*Neuroscience* 133 (2005) 937–945

# EXPRESSION OF P2X PURINOCEPTORS DURING RAT BRAIN DEVELOPMENT AND THEIR INHIBITORY ROLE ON MOTOR AXON OUTGROWTH IN NEURAL TUBE EXPLANT CULTURES

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**Abstract—Extracellular ATP is well known as a neurotransmitter and neuromodulator in the CNS of adults. However, little is known about the involvement of ATP during the development of mammalian brain. In the present study, we have examined the expression pattern of P2X receptor subtype mRNA and protein during perinatal rat brain development (from embryonic day (E) 10 to postnatal day (P) 16 brain). While P2X$_3$ receptors appeared early at E11, they declined in the stages that follow. P2X$_2$ and P2X$_7$ receptors were expressed from E14 onwards, while P2X$_4$, P2X$_5$ and P2X$_6$ receptors were expressed from P1 onwards. P2X$_1$ receptor expression was not observed in any of the developmental ages examined. We investigated the effect of 100 μM ATP and α,β-methylene ATP (α,β-meATP; selective agonist for P2X$_1$, P2X$_{2/3}$ and P2X$_3$ receptors) on motor axon outgrowth in collagen-embedded neural tube explant cultures. Both ATP- and α,β-meATP-treated neural tubes showed a significant reduction in neurite outgrowth compared with the control explants. This inhibitory effect could not be reproduced by uridine triphosphate. In conclusion, all P2X receptor subtypes, except for P2X$_1$, were strongly represented in the developing rat brain. ATP was shown to inhibit motor axon outgrowth during early embryonic neurogenesis, most likely via the P2X$_3$ receptor. It is speculated that P2X$_7$ receptors may be involved in programmed cell death during embryogenesis and that P2X$_4$, P2X$_5$ and P2X$_6$ receptors might be involved in postnatal neurogenesis.** **© 2005 IBRO. Published by Elsevier Ltd. All rights reserved.**

**Key words: ATP, α,β-meATP, receptors, neurons, embryo, neurogenesis.**

ATP was first shown to be released from sensory nerves during antidromic stimulation in 1959 (Holton, 1959). However, it was not until Burnstock et al. (1970) found evidence for the role of ATP as a neurotransmitter in non-adrenergic, non-cholinergic (NANC) nerves supplying the gut and bladder that purinergic signaling was proposed (Burnstock, 1972). It is now known that ATP mediates a wide range of physiological activities by activating two types of receptors: ligand-gated P2X receptors and G protein-coupled P2Y receptors (Ralevic and Burnstock, 1998). To date, seven P2X receptor subunits (P2X$_{1–7}$) (North, 2002) and eight P2Y receptors (P2Y$_{1,2,4,6,11–14}$) have been cloned from mammals (Abbracchio et al., 2003; Burnstock, 2003a).

ATP is released from peripheral and central neurons, where it can act on P2X receptors to elicit fast excitatory neurotransmission (Burnstock, 2003b). Of the seven cloned P2X receptor subunits, P2X$_2$, P2X$_4$, and P2X$_6$ receptor transcripts and proteins are widely distributed in adult rat brains (Nörenberg and Illes, 2000). The P2X$_1$ receptor is localized in the adult cerebral cortex, striatum, hippocampus and cerebellum. The P2X$_5$ receptor shows restricted localization, whereas the mRNA transcript was only found in the mesencephalic trigeminal nucleus. The P2X$_3$ receptor is present in sensory-related areas such as nucleus tractus solitarius (Nörenberg and Illes, 2000). The P2X$_7$ receptor has been reported recently in the hippocampus and medulla oblongata (Deuchars et al., 2001; Sperlágh et al., 2002). Functional analysis showed that ATP acts both as a fast neurotransmitter and as a neuromodulator regulating the release of classical neurotransmitters such as glutamate and GABA (Labrakakis et al., 2000; D'Ambrosi et al., 2001; Sperlágh et al., 2002; see Burnstock, 2003b). Despite a detailed expression of P2X receptors studied in the adult brain, little is known about the expression pattern during neurogenesis.

We have previously identified P2X$_2$ and P2X$_3$ receptor expression in rat embryonic brain (Cheung and Burnstock, 2002). The P2X$_2$ receptor is first expressed weakly in the nucleus tractus solitarius at embryonic day (E) 14, whereas the P2X$_3$ receptor is expressed even earlier in a subpopulation of the neurons and its extended axons in the ventral hindbrain neural tube as early as E11. At E12, the axons expressing the P2X$_3$ receptor grew dorsally, to leave the hindbrain via the large dorsal exit point to trigeminal ganglia, suggesting that the P2X$_3$ receptor immunoreactive cells were branchiomotor (BM) or visceromotor (VM) neurons (reviewed by Jacob et al., 2001). However, expression of the P2X$_3$ receptor was downregulated during further development. According to the spatial and temporal information of P2X$_3$ receptor expression in the ventral neural tube, we propose that the receptor has a role to play in axon outgrowth during hindbrain development.

In the present study, we use reverse transcriptase–polymerase chain reaction (RT-PCR) and immunohistochemistry to examine the expression pattern of all the seven P2X receptor subunits during embryonic rat brain development from E14 to postnatal day (P) 16. In addition, we explore the role of ATP on axon outgrowth during

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*Abbreviations:* BM, branchiomotor; DAB, diaminobenzidine; E, embryonic day; α,β-meATP, α,β-methylene ATP; NCAM, neural cell adhesion molecule; P, postnatal day; RT-PCR, reverse transcriptase–polymerase chain reaction; UTP, uridine triphosphate; VM, visceromotor.

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doi:10.1016/j.neuroscience.2005.03.032

embryonic neurogenesis by culturing hindbrain neural tube explants containing motor neurons in collagen gels in the presence of ATP and its derivative α,β-methylene ATP (α,β-meATP), which is a stable and specific agonist for $P2X_3$ (and $P2X_1$) receptors.

