Localization of P2X₃ Receptors and Coexpression With P2X₂ Receptors During Rat Embryonic Neurogenesis

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ABSTRACT

It is well known that extracellular ATP mediates rapid excitatory signaling by means of the ionotropic P2X receptors. One of its subunits, the $P2X_3$ receptor, is well documented to be associated with sensory innervation in adult animals. It is speculated that the $P2X_3$ receptor may have already been present in the early sensory system. The aim of this study was to investigate the distribution of the $P2X_3$ receptor during neurogenesis by using immunohistochemistry on rat embryos from embryonic day (E)9.5–18.5. The $P2X_3$ receptor was first identified in the hindbrain neural tube and the sensory ganglia in E11–11.5 embryos. At E14.5, the optic tract and retina, nucleus tractus solitarius, mesencephalic trigeminal nucleus, and sensory nerves in both respiratory and digestive tract showed positive staining. The facial nucleus, the prepositus hypoglossal nucleus, and the sympathetic ganglia also showed $P2X_3$ immunoreactivity, even though these are not sensory associated. $P2X_3$ immunoreactivity was detected in the vestibular nucleus, the nerves in mesentery, bladder, and kidney in E16.5 and in nerves in vibrissae in E18.5. $P2X_3$ immunoreactivity in the facial nucleus, spinal trigeminal tract, the mesencephalic trigeminal nucleus, and the vestibular nucleus were undetectable in postnatal day 16 rat brainstem. The $P2X_3$ receptor was coexpressed with the $P2X_2$ receptor in nucleus tractus solitarius, dorsal root ganglion, nodose ganglion, and the taste bud in E16.5 embryo, which was 5 days later than the first appearance of the native $P2X_3$ receptor. In summary, we present a detailed expression pattern of the $P2X_3$ receptor during neurogenesis and report that $P2X_3$ immunoreactivity is down-regulated in early postnatal brainstems. J. Comp. Neurol. 443:368–382, 2002. © 2002 Wiley-Liss, Inc.

Indexing terms: ATP; purinoceptors; central nervous system; sensory ganglia; immunohistochemistry; neurogenesis

Purine compounds such as adenosine 5′-triphosphate (ATP) play important roles in energy metabolism, synthesis of nucleic acids, and regulation of enzymes in living organisms. Nevertheless, the functions of these compounds are not restricted to intracellular actions but also to extracellular signaling. In recent years, the biological functions of extracellular ATP as a neurotransmitter and a neuromodulator have been studied intensively (Burnstock, 1997; Abbracchio and Burnstock, 1998). Extracellular ATP evokes responses by means of two families of P2 receptors, namely P2X and P2Y purinoceptors (Kennedy and Burnstock, 1985; Abbracchio and Burnstock, 1994). The P2X receptors consist of ligand-gated ion channels, which mediate rapid and selective permeability to certain types of cations (Na^+, K^+, Ca^{2+}) (Bean, 1992; Dubyak and El Moatassim, 1993; North, 1996). To date, seven subunits of P2X receptors have been cloned ($P2X_{1-7}$) from different tissues (reviewed by Ralevic and Burnstock, 1998). P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins, with five established subunits in mammals recently proposed (King et al., 2001).

The distribution of the seven cloned P2X receptors in the central nervous system (CNS) has been well studied in adult rat (Kidd et al., 1995; Collo et al., 1996, 1997; Soto et al., 1996; Kanjhan et al., 1999, Yao et al., 2000). Among the P2X receptor subunits, $P2X_3$ is well known to show

strong expression in sensory neurons (Vulchanova et al., 1997; Llewellyn-Smith and Burnstock, 1998; Xiang et al., 1998). Xiang et al. (1998) demonstrated that the $P2X_3$ receptor is highly expressed in trigeminal, nodose, and dorsal root ganglia compared with other P2X receptors studied. Studies of coexpressed $P2X_2$/$P2X_3$ receptors and native P2X receptors in sensory neurons indicate heteropolymerization of $P2X_2$ and $P2X_3$ receptors (Lewis et al., 1995; Vulchanova et al., 1997; Virginio et al., 1998). Homomeric $P2X_3$ receptor is expressed in the capsaicin-sensitive, small dorsal root ganglion neurons, whereas the heteromeric $P2X_{2/3}$ receptor is expressed in the capsaicin-insensitive, medium neurons (Ueno et al., 1999). These results all suggest the involvement of purinergic signaling in sensory function by means of the action of the $P2X_3$ receptor. This has been reinforced in knockout experiments in which $P2X_3$-deficient mice exhibited a marked urinary bladder hyporeflexia and showed a reduction in pain sensation (Cockayne et al., 2000). However, Xiang et al. (1998) have demonstrated the expression of $P2X_3$ receptor protein in scattered neurons in superior cervical and coeliac ganglia. In addition, Glass and Burnstock (2001) have also reported $P2X_3$ receptor expression in thyroid follicular and endothelial cells, suggesting that the $P2X_3$ receptor may have other roles in addition to sensory functioning.

There is growing evidence to suggest that purinergic signaling is involved in early embryonic development. Purinoceptors were shown to be one of the first functionally active membrane receptors in chick embryo cells during gastrulation, in which, by means of purinoceptors, ATP induced rapid accumulation of inositol phosphate and Ca^{2+} mobilization in a similar way and to the same extent as acetylcholine (Laasberg, 1990). Recent reports also implicate ATP as a key regulator of the development of various organs and systems in frog and chick as well as in mammalian embryos (reviewed by Burnstock, 1996, 2001). Responses to ATP have been described in chick ciliary neurons acutely dissociated from day 14 embryonic ciliary ganglia (Abe et al., 1995). In many cases, responses to extracellular ATP have been shown to vary, depending upon the stage of embryonic development. For example, ATP elicits vigorous muscle contraction at embryonic day 6 (E6), but by E17, no effect of ATP on muscle contraction is observed (Wells et al., 1995). Meyer et al. (1999b) demonstrated that two P2X receptor subunits, $P2X_5$ and $P2X_6$, were first expressed at early stages of chick skeletal muscle development and expression disappeared immediately before fusion of myoblasts to form myotubes. These data strongly suggest that P2X receptors play a role in embryonic development. However, although the involvement of P2X receptors has been shown in early development of chick embryo (Meyer et al., 1999a,b), there is little information regarding the expression and function of the P2X receptors in mammalian embryos. One study by Ryten et al. (2001) demonstrated the sequential expression of $P2X_5$, $P2X_6$, and $P2X_2$ receptor subtypes in developing rat skeletal muscles, whereas Kidd et al. (1998) reported $P2X_3$ receptor expression in various nuclei such as spinal trigeminal tract, mesencephalic trigeminal nucleus, and solitary nucleus in late embryonic rat brain; this expression was down-regulated in young adult brain. This finding clearly demonstrates the presence of the $P2X_3$ receptor in the sensory system of the prenatal central nervous system. For the peripheral nervous system, the sensory ganglia such as the trigeminal ganglia and DRG have their primordial tissues present as early as in E10 and in E11 in the rat embryo, respectively (Kaufman and Bard, 1999; Kaufman, 1992). However, it is not currently known whether $P2X_3$ receptors are already expressed during early neurogenesis of the sensory system and, if present, whether $P2X_3$ receptor subtypes are expressed as homomeric receptors or coexpress with other P2X receptor subtypes (especially $P2X_2$ receptors).

It is not currently known whether $P2X_3$ receptors (either homomeric or heteromeric forms) are expressed during early neurogenesis. Thus, in the present study, we investigated the distribution of $P2X_3$ receptors during the development of the nervous system in the rat embryo. Expression of $P2X_3$ receptor protein in the neonatal rat brain was examined to show whether there is a down-regulation of $P2X_3$ receptor protein expression after birth. Coexpression of $P2X_2$ and $P2X_3$ by double labeling was also examined to investigate whether $P2X_2$ and $P2X_3$ receptor coexpression is a common phenomenon during early neurogenesis.

MATERIALS AND METHODS
Tissue preparation

The embryonic expression of $P2X_3$ receptor protein was studied in Sprague-Dawley rat embryos of E9.5–18.5 by using immunohistochemical techniques. Postnatal rat brain on postnatal day (P) 1 and P16 were chosen for examination. The day of identification of the presence of a vaginal plug was designated as day zero (E0). Pregnant Sprague-Dawley rats were killed by asphyxiation with a rising concentration of CO_2 (between 0% and 100%), and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering schedule 1 procedures. Embryos from prenatal day 9.5–18.5 were collected and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.2) at 4°C overnight. Embryos were then washed in 0.1 M phosphate buffered saline (PBS, pH 7.2) and dehydrated by using 10% sucrose, 20% sucrose, and finally 30% sucrose. Thereafter, the embryos were immersed in OCT-embedding medium and frozen in pre-cooled isopropanol (−70°C) for cryosectioning. Frozen sections (12 μm) were cut and mounted on gelatin-coated slides and dried at room temperature. Neonatal rat brains were dissected after cervical dislocation, and the brains were fixed and processed as described above. Frozen sections (15 μm) sections were cut and mounted.

Immunohistochemistry

Immunohistochemistry for P2X receptors was performed by using rabbit polyclonal antibodies against a unique peptide sequence of $P2X_2$ and $P2X_3$ receptor subtypes provided by Roche Bioscience, Palo Alto, CA (Oglesby et al., 1999). The immunogens used for production of polyclonal $P2X_3$ antibody were synthetic peptides corresponding to the carboxyl terminal of the cloned rat $P2X_2$ and $P2X_3$ receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences of the $P2X_2$ and $P2X_3$ receptors are of amino acid sequence 458-472 (QQDSTSTDPKGLAQL) and 383-397 (VEKQSTDSGAY-SIGH), respectively. The polyclonal antibodies were raised by multiple, monthly injections of New Zealand rabbits with the corresponding peptides (prepared by Research Genetics, Huntsville, AL). The specificity of the antisera was verified by immunoblotting with membrane preparation from CHO K1 cells expressing the cloned $P2X_2$ and

P2X$_3$ receptors (Oglesby et al., 1999). As previously reported by Oglesby et al. (1999), no cross-reactivity is observed with these antisera.

