 2002; Boassa *et al.*, 2006). This presents one possible mechanism of cation transfer across the choroid plexus from blood to CSF during development, but it is not known if this current would be sufficient to drive CSF secretion. The results from the present paper showing very early expression and immunohistochemical distribution of several P2 receptors, as early as E15 (Tables 2 and 3 and see below) may indicate that they are important for this early stage of CSF secretion.

P2 receptors in the developing choroid plexus

In the present study, mRNA of all P2X and P2Y receptors investigated was expressed in the adult lateral ventricular choroid plexus. This is the first time the expression of P2Y1,2, 6, 12, 13 and 14 receptors in the choroid plexus has been described. Expression and distribution of P2X receptors in the adult has been studied previously (Xiang & Burnstock, 2005). All of these receptors, except $P2X_7$, were expressed from the earliest time investigated (E15) which is only one day after the first appearance of differentiated choroid plexus epithelial cells in the lateral ventricles (Johansson *et al.*, 2005). However, the pattern of expression of different mRNAs was not uniform during development (Table 2). The level of expression of three P2 receptor mRNAs ($P2X_1$, $P2X_2$ and $P2X_4$) appeared to decrease during development, the expression of two subtypes increased between E15 and adult ($P2X_7$ and $P2Y_2$) and three of the 14 subtypes ($P2X_6$, $P2Y_{12}$ and $P2Y_{13}$) remained at similar expression levels throughout development. The expression pattern for the remaining six receptor subtypes ($P2X_3$, $P2X_5$, $P2Y_1$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$) showed a transient peak in expression at either E18 or P0. The visual comparison of band intensity between different developmental ages normalized to amount of total RNA was used in the present study as a crude guide to mRNA levels at different ages. However, as this approach is only semiquantitative some of the changes in expression levels could also be due to methodological variations, although given the consistency of band intensity between the different pooled samples ($n = 3$ pooled samples) at each age, this seems unlikely.

