Developmental expression of P2X5 receptors in the mouse prenatal central and peripheral nervous systems

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Abstract The functions of P2X purinoceptors (P2X1–7) in the nervous system of adults have been widely studied. However, little is known about their roles during embryonic development. Our previous work has reported an extensive expression of P2X5 receptors in the adult mouse central nervous system. In the present study, we have examined the expression pattern of P2X5 receptor mRNA and protein during prenatal development of the mouse nervous system (from embryonic day E8 to E17). P2X5 receptors appeared in the neural tube as early as E8 and were gradually confined to new-born neurons in the cortical plate and ventral horn of the spinal cord. Heavy signals for P2X5 receptors were also found in dorsal root ganglia (DRG), retina, olfactory epithelium, and nerve fibers in skeletal muscles. In conclusion, P2X5 receptors were strongly represented in the developing

mouse nervous system. The transient high expression pattern of P2X5 receptors in epithelium-like structures suggests a role during early neurogenesis.

Keywords ATP · P2X receptors · P2X5 receptors · Neurogenesis

Introduction

There is strong molecular, cellular, and systems-level evidence that extracellular ATP, via P2 purinoceptors, plays important roles during physiological and pathophysiological activities [1, 2]. The last decade has seen an increase in our understanding of the functions of purinergic signaling during development of the nervous system, including progenitor cell proliferation, cell migration, neuronal and glial maturation and differentiation, and cell death and survival [3–5]. P2 purinoceptors are composed of two major families: a P2X family of ligand-gated ion channel receptors (P2X1–7) and a P2Y family of G protein-coupled receptors (P2Y1, 2, 4, 6, 11, 12, 13, and 14) [6]. A sequential expression of P2X and P2Y receptor subtypes during embryonic rat brain development has been described [7, 8]. However, apart from P2X3 and P2Y1 receptor subtypes, detailed expression and distribution information of other subtypes during the development of the nervous system remains to be identified [9]. Here, we focus on the P2X5 receptor, an electrically quiet ion channel.

P2X5 receptors have been found in all vertebrate species (human [10], mouse [11], rat [12], chick [13], bullfrog [14], and zebrafish [15]), mainly distributed on proliferating cells in the skin, gut, bladder, thymus, and spinal cord, where they are involved in increasing differentiation and thereby inhibiting cell proliferation [16, 17]. P2X5 receptors have

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Fig. 1 Analysis of P2X5 receptor expression pattern during prenatal mouse brain development. **a** RT-PCR analysis of P2X5 receptor mRNA expression. The products (323 bp) are arranged in four lanes corresponding to RT-PCR products of E9, E11, E13, and E17 mouse brain (from left to right). GAPDH was used as the loading control. **b** Quantification of (a) was presented as fold of E9. Results were from three independent experiments. $**P < 0.01$. **c** Western blot analysis of P2X5 receptor protein from the brain. The products (44KD) are arranged in four lanes corresponding to E9, E11, E13, and E17 mouse brain (from left to right). Tubulin was used as a loading control for

protein extracts. **d** Quantification of (c) was presented as fold of E9. Results were from three independent experiments. $*P < 0.05$; $**P < 0.01$. **e** Developmental expression of P2X5 receptor protein in mouse brain at E8 (a), E11 (b), E13 (c), and E17 (d). Immunohistochemistry experiments demonstrated the presence of P2X5 receptor immunoreactivity in the neural tube as early as E8 (a). At E11, P2X5 immunostaining was seen in the hindbrain, midbrain, and forebrain (b). However, as the brain developed, positive signals were confined to the cortical plate (c, d). Abbreviations: IZ intermediate zone, CP cortical plate. Scale bar, a–c 150 and d 75 μ m

been claimed to mediate cell differentiation in the skin [18, 19] and in tumors [20]. It has also been found that activation of P2X5 receptors on muscle satellite cells inhibits cell proliferation and stimulates expression of markers of muscle cell differentiation, including myogenin, p21, and myosin heavy chain, resulting in an increased rate of myotube formation

[21]. In a recent work, Birdsong and his colleagues [22] found that ATP collaborates with acid by increasing the pH sensitivity of the acid-sensing ion channel number 3 to detect lactic acidosis, and they argue that the receptor involved appears to be the P2X5 receptor. However, to date there have been few studies on the role of P2X5 receptors in the nervous system.

