Purinergic Signaling during Rat Embryonic Development

by

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Abstract

Adenosine 5'-triphosphate (ATP) has been shown to be an important extracellular signaling molecule that mediates various physiological activities via the P2 (P2X and P2Y) receptors. However, information on the expression patterns of the P2 receptors during mammalian embryogenesis is limited. We therefore examined the expression patterns of different P2 receptor subtypes in rat embryos. In the hindbrain neural tube, the $P2X_3$ receptor was transiently expressed at embryonic day E11 in the cranial motor neurons and the outgrowing axons. ATP significantly inhibited neurite outgrowth from neural tube explants. $P2X_3$ receptors were also prominently expressed in sensory ganglia at this early stage and were coexpressed with $P2X_2$ receptors in E16.5 embryos. Other P2X receptor subtypes were observed in different brain regions such as subventricular zones, the site of postnatal neurogenesis. In addition, the P2Y receptor expression was detected in the somites and subsequently in the developing skeletal muscle but was downregulated as development proceeded. While the $P2Y_1$ receptor was no longer expressed in the adult skeletal muscle, the expression of $P2Y_2$ receptor was present, although restricted in the satellite cells and the $P2Y_4$ receptor showed reduced expression in adult skeletal muscle. Likewise, the expression of the P2Y receptors was initially expressed throughout the myocardium (E12) but was gradually restricted to the trabeculated myocardium (E14-18). Studies on Ca^{2+} influx showed that

particular P2 receptor subtypes of $P2X_2$, $P2X_4$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors responded to nucleotides in E14 cardiomyocytes. $P2X_7$ receptor expression was detected in developing pancreatic islet cells and later coexpressed with glucagon in α-cells. In addition, transient expression of the $P2X_7$ receptor in insulin-expressing cells was observed in the embryonic, but not in adult, islet cells. Together, the results indicated that widespread and dynamic expression of P2 receptors was found in the three-germ layer-derived embryonic tissues, particularly in some transient embryonic structures during development, which suggested they may be important in embryonic organogenesis

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Abbreviations

2MeSADP	2-Methylthio- adenosine 5'-diphosphate
2MeSATP	2-Methylthio- adenosine 5'-triphosphate
ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
ATP	Adenosine 5'-triphosphate
ATPγS	Adenosine 5'-O-(3-thio)triphosphate
BzATP	2',3'-O-(4-benzoylbenzoyl)- Adenosine 5'-triphosphate
CNS	Central Nervous System
E	Embryonic day
FLIPR	Fluorometric Imaging Plate Reader
IgG	Immunoglobulin G
IP3	Inositol triphosphate
KN62	1-(N,O-bis[5-isoquinolinesulphyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine
MAPK	Mitogen Activated Protein Kinase
MRS2179	2'-deoxy-N^6-methyladenosine-3',5'-bisphosphate
NANC	Nonadrenergic noncholinergic
NF023	8,8'-(carbonylbis(imino-3,1-phenylene carbonylimino)bis(1,3,5-naphthalenetrisulfonic acid)
P	Postnatal day
PGP9.5	Protein Gene Product 9.5
PKC	Protein Kinase C
PLC	Phospholipase C
PNS	Peripheral Nervous System
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Shh	Sonic Hedgehog
TNP-ATP	2',3'-O-(2,4,6-trinitrophenyl)- Adenosine 5'-triphosphate
UDP	uridine 5'-diphosphate
UTP	uridine 5'-triphosphate
αβ-meATP	αβ-methylene ATP
β,γ-meATP	β,γ-methylene ATP

OUTLINE OF CONTENTS

I. General Introduction

I.1 Brief historical perspective on purinergic signaling

I.1.a ATP as an extracellular signaling molecule

Adenosine triphosphate (ATP), a molecule which belongs to the purine family, was first identified in muscle cells independently by Fiske and Subbarow and by Lohmann in 1929 (see Schlenk, 1987; Maruyama, 1991). The chemical structure of ATP is made of a nitrogenous base (adenine), a sugar (ribose) and a chain of three ionized groups (phosphates) bound to the ribose (see Bodin and Burnstock, 2001a). In the field of biochemistry, the most important value of ATP lies on its phosphate groups, which can undergo hydrolytic release in aqueous solution with large negative free energy changes. Therefore ATP was thought of largely as a store of free energy in the cell soon after its discovery, and even till now. Hydrolysis of ATP is used to drive innumerable biochemical reactions, including many that are not phosphorylations. It is a source of energy for cell motility, muscle contraction, and the specific transport of substances across membranes (Mathews and van Holde, 1990). ATP was generated during anaerobic glycolysis in the cytoplasm, within the citric cycle and predominantly during oxidative phosphorylation in the mitochondria (Kalckar, 1969). Hydrolysis of ATP can proceed in either of two ways. Either the terminal phosphate can be cleaved off to produce adenosine diphosphate (ADP) and phosphate (P_i), or cleavage may occur at the second phosphodiester bond to yield adenosine monophosphate

(AMP) and pyrophosphate (PP_i). The former reaction is the more common one in vivo, but the free energy yield is about the same for both. As a result, ATP soon appeared as the most fundamental of all biomolecules and the universal source of readily available chemical energy for all living cells. It has been estimated that ATP participates in more chemical reactions than any other compound on the earth's surface, except water (Burnstock, 1996).

Because of this essential intracellular role, there was a considerable resistance to the concept that ATP could exert any extracellular physiological action, the main argument being that cells would not release a bio-molecule so fundamental to life, a line of thought which was reinforced by the fact that a molecule the size and charge of ATP could not cross the plasma membrane by simple diffusion (Glynn, 1968; Chaudry, 1982). Very soon after the discovery of ATP, however, it became apparent that purine nucleotides were able to exert profound extracellular effects on different organs and tissues.

In the same year of ATP discovery, Drury and Szent-Györgyi (1929) reported the first time that extracellular purines were responsible for a negative chronotropic effect on the heart, a dilatation of coronary vessels and an inhibition of the spontaneous activity of the intestinal smooth muscle, though the crude tissue extracts used contained mostly AMP (see Vassort, 2001). In 1959, Holton showed that ATP was released to the rabbit ear artery

during antidromic stimulation of sensory nerve, resulting in vasodilatation. Hughes and Vane (1967, see Burnstock, 1979) also noted that the responses of the rabbit portal vein to ATP resembled the inhibitory responses to the nonadrenergic, noncholinergic (NANC) intramural nerve stimulation. The NANC nerves, which were characterized by the responses upon the stimulation in the presence of adrenergic and cholinergic neuron blocking agents (or in the absence of adrenergic and cholinergic stimulation), were first defined in 1963 (Burnstock et al, 1963, 1964; Martinson and Muren 1963), although atropine-resistant responses of the urinary bladder were described as far back as 1895 by Langley and Anderson (see Burnstock, 1986). Before ATP was identified to be the actual transmitter for NANC stimulation, other biochemicals were explored as possible candidates, e.g. catecholamines, 5-hydroxytryptamine, cyclic adenosine monophosphate (cAMP), histamine, prostaglandins, amino acids such as glycine and γ–aminobutyric acid (GABA), and polypeptides bradykinin and substance P. However, these substances were rejected by most researchers on the grounds that they were either inactive or did not mimic the nerve-mediated response, or that their action was by stimulation of nerves and not by direct action on smooth muscle (Burnstock et al., 1970). Evidence that ATP was synthesized, stored and released from NANC nerves supplying the intestinal smooth muscle, together with considerable additional evidence available, led Burnstock to propose that ATP was a primary neurotransmitter in NANC nerves in the gut and bladder and thus put forward the ***purinergic***

nerve hypothesis (Burnstock, 1972). In the following years, the purinergic signaling was described in many other tissues and systems (see Burnstock, 1979, 1997).

I.1.b Source and mechanisms of ATP release

Considering that ATP functions as an extracellular signaling molecule, it must have a source of release. Both neuronal and non-neuronal cells have been shown to release ATP. A pilot study for neuronal ATP release has shown that ATP was released from the nerve endings upon antidromic stimulation of the sensory nerve (Holton, 1959). However, it was not until Burnstock (1972) proposed the purinergic hypothesis that ATP became a clear candidate for a neurotransmitter. Burnstock (1976) challenged the principle that nerve cells released one transmitter; his group had shown that ATP might be released from sympathetic nerves, together with noradrenaline in taenia coli (Su et al., 1971), and much supporting evidence has accumulated since then (see Burnstock, 1990, 1999a). Moreover, ATP was shown early on to act as a cotransmitter with acetylcholine in the bladder (Burnstock et al., 1978) and with noradrenaline in the cat nictitating membrane (Langer and Pinto, 1976), vas deferens (Vizi and Burnstock, 1988; Westfall et al., 1978), the rat tail artery (Sneddon and Burnstock, 1984) and the aorta (Sedaa et al., 1990).

There have been an increasing number of studies describing ATP release from

non-neuronal cells. The first hint of ATP release from non-neuronal cells was shown in the circulation of the occluded human forearm in response to effort (Forrester and Lind, 1969). Direct proof came from the investigation on isolated cells present in the vascular system such as platelets (Detwiler and Feinman, 1973), red blood cells (Bergfeld and Forrester, 1989), mononuclear cells (Maugeri et al., 1990), endothelial cells and smooth muscle cells (Pearson and Gordon, 1979; Bodin et al., 1991), which all release ATP mostly upon mechanical stimulation. Other cell types shown to release ATP, including distended uroepithelial cells (Ferguson et al., 1997), pancreatic β cells (Hazama et al., 1998), astrocytes (Contrina et al., 1998), glial cells (Queiroz et al., 1999), Sertoli cells (Lalevée et al., 1999) and chondrocytes (Graff et al., 2000). ATP can be released from non-neuronal cells by a large variety of stimuli, including, among others, hypoxia, acidosis, osmotic shock, receptor stimulation, fluid shear stress and mechanical stimulation (Detwiler and Feinman, 1973; Bergfeld and Forrester, 1989; Bodin and Burnstock, 1996). Recent findings identify transport proteins belonging to the ATP-binding cassette (ABC) family that transport ATP or at least stimulate the activity of a separate ATP channel protein (Schwiebert, 1999). The most common ABC proteins are the cystic fibrosis transmembrane conductance (CFTR), P-glycoprotein and the sulfonylurea receptor (SUR) (see Bodin and Burnstock, 2001a).

I.2 Purinergic receptors

I.2.a Classification of purinoceptors

Extracellular purines and pyrimidines mediate diverse physiological activities by acting on cell surface receptors termed purinergic receptors or purinoceptors (see Abbracchio and Burnstock, 1998). There are two main families of purine receptors, adenosine or P1 receptors, and P2 receptors, recognizing primarily ATP, ADP, UTP and UDP (Burnstock, 1978). Adenosine or P1 receptors have been further subdivided, according to convergent molecular, biochemical, and pharmacological evidence into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, all of which couple to G-proteins (see Ralevic and Burnstock, 1998). Similarly, P2 receptors, based on differences in molecular structure and signal transduction mechanisms were divided into two families: the ligand-gated ion channels (P2X receptors) and G protein-coupled receptors (P2Y receptors) (Abbracchio and Burnstock, 1994). To date, seven P2X receptors subtypes and eight P2Y receptors have been cloned (Burnstock, 2003). Individual subtypes of P2X and P2Y receptors will be discussed separately, with emphasis on the extent of homology between members within the family, tissue distributions (in rat, unless specified) and pharmacological characteristics of individual receptors.

I.2.b P2X receptors

P2X receptors are ligand-gated ion channels opened by micromolar extracellular ATP. The receptors mediate rapid (within 10 ms) and selective permeability to cations (Na^+, K^+,

Ca^{2+}). To date, seven P2X receptor subunits ($P2X_{1-7}$) currently known are encoded by different genes. Each subunit has a topology that is fundamentally different from that of other known ligand-operated channels. P2X receptors are made of proteins, with 379-595 amino acids, inserted in the membrane to form a pore comprising two hydrophobic transmembrane domains with a large extracellular hydrophilic loop. The NH_2 and COOH termini are presumed to be cytoplasmic. The actual P2X receptors are formed from either homomeric or heteromultimeric association of the subunits. The putative extracellular loop of cloned $P2X_{1-7}$ has 10 conserved cysteine and 14 conserved glycine residues as well as 2 to 6 potential N-linked glycosylation sites. Most of the conserved regions are in the extracellular loop, with the transmembrane domains being less well-conserved. Taken together both the extracellular loop and the transmembrane domains, the proteins are from 40 to 55% pairwise identical. The $P2X_4$ sequence is most closely related to more of the other forms, and the $P2X_7$ sequence is least like the others; these observations are true whichever species are considered (see North, 2002). The disulfide bridges present in the extracellular loop are believed to form the structural constraints needed to couple the ATP-binding site to the ion pore. As mentioned above, all the P2X receptor subunits have consensus sequences for N-linked glycosylation (Asn-X-Ser/Thr). Receptors in which any two of the three sites are glycosylated appear at the cell surface and are fully functional, whereas those in which only one site is glycosylated give barely detectable currents in response to ATP, and channels

with no sites glycosylated give no current (see North, 2002).

The recombinant receptors formed from homomeric association of subunits, when expressed in Xenopus oocytes or in mammalian cells have been functionally characterized and show distinct pharmacological profiles (see Ralevic and Burnstock, 1998). Almost all known ionotropic receptors exist as hetero-oligomers (Barnard, 1992). More importantly, the functional and pharmacological characteristics of the receptors are directly determined by the subunit composition, with different subunit combinations yielding different phenotypes (Barnard et al., 1998). Functional studies of native receptors, together with patterns of subunit gene expression, suggest heteromultimeric assembly among members of the P2X receptors (Cook et al., 1997; Lewis et al., 1995). Studies by coimmunoprecipitation have also determined which pairs of subunits are potentially able to coassemble, as not all combinations are possible (**Table 1**) (Torres et al., 1999). Briefly, with the exception of $P2X_6$, all the other P2X receptor subunits formed homo-oligomeric complexes. When co-assembly between pairs of subunits is examined, all are able to form hetero-oligomeric assemblies with the exception of $P2X_7$ receptor. $P2X_5$ receptor is the only receptor subunit that is able to form hetero-oligomeric complexes with all the members within the family.

I.2.b.i $P2X_1$ *receptor*

Using a cDNA library generated from rat vas deferens, the first cDNA encoding the $P2X_1$ receptor was isolated by direct expression in Xenopus oocytes. (Valera et al., 1994). The recombinant receptor, having a single open reading frame encoding 399 amino acids, is activated by agonists with the order of potency as 2MeSATP ≥ ATP > αβ-meATP >> ADP and UTP, and inward currents evoked by these compounds are reversibly blocked by general P2 receptor antagonists like suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Valera et al., 1994). Sustained application of agonist to $P2X_1$ receptors results in rapid channel opening (few milliseconds), fast desensitization (300 ms) and receptor internalization (1-3 min). The receptors reappear at the cell surface soon after agonist application is terminated (see North, 2002).

$P2X_1$ receptor mRNA is expressed in urinary bladder, smooth muscle layers of small arteries and arterioles, and vas deferens, with lower levels in lung and spleen (Valera et al., 1994; Collo et al., 1996). The $P2X_1$ receptor seems to be the most significant P2X receptor subtype in vascular smooth muscle, which was revealed by the similar electrophysiological profiles and pharmacological properties observed in smooth muscle (Bean, 1992). Therefore, it is believed that the vascular smooth muscle P2X receptor is a $P2X_1$ receptor homomer (see Ralevic and Burnstock, 1998). In addition, the $P2X_1$ receptor is also

expressed in myocardium (Hou et al., 1999) and nervous tissues including dorsal root ganglia, trigeminal ganglia, celiac ganglia, spinal cord, and rat brain (Valera et al., 1994, Kidd et al., 1995, Collo et al., 1996; Xiang et al, 1998, Kukley et al., 2001).

1.2.b.ii $P2X_2$ receptor

The $P2X_2$ receptor was first cloned from rat pheochromocytoma PC12 cells (Brake et al., 1994). The homology between $P2X_2$ receptor (472 amino acids) and other members of the family is shown in **Table 2**. ATP, ATP-γS and 2MeSATP are approximately equipotent for $P2X_2$ receptor at eliciting non-selective inward cation currents, whereas αβ-meATP and β,γ-meATP are inactive as agonists or antagonists (Brake et al., 1994). However, there are no agonists or antagonists currently known that are selective for $P2X_2$ receptors. Nevertheless, $P2X_2$ receptor has a special feature of its marked sensitivity to change in pH which makes it distinct from the other P2X subtypes, even from P2Y and P1 receptors (King et al., 1996). When expressed in the oocytes, the receptor shows a maximum response to ATP at pH 6.5. The $P2X_2$ receptor undergoes little or no desensitization. It also differs from the $P2X_1$ receptor in that it is less permeable to Ca^{2+} and shows much higher sensitivity to inhibition by extracellular Ca^{2+} (Evans et al., 1996). Apart from Ca, other divalent cations cause a fast block of the single $P2X_2$ channels with the order of potency as Mn > Mg > Ca > Ba, which is the order of ionic radii. This suggests that the divalent ions are binding

to a charged site within the channel (Ding and Sachs, 1999; 2000). The divalent ions can also inactivate the channel with the order of effectiveness as Ca> Mg > Ba > Mn (Ding and Sachs, 2000).

$P2X_2$ receptor mRNA is distributed in bladder, brain, spinal cord, superior cervical ganglia, adrenal medulla, intestine and vas deferens, with highest levels found in the pituitary gland and vas deferens (Brake et al., 1994). Recently, expression was also reported in retina (Greenwood et al., 1997), cochlea (Housley et al., 1999), vascular smóoth muscle (Nori et al., 1998), thymus (Glass et al., 2000), osteoclasts (Hoebertz et al., 2000), sympathetic ganglia (Schadlich et al., 2001) and skeletal muscle fiber (Ryten et al., 2001). Distinct but restricted patterns of distribution have been described in rat brain (see Nörenberg and Illes, 2000).

A splice variant of a $P2X_2$ receptor has been isolated from rat cerebellum (Brändle et al., 1997; Simon et al., 1997). The receptor protein (called $P2X_{2b}$) has a 69 amino acid deletion of the carboxyl-terminal, shows a similar distribution in the rat central and peripheral nervous system as the original $P2X_2$ receptor, and forms homomeric receptor mediating inward currents to ATP. Although they are equipotent to agonists, $P2X_{2b}$ showed significantly lower antagonist sensitivity and a faster rate of desensitization compared to $P2X_2$ receptor.

1.2.b.iii $P2X_3$ receptor

The $P2X_3$ receptor subunit cDNAs were cloned from rat dorsal root ganglion (Chen et al., 1995; Lewis et al., 1995) and was predicted to be 397 amino acids, with the amino acid sequence exhibiting different degree of identity with other members of the family (**Table 2**). $P2X_3$ receptor shows similar profiles with $P2X_1$ receptor upon agonist activation. It is rapidly activated by agonists with a potency order of 2MeSATP >> ATP > αβ-meATP > ATP-γS >> ADP >> UTP ~ β,γ-meATP, and undergoes rapid desensitization with slow recovery in high ATP concentration (in less than 100ms). These shared characteristics makes the two homomeric receptors distinct from the other homomeric forms. The antagonists suramin, PPADS, and TNP-ATP do not readily distinguish between $P2X_1$ and $P2X_3$ receptors, but NF023 is approximately 20 times less effective at $P2X_3$ than $P2X_1$ receptors. The $P2X_3$ receptor is remarkably insensitive to block by extracellular calcium (Virginio et al., 1998). In contrast, increasing the extracellular Ca^{2+} concentration can greatly accelerate the recovery from desensitization (Cook and McCleskey, 1997; Cook et al., 1998).

The $P2X_3$ receptor has a very restricted tissue distribution in the nervous system. It is expressed only by a subset of sensory neurons (trigeminal, nodose, and dorsal root ganglia), most of them are capsaicin-sensitive neurons involved in nociception (Chen et al., 1995;

Lewis et al., 1995; Vulchanova et al., 1998; Xiang et al., 1998). Accordingly, $P2X_3$ protein or its mRNA was found in cell bodies of sensory neurons, and in their central termination fields (dorsal horn of the spinal cord, nucleus of the solitary tract), as well as in their peripheral endings, e.g. in taste buds and the spiral ganglion of the inner ear. However, $P2X_3$ was also found in other areas such as supraoptic nucleus and sympathetic ganglia (see Nörenberg and Illes, 2000), retina (Wheeler-Schilling et al., 2001) and endothelial cells (Glass and Burnstock, 2001).

However, in other areas, such as certain sensory neurons in the central and peripheral nervous system and sympathetic ganglion cells, the action of ATP is mimicked by αβ-meATP, but slow desensitization. This type of response is later confirmed to be generated by heteromeric $P2X_{2/3}$ receptor (Lewis et al., 1995; Radford et al., 1997; Torres et al., 1999). The heteromer shares some properties with homomeric $P2X_2$ receptor: it is potentiated by low pH, and shows low desensitization. On the other hand, the heteromeric $P2X_{2/3}$ receptor is activated by αβ-meATP, and blocked by TNP-ATP as well as PPADS and suramin, all of which are the properties of $P2X_3$ homomer.

1.2.b.iv $P2X_4$ receptor

The cDNAs for the rat $P2X_4$ receptor has been cloned from rat hippocampus (Bo et al.,

1995) and subsequently from rat brain (Séguéla et al., 1996; Soto et al., 1996), superior cervical ganglia (Buell et al., 1996) as well as pancreatic islet cells (Wang et al., 1996). The amino acid identity between $P2X_4$ receptor (388 amino acids) and other members of the family is shown in **Table 2**. The $P2X_4$ receptor is activated by ATP and partially by 2-MeSATP, and αβ-meATP is either weak or inactive, whereas ADP, UTP and βγ-meATP are inactive. Expression of $P2X_4$ receptor gives an ATP-activated cation-selective channel that has a significantly high Ca^{2+} permeability, and whose agonist sensitivity is increased by Zn^{2+} without altering maximal response (Soto et al., 1996). Desensitization at $P2X_4$ receptors is intermediate between that observed at $P2X_1$ and $P2X_2$ receptor (Séguéla et al., 1996).

The rat $P2X_4$ receptor, but not its human homologue, is unusual in its low sensitivity to blockade by the widely used ATP antagonists, suramin, Reactive blue2 and PPADS and indeed, responses to ATP are even potentiated (Bo et al., 1995). A lysine residue present in the $P2X_1$ and $P2X_2$ receptors, but absent in the $P2X_4$ receptor, is believed to be critical for the binding of antagonists but not agonists (Buell et al., 1996).

$P2X_4$ receptor expression is widely distributed in the brain and other tissues such as spinal cord, sensory ganglia, superior cervical ganglion, lung, bronchial epithelium, thymus, bladder, acinar cells of the salivary gland, adrenal gland, testis and vas deferens (Bo et al.,

1995; Buell et al., 1996; Collo et al., 1996; Séguéla et al., 1996). Recent studies have shown that $P2X_4$ receptor also expressed in vascular smooth muscle (Nori et al., 1998), inner ear tissues (Brändle et al., 1999), pancreas (Hede et al., 1999), oesteoclasts (Hoebertz et al., 2000), endothelial cells and follicular cells in thyroid (Glass and Burnstock, 2001) and retina (Brändle et al., 1998; Wheeler-Schilling et al., 2000; Wheeler-Schilling et al., 2001).

1.2.b.v $P2X_5$ receptor

The $P2X_5$ receptor cDNA was first isolated from cDNA libraries constructed from rat celiac ganglion and heart (Collo et al., 1996; Garcia-Guzman et al., 1996). The extent of amino acid identity between the 417 amino acid-$P2X_5$ receptor protein and the members within the family is shown in **Table 2**. Rapid inward currents are activated by ATP > 2MeSATP > ADP. The most striking feature of the current elicited by ATP in cells expressing the rat $P2X_5$ receptor is their small amplitude, compared with the currents observed with $P2X_1$, $P2X_2$, $P2X_3$, or $P2X_4$ receptors expressed under similar conditions. However, $P2X_5$ receptor shared some similar features with $P2X_2$ receptors: they show little desensitization, are not activated by αβ-meATP, and are blocked by suramin and PPADS at concentrations similar to those effective at $P2X_2$ receptors (Collo et al., 1996; Garcia-Guzman et al., 1996).

$P2X_5$ receptor shows a very restricted expression pattern in the brain, where the expression was only found in the mesencephalic trigeminal nucleus and pineal gland (Collo et al., 1996). In the peripheral nervous system, expression was also detected in the ventral horn of the cervical spinal cord (Collo et al., 1996), sensory ganglia (Xiang et al., 1998, 1999), sympathetic neurons (Schadlich et al., 2001), retinal ganglion cells and (Wheeler-Schilling et al., 2001). Other tissues expressing $P2X_5$ receptor are stratified squamous epithelia of the skin (Gröschel-Stewart et al., 1999a), duodenal globet cells (Gröschel-Stewart et al., 1999b), developing skeletal muscle (Ryten et al., 2001), vascular smooth muscle cells and the follicular cells in the thyroid (Glass and Burnstock, 2001).

Recent papers have reported the existence of heteromeric $P2X_{1/5}$ receptors with unique features (Torres et al., 1998; Haines et al., 1999; Surprenant et al., 2000). The heteromeric receptor is more sensitive to ATP than those with homomeric receptors. Although the $P2X_{1/5}$ receptor is less sensitive to αβ-meATP than the homomeric $P2X_1$ receptor, the heteromer shows a sustained current evoked by αβ-meATP, which is not seen for either of the homomers when expressed separately. The currents generated by the heteromeric receptor are considerably larger than those seen with $P2X_5$ subunits expressed alone, and the current is inhibited by either an increase or a decrease of the extracellular pH. Furthermore, $P2X_{1/5}$ heteromer is much less permeable to Ca^{2+} than the $P2X_1$ homomer.

1.2.b.vi $P2X_6$ receptor

The cDNA for $P2X_6$ receptor was isolated from a rat superior cervical ganglion cDNA library (Collo et al., 1996). The predicted receptor protein has 379 amino acids and the sequence shows homology with other P2X receptors (**Table 2**). Currents evoked by ATP in $P2X_6$ receptor-expressing cells resembled those seen for the $P2X_2$ receptor. Rapid currents are mediated by ATP > 2MeSATP > ADP with little desensitization, whereas αβ-meATP has no effect. Currents are only partially inhibited by antagonist such as suramin or PPADS. Although the currents in cells expressing $P2X_6$ receptor were large, they were observed in only a small fraction of cells examined.

The mRNA transcript of $P2X_6$ receptor is heavily expressed in the CNS, with heaviest staining in cerebellar Purkinje cells and ependyma. The mRNA is also present in the spinal cord, trigeminal, dorsal root and celiac ganglia. In non-neuronal tissues, $P2X_6$ receptor mRNA is prominent in gland cells of the uterus, granulose cells of the ovary, and bronchial epithelia (Collo et al., 1996). Immunoreactivity of the $P2X_6$ receptor protein has been shown in superior cervical and celiac ganglia (Xiang et al., 1998), thymic epithelial cells (Glass et al., 2000), vascular smooth muscle (Glass and Burnstock, 2001) and developing skeletal muscle (Ryten et al., 2001).

Coimmunoprecipitation studies have shown that $P2X_6$ receptor has a very low tendency to form homomeric ion channel due to failure of self co-precipitation (Torres et al., 1999). Instead, accumulated evidence has suggested the presence of two $P2X_6$-possessing heteromeric ion channels, the $P2X_{2/6}$ and $P2X_{4/6}$ heteromeric receptors. Cells expressing the $P2X_{2/6}$ receptors have subtly different responses to ATP than those expressing $P2X_2$ receptors only (King et al., 2000), with the most convincing one being the biphasic inhibition of the current by suramin at pH 6.5; one component has the high sensitivity of homomeric $P2X_2$ receptor whereas the other is less sensitive. For the $P2X_{4/6}$ heteromers, the currents elicited by ATP are larger in cells transfected with $P2X_4$ and $P2X_6$ receptor mRNA than those transfected with $P2X_4$ only (Le et al., 1998).

1.2.b.vii $P2X_7$ receptor

The $P2X_7$ receptor of 595 amino acids cloned from rat macrophages and brain (Surprenant et al., 1996) is the cytolytic P_{2Z} receptor previously described in mast cells, macrophages, fibroblasts, lymphocytes, erythrocytes and erythroleukemia cells. The recombinant receptor has an agonist potency order of BzATP >> ATP >> 2MeSATP > ATP-γS > ADP, whereas αβ-meATP and βγ-meATP are ineffective. The $P2X_7$ receptor, though insensitive to suramin and PPADS, is inhibited by divalent cations, KN62 and

oxidized ATP (see Ralevic and Burnstock, 1998). The unique feature of $P2X_7$ receptors is that under normal physiological conditions, the receptors are selectively permeable to small cations only, whereas in the continued presence of ATP and when divalent cation levels are low, the ion channels can convert to a pore, permeable to both molecules and ions. The currents evoked do not readily desensitize. Instead, longer applications of either BzATP or ATP result in increased permeability to larger organic cations, and the currents take longer to decline after each application. Prolonged stimulations may also result in cell swelling, vacuolization, and cell death by necrosis or apoptosis (Dubyak and el-Moatassim, 1993). $P2X_7$ receptor is structurally similar to other P2X receptors except having a significantly longer intracellular C-terminal (240 amino acids) than the other members of the P2X receptor family, of which the last 177 amino acids are crucial for non-selective pore formation (Surprenant, 1996, Smart et al., 2003). Another interesting feature is that after a brief exposure to ATP, the effectiveness of ADP and AMP is increased, suggesting that a brief initial exposure to ATP caused a long lasting change in the receptor, which subsequently alters its ability to differentiate ATP from ADP and AMP (Chakfe et al., 2002). According to Torres et al (1999), $P2X_7$ receptors do not co-immunoprecipitate with other members of the family.

$P2X_7$ mRNA and protein are distributed in immune cells (Collo et al., 1997), parotid

acinar cells (Tenneti et al., 1998), retina (Brändle et al., 1998), rat duodenal enterocytes and goblet cells and stratified squamous epithelium (Gröschel-Stewart et al., 1999a; 1999b), pancreas (Hede et al., 1999), kidney (Harada et al., 2000), vascular smooth muscle cells and endothelial cells (Glass and Burnstock, 2001), brain (Deuchars et al., 2002; Sperlágh et al., 2002) and cochlea (Nikolic et al., 2003).

1.2.c P2Y receptors

P2Y receptors are purine and pyrimidine nucleotide receptors that are coupled to G proteins. Although the purinergic receptors were originally cloned for receptors that are activated by purine nucleotides such as ATP and ADP, receptors for pyrimidines that are activated specifically by uridine nucleotides (UTP and UDP) were first proposed by Seifert and Schultz (1989). For the current 8 mammalian P2Y receptors cloned, 5 of them ($P2Y_{1,2,4,6,12}$) have their orthologs cloned in rats. The rat P2Y receptors are 328 to 374 amino acid proteins. The typical P2Y receptor protein, like other G protein-coupled receptors, consists of 7 predicted hydrophobic transmembrane domains. However, the P2Y receptor family differs from other known families of G protein-coupled receptors by a high diversity in the amino acid sequences of its different members within the same species, despite a much higher homology found between a particular receptor subtype in different species (see Von Kügelgen and Wetter, 2000). The amino acid similarity of P2Y receptor sequences is

summarized in **Table 3**. Some highly conserved amino acids residues encode for the transmembrane regions 3, 5, 6 and 7 have been suggested to contribute to the agonists binding (Van Rhee et al., 1995).

The signaling pathway of $P2Y_{1,2,4,6}$ receptors is coupling to phospholipase C, leading to formation of inositol 1,4,5-triphosphate (IP_3) and mobilization of Ca^{2+} from intracellular stores (see Von Kügelgen and Wetter, 2000). Increase in IP_3 and intracellular Ca^{2+} can stimulate a variety of pathways including protein kinase C (PKC), phospholipase A_2 and nitric oxide synthase which subsequently can activate other pathways such as phospholipase D and mitogen-activated protein kinase (MAPK). Coupling to adenylate cyclase by $P2Y_{11\text{-}14}$ receptors has also been described (see Ralevic and Burnstock, 1998). The response time of P2Y receptors is longer than that of the P2X receptors because it involves second-messenger systems.

1.2.c.i P2Y$_1$ receptor

The $P2Y_1$ receptor was originally cloned from late embryonic chick brain (Webb et al., 1993) and the orthologs were then detected in several mammalian species. The $P2Y_1$ receptors cloned from different mammalian species are all of 373 amino acids. In all species, the receptor is selective for adenine nucleotides, and especially, nucleotide

diphosphate. However, the receptors present in different species show different agonists with different order of potency. In rat tissue, the recombinant receptor (in rat) is activated by agonists with a potency order of 2MeSADP > ADP > 2MeSATP > ATP, whereas αβ-meATP and UTP are inactive (Tokuyama et al., 1995; Vöhringer et al., 2000; see also Sak and Webb, 2002). The most potent antagonist for $P2Y_1$ receptor is MRS2179 (see Von Kügelgen and Wetter, 2000). The signal transduction pathway of recombinant $P2Y_1$ receptor is the activation of phospholipase C, which involves the G protein $G_{q/11}$ characterized by its insensitivity to pertussis toxin.

$P2Y_1$ receptor is expressed in spleen, lung, liver, kidney, (Tokuyama et al., 1995), heart (Webb et al., 1996), lens fiber cells (Merriman-Smith et al., 1998), astrocytes (Fam et al., 2000), brain (Morán-Jiménez and Matute, 2000), hepatocytes (Dixon et al., 2000), skeletal muscle (Choi et al., 2001) and osteoclasts (Hoebertz et al., 2001).

1.2.b.ii $P2Y_2$ receptor

The first cloned $P2Y_2$ receptor was isolated from mouse neuroblastoma cells (Lustig et al., 1993) and subsequently in rat alveolar type II cells (Rice et al., 1995). The $P2Y_2$ receptor (374 amino acids) is activated by ATP and UTP with approximately equal potency and is insensitive or is only weakly activated by ADP, UDP and other nucleoside

diphosphates, 2MeSATP and αβmeATP (see Sak and Webb, 2002). There are no selective antagonists for $P2Y_2$ receptors, though it is antagonized by suramin and PPADS. $P2Y_2$ receptor can couple via both $G_{i/o}$ and $G_{q/11}$ proteins to mediate phospholipids breakdown and phosphoinositides as well as Ca^{2+} mobilization via PLCβ, an effect which may accordingly be pertussis toxin-sensitive, -partially sensitive, or –insensitive (See Dubyak and el-Moatassim, 1993). For a given signaling pathway, a variety of downstream signaling molecules was reported and seems to be partially dependent on the cell type in which the $P2Y_2$ receptor is expressed (see Ralevic and Burnstock, 1998).

$P2Y_2$ receptor expression is widely distributed, and some of the physiological significance of $P2Y_2$ receptor expression has been reported. $P2Y_2$ receptor present on the endothelium stimulates the synthesis and release of prostacyclin and nitric oxide, leading to vasodilatation. On the other hand, $P2Y_2$ receptors on neutrophils stimulate degranulation and consequently induce aggregation. UTP stimulates mucin secretion from globet cells, increased surfactant released from type II alveolar cells, and increase the beat frequency of cilia in isolated normal and cystic fibrosis epithelial cells (Kellerman, 2002).