# EXPERIMENTAL PROCEDURES

## Animals

Procedures and experiments involving animals and their care conformed to the UK Animals (Scientific Procedures) Act 1986 and associated guidelines on the ethical use of animals. Pregnant rats were killed by asphyxiation with a rising concentration of $CO_2$ and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering Schedule One procedures to minimise suffering. The day of identification of the presence of a vaginal plug was designated as day zero (E0). The minimum number of animals needed for this study was used.

## RT-PCR analysis

Embryonic and neonatal Sprague–Dawley rat brain tissues of different ages were dissected and the meninges were removed to prevent contamination by blood vessels or cells. Total RNA was extracted from whole brain tissues of four developmental ages (E14, E18, P1 and P16) using SV Total RNA Isolation system (Promega, Madison, WI, USA). A non-quantitative reverse transcription and cDNA amplification for all the P2X receptors was carried out with a thermal cycler (Hybaid, Witchford, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Amersham, UK). Every sample was further treated with Amplification Grade DNase I (Sigma Chemical Co., Poole, UK) and tested to confirm the absence of any residual DNA that may generate false positive results. Briefly, 1 μg of total RNA was reverse transcribed using the $pd(T)_{12-18}$ as the first-strand primer at 42 °C for 30 min and the enzyme was denatured at 95 °C for 5 min. The sequence specific primers (Life Technologies, NY, USA) for P2X receptors (Shibuya et al., 1999, see Table 1) were then added to the reaction mixtures and the PCR cycling parameters were 95 °C for 30 s, 58 °C for 1 min (58 °C for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$; 61 °C for $P2X_2$ and 64 °C for $P2X_6$), 72 °C for 1.5 min for 35 cycles, followed by a further cycle of 10-min extension at 72 °C. The resulting PCR products were resolved in a 2% agarose gel and observed under ultraviolet illumination. At least three separate RT-PCR experiments were performed for each P2X receptor on each individual embryonic tissue.

## Immunohistochemistry

Brains collected were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2) at 4 °C, cryoprotected, embedded in Tissue-Tek and kept at −80 °C until cryosectioning. Brains from postnatal animals were divided into at least three portions to improve fixative penetration. Frozen sections (12 μm) were cut in a cryostat and mounted for immunohistochemistry. In immunohistochemical experiments for P2X receptors using diaminobenzidine (DAB) as the chromogen, the procedures were performed according to the protocol previously described (Cheung and Burnstock, 2002). The primary antibodies used were rabbit polyclonal antibodies against $P2X_{1-6}$ receptors (gifts from Roche Palo Alto, CA, USA; 1:200) and the $P2X_7$ receptor (Alomone Laboratories, Jerusalem, Israel, 1:500). For control experiments, the sections were incubated with the primary antibodies pre-adsorbed with the control peptide antigens that were used in raising the antibodies or with normal horse serum only. Double labeling experiments for the $P2X_3$ receptor and the NCAM antibody (1:4000, Sigma) was performed as previously described (Cheung and Burnstock, 2002). All the fluorescence-labeled secondary antibodies were from Jackson ImmunoResearch Laboratory (Westgrove, PA, USA).

## Photomicroscopy

Images of DAB immunohistochemical staining were taken with a Leica DC 200 digital camera (Leica, Heerbrugg, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, USA).

## Neural tube explant culture

Rat embryos were obtained at E12. The hindbrain neural tube explants used were taken between rhombomere 1 (r1) to r4 axial levels (Caton et al., 2000). Motor neuron-containing explants were dissected using Dispase (Roche Pal Alto) and tungsten needles. The bilateral explants were cut open at the dorsal midline and flattened. Only the medial one-third of each side of the neural tube was used for culture to avoid contamination of the roof plate and dorsal neural tube. Tissues were washed in Hanks' Balanced Salt Solution (HBSS, Gibco, Paisley, UK) and kept on ice until needed. Rat tail collagen (Roche Palo Alto) was prepared and made into gels according to Guthrie and Lumsden (1994). Tissues were embedded into gels on four-well plates (Fisher Scientific, Loughborough, Leics, UK) and covered with high glucose-Dulbecco's modified Eagle medium (Gibco) with Glutamax I (Gibco) supple-

**Table 1.** P2X receptor primer sequences

| Receptor | Primer sequence (5′ to 3′) Shibuya et al. (1999) | Primer position | Product length (bp) |
|---|---|---|---|
| $P2X_1$ forward | GAAGTGTGATCTGGACTGGCACGT | 776–801 | 452 |
| $P2X_1$ reverse | GCGTCAAGTCCGGATCTCGACTAA | 1203–1231 | |
| $P2X_2$ forward | GAATCAGAGTGCAACCCCAA | 826–845 | 357 |
| $P2X_2$ reverse | TCACAGGCCATCTACTTGAG | 1183–1164 | |
| $P2X_3$ forward | TGGCGTTCTGGGTATTAAGATCGG | 708–731 | 440 |
| $P2X_3$ reverse | CAGTGGCCTGGTCACTGGCGA | 1126–1147 | |
| $P2X_4$ forward | GAGGCATCATGGGTATCCAGATCAAG | 749–774 | 447 |
| $P2X_4$ reverse | GAGCGGGGTGGAAATGTAACTTTAG | 1170–1195 | |
| $P2X_5$ forward | GCCGAAAGCTTCACCATTTCCATAA | 553–577 | 418 |
| $P2X_5$ reverse | CCTACGGCATCCGCTTTGATGTGATAG | 944–970 | |
| $P2X_6$ forward | AAAGACTGGTCAGTGTGTGGCGTTC | 444–468 | 520 |
| $P2X_6$ reverse | TGCCTGCCCAGTGACAAGAATGTCAA | 938–963 | |
| $P2X_7$ forward | GTGCCATTCTGACCAGGGTTGTATAAA | 384–410 | 354 |
| $P2X_7$ reverse | GCCACCTCTGTAAAGTTCTCTCCGATT | 711–737 | |

Table showing the primer sequences for P2X receptor in RT-PCR analysis.

mented with 1× antibiotic/antimycotic solution and insulin–transferrin–selenium supplements (Gibco). To examine the effects of extracellular nucleotides on the axon outgrowth, 100 μM of ATP, α,β-meATP or uridine triphosphate (UTP; Sigma) was added to the medium at the beginning of the culture period. The collagen-embedded explants were cultured for 4 days in a humidified atmosphere of 5% $CO_2$ at 37 °C. The culture medium was replaced every 2 days with fresh medium containing the same concentration of nucleotides.