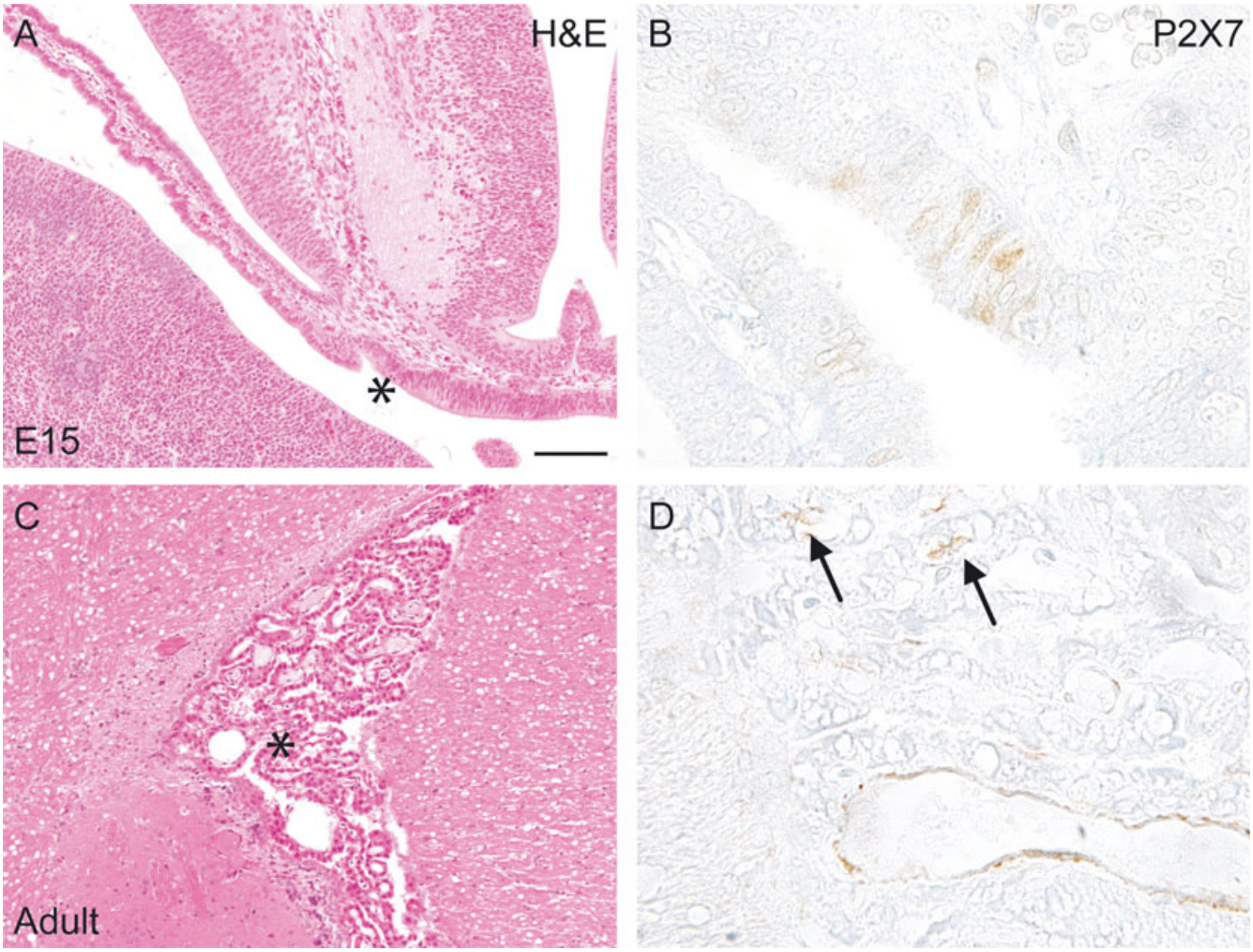

FIG. 4. $P2X_7$ immunoreactivity in the lateral ventricular choroid plexus of the developing rat. (A and C) haematoxylin and eosin (H&E) and (B and D) $P2X_7$ immunoreactivity sections counterstained with toluidine blue. A and B are E15, and C and D are adult. At E15 the stain appeared cytoplasmic but was only present at the very root of the plexus. In the adult the immunoreactivity was present in only a few epithelial cells (arrow) but also in endothelial cells of the choroid plexus blood vessels. Asterisk on A and C sections denotes the approximate location of the choroid plexus in a nearby section shown in B and D at higher magnification. Scale bar, 100 μm (A and C) and 20 μm (B and D).

In the present study the expression of $P2X_3$ mRNA was detected in all samples from E15 onwards, whereas in a previous study of the adult plexus, neither mRNA nor its protein were found in the adult plexus (Xiang & Burnstock, 2005).

Using immunohistochemistry, the P2X receptor proteins ($P2X_{4,5,7}$) as well as two of the three investigated P2Y receptors proteins ($P2Y_{1,4}$) were found in choroid plexus epithelial cells. The presence of P2 receptor proteins in the choroid plexus correlated well with the expression of their mRNAs at the different ages. Both mRNA and protein of $P2X_4$ and $P2X_5$ were detected at E15, the earliest day examined. The number of positive cells increased with age. $P2X_7$ protein, but not its mRNA was found at E15. However, at this age the $P2X_7$ protein was only detected in the root of the choroid plexus (Fig. 4B), which was not included in the sample dissected for RT-PCR to avoid contamination from neuroependymal cells and surrounding brain tissue, which could explain the absence of mRNA at this age.