1.2.c.iii $P2Y_4$ receptor

The $P2Y_4$ receptor was first cloned from human placenta (Communi et al., 1996) and

then from rat heart (Bogdanov et al., 1998b). The cDNA cloned from rat heart gives rise to a predicted protein of 361 amino acids. Although the human $P2Y_4$ receptor is highly selective for uracil nucleotide over adenine nucleotide, the rat counterpart is activated equipotently by the full agonists ATP and UTP, whereas the partial agonists such as 2MeSATP, ADP and ATP-γS are also equipotent. (Bogdanov et al., 1998b). The receptor causes rapid Ca^{2+} mobilization upon agonist stimulation (Webb et al., 1998). The $P2Y_4$ receptor is insensitive to UDP stimulation (see Sak and Webb, 2002). The receptor is reversibly antagonized by Reactive blue 2 but not by either PPADS or suramin, which helps differentiated it from the $P2Y_2$ receptor.

The rat $P2Y_4$ receptor shows a restricted expression pattern. The receptor transcript has been detected in the heart (Bogdanov et al., 1998b), neonatal brain, skeletal muscle and spleen, and adult vas deferens and spinal cord (Webb et al., 1998), pancreas (Hede et al., 1999), cochlear tissues (Teixeira et al., 2000), kidney (Bailey et al., 2000) and bile duct epithelia (Dranoff et al., 2001).

1.2.c.iv $P2Y_6$ receptor

This uridine nucleotide-specific receptor has been cloned from rat aortic smooth muscle (Chang et al., 1995). The rat $P2Y_6$ receptor consists of 328 amino acids, the shortest

sequence among the family. The receptor is activated most potently by UDP, which is approximately 100-fold more potent than UTP, whereas ATP, ADP or 2MeSATP are almost inactive (Nicholas et al., 1996). The $P2Y_6$ receptor is blocked by Reactive blue 2 and suramin. The response is pertussis toxin insensitive, indicating the involvement of $G_{q/11}$ proteins in stimulation of PLC and in the formation of IP_3 (Chang et al., 1995).

The $P2Y_6$ mRNA is found abundantly in various tissues including lung, stomach, intestine, spleen, mesentery and aorta (Chang et al., 1995), heart (Webb et al., 1996), cochlear tissues (Teixeira et al., 2000) and kidney (Bailey et al., 2001). The more widespread distribution of the $P2Y_6$ receptor, compared with the $P2Y_4$ receptor, suggests that this receptor is more likely to account for endogenous uridine nucleotide-specific responses.

1.2.c.v $P2Y_{11}$ receptor

The $P2Y_{11}$ receptor was cloned from human placenta (Communi et al., 1997). The predicted protein possesses 371 amino acids. The receptor coupled to the stimulation of both the phosphinositide and the adenylyl cyclase pathways. The $P2Y_{11}$ receptor is the only P2Y receptor selective for ATP because it is stimulated by agonists with a rank order of potency of ATP > 2MeSATP > ATP-γS >> ADP, with UTP and UDP inactive (Communi et al., 1997). The $P2Y_{11}$ receptor is antagonized by suramin and partially by Reactive blue 2, but

not by PPADS (Communi et al., 1999). Quantitative RT-PCR study has shown that $P2Y_{11}$ receptor is strongly expressed in the brain lymphocytes and spleen (Moore et al., 2001).

1.2.c.vi $P2Y_{12}$ receptor

The human and rat $P2Y_{12}$ receptors were both cloned from platelets cDNA library and presented in the same report (Hollopeter et al., 2001). The rat $P2Y_{12}$ receptor is a 343-amino acid protein which is activated by agonists in the order of potency as 2MeSATP > 2MeSADP > ADP >> ATP (Simon et al., 2002). It has to be noted that 2MeSATP acts as a very potent agonist in recombinant $P2Y_{12}$ receptor, whereas it appears weaker in cells expressing endogenous $P2Y_{12}$ receptor, or even act as antagonists in platelet cells (Simon et al., 2002). The response is antagonized by Reactive blue 2 and suramin, but not by PPADS or MRS2179, which is selective for $P2Y_1$ receptor (Kulick and von Kügelgen, 2002). The receptor couples to pertussis toxin-sensitive G-proteins ($G_{i/o}$) and inhibits adenylyl cyclase upon agonist stimulation (Kulick and von Kügelgen, 2002; Simon et al., 2002). In addition, the receptor coupled to $G_{i/o}$ also interacts with the inward rectifier K^+ channel and the voltage gated Ca^{2+} channel, suggesting that it involves another transduction pathway independent of second messengers and downstream P2Y signaling (Simon et al., 2002). Expression of $P2Y_{12}$ receptor is so far detected in neuronal cells (Unterberger et al., 2002), glioma cells (Czajkowski et al., 2002), endothelial cells (Simon et al., 2002) and platelets (Hollopeter et

al., 2001).

1.2.c.vii $P2Y_{13}$ receptor

An orphan G-protein coupled receptor (SP174) was cloned from a human neutrophil cDNA library and, together with the preexisting mouse ortholog, designated as $P2Y_{13}$ receptors (Zhang et al., 2002). The receptor contains a 1002-base open reading frame encoding 333 amino acid residues, which shows highest homology with $P2Y_{12}$ receptor (45%) and the UDP-glucose receptor (later confirmed to be $P2Y_{14}$ receptor) (43%) (Zhang et al., 2002). The receptor is activated by agonists with a decreasing order of potency of 2MeSADP = 2MeSATP > ADP > ATP. The receptor couples to Gi proteins and adenylyl cyclase pathway.

The mRNA dot blot experiment shows that strong expression is detected in spleen, pancreas, brain and liver (Zhang et al., 2002).

1.2.c.viii $P2Y_{14}$ receptor

An orphan G-protein coupled receptor that specifically responds to UDP-glucose and related sugar-nucleotides (Nomura et al., 1994; Chambers et al., 2000) was recently confirmed to be a human $P2Y_{14}$ receptor (Abbracchio et al., 2003). This receptor is

originally cloned from human myeloid cells and designated an orphan receptor (Nomura et al., 1994). Sequence comparisons reveal that it is structurally related to the known members of the human P2Y receptor family (Chambers et al., 2000), with 18-45% overall identity mainly distributed in the transmembrane domains. A G-protein coupled receptor cloned from ventral tegmentum in rat shows 80% amino acid identity and a fully conserved amino acid motif in the sixth and the seventh transmembrane domains with $P2Y_{14}$ receptor, suggesting that it is the rodent ortholog for $P2Y_{14}$ receptors.

The recombinant $P2Y_{14}$ receptor is responsive to the agonists such as UDP glucose, UDP-galactose, UDP –glucuronic acid and UDP-N-acetylglucosamine, whereas ATP, ADP, UTP or UDP do not activate the receptor. Pertussis toxin completely abolishes agonist responses, indicating the receptor to be coupled to $G_{i/o}$ protein (Wittenberger et al., 2001). The rat $P2Y_{14}$ receptor shows highest expression in cells of haemopoietic origin and clear expression in the brain (Charlton et al., 1997).

I.3 Purinergic signaling during embryonic development

I.3.a Brief introduction on mammalian embryonic development

I.3.a.i From zygote to bilaminar germ disc

Embryonic development starts from the moment when an oocyte is fertilized by a sperm.

The chromosomes of the oocyte and sperm enclosed within the female and male pronuclei respectively fuse with each other to produce a single diploid zygote (see Larsen, 1998). Within a short period of time after fusion of the pronuclei, the zygote undergoes a series of mitotic cell divisions along the journey from the oviduct to the uterus to form a round shaped morula, consisting of around 32 cells enclosed in an acellular layer called zona pellucida. The centrally placed cells (embryoblasts) will form the inner cell mass that gives rise to most of the embryo proper, whereas the cells at the periphery (trophoblasts) are the primary source for the membranes of the placenta. Hydrostatic pressure develops within the morula due to continued fluid entry, resulting in the development of a large cavity within the morula and the embryo is now called blastocyst. When the morula reaches the uterus, the blastocyst hatches from the clear zona pellucida and eventually implants tightly to the uterine wall. Meanwhile, the embryoblast splits into an external columnar cell layer called epiblast and an internal cuboidal cell layer called hypoblast. The two layers together make up the bilaminar germ disc.

I.3.a.ii Gastrulation

The bilaminar germ disc is later converted into a trilaminar germ disc by a process of gastrulation whereby epiblast cell migrate towards and through a region, called the primitive streak, to form the so-called mesoderm (see Larsen, 1998). The primitive streak develops

when a groove (primitive groove) appears along the longitudinal midline of the germ disc and then elongates to occupy about half the length of the embryo. A deeper depression called primitive node develops at the presumptive cranial end of the groove. With the formation of mesoderm, the hypoblast is replaced with a new cell layer derived from epiblast called endoderm, while the epiblast itself is transformed into the ectoderm. Thus the mesoderm separates the overlying ectoderm from the subjacent endoderm. The most important achievement of gastrulation is that it establishes the basic vertebrate body plan: the anteroposterior and the dorsoventral axes, for the developing embryo.

I.3.a.iii Somite formation and development of urogenital system and limb

The mesoderm that forms in the midline gives rise to a transient structure called notochord. The mesoderm adjacent to the notochord forms the paraxial mesoderm, which soon undergoes segmentation to form somitomeres and then to somites. These somites will give rise to the myotomes, sclerotomes and dermatomes that form skeletal muscle, vertebrae and skin, respectively. The intermediate mesoderm forms just lateral to the paraxial mesoderm will later give rise to the urinary system. Three sequential urinary systems develop from the intermediate mesoderm, i.e. i) cervical nephrotomes, which are never functional; ii) a thoracolumbar mesonephric system, which functions briefly during embryonic life; iii) a sacral metanephric system, which ascends to the lumbar region forming

the definitive kidney (see Larsen, 1998). The metanephric system is developed by reciprocal interaction between the ureteric buds that branch from the mesonephric ducts and the metanephric blastema, both of which are derived from intermediate mesoderm (see Larsen, 1998; Vainio and Lin, 2002), resulting in the formation of nephrons differentiated within the metanephric blastema, and the collecting systems formed by bifurcation of the ureteric buds. The most lateral mesoderm, the lateral plate mesoderm, contributes to the formation of the body wall and to somatic and visceral peritoneum (see Larsen, 1998).

Migration of the primordial germ cells to the posterior body wall results in induction of the genital ridges just medial to the mesonephros on each side of the midline. Cells from the mesonephros and the coelomic epithelium invade the mesenchyme in the region of the presumptive gonads to form the primitive sex cords that surround the germ cells. Interaction between the mesonephric and paramesonephric ducts and the primitive sex cords results in the formation of gonads (see Larsen, 1998).

At the stage when the cranial neural tube closure is almost complete, somites induce proliferation of the lateral plate mesoderm in the presumptive limb region to form outgrowths called limb buds, which are covered by surface ectoderm. The limb buds differentiate along three interrelated axes: the proximodistal, the craniocaudal and the dorsoventral axes. The

lateral plate mesoderm also gives rise to the bones, tendons, ligaments and vasculature of the limbs, while the somites migrate to the limb bud and contribute to the muscular component of the limbs. Development of the forelimb buds occurs prior to that of the hindlimb buds (see Larsen, 1998).

I.3.a.iv Neurulation and neural crest migration and differentiation

The axial mesoderm, including notochord, induces thickening of the overlying ectoderm to form a bilateral thickened epithelium, the neural plate. The neural plate appears first at the cranial end of the embryo and differentiates craniocaudally. The neural plate is broad cranially and tapered caudally. Completion of gastrulation is followed by another very important process called neurulation. Neurulation commences as the neural plate begins to fold on either side and come into contact and, eventually, fuse with each other to form a hollow tube along the embryo (see McLachlan, 1994). Neurulation does not take place simultaneously along the whole length of the forming tube: it begins in the region between the developing hindbrain and upper neck and then extends cranially and caudally. Closure of the cranial neural tube occurs prior to that of the caudal neural tube. Before neurulation, several expansions occur in discrete regions of the cranial neural plate. Soon after neurulation, the expansions undergo further enlargement and flexing to form the brain vesicles that give rise to the future brain, with the caudal neural tube forming the future

spinal cord.

Neural crest cells are a special population of cells that arises along the lateral margins of the neural folds (see Larsen, 1998). During neurulation, these cells detach from the neural plate and migrate ventro-laterally to many locations in the body, where they differentiate into a remarkable variety of structures, e.g. in the peripheral nervous system, the neural crest cells differentiated into glia cells and ganglia including dorsal root, sympathetic chain, enteric, preaortic and some cranial ganglia (trigeminal, facio-acoustic, glossopharyngeal and vagal). Other neural crest derivatives include arachnoid and pia mater, dermal bones of skull, pharyngeal arch cartilages, connective tissue surrounding papillary and ciliary muscle in the eyes, odontoblasts and melanocytes, adrenal medulla, truncoconal septum, dermis and hypodermis of face and neck.

I.3.a.v Development of cardiovascular system

The heart is the first organ to function in the embryo. It is originated from a group of mesoderm-derived angioblasts that, subject to induction by the endoderm, differentiated into flattened endothelial cells that join together to form a pair of vascular elements called the endocardial tubes locating cranial and lateral to the neural plate early before neurulation. During embryonic folding, the tubes are brought into the thoracic region, where they meet

along the midline and fuse to form a single primitive heart tube. A series of constrictions and expansions forms along the primitive heart tube that contributes to the various heart chambers (see Larsen, 1998). The heart tube formed soon undergoes looping, which helps bringing the primitive atria and ventricles in their adult positions. Trabeculation takes place in the looped heart, which is believed to be important for myocardial growth (see Sedmera et al., 2000). Remodeling also occurs in the looped heart to establish the proper connection for systemic and pulmonary circulation, followed by septation of the heart and valve formation.

I.3.a.vi Development of respiratory and gastrointestinal system

In early stages, the embryo is a flat, ovoid disc. Due to differential growth of various embryonic structures, the flattened disc is converted into a 3-dimensional embryo (see Larsen, 1998). The endoderm of the trilaminar embryo is destined to give rise to the lining of the respiratory and gastrointestinal tract. Embryo folding also converts the superior and inferior portions of the endoderm into blind-ended tube that gives rise to a cranial blind-ended foregut, a midgut that opens into the yolk sac, and a caudal blind-ended hindgut. The lungs emerged from the outpouching of the endodermal foregut as lung bud. The lung bud begins to grow ventrocaudally through the mesenchyme surrounding the foregut. Soon after, the lung bud bifurcates into right and left primary bronchial buds. The primary bronchial buds

undergo continue branching morphogenesis, followed by mesenchymal thinning, epithelial differentiation and surfactant protein expression (see McMurtry, 2002).

Gut development involves invagination of endoderm and the subsequent growth and differentiation of the subjacent splanchnic mesenchyme (mesodermal). Thus, the gut is composed of an outer mesodermal-derived smooth muscle and an inner endodermal-derived luminal lining flanking the most anterior (mouth) and most posterior (anus) ectodermal-derived epithelium (see Roberts, 2000). The foregut itself will form the thoracic and abdominal esophagus, stomach, and about half the duodenum; the midgut will form half the duodenum, the jejunum and ileum, ascending colon, and two-thirds of the transverse colon. The hindgut will form one-third of the transverse colon, the descending and sigmoid colons, and rectum. Meanwhile, endodermal bud sprouts from the inferior end of the foregut and develops into liver, gallbladder and pancreas, whereas the nearby spleen is formed by mesodermal condensation (see Larsen, 1998).

I.3.b Purinergic signaling during embryonic development

An embryo requires different cell activities such as cell proliferation, differentiation, migration and degeneration that work in orchestra to ensure proper embryonic development. It therefore requires specific signals given by specific signaling molecules at specific time

points. Various signal transduction pathways then cross-talk with each other to provide positional information and induce cell fate specification. Many signaling molecules are well known to regulate various developmental processes in embryos. Examples of those signaling molecules include Sonic hedgehog (Shh), fibroblast growth factors (FGF), bone morphogenic proteins (BMP), Notch and Delta, retinoic acid and Wnt family signaling pathway.

Accumulated evidence suggests that extracellular nucleosides and nucleotides may exert profound effects during embryonic development in different species (see Burnstock, 2001). In early zebrafish embryo, an ionotropic $P2X_3$ receptor is expressed in the trigeminal primordial and the spinal sensory Rohon-Beard cells and in the putative posterior lateral line ganglion (Norton et al., 2000). Studies on *Xenopus* cell culture have shown that extracellular ATP potentiates acetylcholine (ACh) responses at developing neuromuscular synapses during early phase of synaptogenesis (Fu and Poo, 1991; Fu, 1994; Fu and Huang, 1994). In addition, endogenously released ATP acting in concert with various protein kinases, is involved in the maintenance and/or development of the quantum size of synaptic vesicles at embryonic neuromuscular synapses (Fu et al., 1997). A novel P2Y purinoceptor (X1P2Y or $P2Y_8$) was expressed in the neural plate of *Xenopus* embryos from stages 13 to 18 and again at stage 28 when secondary neurulation occurs in the tail bud (Bogdanov et al.,

1997). However, expression ceased after neural tube closure, suggesting that this receptor may be important during early neurogenesis.

ATP, but not pyrophosphate, AMP, ADP or GTP, has been shown to induce precocious evagination of the embryonic chick thyroid, an event which has been hypothesized to be involved in the formation of the thyroid gland from the thyroid primordium (Hilfer et al., 1977). A transmitter-like action of ATP on patched membranes of myoblasts and myotubes cultured from 12-day-old chick embryos was reported (Kolb and Wakelam, 1983). ATP has a potent depolarizing activity on myotubes derived from pectoral muscle cultured from 11-day-old chick embryos (Hume and Hönig 1986). At embryonic day 6 of chick embryo, ATP elicits vigorous contractions in all the muscles tested, but by embryonic day 17 none of the muscles contract in responses to ATP. However, denervation of muscles in newly hatched chicks leads to the reappearance of sensitivity to ATP, suggesting that the expression of ATP receptors is regulated by motor neurons (Wells et al., 1995). Meyer et al (1999b) have shown that expression of two purinoceptors ($P2X_5$ and $P2X_6$ receptors) was detected in developing skeletal muscle of chick embryos. These receptors were first expressed at early stages of skeletal muscle development, whereas expression disappeared immediately before myotube formation i.e. fusion of myoblasts to form myotubes. In the embryonic chick neural retina, ATP or UTP induced an increase in intracellular Ca^{2+} and the response is

blocked by suramin and Reactive blue 2, which suggests that the intracellular Ca^{2+} rise is mediated by $P2Y_2$ or $P2Y_4$ receptors. Interestingly, such increase is dramatically reduced just before synaptogenesis, indicating a very specific temporal effect of extracellular nucleotides (Sugioka et al., 1996). During the first 10 days of embryonic development, $P2Y_1$ receptor is expressed in a developmentally regulated manner in the limb buds, mesonephros, brain, somites and facial primordia of the chick embryos (Meyer et al., 1999a). It is also specifically expressed in the undifferentiated limb mesenchyme in chick embryo, and the expression was lost when the tissue differentiated. Application of ATP or ADP, the potent agonists for $P2Y_1$ receptor, inhibits cartilage formation in micromass culture (Meyer et al., 2001). It was therefore suggested that ATP acting on $P2Y_1$ receptor is involved in the differentiation of limb mesenchymal cells, more specifically in regulating cartilage formation, during embryonic development.

In rat embryos of embryonic day 16, expression of $P2X_3$ receptor is detected in the distinct regions of the brainstem such as mesencephalic trigeminal nucleus, superior and inferior olives, intermediate reticular zone, the spinal trigeminal tract and the prepositus hypoglossal nucleus. The expression, however, is downregulated in the postnatal brain. The $P2X_3$ receptor in the adult animal has been shown to be involved in sensory functioning. The expression in non-sensory related regions in the embryonic brain and subsequent

downregulation as development progresses suggests a role to play during mammalian neurogenesis. Dynamic expression of different P2X receptors ($P2X_5$, $P2X_6$ and $P2X_2$ receptors) was reported in rat developing skeletal muscle (Ryten et al., 2001) in which $P2X_5$ and $P2X_6$ receptors were expressed transiently in the embryonic skeletal muscle, consistent with the results of Meyer et al (1999b). While $P2X_6$ and $P2X_2$ receptor expression was widespread along the myotubes, $P2X_5$ receptor was restricted to regions of muscle close to the myotendinous junctions. The same authors also reported that addition of ATP into rat skeletal muscle satellite cell culture isolated from rat skeletal muscle inhibits cell proliferation, together with an upregulation of markers of muscle cell differentiation (myogenin, p21 and myosin heavy chain) and an increase in the rate of myotube formation. UTP, in contrast, induces an increase in satellite cell proliferation. Addition of ATP also enhances phosphorylation of MAPKs, particularly p38 (Ryten et al., 2002). Inhibition of p38 activity reverses the ATP-induced suppression of cell proliferation. All the findings mentioned above suggest that extracellular nucleotides, by acting via P2 receptors, are likely to play roles during embryonic development.

I.4 Objective of studies

Although it appears that extracellular nucleotides are candidates of signal transduction molecules involved in embryonic development, many of the studies were performed in

non-mammalian species. In addition, early studies on purinergic signaling in embryos were performed prior to receptor cloning, and systematic analysis on expression patterns of purinergic receptors during embryogenesis is lacking. Thus, the present study is mainly focused on the expression patterns of P2 purinoceptors, both P2X and P2Y receptors, during mammalian (rat) embryogenesis. Studies of the expression patterns of purinoceptors in mammalian embryos are crucial to our understanding on the specific roles of extracellular nucleotides in the embryonic development. Particular interest will be given to those receptors that show dynamic expression patterns during embryonic development. The correlation between the spatio-temporal expression of both purinoceptor mRNA transcripts and the receptor proteins and the occurrence of different developmental process will also be examined. Furthermore, with the clues obtained from the expression studies, functional studies will also be carried out to investigate the possible functions of particular purinoceptors during the development of rat embryos.

Table 1 Summary of the potential ability of co-assembly between P2X receptor subunits

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	+	+	+	-	+	+	-
$P2X_2$		+	+	-	+	+	-
$P2X_3$			+	-	+	-	-
$P2X_4$				+	+	+	-
$P2X_5$					+	+	-
$P2X_6$						-	-
$P2X_7$							+

HEK 293 cells transfected with P2X receptor subunits tagged with either FLAG (DYKDDDDK) or the HA (YPYDVPDYA) epitopes into the carboxyl termini were undergone immunoprecipitation. "+", subunits immunoprecipitated with antibody to one epitope could be detected with an antibody to the second epitope. (Data from Torres et al., 1999).

Table 2 Amino acid identity of P2X receptor subunits

	$P2X_1$	$P2X_2$	$P2X_3$	$P2X_4$	$P2X_5$	$P2X_6$	$P2X_7$
$P2X_1$	100 (100)	40.5 (40.6)	46.4 (47.9)	50.6 (50.3)	45.5 (44.7)	46.8 (46.2)	44.9 (45.1)
$P2X_2$		100 (100)	51.5 (51.1)	50.5 (50.5)	46.9 (46.9)	42.7 (42.7)	41.0 (41.0)
$P2X_3$			100 (100)	49.2 (48.6)	47.0 (49.3)	41.4 (43.2)	43.1 (44.7)
$P2X_4$				100 (100)	53.5 (55.4)	47.3 (47.6)	49.8 (48.6)
$P2X_5$					100 (100)	49.2 (48.5)	42.0 (42.0)
$P2X_6$						100 (100)	39.2 (41.0)
$P2X_7$							100 (100)

The identities between amino acid sequences at the transmembrane regions and large extracellular loop of the rat P2X receptor subunits were expressed in percentage. The percentages in parenthesis represent the identities between human P2X receptor subunits.

Table 3 Amino acid identity of P2Y receptors

	$P2Y_1$	$P2Y_2$	$P2Y_4$	$P2Y_6$	$P2Y_{11}$	$P2Y_{12}$
$P2Y_1$	100 (100)	32 (31)	33 (34)	33 (33)	(26)	20 (19)
$P2Y_2$		100 (100)	48 (51)	39 (37)	(25)	22 (20)
$P2Y_4$			100 (100)	41 (38)	(23)	23 (21)
$P2Y_6$				100 (100)	(23)	19 (23)
$P2Y_{11}$					(100)	(15)
$P2Y_{12}$						100 (100)

The identities between amino acid sequences of the rat P2Y receptor subtypes were expressed in percentage. The percentages in parenthesis represent the identities between human P2Y receptor subtypes. Note that rat $P2Y_{11}$ receptor has not yet been cloned.

Chapter II Localization of $P2X_3$ receptors and coexpression with $P2X_2$ receptors during rat embryonic neurogenesis

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.

INTRODUCTION

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 eight established subtypes in mammals recently proposed (Abbracchio et al., 2003).

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., 2001). 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, Burnstock, 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. (1999) 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 dorsal root ganglia 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 precooled isopropanol (-70°C) for cryosectioning. Whole embryos were serially cryosectioned (12μm) and mounted on gelatin-coated slides and dried at room temperature. Every section cut was analyzed for immunoreactivity. Neonatal rat brains were dissected after cervical dislocation, and the brains were fixed and processed as

described above. Frozen sections (15μm) sections were cut and mounted. One in every ten sections was analyzed for immunoreactivity.

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 (VEKQSTDSGAYSIGH), 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), the antisera recognized only one protein of the expected size in the heterologous expression systems and were shown to be receptor-subtype-specific.

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_2O_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 and scored and was confirmed by an independent observer 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 neurons 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 ventral 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).

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 glossopharyngeal-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 have 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 immunoreactivity 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.

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FIGURES & LEGENDS

TABLE 1. Localization of $P2X_3$ receptor immunoreactivity at different stages during rat embryonic development

Embryonic PNS	E9.5-10.5	E11-11.5	E12.5	E14.5	E16.5	E18.5
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	–	–	++
Embryonic CNS						
Forebrain neural tube	–	–	–	na	na	na
Olfactory bulb	na	na	na	–	–	–
Cerebral cortex	na	na	na	–	–	–
Caudate putamen	na	na	na	–	–	–
Amygdala	na	na	na	–	–	–
Internal capsule	na	na	na	–	–	–
Hippocampus	na	na	na	–	–	–
Thalamus	na	na	na	–	–	–
Hypothalamus	na	na	na	–	–	–
Optic (**II**) tract	na	na	na	+	++	+++
Retina	na	na	-	+	++	+++
Superior and inferior colliculus	na	na	na	–	–	–
Mesencephalic trigeminal nucleus of the fifth nerve	na	na	na	++	++	+++
Locus coeruleus	na	na	na	–	–	–
Hindbrain neural tube	–	+	++	na	na	na
Spinal cord	–	–	+	++	+++	+++
Spinal trigeminal tract	na	na	++	+++	+++	+++
Trigeminal motor nucleus	na	na	na	–	–	–
Superior and Inferior olive	na	na	na	–	–	–
Nucleus tractus solitarius	na	na	na	+++	+++	+++
Facial nucleus	na	na	na	+++	+++	+++
Cerebellum	na	na	na	–	–	–

Vagal motor nucleus	na	na	na	−	−	−
Prepositus hypoglossal nucleus	na	na	na	+	+	++
Vestibular spinal nucleus	na	na	na	−	+	++
Dorsal motor nucleus of vagus	na	na	na	−	−	−
Cuneate nucleus	na	na	na	−	−	−
Gracile nucleus	na	na	na	−	−	−

The degree of $P2X_3$ immunoreactivity: +, just detectable; ++, moderate; +++, strong;

-, undetectable; na, not applicable

Figure 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 = 200μm in D (applies to A-D), 50μm in inset of D (applies to insets in A-D), 50μm in inset of E, 200μm in E,F.

Fig 1

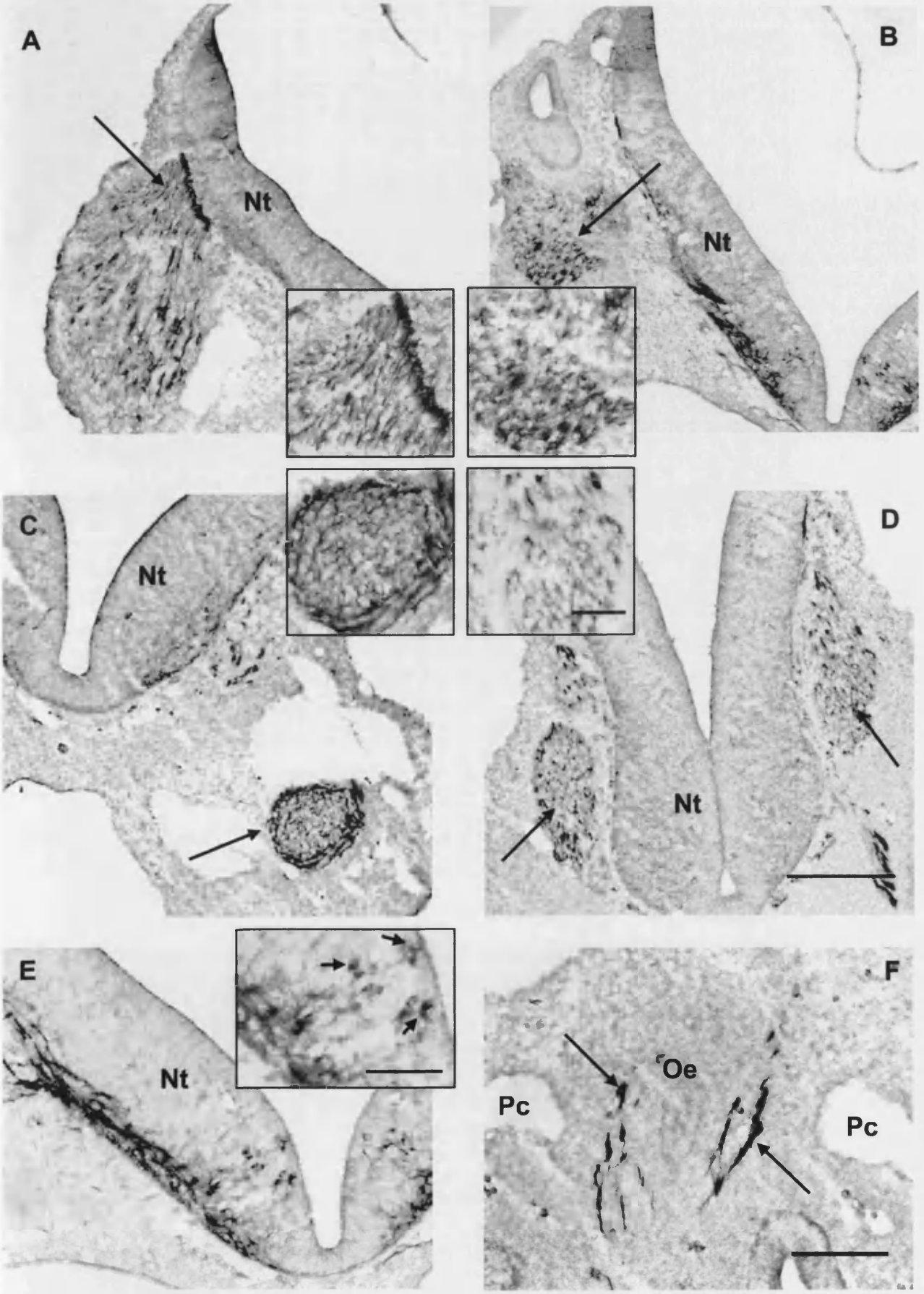


Figure 2 $P2X_3$ immunoreactivity in embryonic day (E) 14.5 rat embryo. **A:** coronal section at the midbrain level showing $P2X_3$ expression in the mesencephalic trigeminal nucleus. **B:** Coronal section at the pontine level showing strong $P2X_3$ expression in the spinal trigeminal tract (Sp5t) 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 = 25μm in A, 50μm in B,E,F, 100μm in C,D,G,H.

Fig 2

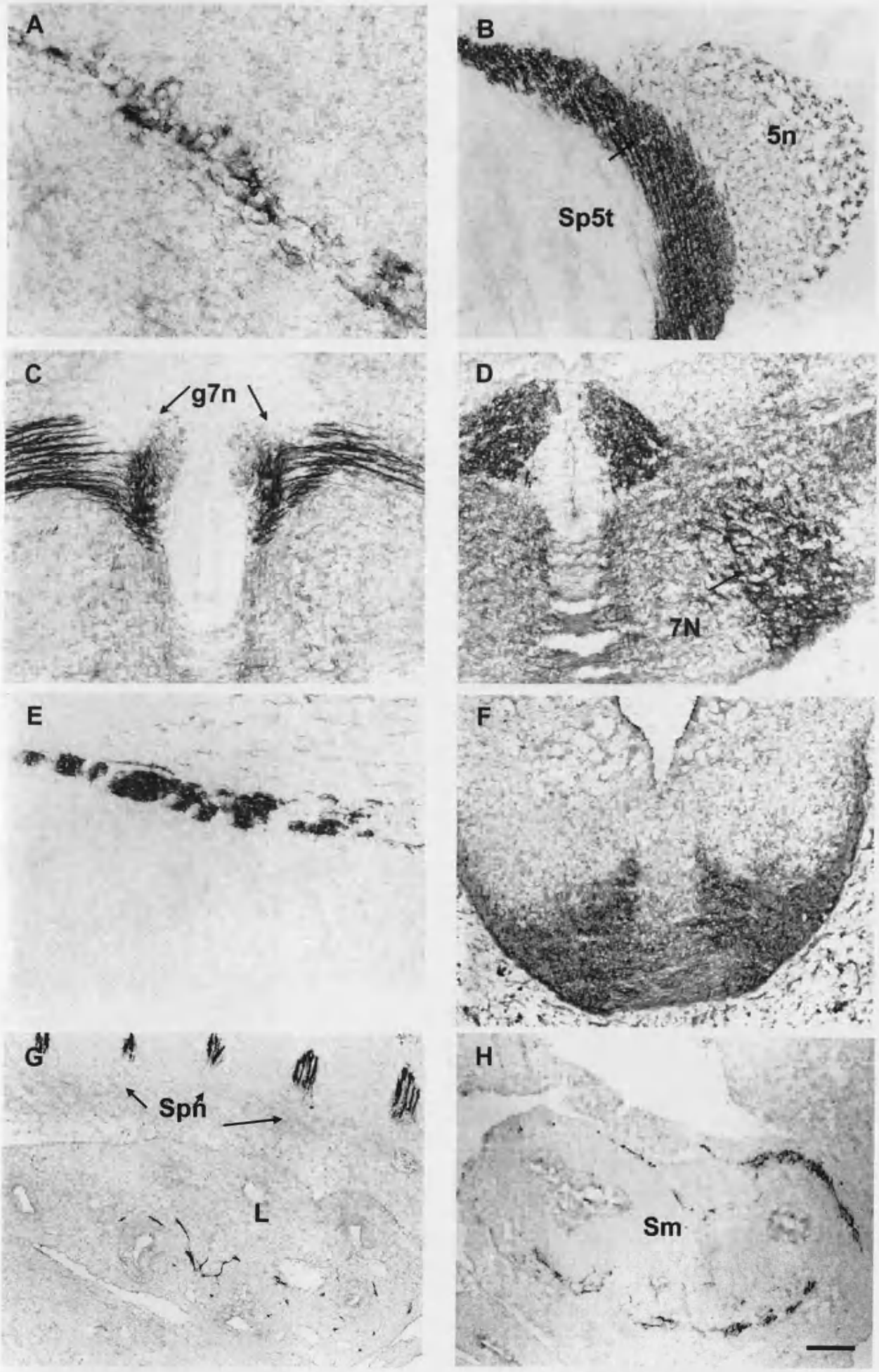


Figure 3 $P2X_3$ immunoreactivity in neural crest-derived ganglia of the embryonic day (E) 18.5 rat embryo. **A-D:** The $P2X_3$ receptor is strongly expressed in trigeminal ganglion (A), dorsal root ganglion (C), vestibulocochlear ganglion (**E**) and nodose ganglion (**F**). Note the peripheral staining 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 = 100μm in H (applies to A-H).