### Immunostaining of collagen gels and quantitation of axon outgrowth

Collagen gels were fixed for immunostaining according to Guthrie and Lumsden (1994) using monoclonal antibody 2H3 (Developmental Studies Hybridoma Bank), which recognizes the 165 kDa neurofilament protein. Gels were mounted under propped coverslips in 90% glycerol/10% PBS and photographed. Images were imported into both Neurolucida (MicroBrightField Inc, Williston, VT, USA) for analysis of neurite length and an analytical package (Metamorphs, Universal Imaging Corporation, Downington, PA, USA) for comparison of the number of neurites projecting from the explants between treatment groups. A total of 50 longest neurites from each explant was taken for comparison of neurite length. The pixels occupied by immunostained neurites were taken as a representative of the number of neurites projecting from the explants for comparison. The pixels occupied by total neurites were then divided by the pixels occupied by the explants in order to determine the neurite/explant ratio. Neural tube explants together with the collagen gels were cryoembedded, sectioned and stained for neural cell adhesion molecule (NCAM) expression using monoclonal NCAM antibody (1:4000; Sigma).

## RESULTS

### Expression pattern of P2X receptors during rat brain development

In keeping with our previous report (Cheung and Burnstock, 2002), the $P2X_3$ receptor was the first purinoceptor to be expressed during neurogenesis. It was detected in the cranial motor neurons early at E11, whereas $P2X_2$ receptor protein was found in the nucleus tractus solitarius at E14. We examined the expression pattern of all the known P2X receptor subtypes during rat brain development using RT-PCR ($P2X_{1-7}$) and immunohistochemistry (for $P2X_{1,4-7}$). The mRNA transcripts of receptor subtypes $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_7$ were detected as early as E14 brain (Fig. 1) and they continued to be expressed, although to different extents, from this stage on to the postnatal period (E14–P16). $P2X_5$ and $P2X_6$ receptor transcripts were expressed weakly at E18 and P1, respectively. The $P2X_1$ receptor transcript was not detected in the brain at any of the stages examined, although in the positive control using uterine tissues, primers for the $P2X_1$ receptor showed strong positive signals (data not shown).

The $P2X_3$ receptor was expressed in neurons and outgrowing axons in the hindbrain neural tubes as early as in E11. Immunoreactivities of other P2X receptor subtypes were not observed at this early stage. The $P2X_2$ receptor was expressed in the nucleus tractus solitarius at E14, confirming the findings from our earlier study (Cheung and Burnstock, 2002). To obtain spatial information of expression of the P2X receptors, we examined the distribution of $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptor protein immunohistochemically during rat brain development. Consistent with the RT-PCR results, $P2X_7$ receptor immunoreactivity was observed at E14. Expression was localized in the lateral hypothalamus (Fig. 2) and the isthmus. At E18, the $P2X_7$ receptor was detected in the lateral olfactory tract and the optic tract, and widespread throughout the diencephalon (thalamus and lateral hypothalamus), cerebellum and brainstem (midbrain, pons and medulla) and this expression persisted after birth (data not shown). $P2X_4$ receptor immunoreactivity was not observed until P1, although its mRNA transcript was expressed early at E14. The $P2X_4$ receptor was expressed in distinct subpopulations of cells rather than the widespread expression seen in the adult (see review Nörenberg and Illes, 2000) (Fig. 3). $P2X_4$ receptor-immunopositive cells showed cytoplasmic staining and were located in the striatal subventricular zone, mesencephalic trigeminal nucleus, parabrachial nucleus, lateral deep cerebellar nucleus and cuneate nucleus (Fig. 3A–D). Likewise, both $P2X_5$ and $P2X_6$ receptor pro-