In the present study, $P2Y_1$ receptor mRNA was detected at E15 whereas the protein was not detected until the next investigated age, E18. At this age, the largest number of positive epithelial cells was found. At E18 and onwards the appearance of the $P2Y_1$ immunostaining was diffuse cytoplasmic. Both $P2Y_4$ mRNA and protein were detected at E15 with an increase in both levels until P0 and then a decrease in the adult. The $P2Y_4$ reaction product was localized to the cytoplasm, showed a distinct punctate pattern and a concentration of immunopositive epithelial cells were seen at the root of the plexus during development. The $P2Y_6$ receptor protein was only detected in blood vessels endothelial cells in the adult choroid plexus stroma. In addition, $P2X_7$ and $P2Y_1$ receptor protein were found in adult blood vessels of the choroid plexus.

The localization of the $P2X_4$ receptor protein appeared to shift from apical at E15 to a diffuse cytoplasmic localization at E18. The reasons for this change could be a functional shift or it could be due to changes in receptor recycling and internalization, which have been demonstrated in both P2X (Bobanovic *et al*., 2002; Jensik & Cox, 2002; Feng *et al*., 2005) and P2Y (Sromek & Harden, 1998; Brinson & Harden, 2001; Tulapurkar *et al*., 2006) receptor subtypes. The internalization of the P2 receptors is also the most likely explanation for the cytoplasmic localization seen in the present and previous studies of purinergic receptor localization. However, electron microscopy would be needed

