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REVIEW ARTICLE

Purinergic signalling during development and ageing

Geoffrey Burnstock[1,2] · **Nicholas Dale**[3]

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Abstract Extracellular purines and pyrimidines play major roles during embryogenesis, organogenesis, postnatal development and ageing in vertebrates, including humans. Pluripotent stem cells can differentiate into three primary germ layers of the embryo but may also be involved in plasticity and repair of the adult brain. These cells express the molecular components necessary for purinergic signalling, and their developmental fates can be manipulated via this signalling pathway. Functional P1, P2Y and P2X receptor subtypes and ectonucleotidases are involved in the development of different organ systems, including heart, blood vessels, skeletal muscle, urinary bladder, central and peripheral neurons, retina, inner ear, gut, lung and vas deferens. The importance of purinergic signalling in the ageing process is suggested by changes in expression of A_1 and A_2 receptors in old rat brains and reduction of P2X receptor expression in ageing mouse brain. By contrast, in the periphery, increases in expression of P2X3 and P2X4 receptors are seen in bladder and pancreas.

Keywords Stem cells · Zebrafish · Heart · Bladder · Retina · Cochlea · Lung · Skeletal muscle · ATP · Adenosine

✉ Geoffrey Burnstock
g.burnstock@ucl.ac.uk

[1] Autonomic Neuroscience Centre, University College Medical School, Rowland Hill Street, London NW3 2PF, UK

[2] Department of Pharmacology and Therapeutics, The University of Melbourne, Melbourne, Australia

[3] School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK

Synopsis

Introduction

Most early studies of the roles of nucleotide in development have been discussed in terms of their intracellular roles and as a source of energy. However, purines and pyrimidines are now

generally accepted to have potent extracellular actions mediated by the activation of specific membrane receptors (see [1]); many of these previous studies can now be reinterpreted. ATP and adenosine play key roles from the very beginning of life, i.e. the moment of conception. Adenosine 5′-triphosphate (ATP) is obligatory for sperm movement [2] and is a trigger for capacitation, the acrosome reaction necessary to fertilize the egg [3]. Extracellular ATP also promotes a rapid increase in Na^+ permeability of the fertilized egg membrane through the activation of a specific ATP receptor [4]. Mg^{2+}–ATPase activity has been localized on the entire surface of unfertilized eggs and in preimplantation and postimplantation embryos [5, 6]. Together with the demonstration that ATP-activated spermatozoa show very high success rates in fertilization tests [3], this strongly suggests that ATP is a key sperm-to-egg signal in the process of fertilization.

In this review, the extracellular roles of purines and pyrimidines will be considered as signalling molecules in both embryological and postnatal development in a wide variety of systems in amphibians, birds and mammals, including humans. Readers are referred to reviews of the earlier literature about the involvement of purinergic signalling in both embryonic and postnatal development [7, 8] and very good reviews specifically about the development of the nervous system [9–11]. There have been relatively few reports about changes in purinergic transmission during ageing, and these are mostly concerned with the brain and cardiovascular system.

Together with muscarinic cholinergic receptors, extracellular receptors to ATP were shown to be the first functionally active membrane receptors in chick embryo cells at the time of germ layer formation. In gastrulating chick embryo, ATP caused rapid accumulation of inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} mobilization in a similar way and to the same extent as acetylcholine (ACh), whereas other neuroendocrine substances such as insulin and noradrenaline (NA) had much weaker effects. This suggests that, alongside ACh, other phylogenetically old and universal regulators of cell metabolism such as ATP (and perhaps nitric oxide (NO)) might play leading roles in the functional regulation of gastrulation via the activation of specific receptors triggering Ca^{2+} mobilization.

Early embryos

The expression patterns of purinergic receptors and the ectonucleotidase enzymes that convert ATP to downstream nucleotides and nucleosides vary during development. This suggests, in turn, that these molecules may have important developmental roles, either to mediate particular physiological functions at different stages of development or to control the developmental processes themselves [12].

Zebrafish

Trigeminal ganglia of zebrafish express P2X3 receptors from a very early stage of development, most likely in neural crest-derived trigeminal cells rather than placode-derived cells [13]. Spinal sensory Rohon–Beard cells also expressed P2X3 receptors, also in the putative lateral line ganglion and in the early developing zebrafish. Ectodermal p2rx3.1 receptor, a paralog of the mammalian P2X3 receptor, is expressed in subpopulations of both neural and ectodermal cells in the embryonic head of zebrafish [14]. Knockdown of expression of this receptor disrupted craniofacial development, suggesting a critical role in this process.