For immunostaining of cryosections, the standard avidin-biotin complex (ABC) technique was used. Sections were post-fixed with 4% paraformaldehyde for 2 minutes at room temperature. Endogenous peroxidase was blocked by 0.5% H$_2$O$_2$ and 50% methanol (methanol:PBS, 1:1) for 20 minutes. The P2X$_3$ primary antibody was used at a concentration of 1:200 prepared in 10% normal horse serum (NHS) containing 0.2% Triton X-100. For P2X$_2$ immunohistochemistry, the primary antibody was used at a dilution of 1:200 prepared in 10% NHS only. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA) at a dilution of 1:500 in PBS containing 1% NHS for 1 hour. The sections were then incubated in ExtrAvidin peroxidase diluted 1:1000 in PBS for 30 minutes at room temperature. For color reactivity, a solution containing 0.05% 3,3′-diaminobenzidine (DAB), 0.04% nickel ammonium sulfate, 0.2% β-D-glucose, 0.004% ammonium nitrate, and 1.2 U/ml glucose oxidase in 0.1 M PB (pH 7.2) was applied. Sections were washed three times with 0.1 M PBS after each of the above steps (except for serum preincubation). Slides were mounted with Eukitt (BDH Laboratory, UK) and examined with light microscopy. The control experiments were carried out with the primary antibodies preadsorbed with the peptides for immunizing the rabbits or the primary antibody replaced with the normal horse serum.

Immunofluorescence double labeling

In colocalization studies investigating the coexpression of P2X$_2$ and P2X$_3$ receptors, P2X$_2$ receptor immunoreactivity was enhanced with tyramide amplification, which allows high sensitivity and low background specificity (Renaissance, TSA indirect, NEN, USA). Tyramide amplification was performed after the primary antibody, specific for the P2X$_2$ receptor (1:800), was coupled to biotinylated donkey anti-rabbit IgG and ExtrAvidin peroxidase as described above. The immunoreactivity was detected with Streptavidin–fluorescein isothiocyanate (FITC) (Amersham, UK). Polyclonal rabbit antibody against P2X$_3$ receptor subtype (1:150) was applied as a second primary antibody and detected with Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA). To prevent the generation of artifacts due to nonspecific labeling after tyramide amplification, double labeling was also performed by using anti-P2X$_3$ receptor antibody as the first primary antibody and the anti-P2X$_2$ receptor antibody as the second primary antibody, to confirm the expression pattern.

To demonstrate the colocalization of the P2X$_2$ receptor with embryonic heart muscle, the P2X$_2$ receptor antibody (1:800) was used as the first primary antibody, enhanced with tyramide amplification and detected with Streptavidin-FITC as described above. Mouse monoclonal antibody against α-smooth muscle actin (1:400; Sigma, UK) was applied as the second primary antibody and the immunoreactivity was then detected with rhodamine (TRITC)-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Lab, West Grove, PA).

Photomicroscopy

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

Data analysis

Analysis was performed by two independent observers who examined the slides in a blind manner. Scores for P2X$_3$ immunohistochemical staining were made by using a subjective, graded scale varying from −, undetectable staining; +, weak staining but distinguishable from background, or scattered cells with moderate intensity staining; ++, moderate intensity staining in over 50% of cells; +++, very intense immunoreactivity in over 50% of cells.

RESULTS

P2X$_3$ immunoreactivity in the rat embryonic nervous system

Immunohistochemistry was performed to investigate the pattern of P2X$_3$ receptor protein expression during rat embryonic development. By using the standard ABC method with nickel and DAB as chromogens, black staining indicated positive immunoreactivity. In this study, rat embryos of E9.5–18.5 were chosen to investigate P2X$_3$ immunoreactivity and the results are summarized in Table 1.

No detectable P2X$_3$ immunoreactivity was observed in E9.5–E10.5 embryos. Weak P2X$_3$ immunolabeling was first identified in the trigeminal preganglia, facioacoustic ganglion complex, glossopharyngeal-vagal ganglion complex, and the ventrolateral region of the hindbrain neural tube in E11–11.5 rat embryos (data not shown). At E12.5, stronger P2X$_3$ staining intensity was found in the same embryonic tissues as E11.5 rat embryos (Fig. 1). For the trigeminal preganglion in E11–11.5 rat embryos, P2X$_3$ was found in the neurons and fibers only, which were separated from the neural tube. In E12.5 rat embryos, when the spinal trigeminal tract has begun to appear between the trigeminal ganglion and the neural tube, the spinal trigeminal tract was stained with P2X$_3$ antibody (Fig. 1). In addition, the dorsal root ganglia and the vagal trunk also started expressing P2X$_3$ receptors in E12.5 rat embryos (Fig. 1). Unlike the E11.5 embryo, where P2X$_3$ receptors were expressed in only a few neurons and fibers in the neural tube, extensive neurons and fibers in the ventrolateral region of the neural tube showed stronger P2X$_3$ immunoreactivity in E12.5 embryos (Fig. 1). Although the expression was strong in the hindbrain neural tube, weak expression was also found in the spinal neural tube. However, expression of P2X$_3$ protein was not found in the forebrain neural tube.

For E14.5 rat embryos, more embryonic organs showed P2X$_3$ neural immunoreactivity (Fig. 2). The central nervous system (CNS), which differentiates from neural tube, was shown to have P2X$_3$ immunoreactivity at this stage. The P2X$_3$ receptor was expressed in the mesencephalic trigeminal nucleus, spinal trigeminal tract, nucleus tractus solitarius, and the spinal cord. In the pontine region, P2X$_3$ receptor expression was strong in the facial nerves, and both the settled and migrating facial neurones of the facial nucleus. The prepositus hypoglossal nucleus, however, showed very weak P2X$_3$ receptor staining. In the spinal cord, the P2X$_3$ receptor was expressed in the ven-

TABLE 1. Localization of P2X$_3$ Receptor Immunoreactivity in the Rat Embryonic PNS and CNS[1]

Embryonic Nervous System	Embryonic day					
	9.5–10.5	11–11.5	12.5	14.5	16.5	18.5
Peripheral						
Trigeminal (V) ganglia and its nerve	−	+	++	+++	+++	+++
Facial (VII) ganglia and its nerve	−	+	++	+++	+++	+++
Vestibulocochlear (VIII) ganglia and its nerve	−	+	++	+++	+++	+++
Glossopharyngeal (IX) ganglia and its nerve	−	+	+++	+++	+++	+++
Dorsal root ganglia and its nerve	−	−	++	+++	+++	+++
Vagal (X) ganglia and vagal trunk	−	−	++	+++	+++	+++
Sympathetic trunk	−	−	−	+	++	++
Sympathetic ganglia	−	−	−	+	+	+
Nerves supplying the internal organs:						
Stomach and intestine	−	−	−	++	++	+++
Lung and trachea	na	−	−	++	++	+++
Tongue and pharynx	na	na	na	++	+++	+++
Mesentery	na	na	na	−	+++	+++
Kidney	na	na	na	−	+	+
Bladder	na	na	na	−	++	++
Nerves in mesentery	−	−	−	−	++	+++
Nerves around vibrissae	na	na	na	−	−	++
Central						
Optic (II) tract	na	na	na	+	++	+++
Retina	na	na	−	+	++	+++
Mesencephalic trigeminal nucleus of the fifth nerve	na	na	na	++	++	+++
Hindbrain neural tube	−	+	++	na	na	na
Spinal cord	−	−	+	++	+++	+++
Spinal trigeminal tract	na	na	++	+++	+++	+++
Nucleus tractus solitarius	na	na	na	+++	+++	+++
Facial nucleus	na	na	na	+++	+++	+++
Prepositus hypoglossal nucleus	na	na	na	+	+	++
Vestibular spinal nucleus	na	na	na	−	+	++

[1]Degree of P2X$_3$ immunoreactivity: +, weak; ++, moderate; +++, strong; −, undetectable; na, not applicable. CNS, central nervous system; PNS, peripheral nervous system.

tral region of the spinal cord (Fig. 2). The optic nerve and retina (neural layer) showed very weak P2X$_3$ immunoreactivity (data not shown). In the peripheral nervous system, trigeminal and dorsal root ganglia (which were more differentiated than in previous stages) showed strong P2X$_3$ receptor expression. Vestibulocochlear ganglia, which are derived from the facioacoustic ganglion complex, also expressed P2X$_3$ receptor protein. In addition to the cell bodies of the ganglia showing P2X$_3$ expression, nerve fibers in the ganglion (e.g., trigeminal nerve, facial nerve, vestibular nerve, glossopharyngeal nerve, spinal nerve, vagal trunk, and sympathetic trunk) also expressed P2X$_3$ protein (data not shown). The innervation of visceral organs such as lung, esophagus and stomach (Fig. 2), and the nerves in tongue and pharynx were P2X$_3$ receptor-positive.

In E16.5 rat embryos, vestibular nucleus in the medulla started to show weak P2X$_3$ receptor staining (data not shown). Peripheral nerves such as the mesenteric nerves (especially those surrounding blood vessels), nerves in the bladder and intestine did not show P2X$_3$ receptor expression until E16.5. However, P2X$_3$ immunoreactivity was not detected in the ventral spinal cord in E16.5 embryos. Unlike the sensory ganglia (e.g., trigeminal ganglion, dorsal root ganglia, and nodose ganglia) that showed strong P2X$_3$ immunoreactivity, only scattered ganglionic cells in superior cervical ganglion and the sympathetic ganglion were stained weakly with P2X$_3$ receptor antibody in E18.5 embryos (Fig. 3). The sympathetic trunk, however, showed relatively stronger P2X$_3$ receptor expression (Fig. 3). In the CNS, P2X$_3$ immunoreactivity was strong in the dorsal spinal cord (Fig. 4) and the mesencephalic trigeminal nucleus (Fig. 5). P2X$_3$ receptor expression was also found along the whole optic tract running from the neural layer of the retina through the optic chiasm to the lateral geniculate of the diencephalon. However, the lateral geniculate nucleus in the diencephalon appeared to have no P2X$_3$

immunoreactivity. In the retina, high magnification revealed that P2X$_3$ expression was restricted to the retinal ganglion cells in the inner neural layer of the retina and was not seen in the outer pigmental layer (Fig. 4). The prepositus hypoglossal nucleus in the medulla still expressed weak P2X$_3$ receptor protein. Strong P2X$_3$ immunoreactivity was observed in the peripheral nerves supplying the tongue, mesentery, and vibrissae (Fig. 4). There was no immunostaining observed in any of the following regions: forebrain (olfactory bulb, cerebral cortex, caudate putamen, amygdala, internal capsule), diencephalon (thalamus, hypothalamus), cerebellum and brainstem (superior and inferior colliculus, superior and inferior olive, vagal motor nucleus, dorsal motor nucleus of vagus, trigeminal motor nucleus, cuneate nucleus, gracile nucleus). There was no visible staining in control experiments where the P2X$_3$ antibody was replaced with NHS (data not shown) or preadsorbed with peptide (Fig. 4).