Fig. 2 Colocalization of P2X5 receptor-immunoreactive (ir) and TUJ-1-ir cells in mouse brain at E8 (**a–c**) and E11 (**d–f**). **a** P2X5 receptor-ir cells (*red*) in the neural tube. **b** TUJ-1-ir cells (*green*) in the neural tube from the same section as (**a**). **c** Merged image from (**a**) and (**b**). Double-labeled cells are *yellow* in color and most of the P2X5 receptor-ir cells are only labeled with *red*. **d** P2X5 receptor-ir cells (*red*) in the neural tube. **e** TUJ-1-ir cells (*green*) in the area of CP from the same section as (**d**). **f** Merged image from (**d**) and (**e**). Double-labeled cells are *yellow* in color in the area of CP and most of the P2X5 receptor-ir cells are only labeled with *red*. Abbreviations: *LV* lateral ventricle, *VZ/SVZ* ventricular zone/subventricular zone, *IZ* intermediate zone, *CP* cortical plate. Scale bar, **a–c** 75 and **d–f** 75 μ m

P2X5 receptor mRNA or protein have been detected in rostral ventrolateral medulla [23], solitary tract nucleus [24], compact division of the nucleus ambiguus [25], paraventricular nucleus [26], mesencephalic trigeminal nucleus, spinal cord [12], hypothalamus [27–30], cerebellum [31, 32], and choroid plexus [33] of the adult mouse brain. Our previous work investigating the combined distribution of P2X5 receptor protein and mRNA by using immunohistochemistry and in situ hybridization histochemistry, has found an extensive expression of P2X5 receptors in the mouse central nervous system (CNS) [34]. In the present study, we investigated the distribution of P2X5 receptors during the development of the nervous system of the mouse embryo.

Materials and methods

Tissue preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the

Second Military Medical University and conformed to the UK Animals (Scientific Procedures) Act 1986 and associated guidelines on the ethical use of animals. The day of identification of the presence of a vaginal plug was designated as embryonic day zero (E0). Pregnant C57BL/6J mouse were killed by asphyxiation with a rising concentration of CO₂ (between 0 and 100 %). Embryos from E8 to E17 were collected and divided into three groups for immunohistochemistry or western blotting or reverse transcription polymerase chain reaction (RT-PCR) experiments. Samples were examined from brain, spinal cord, dorsal root ganglia (DRG), olfactory epithelia, gut, blood vessels, lung, and skeletal muscle of the forelimb.

Immunohistochemistry

The collected embryos were fixed in 4 % paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2) at 4 °C overnight. Embryos were then washed in 0.1 M PBS (pH 7.2) and dehydrated by using 20 % sucrose. Thereafter, the embryos were immersed in OCT-embedding medium and frozen in

Fig. 3 Colocalization of P2X5 receptor-ir and TUJ-1-ir cells in the spinal cord at E9 (**a–c**) and E11 (**d–f**). **a** P2X5 receptor-ir cells (*red*) in the spinal cord. Note the spinal cord was uniformly labeled in *red*. **b** TUJ-1-ir cells (*green*) in the spinal cord from the same section as (**a**). **c** Merged image from (**a**) and (**b**). Double-labeled cells are *yellow* in color and located in the basal plate. **d** P2X5 receptor-ir cells (*red*) in the spinal cord. Note only the ventral horn was labeled with *red*. **e** TUJ-1-ir cells (*green*) in ventral horn from the same section as (**d**). **f** Merged image from (**d**) and (**e**). Double-labeled cells are *yellow* and most of the P2X5 receptor-ir cells are labeled with *green*. Abbreviations: DRG dorsal root ganglia. Scale bar, **a–c** 75 and (**d–f**) 75 μ m

precooled isopropanol (-70°C) for cryosectioning. Frozen sections ($8\ \mu\text{m}$) were cut and mounted on gelatin-coated slides and dried at room temperature. The preparations were washed 3–5 min in PBS and then preincubated in antiserum solution 1 (10 % normal bovine serum albumin (BSA), 0.2 % Triton X-100, and 0.4 % sodium azide in 0.01 mol/l PBS at pH 7.2) for 30 min followed by incubation with P2X5 antibody (rabbit polyclonal), which was provided by Roche (Palo Alto, CA); the specificity of the P2X5 receptor antibody has been tested and established in our previous work [34]. The antibody was diluted 1:200 in antiserum solution 2 (1 % normal BSA, 0.2 % Triton X-100, and 0.4 % sodium azide in 0.01 mol/l PBS at pH 7.2), at room temperature overnight. Subsequently, the preparations were incubated with FITC-conjugated donkey anti-rabbit IgG diluted 1:200 for 2 h. For double immunostaining of P2X5 receptors with β -tubulin III (TUJ-1) (1:400, mouse anti-rat; Chemicon), Cy3-conjugated donkey anti-rabbit IgG (1:400) and FITC-conjugated donkey anti-mouse