Frog embryos

A novel P2Y receptor (p2y8) was cloned and sequenced and was expressed (as seen by Northern blots and in situ hybridization) 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 [15]. It differs from other members of the P2Y receptor family having an intracellular C terminus with 216 amino acid residues (compared to 16 to 67 in the known P2Y receptors). It shows equipotent responses to the triphosphates ATP, uridine 5′-triphosphate (UTP), inosine triphosphate, cytidine triphosphate and guanosine triphosphate (GTP) and smaller responses to diphosphates and tetraphosphates when expressed as a recombinant receptor in *Xenopus* oocytes but is not responsive to inorganic phosphates. Activation of the p2y8 receptor evoked long duration responses (40–60 min). It was suggested that this novel P2Y receptor may be involved in the early formation of the nervous system.

Suramin and trypan blue, both substances that are known to be antagonists at P2 receptors (see [16]) as well as having other actions, have been shown to interfere with gastrulation [17]. If injected early when the dorsal lip is first invaginating, the *Xenopus* embryo develops no head or trunk and sometimes no tail; somites and notochord are also missing. If they are injected midway in gastrulation, embryos develop without heads, but with trunks and tails, while if injected at the end of gastrulation, the embryo is completely unaffected.

The nicotinic channels in myotomal muscle cells cultured from *Xenopus* embryos at stages 19–22 were shown to be opened by micromolar concentrations of exogenous ATP [18], following the earlier demonstration that ATP increases the sensitivity of receptors in adult frog skeletal muscles without increasing the affinity of ACh for the receptor or inhibitory acetylcholinesterase [19]. Since then, there have been a number of studies of the actions of ATP in developing *Xenopus* neuromuscular synapses (see [20]). Extracellular applications of ATP to developing *Xenopus* neuromuscular synapses in culture potentiate ACh responses of developing muscle cells during the early phase of synaptogenesis [21–23]. The

possibility that extracellular ATP coreleased with ACh may serve as a positive trophic factor at developing neuromuscular synapses has also been raised [20, 21]. It is further suggested that calcitonin gene-related peptide (CGRP) and ATP coreleased with ACh from the nerve terminal may act together to potentiate postsynaptic ACh channel activity during the early phase of synaptogenesis [24]; it is claimed that CGRP actions are mediated by cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PK) A, while ATP exerts its effects via PKC. In a later report from this group [25], they present results that suggest that endogenously released ATP, acting in concert with various PKs, is involved in the maintenance and/or development of the quantum size of synaptic vesicles at embryonic neuromuscular synapses.

Purine-mediated signalling triggers eye development in *Xenopus* [26]. The authors have shown that overexpression of ecto-NTPDase2 (that converts ATP to adenosine 5′-diphosphate (ADP)) caused ectopic eye-like structures and occasionally complete duplication of the eye. Expression of ecto-NTPDase2 is essential for the expression of key genes required for eye development such as *Rx1* and *Pax6* (Figs. 1 and 2). It appears that the ecto-NTPDase2 produces ADP from ATP which is necessary for activation of the $P2Y_1$ receptor. Although knockdown of $P2Y_1$ expression had relatively mild effects, combined knockdown of ecto-NTPDase2 and the $P2Y_1$ receptor could completely prevent eye development (Fig. 2). All the key components of purinergic signalling are present around stage 12 of embryogenesis when the key determination of eye development occurs (Fig. 3). In the eye field (the site of the future development of the eye and where key genes are activated to achieve this), a period of transient ATP release was observed (Fig. 3). However, this mechanism may be specific to lower vertebrate embryos, as knock out of NTPDase2, even in combination with knock out of the $P2Y_1$ receptor, in mice does not prevent or alter eye development [27].

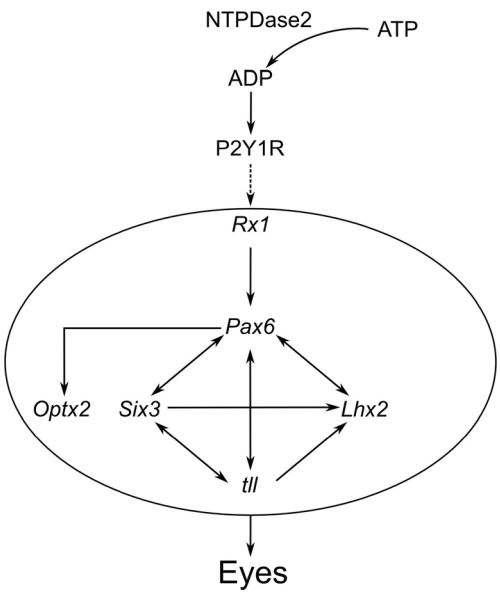

Fig. 1 Purinergic signalling is upstream of the eye field transcription factors (EFTFs) that are expressed very early in development of the frog embryo and to control development of the eye (see [364]). ATP released in the presumptive eye field is converted to ADP via NTPDase2. The ADP can activate the P2Y1 receptor (P2Y1R), which then triggers expression of the transcription factor *Rx1*