P2X$_3$ receptor immunoreactivity in neonatal rat brain

In P1 rat brain, P2X$_3$ receptor expression was localized to a subpopulation of the mesencephalic trigeminal nucleus. At this developmental stage, weaker P2X$_3$ receptor expression was seen in spinal trigeminal tract, spinal trigeminal nucleus, facial nucleus and facial nerve, vestibular nucleus, nucleus tractus solitarius, and in the prepositus hypoglossal nucleus when compared with expression in embryonic brain (Fig. 5). In P16 rat brain, no P2X$_3$ receptor immunoreactivity was detected in the mesencephalic trigeminal nucleus, spinal trigeminal tract, facial nerves, and facial nucleus or the vestibular nucleus. Only the nucleus tractus solitarius and the hypoglossal nucleus remained P2X$_3$ immunopositive at this time point (data not shown).
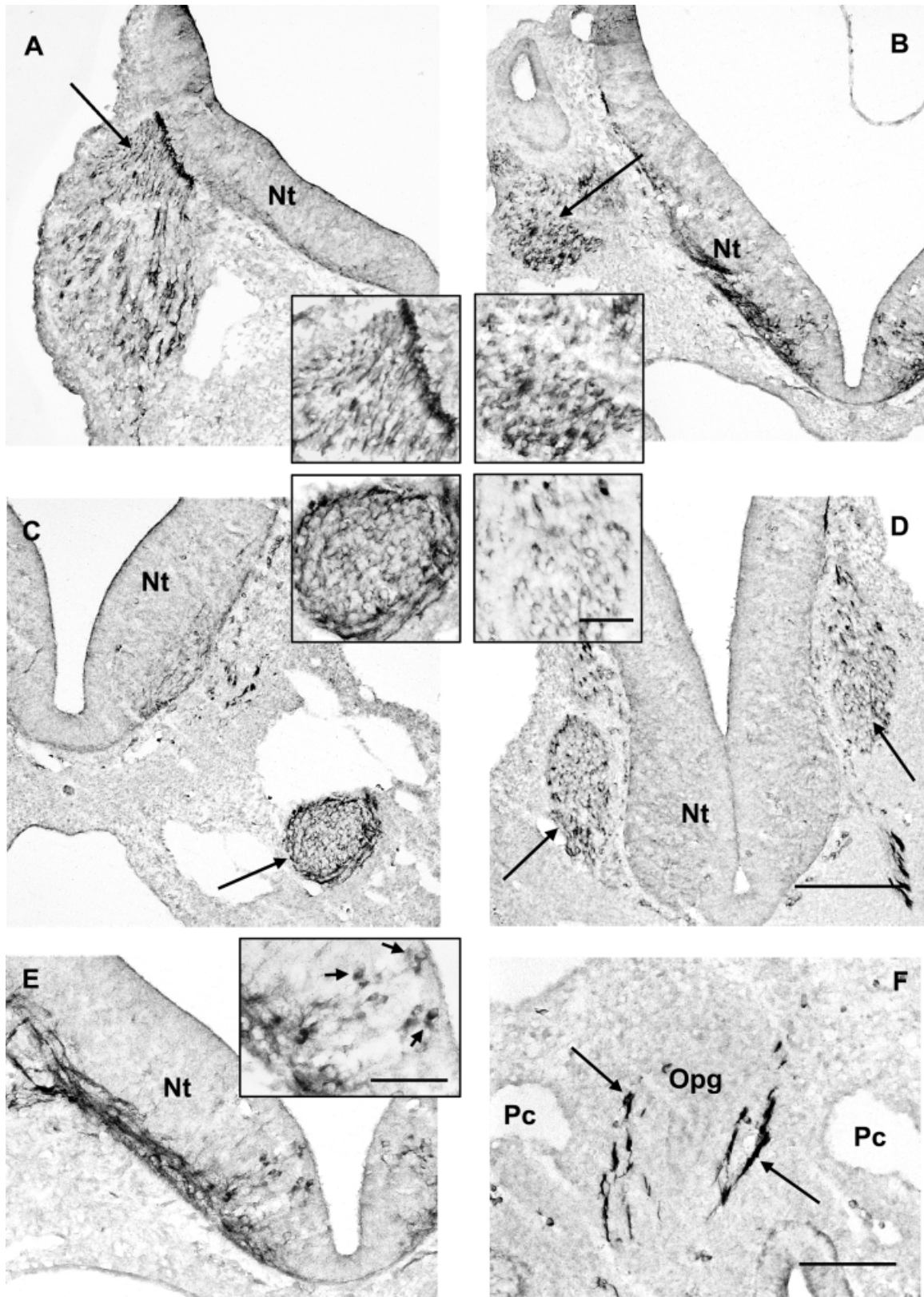
Fig. 1. P2X$_3$ immunoreactivity in embryonic day (E) 12.5 rat embryos. **A–C:** Transverse sections at the first (A), second (B), and third (C) branchial arch levels showing P2X$_3$ immunoreactivity (arrow) in the trigeminal ganglion, facioacoustic ganglion complex, and glossopharyngeal ganglion, respectively. Note the expression of P2X$_3$ in the primitive spinal trigeminal tract between the trigeminal ganglion and the neural tube (Nt). **D:** Transverse section at the caudal part of the embryo showing P2X$_3$ expression (arrow) in the dorsal root ganglia on both sides of the neural tube. Insert figures show enlargements of the areas indicated by arrows in the corresponding figures. **E:** Transverse section at the hindbrain neural tube showing P2X$_3$-positive neurons (arrows in the insert) and nerve fibers in the ventrolateral region of the neural tube. **F:** Transverse section at the pericardio-peritoneal canal (Pc) showing P2X$_3$ expression in the vagal trunk (arrows) on both sides of the esophagus (Opg). Note also that P2X$_3$ receptor expression in the hindbrain neural tube shown in both B and E was much stronger than the spinal neural tube shown in C and D. Scale bar = 200 μm in D (applies to A–D), 50 μm in inset of D (applies to insets in A–D), 50 μm in inset of E, 200 μm in E,F.