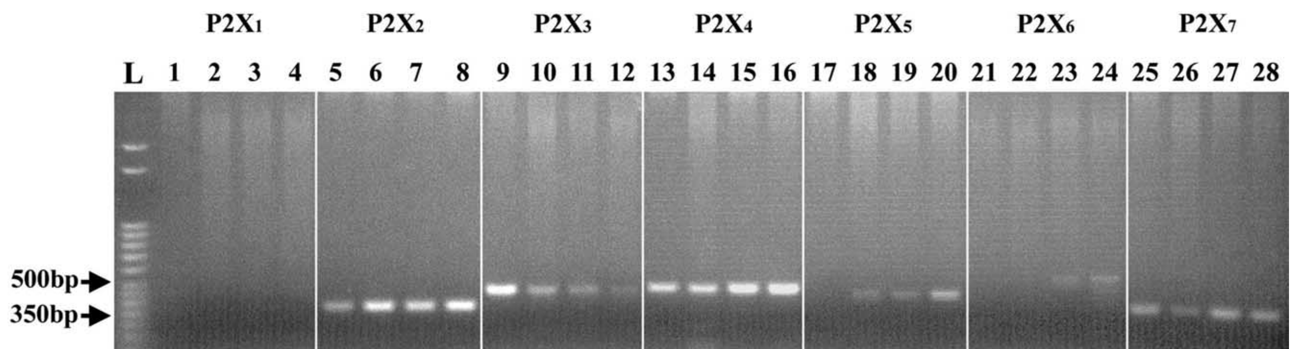


**Fig. 1.** RT-PCR analysis of P2X receptor mRNA expression during prenatal rat brain development. Total RNA from brain samples of four developmental ages (E14, E18, P1 and P16) was reverse-transcribed, and the resulting cDNA was PCR-amplified using gene-specific P2X receptor primers (see Experimental Procedure). Lane L represents 100 bp-DNA ladder. The amplified products are arranged in four lanes corresponding to RT-PCR products of E14, E18, P1 and P16 rat brains (from left to right) for each P2X receptor examined, i.e. $P2X_1$ (lanes 1–4); $P2X_2$ (lanes 5–8); $P2X_3$ (lanes 9–12); $P2X_4$ (lanes 13–16); $P2X_5$ (lanes 17–20); $P2X_6$ (lanes 21–24) and $P2X_7$ (lanes 25–28). The $P2X_1$ receptor primers that detect no expression in brain samples showed strong positive signals using rat uterus as a positive control (data not shown). The expression pattern shown here was confirmed in three independent experiments.

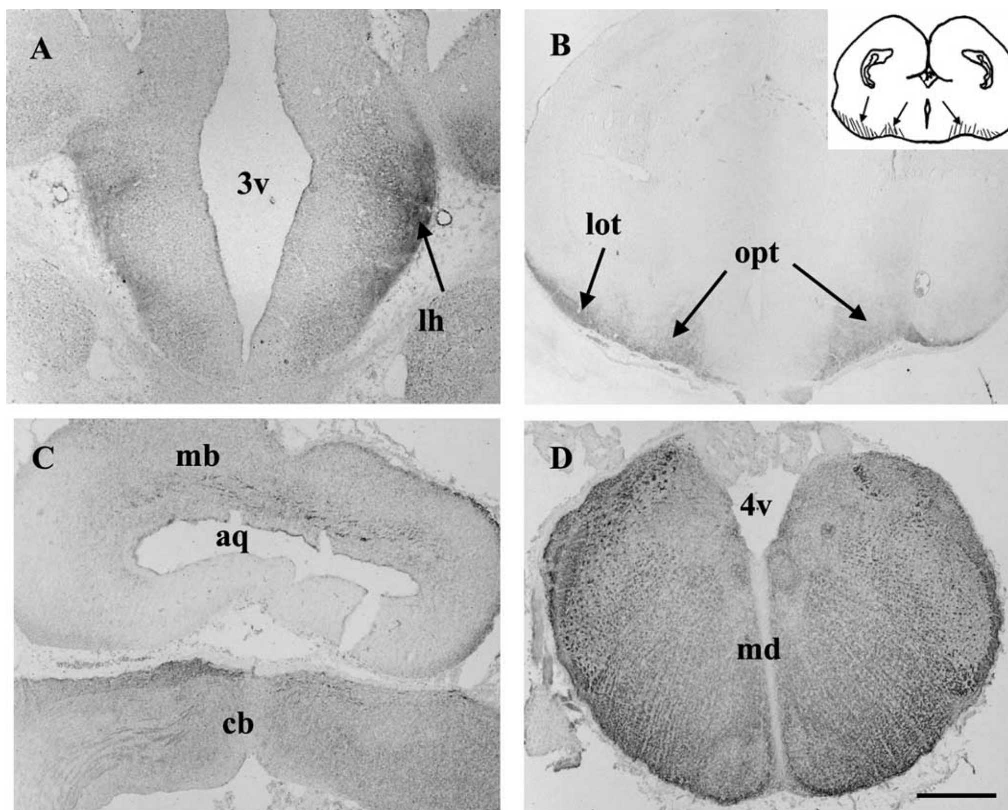


**Fig. 2.** Immunoreactivity of $P2X_7$ receptor protein in the developing brain. $P2X_7$ immunoreactivity was detected in: (A), the lateral hypothalamus (lh) at E14 (B), the lateral olfactory tract (lot) and optic tract (opt). Diagram inset in B outlines the region represented (arrows). (C) Cerebellum and midbrain (cb and mb) and (D) medulla (md) at E18. aq, aqueduct; 3v, third ventricle; 4v, fourth ventricle. Scale bar=250 μm (A, C, D) and 500 μm (B).

tein was detected in P1 brain. The $P2X_5$ receptor was expressed in the supraoptic nucleus, striatal subventricular zone and cuneate nucleus and in cells scattered over the cortex and diencephalons (Fig. 3E–G). Similar to $P2X_4$ receptor staining, the $P2X_5$-immunopositive cells in the supraoptic nucleus were spherical and showed cytoplasmic staining, whereas the scattered $P2X_5$-immunoreactive cells in the cortex and those in the striatal subventricular zone were pyramidal in shape (Fig. 3G). The $P2X_6$ receptor was, however, expressed weakly in the lateral hypothalamus (Fig. 3H). Specific immunoreactivity for the $P2X_1$ receptor was not detected at any of the stages examined.

Based on the timing of the initial appearance of the receptors, the sequential expression for P2X receptors during brain development is summarized in Fig. 4.