Chick embryos

In the chick, the thyroid gland most likely forms from the thyroid primordium via a process of evagination that appears to be induced by ATP [28]. The requirement for ATP was very precise, since it could not be replaced by pyrophosphate, AMP or ADP nor by GTP, suggesting a high degree of specificity of the ATP-induced effect. Together with muscarinic cholinergic receptors, extracellular receptors to ATP were shown to be the first functionally active membrane receptors in chick embryo cells at the time of germ layer formation [29]. In gastrulating chick embryo, ATP causes rapid accumulation of inositol phosphate and Ca^{2+} mobilization in a similar way and to the same extent as ACh, whereas other neuroendocrine substances such as insulin and NA have much weaker effects [29]. This suggests that, alongside ACh, other phylogenetically old and universal regulators of cell metabolism such as ATP (and perhaps NO) might play a leading role in the functional regulation of gastrulation via the activation of specific receptors triggering Ca^{2+} mobilization. Wide distribution of $P2Y_1$ receptors in the 1-day-old chick brain was reported.

ATP acts on embryonic and developing cells of both nervous and non-nervous systems by increasing intracellular Ca^{2+} concentrations. Release of Ca^{2+} from intracellular stores is evoked in the otocyst epithelium of the early embryonic chick, incubated for 3 days (stage 18 to 19) [30] (Fig. 4), in developing chick myotubes [31] and in dissociated cells from whole early embryonic chicks [29, 32]. Programmed cell death was demonstrated in proliferative regions of chick optic tectum during early development, particularly in the ventricular zone between stages E7.5 and E8. This is of interest since P2X7 receptors mediate apoptosis. A P2X receptor subunit in embryonic chick brain has been cloned and characterized, which is highly homologous to the mammalian P2X4 receptor (human and rat) with approximately 75 % sequence identity [33].

In a study, the expression of the G protein-coupled $P2Y_1$ receptor during embryonic development of the chick was described [34]. During the first 10 days of embryonic development, the $P2Y_1$ receptor is expressed in a developmentally regulated manner in the limb buds, mesonephros, brain, somites and facial primordia (Fig. 5), suggesting that there may be a role for ATP and $P2Y_1$ receptors in the development of these systems.