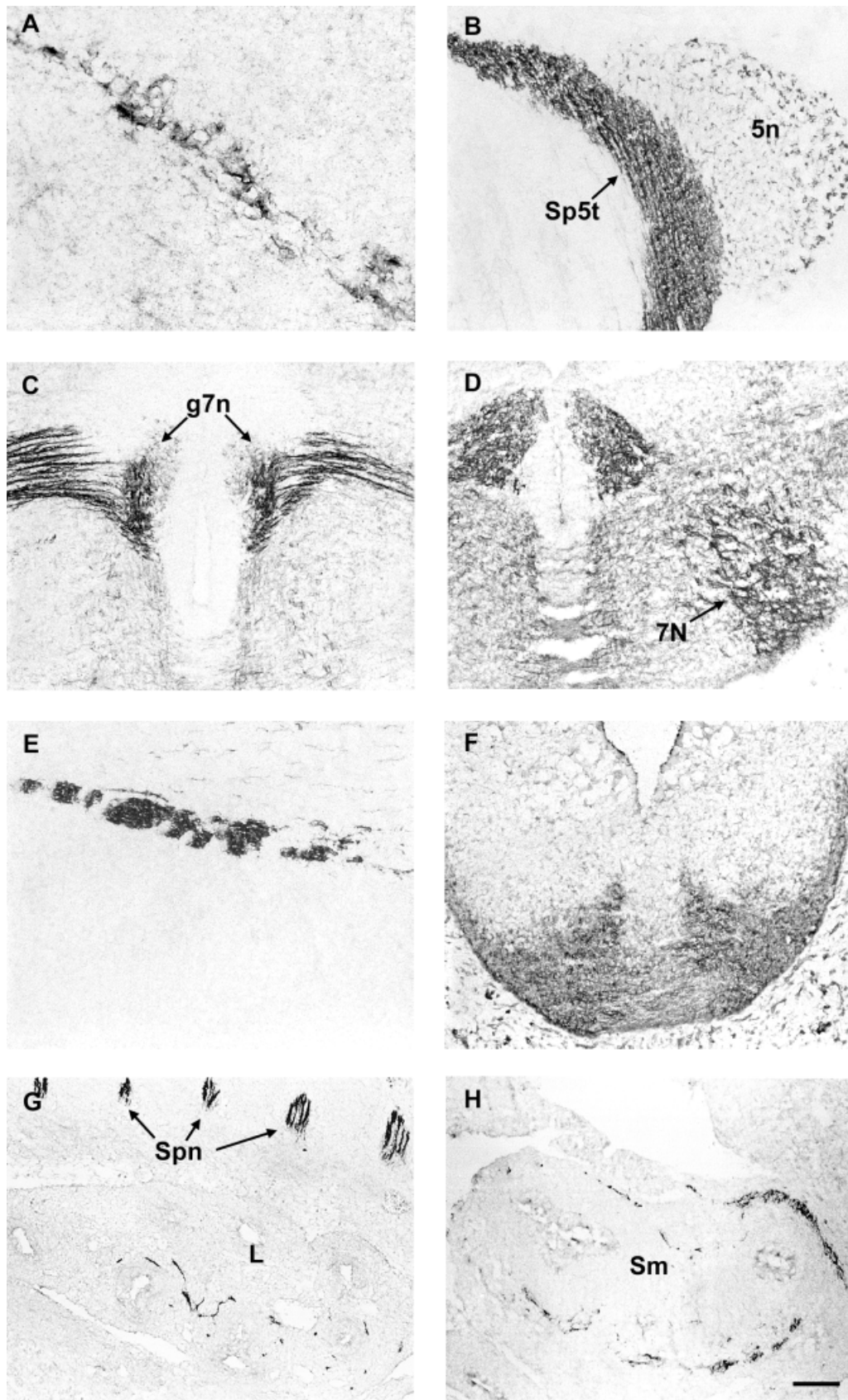
Fig. 2. P2X$_3$ immunoreactivity in embryonic day (E) 14.5 rat embryo. **A:** coronal section at the midbrain level showing P2X$_3$ expression in the mesencephalic trigeminal nucleus. **B:** Coronal section at the pontine level showing strong P2X$_3$ expression in the spinal trigeminal tract (Sp5t) and the longitudinal running trigeminal nerve (5n). **C,D:** Coronal section at the pontine level showing the genu of the facial nerve (g7n) and the facial nucleus (7N) stained strongly with P2X$_3$ receptor antibody. **E:** Coronal section at the medulla level showing the P2X$_3$ stained nucleus tractus solitarius. **F:** Transverse section showing P2X$_3$ receptor expression in the ventral spinal cord. **G,H:** Spinal nerves (Spn), and nerves found in lung (L) (G) and stomach (Sm) (H) at this stage showing P2X$_3$ immunoreactivity. Scale bar in H = 25 μm in A, 50 μm in B,E,F, 100 μm in C,D,G,H.
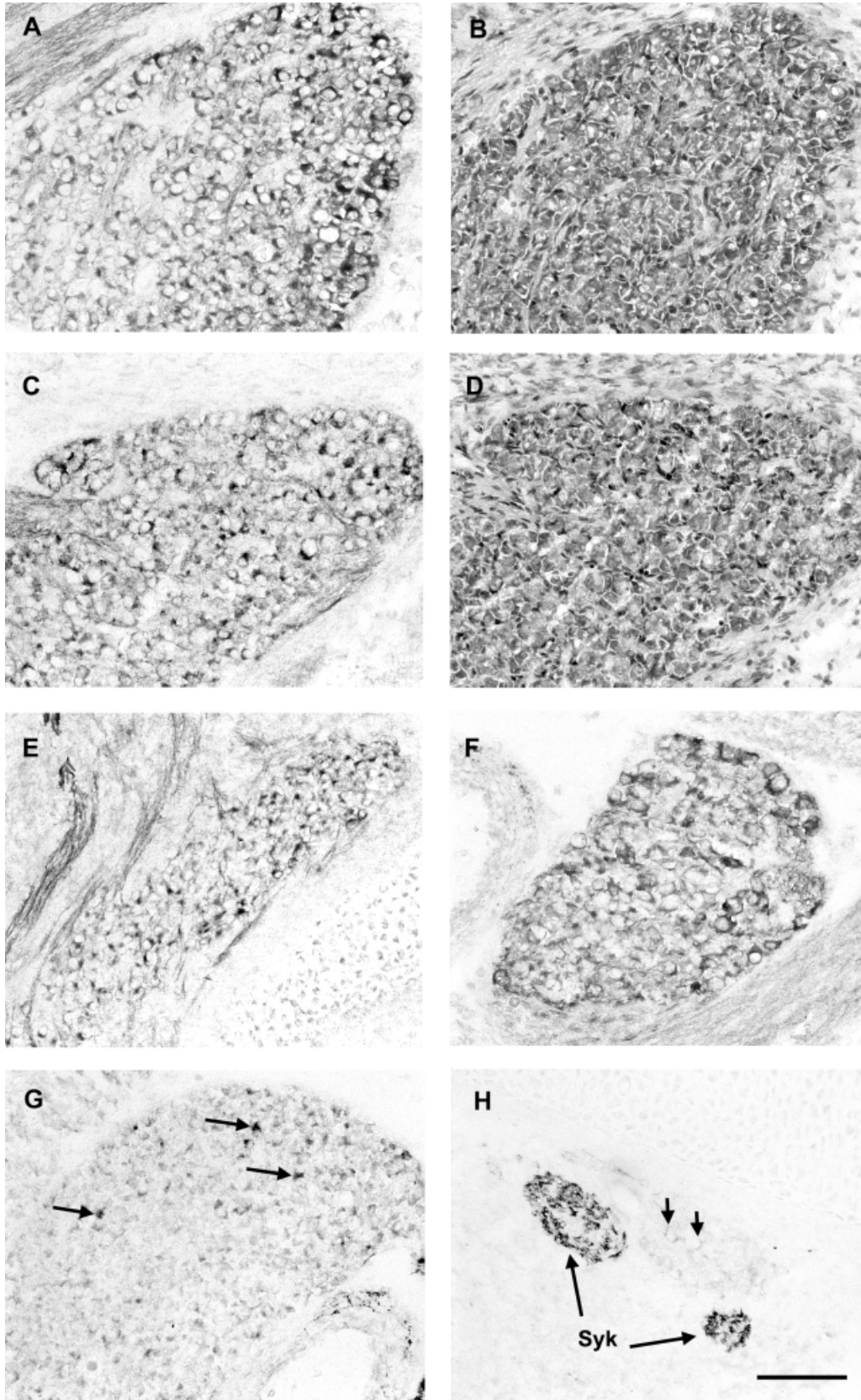
Fig. 3. P2X$_3$ immunoreactivity in neural crest-derived ganglia of the embryonic day (E) 18.5 rat embryo. **A–D:** The P2X$_3$ receptor is strongly expressed in trigeminal ganglion (A), dorsal root ganglion (C), vestibulocochlear ganglion (**E**) and nodose ganglion (**F**). Note the peripheral staining of P2X$_3$ immunoreactivity in the ganglionic neurons. (B) and (D) H&E staining showing the cellular structures of the trigeminal (B) and dorsal root ganglia (D) (2–3 sections from the corresponding P2X$_3$ immunostained sections). **G:** Scattered ganglionic cells (arrows) in superior cervical sympathetic ganglion also express P2X$_3$ protein. **H:** Strong P2X$_3$ receptor expression was detected in the nerve fibres of the sympathetic trunk (syk). Only scattered neurons in the sympathetic ganglion showed weak P2X$_3$ immunoreactivity. Note also the uneven localization of the P2X$_3$ immunoreactivity on the surface of the neuronal membrane. Scale bar = 100 μm in H (applies to A–H).
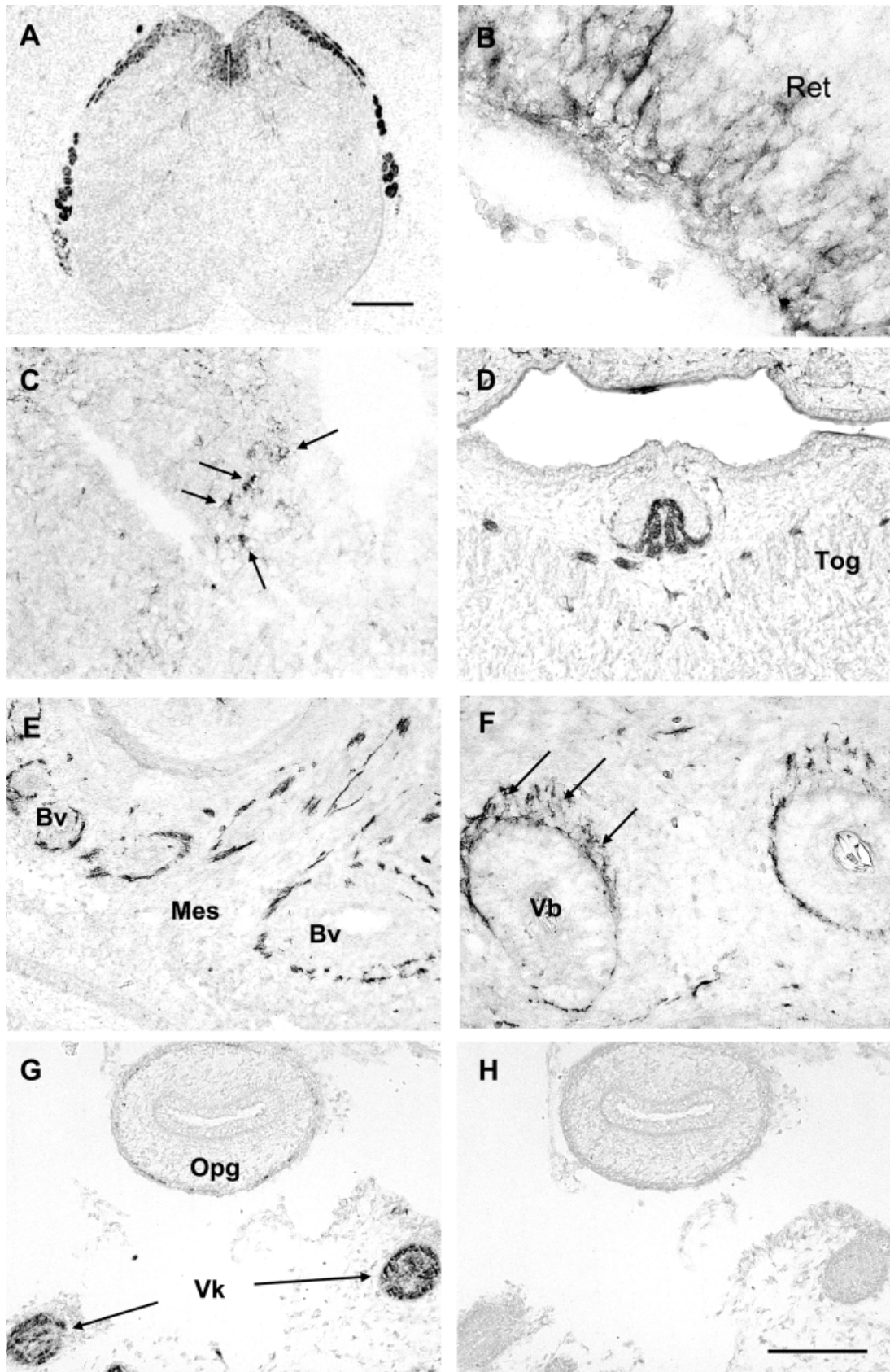
Figure 4