### Neural tube explant culture and axon outgrowth

$P2X_3$ immunoreactivity was also localized in the motor neurons and its outgrowing nerves in the hindbrain neural tube at E11–E12 (Fig. 5C). In order to investigate if ATP, the receptor ligand, also participated in motor axon outgrowth, the collagen-embedded cranial neural tubes containing motor neurons were treated with ATP and related compounds. In a control experiment where the collagen gel was bathed with medium only, motor axon outgrowth extended from the lateral sides as well as the rostral and caudal borders of the explants. The outgrowing motor axons were visualized with immunostaining using monoclonal 2H3 antibody. After staining, the axons appeared dark brown in color. In the ATP-treated group, the extent of the axon outgrowth was significantly reduced compared with the control group (Figs. 5, 6). There was a 46% reduction of neurite length in the ATP-treated group (Fig. 6A). Similarly, α,β-meATP, a stable analog of ATP and also the agonist for $P2X_3$ receptors, caused a 41% decrease in axon length (Fig. 6A). Application of UTP did not show a significant inhibitory effect on neurite outgrowth from neural tube explants compared with control explants, eliminating the possibility of $P2Y_2$ and $P2Y_4$ receptor involvement (Fig. 6A). We subsequently compared the area occupied by all the outgrowing neurites (excluding the neural tube explants) between treatment groups. Both ATP and α,β-meATP caused a reduction of 61% and 62%, respectively, of the total area occupied by the neurites (Fig. 6B). To minimize the variation in neurite outgrowth due to the difference in the sizes of the neural tube explants, the neurite-occupied areas were then divided by the area occupied by the explants. A reduction of 47% in total neurite area was observed in both ATP-treated groups and 46% in α,β-meATP-treated groups (Fig. 6C). ATP and α,β-meATP appeared to reduce the length and probably the number of extending neurites, too.

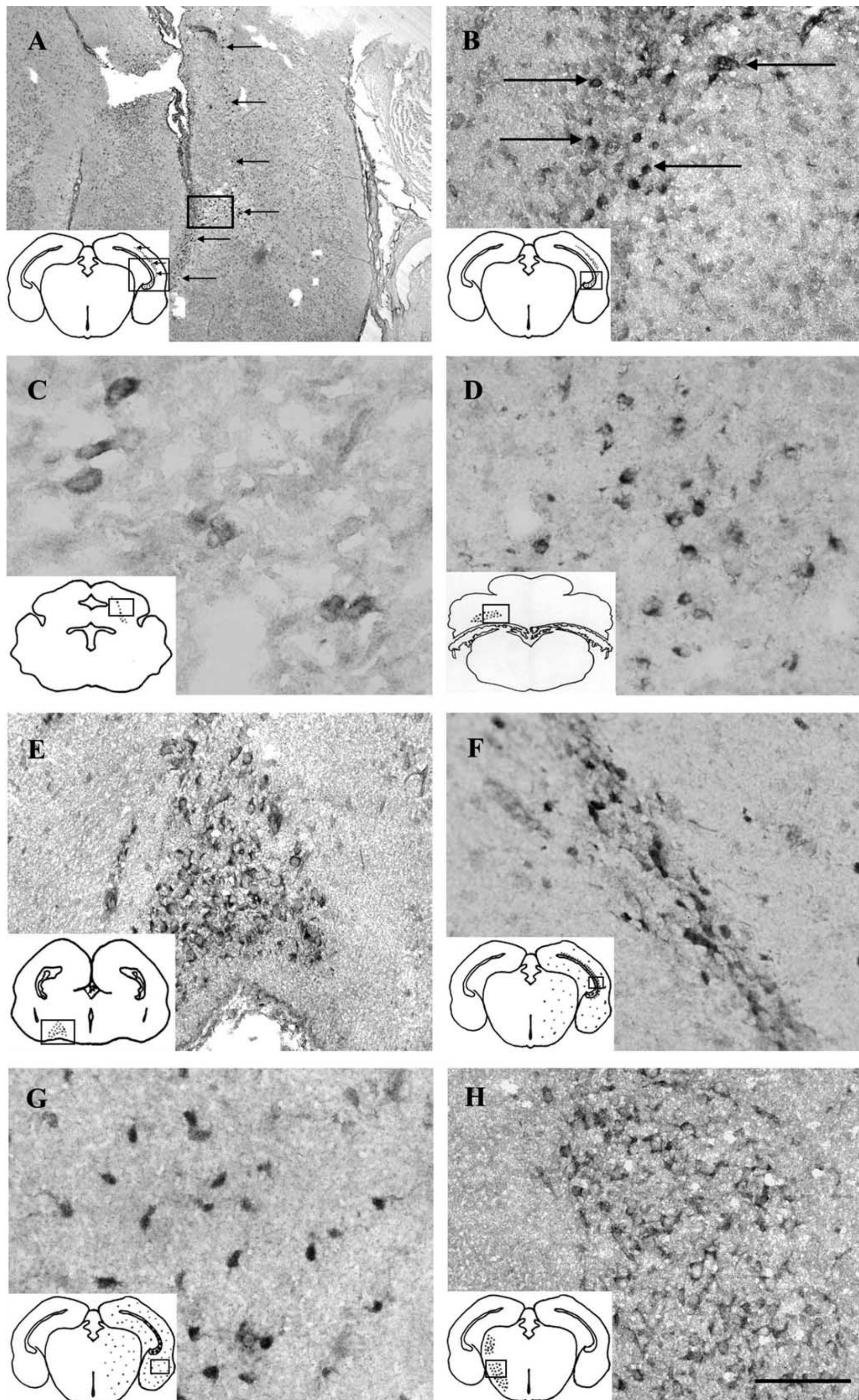


**Fig. 3.** Immunoreactivity of P2X receptors in neonatal (P1) brain. Rectangular boxes in the insets outline the regions shown in the corresponding pictures and the black dots in some of the inserts represent P2X immunoreactivity. (A) $P2X_4$ receptor-positive cells (arrows) were detected in regions along the striatal subventricular zone. (B) A high magnification of the rectangular box is shown in (A) with immunopositive cells indicated by arrows. (C) $P2X_4$ receptor immunoreactivity was also detected in mesencephalic trigeminal nucleus and (D) lateral deep nucleus of the cerebellum. (E) $P2X_5$ receptor expression was located in the supraoptic nucleus and (F) in cells along the striatal subventricular zone. (G) Scattered $P2X_5$ immunoreactive cells were also observed throughout the cortex (G). (H) $P2X_6$ receptor expression was detected in the lateral hypothalamus. Scale bar=750 $\mu$m (A), 100 $\mu$m (B, E, H) and 200 $\mu$m (C, D, F, G).