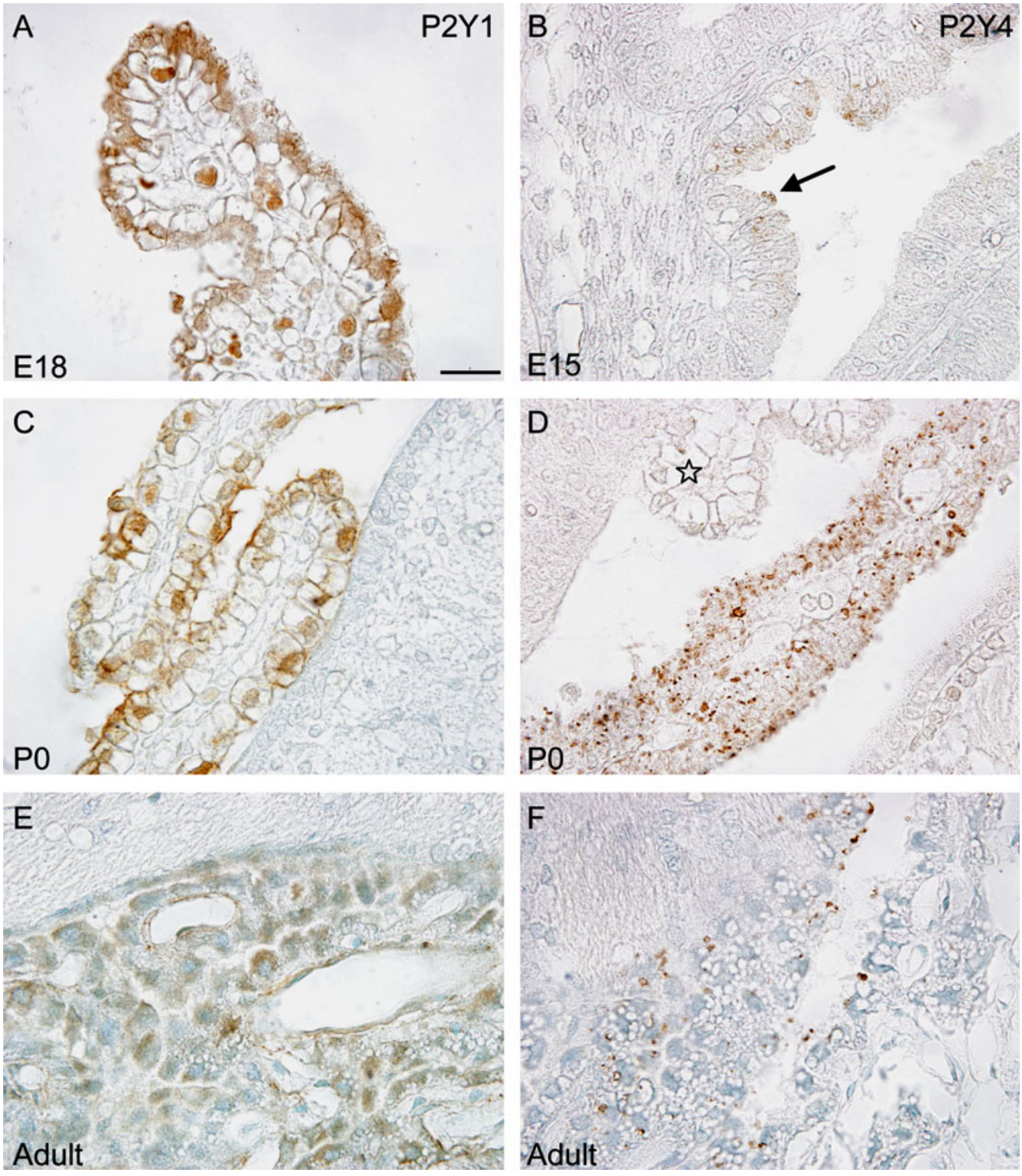

FIG. 5. $P2Y_1$ and $P2Y_4$ immunoreactivity in the lateral ventricular choroid plexus of the developing rat. $P2Y_1$ immunoreactivity (A, C and E) was first detected in the choroid plexus at E18 (A), and was also present at P0 (C) and in the adult (E). The immunoreactivity appeared cytoplasmic at all ages. In the adult, endothelial cells in the stromal blood vessels were also positive. $P2Y_4$ immunoreactivity (B, D and F) was present at all ages examined from E15 to adult. At E15 only a few cells at the root of the choroid plexus were positive (arrow, B). At P0 (D), the number of positive cells had greatly increased and appeared to be localized primarily at the root of the plexus. Note the tip of the plexus, folded down next to the root (star) does not show any immunoreactivity. In the adult (F) the staining was still punctate but in fewer cells that were sparsely distributed along the length of the plexus. Counterstained with toluidine blue. Scale bar, 100 μm.