Fig 3

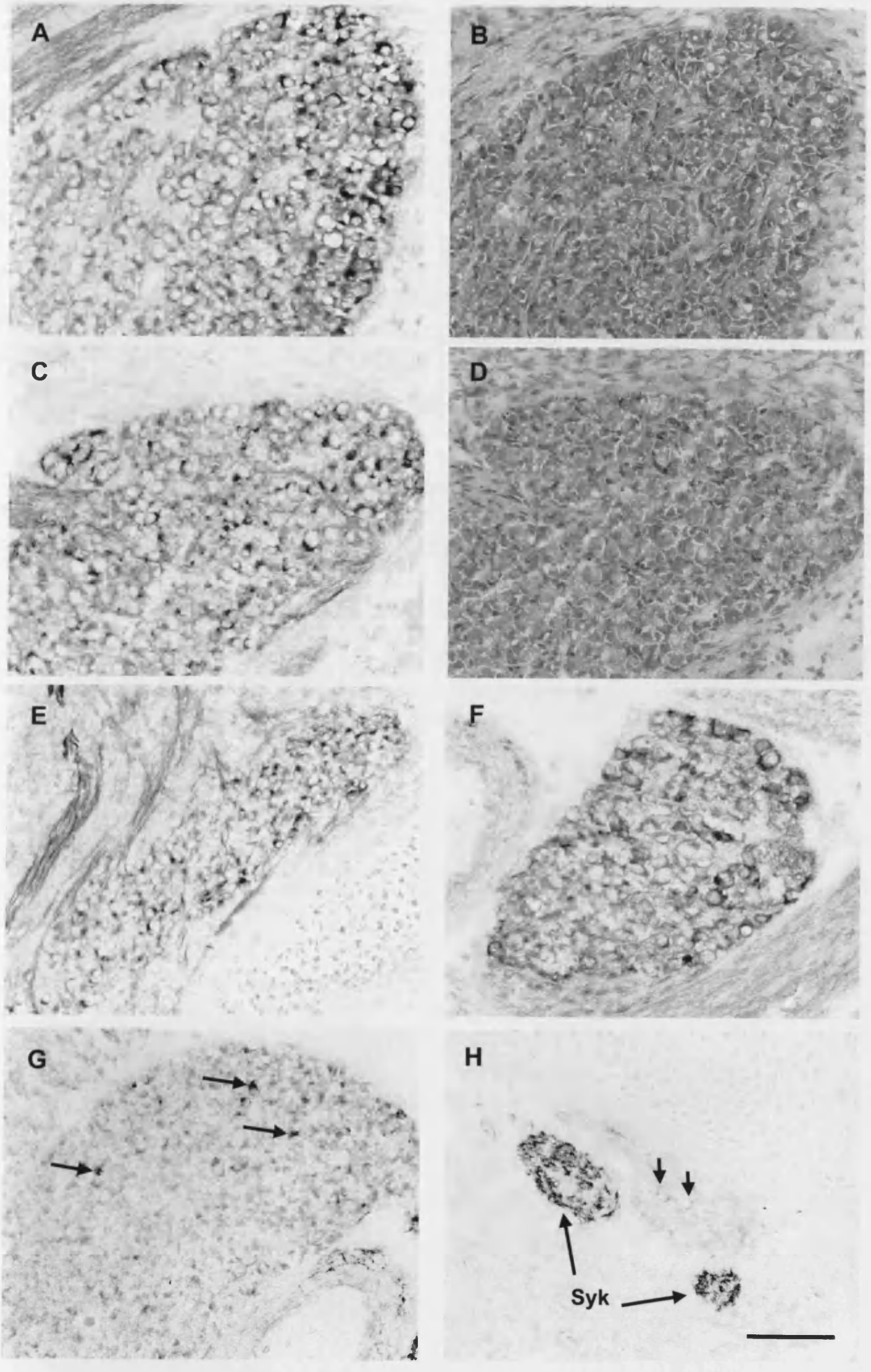


Figure 4 $P2X_3$ immunoreactivity in embryonic day (E) 18.5 rat embryo. **A:** Transverse section showing $P2X_3$ immunoreactivity in the dorsal spinal cord in the lumbar region. **B:** High-magnification image showing the retinal ganglion cells in the neural retina (Ret) expressing $P2X_3$ receptor 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 = 200μm, scale bar in H = 200μm in C,D,F, 100μm in E,G,H, 50μm in B.

Fig 4

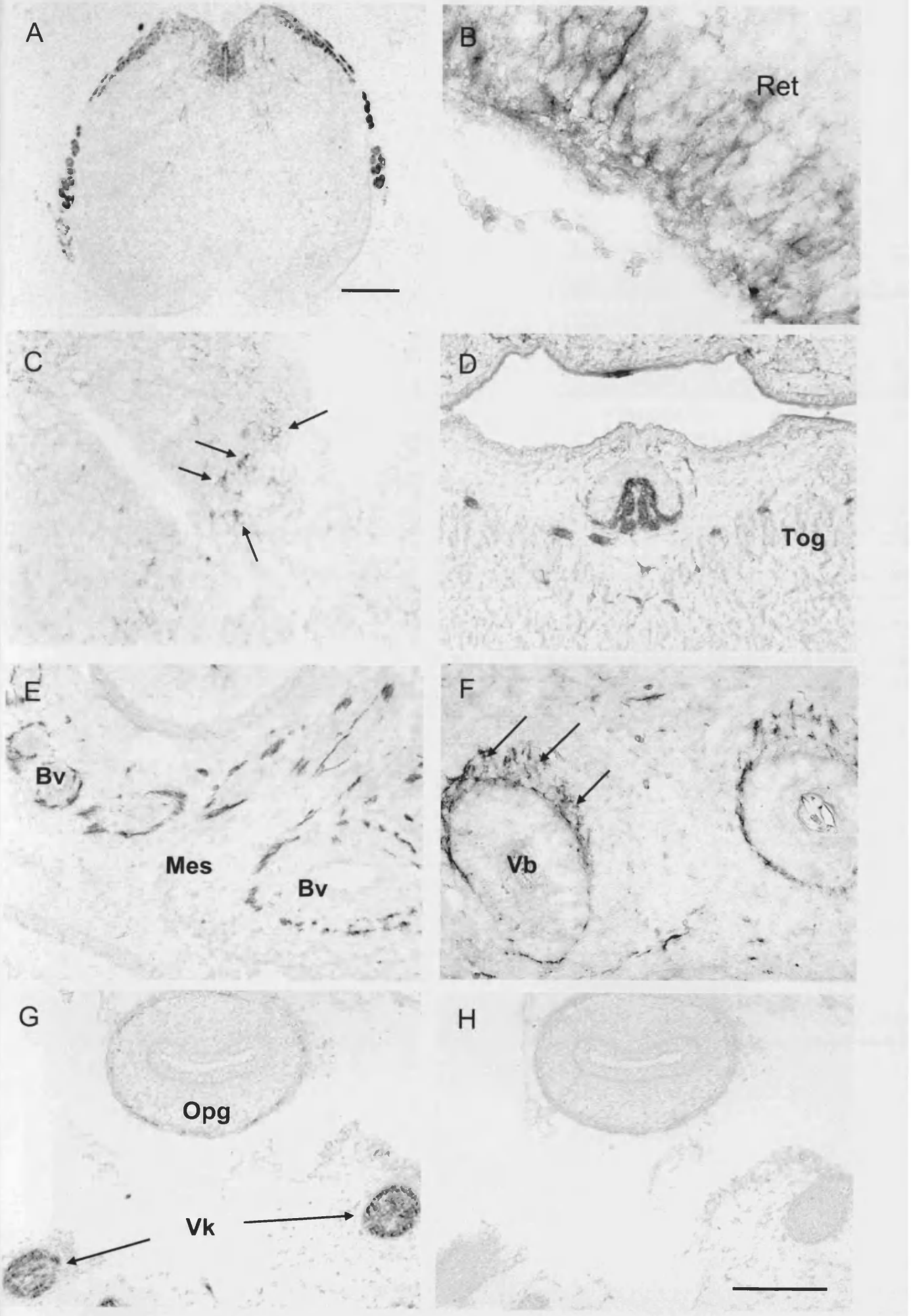


Figure 5 $P2X_3$ immunoreactivity in late embryonic and neonatal rat brainstem. **A,C,E:** $P2X_3$ receptor expression in the mesencephalic trigeminal nucleus in embryonic day (E) 18.5, postnatal day (P) 1, and P16 rat brainstem, respectively. Note that only a subpopulation of the neurons in the mesencephalic 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 = 100μm in E (applies to A,C,E), 500μm in D (applies to B,D), 200μm in F.

Fig 5

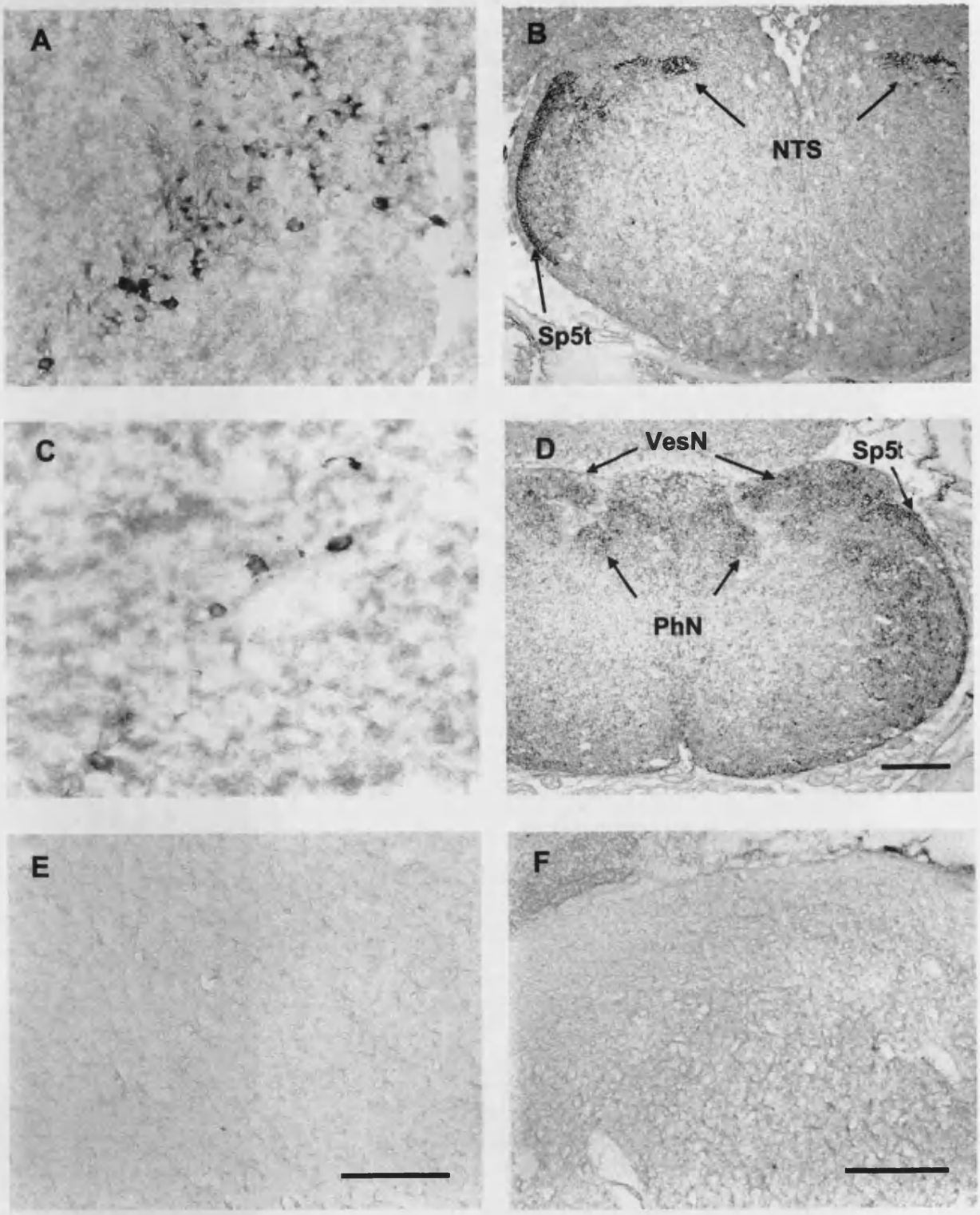


Figure 6 $P2X_2$ immunoreactivity at different stages of rat embryo development. **A:** Transverse section in the spinal neural tube (Nt) in embryonic day (E) 12.5 embryos showing $P2X_2$ receptor expression in the notochord. **B:** Adjacent section of A stained with $P2X_3$ receptor antibody. $P2X_3$ 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 = 500μm in C. Scale bar in I = 200μm in E, 100μm in H, 50μm in A,B,D,F,G.

Fig 6

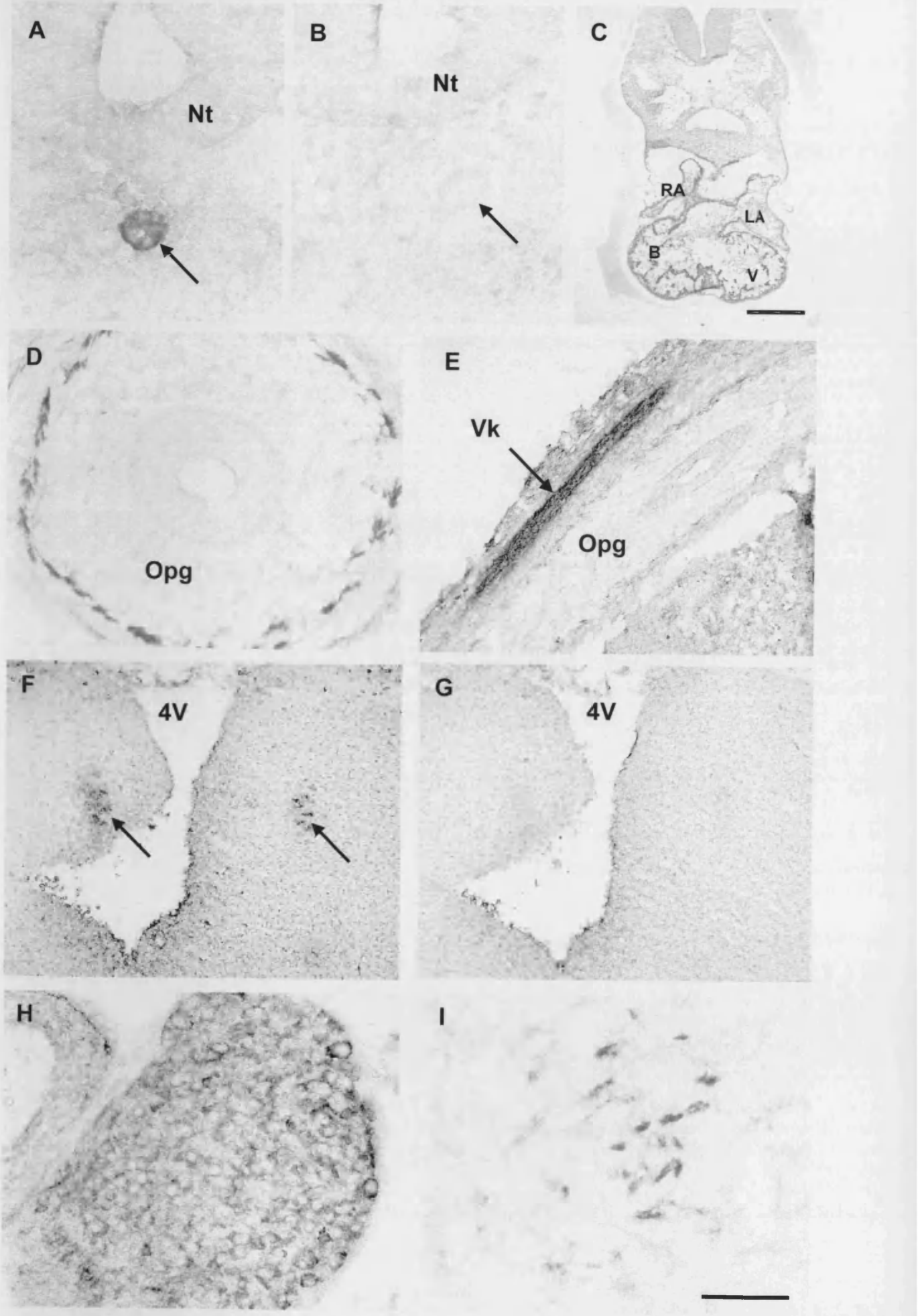
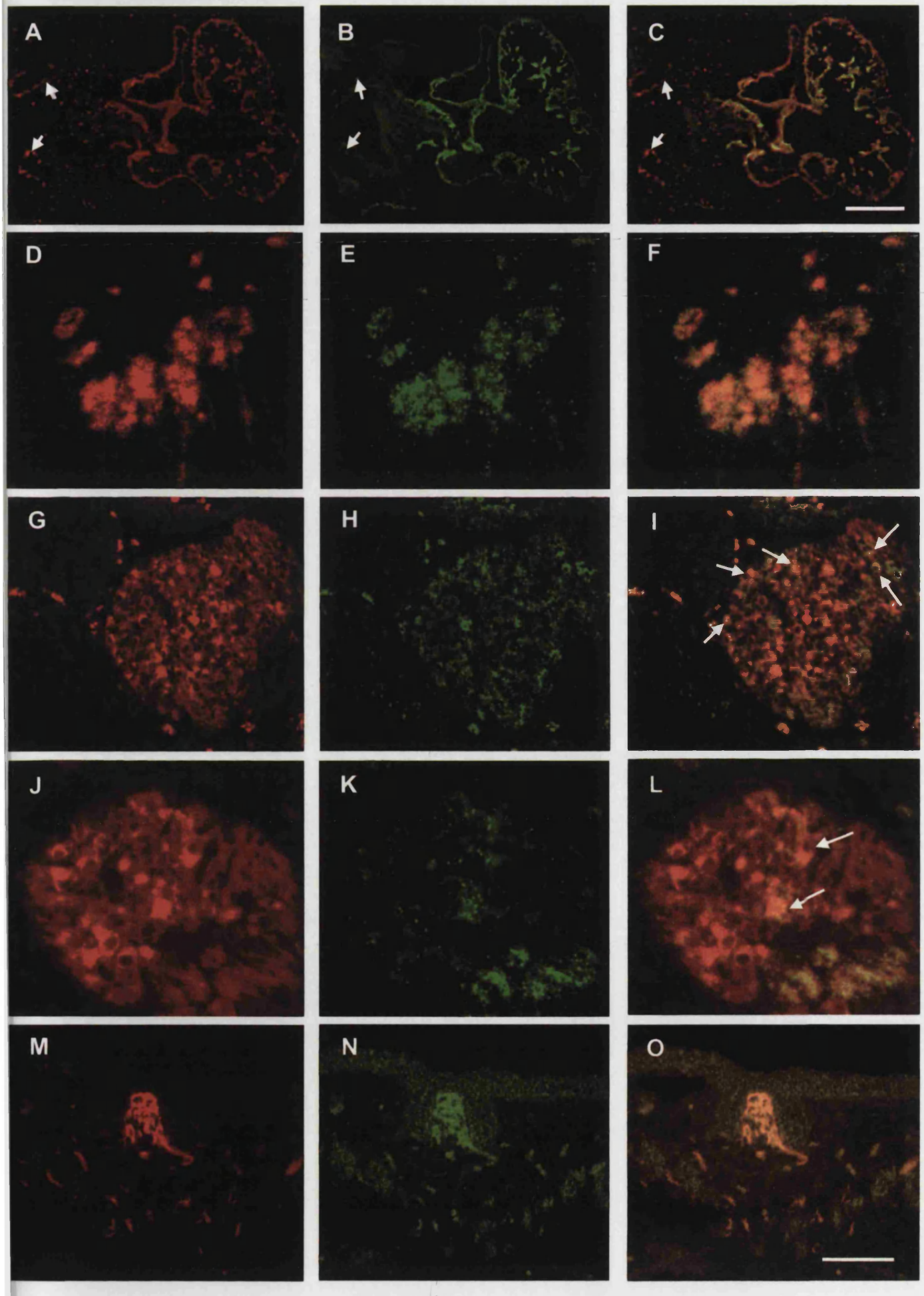


Figure 7 Immunofluorescence double labeling for the $P2X_2$ receptor and α-smooth muscle actin (A-C), and for $P2X_2$ and $P2X_3$ receptors (D-O) in rat embryos. **A:** In embryonic day (E) 12.5 heart, the cardiac muscles in the heart and the smooth muscle in the dorsal aorta (arrows) showed α-smooth 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

Fig 7



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 = 100μm C (applies to A-C). Scale bar in O = 100μm in G-I,M-O, 50μm in D-F,J-L.

Chapter III Abundant and dynamic expression of G protein-coupled P2Y receptors in mammalian development

ABSTRACT

Extracellular ATP mediates diverse biological effects by activating two families of receptors, the P2X and P2Y receptors. There is growing evidence to show that activation of G protein-coupled P2Y receptors can produce trophic effects in many cell types. Yet the expression and function of the P2Y receptors in development has rarely been studied, and has never been investigated in mammalian development. This study used the reverse transcriptase-polymerase chain reaction and immunohistochemistry to demonstrate the abundant and dynamic expression of P2Y receptors in rat development. These receptors were expressed in a wide range of embryonic structures, notably somites, skeletal muscle, the central and peripheral nervous system, the heart, lung and liver. All the P2Y receptors studied were expressed as early as E11, when most embryonic organs were far from being functional and still in the process of being formed. P2Y receptor proteins were strongly expressed in temporary, developmental structures that do not have a correlate in the adult animal, including the somites ($P2Y_1$, $P2Y_2$ and $P2Y_4$) and the floor plate of the neural tube ($P2Y_1$). P2Y receptors were also dynamically expressed,

with receptor mRNA and protein being both up- and down-regulated at different developmental stages. The down-regulation of the $P2Y_{1, 2}$ and $_4$ receptor proteins in skeletal muscle and heart, and the disappearance of the $P2Y_4$ receptor from the brainstem and ventral white matter of the spinal cord postnatally, demonstrated that many P2Y receptors were likely to be involved in functions specific to embryonic life. Thus, these findings strongly suggest that P2Y receptors play an important role in the development of many tissues, and pioneer further studies into the role of purinergic signaling in development.

INTRODUCTION

Extracellular nucleotides evoke responses by activation of two families of purinoceptors, namely the P2X and P2Y receptors (Abbracchio and Burnstock, 1994). Whereas P2X receptors are ligand-gated ion channels, P2Y receptors are G protein-coupled receptors. Eight P2Y receptor subtypes have been cloned to date in mammals ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) (Abbracchio et al., 2003). These receptors couple predominantly to phospholipase C activation, leading to the formation of inositol phosphate and mobilization of intracellular Ca^{2+} (Ralevic and Burnstock, 1998; Vassort, 2001). In addition to increased intracellular Ca^{2+}, a variety of signal transduction pathways, involving protein kinase C, phospholipase A2 and the mitogen-activated protein kinases, mediate the effects of P2Y receptors. Thus, activation of the P2Y receptors can cause long-term, trophic effects on cell activity (Neary et al., 1996; Abbracchio and Burnstock, 1998, Burnstock, 2002a).

P2Y receptors have been implicated in the regulation of cell proliferation and differentiation. Activation of P2Y receptors on a variety of cell types, including astrocytes and vascular smooth muscle cells, results in cell proliferation (Burnstock, 2002b; Neary et al., 1998; 1999; Neary, 2000). In contrast, activation of the $P2Y_{11}$ receptor has been strongly implicated in neutrophil differentiation (Jiang et al., 1997; Conigrave et al, 1998; Communi et al., 2000). Although these

findings strongly suggest a role for purinergic signaling in development, there have been few studies focusing on the expression and function of P2Y receptors in embryonic and postnatal development.

The *Xenopus* P2Y receptor, the XIP2Y (also called $P2Y_8$), was shown in the neural plate during neurulation, but not detected after neural tube closure (Bogdanov et al., 1997), suggesting a role for this receptor in the process of neurulation. More recently, expression of $P2Y_1$ receptor ($cP2Y_1$) mRNA and protein has been demonstrated during chick embryonic development (Meyer et al., 1999a; Choi et al., 2001). Meyer et al. (1999a) reported strong expression of $cP2Y_1$ receptor mRNA in undifferentiated limb mesenchyme cells, but expression was lost as the cells differentiated. The same group also demonstrated that ATP acting by means of $cP2Y_1$, significantly inhibited cartilage formation in micromass cultures (Meyer et al., 2001).

These studies are limited to the $P2Y_1$ and $P2Y_8$ receptors. Other P2Y receptor subtypes have rarely been studied in development, and none have ever been studied in mammalian *in vivo* development. In this chapter we used the reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry to investigate the expression of four P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$, in rat embryonic and postnatal development. Expression of all the P2Y receptors cloned in rat to date was studied by RT-PCR. In addition, by using specific antibodies

that have only recently become commercially available, the localization of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor proteins was investigated. Thus, it has been possible to demonstrate for the first time, the abundant and dynamic expression of P2Y receptors in mammalian development.

MATERIALS AND METHODS

RT-PCR analysis

Total RNA was extracted from fresh embryonic tissues at E11, E12, E14 and E18 by using SV Total RNA Isolation system (Promega, Madison, WI). Reverse transcription and cDNA amplification for all the P2Y receptors was carried out with a thermal cycler (Hybaid, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech, Buckinghamshire, UK). Every sample was further treated with Amplification Grade DNase I (Sigma, UK) to remove any residual DNA present that could generate false positive results. Briefly, 1μg 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, Bethesda, MD) for P2Y receptors (Bailey et al., 2000; 2001; see **Appendix**) 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 $P2Y_1$, 60°C for $P2Y_2$ and $P2Y_6$, 64°C for $P2Y_4$), 72°C for 1.5 min for 35 cycles (40 cycles for $P2Y_4$), followed by a further stage of 10-min extension at 72°C. The resulting PCR products were resolved in a 2% agarose gel containing ethidium bromide and observed under ultraviolet illumination.

RT-PCR results for all tissues were confirmed by repetition with at least three separate RNA samples, prepared from embryonic tissues obtained from separate rat litters. Furthermore, a minimum of three RT-PCR experiments were performed for each P2Y receptor on each individual embryonic tissue. Control experiments were conducted by denaturing the reverse transcriptase (95°C for 15 min) before the RT-PCR reaction. These experiments demonstrated that, on denaturation of the reverse transcriptase, no P2 receptor mRNA could be detected. Thus, we were able to verify that the results obtained were due to the presence of P2 receptor mRNA in a sample and not as a result of genomic DNA contamination.

Tissue preparation

Please refer to the section of **Materials and Methods** on Chapter II. Tibialis anterior muscles from both 3 –week- and 2-month-old animals were collected and freshly embedded in Tissue-Tek. Frozen sections (12μm) were cut in a cryostat and mounted.

DAB Immunohistochemistry and Immunofluorescence double labeling

Please refer to the section of **Materials and Methods** on Chapter II for immunohistochemistry (using DAB as chromogen). For double labeling using antibodies raised from different species, the primary antibodies were applied to the tissue sections at the

same time for overnight incubation at room temperature. After washing with PBS, secondary antibodies were applied to the tissue sections at the same time for 1 hour incubation at room temperature. The sections were washed and mounted with glycerol:PBS (9:1). For double labeling using antibodies raised from same species (e.g. rabbit), the first primary antibody was applied to the tissue sections for overnight incubation at room temperature. After washing and secondary antibody incubation, sections were washed and the second primary antibody was then applied to the same tissue sections for overnight incubation at room temperature. After washing with PBS, second secondary antibodies were applied to the tissue sections for 1 hour incubation at room temperature. The sections were washed and mounted with glycerol:PBS (9:1). The primary antibodies used were rabbit anti-$P2Y_{1,2,4}$ (diluted 1:200 for $P2Y_1$ and $P2Y_2$; 1:100 for $P2Y_4$; Alomone, Israel; see **Appendix**), rabbit anti-PGP9.5 (UltraClone Ltd., UK), mouse anti-α-smooth muscle actin (1:400, Sigma) and mouse anti-MF20 (1:100, Developmental Studies Hybridoma Bank). For labeling of acetylcholine receptors, sections were incubated with Texas Red-conjugated α-bungarotoxin (1:800, Sigma). The secondary antibodies used were either fluorescein Isothiocyanate-conjugated or Cy3-conjugated (Jackson Immunoresearch Laboratories, Inc., WestGrove, PA).

Photomicroscopy

Please refer to the section of **Materials and Methods** on Chapter II.

RESULTS

RT-PCR for P2Y receptors

By using RT-PCR, the developmental expression profiles for $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ were studied in rat embryos (**Fig. 1**, **Table 1, 2**). All the P2Y receptor subtypes examined were detected as early as embryonic day 11 (E11), and mRNA transcripts were present throughout development (E12-E18). From E11 onward, clear bands were observed for $P2Y_1$ and $P2Y_4$ mRNA transcripts (**Fig 1**). $P2Y_2$ receptor expression was very weak at E11 but became progressively stronger with development. Although $P2Y_6$ receptor expression was present as early as E11, expression was only barely detectable and strong expression was observed only at E18.

To determine more precisely the location of P2Y receptor expression, brain, heart, liver, lung and muscle were taken from both E14 and E18 embryos for RT-PCR analysis (**Fig. 1, Table 2**). All embryonic tissues examined expressed at least one P2Y receptor subtype. However, different embryonic organs demonstrated different P2Y receptor profiles. For example, at E14 only $P2Y_4$ receptor mRNA was detected in the brain, whereas $P2Y_1$, $P2Y_2$ and $P2Y_4$ were expressed in lung, and all the P2Y receptors tested ($P2Y_{1,\ 2,\ 4,\ 6}$) were expressed in heart.

Furthermore, the expression of P2Y receptor mRNA was up- and down-regulated during the course of embryonic development. In the brain, no $P2Y_1$ was detected at E14, but at E18, RT-PCR demonstrated strong expression. Similarly in muscle, $P2Y_6$ expression only began at E18. In contrast, whereas $P2Y_4$ was expressed in the lung at E14, no $P2Y_4$ transcripts were detected at E18.

Immunohistochemistry demonstrated expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor protein in somites

P2Y receptor immunoreactivity was first detected in embryos at E11. At this stage $P2Y_1$ and $P2Y_4$ receptor proteins were detected in the somites. Whereas the $P2Y_4$ receptor was detected in the dermomyotome, $P2Y_1$ receptor expression appeared to be restricted to the myotome (**Fig. 2 A, C and D**). The $P2Y_2$ receptor was also expressed in the somites at E12 (**Fig. 3 A**), a day later than the expression of $P2Y_1$ and $P2Y_4$. By using MF20 as a marker for the myogenic cells of the myotome (**Fig. 3 B**), the expression of all the P2Y receptors was demonstrated specifically in this area of the somite. As in the case of $P2Y_1$ and $P2Y_4$, double-labeling for $P2Y_2$ and MF20 showed co-localization of these proteins in the myotome as opposed to the sclerotome (**Fig. 3 C**).

Immunohistochemistry demonstrated expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor protein in skeletal muscle

Consistent with the expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ in the myotome, the primary skeletal muscle fibers were also immunopositive for these P2Y receptors (**Fig. 2 B, E and F**). However, in the case of the $P2Y_1$ and $P2Y_2$ receptors, expression was not maintained. By E18, no $P2Y_1$ or $P2Y_2$ immunoreactivity was detected in skeletal muscle fibers (**Fig. 2 G**). In contrast, at this stage strong $P2Y_4$ receptor expression was found in all skeletal muscle masses (**Fig. 2 H**).

Examination of postnatal skeletal muscles (3-week and 2-month old rats) demonstrated the down-regulation of $P2Y_4$ receptor expression. Immunoreactivity for $P2Y_4$ was detected only in the peripheral muscle fibers. $P2Y_1$ and $P2Y_2$ receptor expression was detected in cells in between muscle fibers. The smooth muscle layer of blood vessels showed strong $P2Y_1$ receptor staining (**Fig 3 D**), as identified by double labeling with smooth muscle actin (data not shown). The $P2Y_2$ receptor was expressed in scattered cells adjacent to the skeletal muscle fibers (**Fig 3 E**). Although the identity of the cells was not confirmed, this expression pattern would suggest that these cells were skeletal muscle satellite cells. None of the P2Y receptors showed any immunoreactivity at the neuromuscular junction (identified by staining with Texas Red-labeled α-bungarotoxin) in either the prenatal or postnatal skeletal muscle examined (**Fig 3 D-F**).

Immunohistochemistry demonstrated expression of only $P2Y_1$ and $P2Y_4$ receptor protein in the brain

Consistent with RT-PCR results, which demonstrated the expression of $P2Y_4$ receptor mRNA alone at E14, only $P2Y_4$ receptor protein was detected in embryonic brain at this age (**Fig. 4 A- F**). Receptor expression was demonstrated in the olfactory system, diencephalons, amygdala and brainstem. In the olfactory system, $P2Y_4$ receptor staining was detected in the olfactory nerve and lateral olfactory tract (**Fig. 4 A and B**). In the diencephalons, expression was restricted to the anterior hypothalamus, dorsal geniculate nucleus and lateral hypothalamic area (**Fig. 4 C and D**). In the amygdala, only the cortical amygdaloid nucleus showed $P2Y_4$ receptor expression (**Fig. 4 D**). $P2Y_4$ receptor expression in the brainstem was widespread. The midbrain, pons and medulla all showed $P2Y_4$ receptor immunoreactivity (**Fig. 4 E and F**). The cerebral cortex, the basal ganglia, the hippocampus and the cerebellum did not show any $P2Y_4$ receptor staining at E14.

Expression of $P2Y_4$ receptor protein was not maintained in all parts of the brain postnatally. In the neonatal brain (postnatal day 1, P1), $P2Y_4$ receptor expression disappeared from the midbrain, isthmus and medulla. However, receptor protein was still detected in the olfactory system, the amygdala, the diencephalons and the pons. In addition, areas such as the septum and the neuroepithelium (adjacent to the ventricles), which had previously not showed any staining

for $P2Y_4$ became immunopositive for this receptor (**Fig. 4 G and H**). Consistent with the up-regulation of $P2Y_1$ receptor mRNA in late embryonic development, $P2Y_1$ receptor protein was also detected in the P1 brain. Receptor expression was restricted to the cerebral peduncle (**Fig. 4 I**). As would be predicted from RT-PCR no $P2Y_2$ receptor protein was expressed at any stage examined.