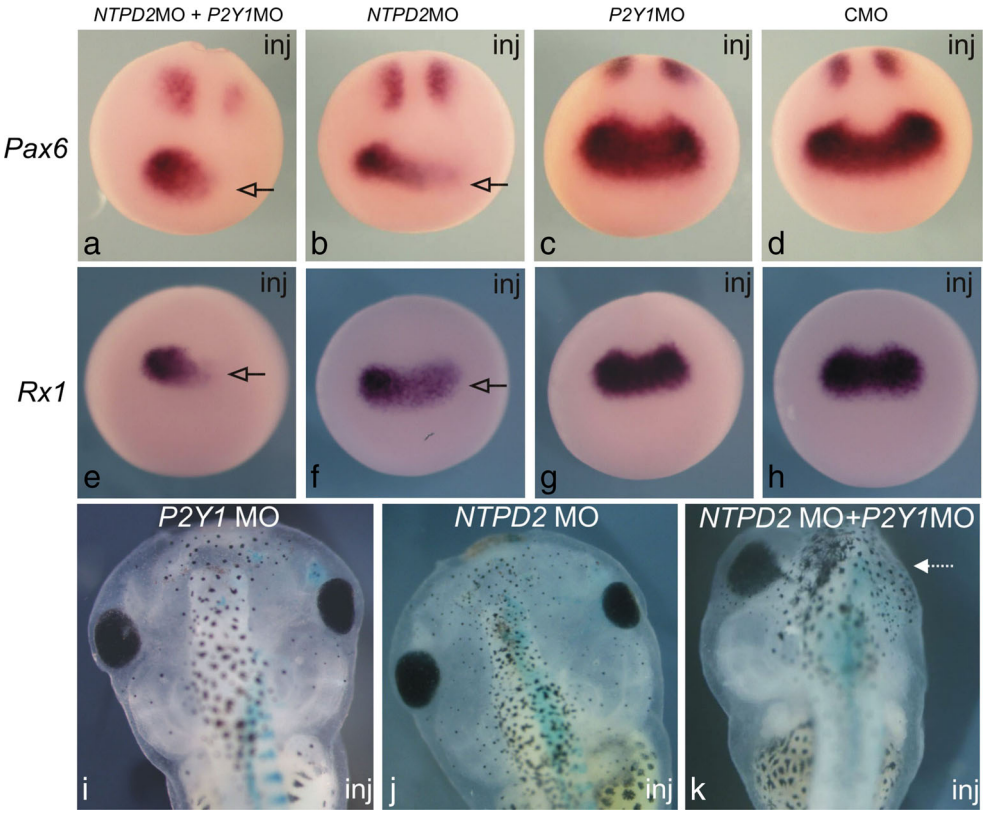

Fig. 2 Morpholino oligonucleotide knockdown of NTPDase2 (*NTPD2MO*) or the P2Y1 receptor (*P2Y1MO*) alters the expression of *Pax6* and *Rx1* (**a**–**h**). The morpholino constructs were injected on just one side of the early embryo to facilitate a comparison with the uninjected side as an internal control. *NTPD2MO* either on its own or in combination with *P2Y1MO* reduced *Pax6* and *Rx1* expression only on the injected side (*arrows*). However, injection of *P2Y1MO* on its own or a control morpholino (CMO) has no effect on expression of *Pax6* and *Rx1* (**i**–**k**). This reduced expression of *Pax6* and *Rx1* results in smaller eyes on the injected side (indicated by lacZ staining) or, in the case of the dual morpholino knockdown, no eyes (**k**, *arrow*) (reproduced with permission from [26])

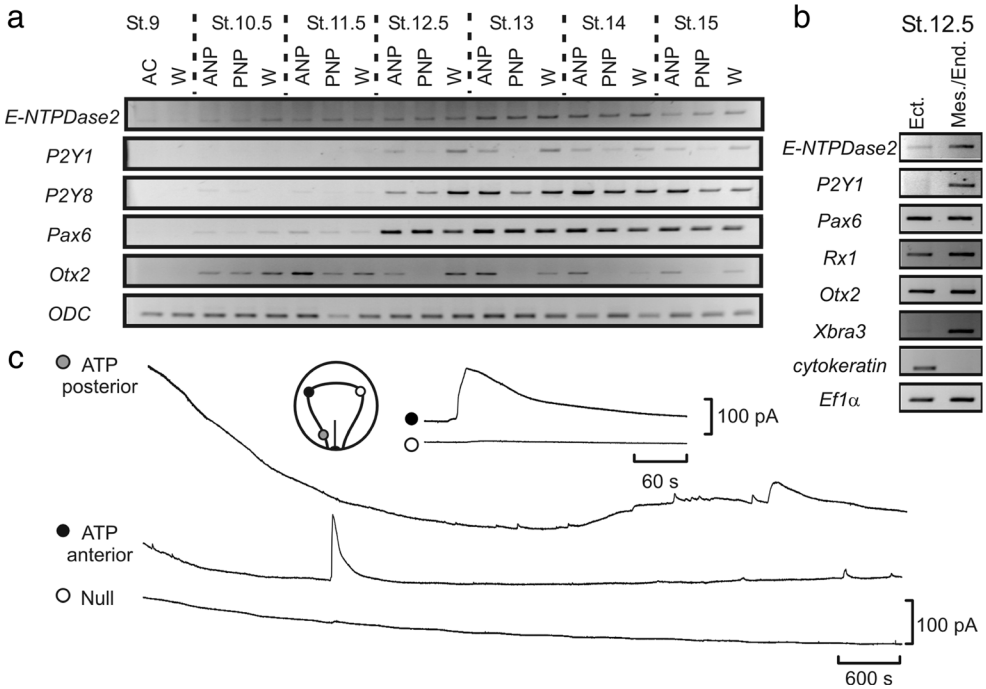

Fig. 3 The expression of the components of purinergic signalling and Pax6 and Otx2 during frog embryonic development. **a** *NTPDase2* expression precedes induction of *Pax6*, and expression of the P2Y1R is coincident with the upregulation of Pax6 that occurs at St 12.5. **b** Both NTPDase2 and P2Y1 are expressed more strongly in the mesoderm/endoderm, suggesting that it is this tissue layer that initiates expression of the EFTFs in the ectoderm. **c** ATP is released from the neural plate during development. The *inset diagram* indicates the positioning of ATP biosensors in the very early frog embryo. A large transient signal is seen in the anterior region—the site of the eye field. ATP signalling is also seen in the posterior regions. The ATP release event is blown up in the *inset traces* (reproduced with permission from [26])