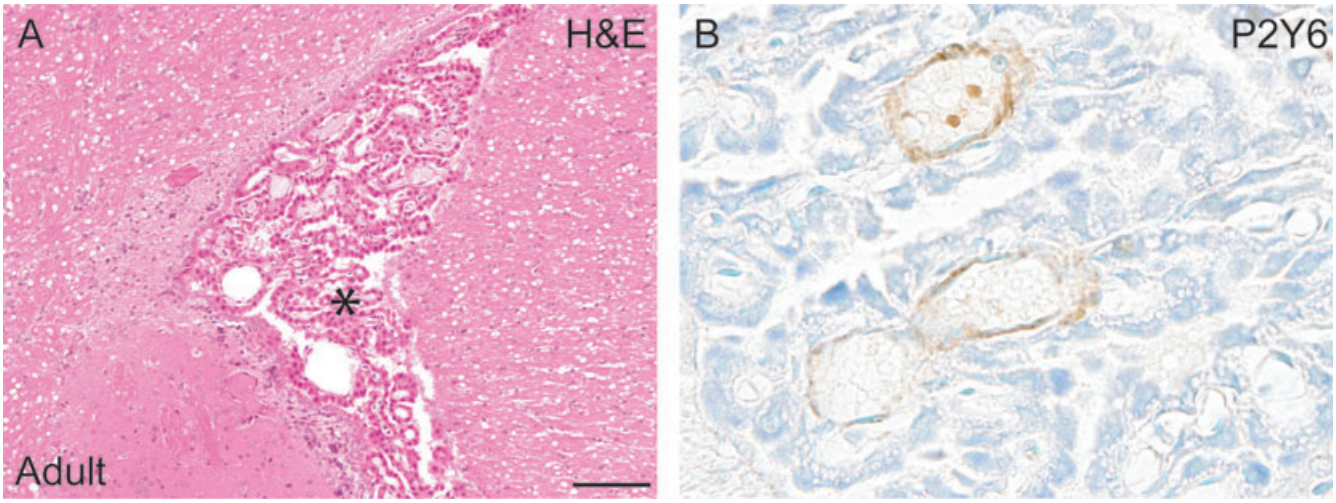

FIG. 6. $P2Y_6$ immunoreactivity in the lateral ventricular choroid plexus of adult rat. (A) Haematoxylin and eosin (H&E) and (B) $P2Y_6$ immunoreactivity section counterstained with toluidine blue. In the adult, $P2Y_6$ immunoreactivity was present in endothelial cells of the choroid plexus blood vessels (B). Weak endothelial staining was sometimes also seen at other ages (not illustrated). Epithelial immunoreactivity was not seen. Asterisk on H&E section denotes the approximate location of the choroid plexus in a nearby section shown in B at higher magnification. Scale bar, 100 μm (A) and 20 μm (B).

to determine the subcellular localization of the P2 receptors in the choroid plexus epithelial cells.

P2 receptors during development of other organs

The ontogeny, and especially the postnatal profile of P2 receptors have been investigated in many organs and structures (Burnstock, 2001). In most systems there appears to be a decrease in expression or signalling during the postnatal period. In one study that investigated the presence of $P2X_3$ in the central nervous system during rat development, the receptor was present from as early as E11 in the neural tube (Cheung & Burnstock, 2002). The relative amount of positive P2X immunoreactivity was reported to increase during embryonic development and then decreased in the early postnatal period (Cheung & Burnstock, 2002). Similarly, in the superior cervical ganglia both the response to the $P2X_3$ receptor agonist and immunohistochemical staining for the receptor was much stronger in P1 compared to P17 rats (Dunn *et al*., 2005). In the rat tail artery, both the responses to agonists and immunohistochemical signals decreased with age (from 4 weeks postnatally) for $P2Y_1$, $P2Y_2$, $P2X_1$ and $P2X_4$ (Wallace *et al*., 2006).

Although a decrease in P2 receptor expression and signalling during development was observed in several studies, other developmental patterns have also been reported. For some P2 receptors (e.g. $P2X_2$ in superior cervical ganglia Dunn *et al*., 2005 or $P2Y_4$ in the rat tail artery Wallace *et al*., 2006), expression remained similar throughout development. Other receptors such as $P2X_1$, $P2X_2$ and $P2Y_4$ in the vascular and longitudinal muscles of mouse ileum and colon (Giaroni *et al*., 2006) increased during development. All these different developmental patterns were observed in the choroid plexus in the present study, confirming the complexity of P2 receptor signalling system even within the same structure. Investigation of the developmental profile is further complicated by possible changes in the composition of the hetero-multimers that certain nucleotide receptors tend to form (Dunn *et al*., 2005). In a recent paper (Giaroni *et al*., 2006) it was found that in the developing mouse gastrointestinal tract the response to $P2Y_1$ receptor stimulation changed from contraction to relaxation, a finding that further complicates interpretation of functional aspects of P2 receptors during development. However, the results from the present study do suggest specific roles for different subtypes in the complex series of events occurring during development.