IgG (1:200) were used. All incubations and reactions were separated by 5–10 min washes in PBS.

Images were taken with a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The density of the P2X5 receptor protein immunostaining signal was scored as: absent (–), weak (+), moderate (++), or heavy (+++) [12, 34].

RT-PCR

Total RNA was isolated using trizol reagents (Invitrogen) from cultures. To avoid DNA contamination, a digestion with RNase-free DNase I (Qiagen) was performed during the purification protocol. The cDNA synthesis was performed using thermoscript RT-PCR system (Invitrogen). The following oligonucleotide primers were used: for mouse P2X5, the forward primer was 5'-

Fig. 4 Colocalization of P2X5 receptor-ir and TUJ-1-ir cells in DRG at E11 (**a–c**) and E13 (**d–f**). **a** P2X5 receptor-ir cells (*red*) in DRG. Note that the P2X5 receptor expression appeared to be uniform in all DRG neurons. **b** TUJ-1-ir cells (*green*) in DRG from the same section as (**a**). **c** Merged image from (**a**) and (**b**). Double-labeled cells are *yellow*. **d** P2X5 receptor-ir cells (*red*) in DRG. Note that the P2X5 receptor expression appeared to be uniform in all DRG neurons. **e** TUJ-1-ir cells (*green*) in DRG from the same section as (**d**). **f** Merged image from (**d**) and (**e**). Double-labeled cells are *yellow* in color. Scale bar, **a–c** 75 and **d–f** 75 μ m

TCCCGGATGGCGAGTGTTCAG-3', and the reverse primer was 5'-GATGGGGCAGTAGAGATTGGTGGAG-3'; for mouse GAPDH, the forward primer was 5'-AACGACCCCTTCATTGACC-3', and the reverse primer was 5'-GTAGACTCCACGACATACTCAGC-3'.

Western blot

Brains were rapidly removed and lysed with 20 mM Tris-HCl buffer, pH 8.0, containing 1 % NP-40, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.1 % L-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the BCA protein assay method using BSA as standard. One hundred micrograms of protein samples from brain was loaded per lane, separated by SDS-PAGE (12 % polyacrylamide gels) and then was electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10 % nonfat dry milk in Tris-buffered saline for 1 h and incubated overnight at 4 °C with P2X5 antibody (1:1,000; Roche) or Tubulin antibody (1:5,000; Beyotime) diluted in 2 % BSA in PBS. The membranes were then incubated with alkaline phosphatase-

conjugated goat anti-rabbit IgG (Sigma) or goat anti-mouse IgG (Sigma) diluted 1:5,000 in 2 % BSA in PBS for 1 h at room temperature. The color development was performed with 400 μ g/ml Nitro-Blue Tetrazolium, 200 μ g/ml 5-bromo-4-chloro-3-indolyl phosphate, and 100 mg/ml levamisole in TSM2 (0.1 mol/l Tris-HCl₂ buffer, pH 9.5, 0.1 mol/l NaCl and 0.05 mol/l MgCl₂) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories)

Statistical analysis

All data are presented as mean $\pm$ SD. Statistical analysis was performed using unpaired Student's *t* test. Statistical significance was calculated with a *P* value of <0.05.