Expression of $P2X_2$ and $P2X_{2/3}$ receptor protein

$P2X_2$ receptor expression was first identified in the E11–11.5 rat embryo. Immunostaining was observed in the notochord at the level of the spinal neural tube and the myocytes of the heart. The expression of the $P2X_2$ receptor in notochord persisted in E12.5 embryo (Fig. 6). However, $P2X_3$ receptor protein was not detected in the notochord or the heart at this stage (Fig. 6). In the heart, cardiomyocytes stained strongly with $P2X_2$ receptor antibody. α-Smooth muscle actin, which is known to be strongly expressed during early development of cardiac muscle (Woodcock-Mitchell et al., 1988; Sawtell and Lessard, 1989), was used as a marker for early stage cardiac muscle. Double-labeling experiments showed that $P2X_2$ was coexpressed with α-smooth muscle actin in both E11.5 and E12.5 heart, including the truncus arteriosus, right and left atria, ventricle, and bulbus cordis (Fig. 7). The smooth muscle layer of the dorsal aorta was continuous with the embryonic heart. Together with the heart muscles, the smooth muscle layer at these stages also expressed α-smooth muscle actin. However, the aortic muscle layer did not show any $P2X_2$ immunoreactivity (Fig. 7). In E11.5 and 12.5 rat embryos, no detectable $P2X_2$ immunoreactivity was seen in the neural tubes. In the central nervous system, very weak $P2X_2$ immunoreactivity was first identified in the nucleus tractus solitarius and the spinal nerves emerging from the dorsal root ganglia of E14.5 embryo. However, the dorsal root ganglia did not show any $P2X_2$ immunoreactivity. At E16.5, the smooth muscle of the dorsal aorta, trachea and bronchi, esophagus, stomach, intestine, and bladder clearly stained positive with the $P2X_2$ receptor antibody. In addition, the nodose ganglia and the vagal trunk showed weak and moderate $P2X_2$ immunoreactivity (Fig. 6), respectively. The peripheral nerves supplying the tongue also showed $P2X_2$ immunoreactivity. The nucleus tractus solitarius and the nodose ganglia in E18.5 embryos showed obvious $P2X_2$ receptor expression (Fig. 6). Most of the skeletal muscles in E18.5 embryos were found to be $P2X_2$ receptor positive (data not shown). In all the $P2X_3$-immunopositive neural tissue examined, the $P2X_2$ receptor was expressed in the nucleus tractus solitarius and spinal nerves from E14.5 and the nodose ganglion, the vagal trunk, and the peripheral nerves supplying the tongue from E16.5. However, at E14.5, the staining was very weak even after tyramide amplification. Thus, coexpression of $P2X_2$ and $P2X_3$ receptors could only be detected clearly at E16.5. Double labeling experiments (Fig. 7) showed that in the nodose ganglia, only a subpopulation of neurons showed $P2X_{2/3}$ colocalization. After tyramide amplification, the dorsal root ganglia showed $P2X_2$ immunoreactivity; however, high magnification revealed the staining to be localized in the spinal nerves. Only few neurons in the dorsal root ganglia showed $P2X_2$ receptor staining, and this was coexpressed with $P2X_3$ receptor protein. A high degree of $P2X_2$ and $P2X_3$ coexpression was detected in the peripheral nerves ending in tongue. The strongest staining of both $P2X_2$ and $P2X_3$ immunoreactivity was found in the median circumvallate papilla. Quantitatively, however, $P2X_2$-positive nerves in the tongue were more frequently identified than $P2X_3$-positive nerves. Apart from taste buds in tongue, the nucleus tractus solitarius in E16.5 showed a high degree of $P2X_2$ and $P2X_3$ colocalization (Fig. 7).

Preliminary studies in our laboratory have shown that, except for $P2X_3$, all the other P2X receptors, including $P2X_2$, were absent in neural tubes and the sensory ganglia from E11 to E12.5 embryos. They were first detected in the embryonic brain and spinal cord in E14 embryos (unpublished data). In addition, of all the P2X receptors examined, only $P2X_2$ receptor subtypes were found in the heart and the notochord at this stage.

DISCUSSION

P2X receptors are ligand-gated ion channels activated by extracellular ATP that mediate rapid cation permeability and fast excitatory neurotransmission in both the central and peripheral nervous systems (reviewed by Ralevic and Burnstock, 1998). One of the P2X receptor subunits, $P2X_3$, was cloned from rat dorsal root ganglia (Chen et al., 1995; Lewis et al., 1995) and is known to be largely restricted to a subset of sensory neurons (trigeminal, nodose, and dorsal root ganglia). In early reports, $P2X_3$ was not detected in sympathetic, enteric, and CNS neurons (Chen et al., 1995; Collo et al., 1996), although the presence of the $P2X_3$ receptors has been demonstrated in superior cervical and celiac ganglia (Xiang et al., 1998; Zhong et al., 2000) and nucleus tractus solitarius (Vulchanova et al., 1997; Llewellyn-Smith and Burnstock, 1998; Yao et al., 2000). In this study, we present the detailed expression pattern of the $P2X_3$ receptor at different stages of rat embryonic development. In addition, the coexpression of $P2X_2$ and $P2X_3$ receptors was also examined to investigate whether $P2X_{2/3}$ receptor heteromerization is an early event during embryo development. The results obtained have shown that the $P2X_3$ receptor is the dominant receptor subtype among the P2X receptor family in the early embryonic nervous system and that $P2X_3$ receptor expression is down-regulated in the neonatal rat brain. $P2X_{2/3}$ expression appeared late in development compared with the individual receptor subtypes.

During E11–11.5, when gastrulation is complete and the neural tube has closed, gangliogenesis occurs (Kaufman and Bard, 1999). At this stage, the trigeminal ganglion and facioacoustic ganglion complex is formed and the glossopharyngeal-vagal preganglion is developing. At this time, $P2X_3$ protein first appears during rat embryonic development and is localized in the developing trigeminal preganglia, facioacoustic ganglion complex, and glosso-