P2 receptors, epithelial transport and implications for CSF secretion

The P2 receptors have been found to influence epithelial transport in several other epithelia (Dubyak, 2003; Leipziger, 2003). The $P2Y_{2,4,6}$ receptors have been found to increase Cl^- secretion in many epithelia such as the rat pancreatic duct, bile duct and collecting ducts by activation of different Cl^- channels such as calcium independent outwardly rectifying Cl^- channels, the cystic fibrosis transmembrane conductance regulator as well as calcium dependent Cl^- channels (Hwang *et al*., 1996; Zeng *et al*., 1997; Leipziger, 2003). $P2Y_2$ receptors have been found to both increase K^+ secretion and decrease Na^+ absorption in the colon (Kerstan *et al*., 1998). In rat isolated bile duct, increased HCO_3^- secretion and a subsequent increase in pH was seen in response to nucleotide stimulation (Dranoff *et al*., 2001). The movements of Na^+, K^+, Cl^- and HCO_3^- ions are crucial for CSF secretion (e.g. Brown *et al*., 2005) and may be regulated by nucleotide receptors in the epithelial cells. In the present study $P2Y_1$ and $P2Y_4$, but not $P2Y_6$, were found in the early developing choroidal epithelium, further indicating their role in CSF secretion. Similarly, the P2X receptors have been shown to influence ion currents in other epithelia. An increase in chloride ion current has been shown in both rat salivary gland duct (Lee *et al*., 1997; Turner *et al*., 1998) and in mouse trachea (Taylor *et al*., 1999) in response to $P2X_7$ and $P2X_{4/5}$ receptor activation, respectively. The effect of specific P2 receptor agonists and antagonists on CSF secretion has not yet been investigated in either adult or during development. However, the appearance or up-regulation of the receptors at different developmental stages seems to indicate that they play roles in specific aspects of development of both CSF secretion and CSF flow. The first of these events is the appearance of the choroid plexuses, which in the lateral ventricles occurs around E14 (Johansson *et al*., 2005). During the first few days after the appearance of the choroid plexuses, the fluid in the