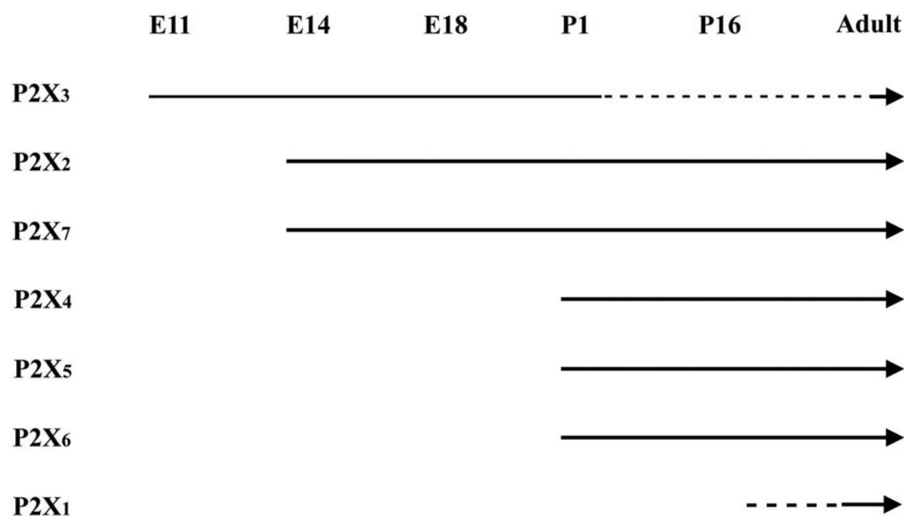


**Fig. 4.** Summary of the sequential expression of P2X receptors during rat neurogenesis. P2X receptors are arranged from top to bottom according to the chronological order of expression during rat brain development from E11 to Adult. While $P2X_3$ receptors appeared early, they declined in the stages that followed (represented by dotted line). $P2X_2$ and $P2X_7$ receptors were expressed from the same day (E14) onwards, while $P2X_4$, $P2X_5$ and $P2X_6$ receptors were expressed from P1 onwards. Initial dotted line for $P2X_1$ receptor represents unknown starting point, since expression of $P2X_1$ receptor was not observed in any of the developmental ages examined in this study.

The neural tube explants were stained with the $P2X_3$ receptor antibody and NCAM, a marker for neural cell adhesion and neurite outgrowth. Results of immunofluorescence showed that both $P2X_3$ and NCAM were expressed in the neurons as well as the outgrowing neurites (Fig. 5D–F). Double labeling experiments showed that colocalization of the $P2X_3$ receptor and NCAM was frequently seen in the outgrowing neurites, although neurites showing NCAM, but not $P2X_3$ receptor expression, were also observed (Fig. 5F).

## DISCUSSION

The idea of extracellular ATP acting as a neurotransmitter or neuromodulator has been examined extensively in both the CNS and peripheral nervous system in adult animals, and a large body of evidence shows that the ATP-induced fast synaptic response is mediated by activation of ATP-gated ion channels, the P2X receptors (Burnstock, 1972, 2003b; Burnstock and Kennedy, 1985; Nörenberg and Illes, 2000; North, 2002). The present study shows for the first time the developmental expression of six of the seven P2X receptor subtypes during perinatal brain development.

We have demonstrated that the neuronal $P2X_3$ and $P2X_2$ receptors are first expressed in the embryonic rat CNS at E11 and E14, respectively, confirming our earlier findings (Cheung and Burnstock, 2002). $P2X_7$ receptors in the embryonic brain were also expressed from E14 onwards. $P2X_7$ receptors have been shown to be involved in programmed cell death or to induce cytotoxicity at a high concentration of ATP released during inflammation or upon tissue trauma (Le Feuvre et al., 2002). Sperlágh et al. (2002) demonstrated that ATP regulates glutamate release via activation of the $P2X_7$ receptor, and excessive glutamate release altered $Ca^{2+}$ homeostasis and resulted in activation of the apoptosis-related gene, caspase (Le Feuvre et al., 2002). Since apoptosis is a common event in developing brain, it seems likely that activation of $P2X_7$ receptors is also involved in cell death during neurogenesis.

Although the expression of $P2X_4$, $P2X_5$ and $P2X_6$ receptors appeared late during development, $P2X_4$ and $P2X_5$ receptors were both expressed in the subventricular zone, the site of postnatal neurogenesis (see review by Conover and Allen, 2002). It has been claimed that astrocytes in the subventricular zone serve as neural stem cells (Doetsch et al., 1999) that give rise to both neurons and glia. An opposing report by Johansson et al. (1999), however, suggests that it is the ependymal cells adjacent to subventricular zone that generate multipotent neurospheres. It is as yet unknown whether the P2X receptor-positive cells present in the subventricular zone are neural stem cells or migrating neuroblasts. $P2X_4$ and $P2X_5$ may constitute two different types of cells in terms of the morphology and area of expression. $P2X_4$ receptor-expressing cells appeared round and resided in the subventricular zone, while cells positive for $P2X_5$ receptor looked pyramidal in shape and were scattered throughout different layers of the cortex as well as being located in the subventricular zone. An investigation of the identity of the $P2X_4$ and $P2X_5$ receptor-expressing cells in the subventricular zone is in progress. Little is known about the role of $P2X_6$ receptors in the neonatal hypothalamus, but the $P2X_6$ receptor has been implicated in hormone release from axon terminals as well as membrane recycling of the granular vesicles and microvesicles in the adult hypothalamo–neurohypophysial system (Loesch and Burnstock, 2001). It is therefore suggested that such expression may not relate to development. The $P2X_1$ receptor, according to the present results, was not expressed in any