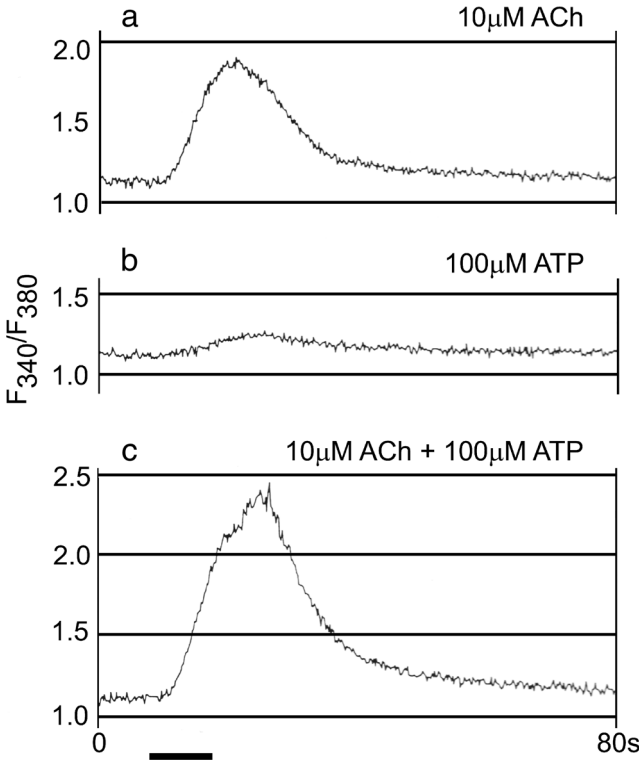

Fig. 4 Interaction between acetylcholine (ACh) and ATP recorded in an otocyst from chick embryo. **a** The response to 10 μM ACh. **b** The response to 100 μM ATP. **c** The response to the coapplication of 10 μM ACh and 100 μM ATP. The records in **a**–**c** were taken in this order at 5-min intervals. The bath solutions contained 25 mM Ca^{2+} (reproduced with permission from [30])

Adenosine has been implicated in growth regulation of the vascular system in the chick embryo [35], in common with a similar role claimed for experimental angiogenesis in the chorioallantoic membrane [36–38].

Mammalian embryos

Puff-applied ATP has been shown to have two main effects on a mouse mesodermal stem cell line: an increase in intracellular Ca^{2+} concentrations and a subsequent hyperpolarization due to Ca^{2+}-activated K^+ conductance [39] (Fig. 6). The author speculated that the transient increase in intracellular Ca^{2+} may influence mesodermal cell differentiation, particularly in relation to muscle differentiation. In a subsequent paper [40], two myoblastic cell lines, one from rat, the other from mouse, showed similar properties to those of the myogenic clonal cells derived from the mouse mesodermal stem cell line described above.

ATP and ADP have been shown to enhance, reduce or have no effect (depending on the dose used) on the incidence of trypan blue-induced teratogenic malformations in the rat foetus at day 20 [41]. Concomitant administration of ATP and cortisone in mice either decreases the teratogenic effect of cortisone (50 μg ATP) or enhances its teratogenic effect (>100 μg ATP) [42]. Mouse heads of embryos from 14 to 24 pairs of body somites exposed to an ATP-containing medium have been demonstrated to undergo rapid epithelial thickening and invagination, a process that appears to take

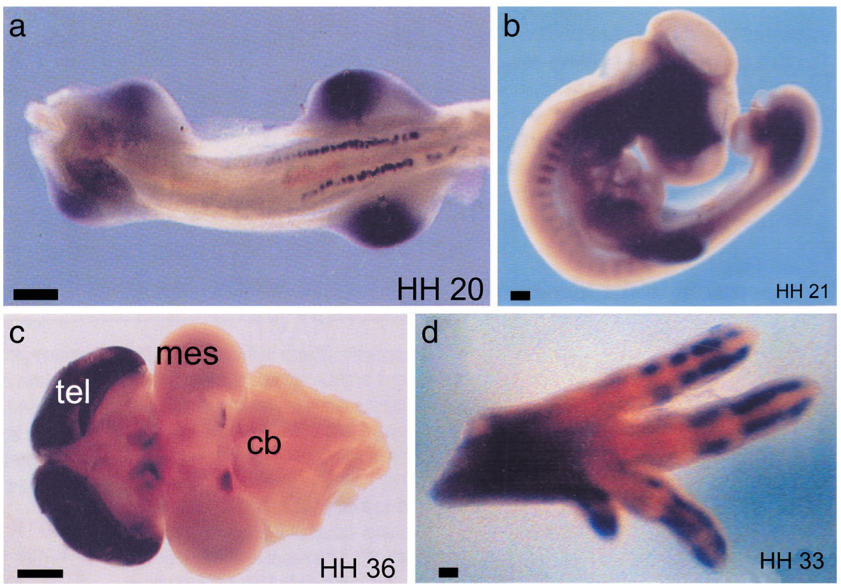

Fig. 5 Expression of $P2Y_1$ receptors during embryonic development of the chick as visualized by whole-mount in situ hybridization. Stages of development are shown in *bottom right corner*. **a** Ventral view of stage 20 embryo showing $P2Y_1$ expression in mesonephros and limb buds (*scale bar*=200 μm). **b** Lateral view of the chick somite at stage 21 showing $P2Y_1$ expression in the anterior region. The *dark area in the head region* is due to an artefact of photography (*scale bar*=200 μm). **c** Dorsal view of stage 36 brain (*anterior to the left*), showing increased levels of expression in telecephlon (*tel*), dorsal diencephlon and posterior midbrain. *mes* mesencephalon, *cb* cerebellum (*scale bar*=1 mm). **d** An anterior-uppermost view of a leg at embryonic stage 33. Expression of $P2Y_1$ is seen in the digits, but not in areas of joint formation. The same expression pattern is also seen in the wing (*scale bar*=100 μm) (reproduced with permission from [34])