Immunohistochemistry demonstrated expression of P2Y receptor protein in the spinal cord and peripheral nervous

$P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor proteins were all expressed in the neural tube and peripheral nervous system from E12. At this stage, $P2Y_1$ receptor protein was detected in the floor plate of the spinal neural tube (**Fig. 5 A**). Subsequently, expression was detected in the ventral commissure of both E14 and E18 spinal cord (**Fig. 5 B**). $P2Y_1$ receptor protein was also detected weakly in the gray matter, but not the white matter at E18 (**Fig. 5 B**). The $P2Y_2$ receptor was first detected in the spinal motor nerves in E12 embryos (**Fig. 5 C**). At E14, $P2Y_2$ receptor protein was expressed heavily in the white matter of the intermediate and ventral horns, and the dorsal column of the spinal cord (**Fig. 5 D**). The gray matter showed clear but relatively weaker $P2Y_2$ immunoreactivity. This pattern of expression was maintained in late embryonic development (**Fig. 5 E**). At E18, the presence of $P2Y_2$ receptor expression in the dorsal root ganglia was also clearly apparent (**Fig. 5 E**). $P2Y_4$ immunoreactivity was first weakly detected

in the ventral horn of the spinal neural tube at E12. Staining for $P2Y_4$ in the ventral horns increased in strength at E14. Both the spinal motor neurons and the white matter showed immunoreactivity for this receptor (**Fig. 3 G**). However, double labeling with the neural marker PGP9.5 showed that $P2Y_4$ was not expressed in any of the peripheral nerves (**Fig. 3 J-L**). In the E18 spinal cord, $P2Y_4$ expression disappeared from the ventral white matter and was only weakly expressed in the gray matter and dorsal root ganglia (**Fig. 5 F**).

Immunohistochemistry demonstrated expression of P2Y receptor proteins in the embryonic cardiovascular system, liver, lung and lens

$P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor proteins were strongly expressed in the cardiovascular system, liver, lungs and lens. The $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were first weakly expressed in the heart at E11 and expression became stronger at E12 (**Fig. 6 A and B**). Immunostaining was localized to both the primitive atria and ventricles at this stage. By E14, the P2Y receptors ($P2Y_1$, $P2Y_2$ and $P2Y_4$) were detected only in the atria and the inner trabecular layer of the ventricles (**Fig. 6 C**). The outer myocardial layer and the interventricular septum showed either weak or no expression. At E18, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptor expression was restricted to the atria and the inner trabecular layer of the ventricles. No P2Y receptor proteins were detected in the myocardium and interventricular septum (**Fig. 6 D**). In the case of all three P2Y receptors detected, $P2Y_1$, $P2Y_2$ and $P2Y_4$, expression appeared to be restricted to the myocardium, with no

immunostaining present in the endocardium. This was confirmed by double-labeling experiments, performed for $P2Y_{1,2,4}$ and muscle myosin/ von Willebrand factor (a marker for endothelial cells), which showed that expression of the P2Y receptors was localized to the myocardial and not the endocardial cells (data not shown).

In the case of $P2Y_1$, receptor protein was also detected in the blood vessels from E12 onwards (**Fig. 6 E and F**). Colocalization experiments, performed for $P2Y_1$ and smooth muscle actin/ von Willebrand factor (a marker for endothelial cells), confirmed that expression of the $P2Y_1$ receptor was localized to the smooth muscle cells of the dorsal aorta and not the endothelial cells (data not shown).

P2Y receptor expression in the liver began at E14 (**Fig. 7 A**). At this stage only the $P2Y_1$ receptor was expressed. By E18, $P2Y_2$ receptor expression could also be detected. Whereas the $P2Y_1$ receptor showed distinct staining in scattered cells (**Fig. 7 B**), very weak $P2Y_2$ receptor expression was detected in the general embryonic liver parenchyma (data not shown). The $P2Y_4$ receptor was not detected in the liver in any of the stages examined.

In the lung, the smooth muscle layer beneath the bronchial epithelium showed $P2Y_1$

receptor immunoreactivity weakly in E14 and clearly in E18 embryos (**Fig. 7 C**). The $P2Y_2$ receptor was not expressed in the lung until E18. Some, but not all, of the epithelial cells of the bronchus within the fetal lung showed $P2Y_2$ immunoreactivity (**Fig. 7 D**). $P2Y_4$ receptor expression was absent from the lung (**Fig 7 E**).

The $P2Y_1$ receptor was strongly expressed in the primary lens fibers at E14 (**Fig. 7 F**). Although these fibers differentiate from the epithelial cells in the anterior part of the lens (between the lens capsule and the elongating primary lens fibers), the epithelial cells showed no $P2Y_1$ receptor immunostaining. Immunoreactivity for the $P2Y_1$ receptor was maintained in the lens fibers postnatally (**Fig. 7 G**). At this stage, the $P2Y_4$ receptor was also expressed in the lens fibers but was absent from lens epithelial cells (**Fig. 7 H**). No $P2Y_2$ receptor expression was detected in the lens at any stage examined.

DISCUSSION

Despite that P2Y receptors have been shown to regulate cell proliferation and differentiation, key processes in tissue formation, few studies have investigated the expression and function of P2Y receptors in embryonic development. Before this study, the expression of the $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors had never been investigated in development and none of the P2Y receptors had ever been studied in mammalian development. Using RT-PCR and immunohistochemistry, it was possible to demonstrate the abundant and dynamic expression of the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in many tissues and organs, including skeletal muscle, heart, brain, spinal cord, liver, lung and lens, in rat embryonic and postnatal development. The pattern and timing of receptor expression strongly suggested a role for these receptors in development.

All the P2Y receptors studied were expressed as early as E11, when most embryonic organs far from being functional, were still in the process of being formed. Furthermore, P2Y receptor proteins were strongly expressed in transient, developmental structures, including the somites ($P2Y_1$, $P2Y_2$ and $P2Y_4$) and the floor plate of the neural tube ($P2Y_1$). While both these structures play an essential role in embryonic development, the floor plate in patterning the ventral neural tube and the somites in the formation of mesodermal tissues, neither are retained. P2Y receptors were also dynamically expressed, with receptor mRNA and protein being both up- and

down-regulated. The down-regulation of the $P2Y_{1,\ 2}$ and $_4$ receptor proteins in skeletal muscle and heart, and the disappearance of the $P2Y_4$ receptor from the brainstem and ventral white matter of the spinal cord postnatally demonstrated that many P2Y receptors were developmentally regulated and were involved in functions specific to embryonic life. Thus these findings strongly suggest that whereas there are many well-recognized functions for P2Y receptors in mature, adult tissues, P2Y receptors may also play a role in tissue formation and development.

Skeletal muscle

The transient expression of the $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in skeletal muscle strongly suggests a role for these receptors in the formation and differentiation of skeletal muscle. The onset of $P2Y_1$ and $P2Y_4$ receptor expression (E11) was similar to that of MyoD and Myf5, the two myogenic transcription factors responsible for defining the muscle precursor cells (myoblasts) (Buckingham, 2001). The expression of both receptors was ultimately confined to the myotome, as opposed the dermatome that will form the dermis of the skin. It is still difficult to determine whether the expression of $P2Y_1$ and $P2Y_4$ receptors was required for myogenic specification, or the expression follow the specification, unless the P2Y receptor expression has been positioned in the signaling pathway of myogenesis. The $P2Y_2$ receptor was expressed a day later (E12) in the myotome. P2Y receptor expression in muscle was not maintained. Staining for the $P2Y_1$ and $P2Y_2$ receptors disappeared by E18 and $P2Y_4$ receptor

expression was down-regulated postnatally. These findings suggested that while the P2Y receptors were unlikely to be involved in myogenic specification, these receptors could regulate subsequent processes in muscle formation including the proliferation of myoblasts, migration or fusion to form primary and secondary myotubes (Buckingham, 2001). In fact, a recent study by Ryten et al. (2002) demonstrates that myoblast proliferation *in vitro* can be potentiated by application of UTP, an agonist at both $P2Y_2$ and $P2Y_4$ receptors. P2Y receptors have also been implicated in the migration of several cell types, including vascular smooth muscle cells and human dendritic cells (Idzko et al., 2002; Pillois et al., 2002).

The pattern of P2Y receptor expression in skeletal muscle also suggested a role specifically for the $P2Y_4$ receptor in myotube maturation and differentiation. $P2Y_4$ receptor expression was maintained during postnatal development of muscle fibers, which included muscle fiber hypertrophy and maturation of the neuromuscular junction. Since these processes are largely dependent on changes in intracellular Ca^{2+} concentration and P2Y receptor activation will result in the mobilization of intracellular calcium, this receptor has the potential to be involved in any of these processes (Olson and Williams, 2000a; 2000b; Sanes and Lichtman, 2001). However, because neither the $P2Y_4$ receptor, nor for that matter the $P2Y_1$ and $P2Y_2$ receptors, were found to be expressed specifically at the neuromuscular junction, it is more likely that $P2Y_4$ receptor activation plays a role in muscle fiber hypertrophy or fiber-type determination. Although these

findings do not entirely agree with a recent study by Choi et al. (2001), they are consistent with the work of Meyer et al. (1999a). Choi et al. (2001) have demonstrated the up-regulation of the $P2Y_1$ receptor during the course of embryonic muscle development in chick and expression of $P2Y_1$ receptor protein at the adult neuromuscular junctions in chick and rat, whilst our findings and those of Meyer et al. (1999a) suggest that this receptor is down-regulated in development and is not expressed at the neuromuscular junction.

Heart

The dynamic expression of the P2Y receptors in embryonic heart also suggested a role for these receptors in the development of cardiac muscle. Immunoreactivity for the $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors was detected in both the primitive atria and ventricles of the embryonic heart from E11, just before trabeculations first become evident along the inner myocardial layers (E11.5) (Sedmera et al., 2000). At E14, when trabeculations develop and become compressed within the ventricular wall, P2Y receptor expression was restricted to the trabeculated layers of the atria and ventricles, no immunoreactivity being found in the compact layer of the ventricular myocardium. Consistent with previous reports, P2Y receptors were down-regulated with further development (Webb et al., 1996).

Because formation of the trabeculated layer of the heart and its fusion with the compact layer is vital to heart development, the expression of the P2Y receptors in the trabeculated layer

specifically, is likely to be of functional significance. In fact, trabeculations are so vital to cardiac morphogenesis that the absence of these structures in neuregulin null mice results in embryonic death at E11 (the tubular heart stage) (Gassmann et al., 1995; Lee et al., 1995). Thus, the pattern and timing of P2Y receptor expression in the heart might suggest a role for these receptors in the differentiation of the trabeculated layer and the formation of the ventricular myocardium.

Nervous system

Expression of the P2Y receptor proteins, and particularly the $P2Y_4$ receptor, was prominent in the embryonic nervous system. Among all the P2Y receptors examined, the $P2Y_4$ receptor was the first to be expressed in the embryonic brain at E14 on the basis of both RT-PCR and immunohistochemistry. Instead of a general or weak expression throughout the whole brain, the receptor expression was intense and localized. At E14, $P2Y_4$ immunoreactivity was detected in the telencephalon (olfactory system, pallidum and amygdala), diencephalons (lateral hypothalamic area and dorsal geniculate nucleus) and brainstem (midbrain, pons and medulla). After birth, additional regions of the brain, such as the septum and the neuroepithelium adjacent to the ventricles showed $P2Y_4$ receptor expression, and $P2Y_1$ receptor staining was detected in the cerebral peduncle. However, of greatest developmental significance was the disappearance of $P2Y_4$ immunoreactivity from the brainstem after birth. Thus, the $P2Y_4$ receptor appeared to be

the dominant P2Y receptor present early in the brain. The early expression of $P2Y_4$ receptor in specific brain regions and its subsequent down-regulation in some areas, suggested that this receptor has role to play in prenatal brain development, particularly within the brainstem.

The spinal cord showed immunoreactivity for the $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors. Expression of all these receptors was related either directly or indirectly to motor neuron development. The $P2Y_2$ and $P2Y_4$ receptors were expressed in the ventral horns of the embryonic spinal cord, in the case of the $P2Y_4$ receptor only transiently (E14-E18). Both $P2Y_2$ and $P2Y_4$ receptor expression was mainly localized to the white matter of the ventral horns, although the spinal motor neurons also showed weak expression. $P2Y_1$ expression was localized to the floor plate of the spinal neural tube and subsequently the ventral commissure of the spinal cord, the former an important structure in the differentiation of the ventral neural tube. The floor plate expresses a powerful signaling molecule, Sonic hedgehog (Shh), which specifies the identities of motor neurons and interneurons in a concentration-dependent manner (Dodd et al., 1998). Thus the expression of the $P2Y_1$ receptor in floor plate, and the $P2Y_2$ and $P2Y_4$ receptors in the ventral horns, suggests that purinergic signaling could regulate motor neuron development at multiple sites.

It seems likely that not all P2Y receptor expression had a role in embryonic development,

but rather some receptor expression was related to adult function. The lack of dynamic or transient P2Y receptor expression in the embryonic and fetal liver suggested that P2Y receptors were not involved in liver formation. As in adult hepatocytes (Dixon et al., 2000), experiments conducted on embryonic liver demonstrated the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA transcripts and immunoreactivity for $P2Y_1$ and, to a lesser extent, the $P2Y_2$ receptor. Expression of the $P2Y_1$ and $P2Y_4$ receptors in the lens fibers is also unlikely to be involved in lens development. The $P2Y_1$ receptor is strongly expressed in both the adult (Merriman-Smith et al., 1998) and embryonic lens, but the $P2Y_4$ receptor was only expressed postnatally. Nonetheless this study does demonstrate for the first time the expression of the $P2Y_4$ receptor in lens fibers, and expression of this receptor could account for the reports of responses of adult lens cells to ATP (Churchill and Louis, 1997; Collison and Duncan, 2001). Similarly, expression of the $P2Y_1$ and $P2Y_2$ receptors in the fetal lung is unlikely to be related to lung development. In the fetal (E18) lung, the $P2Y_2$ receptor was expressed in the epithelial cells of the bronchi. Adult lung epithelial cells also express this receptor and can respond to the $P2Y_2$ receptor agonist UTP, with an increase in transepithelial chloride secretion and mucus secretion (Rice and Singleton, 1987; Rice et al., 1995; Burnstock, 2002b). The $P2Y_1$ receptor, which is known to be expressed on many types of smooth muscle cells, was also expressed in the smooth muscle layer of the bronchi. Thus, although P2Y receptors are undoubtedly essential for proper lung function, it is very unlikely that embryonic P2Y receptor expression is related to

organogenesis of the lung.

The question of the source(s) of ATP involved in activating these P2 receptors should next be raised. Many cell types are known to release ATP in response to mechanical disturbance (Burnstock, 1999b; Bodin and Burnstock, 2001a,b) and because there is much cellular movement during embryogenesis, several cell types might be releasing ATP. In addition, the presence of apoptotic cells in several developing tissues might represent another source of ATP.

In summary, we have demonstrated for the first time the early and dynamic expression of the $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors during rat embryonic development. Although not all P2Y receptor expression is likely to be related to embryonic development, these findings suggest the involvement of purinergic signaling in skeletal muscle, heart and central nervous system development. Thus, purinergic signaling is likely to be an important signaling system in embryonic development and in particular organogenesis.

The work of this chapter has been published in:

Cheung KK, Ryten M and Burnstock G. (2003). Abundant and dynamic expression of G protein-coupled P2Y receptors in mammalian development. *Dev Dyn.* (in press)

FIGURES & LEGENDS

Figure 1 RT-PCR analysis to investigate the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor transcripts during rat development from embryonic day 11 (E11) to E18. Total RNA from whole embryos (E11, E12, E14 and E18) or isolated embryonic organs (brain, heart, liver, lung and muscle) was extracted and subsequently used for RT-PCR experiments.

Fig 1

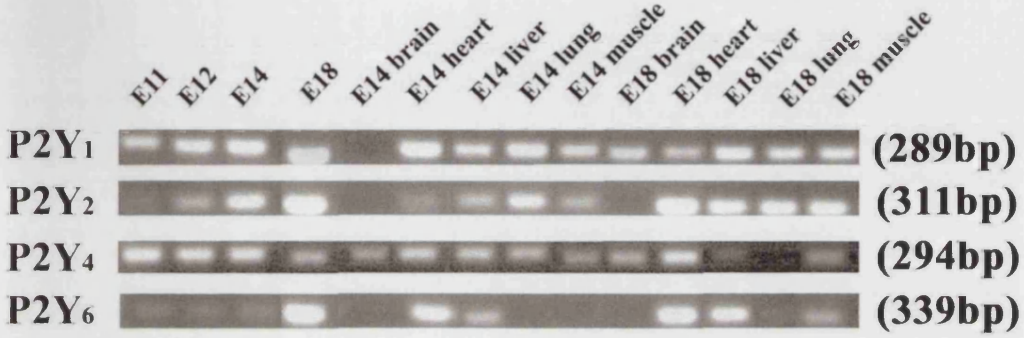

E11
E12
E14
E18
E14 brain
E14 heart
E14 liver
E14 lung
E14 muscle
E18 brain
E18 heart
E18 liver
E18 lung
E18 muscle
P2Y1
(289bp)
P2Y2
(311bp)
P2Y4
(294bp)
P2Y6
(339bp)

Table 1 Summary of the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA at different developmental ages (E11, E12, E14 and E18). Key: (+) indicates clear positive bands, (-) indicated absence of expression and (+/-) indicates barely detectable bands.

Table 2 Summary of the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor mRNA in brain, heart, liver, lung and skeletal muscle taken from E14 and E18 embryos. Key: (+) indicates clear positive bands, (-) indicated absence of expression and (+/-) indicates barely detectable bands.

Table 1

	E11	**E12**	**E14**	**E18**
$P2Y_1$	+	+	+	+
$P2Y_2$	+/-	+	+	+
$P2Y_4$	+	+	+	+
$P2Y_6$	+/-	+/-	+/-	+

Table 2

	Brain		**Heart**		**Liver**		**Lung**		**Muscle**	
	E14	E18	E14	E18	E14	E18	E14	E18	E14	E18
$P2Y_1$	-	+	+	+	+	+	+	+	+	+
$P2Y_2$	-	-	+	+	+	+	+	+	+	+
$P2Y_4$	+	+	+	+	+	+/-	+	-	+	+
$P2Y_6$	-	-	+	+	+	+	-	+/-	-	+

Figure 2: Immunoreactivity for P2Y receptors in somites and developing skeletal muscle. **A,** Transverse section with haematoxylin and eosin staining showing the morphology of E11 somites (sm). **B,** Transverse section showing haematoxylin and eosin staining of an E14 embryo at the mid-liver level. The rectangular insert shows an area of developing skeletal muscle. **C,** Transverse section taken at the level of the embryonic heart at high magnification showing limited $P2Y_1$ receptor expression in E11 somites (sm). In order to aid orientation the ventral (v) and dorsal (d) aspects are marked. $P2Y_1$ receptor expression was detected in the myotome. **D,** Transverse section taken at the caudal region at high magnification showing widespread $P2Y_4$ receptor expression in E11 somites (sm). $P2Y_4$ receptor expression was detected in the dermomyotome. **E,** Higher magnification (of the rectangular insert shown in B) demonstrating $P2Y_1$ receptor expression in the developing skeletal muscle (skm). **F,** Higher magnification (of the rectangular insert shown in B) demonstrating $P2Y_4$ receptor expression in the developing skeletal muscle (skm). **G, H,** Transverse sections of E18 embryo showing the intercostal muscle between ribs (rb). The $P2Y_1$ receptor was not detected in the skeletal muscle fibers (**G**), whereas strong $P2Y_4$ receptor immunoreactivity (**H**) was detected in the intercostal muscle. Scale bar = 50μm (**C, D**), 200μm (**A, E-H**) and 750μm (**B**).

Fig 2

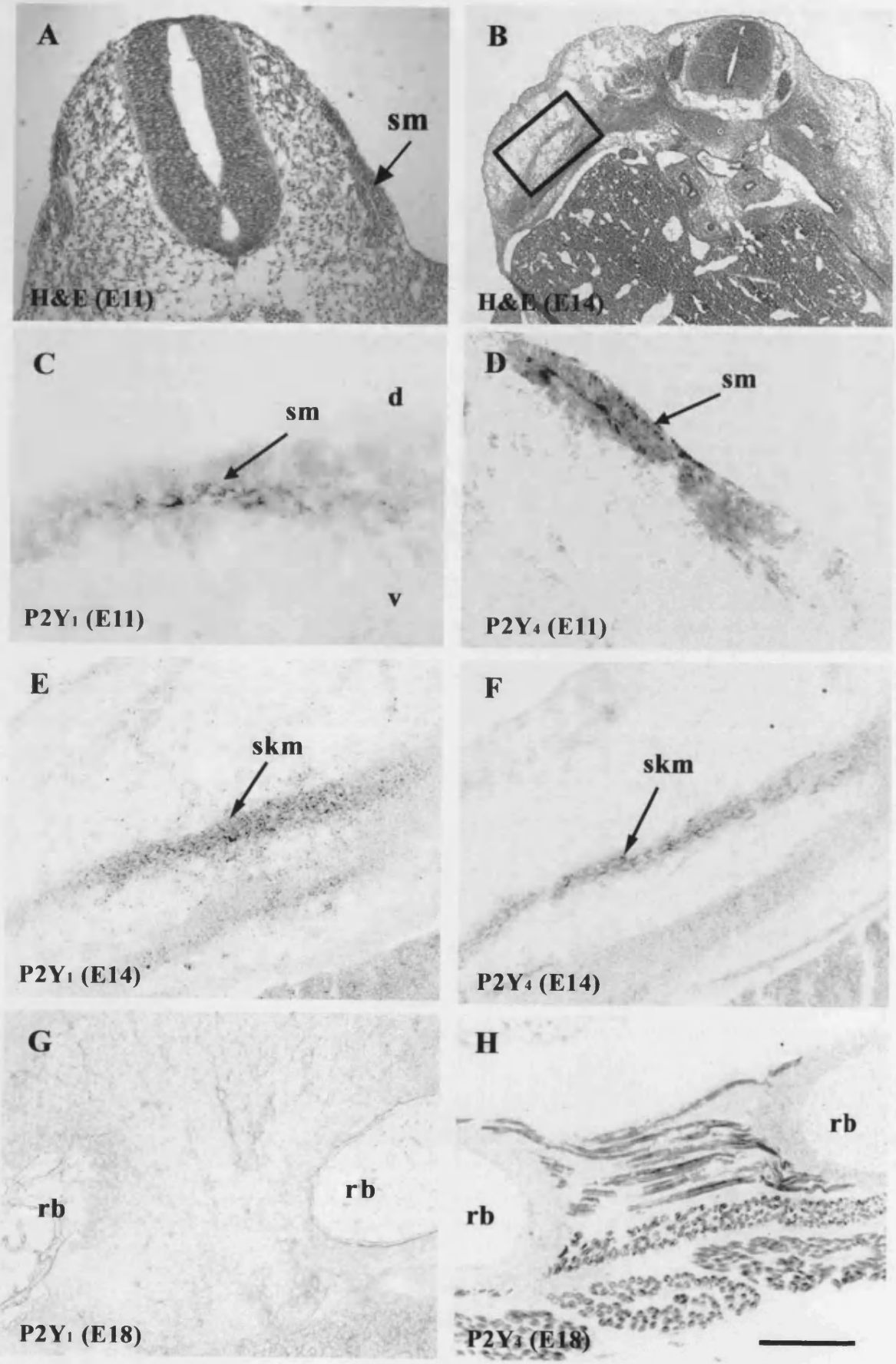
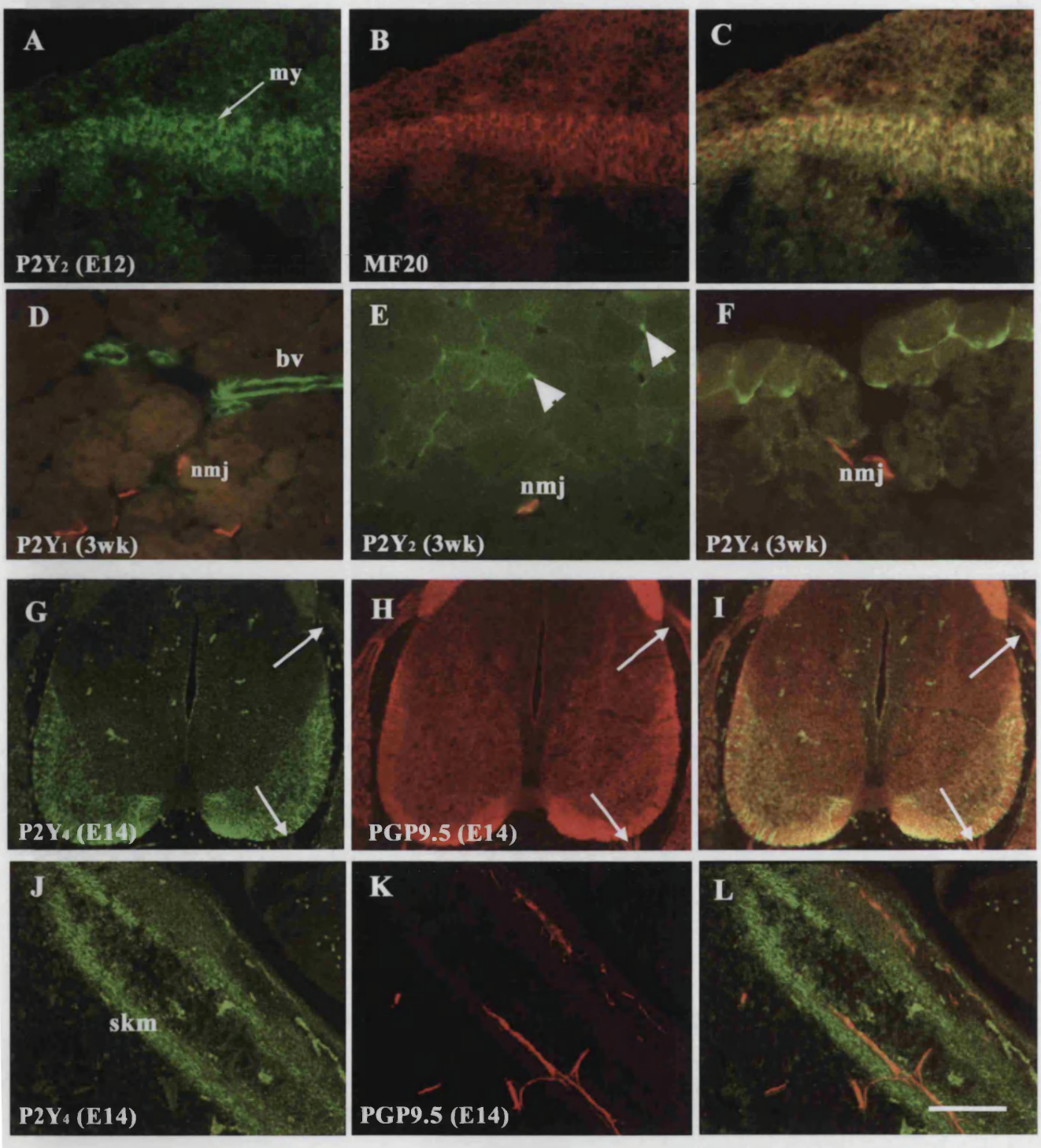

A
sm
H&E (E11)
B
H&E (E14)
C
sm
d
v
P2Y1 (E11)
D
sm
P2Y4 (E11)
E
skm
P2Y1 (E14)
F
skm
P2Y4 (E14)
G
rb
rb
P2Y1 (E18)
H
rb
rb
P2Y4 (E18)

Figure 3: Immunofluorescence of P2Y receptors during skeletal muscle development and in the embryonic spinal cord. **A,** Transverse section showing $P2Y_2$ receptor immunoreactivity (green) in E12 myotomes (my). **B,** The same section also showed immunoreactivity for MF20 (red), a marker of the differentiated myogenic cells. **C,** Double labeling showing that the $P2Y_2$ receptor and MF20 were co-expressed in the myotome (yellow). **D-F,** Double labeling experiments for P2Y receptors (green) and acetylcholine receptors (red) on the tibialis anterior muscle of 3-week old animal. **D,** $P2Y_1$ receptor immunoreactivity was localized at the smooth muscle layer of the blood vessel (bv) between the skeletal muscle fibers. **E,** $P2Y_2$ receptor immunoreactivity was detected in scattered cells (arrowhead) located between skeletal muscle fibers. **F,** $P2Y_4$ receptor protein was detected on the membranes of the peripheral, but not the central muscle fibers. None of the P2Y receptors were detected at the neuromuscular junctions, as demonstrated by acetylcholine receptor expression (red). **G,** Transverse section showing $P2Y_4$ receptor immunoreactivity (green) in the spinal cord of an E14 embryo. Arrows showing the spinal nerves emerging from dorsal (dh) and ventral horns (vh). **H,** PGP9.5 immunoreactivity (red) on the same section (**G**). **I,** Double labeling showing the co-localization (yellow) of the $P2Y_4$ receptor and the PGP9.5 in the ventral spinal horn but not the dorsal spinal cord. **J,** Transverse section showing expression of the $P2Y_4$ receptor (green) in embryonic skeletal muscle (skm). **K,** PGP9.5 stains nerves (red) innervating the muscles in the same section. **L,** Double labeling experiment showing that $P2Y_4$ receptor protein was expressed in the muscles only but not in the

Fig 3


A
my
P2Y2 (E12)
B
MF20
C
D
bv
nmj
P2Y1 (3wk)
E
nmj
P2Y2 (3wk)
F
nmj
P2Y4 (3wk)
G
P2Y4 (E14)
H
PGP9.5 (E14)
I
J
skm
P2Y4 (E14)
K
PGP9.5 (E14)
L

peripheral nerves innervating the muscles. Scale bar = 105μm (**A-C**), 50μm (**D-F**), 200μm (**G-L**).

Figure 4: P2Y receptor immunoreactivity in embryonic and neonatal rat brain. Coronal sections of an E14 embryonic brain are shown in an antero-posterior direction (**A to F**). **A, B,** $P2Y_4$ immunoreactivity was detected in the olfactory tubercle (**A**, ot) and the lateral olfactory tract (**B**, lot). **C,D**, In the diencephalon, $P2Y_4$ receptor protein was expressed in the anterior hypothalamus (**C**, ah), the dorsolateral geniculate nucleus (**D**, dgn) and the lateral hypothalamic area (**D**, lh). The cortical amygdaloid nucleus also stained for $P2Y_4$ (**D**, ca). **E, F,** In the brainstem, the $P2Y_4$ receptor expression was detected in the midbrain (**E**, mb), the pons (**E**, pn), the isthmus (**F**, is) and the medulla (**F**, md). **G, H,** Coronal sections of a neonatal rat brain showing $P2Y_4$ receptor immunoreactivity in the septum (**G**) and the neuroepithelium adjacent to the ventricles (**H**). **I,** Coronal section showing $P2Y_1$ receptor immunoreactivity in the cerebral peduncle. Scale bar = 500μm (**A-F**), 100μm (**G**) and 200μm (**H-I**).

Fig 4

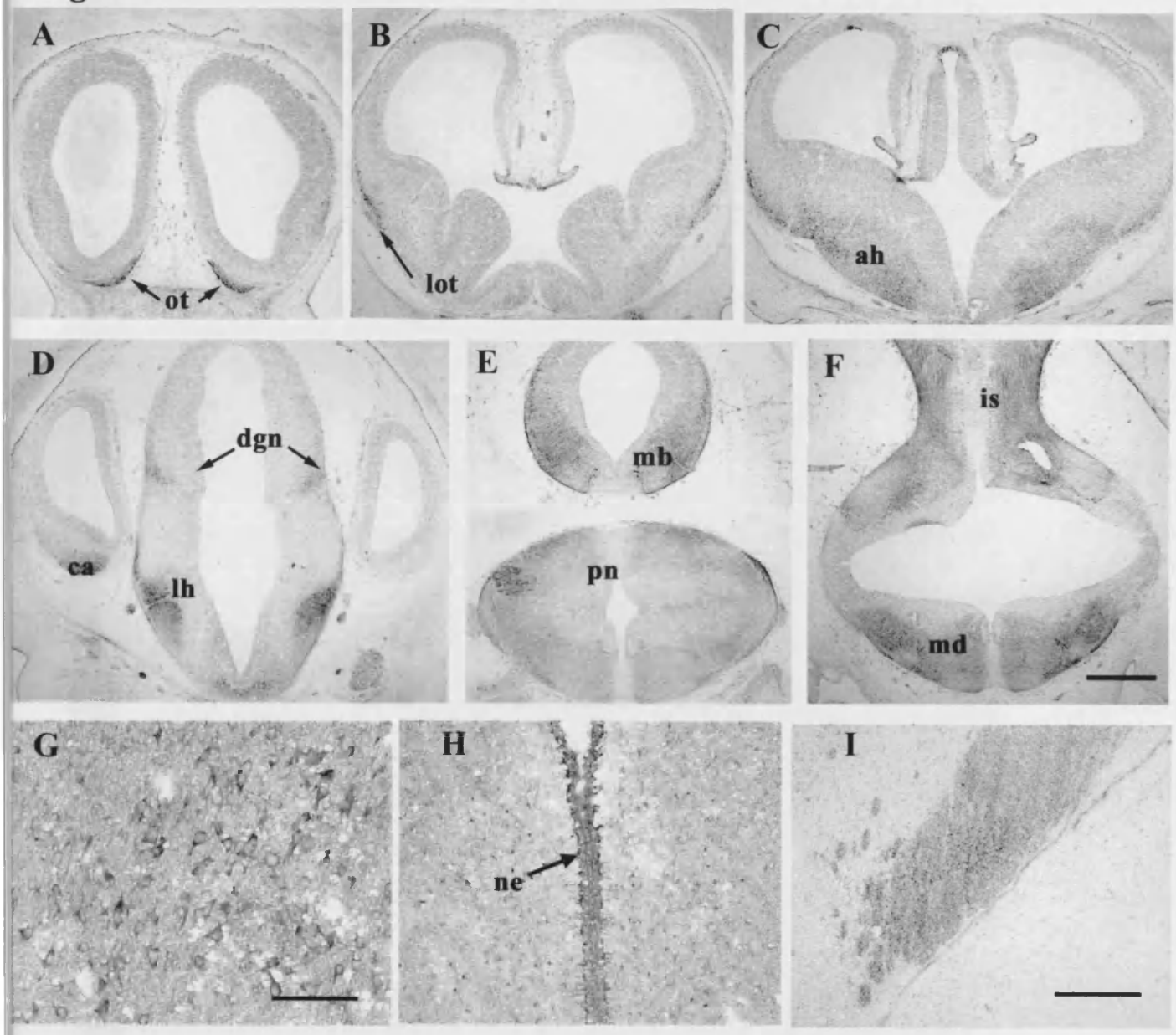


Figure 5: Immunohistochemical expression of P2Y receptors in the embryonic nervous system. **A**, Transverse section of E12 embryos (at the level just caudal to the hindbrain neural tube) showing $P2Y_1$ receptor immunoreactivity specifically located in the floor plate region (fp, arrow). **B,** Transverse section showing $P2Y_1$ receptor expression in the ventral commissure (arrowhead) of the E18 spinal cord. Note the very weak $P2Y_1$ immunoreactivity in the gray matter (gm). **C,** Transverse section of E12 embryos demonstrating $P2Y_2$ receptor expression in spinal nerves (arrows). **D,** In E14 spinal cord $P2Y_2$ receptor protein was heavily expressed in the white matter of the ventral horns (vh) and in the peripheral nerves. The gray matter and dorsal root ganglia (drg) also stained weakly for the $P2Y_2$ receptor. **E,** At E18, widespread $P2Y_2$ receptor expression was detected in the spinal cord, with very strong immunoreactivity in the white matter of the intermediate and ventral horns (arrows). Immunostaining for $P2Y_2$ was also detected in the dorsal root ganglia. **F,** The $P2Y_4$ receptor was expressed in the gray matter of the spinal cord at E18. Weak staining was also observed in the white matter and dorsal root ganglia at this stage. Scale bar = 50μm (**A**), 200μm (**C**), 450μm (**B, E-F**) and 750μm (**D**).