Results

By using RT-PCR, the developmental expression profile for P2X5 receptor mRNA was studied in mouse brain from E9. From E9 onwards, clear bands were observed throughout development (Fig. 1a). The expression level increased

Fig. 5 Colocalization of P2X5 receptor-ir and TUJ-1-ir cells in the mouse eye at E11. **a** P2X5 receptor-ir cells (*red*) in the eye. Note the areas of *re* and *op* were heavily labeled in *red*. **b** TUJ-1-ir cells (*green*) in the eye from the same section as **(a)**. **c** Merged image from **(a)** and **(b)**. Double-labeled cells are *yellow* in color and the P2X5 receptor-ir cells in basal plate are only labeled with *red*. **d, e** High-power images of the areas marked by a *rectangle* in **(a)** and **(b)**, respectively. **f** Merged image of **(d)** and **(e)**. Abbreviations: *re* retina, *op* optic nerve. Scale bar: **a–c** 150 and **d–f** 75 μ m

gradually during E9–E13 and then declined from E13 to E17 (Fig. 1b). Protein for P2X5 receptors was also detected in brain extracts from E9 onwards by western blotting experiments (Fig. 1c); the protein level increased during E9–E13 and then declined rapidly after E13 (Fig. 1d).

Corresponding to the RT-PCR results, immunohistochemistry experiments demonstrated the presence of P2X5 receptor protein in neural tube as early as E8 (Figs. 1e(a) and 2a). At the early stage of neurogenesis, the immunolabeling was mainly located in neuroectoderm. At E11, P2X5 immunostaining was seen in the hindbrain, midbrain and forebrain (Figs. 1e(b) and 2d). However, as the brain developed, positive signals were confined to the cortical plate (Fig. 1e(c, d), where new-born neurons had begun to differentiate. In the spinal cord, a similar expression pattern was found. P2X5 positive signals were detected in the whole spinal cord at E9 (Fig. 3a). However, the signals were confined to the area of the ventral horn (the presumed motor neuron zone) at E11 (Fig. 3d).

Previous works have reported an extensive expression of P2X5 receptors in neurons in the adult CNS in mammals, so we used the antibody for TUJ-1, a popular neuronal marker,

to examine the type of P2X5-positive cells during embryonic development by double immunostaining experiments. At E8 in the neural tube, very few (about 5 percent of all cells, not showed) P2X5 positive cells colocalized with TUJ-1-positive cells (Fig. 2a–c). A similar pattern was found in the spinal cord at E9 (Fig. 3a–c). However, at E11 when the P2X5-positive cells were confined to the cortical plate and the ventral horn, most of the P2X5-positive cells colocalized with TUJ-1-positive cells (Figs. 2d–f and 3d–f).

We detected P2X5 receptor expression in embryonic mouse DRG as early as E9 and from that time P2X5 expression appeared to be uniform in all DRG neurons and all of the P2X5-positive cells colocalized with TUJ-1-positive cells (Fig. 4a–f).

Heavy P2X5 labeling was found in the neural layer of the retina and also in the optic nerve at E11, and most P2X5-positive cells colocalized with TUJ-1-positive cells (Fig. 5a–f). Heavy P2X5 labeling was also found in the olfactory epithelium at E11 (Fig. 6a–f).

In the gut, all P2X5-positive cells colocalized with TUJ-1-positive cells at E11 (Fig. 7a–c). We also have found

Fig. 6 Colocalization of P2X5 receptor-ir and TUJ-1-ir cells in olfactory epithelium at E11. **a** P2X5 receptor-ir cells (*red*) in the olfactory epithelium. **b** TUJ-1-ir cells (*green*) in olfactory epithelium from the same section as (**a**). **c** Merged image from (**a**) and (**b**). Double-labeled cells are *yellow* in color. **d, e** High-power images of the areas marked by a *rectangle* in (**a**) and (**b**), respectively. **f** Merged image of (**d**) and (**e**). *Scale bar, a–c* 150 and *d–f* 75 μ m

extensive labeling of nerve fibers scattered throughout skeletal muscle of the forelimb (Fig. 7d–f), the wall of blood vessels and the lung (not shown).

We have summarized the expression pattern of P2X5 receptors in the various regions of the nervous system at E8, E9, E11, E13, and E17 in Table 1. The positive signals in all regions examined became gradually weaker as the mouse embryo developed.

Discussion

In this study, for the first time, a detailed expression pattern of P2X5 receptors in both central and peripheral nervous systems during early embryological development of the mouse has been described. While there are a number of investigations of P2X5 receptors in adult mammals, little evidence about the development of P2X5 receptors during embryonic development is available. Meyer and his colleagues found that chick P2X5 receptors appeared at various sites during early-stage embryonic development (stages 28–

30), from nerves supplying developing skeletal muscle, in some DRG cells and in the dorsal and ventral spinal cord [35]. However, in the rat brain, P2X5 receptors were only found to be expressed from P1 onwards [8]. In our previous work, altered expression of P2X5 receptors during postnatal development of the rat cerebellum, from P3 to P60, was described in detail [32].