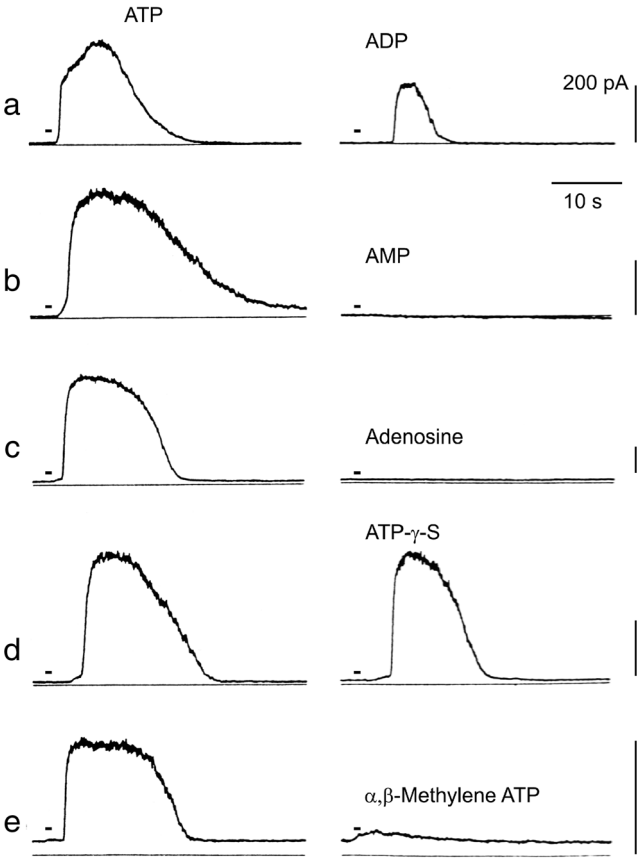

Fig. 6 K^+ responses to ATP analogues of a mouse mesodermal cell line. Each of the two traces was obtained from the same cell (**a**–**e**). The responses induced by ATP (*left traces*) and ATP analogues (*right traces*) are shown. The names of the analogues are shown near the traces. Each drug was applied at 20 μM, and the holding potentials were 0 mV (reproduced with permission from [39])

part in the shaping of nasal pits and formation of the primary palate [43]. The trophoblast appears at the blastocyst stage of embryogenesis and contributes to placentation. ATP increases $[Ca^{2+}]_i$ via a P2Y receptor in proliferating bovine trophoblast cells [44].

Besides ATP, a number of reports implicate adenosine as one of the endogenous effectors that can selectively modulate cell growth during embryonic development. For example, adenosine is shown to potentiate the delaying effect of dibutyryl cAMP (a membrane-permeable analogue of cAMP) on meiosis resumption in denuded mouse oocytes [45]. The role of adenosine has been particularly well characterized in the morphogenetic outgrowth of vertebrate limb buds [46]. Embryonic limb development in the mouse is driven by rapid mesenchymal cell proliferation induced by trophic substances secreted by the apical ectodermal ridge. This interaction can be restricted experimentally by pharmacological agents that elevate intracellular cAMP levels or physiologically by the onset of programmed cell death triggered by naturally occurring negative regulators of growth. Mutations that affect the pattern of limb/bud outgrowth provide invaluable experimental means to investigate these growth-regulatory processes. Knudsen and Elmer [46] studied the regulation of polydactylous outgrowth (an expression of the Hemimelia-extra toe ($Hm^{X/+}$) mutant phenotype) in hindlimb bud explants. Polydactylous growth is enhanced by exogenous adenosine deaminase, the enzyme which catalyzes the inactivation of endogenous adenosine. This effect was reversed by coexposure to hydrolysis-resistant adenosine analogues. Since the P1 receptor antagonist, caffeine, could completely prevent suppression of polydactylous outgrowth, the effects of an adenosine analogue were most likely mediated by activation of specific extracellular receptors.

Micromolar concentrations of adenosine, inosine and hypoxanthine, but not guanosine, block the second or third cleavage of mouse embryos developing in vitro [47]. Zygotes or early two-cell embryos, cultured in a purine-containing medium for 24 h, resume development following transfer to purine-free conditions. The precise mechanism of the purine-sensitive process is not known, but embryos conceived in vivo are sensitive until approximately 28–30 h after fertilization and are no longer sensitive by 34 h [48]. However, a later study by this group has shown that the purine-induced block can be reversed by compounds that elevate cAMP [49]. In mouse embryonic development, adenosine deaminase increased 74-fold between days 7 and 9; deoxyadenosine kinase increased 5.4-fold during the same period; adenosine kinase, deoxyguanosine kinase and purine nucleoside phosphorylase exhibited less than 2-fold changes in activity between days 7 and 13 [50]. The authors concluded that while phosphorylation of adenosine was the principal route of metabolism up to day 9, after which, there is a switch to deamination. The possible role of ecto-adenosine deaminase in the development of the nervous system and the neurological abnormalities that occur in adenosine deaminase-deficient patients is discussed in a review by Franco et al. [51].