Fig 5

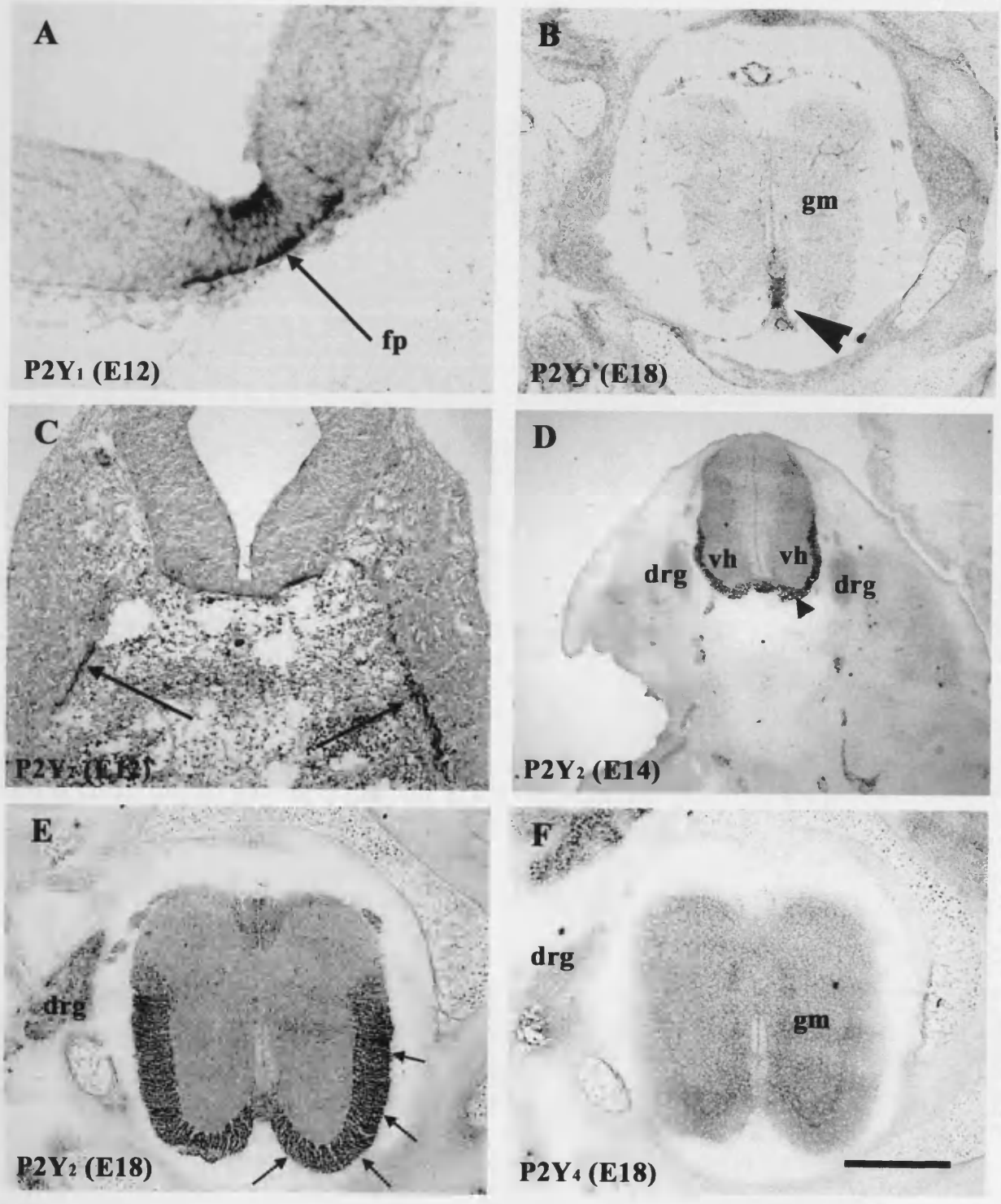

A
fp
P2Y1 (E12)
B
gm
P2Y1 (E18)
C
P2Y2 (E14)
D
drg
vh
vh
drg
P2Y2 (E14)
E
drg
P2Y2 (E18)
F
drg
gm
P2Y4 (E18)

Figure 6: Immunoreactivity for the P2Y receptors in the embryonic cardiovascular system. **A,** Transverse section showing the expression of the $P2Y_2$ receptor in the developing heart at E12. **B,** Transverse section showing the expression of the $P2Y_4$ receptor in the developing heart at E12. **C, D,** Transverse sections showing $P2Y_4$ receptor immunoreactivity in the hearts of E14 (**C**) and E18 (**D**) embryos demonstrate that this receptor was specifically expressed in the trabeculated layer of the ventricles and atria of the developing hearts, but was absent from the interventricular septum (ivs). **E, F,** Transverse sections showing $P2Y_1$ receptor immunoreactivity in the smooth muscle layers of dorsal aorta at E12 (**E**) and E18 (**F**). Scale bar = 200μm (**A, B**), 450μm (**C**), 750μm (**D**), 50μm (**E**) and 200μm (**F**).

Fig 6

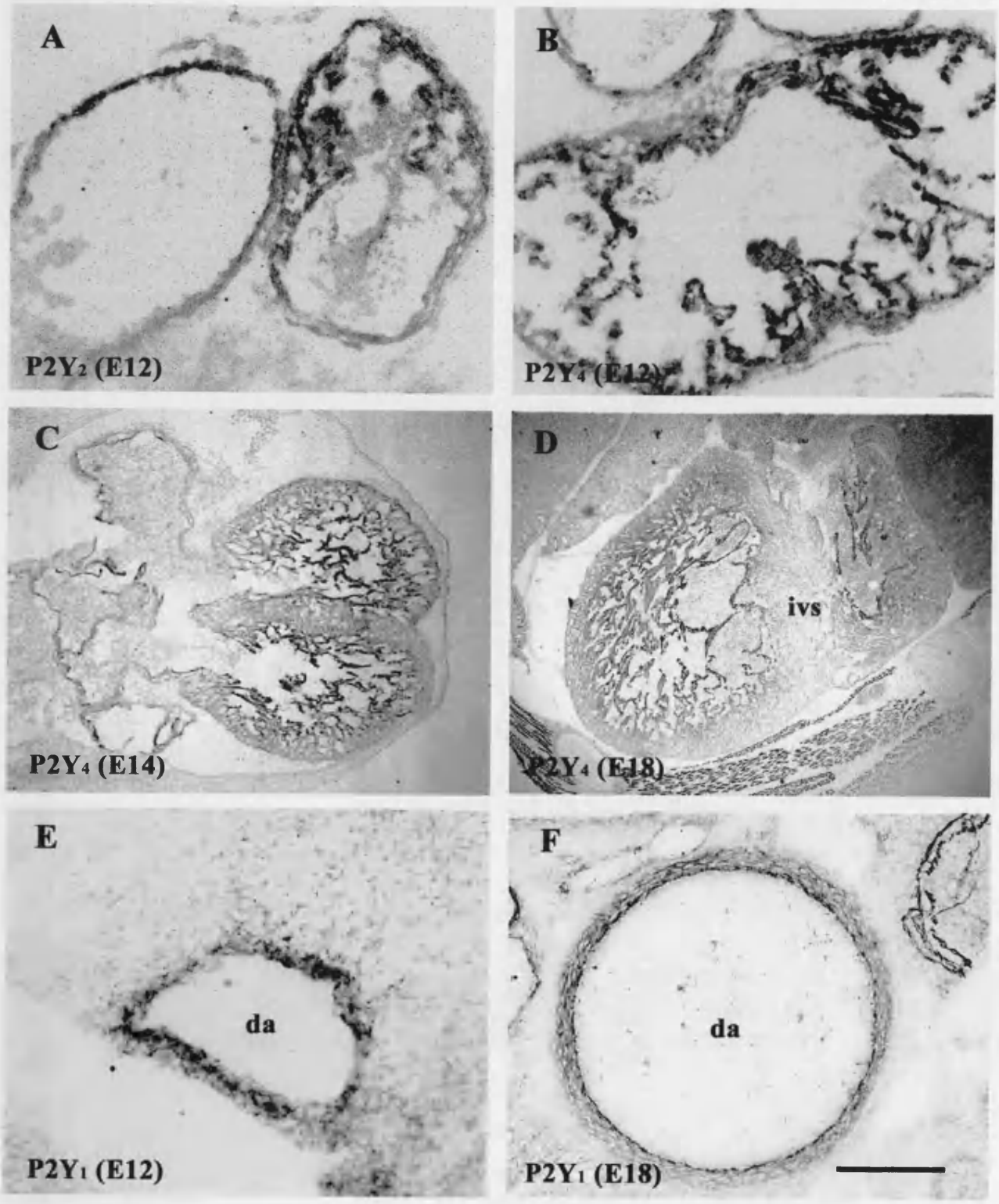
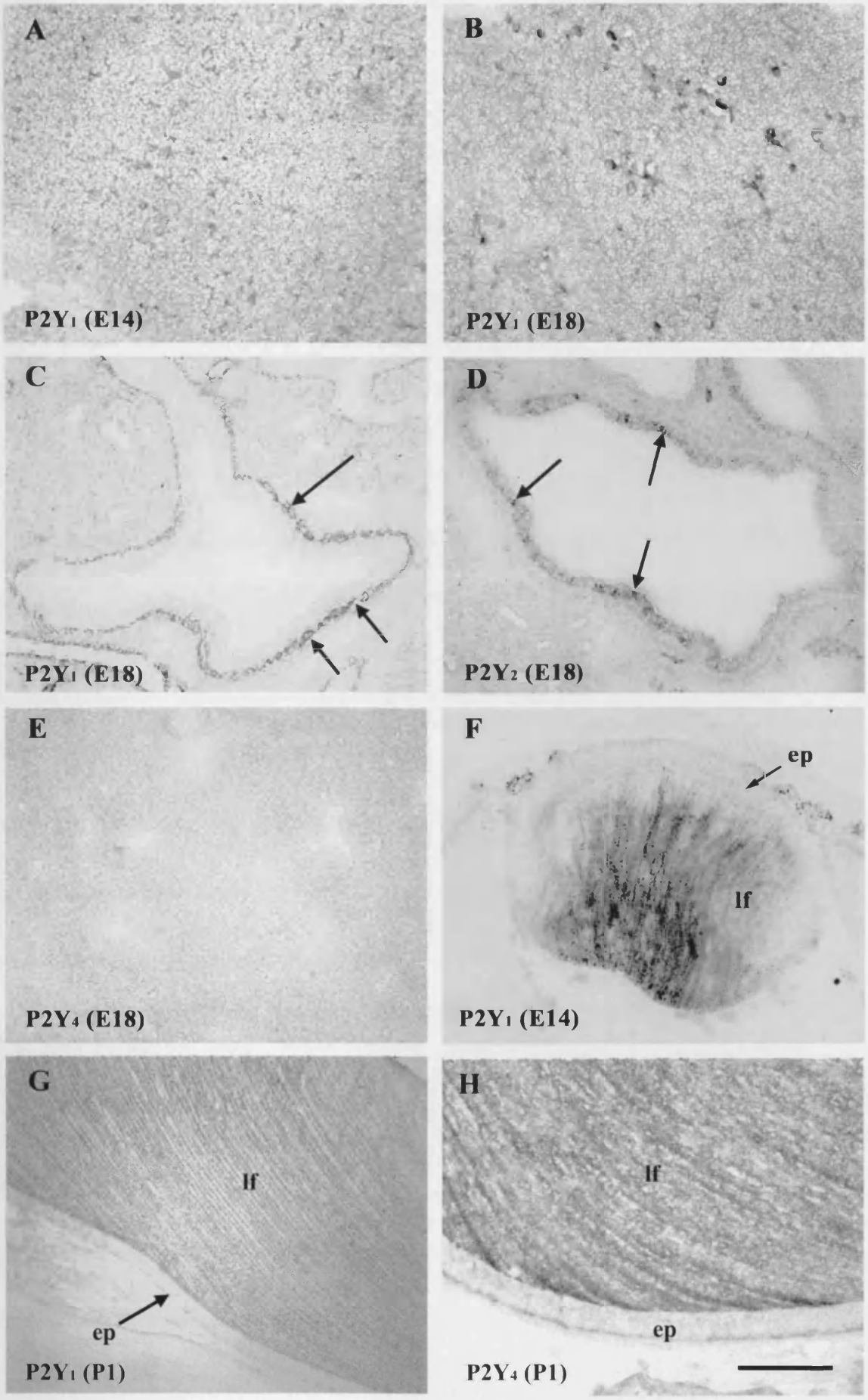

A
P2Y2 (E12)
B
P2Y4 (E12)
C
P2Y4 (E14)
D
ivs
P2Y4 (E18)
E
da
P2Y1 (E12)
F
da
P2Y1 (E18)

Figure 7: Immunoreactivity of P2Y receptors on embryonic liver, lung and lens. **A, B,** Transverse sections showing $P2Y_1$ receptor expression in E14 (**A**) and E18 (**B**) liver. The $P2Y_1$ receptor was expressed weakly in E14 (**A**) and clearly in E18 (**B**) liver. Scattered cells showed clear $P2Y_1$ receptor immunoreactivity in E18 liver. **C, D, E,** Transverse sections showing $P2Y_1$ (**C**), $P2Y_2$ (**D**) and $P2Y_4$ (**E**) receptor-stained E18 lung tissues. Note that the $P2Y_1$ receptor was expressed in the smooth muscle layer of the bronchi (arrows, **C**) and the $P2Y_2$ receptor expressed in the epithelial cells (arrows, **D**). The $P2Y_4$ receptor was not expressed in the lung (**E**). **F, G,** Transverse sections of the lens at E14 (**F**) and P1 (**G**) show that the $P2Y_1$ receptor was expressed in the elongating lens fiber cells (lf), but not the epithelial cells (ep) inside the anterior capsule of the developing lens. **H,** The $P2Y_4$ receptor was also expressed in the elongating lens fiber cells at P1 (lf). Scale bar = 200μm (**A-E**), 50μm (**F**), 100μm (**G**) and 50μm (**H**).

Fig 7


A
P2Y1 (E14)
B
P2Y1 (E18)
C
P2Y1 (E18)
D
P2Y2 (E18)
E
P2Y4 (E18)
F
ep
lf
P2Y1 (E14)
G
lf
ep
P2Y1 (P1)
H
lf
ep
P2Y4 (P1)

Chapter IV Expression of P2X purinoceptors during rat brain development and their inhibitory role on motor axon outgrowth in neural tube explant cultures

ABSTRACT

Extracellular ATP is well known as a neurotransmitter and neuromodulator in adult central and peripheral nervous systems. ATP mediates rapid synaptic responses via ligand-gated P2X receptors. However, little is known about the involvement of ATP during the development of mammalian embryos. We studied the expression pattern of P2X receptor subtype mRNA and protein during prenatal rat brain development (from E10 to P1 brain) using RT-PCR and immunohistochemical methods. It was shown that the $P2X_3$ receptor was the first to be expressed in the neural tube at E11, followed by the $P2X_2$ and $P2X_7$ in the embryonic brain at E14, and later the $P2X_4$, $P2X_5$ and $P2X_6$ receptors at P1. $P2X_7$ receptor was expressed in the lateral hypothalamus and isthmus in the E14 brain, and subsequently expressed throughout the brain as development proceeded. Expression of $P2X_4$ receptor was observed in the striatal subventricular zone, mesencephalic trigeminal nucleus, parabrachial nucleus, lateral deep cerebellar nucleus and cuneate nucleus, whereas $P2X_5$ receptor was detected in the striatal

subventricular zone, supraoptic nucleus and cuneate nucleus. $P2X_5$ immunoreactivity was also observed in cells scattered over the cortex and the diencephalon. $P2X_6$ receptor expression was detected weakly but widespread in the lateral hypothalamus. $P2X_1$ receptor expression was not observed in the brain at any of the stages examined. The sensory-related $P2X_3$ receptor was also expressed in a subpopulation of motor neurons and the outgrowing motor axons in the ventral neural tube of rat embryos at E11-12 and the expression was downregulated as development progressed, whereas the outgrowing axons emerged from somatomotor neurons did not express $P2X_3$ receptor. It was therefore speculated that ATP might be involved in motor axon outgrowth. Thus we then investigated the effect of ATP on motor axon outgrowth in collagen-embedded neural tube explant cultures. Motor neurons-containing neural tube explants at E12 were cultured for 4 days in a 3-dimensional collagen gel bathed with 100μM ATP or αβ-meATP (selective agonist for $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors). Both ATP- and αβ-meATP-treated neural tubes showed a significant reduction in neurite outgrowth compared with the control explants. The inhibitory effect could not be induced by UTP. In conclusion, all P2X receptor subtypes, except for $P2X_1$, are strongly represented in the developing rat embryonic brain. The possibilities will be discussed that: ATP is involved in inhibition of motor axon outgrowth during early embryonic neurogenesis, most likely via the $P2X_3$ receptor; 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.

INTRODUCTION

Extracellular 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 nonadrenergic, noncholinergic (NANC) nerves supplying the gut and bladder that purinergic signaling was hypothesized (Burnstock, 1972). It is now well known that ATP mediates wide ranges 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 ($P2Y_{1,2,4,6,11,12,13,14}$) have been cloned from mammals (Burnstock, 2003a; Abbracchio et al., 2003).

ATP is released from the 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). $P2X_1$ receptor is localized in the adult cerebral cortex, striatum, hippocampus and cerebellum. $P2X_5$ shows restricted localization, where the mRNA transcript was only found in the mesencephalic trigeminal nucleus. $P2X_3$ is present in the sensory-related area such as nucleus tractus solitarius

(Nörenberg and Illes, 2000). $P2X_7$ has been reported recently in the hippocampus and medulla oblongata (Deuchars, 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 like glutamate and GABA (Labrakakis et al., 2000; D'Ambrosi et al., 2001; Sperlágh et al., 2002; see Burnstock, 2003b).

We have previously identified the $P2X_2$ and $P2X_3$ receptor expression in rat embryonic brain (Cheung and Burnstock, 2002). $P2X_2$ receptor is first expressed weakly in the nucleus tractus solitarius at embryonic day 14 (E14), whereas $P2X_3$ 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 $P2X_3$ receptor grew dorsally to leave the hindbrain via the large dorsal exit point to trigeminal ganglia, suggesting that the $P2X_3$ immunoreactive cells were branchiomotor (BM) or visceromotor (VM) neurons (Jacob et al., 2001). However, expression of $P2X_3$ receptor was downregulated during further development. According to the spatial and temporal information of $P2X_3$ receptor expression in the ventral neural tube, we proposed that the receptor has a role to play in axon outgrowth during hindbrain development.

In the present study, we use 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 16 (P16). In addition, we have explored the role of ATP on axon outgrowth during embryonic neurogenesis by culturing hindbrain neural tube explants containing motor neurons in collagen gels in the presence of ATP and its derivative αβ-meATP, which is a stable and selective agonist for $P2X_3$ receptors (and $P2X_1$ receptors).

MATERIALS AND METHODS

RT-PCR analysis

Embryonic and neonatal Sprague-Dawley rat brain tissues of different ages were dissected and the meninges were removed. Total RNA was extracted from the whole brain tissues of four developmental ages (E14, E18, P1 and P16) according to the sections of **Materials and Methods** in Chapter III. The sequence specific primers (Life Technologies, NY, USA) for P2X receptors (Shibuya et al., 1999; see **Appendix**) 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 3 separate RT-PCR experiments were performed for each P2X receptor on each individual embryonic tissue.

Tissue processing and immunohistochemistry

Please refer to the sections of **Materials and Methods** on Chapter II. The primary antibodies used were rabbit polyclonal antibodies against $P2X_{1-6}$ receptors (gifts from Roche Bioscience, USA, 1:200; see **Appendix**) and $P2X_7$ receptor (Alomone, Israel, 1:500 see

Appendix). For control experiments, the sections were incubated with the primary antibodies pre-adsorbed with the control peptide antigens that used in raising the antibodies or with normal horse serum only. Double labeling experiment of $P2X_3$ and Islet-1 antibody (1:700, Developmental Studies Hybridoma Bank, DSHB) was performed as previously described in the section of Materials and Methods in Chapter III.

Photomicroscopy

Images of DAB immunohistochemical staining were taken with a 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).

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 Bioscience) 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 Hank's Balanced Salt Solution (HBSS, Gibco, UK) and kept on ice until needed. Collagen gel was freshly made by mixing the rat tail

collagen (Roche Bioscience, USA) with 7.5% sodium bicarbonate solution and 10X Minimum Essential Medium (MEM) in a ratio of 8:1:1 (collagen: sodium bicarbonate: 10X MEM). A thin layer of collagen gel was laid and allowed to set in the culture well of 4-well pates (Nunc). The neural tube explant was transferred onto the thin collagen layer and a large drop of collagen gel was added to embed the explants. The collgen gel with embedded neural tube explant was covered with high glucose-Dulbecco's modified Eagle medium (DMEM) with Glutamax I (Gibco) supplemented with 1X antibiotic/antimycotic solution and insulin-transferrin-selenium supplements (Gibco). To examine the effects of extracellular purines on the axon outgrowth, 100μM of ATP, αβ-meATP or UTP (Sigma, USA) 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 with fresh medium containing same concentration of the nucleotides every 2 days.

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, USA) for analysis of neurite length and an analytical package

(Metamorphs, Universal Imaging Corporation, USA) for comparison of the number of neurites projecting from the explants between treatment groups. A total of 50 longest neurites from each explant were 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 pixel occupied by the explants to determine the neurite/explant ratio. Neural tube explants together with the collagen gel 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

Previously, we have shown that $P2X_3$ was the first purinoceptor to express 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 (Cheung and Burnstock, 2002). Here we examined the expression pattern of all the known P2X receptor subtypes during rat brain development using RT-PCR and immunohistochemistry. 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 express, though to different extents, from this stage onward into the postnatal period (E14 to P16). The abundance of the $P2X_2$ and $P2X_7$ receptor transcripts appeared approximately the same throughout the stages examined. A gradual decrease in expression of $P2X_3$ receptor was observed, where the expression was high at E14 and E18 but appeared to be lower at P1 and P16. In contrast, the intensity of $P2X_4$ receptor transcripts amplified was gradually increased from E14 to P16. $P2X_5$ and $P2X_6$ receptor transcripts started to express weakly at E18 and P1, respectively. $P2X_1$ receptor transcript was not detected in the brain at all the stages examined although in the positive control using uterine tissues, the primers for $P2X_1$ receptor showed strong positive signals (data not shown).

$P2X_3$ receptor was expressed in the neurons and the outgrowing axons in the hindbrain neural tubes as early as in E11 confirming our earlier report (Cheung and Burnstock, 2002). Immunoreactivities of other P2X receptor subtypes were not observed at this early stage. $P2X_2$ receptor was expressed in the nucleus tractus solitarius at E14. To obtain the spatial information of expression of the P2X receptors, we used immunohistochemistry to examine the distribution of the $P2X_1$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$ receptor protein during rat brain development. Consistent with the RT-PCR results, $P2X_7$ immunoreactivity was observed at E14. Expression was localized in the lateral hypothalamus (**Fig. 2**) and the isthmus. At E18, $P2X_7$ 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 the expression persisted after birth (data not shown). The $P2X_4$ immunoreactivity was not observed until P1, although its mRNA transcript was expressed early at E14. $P2X_4$ receptor was expressed in distinct populations of cells instead of a widespread expression in adult (see review Nörenberg and Illes, 2000) (**Fig. 3**). $P2X_4$ 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. 3**). Likewise, both the receptor proteins of $P2X_5$ and $P2X_6$ receptors were also detected in P1 brain. $P2X_5$ receptor was expressed in the supraoptic nucleus, striatal subventricular zone and cuneate nucleus and in cells scattered over

the cortex and diencephalons (**Fig. 3**). Similar to $P2X_4$ receptor staining, the $P2X_5$-immunopositive cells in the supraoptic nucleus showed cytoplasmic staining and round in shape, whereas the scattered $P2X_5$-immunoreactive cells in the cortex and those in the striatal subventricular zone appeared pyramidal in shape (**Fig. 3**). $P2X_6$ receptor was, however, expressed weakly in the lateral hypothalamus (**Fig. 3**). Specific immunoreactivity for $P2X_1$ receptor was not detected at all the stages examined.

Based on the timing of the initial appearance of the receptors, the sequential expression for P2X receptors during brain development was summarized as 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 ventral 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 to the control group (**Fig 5, 6**). There was a 46% reduction of neurite length in the ATP-treated group (**Fig 6**). Similarly, αβ-meATP, a stable analog of ATP and also the agonist for $P2X_3$ receptor, caused a 41% decreased in axon length (**Fig 6**). Application of UTP did not show significant inhibitory effect on neurite outgrowth from neural tube explants compared to control explants, eliminating the possibility of $P2Y_2$ and $P2Y_4$ receptor involvement (**Fig 6**). We subsequently compared the area occupied by all the outgrowing neurites (excluding the neural tube explants) between treatment groups. Both ATP and αβ-meATP caused a reduction of 61% and 62%, respectively, of the total area occupied by the neurites (**Fig 6**). To minimize the variation in neurite outgrowth due to the difference in the sizes of the neural tube explants, the neurites-occupied areas were then divided by the area occupied by the explants. Reduction of 47% of total neurite area was observed in both ATP-treated group and 46% in αβ-meATP-treated groups (**Fig 6**). ATP and αβ-meATP appeared to reduce the length and probably the number of extending neurites, too.

The neural tube explants were stained with $P2X_3$ receptor 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 5**). Double labeling experiment showed that co-localization of $P2X_3$ receptor and NCAM was frequently

seen in the outgrowing neurites, although neurites showing NCAM but not $P2X_3$ receptor expression were also observed (**Fig 5**).

DISCUSSION

The idea of extracellular ATP acting as a neurotransmitter or neuromodulator has been examined extensively in both the central 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; Burnstock and Kennedy, 1985; Nörenberg and Illes, 2000; North, 2002; Burnstock, 2003b). We have previously found that ATP-gated $P2X_3$ purinoceptor was expressed in the early embryonic nervous system (Cheung and Burnstock, 2002). The present study shows for the first time the developmental expression of six of the seven P2X receptor subtypes during prenatal brain development, and the possible role of ATP and the $P2X_3$ receptor during embryonic neurogenesis.

We demonstrated that the neuronal $P2X_3$ and $P2X_2$ receptors start to express in the embryonic rat central nervous system (CNS) at E11 and E14, respectively, in confirmation of our earlier findings (Cheung and Burnstock, 2002). Here we showed that in addition to the $P2X_2$ receptor, the $P2X_7$ receptor in the brain was 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 (reviewed

by Le Feuvre et al., 2002). Sperlágh et al. (2002) demonstrated that ATP regulates glutamate release via activation of $P2X_7$ receptor, and excessive glutamate release altered the Ca^{2+} homeostasis, resulted in activation of apoptosis-related gene (e.g caspase) (see review by Le Feuvre et al., 2002). Since apoptosis is a common event in developing brain, it is therefore likely that the activation of $P2X_7$ receptor is also involved in cell death during neurogenesis, but this possibility awaits further investigation.

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 Conover and Allen, 2002). It has been claimed that astrocytes in the subventricular zone served 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 believed that such expression may not relate to development. The $P2X_1$ receptor, according to the present results, did not express throughout the perinatal stages examined. However, previous studies have shown $P2X_1$ receptor expression in the cerebellum (Loesch and Burnstock, 1998) and $P2X_1$ receptor up-regulation in the CNS after injury (Florenzano et al., 2002; Franke et al., 2001).

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. Among all the P2X receptors examined, homomeric $P2X_3$ receptor was the first to express during neurogenesis in both central and peripheral nervous system. $P2X_3$ immunoreactivity is detected in the cranial motor neurons as early as E11, by the time when 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 $P2X_3$ receptors expressing cells are the BM and/or VM neurons, instead of 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 to the weak staining in the spinal neural tube suggests that the BM neurons also expresses the receptor.

We have shown that both ATP and αβ-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, the αβ-meATP was used in the present study as a stable analog of ATP to exclude any possible P1 receptor involvement. In addition, since αβ-meATP is a selective agonist only for $P2X_1$, $P2X_{2/3}$ and $P2X_3$ receptors. Since neither RT-PCR and 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 αβ-meATP was mediated via $P2X_3$ receptor. 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 instead of 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 axons growing out from the neural tube at E12 expressed NCAM. Some but not all of the outgrowing axons showed NCAM and $P2X_3$ receptor coexpression, implicating that ATP-mediated inhibition in neurite outgrowth might act, at least partly, through the NCAM signaling system.

In summary, we present the first report to show the developmental expression profile of P2X receptors subtypes during prenatal brain development in the rat. Results from the present study indicated that different P2 receptor subtypes may participate in different developmental processes such as neurite outgrowth (involving $P2X_3$ receptor), postnatal neurogenesis (relating to $P2X_4$ and $P2X_5$ expression) and cell death (possibly involving $P2X_7$

receptor), whereas others might not be involved 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 the interaction between $P2X_3$ receptor and NCAM.

FIGURES & LEGENDS:

Figure 1 RT-PCR analysis on 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 100bp-DNA ladder. The amplified products are arranged in 4 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. $P2X_1$ (lane 1-4); $P2X_2$ (lane 5-8); $P2X_3$ (lane 9-12); $P2X_4$ (lane 13-16); $P2X_5$ (lane 17-20); $P2X_6$ (lane 21-24) and $P2X_7$ (lane 25-28). The $P2X_1$ receptor primers that detects no expression in brain samples shows strong positive signals using rat uterus as positive control (data not shown). Note the gradual decline in $P2X_3$ mRNA expression from E14 to P16. The expression pattern shown here represents results from at least three independent experiments.

Fig 1

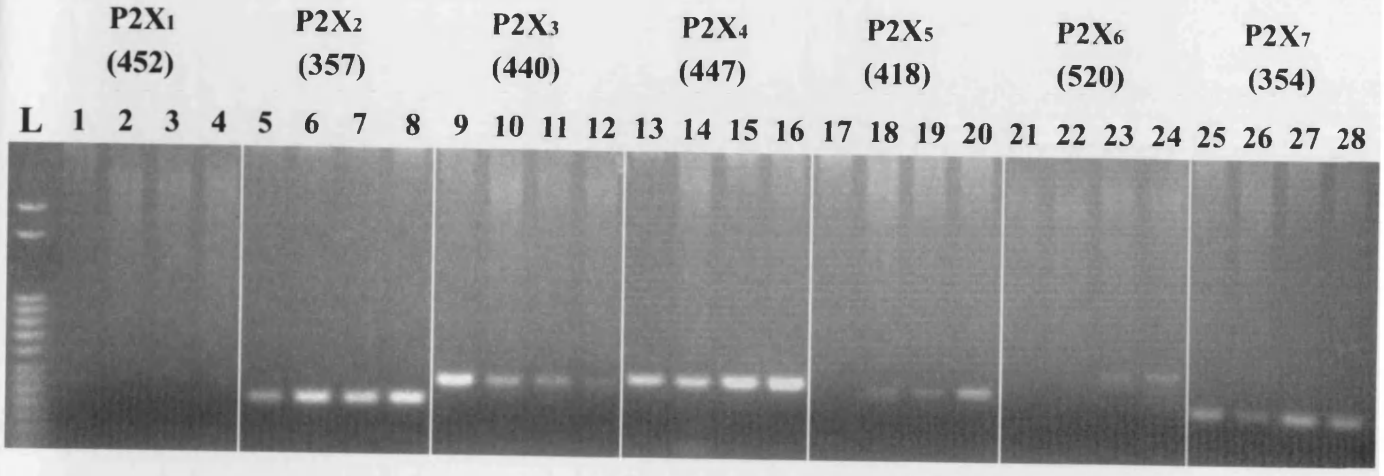

P2X1 (452)
P2X2 (357)
P2X3 (440)
P2X4 (447)
P2X5 (418)
P2X6 (520)
P2X7 (354)
L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

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

Fig 2

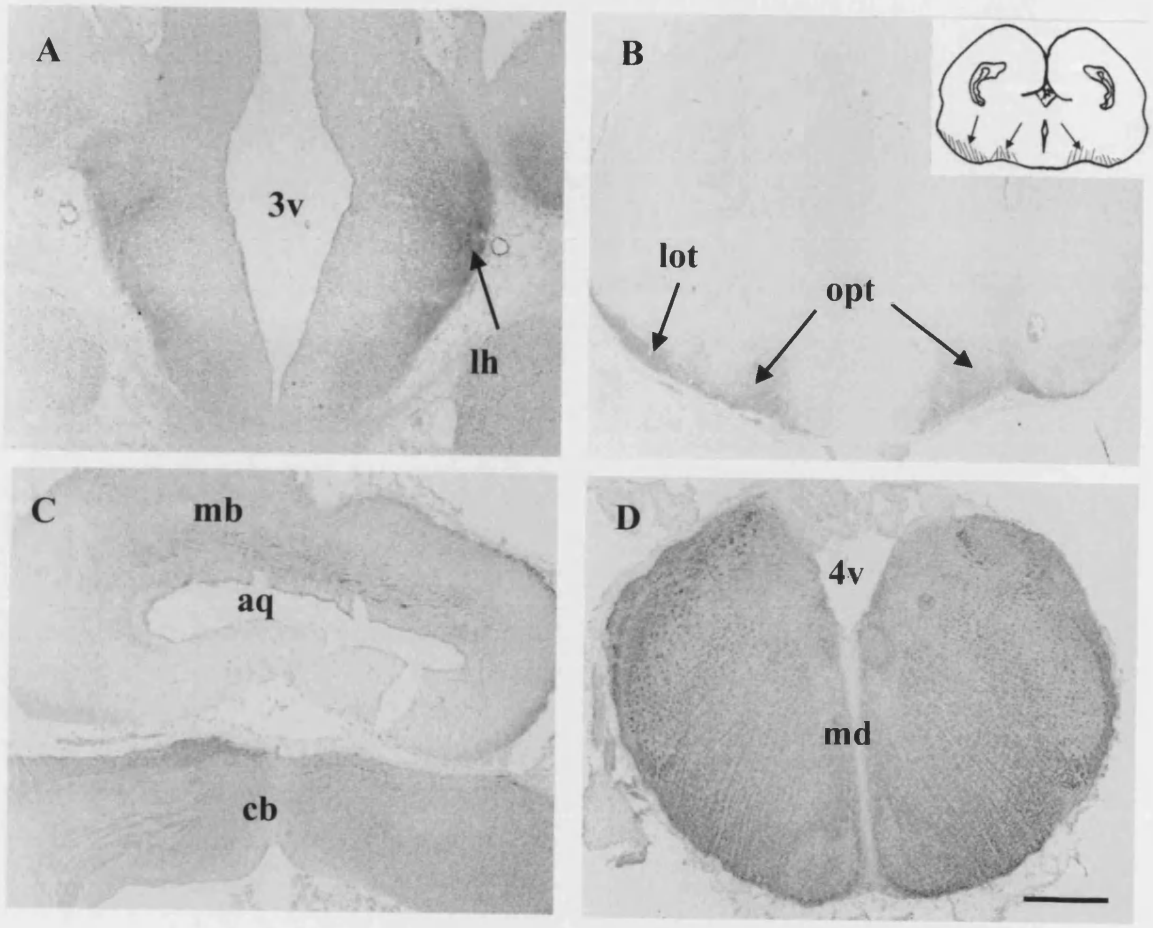


Figure 3 Immunoreactivity of P2X receptors in neonatal (P1) brain. Rectangular boxes in the inserts outline the regions shown in the corresponding pictures and the black dots in some of the inserts represent P2X immunoreactivity. (**A**) $P2X_4$ receptor-positive cells (arrows) are detected in regions along the striatal subventricular zone. (**B**) A high magnification of the rectangular box is shown in (**A**). (**C**) $P2X_4$ receptor immunoreactivity is also detected in mesencephalic trigeminal nucleus and (**D**) lateral deep nucleus of the cerebellum. (**E**) $P2X_5$ receptor expression is located in the supraoptic nucleus and (**F**) the cells along the striatal subventricular zone. (**G**) Scattered $P2X_5$ immunoreactive cells are also observed throughout the cortex (**G**). Note that the whole cell bodies of the $P2X_5$ receptor-positive cells shown in **F** and **G** are labeled. (**H**) $P2X_6$ receptor expression is detected in the lateral hypothalamus. Scale bar = 750μm (**A**), 100μm (**B**, **E**, **H**) and 200μm (**C**, **D**, **F**, **G**).