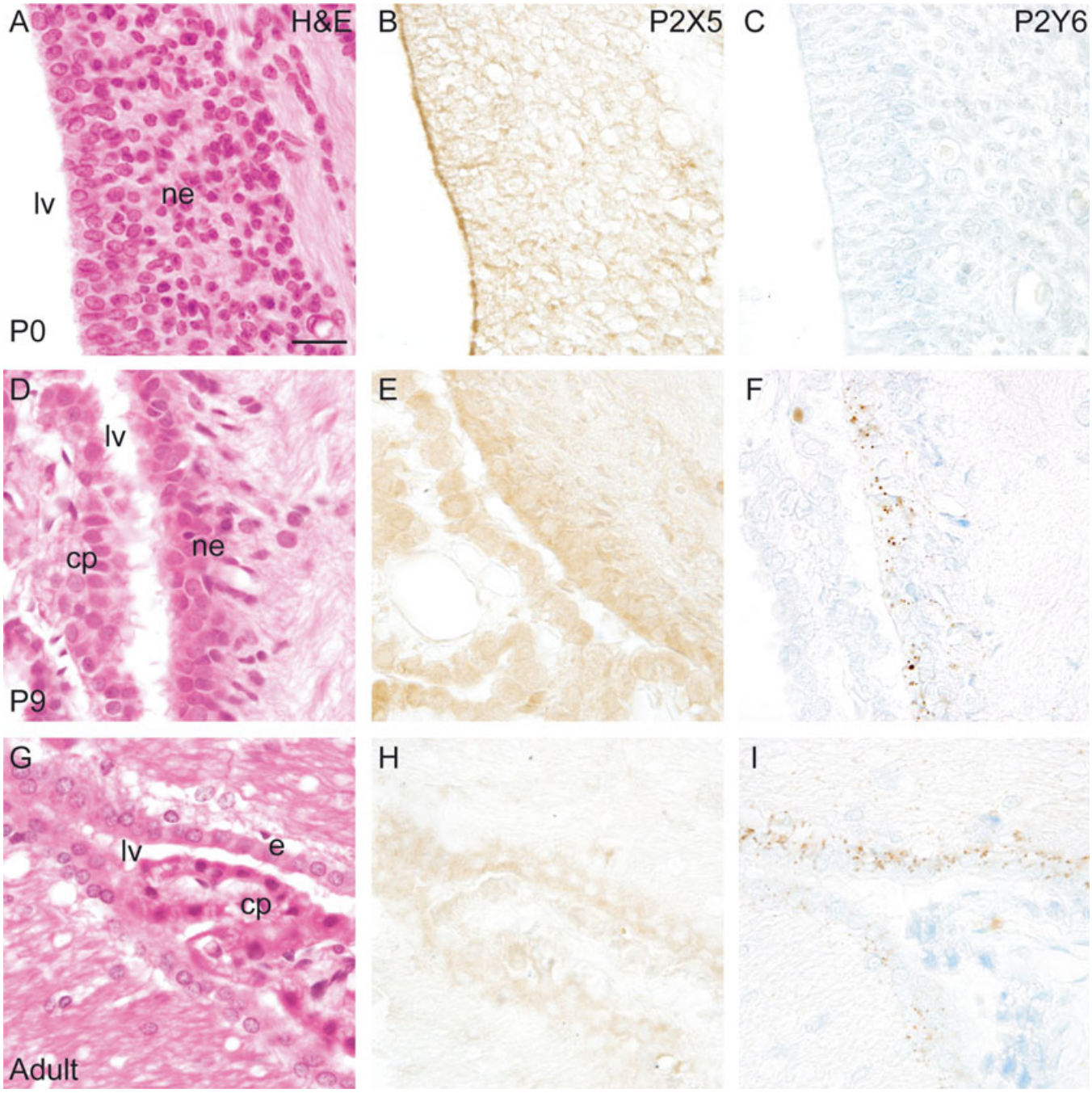

FIG. 7. $P2X_5$ and $P2Y_6$ immunoreactivity in the developing neuroependyma. At P0 (A) the developing neuroependyma appears pseudostratified; at P9 (D) the neuroependyma still appears pseudostratified but is less deep and in the adult (G) the ependyma is a single cell layer lining the ventricles. $P2X_5$ immunoreactivity (B, E and H) appeared in the developing neuroependyma at P0 (B) and was present at both P9 (E) and in adult (H). Note that the localization appears to change from the apical membrane at P0 to cytoplasm at P9 and in adult. $P2Y_6$ immunoreactivity (C, F and I) was not detected at P0 but was detected at P9 (F) and in the adult (I). The immunoreactivity exhibited a punctate distribution in the lateral cell membrane, possibly associated with intercellular junctions. Note the absence of $P2Y_6$ staining in the adjacent choroid plexus and brain parenchyma. Lv, lateral ventricle, ne, neuroependyma; e, ependyma; cp, choroid plexus. $P2X_5$ and $P2Y_6$ sections counterstained with toluidine blue. Scale bar, 20 μm.

cerebral ventricles is enclosed and does not, as in the adult, communicate with the fluid in the subarachnoid spaces. During this period the ventricles expand, doubling their volume between E14 and E16, after which the ventricles decrease in size in the rat (Johansson *et al.*, 2006). Ventricular expansion appears to be driven by the increase in pressure caused by the secretion of fluid into the enclosed space and is necessary for normal brain development (Desmond & Jacobson, 1977; Van Essen, 1997). In the present study $P2X_4$, $P2X_5$, $P2X_7$ and $P2Y_4$ were found in the choroid plexus during this period and could therefore be implicated in early fluid secretion and the expansion of the ventricles. Around E17–E18, pores are formed in the roof of the fourth ventricle rendering the fluid in the ventricles and the subarachnoid spaces continuous (Jones, 1980; Jones & Sellars, 1982). In the present study $P2Y_1$ was present

for the first time at this developmental stage and the number of cells positive for $P2X_4$, $P2X_5$ and $P2Y_4$ had greatly increased, compared to E15. This suggests an important role for nucleotide receptors in the regulation and secretion of CSF. Immunoreactivity for $P2X_5$, $P2X_7$ and $P2Y_4$ appeared to be preferentially located at the root of the plexus from where it grows. Thus the preferential location of $P2X_5$, $P2X_7$ and $P2Y_4$ may reflect a role for these receptors in the growth and differentiation of the lateral ventricular choroid plexus.