Fig. 4. $P2X_3$ immunoreactivity in embryonic day (E) 18.5 rat embryo. **A:** Transverse section showing $P2X_3$ immunoreactivity in the dorsal spinal cord in the lumbar region. **B:** High-magnification image showing the retinal ganglion cells in the neural retina (Ret) expressing $P2X_3$ receptor protein. No $P2X_3$ immunoreactivity was found in the pigmental layer of the retina. **C:** Coronal section showing very weak $P2X_3$ receptor expression (arrows) in the prepositus hypoglossal nucleus ventrolateral to the fourth ventricle. **D:** Transverse section showing strong $P2X_3$ expressing in the taste bud of the tongue (Tog). **E:** Transverse section showing the mesentery (Mes) of the gut. $P2X_3$ immunoreactivity was found in the nerves surrounding the blood vessels (Bv) in the mesentery. **F:** Transverse section showing the vibrissae (Vb). $P2X_3$-positive nerve fibers (arrows) are found innervating the hair follicle of the vibrissae. **G:** Transverse section showing $P2X_3$ receptor expression in the nerves surrounding the esophagus (Opg); the nearby vagal trunk (Vk) also showed strong $P2X_3$ immunoreactivity. **H:** Adjacent section of G incubated with $P2X_3$ receptor preadsorbed with the preimmune peptide did not show any $P2X_3$ receptor staining. Scale bar in A = 200 µm, scale bar in H = 200 µm in C,D,F, 100 µm in E,G,H, 50 µm in B.
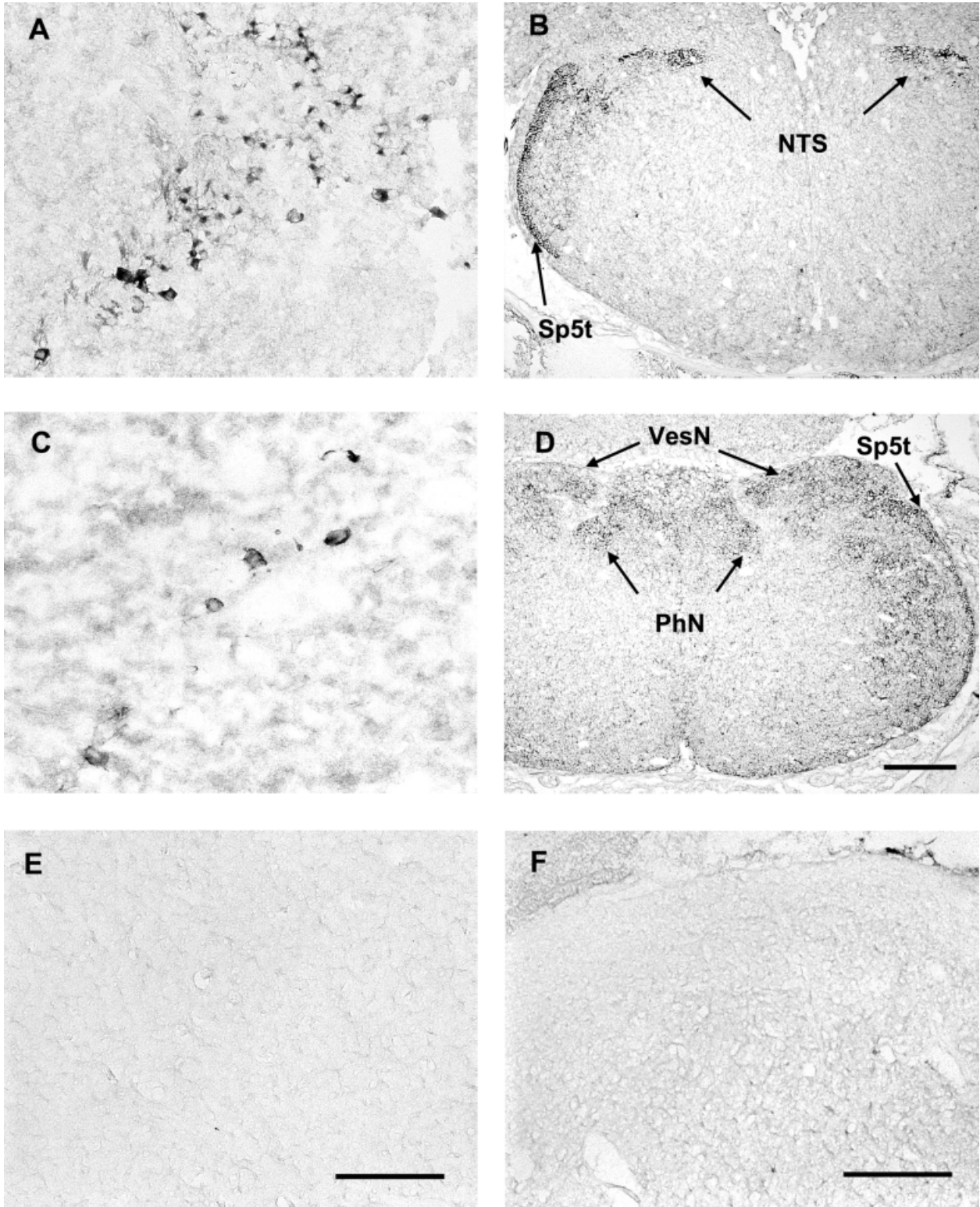
Fig. 5. P2X$_3$ immunoreactivity in late embryonic and neonatal rat brainstem. **A,C,E:** P2X$_3$ receptor expression in the mesencephalic trigeminal nucleus in embryonic day (E) 18.5, postnatal day (P) 1, and P16 rat brainstem, respectively. Note that only a subpopulation of the neurons in the mesencephalic trigeminal nucleus showed positive immunostaining in P1 brainstem (C). No P2X$_3$ immunoreactivity was detected in P16 brainstem (E). The spinal trigeminal tract, the nucleus tractus solitarius, the vestibular nucleus, and the prepositus hypoglossal nucleus in P1 brainstem clearly expressed P2X$_3$ receptor (**B,D**). **F:** In P16 medulla, P2X$_3$ receptor in the spinal trigeminal tract was not detected. Scale bars = 100 μm in E (applies to A,C,E), 500 μm in D (applies to B,D), 200 μm in F.
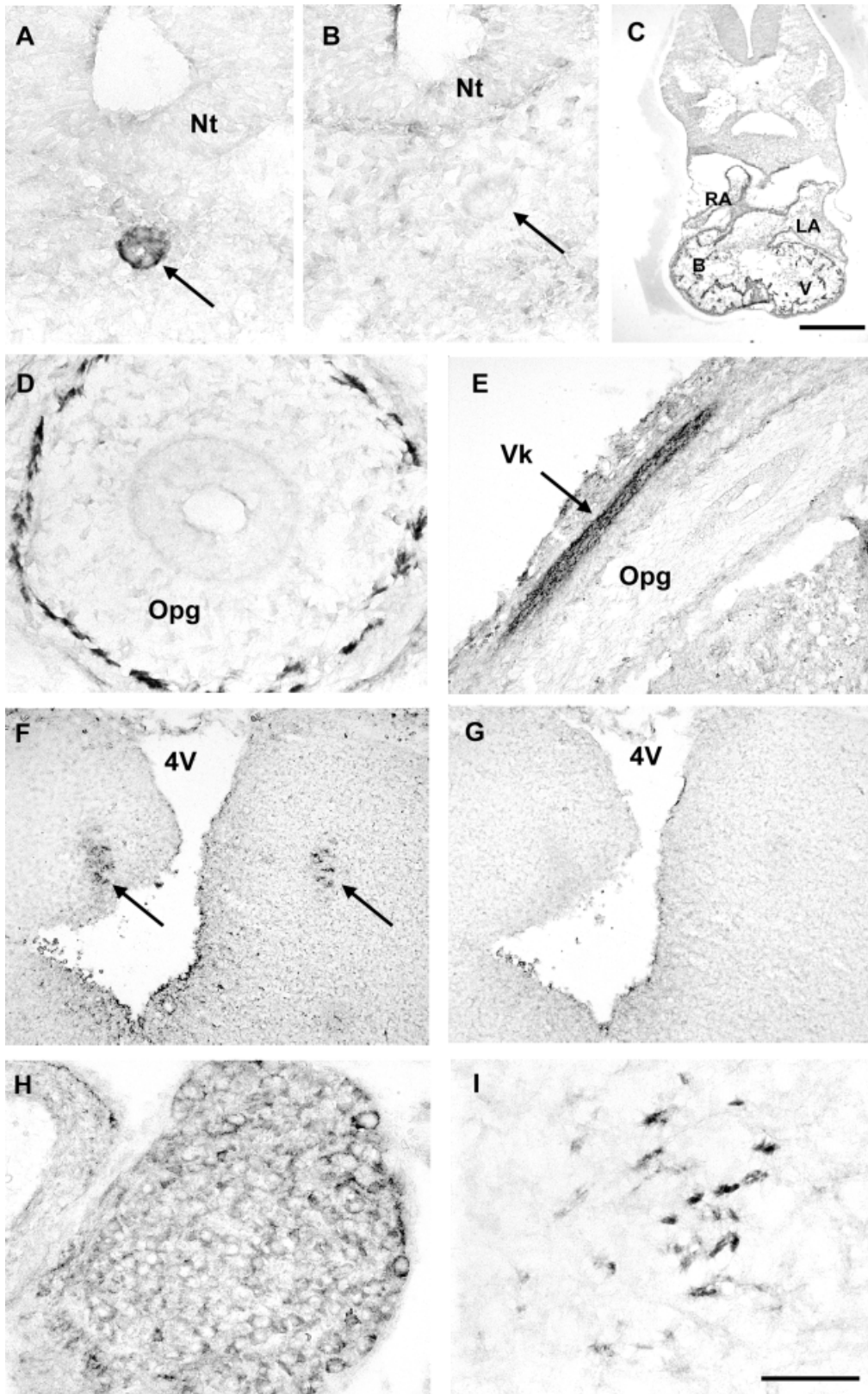
Fig. 6. P2X$_2$ immunoreactivity at different stages of rat embryo development. **A:** Transverse section in the spinal neural tube (Nt) in embryonic day (E) 12.5 embryos showing P2X$_2$ receptor expression in the notochord. **B:** Adjacent section of A stained with P2X$_3$ receptor antibody. P2X$_3$ receptor protein was absent in the notochord. **C:** Transverse section at the heart level showing P2X$_2$ receptor expression in the E12.5 heart, including the left and right atria (LA and RA), bulbus cordis (B), and the primitive ventricle (V). **D:** Transverse section showing the esophagus in E16.5 rat embryo. The P2X$_2$ receptor was localized in the smooth muscle of the esophagus. **E:** Sagittal section showing the trunk region of the E16.5 embryo. The P2X$_2$ immunoreactivity was found in the vagal trunk (Vk) at the periphery of the esophagus (Opg). **F,G:** The nucleus tractus solitarius in E16.5 showed P2X$_2$ immunoreactivity (F) and the preadsorption control in the adjacent section did not show any P2X$_2$ receptor staining (G). 4V, fourth ventricle. **H,I:** P2X$_2$ immunoreactivity was found in the nodose ganglion (H) and the nucleus tractus solitarius (I) in E18.5 rat embryo. Scale bar = 500 µm in C. Scale bar in I = 200 µm in E, 100 µm in H, 50 µm in A,B,D,F,G.
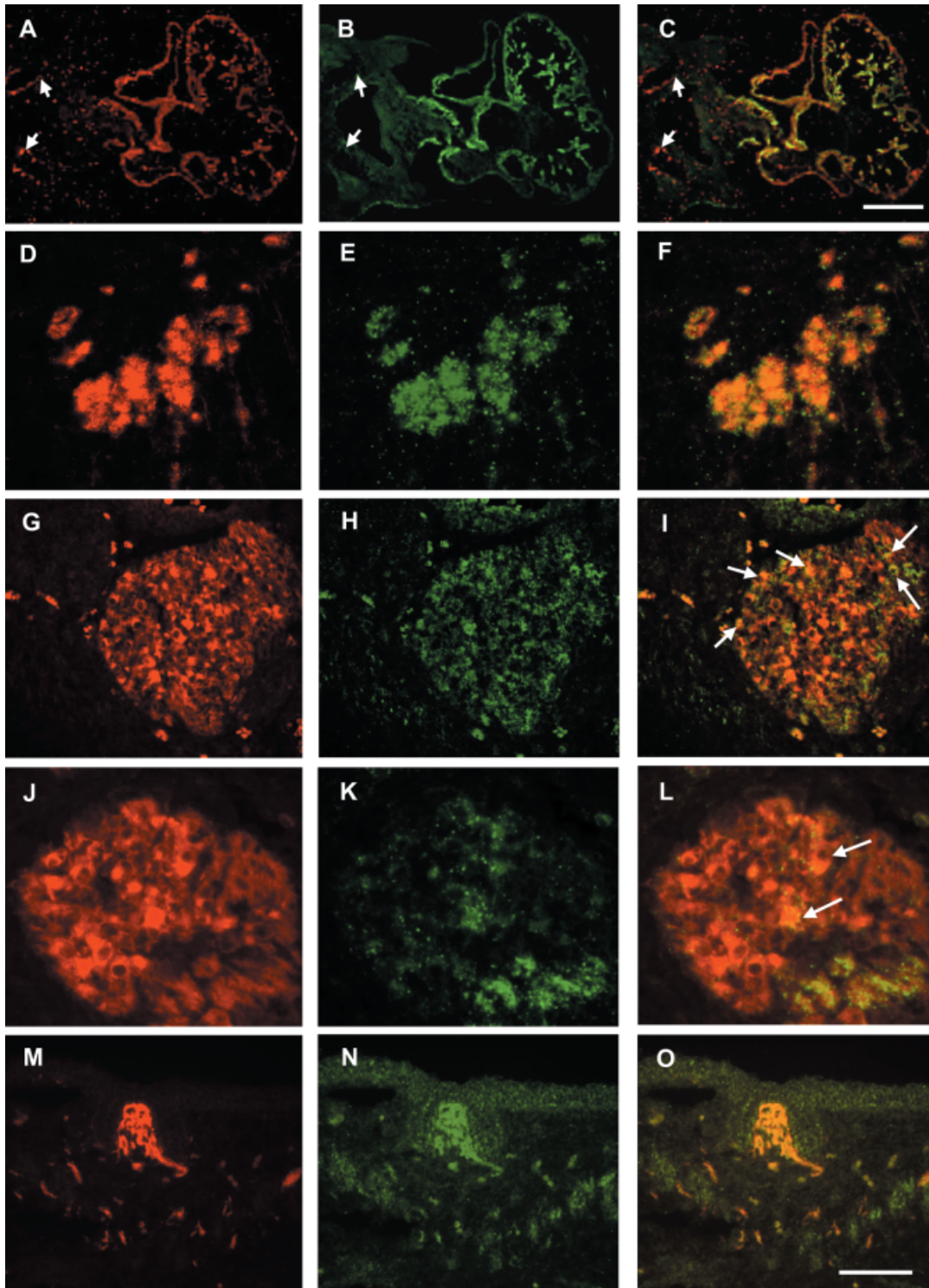
Figure 7

pharyngeal-vagal ganglion complex. At E12.5, the spinal trigeminal tract, which acts as a connection between the peripheral nervous system and CNS in the trigeminal system, showed strong $P2X_3$ immunoreactivity. $P2X_3$ receptors were also found in the vagal trunk and dorsal root ganglia at this stage.

The ganglia mentioned above are either wholly or partially derived from neural crest cells (Le Douarin and Kalcheim, 1999). These ganglia are also involved in sensory function. It is not known whether expression of the $P2X_3$ receptor has any association with neural crest development or whether it is only associated with sensory function. From E14.5 onward, the mesencephalic trigeminal nucleus, the nucleus tractus solitarius and the prepositus hypoglossal nucleus in the CNS showed $P2X_3$ immunoreactivity. Although the mesencephalic trigeminal nucleus belongs to the CNS, it is not derived from the neural tube. Instead, studies on the heterospecific grafting between quail and chick embryos have shown that the mesencephalic trigeminal nucleus is actually derived from neural crest cells in which the precursor cells of the nucleus migrate from the crest toward the ventricular surface of the neuroepithelium (Narayanan and Narayanan, 1978). This finding further increases the association between the neural crest cells and $P2X_3$ receptor expression. $P2X_3$ receptors are constantly expressed in the above ganglia throughout prenatal development (from E11.5 to E18.5), and most of the ganglia still express $P2X_3$ receptor even in adulthood (Xiang et al., 1998, 1999), so they would appear to be involved in development, maturation, and normal sensory functioning of the ganglia. $P2X_3$ receptors were expressed not only in sensory ganglia but also in the superior cervical ganglia and sympathetic ganglia, although the immunostaining appeared scattered and weak. Xiang et al. (1998) showed scattered ganglionic cells expressing $P2X_3$ receptor protein in the superior cervical ganglia, which is consistent with the present study and suggests that the pattern of $P2X_3$ immunoreactivity in the superior cervical ganglion persists from the embryonic stage to adulthood.