Fig 3

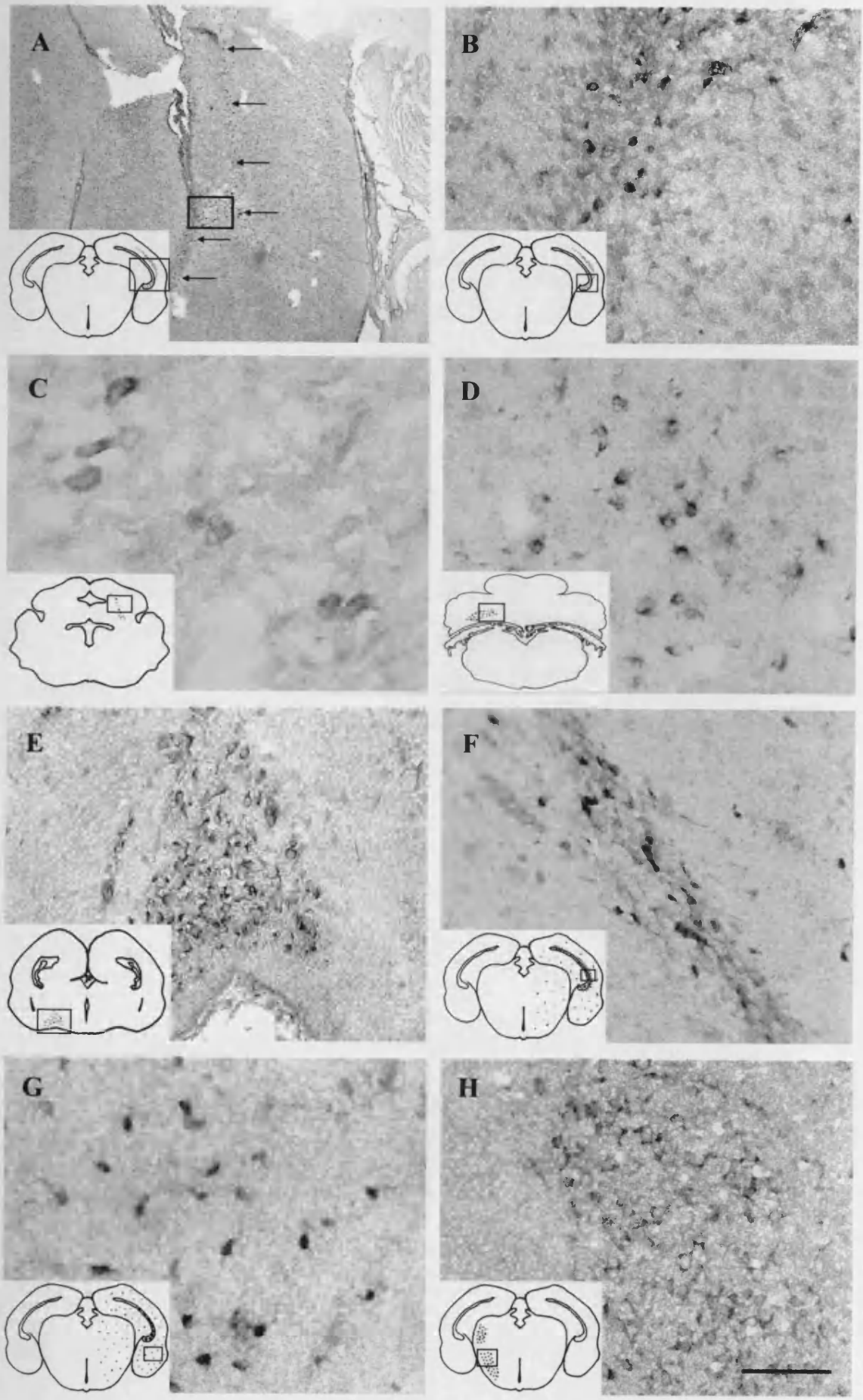


Figure 4 Summary of the sequential expression of P2X receptors during rat neurogenesis. P2X receptors are arranged from top to bottom according to the chronological order of expression during rat brain development from E11 to Adult. While $P2X_3$ receptors appear early, they decline in the stages that follow. $P2X_2$ and $P2X_7$ receptors are expressed from the same day (E14) onwards, while $P2X_4$, $P2X_5$ and $P2X_6$ are also expressed from P1 onwards. 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.

Fig 4

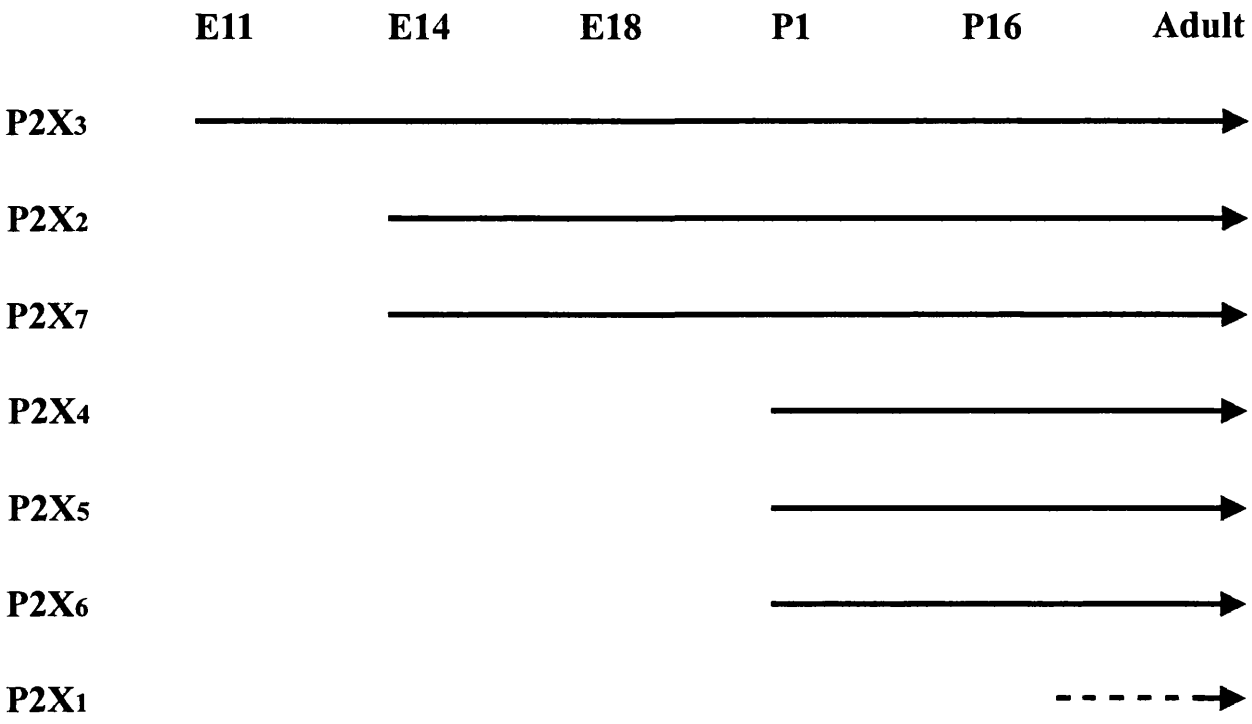


Figure 5 Neural tube explants in collagen gel after treatment with 100μM of (**A**) ATP and (**B**) control. (**C**) Cross section from E12 hindbrain neural tube showing $P2X_3$ receptor immunoreactivities (red) in the motor neurons and the motor fibers in the ventral neural tube. (**D** and **E**) Neural 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 = 500μm (**A-B**) and 100μm (**C-F**).

Fig 5

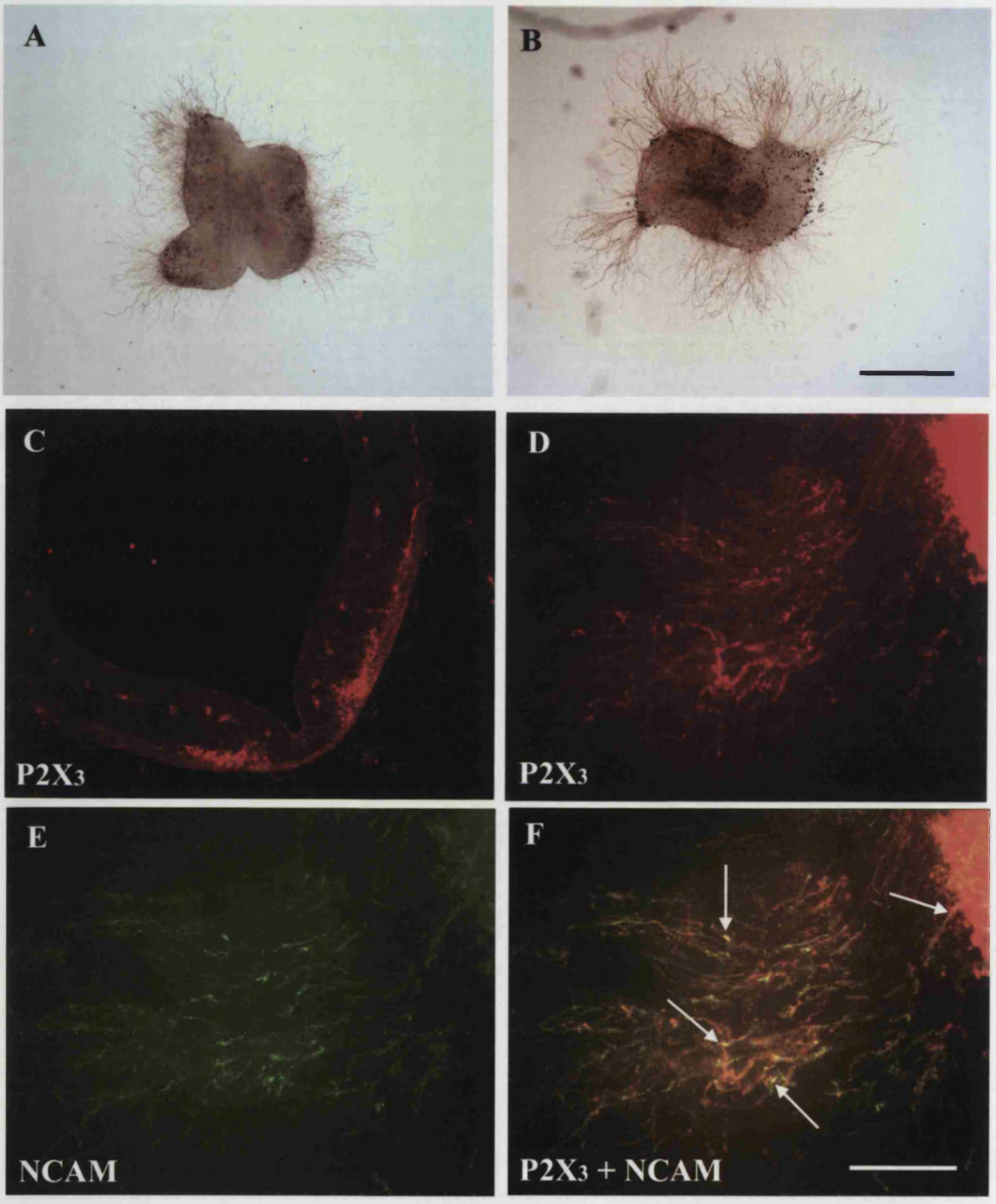
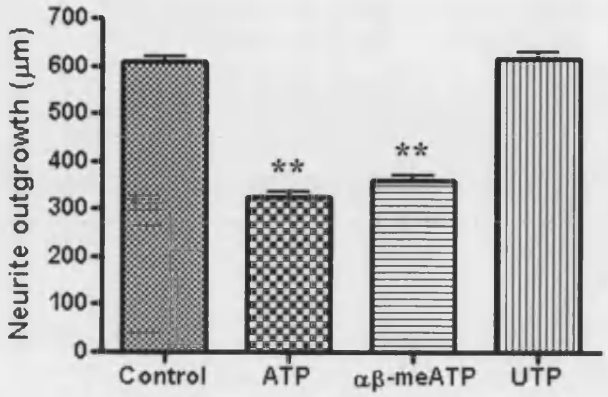


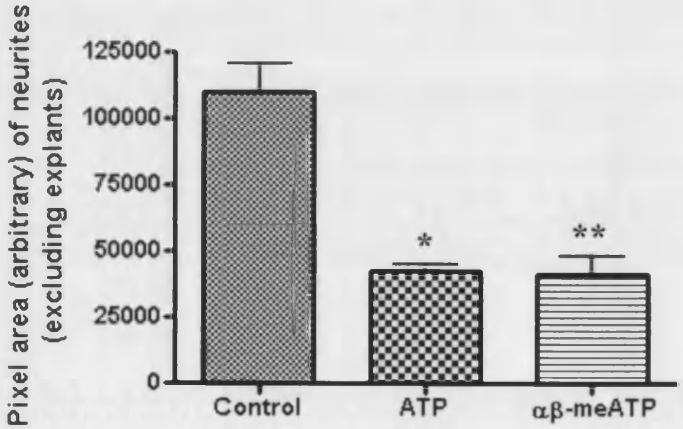
Figure 6 Quantitation of neurite outgrowth from collagen embedded neural tube explant culture. Neural tube explants were bathed with 100μM of extracellular nucleotides (ATP, αβ-meATP and UTP). Control explants were treated with medium only. Images of treated neural tube explants were imported into Neurolucida 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 represents standard error. (**B**) Images of treated neurite tube explants were imported into Metamorph and the total pixel area (arbitrary units) of the neurites (excluding the explants) were 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) were 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.

Fig 6

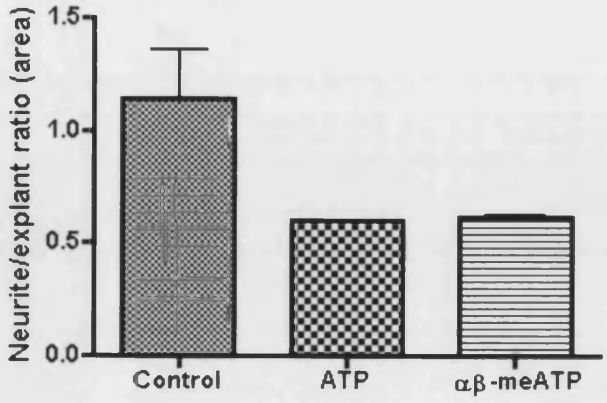
A


Neurite outgrowth (μm)
700
600
500
400
300
200
100
0
**
**
Control
ATP
αβ-meATP
UTP

B


Pixel area (arbitrary) of neurites (excluding explants)
125000
100000
75000
50000
25000
0
*
**
Control
ATP
αβ-meATP

C


Neurite/explant ratio (area)
1.5
1.0
0.5
0.0
Control
ATP
αβ-meATP

Chapter V Pharmacological and molecular characterization of functional P2 receptors in rat embryonic cardiomyocytes

ABSTRACT

Purinergic receptors activated by extracellular nucleotides (ATP, ADP, UTP and UDP) are well known to exert physiological effects on the cardiovascular system, including cardiomyocytes. However, the possibility that nucleotides participate in embryonic heart development is not clear. We have investigated the responsiveness of embryonic cardiomyocytes to P2 agonists by means of the fluorometric imaging plate reader (FLIPR), which measures Ca^{2+} influx. Cardiomyocytes taken from embryonic day (E) 12 did not respond to P2 agonists, but responses were detected in cardiomyocytes taken from E14 and E18 rats. ATP, 2MeSADP, UTP, UDP and BzATP, but not αβ-meATP, were able to induce Ca^{2+} mobilization. PPADS blocked the ATP-induced responses of E14 embryonic cardiomyocytes but not those produced in E18 hearts. RT-PCR showed a dominant presence of $P2X_2$ and $P2X_4$ mRNA transcripts on E14 cardiomyocytes with a lower expression of $P2X_3$ and $P2X_7$. $P2X_1$ and a low level of $P2X_5$ receptor mRNA were also

expressed at E18. Immunocytochemical data indicated that only $P2X_2$ and $P2X_4$ receptor protein was expressed in E14 cardiomyocytes. All the P2X receptor subtype protein was expressed in E18 cardiomyocytes except for $P2X_3$ and $P2X_6$. Responses mediated by agonists specific for P2Y receptors subtypes (2MeSADP, UTP and UDP) showed that P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) were also present in both E14 and E18 cardiomyocytes. Our results provide evidence that specific P2 receptor subtypes are present in embryonic cardiomyocytes, including $P2X_7$ and $P2Y_4$ receptors which have not been identified in adult rat cardiomyocytes. Some of the receptors have shown responsiveness to the ATP stimulation even before birth, suggesting that ATP may be an important messenger in embryonic as well as adult hearts.

INTRODUCTION

Extracellular purines and pyrimidines (ATP, ADP and UTP) are well known to act as agonists in regulating a variety of physiological functions in many different cell types (Burnstock, 1997). In 1978, Burnstock proposed that there were specific receptors for adenine nucleosides (P1) and nucleotides (P2 receptors). Based on cloning and second messenger systems P2 receptors were divided into two receptor families, ionotropic P2X receptors and metabotropic P2Y receptors (Abbracchio and Burnstock, 1994). P2X receptors are ligand-gated ion channels formed from either homomeric or heteromeric association of 7 receptor subunits ($P2X_{1-7}$) (North, 2002). P2Y receptors are G protein-coupled receptors, with 8 mammalian subtypes ($P2Y_{1,2,4,6,11,12,13,14}$) being cloned (Burnstock, 2003a).

ATP is known to mediate various physiological activities in the adult heart of different species (Olsson and Pearson, 1990; Ralevic and Burnstock, 1991, Vassort, 2001). However, only a few studies focused specifically on P2 receptor expression in cardiomyocytes (Vulchanova et al., 1996; Webb et al., 1996; Nori et al., 1998; Hansen et al., 1999). The mRNA transcripts of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are all expressed in the neonatal cardiomyocytes, whereas $P2Y_4$ receptor transcript is no longer detected in the adult myocytes

(Webb et al., 1996), suggesting that dynamic changes in expression of P2Y receptors were occurring during heart development. While it was demonstrated that the $P2X_1$ receptor protein was specifically expressed in the intercalated disks of cardiac muscle (Vulchanova et al., 1996), Nori et al (1998) also detected the mRNA transcripts for $P2X_1$, $P2X_2$ and $P2X_4$ receptors from the micro-dissected tissues in various areas of the heart, with strong signals in the atria and only the typical band for $P2X_4$ receptor transcripts in ventricles. In addition, Hansen et al (1999) showed an abundance of colocalized $P2X_2$ and $P2X_5$ receptor clusters on the sarcolemma of neonatal heart.

The heart is the first organ to function in developing embryos (Fishman and Chien, 1997). The tubular heart undergoes sequential morphological changes such as looping, segmentation, trabeculation, septation etc (Franco et al., 1998). As with skeletal or smooth muscle, the arrangement of the myocardial cells is not random, but shows certain patterns which change during ontogenetic development. Such arrangement is of great importance to ensure the efficiency of the myocardial pump. It is therefore considered that dynamic changes in spatiotemporal patterns of gene expression resulting in complex structural changes during heart ontogeny are involved in the developing embryo (Olson and Srivastava, 1996; Zhao and Rivkees, 2000); thus the gene expression may be different between different developmental stages and from that of the adult (Franco et al., 1998). Nevertheless, identifying the presence

of gene expression may not provide the information of whether the gene products are in functional status or not. There have been some studies of P2 receptors gene expression in embryonic heart. Previously, the expression of some of the purinergic receptors subtypes ($P2X_2$ and $P2Y_{1,2,4,6}$) in the embryonic rat heart have been identified (Cheung and Burnstock, 2002; Cheung et al., 2003), yet the functional status of the receptors remained unclear. There have also been reports of the expression of P2X receptor subtypes in the chick embryonic heart where $P2X_4$ and $P2X_5$ mRNA has identified (Ruppelt et al., 1999, 2001; Hu et al., 2002) and in the human embryonic heart, where mRNA for $P2X_1$, $P2X_3$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were expressed (Bogdanov et al., 1998a). However, information on the developmental expression profile together with the functional status of other P2X receptor subunits in rat embryo is lacking. To fill this gap, we have used the fluorometric imaging plate reader (FLIPR) together with RT-PCR analysis and immunocytochemistry to study the expression profile of the purinergic receptors during the development of rat cardiomyocytes. Such combinatorial information on the gene expression and pharmacological analysis may clarify the specific receptors responsible for ATP-mediated activities during cardiac embryogenesis.

MATERIALS AND METHODS

Cell Culture and Ca^{2+} mobilization analysis

Cardiomyocytes were prepared from embryonic Sprague-Dawley rats according to Burton et al (1999). Briefly, female time-mated rats were sacrificed using carbon dioxide inhalation and cervical dislocation. The day of identification of the presence of a vaginal plug was designated as day zero (E0). Embryos were removed and placed into ice-cold phosphate-buffered saline (PBS, pH 7.4). The hearts were dissected out, placed in Ca^{2+}-free PBS at 4°C, washed, and the heart tissues were digested in type II collagenase (Worthington; 1 mg/ml in the same medium) at 37°C. The hearts were then gently triturated until all tissue fragments disappeared. The cell suspension was then mixed with Dulbecco's modified Eagle's medium (DMEM) with GLUTAMAX I containing 10% (v/v) fetal calf serum (FCS) and centrifuged at 700g for 6 min. The cell pellet was resuspended in DMEM-FCS containing penicillin (100U/ml), streptomycin (0.1mg/ml), and amphotericin B (0.25 μg/ml) and passed sequentially through a 21- and 25-gauge needle. Cells were allowed to preplate (twice, 1 hr each) and the nonadherent cells were then plated at 1 x 10^4 cells per well into 96-well black-wall, clear bottom microtitre plates (BD Biosciences, Franklin Lakes, NJ), which had been previously coated with a solution of 1% (w/v) gelatin in water. These cardiomyocyte preparations were >98% pure as judged by immunocytochemical staining with

monoclonal antibodies against sacromeric actin (Sigma; diluted 1/500). The myocytes were cultured in the same medium plus insulin-transferrin-selenium supplement at 37°C in 5% CO_2 for one day. The growth medium was then aspirated and replaced with 100 µl of loading medium (PBS containing 1mM Fluo-4-AM, 10 % pluronic acid and 2.5 mM probenicid) and incubated for one hour at room temperature. The cells were subsequently washed three times with PBS, and 100 µl of PBS supplemented with 1 mM $CaCl_2$ were added to each well. The cells were then placed in a Fluorometric Imaging Plate Reader (FLIPR; Molecular Devices Corp., Sunnyvale, CA), and changes in cellular fluorescence were recorded after the addition of 50 µl control buffer or 50 µl of the ATP diluted in PBS. Maximum change in fluorescence over baseline was used to determine the response towards ATP. In addition, P2 agonists such as 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP), αβ-methylene ATP (αβ-meATP), 2-Methylthio-ATP (2MeSADP), uridine 5'-triphosphate (UTP) and uridine 5'-diphosphate (UDP) were also used for examining the Ca^{2+} mobilizing response on the cardiomyocytes. To investigate if pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), a P2 receptor selective antagonist, blocked the ATP-induced response, cells were treated with PPADS (30µM) for 30 min before addition of ATP. ATP and related nucleotides were obtained from Sigma Chemical Co. (UK) except for PPADS, which was from Tocris Cookson, UK.

Double labeling immunocytochemistry

Cardiomyocytes were grown on gelatin-coated 8-well chambered slides (Nunc). The cells were fixed in 4% paraformaldehyde in phosphate buffer (PB, pH 7.4) for 2 hr at room temperature. Cells were washed with PBS, blocked with 10% normal horse serum in 0.1% Triton, and the cells were stained with the monoclonal antibody against sacromeric actin (1:500; Sigma), together with the polyclonal antibodies against $P2X_{1-6}$ (1:200; Roche Bioscience) and $P2X_7$ receptors (1:500; Alomone) followed by an FITC-conjugated donkey anti-mouse IgG antibody and cy3-coupled donkey anti-rabbit IgG antibody (1:200; Jackson ImmunoResearch Lab) for 1 hr. Please refer to the sections of **Materials and Methods** on Chapter II for the details for Photomicroscopy

RT-PCR analysis

Embryonic hearts at E14 and E18 were dissected and total RNA was extracted from the dissected hearts according to the sections of **Materials and Methods** on Chapter III. The sequence specific primers (Life Technologies, NY, USA) for P2X receptors (Shibuya et al., 1999) 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 stage of 10-min extension at 72°C.

RESULTS

Effects of agonists on Ca^{2+} mobilization of cardiomyocytes

We used FLIPR to study the responses of purinergic receptors that are present in cultured embryonic cardiomyocytes upon exposure to ATP and its derivatives. Positive responses were represented by an increase in fluorescence units, indicating Ca^{2+} mobilization in response to receptor activation. Cardiomyocytes from three embryonic ages (E12, E14 and E18) were taken for analysis. All the agonists were in a concentration of 100μM. No apparent change was observed in control group when Ca^{2+}-containing PBS was added into the cells (**Fig 1B, D, G**). Cardiomyocytes taken from E12 did not show any significant response in Ca^{2+} mobilization against ATP (**Fig 1A**) or any of the ligands tested. In contrast, significant responses were detected with cardiomyocytes taken from E14 and E18 embryonic hearts when the cells were treated with rapid application of ATP (**Fig 1C, 1F**). Rapid increase in fluorescence occurred in a second. We then investigated if the P2 antagonist PPADS blocked the ATP-triggered response. The ATP-induced response on E14 cardiomyocytes was antagonized by PPADS at a concentration of 30μM (**Fig 1D**), whereas the antagonizing effects of PPADS on E18 cardiomyocytes was not significant (**Fig 1H**). On the other hand, the P2Y receptor agonists such as 2MeSADP, UTP and UDP all induced significant response in Ca^{2+} mobilization on cardiomyocytes taken at both embryonic ages (**Fig 2A-F**). BzATP induced a

weak response on E14 cardiomyocytes, and the response was increased in E18 cardiomyocytes (**Fig 3A, 3B**). αβ-meATP did not induce a significant response on E14 cardiomyocytes (**Fig 3C**). The response by αβ-meATP, though very weak, was observed in E18 cardiomoyocytes (**Fig 3D**).

RT-PCR analysis

Two developmental ages of embryonic heart at E14 and E18 were studied for mRNA expression of all the seven P2X receptors ($P2X_{1-7}$). Results of the mRNA expression were summarized in **Fig 4**. For all the P2X receptors examined, $P2X_2$, $P2X_3$, $P2X_4$ and $P2X_7$ receptors showed mRNA expression in the heart at both E14 and E18. $P2X_1$ and $P2X_5$ receptor was expressed at E18 but not at E14, though the expression of $P2X_5$ in the heart was barely detectable at E18. The $P2X_6$ receptor, however, was not expressed in either E14 or E18 hearts.

Double-labeling immunocytochemistry

Fluorescence immunocytochemical staining has shown that among all the P2X receptors examined, only $P2X_2$ and $P2X_4$ receptors were expressed in the cardiomyocytes at E14 (**Fig 5A-F**), as were shown in the double-labeled immunocytochemical staining with α-sarcomeric actin. None of the other P2X receptor subunits was coexpressed with α-sarcomeric actin (**Fig**

5G-K). Similar to E14, $P2X_2$ and $P2X_4$ receptors were detectable in E18 cardiomyocytes labeled with α-sarcomeric actin shown by double-labeling (**Fig 6A-F**). $P2X_1$, $P2X_5$ and $P2X_7$ receptors were also expressed in E18 cultured cardiomyocytes (**Fig 6G-O**). However, immunoreactivity of $P2X_3$ and $P2X_6$ was not detected in any of the stages examined.

DISCUSSION

It is well known that extracellular ATP exerts a wide range of activities on the adult myocardium (for reviews, see Olsson and Pearson, 1990; Ralevic and Burnstock, 1991; Pelleg and Belardinelli, 1998; Vassort, 2001). However, combined functional, molecular and immunocytochemical information about P2 receptor expression in the embryonic heart is lacking, which is needed for our understanding of purinergic signaling during embryonic development. The present study focused on the expression pattern of P2 receptors in the rat heart during embryonic development.

With the use of FLIPR, the responsiveness of cardiomyocytes taken at different embryonic ages towards ATP was analyzed. Indeed, cells at different embryonic ages responded differently to ATP. In addition to the previous reports demonstrating the presence of functional P1 adenosine receptors in mammalian embryonic cardiomyocytes (Hofman et al., 1997; Zhao and Rivkees, 2001; Zhao et al., 2002), the results of the present study clearly indicated the presence of functional P2X and P2Y receptors in rat cardiomyocytes from E14 onwards. Taken together the results of RT-PCR and immunocytochemical staining showed that $P2X_2$ and $P2X_4$ receptors were the most likely P2X receptor subtypes responding to ATP at E14. The discriminating feature between the $P2X_2$- and the $P2X_4$-receptor is the blockade of

the latter but not the former by PPADS (Ralevic and Burnstock, 1998). However, the ATP-induced response at E14 was almost completely blocked by PPADS, which suggested that it was mostly the $P2X_2$ receptor that accounted for the ATP-mediated response at E14 cardiomyocytes. RT-PCR studies were consistent with this conclusion, with the $P2X_2$ mRNA band dominant. In addition, the $P2X_2$ receptor was also expressed in the embryonic rat heart as early as at E12 (Cheung and Burnstock, 2002), the stage at which none of the other P2X receptors were detected (unpublished data). In contrast, the ATP-induced response at E18 cardiomyocytes was sustained after treating with PPADS, suggesting the involvement of $P2X_4$, $P2X_6$ and $P2X_7$ receptors (see Ralevic and Burnstock, 1998). However, absence of $P2X_6$ mRNA transcript in the present study ruled out the possibility of $P2X_6$ receptor involvement. RT-PCR and immunocytochemistry indicated the presence of $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_7$ receptors at E18 hearts. In addition, BzATP, a $P2X_1$ and $P2X_7$ receptor-sensitive agonist, triggered Ca^{2+} mobilization in E18 cardiomyocytes. Although $P2X_1$ receptor is also sensitive to BzATP, αβ-meATP, a $P2X_1$-specific agonist, did not induce significant responses in both E14 and E18 cardiomyocytes. It is therefore suggested that the BzATP-induced response is mostly contributed by activation of functional $P2X_7$ rather than $P2X_1$ receptors; this has not been reported previously. In contrast, $P2X_4$ receptor expression has been reported in human fetal and chick embryonic heart (Bogdanov et al., 1998a; Ruppelt et al., 1999; Hu et al., 2002). ATP is known to stimulate a large increase in cytosolic calcium transient and myocyte

contractile amplitude (see review by Vassort, 2001). Transgenic mouse overexpressing human $P2X_4$ receptors demonstrated an increase in contractility in cardiomyocytes subject to ATP stimulation (Hu et al., 2001). In contrast to $P2X_4$ receptor, little is known about the function of $P2X_2$ and $P2X_7$ receptor in the myocardium, although it has been shown previously that activation of $P2X_7$ receptors induced contraction of human saphenous vein smooth muscle (Cario-Toumaniantz et al., 1998). It is still not clear what factors underlie early embryonic changes in contractility in rat, as both changes in Ca^{2+} sensitivity and troponin isoforms occur after 7 days postnatal (Reiser et al., 1994; Jin, 1996). However, the possibility of increase in contractility in embryonic rat as a result of $P2X_4$ and $P2X_7$ receptor activation (and probably $P2X_2$ receptor as well) mediated by ATP is worth investigating.

The presence of receptor transcripts of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in embryonic rat heart has been demonstrated previously (Cheung et al., 2003), as well as in human fetal heart for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Bogdanov et al., 1998a). The present study helps to uncover the functional status of the receptors on cardiomyocytes during heart development. The P2Y receptor agonists all induced Ca^{2+} mobilization on cardiomyocytes at E14. 2MeSADP, which is a stable analog to ADP, induced a $P2Y_1$-specific response on Ca^{2+} mobilization on cardiomyocytes. The uracil nucleotides that preferentially activate $P2Y_2$ and $P2Y_4$ receptors (by UTP) and $P2Y_6$ receptor (by UDP) also induced Ca^{2+} mobilization on

cardiomyocytes. Although the receptor proteins were expressed as early as in E12 (Cheung et al., 2003), the receptors were shown to be responsive to the agonists in the cardiomyocytes from E14 onwards. We have previous shown that the P2Y receptor proteins expressed initially all over the heart but soon restricted to the trabeculated layers; the expression was absence in compact myocardium after E14 (Cheung et al., 2003). Trabeculation differs from compact myocardium in terms of extent of differentiation, rate of contractions and impulse conduction (Franco et al., 1998). P2Y receptor expression in the trabeculated layer, and the responsiveness towards agonists from E14 onwards suggested that the receptor may have something to do with either the differentiation or contraction. Small spontaneous responses were occasionally observed in E14 and E18 cardiomyocytes. Such responses appeared to be artifacts, as the spontaneous responses retained in the presence of PPADS. It is probably due to the contractile property of the cardiomyocytes that generate the responses.

Bogdanov et al (1998a) have shown the presence of $P2X_1$ and $P2X_3$ mRNA transcripts in human fetal heart. Here we were also able to amplify the mRNA transcripts of $P2X_1$ and $P2X_3$ receptors in E18 hearts. However, compared to ATP, BzATP and the P2Y receptor agonists, αβ-meATP only triggered a very small response in E18 cardiomyocytes. It is therefore unlikely that $P2X_1$ and $P2X_3$ receptors are highly involved in the development of cardiomyocytes in rats.

Ruppelt et al. (2001) demonstrated the mRNA expression of the chick $P2X_5$ receptor subtypes in the embryonic chick heart. Although the results of RT-PCR from the present study showed very weak $P2X_5$ mRNA transcript expression in E18 rat heart, double labeling experiments confirmed the presence of $P2X_5$ receptor protein in the cardiomyocytes.

Although expression profile of P2 receptors on heart development has been examined previously, expression pattern together with functional analysis on embryonic cardiomyocytes have not been studied previously. Our observations support the hypothesis that ATP acts on particular P2 receptor subtypes during different stages of development, suggesting that complex mechanisms are involved in governing sequential P2 receptor subtype expression. In summary, we have identified P2X receptor subtypes ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$ and $P2X_7$) and P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$) in rat embryonic cardiomyocytes. All these receptor subtypes, except for $P2X_7$ and $P2Y_4$ have been described in adult rat cardiomyocytes. Therefore it appears that $P2X_7$ and $P2Y_4$ play additional roles in the embryo. Complex remodeling occurs during embryonic development of the heart, but further investigations are needed to uncover the precise roles played by the various P2 receptor subtypes.