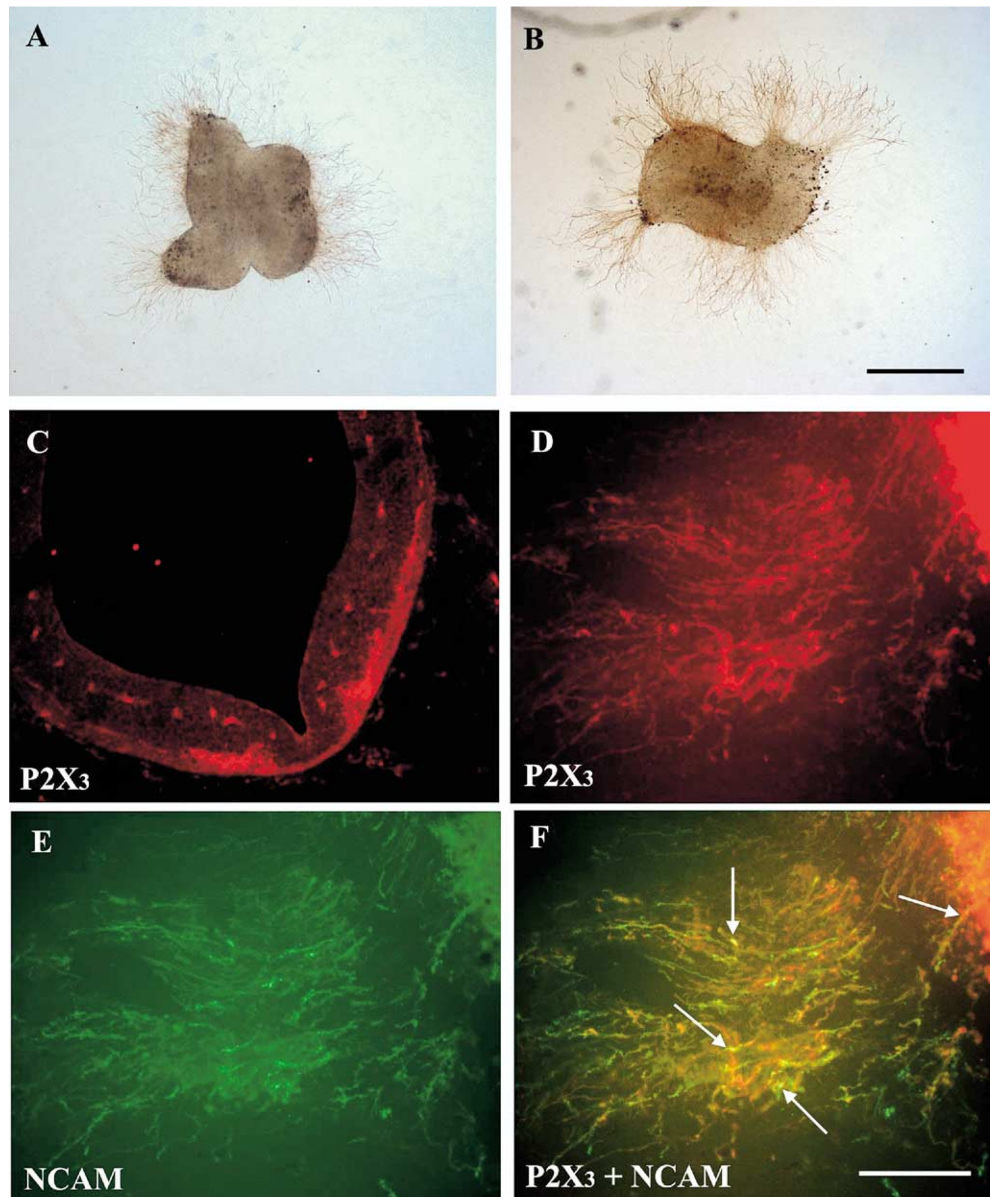


**Fig. 5.** Neural tube explants in collagen gel after treatment with 100 μM of (A) ATP and (B) control. (C) Cross-section from E12 hindbrain neural tube showing $P2X_3$ receptor immunoreactivities (red) in the motor neurons and the motor fibers in the ventral neural tube. (D, E) Neural tube explants are cryosectioned and immunostained with the $P2X_3$ receptor (red) and NCAM (green). (F) Image merged from D and E showing co-localization (yellow/orange) of $P2X_3$ and NCAM (arrows). Scale bar=500 μm (A, B) and 100 μm (C–F).

of the perinatal stages examined. However, previous studies have shown $P2X_1$ receptor expression in the adult cerebellum (Loesch and Burnstock, 1998) and $P2X_1$ receptor up-regulation in the CNS after injury (Franke et al., 2001; Florenzano et al., 2002). The presence of molecules that are involved in both embryonic neurogenesis and regeneration after injury is not unusual (Waschek, 2002). However, the possibility that ATP is involved in both processes is yet to be established (but see Volonté et al., 1999; Burnstock, 2003b; Viscomi et al., 2004).