$P2X_3$ receptor expression was not only detected in the sensory and sympathetic ganglia but also in the neural tube, which gives rise to the CNS. The results from this study showed that, in early stage embryos, intense $P2X_3$ receptor expression was observed in the anterior part of the hindbrain neural tube (ventral neural tube) and that the intensity of $P2X_3$ immunoreactivity decreased with increasing distance along the spinal cord. The anterior hindbrain neural tube gives rise to the metencephalon, which consists of a dorsal region that develops into the cerebellum and a ventral region that will form the pons. The posterior hindbrain neural tube forms the myelencephalon which gives rise to the medulla oblongata. Our results show that $P2X_3$ receptors are expressed in the ventral part of the prospective metencephalon and myelencephalon, thus, defining the $P2X_3$ immunopositivity in the pons and medulla in the late stage embryos. Later during development, $P2X_3$ receptor protein was expressed in brainstem nuclei such as mesencephalic trigeminal nucleus, nucleus tractus solitarius, and prepositus hypoglossal nucleus. These results are in agreement with a study demonstrating $P2X_3$ immunoreactivity in E16 rat embryos reported by Kidd et al. (1998), except that we did not identify $P2X_3$ receptor expression in the superior and inferior olives. $P2X_3$ receptors were also expressed in the neural layer of the retina and the optic tract that extends all the way to the lateral geniculate in the diencephalon. The $P2X_3$ receptor expression in the retina appeared to be present even in the adult (Brändle et al., 1998). Our results show that $P2X_3$ immunoreactivity was present in the ventral spinal cord at E12.5–E14.5. However, $P2X_3$ receptor expression was not observed in the ventral spinal cord at E16.5. Instead, strong $P2X_3$ immunoreactivity was localized in the dorsal spinal cord. Surprisingly, $P2X_3$ receptors were present in the facial motor nucleus, which is known to be involved in motor function. Our results show that both the ventral spinal cord and facial motor nucleus are down-regulated during prenatal and postnatal development, respectively. Such changes in expression pattern together with the $P2X_3$ receptor expression in the sympathetic nervous system suggests that the $P2X_3$ receptor has a role other than a sensory function during early development of the nervous system. Although intense $P2X_3$ receptor protein expression was detected in the rat embryonic brain and spinal cord, previous studies have shown reduced expression of the $P2X_3$ receptor in the adult rat brain (Kidd et al., 1998). The nucleus tractus solitarius and the spinal cord has been described in the adult rat CNS (Vulchanova et al., 1997; Kidd et al., 1998; Llewellyn-Smith and Burnstock, 1998). In addition, a recent report also described strong $P2X_3$ receptor immuno-

Fig. 7 (Overleaf). Immunofluorescence double labeling for the $P2X_2$ receptor and α-smooth muscle actin (A–C), and for $P2X_2$ and $P2X_3$ receptors (D–O) in rat embryos. **A:** In embryonic day (E) 12.5 heart, the cardiac muscles in the heart and the smooth muscle in the dorsal aorta (arrows) showed α-smooth muscle actin expression (red). **B:** On the same section immunostained with $P2X_2$, the heart also showed $P2X_2$ receptor expression (green). However, no $P2X_2$ immunoreactivity was found in the dorsal aorta, indicated by arrows. **C:** Double-labeling images showed the colocalization of the $P2X_2$ receptor and α-smooth muscle actin in the cardiac muscle but not the dorsal aorta (arrows). **D–F:** $P2X_3$ (D) and $P2X_2$ (E) immunoreactivities in nucleus tractus solitarius in E16.5 embryos. Most of the cells in the nucleus tractus solitarius showed $P2X_3$ and $P2X_2$ coexpression (F), although the staining intensity of $P2X_3$ is much stronger than $P2X_2$ (even after tyramide amplification). **G–I:** $P2X_3$ and $P2X_2$ immunoreactivities in nodose ganglion in E16.5 embryos. Most of the neurons in nodose ganglion showed $P2X_3$ receptor expression (G), whereas only scattered $P2X_2$ receptor expression was detected (H). I: Double-labeling revealed a subpopulation of nodose ganglion showing $P2X_2$ and $P2X_3$ receptor coexpression (arrows). Although $P2X_2$ immunoreactivity was not as strong as that of $P2X_3$, scattered neurons did show $P2X_2$ receptor protein only, instead of $P2X_2$ and $P2X_3$ heteromers. **J–L:** $P2X_3$ and $P2X_2$ immunoreactivities in the E16.5 dorsal root ganglion. Neurons in the dorsal root ganglion showed strong $P2X_3$ receptor expression (J). Only very few neurons in the dorsal root ganglion showed $P2X_2$ immunoreactivity (K), and they coexpressed with $P2X_3$ receptors indicated by arrows (L). K: The $P2X_2$ receptor, appeared to be mainly expressed in spinal nerves instead of the neurons. Note that the stronger $P2X_2$ receptor expression in the spinal nerves compared with $P2X_3$ receptor was due to tyramide amplification. For 3,3′-diaminobenzidine immunostaining, $P2X_3$ receptor protein showed stronger expression than that of the $P2X_2$ receptor (data not shown). **M–O:** $P2X_3$ and $P2X_2$ immunoreactivities in the taste bud in E16.5 embryo. $P2X_3$ and $P2X_2$ receptors were coexpressed in the taste bud in the tongue (O). The nearby peripheral nerves showed $P2X_2$ and $P2X_3$ coexpression, although some of the nerves also showed $P2X_2$ receptor expression only. Scale bar = 100 μm C (applies to A–C). Scale bar in O = 100 μm in G–I,M–O, 50 μm in D–F,J–L.

reactivity in nucleus tractus solitarius, and medial and lateral parabrachial nucleus by using antibodies recognizing the extracellular domain of the $P2X_3$ receptor protein (Yao et al., 2000). In the present study, we show that $P2X_3$ immunoreactivity in the spinal trigeminal tract, facial nucleus, mesencephalic trigeminal nucleus, and vestibular nucleus is absent in P16 rat brain. Together, the results suggest that the expression of the $P2X_3$ receptor is developmentally regulated and the transient expression of $P2X_3$ in these tissues may indicate a role in neurogenesis.

In addition to the CNS, $P2X_3$ immunoreactivity is also found in nerve fibers innervating the developing visceral organs, including tongue, vibrissae, lung, bladder, stomach, and intestine. Chen et al. (1995) claimed that the $P2X_3$ receptor was absent from the lung, bladder, stomach, intestine by using Northern analysis, a technique that is not sensitive for detecting low levels of mRNA expression. Cockayne et al. (2000) have shown recently that $P2X_3$ knockout mice suffered from urinary bladder hyporeflexia, demonstrating the importance of $P2X_3$ receptors in somatic and visceral sensory function.

In the present study, we show that during organogenesis (i.e., E11–E12.5), $P2X_3$ and $P2X_2$ receptors are differentially expressed in embryonic tissues, whereas the $P2X_3$ receptor is expressed in both the central and peripheral nervous system, the $P2X_2$ receptor is mainly expressed in the cardiac muscle and notochord at the level of the spinal neural tube. The notochord, a mesodermally derived structure, is well known for its inductive effect on neural tube patterning (Roelink et al., 1994) in both invertebrates and vertebrates. It is not yet known whether ATP has any effect on spinal cord patterning by means of the $P2X_2$ receptor. Therefore, it would be of interest to investigate in future studies whether there is any interaction between the ATP effects on the neural tube by means of $P2X_3$ and that on the notochord by means of $P2X_2$. The findings from the present study indicate that $P2X_3$ and $P2X_2$ are expressed as homomeric receptors. During embryonic development, $P2X_3$ receptor protein expression is restricted to the nervous system, whereas $P2X_2$ receptor protein is also expressed in developing muscle tissues. The $P2X_2$ receptor is expressed in cardiac muscle, visceral smooth muscle, and in skeletal muscle. A recent study from our laboratory has shown the expression of $P2X_2$ in rat skeletal muscle in the late embryonic stages (Ryten et al., 2001). In this study, although $P2X_3$ and $P2X_2$ homomeric receptors were individually expressed as early as in E11.5, the coexpression of the two receptor subtypes could only be detected clearly at E16.5, which was 5 days later than the first appearance of individual receptor expression. Despite the strong expression of $P2X_3$ receptor found in dorsal root ganglion, only scattered neurons showed $P2X_2$ immunoreactivity in the dorsal root ganglion, which previously has been shown to express heteromeric $P2X_{2/3}$ receptor expression (Lewis et al., 1995; Ueno et al., 1999). The nodose ganglion, which is also known to express $P2X_{2/3}$ receptors, also showed $P2X_2$ and $P2X_3$ coexpression in a subpopulation of neurons at E16.5. These data are in agreement with previous functional studies from our laboratory that showed $P2X_{2/3}$ heteromers are expressed in a subpopulation of nodose ganglia (Lewis et al., 1995; Dunn et al., 2000). Functional studies in the $P2X_3$ knockout animal have shown that it is mainly the $P2X_3$ receptor that responds to ATP in the dorsal root ganglion, whereas the $P2X_2$ and $P2X_{2/3}$ receptors appear more important in the nodose ganglion (Cockayne et al., 2000). In the present study, the distribution in the $P2X_2$ and $P2X_3$ receptors in the dorsal root ganglion and the nodose ganglion seem to be consistent with the functional studies mentioned above. Previous reports have shown that the $P2X_2$ receptor is expressed in the retinal ganglion cells in rat (Greenwood et al., 1997). In contrast, our study shows that $P2X_3$ receptor protein is only detected in the retinal ganglion cells from E14.5 onward; furthermore, $P2X_2$ receptor expression was not detected in the retina at any of the prenatal stages examined (data not shown).

The results obtained may indicate that the role of the $P2X_3$ receptor as a sensory-involving molecule in the retina may be replaced, at least in part, by $P2X_2$ receptor expression during postnatal development. Both the taste buds in the tongue in the peripheral nervous system and the nucleus tractus solitarius in the central nervous system showed a high degree of $P2X_2$ and $P2X_3$ coexpression. In tongue, the median circumvallate papilla showed strong $P2X_3$ and $P2X_2$ immunoreactivity which is in agreement with the results demonstrated by Bo et al. (1999). Therefore, it is not surprising that the nucleus tractus solitarius also showed a high degree of $P2X_2$ and $P2X_3$ coexpression, as the sensory fibers that receive input from the taste cells run in cranial nerves VII, IX, and IX and enter the solitary tract in the medulla. Despite the fact that dorsal root ganglion, nodose ganglion, taste buds, and nucleus tractus solitarius have sensory functions in the peripheral and central nervous systems, different degrees of $P2X_3$ and $P2X_2$ receptor expression in different sensory neurons and nerves account for the different responses reported in functional studies (reviewed by Nörenberg and Illes, 2000). Preliminary immunohistochemical studies in our laboratory have shown that the $P2X_3$ receptor protein is the only receptor subtype among the P2X receptor family present in the early central and peripheral nervous system before E14.5. These data suggest that the homomeric $P2X_3$ is the dominant P2X receptor during early neurogenesis, despite the fact that $P2X_2$ and $P2X_4$ are the dominant receptor subtypes in the adult central nervous system (reviewed by Nörenberg and Illes). In addition, our data also indicate that the $P2X_3$ receptor is expressed as a homomeric receptor rather than the heteromeric $P2X_{2/3}$ form in early stage embryos.