FIGURES & LEGENDS

Figure 1 Ca^{2+} mobilization of embryonic cardiomyocytes. The response of Ca^{2+} mobilization is represented by an increase in the mean of fluorescent units (n = 3; error bars represent standard error of the mean). ATP-induced response in cultured cardiomyocytes taken from E12 (**A**), E14 (**C**), E18 (**F**) and the corresponding controls (**B**, **D**, **G**, respectively). **E** and **H** show the ATP-induced response after the preincubation with PPADS.

Fig 1

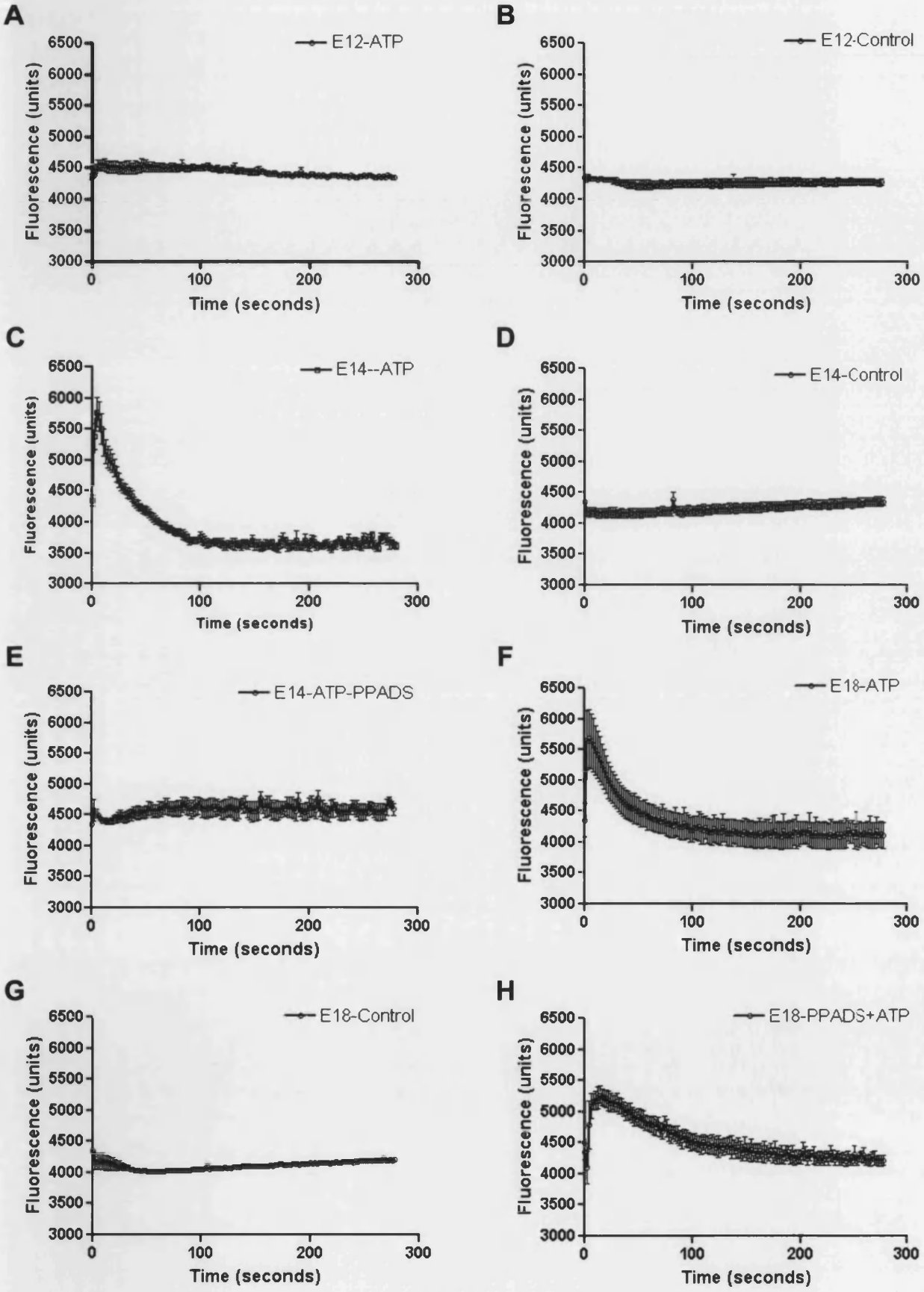

A
E12-ATP
B
E12-Control
C
E14--ATP
D
E14-Control
E
E14-ATP-PPADS
F
E18-ATP
G
E18-Control
H
E18-PPADS+ATP
Fluorescence (units)
Time (seconds)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300

Figure 2 Ca^{2+} mobilization of embryonic cardiomyocytes. The response of Ca^{2+} mobilization is represented by an increase in the mean of fluorescent units (n = 3; error bars represent standard error of the mean). **A, C, E** show responses of E14 cardiomyocytes induced by 2MeSADP, UTP and UDP, respectively; whereas **B, D, F** show responses of E18 cardiomyocytes induced by corresponding agonists.

Fig 2

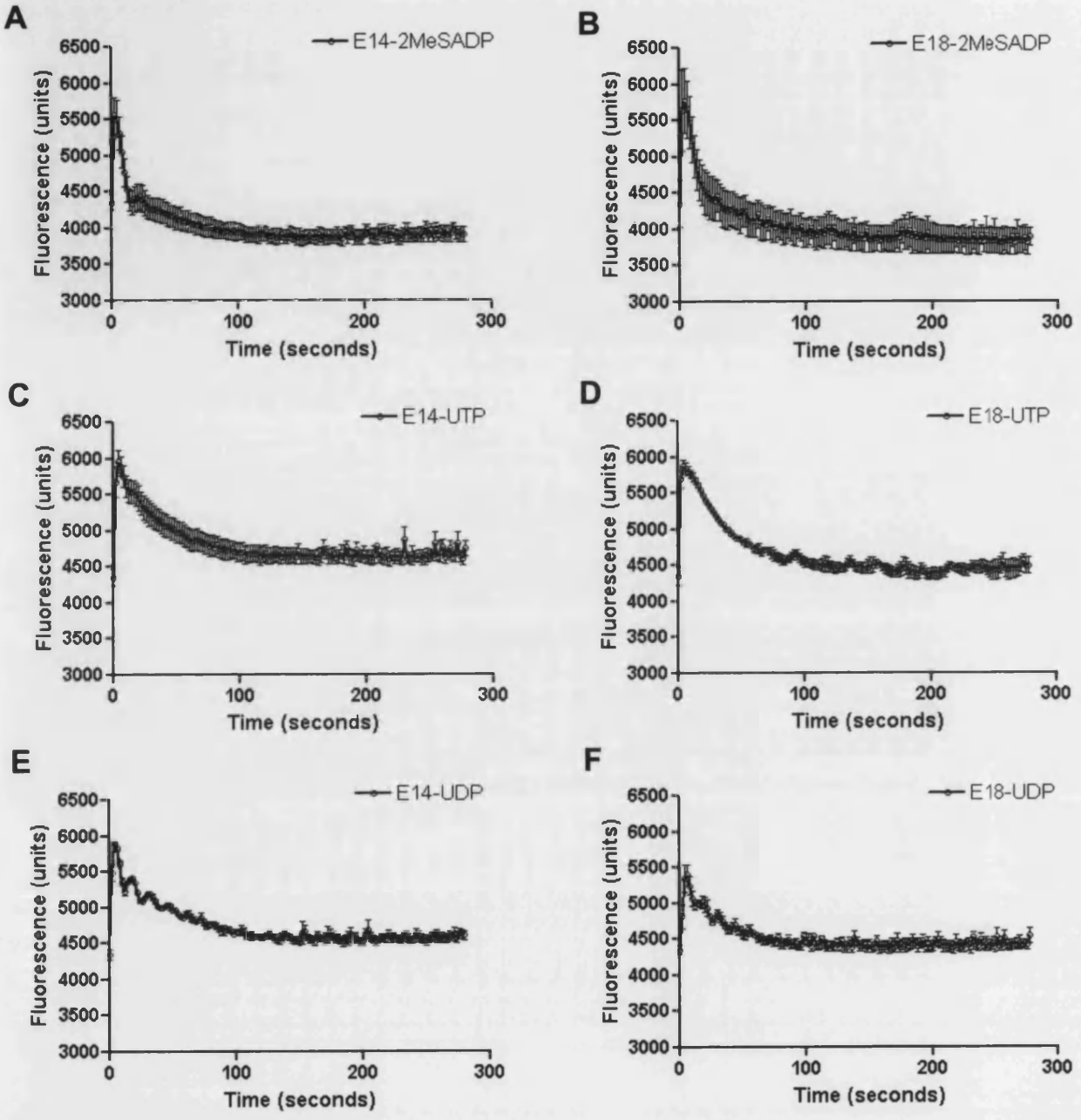

A
E14-2MeSADP
B
E18-2MeSADP
C
E14-UTP
D
E18-UTP
E
E14-UDP
F
E18-UDP
Fluorescence (units)
Time (seconds)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300

Figure 3 Ca^{2+} mobilization of embryonic cardiomyocytes. The response of Ca^{2+} mobilization is represented by an increase in the mean of fluorescent units (n = 3; error bars represent standard error of the mean). **A, B** show responses induced by BzATP on E14 and E18 cardiomyocytes, respectively. **C, D** show responses induced by αβ-meATP on E14 and E18 cardiomyocytes, respectively.

Fig 3

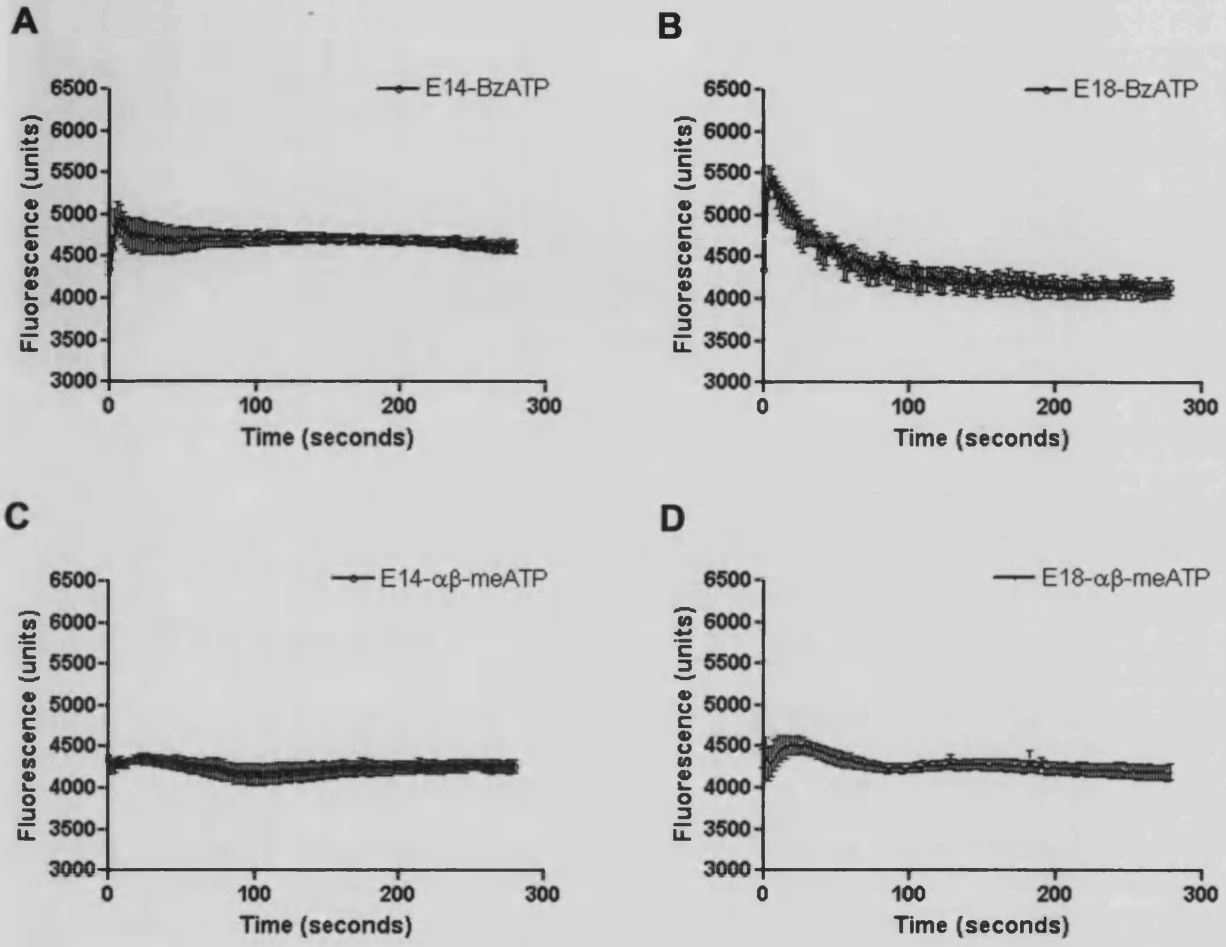

A
E14-BzATP
Fluorescence (units)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300
Time (seconds)
B
E18-BzATP
Fluorescence (units)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300
Time (seconds)
C
E14-αβ-meATP
Fluorescence (units)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300
Time (seconds)
D
E18-αβ-meATP
Fluorescence (units)
6500
6000
5500
5000
4500
4000
3500
3000
0
100
200
300
Time (seconds)

Figure 4 RT-PCR analysis for $P2X_{1-7}$ receptors on E14 and E18 heart tissues. Primers for $P2X_6$ receptors that gave negative results at both stages showed positive signals using rat brain tissue extract (data not shown).

Fig 4

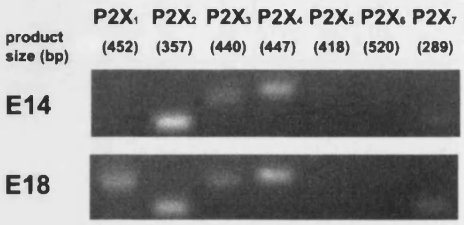

P2X1 P2X2 P2X3 P2X4 P2X5 P2X6 P2X7
product size (bp)
(452) (357) (440) (447) (418) (520) (289)
E14
E18

Figure 5 P2X receptor subtype expression in cultured E14 cardiomyocytes. Cardiomyocytes were identified by monoclonal antibody against α-sacromeric actin (α-SMA, green) (**A** and **D**). P2X receptor-immunoexpressing cells were shown in red (**B** and **E**). Cardiomyocytes showing coexpression (yellow) of P2X receptors ($P2X_2$ and $P2X_4$) with the α-SMA (**C** and **F**). Cardiomyocytes at this stage did not express $P2X_1$, $P2X_3$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors (**G-K**, respectively). Scale bar = 25μm.

Fig 5

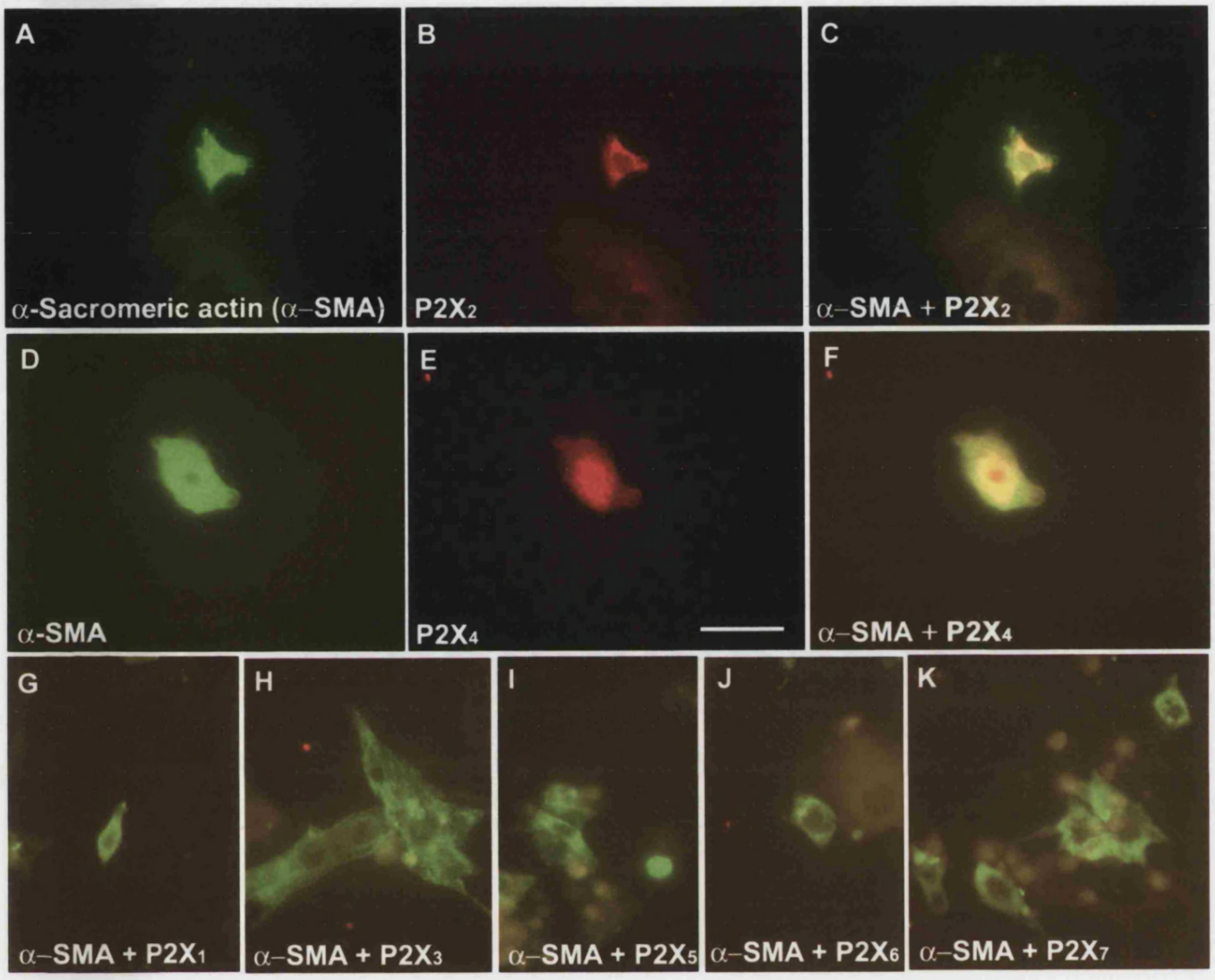
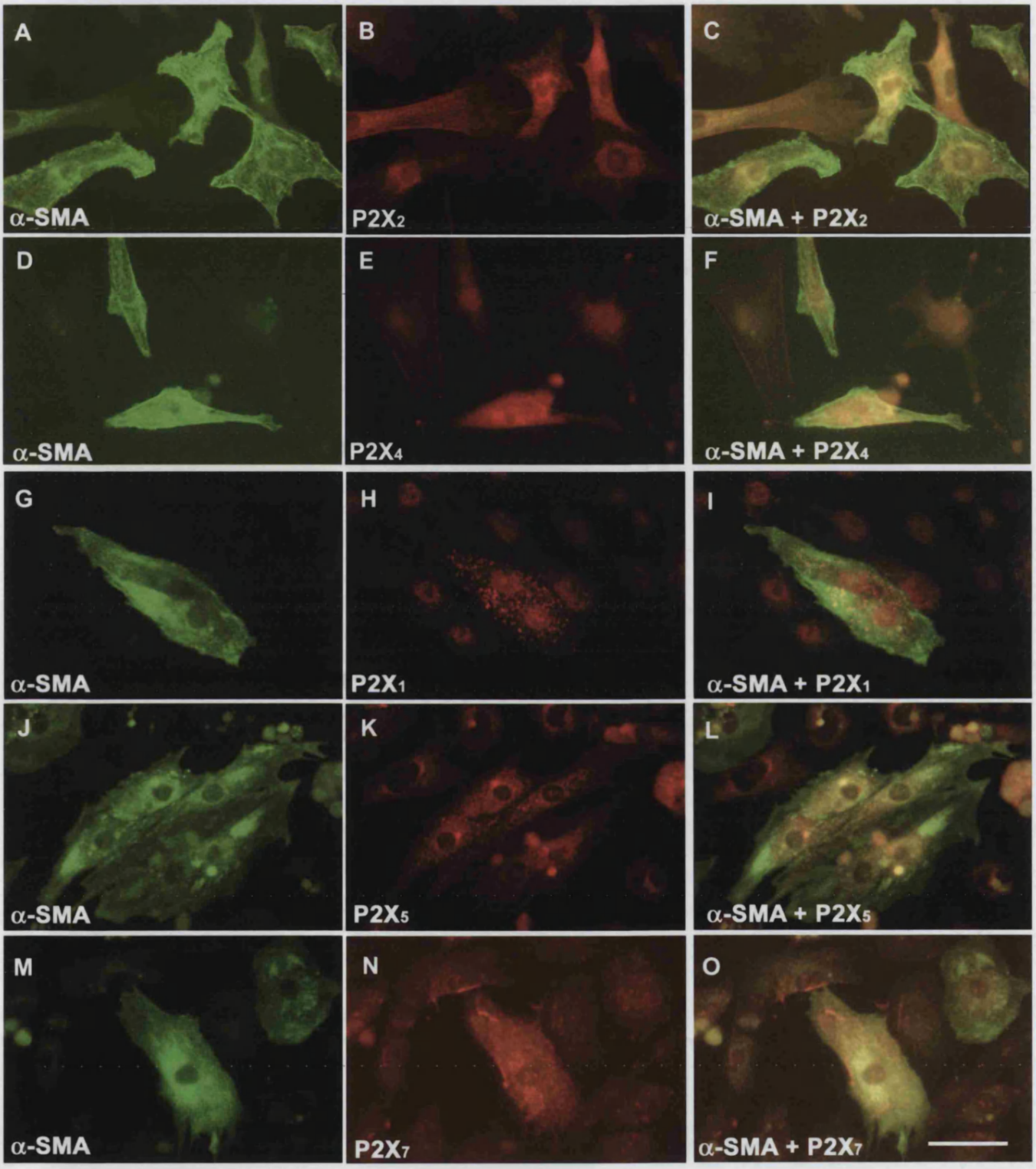

A
α-Sacromeric actin (α–SMA)
B
P2X2
C
α–SMA + P2X2
D
α-SMA
E
P2X4
F
α–SMA + P2X4
G
α–SMA + P2X1
H
α–SMA + P2X3
I
α–SMA + P2X5
J
α–SMA + P2X6
K
α–SMA + P2X7

Figure 6 P2X receptor expression in cultured E18 cardiomyocytes. Cardiomyocytes were identified by monoclonal antibody against α-sacromeric actin (α-SMA, green) (**A**, **D**, **G**, **J** and **M**). P2X receptor-expressing cells are shown in red ($P2X_2$ (**B**), $P2X_4$ (**E**), $P2X_1$ (**H**), $P2X_5$ (**K**) and $P2X_7$ (**N**)). P2X receptor subtypes coexpressing with α-SMA on cardiomyocytes were shown in yellow (**C**, **F**, **I**, **L** and **O**). $P2X_3$ and $P2X_6$ receptors were not expressed in E18 cardiomyocytes (data not shown). Scale bar = 50μm.

Fig 6


A
α-SMA
B
P2X2
C
α-SMA + P2X2
D
α-SMA
E
P2X4
F
α-SMA + P2X4
G
α-SMA
H
P2X1
I
α-SMA + P2X1
J
α-SMA
K
P2X5
L
α-SMA + P2X5
M
α-SMA
N
P2X7
O
α-SMA + P2X7

Chapter VI Early expression of ATP-gated $P2X_7$ receptor in the developing rat pancreas

ABSTRACT

Extracellular ATP modulates the functions of the adult pancreas via two nucleotide receptor families, the P2X and P2Y receptors. Recently, expression of the $P2X_7$ receptor was demonstrated in islet cells of the pancreas, particularly the mature α cells that secrete glucagon. In streptozotocin-induced diabetic model, loss of insulin-secreting cells was accompanied by an increase in α cells that expressed the $P2X_7$ receptor. In the present study we have examined the expression of $P2X_7$ receptor in the developing pancreas from embryonic days (E) 11-18. We detected $P2X_7$-immunoreactive cells in pancreatic islets cells as early as at E11, prior to glucagon expression. Subsequently $P2X_7$ receptor was expressed in glucagon-secreting cells at E12 and complete colocalization was observed at E14. Occasional colocalization of $P2X_7$ receptor and insulin was observed in scattered cells at E12 and E14 but not at E18, when the glucagon-secreting and insulin-secreting cells were almost completely segregated. In summary, it was found that $P2X_7$ receptor was expressed early in a subpopulation of glucagon- and insulin-immunopositive cells in developing islets and subsequently became restricted to glucagon-expressing cells as development proceeded.

INTRODUCTION

The functional units of the endocrine pancreas are the islets of Langerhans composed of four cell types: α, β, δ and PP cells. The insulin-secreting β cells constitute the majority of the endocrine cell population and form the core of the islet, whereas α, δ and PP cells secreting glucagon, somatostatin and a pancreatic polypeptide respectively make up the rest of the islet cell population. In mammals the embryonic pancreas develops from the primitive foregut as two outpocketings (the dorsal and ventral buds), each of which consists of an inner endodermal epithelium surrounded by the mesenchyme (Pictet and Rutter, 1972). Both buds subsequently proliferate to form multiple branches and fuse together to make a functional organ. Among the hormones secreted from the endocrine portion of the pancreas, glucagon is the first peptide expressed in the developing pancreatic epithelium at embryonic day (E) 9.5 in the mouse (Rall et al., 1973; Guz et al., 1995). The functional significance of this early appearance of glucagon in the pancreas has not been resolved, although a recent study suggests that glucagon regulates the differentiation of insulin-secreting cells (Prasadan et al., 2002). However, the relationship of these endocrine cells in embryos and how the differentiation of these various types of pancreatic cells is regulated is still far from clear. The histogenesis of pancreatic endocrine cells requires complex and dynamic gene expression (Larsson, 1998; St-Onge et al., 1999).

Receptors responsible for ATP-mediated activities are subdivided into ionotropic P2X and metabotropic P2Y receptor families (Ralevic and Burnstock, 1998). P2X receptors mediate rapid non-selective passage of small cations across the cell membrane resulting in an increase in intracellular Ca^{2+} and depolarization (see Ralevic and Burnstock, 1998). Among all the P2X receptors cloned, the $P2X_7$ receptor is structurally and functionally unique in that the cation channel of the $P2X_7$ receptor, under the continuous presence of ATP and a low level of divalent cations, converts to a pore permeable to small molecules as well as ions (North, 2002). The significantly longer intracellular C-terminus than other P2X receptors is associated with the induction of the non-selective pore (Surprenant et al., 1996). In addition, the $P2X_7$ receptor is the only P2X receptor subunit that is unable to form heteromeric assemblies with other P2X receptor subunits (Torres et al., 1999), and thus the $P2X_7$ receptor only exists as a homomer. $P2X_7$ receptor has also been implicated in mediating apoptosis in some tissues (Surprenant et al., 1996; Di Virgilio, 2000; North, 2002).

Exogenous nucleotides modulate the functions of the adult pancreas (Loubatières-Mariani and Chapal, 1988; Petit et al., 2001; Novak et al., 2002). It has been demonstrated that ATP stimulates insulin secretion, while adenosine inhibits insulin secretion from β cells and stimulates glucagon secretion from α cells.

The $P2X_7$ receptor has been recently detected in α cells but not the other pancreatic endocrine cell types (Coutinho-Silva et al., 2001, 2003). In the present study, we have investigated the developmental expression pattern of the $P2X_7$ receptor. We have made use of specific antibodies and an immunofluorescent double labeling method to study the spatial-temporal expression of $P2X_7$ receptor in the early developing pancreas in order to find out its relationship with the glucagon and insulin secreting cells.

MATERIALS AND METHODS

Tissue preparation

Please refer to the section **Materials and Methods** on Chapter II.

Immunofluorescence double labeling

Please refer to the section **Materials and Methods** on Chapter III. The primary antibodies used were rabbit anti-$P2X_7$ (1:200; Roche Bioscience), goat anti-glucagon (1:50; Santa Cruz Biotechnology) and guinea pig anti-insulin (1:500; Inestar Stillwater).

RESULTS

$P2X_7$ receptor immunoreactivity was detected in pancreatic islets at E11-E18. The $P2X_7$ receptor was first expressed at E11 (**Fig 1A**), the stage at which glucagon and insulin immunoreactivities were not yet detectable (also see Roberts et al., 2001). $P2X_7$ receptor-immunoreactive cells were found in the islets, which appear as ovoid cell clusters in the pancreas. At E12, both glucagon and insulin started to express in the islets (**Figs 1B, 1C**). Double immunofluorescence labeling showed that all the insulin-expressing cells were glucagon-positive, whereas some pancreatic cells expressed glucagon only (**Fig 1D**). Insulin-expressing cells constituted only a small fraction of cells within the glucagon-expressing population at E12 (**Fig 1D**). Double labeling also showed that all the $P2X_7$ receptor-expressing cells were glucagon positive (**Fig 1F**). Some of the glucagon-expressing cells, however, were $P2X_7$ receptor-negative (**Fig 1F**). At this stage, a small number of islet cells were observed co-expressing $P2X_7$ receptor and insulin, whereas some populations expressing either $P2X_7$ receptor or insulin only were also identified (**Fig 1J**).

At E14, expression of glucagon and insulin was detected. Unlike E12, where all the insulin-expressing cells were glucagon-immunopositive, cells that express insulin but not glucagon were first appeared at this stage (**Fig 2A-2C**). All the glucagon-immunoreactive

cells showed complete co-localization with the $P2X_7$ receptor (**Fig 2D-2F**). Similar to that observed at E12, some but not all of the $P2X_7$ receptor-expressing cells were colocalized with insulin-immunopositive cells. Cells expressing either $P2X_7$ receptor or insulin were also observed.

A sharp increase in the population of insulin-expressing cells took place after E14 (Larsson, 1998). At E18, when insulin-secreting β cells became the dominant endocrine cell type and outnumbered the glucagon-immunoreactive cells (**Fig 3A-3B**), the glucagon-positive and insulin-positive cells were completely segregated (**Fig 3C**). The $P2X_7$ receptor was expressed in the glucagon-expressing islet cells, indicated by colocalization of $P2X_7$ receptor and glucagon (**Fig 3D-3F**). Glucagon-positive, $P2X_7$ receptor negative cells, though present, were very rare (**Fig 3F**). At this stage, none of the insulin-expressing cells were $P2X_7$ receptor-immunopositive (**Fig 3G-3I**).

DISCUSSION

The current experiments used a double immunohistochemical method to examine the expression of ATP-gated $P2X_7$ receptor in glucagon and insulin expressing cells during early development in the rat pancreas. Glucagon and insulin are secreted hormonal peptides as well as common markers for α cells and β cells respectively in both adult and embryonic islets in pancreas. Surprisingly, we found that the $P2X_7$ receptor, which has previously been shown to be expressed specifically in glucagon-expressing cells in postnatal as well as in adult pancreas (Coutinho-Silva et al., 2001), was detected as early as at E11, the stage at which pancreatic cells are still glucagon- and insulin-negative. The early appearance of $P2X_7$ immunoreactivity prior to glucagon expression indicates that expression of the $P2X_7$ receptor does not require the presence of glucagon. Whether or not the $P2X_7$ receptor participates in regulation of glucagon expression, however, is not known. Subsequent expression of the $P2X_7$ receptor was shown to be largely associated with glucagon-positive cells but some insulin staining cells also expressed $P2X_7$ receptors. Eventually, $P2X_7$ receptor expression was only detected in mature α cells containing glucagon (Coutinho-Silva et al., 2001). It has been shown that the early islet cell population (before E14-E15) consists of glucagon-positive cells and glucagon/insulin-coexpressing cells (Larsson, 1998). Our present study and also other reports (Herrera et al., 1991; Teitelman et al., 1993; Larsson, 1998) show that all insulin

expressing cells prior to E14 belong to the glucagon/insulin-coexpressing cell subpopulation. Our results also show that the $P2X_7$ receptor was expressed in both glucagon-positive and glucagon/insulin-coexpressing cells. In view of the finding in the adult pancreas that the $P2X_7$ receptor was restricted to mature α cells that secrete glucagon only (Coutinho et al., 2001, 2003), the expression of the $P2X_7$ receptor in glucagon/insulin expressing cells appears to be novel. It is however known that while the glucagon-positive cells will give rise to the mature α cells, the glucagon/insulin expressing cells will not give rise to any of the mature α- or β-cells (Herrera, 2000), which are thought to be eliminated by apoptosis (Larsson, 1998). Whether the $P2X_7$ receptor is involved in the elimination of the glucagon/insulin expressing cells through apoptosis needs further investigation, but the finding that the $P2X_7$ receptor are not found in all of the glucagon/insulin expressing cells and are observed in glucagon-positive cells indicates that the involvement of the $P2X_7$ receptor in the pancreatic cell death is highly unlikely.

Previous studies demonstrated the first appearance of ultrastructurally recognizable β cells with a characteristic of insulin-positive and glucagon-negative immunoreactivity at E14-E15 (Larsson, 1998). Our present study also found that the insulin-positive and glucagon-negative cells first appeared at E14.

In summary, we have shown the expression of the $P2X_7$ receptor early in the embryonic pancreas even before expression of hormone peptides, suggesting that expression of $P2X_7$ receptor did not depend on the presence of glucagon. Subsequent expression was detected in islet cells expressing different endocrine markers. Thus the $P2X_7$ receptor appears to be one of the earliest markers during islet cell development. Besides, transient expression of $P2X_7$ receptors in the glucagon and insulin co-expressing cells offers additional insight into whether ATP is involved during pancreatic development, in addition to its role in modulating insulin secretion.

FIGURES & LEGENDS

Figure 1 Immunohistochemistry of $P2X_7$ receptor, glucagon and insulin in islets of the E11-E12 pancreas. (**A**) $P2X_7$ receptor-immunoreactive cells (red) are expressed in clusters in E11 pancreas. (**B**) Immunoreactivity of glucagon-expressing cells (red); (**C**) insulin-expressing cells (green), and (**D**) colocalization of glucagon and insulin (yellow). (**E-G**) Double labeling of glucagon and $P2X_7$ receptor. (**E**) Glucagon expression (red) and (**F**) $P2X_7$ receptor expression (green). (**G**) Colocalization of glucagon and $P2X_7$ receptor (yellow). (**H**) $P2X_7$ receptor expression (red); (**I**) insulin expression (green) and (**J**) colocalization of $P2X_7$ receptor and insulin (yellow). Scale bar = 100μm (**A, E-J**) and 114μm (**B-D**).