Among all the P2X receptors examined, the homomeric $P2X_3$ receptor was the first to be expressed during neurogenesis in both CNS and peripheral nervous systems. $P2X_3$ immunoreactivity was detected in the cranial motor neurons as early as E11, by the time the neurons finish their final mitosis, exit the cell cycle, and start axon outgrowth (Ericson et al., 1992; Yamada et al., 1993). Based on the locations of immunoreactivities which are overlapping with dorsal axon trajectories and the dorsal migratory pathway of motor neurons from the ventral neural tube, it was suggested that the $P2X_3$ receptor-expressing cells are the BM and/or VM neurons, rather than somatomotor (SM) neurons (see review Jacob et al., 2001). Although the $P2X_3$ immunoreactivity in the spinal neural tube suggests that the VM neurons express the receptor, the intense staining in the hindbrain neural tube compared

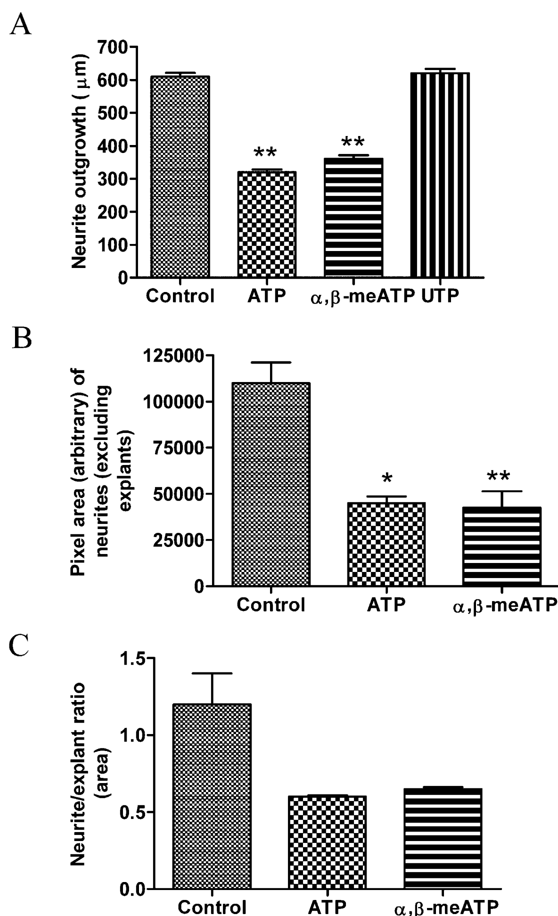


**Fig. 6.** Quantitation of neurite outgrowth from collagen embedded neural tube explant culture. Neural tube explants were bathed with 100 μM of extracellular nucleotides (ATP, α,β-meATP and UTP). Control explants were treated with medium only. Images of treated neural tube explants were imported into Neurolucida and neurite outgrowth was analyzed by measuring the 50 longest neurites from each explant. (A) Results expressed as the average length of neurites counted with error bar representing the standard error. (B) Images of treated neural tube explants were imported into Metamorph and the total pixel area (arbitrary units) of the neurites (excluding the explants) was computed (B). (C) To minimize the variations of the neurite outgrowth due to the differences in individual explant size, the total pixel area occupied by the neurites (excluding the explants) was divided by the total pixel area occupied by the corresponding explants and the ratios were compared between groups. Significant difference is represented by ** ($P<0.001$) or * ($P<0.05$) analyzed by unpaired *t*-test.

with the weak staining in the spinal neural tube suggests that the BM neurons also express the receptor.

We have shown that both ATP and α,β-meATP significantly reduced neurite extension from motor neuron-containing neural tube explants at E12. ATP is a non-selective agonist for most of the P2X and P2Y receptor subtypes. Although it is possible that P1 adenosine receptors may also be involved after adenosine is produced following ectoenzymatic breakdown of ATP, adenosine receptors were not included in our present study. Instead, α,β-meATP, a stable analog of ATP, was used to exclude any possible P1 receptor involvement, since it is a selective agonist for $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors. Since neither RT-PCR nor the immunohistochemical data showed any $P2X_1$ receptor expression in embryonic and postnatal brains and $P2X_2$ receptor expression was not observed in the brain until E14, it is most likely that the effect of α,β-meATP was mediated via $P2X_3$ receptors. In addition to the $P2X_3$ receptor, $P2Y_2$ and $P2Y_4$ receptors, which are UTP-activated receptors, have also been demonstrated previously in motor neurons in the ventral neural tube (Cheung et al., 2003). However, the P2Y receptors reside mainly in the spinal, rather than cranial, neural tube. Furthermore, the present result also showed that the inhibitory effect was not observed when UTP was used instead of ATP, thus excluding the involvement of $P2Y_2$ and $P2Y_4$ receptors.

ATP has previously been shown to inhibit neurite outgrowth in hippocampal neurons transfected with NCAM, a marker for neural cell adhesion and neurite outgrowth, and the inhibitory effect of ATP was lost when the hippocampal neurons did not express NCAM, suggesting that ATP regulates neurite extension via NCAM (Skladchikova et al., 1999). Immunohistochemical studies showed that most of the axons growing out from the neural tube at E12 coexpressed NCAM and $P2X_3$ receptors, suggesting that ATP-mediated inhibition in neurite outgrowth might act, at least partly, through the NCAM signaling system. Note, $P2X_1$ receptors have been shown recently to be expressed in microglia isolated from developing rat brain (Xiang and Burnstock, 2005), but the expression was too dispersed and low to be seen in the present study of brain sections.

## CONCLUSION

In summary, this study demonstrates for the first time, the developmental expression profile of P2X receptors subtypes during perinatal brain development in the rat. Our results indicate that different P2 receptor subtypes may participate in different developmental processes such as neurite outgrowth (involving $P2X_3$ receptors), postnatal neurogenesis (related to $P2X_4$ and $P2X_5$ receptor expression) and cell death (possibly involving $P2X_7$ receptors), whereas others may not play a role in development ($P2X_1$ and $P2X_6$ receptors). Furthermore, extracellular ATP may function as a signaling molecule to inhibit motor axon outgrowth in the embryonic neural tube, most likely via $P2X_3$ receptors acting through the NCAM signaling system.

*Acknowledgments—We thank Dr. Chrystalla Orphanides for excellent editorial assistance and Jenny Hou, Corinna Au, Tim Robson and Terence Tam for technical assistance. We are also grateful for the gift of P2X receptor antisera from Roche Palo Alto. The work described in this paper was partially supported by a grant to W.Y.C. from the Research Grant Council of the Hong Kong Special Administrative Region (Project no. CUHK4275/99M).*

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*(Accepted 9 March 2005)*