In summary, we have shown a detailed expression pattern of $P2X_3$ in both central and peripheral nervous systems at different stages of rat embryonic development. Strong association was observed between $P2X_3$ expression and neural crest derivatives. The $P2X_3$ receptors appear to have a major role in sensory function in the early stage embryos but these receptors may also be involved in somatic and autonomic motor systems. $P2X_3$ and $P2X_2$ receptors are expressed separately during organogenesis and the $P2X_2$ and $P2X_3$ receptor coexpression appeared later in development than the individual native P2X receptors. Absence of other P2X receptor expression in the nervous system of the early stage embryos indicates that $P2X_3$ may be the only receptor subtype involved in fast excitatory signaling mediated by ATP during embryonic neurogenesis. The precise function of purinergic signaling by means of the $P2X_3$ receptor in the embryo still remains to be elucidated. However, the transient expression of $P2X_3$ receptor protein reported here clearly suggests a role in the development of the nervous system in the mammalian embryo. The involvement of other P2X and P2Y receptor subtypes in early mammalian embryogenesis remains to be explored.

REFERENCES

Abbracchio MP, Burnstock G. 1994. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Ther 64:445–475.

Abbracchio MP, Burnstock G. 1998. Purinergic signalling: pathophysiological roles. Jpn J Pharmacol 78:113–145.

Abe Y, Sorimachi M, Itoyama Y, Furukawa K, Akaike N. 1995. ATP responses in the embryo chick ciliary ganglion cells. Neuroscience 64:547–551.

Bean BP. 1992. Pharmacology and electrophysiology of ATP-activated ion channels. Trends Pharmacol Sci 13:87–90.

Bo X, Alavi A, Xiang Z, Oglesby I, Ford A, Burnstock G. 1999. Localization of ATP-gated P2X$_2$ and P2X$_3$ receptor immunoreactive nerves in rat taste buds. Neuroreport 10:1107–1111.

Brändle U, Guenther E, Irrle C, Wheeler-Schilling TH. 1998. Gene expression of the P2X receptors in the rat retina. Brain Res Mol Brain Res 59:269–272.

Burnstock G. 1996. Purinoceptors: ontogeny and phylogeny. Drug Dev Res 39:204–242.

Burnstock G. 1997. The past, present and future of purine nucleotides as signalling molecules. Neuropharmacology 36:1127–1139.

Burnstock G. 2001. Purinergic signalling in development. In: Abbracchio MP, Williams M editors. Handbook of experimental pharmacology. Vol. 151/I. Purinergic and pyrimidinergic signalling I: Molecular, nervous and urogenitary system function. Berlin: Springer-Verlag. p 89–127.

Chen CC, Akopian AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN. 1995. A P2X receptor expressed by a subset of sensory neurons. Nature 377:428–430.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic A, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford APDW. 2000. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X$_3$-deficient mice. Nature 407:1011–1015.

Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A. Buell G. 1996. Cloning of P2X$_5$ and P2X$_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. J Neurosci 16:2495–2507.

Collo G, Neidhart S, Kawashima E, Kosco-Vilbois M, North RA, Buell G. 1997. Tissue distribution of the P2X$_7$ receptor. Neuropharmacology 36:1277–1283.

Dubyak GR, El Moatassim C. 1993. Signal transduction via P$_2$-purinergic receptors for extracellular ATP. Am J Physiol 265:C577–C606.

Dunn PM, Liu M, Zhong Y, King BF, Burnstock G. 2000. Diinosine pentaphosphate: an antagonist which discriminates between recombinant P2X$_3$ and P2X$_{2/3}$ receptors and between two P2X receptors in rat sensory neurones. Br J Pharmacol 130:1378–1384.

Glass R, Burnstock G. 2001. Immunohistochemical identification of cells expressing ATP-gated cation channels (P2X receptors) in the adult rat thyroid. J Anat 198:569–579.

Greenwood D, Yao WP, Housley GD. 1997. Expression of the P2X$_2$ receptor subunit of the ATP-gated ion channel in the retina. Neuroreport 8:1083–1088.

Kanjhan R, Housley GD, Burton LD, Christie DL, Kippenberger A, Thorne PR, Luo L, Ryan AF. 1999. Distribution of the P2X$_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system. J Comp Neurol 407:11–32.

Kaufman MH. 1992. The atlas of mouse development. London: Academic Press.

Kaufman MH, Bard JBL. 1999. The anatomical basis of mouse development. London: Academic Press.

Kennedy C, Burnstock G. 1985. Evidence for the presence of two types of P2 purinoceptor in the longitudinal muscle of rabbit portal vein. Eur J Pharmacol 111:49–56.

Kidd EJ, Grahames CB, Simon J, Michael AD, Barnard EA, Humphrey PPA. 1995. Localization of P2X purinoceptor transcripts in the rat nervous system. Mol Pharmacol 48:569–573.

Kidd EJ, Miller KJ, Sansum AJ, Humphrey PPA. 1998. Evidence for P2X$_3$ receptors in the developing rat brain. Neuroscience 87:533–539.

King BF, Burnstock G, Boyer JL, Boeynaems J-M, Weisman GA, Kennedy C, Jacobson KA, Humphries RG, Abbracchio MP, Miras-Portugal MT.

2001. The P2Y receptors. In: Girdlestone D, editor. The IUPHAR compendium of receptor characterization and classification. London: IUPHAR Media, Ltd. (in press).

Laasberg T. 1990. Ca^{2+}-mobilizing receptors of gastrulating chick embryo. Comp Biochem Physiol C 97:1–12.

Le Douarin NM, Kalcheim C. 1999. The neural crest. 2nd ed. Cambridge: Cambridge University Press.

Lewis C, Neidhart S, Holy C, North RA, Buell G, Suprenant A. 1995. Coexpression of P2X$_2$ and P2X$_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–434.

Llewellyn-Smith IJ, Burnstock G. 1998. Ultrastructural localization of P2X$_3$ receptors in rat sensory neurons. Neuroreport 9:2545–2550.

Meyer MP, Clarke JDW, Patel K, Townsend-Nicholson A, Burnstock G. 1999a. Selective expression of purinoceptor cP2Y1 suggests a role for nucleotide signalling in development of the chick embryo. Dev Dyn 214:152–158.

Meyer MP, Gröschel-Stewart U, Robson T, Burnstock G. 1999b. Expression of two ATP-gated ion channels, P2X$_5$ and P2X$_6$, in developing chick skeletal muscle. Dev Dyn 216:442–449.

Narayanan CH, Narayanan Y. 1978. Determination of the embryonic origin of the mesencephalic nucleus of the trigeminal nerve in birds. J Embryol Exp Morphol 43:85–105.

Nörenberg W, Illes P. 2001. Neuronal P2X receptors: localisation and functional properties. Naunyn Schmiedebergs Arch Pharmacol 362: 324–339.

North RA. 1996. P2X purinoceptor plethora. Semin Neurosci 8:187–194.

Oglesby IB, Lachnit WG, Burnstock G, Ford APDW. 1999. Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, P2X$_1$ through P2X$_7$. Drug Dev Res 47:189–195.

Ralevic V, Burnstock G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492.

Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz I, Altaba A, Tanabe Y, Placzek M, Edlund T, Jessell TM, Dodd J. 1994. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell 76:761–775.

Ryten M, Hoebertz A, Burnstock G. 2001. Sequential expression of three receptor subtypes for extracellular ATP in developing rat skeletal muscle. Dev Dyn 221:331–341.

Sawtell NM, Lessard JL. 1989. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the α-vascular but not the γ-enteric isoform in differentiating striated myocytes. J Cell Biol 109:2929–2937.

Soto F, Garcia-Guzman M, Gomez-Hernandez JM, Hollmann M, Karschin C, Stühmer W. 1996. P2X$_4$: an ATP-activated ionotropic receptor cloned from rat brain. Proc Natl Acad Sci U S A 93:3684–3688.

Ueno S, Tsuda M, Iwanaga T, Inoue K. 1999. Cell type-specific ATP-activated responses in rat dorsal root ganglion neurons. Br J Pharmacol 126:429–436.

Virginio C, North RA, Suprenant A. 1998. Calcium permeability and block at homomeric and heteromeric P2X$_2$ and P2X$_3$ receptors, and P2X receptors in rat nodose neurones. J Physiol 510:27–35.

Vulchanova L, Riedl MS, Shuster SJ, Buell G, Surprenant A, North RA, Elde R. 1997. Immunohistochemical study of the P2X$_2$ and P2X$_3$ receptor subunits in rat and monkey sensory neurons and their central terminals. Neuropharmacology 36:1229–1242.

Wells DG, Zawisa MJ, Hume RI. 1995. Changes in responsiveness to extracellular ATP in chick skeletal muscle during development and upon denervation. Dev Biol 172:585–590.

Woodcock-Mitchell J, Mitchell JJ, Low RB, Kieny M, Sengel P, Rubbia L, Skalli O, Jackson B, Gabbiani G. 1988. α-Smooth muscle actin is transiently expressed in embryonic rat cardiac and skeletal muscles. Differentiation 39:161–166.

Xiang Z, Bo X, Burnstock G. 1998. Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. Neurosci Lett 256:105–108.

Xiang Z, Bo X, Burnstock G. 1999. P2X receptor immunoreactivity in the rat cochlea, vestibular ganglion and cochlear nucleus. Hear Res 128: 190–196.

Yao ST, Barden JA, Finkelstein DI, Bennett MR, Lawrence AJ. 2000. Comparative study on the distribution patterns of P2X$_1$-P2X$_6$ receptor immunoreactivity in the brainstem of the rat and the common marmoset (Callithrix jacchus): association with catecholamine cell groups. J Comp Neurol 427:485–507.

Zhong Y, Dunn PM, Burnstock G. 2000. Pharmacological comparison of P2X receptors on rat coeliac, mouse coeliac and mouse pelvic ganglion neurons. Neuropharmacology 39:172–180.