Fig 1

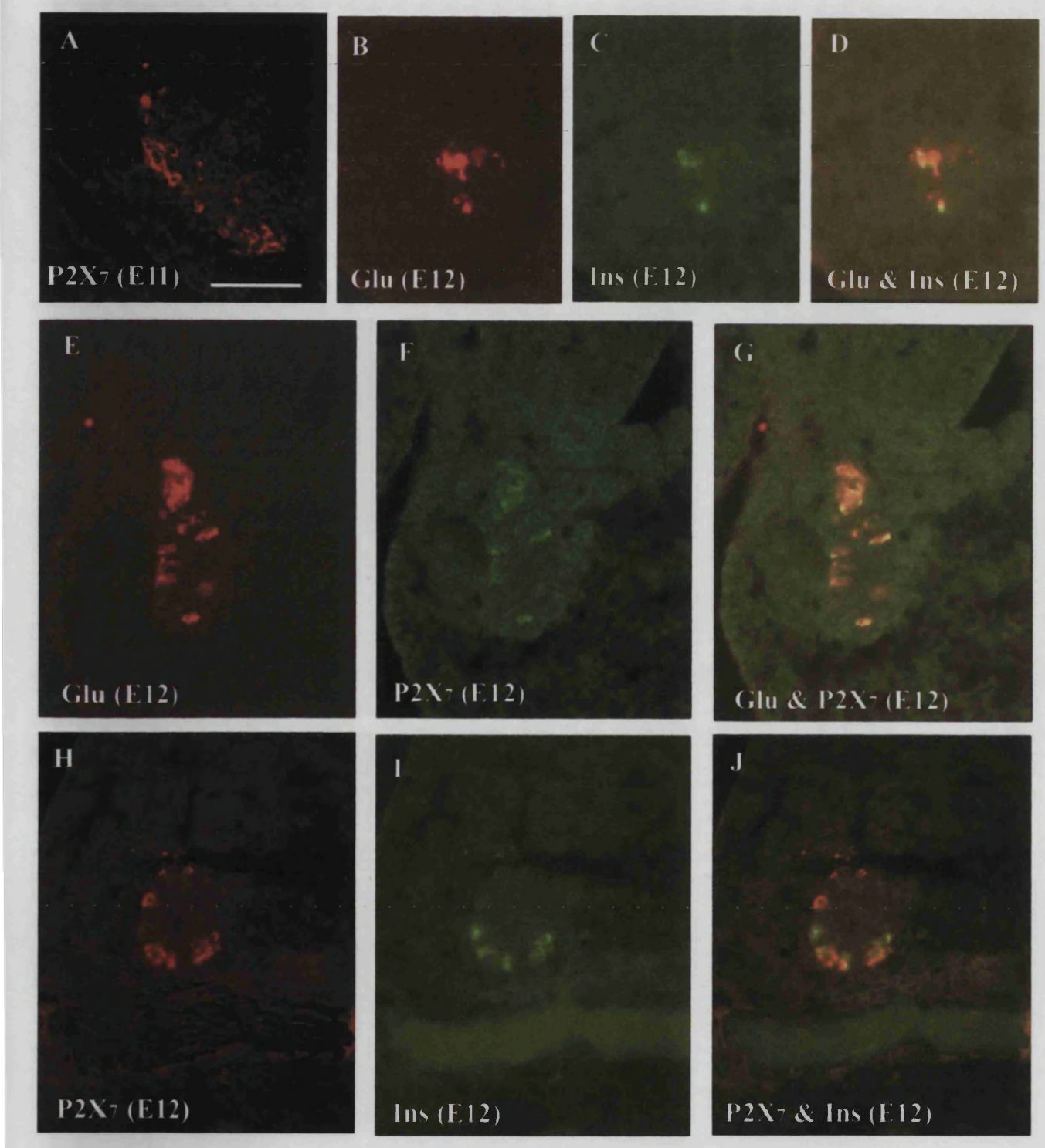

A
P2X7 (E11)
B
Glu (E12)
C
Ins (E12)
D
Glu & Ins (E12)
E
Glu (E12)
F
P2X7 (E12)
G
Glu & P2X7 (E12)
H
P2X7 (E12)
I
Ins (E12)
J
P2X7 & Ins (E12)

Figure 2 Immunohistochemistry of $P2X_7$ receptor, glucagon and insulin in islets of the *E14 pancreas*. (**A**) Immunoreactivity of glucagon-expressing cells (red), (**B**) insulin-expressing cells (green). (**C**) Colocalization of glucagon- and insulin-expressing cells (yellow). (**D**) $P2X_7$ receptor immunoreactive cells (red). (**E**) Glucagon-expressing cells (green) and (**F**) colocalization of glucagon and $P2X_7$ receptor (yellow). (**G**) $P2X_7$ immunostaining (red), (**H**) insulin immunostaining (green). (**I**) Colocalization of $P2X_7$ receptor (yellow). Scale bar = 100μm.

Fig 2

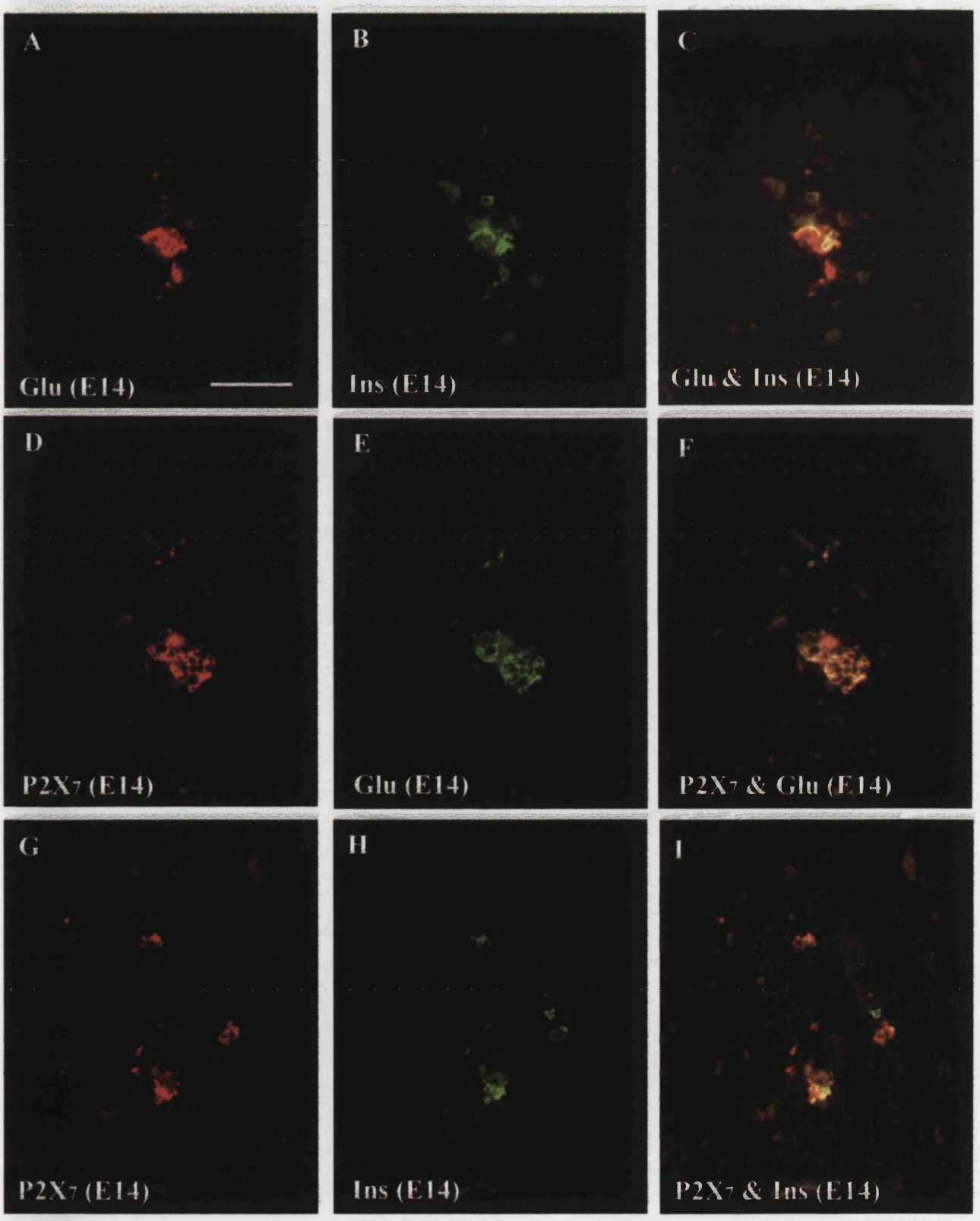
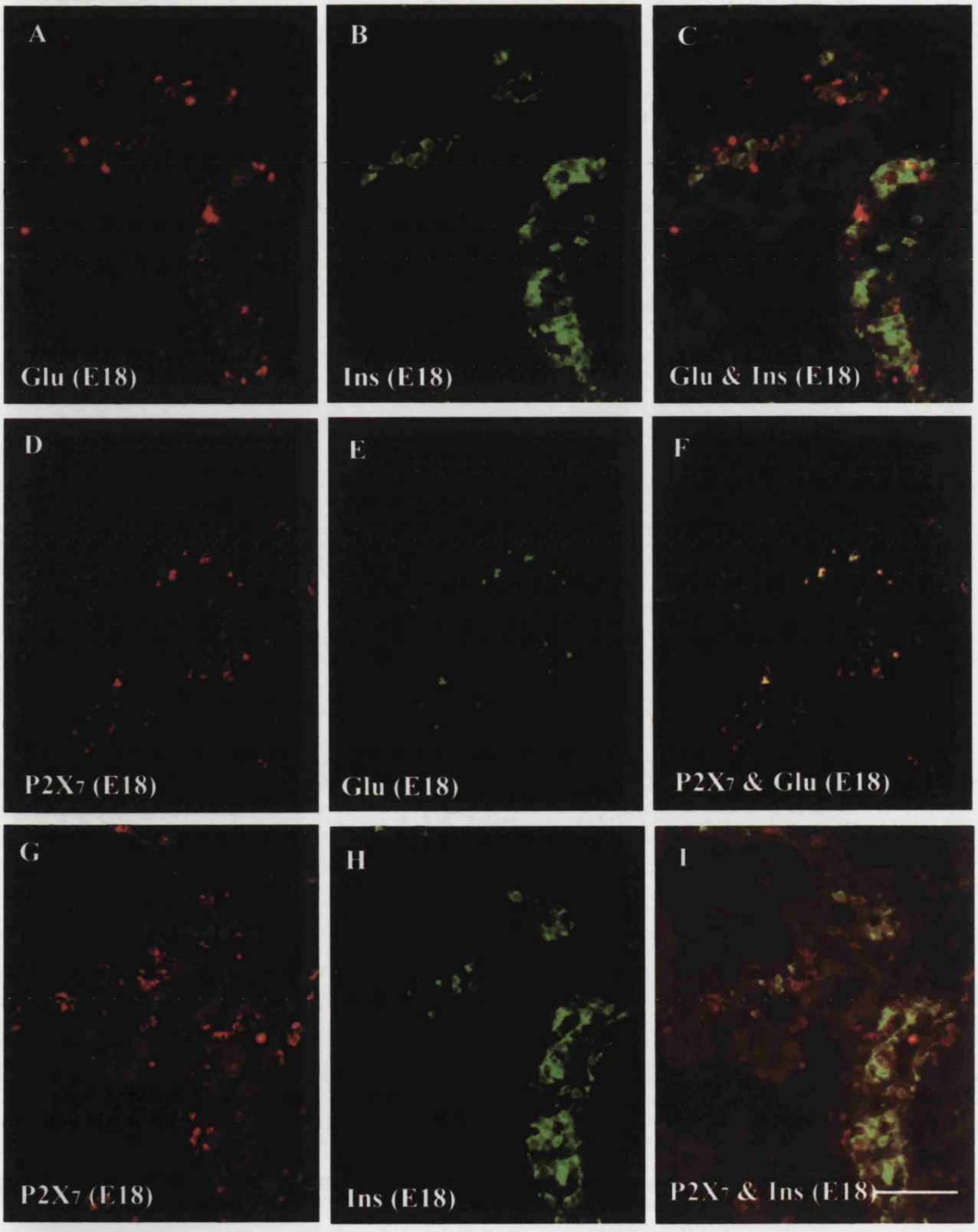

A
Glu (E14)
B
Ins (E14)
C
Glu & Ins (E14)
D
P2X7 (E14)
E
Glu (E14)
F
P2X7 & Glu (E14)
G
P2X7 (E14)
H
Ins (E14)
I
P2X7 & Ins (E14)

Figure 3 Immunohistochemistry of $P2X_7$ receptor, glucagon and insulin in islets of the *E18 pancreas*. (**A**) Immunoreactivity of glucagon-expressing cells (red), (**B**) insulin-expressing cells (green), and (**C**) colocalization of glucagon and insulin (yellow); (**D**) $P2X_7$ receptor immunoreactive cells (red), (**E**) glucagon and insulin expressing cells (green), and (**F**) colocalization of $P2X_7$ and glucagon (yellow); (**G**) $P2X_7$ immunoreactive cells (red), (**H**) Insulin expressing cells (green) and (**I**) colocalization of $P2X_7$ and insulin (yellow). Scale bar = 100μm.

Fig 3


A
Glu (E18)
B
Ins (E18)
C
Glu & Ins (E18)
D
P2X7 (E18)
E
Glu (E18)
F
P2X7 & Glu (E18)
G
P2X7 (E18)
H
Ins (E18)
I
P2X7 & Ins (E18)

Chapter VII. General Discussion and Conclusion

VII.1 Introduction

Fundamental questions in developmental biology concern (1) what genes are expressed; (2) where and when they are expressed and turned off; (3) the regulatory programs that govern the gene expression; (4) and ultimately, why they are expressed. The vast majority of events take place in embryos, with many genes expressed in a dynamic sequential manner, making development a complex and highly integrated process. ATP has an intracellular role in energy storage and release, as well as extracellular roles in mediating wide ranges of physiological activities (see review Abbracchio and Burnstock, 1998; Burnstock, 2002b). Extracellular activities of ATP and related nucleotides (ADP, UTP and UDP) are mediated by activation of two families of P2 receptors, the ionotropic P2X receptors and the metabotropic P2Y receptors (Abbracchio and Burnstock, 1994). Cloning of over 50 P2 receptor subtypes in different species for the past decade (Burnstock, 2003a) has increased our knowledge of the P2 receptor-mediated signaling. It is expected that purinergic signaling pathways would be involved in embryonic development (see Burnstock, 2001.

During development of the early mammalian embryos, the process of gastrulation determines the embryonic body axis and, in addition, generates three distinctive germ layers, namely ectoderm, mesoderm and endoderm. There are two types of ectodermal tissues, the

neuroectoderm and the surface ectoderm. While the neuroectoderm gives rise to tissues of both the central and peripheral nervous system, the surface ectoderm will form the skin. Mesoderm gives rise to several types of tissues, such as musculo-skeletal, cardiovascular and urogenital tissues. Endoderm gives rise mainly to the tissues of the viscera, including those of the digestive and respiratory systems. The results of the present study have demonstrated that P2 receptors are widely expressed throughout the three germ layers during development. The spatial-temporal expression pattern of the P2 receptors in each germ layer, together with the possible roles during development, will be discussed below.

VII. 2 P2 receptors in ectoderm-derived tissues

Nervous system

Activation of P2X receptors involves depolarization of plasma membrane and activation of voltage-dependent Ca^{2+} influx, resulting in an increase in intracellular free Ca^{2+} concentration. In addition, during early development changes in intracellular Ca^{2+} can alter growth cone motility and the rate of neurite outgrowth (Mattson and Kater, 1987), neural migration (Komuro and Rakic, 1996) and neural phenotype (Marty et al., 1996). Therefore, we investigated the expression pattern of P2X receptors during embryonic neurogenesis. Surprisingly, the $P2X_2$, $P2X_4$ and $P2X_6$ receptors, which are widely distributed in adult brains (see Burnstock, 2003b), were absent in early embryonic neural tube. It was the homomeric

$P2X_3$ receptor that showed early expression in embryonic branchiomotor/visceromotor (BM/VM) neurons in the neural tube as well as in cranial ganglia. As development proceeded, the $P2X_3$ receptor was detected in the facial motor nucleus in the hindbrain, which raised the question of whether the cranial motor neurons showing $P2X_3$-immunoreactivity were the facial branchiomotor neurons. The spatial-temporal expression pattern of $P2X_3$ receptors in the cranial motor neurons suggested that it might be involved in neurite outgrowth. Both αβ-meATP and ATP, agonists to the $P2X_3$ receptor, showed significant inhibition of neurite extension. UTP did not mimic the inhibitory effect of ATP; therefore, it is unlikely that $P2Y_2$ and $P2Y_4$ receptors were involved, even though these receptors were expressed in the embryonic spinal nerves. The stability of αβ-meATP also ruled out the possibility of an adenosine-mediated response. ATP has been shown to inhibit NCAM-expressing neurites (Skladchikova et al., 1999). Our results showed that the embryonic cranial motor axons expressed NCAM, and the ATP-mediated inhibitory effects on NCAM-expressing axons further indicated that ATP might interact with NCAM in the process of axon outgrowth in embryonic cranial motor neurons, most likely via $P2X_3$ receptor. The process of axon guidance appears to be very complex, involving different types of signaling molecules acting in concert to direct pathfinding (Tessier-Lavigne and Goodman, 1996). Our results demonstrated that ATP has an inhibitory effect on neurite outgrowth from cranial neural tube. However, no experiments were carried out to see if ATP had any effect on axon guidance.

Several questions arise: does the inhibitory effect of ATP reflect the rejection of cranial motor axons to particular tissues from the environment that release ATP? What is the source of ATP surrounding the neural tube? Is this inhibitory effect due to a reduction in length of BM/VM axons? Identifying the *in vivo* source of ATP together with the use of specific markers for different types of motor axons will be crucial in uncovering the role of purinergic signaling in embryonic axon pathfinding.

The expression of $P2X_3$ receptor was also prominent in a number of neural crest-derived tissues, including trigeminal, facioacoustic, glossopharyngeal-vagal, dorsal root and sympathetic ganglia, mesencephalic trigeminal nucleus. Enteric neurons in the adult guinea pig also show $P3X_3$ receptor expression (Van Nassauw, et al., 2002; see Burnstock, 2003b). Other neural-crest derived tissues which are non-neural, such as melanocytes, dermal bones of skull, odontoblasts and the pharyngeal arches cartilages, all show absence of $P2X_3$ receptor expression throughout life. It seems that the onset of $P2X_3$ receptor expression begins after the migrating neural crest cells arrive at their presumptive destination and are committed into a neural fate. $P2X_3$ receptor-deficient mice showed an impaired response towards inflammatory pain (Cockayne et al., 2000) and peristalsis (Bian et al., 2003), whereas loss or atrophy of neurons was never reported in the transgenic model, suggesting that $P2X_3$ receptor expression is important for the function, but not the survival of neurons.

The $P2X_2$ and receptor expression was detected in the nucleus tractus solitarius in E14 rat brain. Results from double labeling experiments indicated that the $P2X_2$ receptor was largely, if not entirely, colocalized with the $P2X_3$ receptor. It is very likely that the two receptors formed $P2X_{2/3}$ heteromers in the nucleus tractus solitarius. In contrast, the nodose and the dorsal root ganglia showed an incomplete colocalization of $P2X_2$ and $P2X_3$ receptors. It is known that the $P2X_{2/3}$ heteromers differ from the homomeric $P2X_2$ and $P2X_3$ receptors in the response towards ATP, especially in the aspects of desensitization (Liu et al., 2001, Koshimizu et al., 2002). The variable extent of involvement between the $P2X_2$ and $P2X_3$ in the formation of the $P2X_{2/3}$ heteromers may thus contribute to different effects of ATP-mediated activities.

The $P2X_7$ receptor was expressed in the rat hypothalamus from E14 onwards, slightly earlier than the appearance of the functional glutamate receptor in the embryonic hypothalamus (at E15) (van den Pol et al., 1995). Sperlágh et al. (2002) demonstrated that ATP regulates glutamate release via activation of $P2X_7$ receptors, and excessive glutamate release altered the Ca^{2+} homeostasis, resulting in activation of apoptosis-related gene (e.g caspase) (see review Le Feuvre et al., 2002). In vitro studies on the embryonic hippocampal neurons also showed that glutamate release upon reactive oxygen species induced activation of glutamate receptor

(NMDA receptor), which then activated caspases and in turn triggered apoptosis (Ishikawa et al., 1999). Given that ATP-mediated apoptosis involved activation of $P2X_7$ receptor (see review Le Feuvre et al., 2002), it seems likely that the activation of $P2X_7$ receptor is also involved in cell death during neurogenesis, but this possibility awaits further investigation.

The $P2X_4$, $P2X_5$ and $P2X_6$ receptors were not detected in the developing brain until the late embryonic period, between E18-P1. $P2X_6$ receptor expression was shown in the lateral hypothalamus of P1 brain. $P2X_6$ receptors have been implicated in hormone release from axon terminals as well as recycling of the granular vesicles and microvesicles in the adult hypothalamo-neurohypophysial system (Loesch and Burnstock, 2001). $P2X_4$ and $P2X_5$ receptors were both detected in the subventricular zone (SVZ) of the embryonic striatum. The SVZ was previously demonstrated to be a site of postnatal neurogenesis (Conover and Allen, 2002). $P2X_4$ and $P2X_5$ receptors labeled two different types of cells in the SVZ; the round-shaped, cytoplasmic-stained, $P2X_4$ receptor immunoreactive cells, and the pyramidal-shaped, nuclear and cytoplasmic-stained, $P2X_5$ receptor immunoreactive cells. Doetsch et al. (1999) and Laywell et al. (2000) showed that the subventricular (SV) astrocytes are the neural stem cells that give rise to both neurons and glia. Other results suggest that it is the ependymal cells that generate multipotent neurospheres (Johansson et al., 1999). It would be interesting to use double-labeling to examine the identity of the $P2X_5$

receptor-immunoreactive cells in the SVZ to see if they are SV astrocytes or ependymal cells. $P2X_4$ receptor-immunoreactive cells, however, resemble migrating neuroblasts rather than the morphologically distinctive astrocytes or ependymal cells. The $P2X_1$ receptor, according to our result, was not expressed before P16. It has been demonstrated previously that $P2X_1$ receptors are expressed in the adult rat cerebellum (Loesch and Burnstock, 1998; Florenzano et al., 2002) and upregulation of the receptor was observed in the precerebellar neurons that survived after cerebellar injury (Florenzano et al., 2002). It is not clear whether the upregulation is important for post-injured cell survival or a consequence of axotomy. Although $P2X_1$ receptor expression was unlikely to be involved in embryonic neurogenesis, its potential role in either cell survival or regeneration after injury would be worth investigating.

In addition, the P2X receptor involvement in embryonic neurogenesis, G-protein coupled P2Y receptor expression was also detected in the embryonic nervous system. We have observed an early expression of $P2Y_2$ and $P2Y_4$ receptors in the ventral neural tube. The $P2Y_2$ receptor was expressed in the spinal motor neurons and the ventrally migrating motor axons. The $P2Y_4$ receptors, however, were only found in the spinal motor neurons. Absence of inhibitory effects by UTP suggested that ATP-induced effects were not mediated by $P2Y_2$ or $P2Y_4$ receptors. Nevertheless, the distinct expression pattern of $P2X_3$, $P2Y_2$ and $P2Y_4$ receptors in the ventral neural tube raises the question of whether there is any interaction

between ATP signaling and the well known ventral-patterning signals such as Sonic Hedgehog (Shh) and Pax 6 (Goulding et al., 1993; Mansouri et al., 1996; Dodd et al., 1998, Litingtung and Chiang, 2000). Among the P2Y receptor examined, the $P2Y_4$ receptor appeared to be the dominant receptor in the developing brain. Although the exact function of the $P2Y_4$ receptor during neurogenesis was unknown, the transient expression (at least in the medulla) suggests a role during development.

Taken together, our results suggest that ATP is involved during neurogenesis in both prenatal and postnatal animals. While the $P2X_2$, $P2X_4$ and $P2X_6$ receptors are dominant in the neonatal as well as adult brain, $P2X_3$ receptors appeared to play a more important role during embryonic neurogenesis. Despite a transient and dynamic expression of $P2X_3$ receptors in the embryonic brain especially in the motor neurons, the early expression in the cranial sensory ganglia persists after birth. Nevertheless, purinergic signaling via P2X receptor activation appears to be complex and fine-tuned, for each of the receptors are expressed in a spatial-temporal manner.

The present study also showed distinct localization of $P2X_2$ and $P2Y_1$ receptors in the notochord and the floor plate, respectively. The notochord is situated just beneath the neural tube, while the floor plate is at the ventral midline. Although the notochord and the floor

plate were not considered to be neuronal structures, they are known to exert major effects on neurogenesis (Placzek, 1995; Bronner-Fraser and Fraser, 1997). Both the notochord and the floor plate secrete Shh, which is a powerful signaling molecule involved in ventral patterning of the neural tube (Goulding et al., 1993; Dodd et al., 1998, Litingtung and Chiang, 2000). Shh first secretes from the notochord and then from the floor plate. Shh regulates one of the transcription factors *Pax* 6 gene, and both of them act in a concentration dependent manner in promoting the differentiation of the neurons in the ventral neural tube to become motor neurons. This is the first report demonstrating P2 receptor expression in the notochord and floor plate. It would be interesting to look at the interaction between the Shh, Pax and purinergic signaling.

VII.3 P2 receptors in mesoderm-derived tissues

VII.3.a Somites and skeletal muscles

Following gastrulation, the paraxial mesodermal tissues on either side of the embryos in a position lateral to the neural tube segregate from the rest of the mesoderm and form the well-defined tissue blocks, the somites. Shortly after it has formed, each somite undergoes a mesenchyme-to-epithelial transition, and the epithelialized somite becomes partitioned into presumptive sclerotome (ventromedial) and dermomyotome (dorsolateral) regions, in a rostral-caudal direction. The dermomyotome will give rise to myotome (forming skeletal

muscle) and the dermatome (forming dermis).

Although the $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in our study were all expressed early in the myotome and later in the developing skeletal muscle, their fate appeared to be quite different. Whereas $P2Y_4$ receptor expression was sustained in postnatal skeletal muscle, the $P2Y_1$ and $P2Y_2$ receptors were no longer expressed. Instead, $P2Y_1$ receptor was located in the smooth muscle of the blood vessels and $P2Y_2$ receptors appeared to be expressed in satellite cells. Neonatal satellite cells showed a concentration-dependent proliferation upon UTP treatment (Ryten et al., 2002), suggesting that $P2Y_2$ receptor activation by UTP was likely to participate in satellite cell proliferation, which is very important in the response to muscle damage. It is equally possible that UTP (and possibly ADP via $P2Y_1$ receptors) are also involved in proliferation during embryonic myogenesis. The present results suggest future studies of the interaction between extracellular nucleotides and myogenic signaling molecules such as Pax 3, MyoD and Myf5 (Tajbakhsh et al., 1997; Buckingham, 2001; Chi and Epstein, 2002; Buckingham et al., 2002).

Choi et al (2002) showed a colocalization of $P2Y_1$ receptor with the acetylcholine receptor. In the present study, however, colocalization of P2Y receptor subtypes with acetylcholine receptors in the neuromuscular junction was not observed. It may be that the

difference in the antibodies used in the two studies accounts for the discrepancy. We used a commercial rat-specific antibody, while the one used by Choi et al (2002) was prepared against chick receptor. The $P2Y_1$ receptor expression in our study was downregulated in the embryonic skeletal muscle as development proceeded, which was in agreement with a downregulation of chick $P2Y_1$ receptor mRNA transcript in embryonic skeletal muscle reported by Meyer et al. (1999a).

In addition to expression studies, functional studies also showed that upon ATP treatment, the isolated skeletal muscle cells from mouse embryos demonstrated P2X receptor-like responses (Collet et al., 2002). The response to ATP was developmentally regulated, as the amplitude of responses was decreased during early postnatal stages. The responses were completely lost in adult skeletal muscle cells, indicating that the ATP-induced responses were stage-specific. Altogether, the dynamic expression of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in somites and in the developing skeletal muscle, the transient expression of the $P2X_5$ receptor in the embryonic skeletal muscle tissue and its role in inducing satellite cell differentiation (Ryten et al., 2001; 2002), strongly suggests that ATP is involved in regulation of skeletal muscle development as well as regeneration.

VII.3.b Heart

The heart originates from paired cardiac mesodermal primordia which fuse in the midline to produce a primitive heart tube (Yutzey and Bader, 1995). The heart tube then undergoes a series of remodeling steps, including looping, trabeculation, septation, and development of spiral outflow tracts (Larsen, 1998). Extracellular ATP and adenosine are known to have powerful actions on cardiomyocytes (Olsson and Pearson, 1990; Ralevic and Burnstock, 1991; Pelleg and Belardinelli, 1998; Vassort, 2001). Previous studies showed that subtypes of P1 adenosine receptors were expressed in the embryonic cardiomyocytes (Hofman et al., 1997; Zhao and Rivkees, 2001; Zhao et al., 2002). It was therefore interesting to investigate if subtypes of P2 receptor were also expressed in particular stages of heart development. The present results showed that $P2X_2$ and $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors were expressed in the myocardium at E12, the stage at which trabeculation and septation are in progress (Franco et al., 1998). Studies on Ca^{2+} influx, together with RT-PCR and immunocytochemistry, have identified particular subtypes of P2X ($P2X_2$, $P2X_4$, $P2X_7$) and P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$) receptors in embryonic cardiomyocytes at E14, followed by $P2X_1$, $P2X_3$ and $P2X_5$ receptors at E18. Identification of $P2X_7$ receptor in the cardiomyocytes appears to be novel. Although P2Y receptor-mediated responses were observed in the cardiomyocytes at both E14 and E18, immunohistochemical studies showed that the $P2Y_1$, $P2Y_2$, $P2Y_4$ receptor subtypes, while widely expressed throughout the myocardium, gradually became restricted to the trabeculated myocardium but not to the compact myocardium. Differences in the proliferation capacity

between the compact layer and the trabeculated layer of the myocardium (Sedmera et al., 2000) may provide a clue to the functional role of P2Y receptor activation. Trabeculations are important in enhancing contractility (Challice and Viragh, 1973) and in coordinating intraventricular conduction (de Jong et al., 1992). However, preliminary results have shown that treatment of cultured embryonic cardiomyocytes with ATP, ADP, UTP and UDP did not affect cell proliferation (unpublished data). P2X receptors are expressed in embryonic as well as adult cardiomyocytes. Persistent expression of P2X receptors did not necessarily reflect the absence of dynamic expression patterns. *In situ* labeling of the P2X receptors in the embryonic hearts during development should further be examined. It is possible that P2X receptors regulate particular physiological activities which are important in embryonic as well as in adult cardiomyocytes (Pelleg and Belardinelli, 1998).

VII.4 P2 receptors in endodermal-derived tissues

Pancreas

We have shown in the present study that one of the P2X receptors, the $P2X_7$ receptor, was expressed in developing endodermal tissue, the embryonic pancreas. The study was focused on the endocrine tissues, i.e. the islets of Langerhans. The major hormones present are insulin secreted from β cells, somatostatin from δ cells and glucagon from α cells. Previously, our group has demonstrated the expression of $P2X_7$ receptor to be restricted to α cells raising the

question of whether $P2X_7$ receptor expression depends upon the presence of glucagon. The present results showed that i) the possibility of glucagon-driven $P2X_7$ receptor expression was ruled out; ii) $P2X_7$ receptor expression was not limited to glucagon-expressing cells during early development; and iii) colocalization of $P2X_7$ receptor with insulin was transient. It is reasonable to speculate that the $P2X_7$ and glucagon positive cells (insulin negative) at both E12 and E14 would give rise to α cells, whereas the insulin-positive and $P2X_7$ negative (also glucagon negative) cells would give rise to β cells. Given that the early islet cells are all glucagon-positive (at E12), two additional types of cells were identified which were the glucagons/insulin/$P2X_7$ receptor coexpressing cells (i.e $P2X_7$ receptor-positive) and glucagons/insulin coexpressing cells (i.e. $P2X_7$ receptor-negative). Information is missing regarding to the identity of these cells, although these multihormonal cells would not give rise to either mature α or β cells and appeared to be eliminated in later development (Larsson, 1998; Herrera, 2000). The possibility of $P2X_7$ receptor control of glucagon-release appears to be unlikely, and previous studies have shown that glucagon release is regulated by adenosine, while insulin secretion is stimulated by ATP via P2Y receptor-mediated pathway (Loubatières-Mariani and Chapal, 1988; Loubatières-Mariani et al., 1997). Previous studies on $P2X_7$ receptor-deficient mice did not report any pancreatic-related abnormalities (Labasi et al., 2002; Ke et al., 2003). Further studies using in vitro culture systems would be an appropriate approach, together with the application of ATP or manipulation of $P2X_7$ receptor

expression (either overexpressed receptor transcripts, or suppressed transcripts by means of RNA interference techniques) to examine the roles of $P2X_7$-mediated signaling. Coutinho et al (2003) have demonstrated the expression of the $P2Y_4$ receptor in both α and β cells in adult pancreas. Examination on the $P2Y_4$ receptor expression in the developing pancreas will probably provide a more complete picture about how ATP regulates the development of pancreatic islets.

VII.5 Concluding Remarks

In conclusion, our study has demonstrated for the first time the widespread expression pattern of P2X and P2Y receptor subtypes during rat embryonic development. The expression pattern of P2 receptor subtypes was not restricted to a particular tissue or system but included all the three germ layers. Dynamic and transient expression for particular receptors was observed in different tissues (for example, the $P2X_3$ receptor in cranial motor neurons; the $P2X_2$ receptor in notochord; the $P2X_7$ receptor in pancreatic insulin-expressing cells; the $P2Y_1$ receptor in the floor plate; the $P2Y_4$ receptor in the brainstem; the $P2X_5$, $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in somites in developing skeletal muscle; and the $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in the compact layer of myocardium). It appears that ATP acting on P2X and P2Y receptor subtypes regulates organogenesis during embryonic development.

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Appendix

Table 1: Primer sequence for P2X and P2Y receptors in RT-PCR analysis

Receptor	Primer sequence (5' to 3')	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	
$P2Y_1$ forward	ACGTCAGATGAGTACCTGCG	1235-1254	289
$P2Y_1$ reverse	CCCTGTCGTTGAAATCACAC	1504-1523	
$P2Y_2$ forward	CTGGTCCGCTTTGCCCGAGATG	1417-1438	311
$P2Y_2$ reverse	TATCCTGAGTCCCTGCCAAATGAGA	1703-1727	
$P2Y_4$ forward	TGTTCCACCTGGCATTGTCAG	1586-1586	294
$P2Y_4$ reverse	AAAGATTGGGCACGAGGCAG	1840-1859	
$P2Y_6$ forward	TGCTTGGGTGGTATGTGGAGTC	868-889	339
$P2Y_6$ reverse	CAATAGTCGAAGGACGGAAAGGT	1184-1206	

Shibuya et al (1999); Bailey et al. (2000, 2001)

Table 2: Peptide sequence for raising the polyclonal antibodies against P2X and P2Y receptor subtypes

Receptor antibody	Peptide position	Amino acid sequence
Roche Antibodies		
Rabbit-anti-rat $P2X_1$	385-399	ATSSTLGLQENMRTS
Rabbit-anti-rat $P2X_2$	458-472	QQDSTSTDPKGLAQL
Rabbit-anti-rat $P2X_3$	383-397	VEKQSTDSGAYSIGH
Rabbit-anti-rat $P2X_4$	374-388	YVEDYEQGLSGEMNQ
Rabbit-anti-rat $P2X_5$	437-451	RENAIVNVKQSQILH
Rabbit-anti-rat $P2X_6$	357-371	EAGFYWRTKYEEARA
Rabbit-anti-rat $P2X_7$	555-569	TWRFVSQDMADFAIL
Alomone antibodies		
Rabbit-anti-rat $P2Y_1$	242-258	RALIYKDLDNSPLRRKS
Rabbit-anti-rat $P2Y_2$	227-244	KPAYGTTGLPRAKRKSVR
Rabbit-anti-rat $P2Y_4$	337-350	HEESISRWADTHQD
Rabbit-anti-rat $P2X_7$	576-595	KIRKEFPKTQGQYSGFKYPY