Taken together, these results point to a role for purines in both physiological fertilization and normal development and also underline that alterations of the purinergic regulation of embryonal growth might be involved in the onset of morphological malformations. Depending upon the purine derivative, and probably upon the purinoceptor involved as well, ATP and adenosine can act as both positive and negative regulators of growth. This is also consistent with data obtained from in vitro cell lines which implicates purines in both cell proliferation and apoptosis. Further studies are needed to better characterize the receptor subtypes involved and also to identify more precisely the developmental events specifically controlled by purines.

Human embryos

A few studies have been made of receptors to purines and pyrimidines in human embryos. Human embryonic kidney

cells (HEK293) endogenously express $P2Y_1$ and $P2Y_2$ receptors [52]. These embryonic cells have also been shown to express an endogenous A_{2B} receptor [53]. ATP and adenosine-5′-*O*-3-thiotriphosphate were shown to stimulate DNA synthesis in human foetal astrocyte cultures [54]. In addition, ATP stimulated a mitogen-activated protein kinase (MAPK) termed extracellular signal-regulated protein (ERK), a key component of signal transduction pathways involved in cellular proliferation and differentiation. The activation of MAPK was mediated, at least in part, by P2 receptors since suramin produced 50 % block.

There has been a study of plasma ATP levels in the foetus at the time of obstetrical delivery, samples being collected immediately after clamping of the cord [55], and the results showed that the plasma ATP was significantly higher in arterial compared to venous or maternal venous blood. It was suggested, therefore, that the ATP in arterial blood was of foetal origin and that the levels decrease in response to stress during vaginal delivery and correlate with the oxygen supplied from the placenta.

In a study of human fibroblasts, differential sensitivity to adenosine was demonstrated in donors of different ages [56]. Foetal fibroblasts were the most sensitive to adenosine, which produced inhibition of growth and RNA synthesis; in contrast, fibroblasts taken from 4-year-old donors showed growth stimulation to adenosine. Activation of A_2 receptors by adenosine stimulates L-arginine transport and NO synthase in human foetal endothelial cells [57]. Adenosine selectively inhibits tumour necrosis factor-α production in the human newborn [58]. Expression of purinergic P2X4, P2X7 and $P2Y_2$ receptors and their activation led to increase in $[Ca^{2+}]_i$, which is involved in the changing demand for calcium with increased foetal growth over gestation in the first trimester and term human placenta [59].

Overall, our survey of purinergic signalling during early development in a range of species provides substantial evidence favouring its involvement in a variety of developmental processes. The current state of the literature therefore suggests that, in future, more precise molecular genetic manipulations of specific components of purinergic signalling to determine their roles in the regulation of development would be well worthwhile.

Development in different systems

Cardiovascular system

Heart

Studies of the development of pharmacological sensitivity to adenosine analogues in embryonic chick heart [60, 61] show that pharmacological sensitivity to A_1 receptor agonists begins at embryonic day 7 and then increases continuously to day 12 when the atria became fully responsive. Ligand binding shows that A_1 receptors are present at days 5 and 6, but are not responsive to adenosine, and the author concluded that the development of sensitivity to A_1 receptor-mediated negative chronotropic responses was not paralleled by developmental changes in adenosine inhibition of adenylyl cyclase or by the development of sympathetic and parasympathetic innervation. Chronic exposure of the embryonic chick heart (15–17 days old) to R-N^6-2-phenylisopropyladenosine (R-PIA) produces down-regulation of A_1 receptors and desensitization of the negative inotropic response to adenosine [62]. A study of ventricular cells cultured from chick embryos 14 days *in ovo* showed that a functional A_{2A} receptor is expressed and mediates augmentation of myocyte contractility [63]. The A_{2A} receptor coexists with an A_{2B} receptor, although it has 50-fold higher affinity, and the authors suggest that high affinity A_{2A} receptors play an important modulatory role in the presence of low levels of adenosine, while the low affinity A_{2B} receptor becomes functionally important when the adenosine level is high.