One of the notable discoveries is the expression of P2X5 receptors in neuroepithelial cells, which are a type of neural stem cells, believed to be important during development [36]. The expression of receptors on and the function of purinergic signaling in stem cells have provoked a number of studies [5, 37]. However, much of the information has been obtained from an in vitro neurosphere model, which consists of aggregates of neural progenitor cells. Our work, correlates well with a recent study, which found the expression of P2X2-7 receptor subunits, as well as P2Y1, 2, 4, and 6 metabotropic receptors in neurospheres obtained from foetal rat brain [38]. This suggests a need for further investigation of the role of P2X5 receptors in neuroepithelial cells.

Fig. 7 Colocalization of P2X5 receptor-ir and TUJ-1-ir cells in the gut (**a–c**) and skeletal muscle (**d–f**) at E11. **a** P2X5 receptor-ir cells (*red*) in the gut. **b** TUJ-1-ir cells (*green*) in the gut from the same section as (**a**). **c** Merged image from (**a**) and (**b**). Double-labeled cells are *yellow* in color. **d** P2X5 receptor-ir cells (*red*) in skeletal muscle of the forelimb. **e** TUJ-1-ir cells (*green*) in skeletal muscle from the same section as (**d**). **f** Merged image from (**d**) and (**e**). Double-labeled cells are *yellow* in color and only fibers were labeled in *green*. Scale bar, **a–c** 75 and **d–f** 75 μ m

We have found that P2X5 receptors are gradually limited to specific new-born neurons during early development. The potential of nucleotides to increase or inhibit neuronal differentiation and neurite or axon outgrowth has received attention

[4]. Sequential expression of P2X receptor mRNA and protein during neurogenesis in the rat brain has been described, indicating that P2X3 receptors appeared as early as E11 and P2X2 and P2X7 receptors were expressed from E14 onwards. It is

Table 1 Expression of P2X5 receptor in the mouse embryo between E8 and E17

Structures	Immunoreactivity for P2X5 receptor age				
	E8	E9	E11	E13	E17
Neuroectoderm of brain vesicles	+++	+++	+++	++	+
Cortical plate of brain	NE	NE	+++	+++	++
Ventral horn of the spinal cord	+++	+++	+++	+++	++
Dorsal horn of the spinal cord	+++	+++	++	+	–
Dorsal root ganglia	NE	+++	+++	++	++
Retina	NE	NE	+++	+++	+
Optic nerve	NE	NE	+++	+++	+
Olfactory epithelium	NE	NE	++	++	+
Neurons in gut	NE	NE	++	++	+
Nerve fiber in muscle	NE	NE	++	++	+
The wall of blood vessels	NE	NE	++	++	+
Neurons in lung	NE	NE	++	++	+

The intensity of the immunoreactivity has been subjectively evaluated as: high (+++), moderate (++), low (+), absent (–), and not examined (NE)

widely believed that P2X5 receptors may form heteromeric multimers with other P2X receptors to play functional roles [39], so how the receptor works during early development requires more direct evidence.

It was surprising to note the sharp fall in P2X5 receptor signals in the spinal dorsal horn at E17, especially in view of our previous work that indicated a moderate level of straining for P2X5 receptors in the same area in the adult mouse [34]. In brain development, we found that the levels of mRNA and protein for P2X5 receptors displayed a trend in an initial increase followed by a decline. We speculate that the levels in the spinal cord may follow the same trend, implying a role for the function of P2X5 receptors in neurogenesis in the CNS. In the later stages of neurogenesis in the spinal dorsal horn, the protein level may have fallen to undetectable immunohistochemical levels in the present study.

The finding that P2X5 receptors are expressed during early-stage eye development suggests a potential role for ATP in this process. ATP, acting on P2 receptors, is involved in the regulation of retinal progenitor cell proliferation at early embryonic stages, perhaps in collaboration with growth factors. A recent important study has shown that purine-mediated signaling triggers eye development in *Xenopus* [40].

In summary, we have found that P2X5 receptors are highly transiently expressed in the early stages of neural development and are gradually confined to new-born neurons. It is suggested that the P2X5 receptors may be involved in the early development of the nervous systems.

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