Source of ATP in the choroid plexus

The source of ATP to activate the P2 receptors in the choroid plexus in not yet known. There are three main possible sources of nucleotides: the epithelial cells, the endothelial cells or through cotransmission by neurons innervating the choroid plexus, all of which have been demonstrated in other organs. Release from both epithelial and endothelial cells has been repeatedly demonstrated both *in vivo* and *in vitro* (Bodin & Burnstock, 2001; Lazarowski *et al*., 2003; Schwiebert & Zsembery, 2003). In one study, using cultured rat cholangiocytes, ATP was found to be released across both the apical and basolateral membranes (Salter *et al*., 2000). This is supported by the finding of ATP in CSF from adult guinea-pigs (Munoz *et al*., 1995) and dogs (Yin *et al*., 2002). As ATP is rapidly degraded by ecto-nucleotidases, its presence in CSF indicates local release directly into the ventricles from either the epithelial cells or the neuroependymal cells lining the ventricles, rather than from more distant release in the brain parenchyma. To our knowledge, no measurements of ATP concentrations in CSF during development have been published. The choroid plexus is innervated by both adrenergic and cholinergic neurons (Lindvall & Owman, 1981). Co-transmission of ATP in both types of neuron has been well documented in the central and peripheral nervous systems (e.g. Burnstock, 2003) and may be a source of ATP in the choroid plexus. However, in a study investigating noradrenergic innervation in the developing rabbit choroid plexus, adrenergic nerves were not seen until after birth (Lindvall & Owman, 1978).

P2 receptors in the neuroependymal cells lining the ventricles

Unlike the adult brain, the CSF–brain interface in the embryo is impermeable to proteins and possibly also small molecules (Saunders, 1992; Saunders *et al*., 1999). The barrier consists of specialized junctions, 'strap junctions', which are present between the neuroependymal cells and disappears during development being replaced with gap junctions as seen in the adult (Møllgård *et al*., 1987; Monroe & Holmes, 1982). The CSF–brain barrier has mostly been characterized in the sheep and is found to restrict the passage of large molecules such as protein from the CSF to the brain and is also thought to stabilize the ventricles during a period of rapid cellular growth, differentiation and migration (Dziegielewska *et al*., 1979; Fossan *et al*., 1985; Møllgård *et al*., 1987). Junctions similar to those described in sheep were also found in newborn rats at P0 but not at P10 (Monroe & Holmes, 1982). In the present study $P2Y_6$ was not present between the neuroependymal cells until P9 (Fig. 7B) when it appeared as a punctate noncontinuous deposit between cells. The appearance of this staining pattern also coincides with the appearance of the gap-junctional protein connexin-43, found to be present in the neuroependyma at P5 but not at P0 (Yamamoto *et al*., 1992). The time of appearance and localization of P2Y6 in the developing neuroependyma suggest a possible link between $P2Y_6$ and gap junctions not previously described.

Conclusions

The mechanism underlying the rapid expansion of the lateral ventricles in the rat embryo between E14 and E16 (Johansson *et al*., 2006) is unknown. The presence of P2 receptors in the lateral ventricular choroid plexuses only one day after the first appearance of differentiated plexus epithelial cells strongly suggests that they contribute to CSF secretion, which is thought to be involved in the expansion of the embryonic ventricular system. This further supports previous work showing that the choroid plexuses are functional from their earliest appearance and are important for normal brain development. The transient peaks in expression and immunoreactivity during development of the choroid plexus of some P2 receptors subtypes, may indicate roles in major events such as the start of CSF secretion, expansion of the ventricles and onset of CSF flow from the ventricles into the subarachnoid spaces. The down-regulation of most of the P2 receptors subtypes seen in the postnatal period further reinforces the suggestion that purinergic and pyrimidinergic signalling have significant transient roles during development.

Acknowledgements

K.M.D. was supported by NIH grant RO1NS043949-01A1.

Abbreviations

CSF, cerebrospinal fluid; E, embryonic day; P, postnatal day.

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