In foetal sheep, centrally administered adenosine influences cardiac function [64]. The ontogeny of A_1 receptors was studied in rats with binding assays (using [^{3}H]1,3-dipropyl-8-cyclopentylxanthine (DPCPX)), an A_1 antagonist, and by in situ hybridization of messenger RNA (mRNA) [65]. In a later study of mouse embryo cardiac function [66], adenosine, via A_1 receptors, was shown to potently regulate heart rate via multiple effector systems at very early stages of prenatal development (9–12 days postconception). At gestational days 8–11, mRNA expression for A_1 receptors was detected in the atrium (one of the earliest G protein-coupled receptor genes to be expressed in the heart), but not in other foetal structures, while at gestational day 14, A_1 mRNA was present in the CNS (thalamus, ventral horn of spinal cord) as well as the atrium; by gestational age 17, patterns of A_1 receptor expression in the brain were similar to those observed in adults [67, 68]. Determination of A_1 receptor density in developing rat heart using [^{3}H]DPCPX showed that functional A_1 receptors are present in greater numbers in the immature perinatal heart than in the adult heart [69].

Intravenous infusion of adenosine analogues into foetal lambs produced dose-dependent bradycardia and hypotension [70–72]. In contrast, in the newborn, 5′-*N*-ethylcarboxamidoadenosine (NECA) produced dose-dependent tachycardia, while R-PIA and cyclohexyladenosine produced dose-dependent bradycardia. Although adenosine causes cardiovascular changes in pregnant ewes, the effects are well tolerated and do not significantly affect the cardiorespiratory status of the foetus [73].

P2 receptors are expressed in human foetal heart [74]. P2X1, P2X3 and P2X4 receptor subtypes as well as $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors were present. A subunit of the

P2X receptor family was isolated from cardiomyocytes and brain from 14-day-old chick embryos that had 75 % identity with the rat and human P2X4 receptor [33].

The postnatal development of A_1 receptors in the heart has been studied extensively (see [7]). In preinnervated immature rat myocytes, A_1 receptors are present in greater numbers compared to the adult and they are functionally coupled at their effector sites [69].

Adenosine formation and release by neonatal rat ventricular myocytes in culture have been described [75, 76]. Adenosine and hypoxia effects on the atrioventricular node of neonatal rabbit hearts have been reported [77]. The adenosine agonist NECA increases cardiac output in developing *Xenopus* tadpoles through a combination of increased filling and accelerated growth of heart and vessels [78]. Adenosine was claimed not to be as effective as a vasodilator of internal carotid arteries in the newborn pig as it is in the adult. In foetal sheep, centrally administered adenosine influences cardiac function. Expression of A_1 and A_{2A} receptor genes in rat peripheral arterial chemoreceptors is differential during postnatal development [79, 80].

Expression of P2Y receptor subtypes in myocytes changes from neonate rat heart to the adult [81]. Expression of $P2Y_1$ receptors is higher in comparison to $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors in the neonatal myocyte, while $P2Y_4$ receptors were not detected in adult myocytes. In neonatal heart fibroblasts, $P2Y_1$ and $P2Y_6$ receptors were expressed at higher levels than $P2Y_2$ and $P2Y_4$ receptors. RT-PCR studies of the foetal human heart showed that P2X1, P2X3 and P2X4 subtypes were expressed together with $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors [74]. ATP and α,β-methylene ATP (α,β-meATP) intravenous injection increased heart rate via P2X receptors in rats aged 21, 56 and 100 days, with the most potent effect in 21-day-old animals [82]. It was claimed that P2Y receptor-mediated changes were more prominent in the later developmental stages [83]. ATP increased expression of the immediate-early genes *c-fos* and jun B in cultured neonatal cardiac myocytes by a different pathway from that produced by NA [84]. UTP caused hypertrophic growth in neonatal rat cardiomyocytes, while prolonged exposure to ATP had hypertrophic growth inhibitory effects [85]. $P2Y_4$ receptors were shown to be involved in myocardial contractile activity in rats during postnatal development [86].

Blood vessels

Adenosine was claimed not to be as effective as a vasodilator of internal carotid arteries in the newborn as it was in the adult pig [87]. It was suggested that neonatal brain adenosine may play a role in regulating blood flow during hypoxia [88]. Adenosine appears to play an important role in the regulation of coronary blood flow in the newborn lamb [89]. Adenosine-induced vasodilatation of the guinea pig coronary vascular bed is greater in immature guinea pig heart (5 days) compared to mature heart (1–2 months) [90]. Responses of isolated mesenteric arteries of the beagle to both β-adrenoceptors and P1 receptors are less in old compared to young animals [91].

In the mesenteric artery of the rat, the sympathetic and sensory nerve fibre plexuses develop over the first three postnatal weeks, but functionally mature nerve-mediated contractile responses cannot be elicited before 14 days postnatal [92], correlating with the appearance of adult-like excitatory junction potentials (EJPs) [93]. Prior to this period, intracellular recordings from animals aged 4 to 9 days old showed slow depolarizing potentials which were mediated by α-adrenoceptors. From day 9 onwards, EJPs, which were resistant to α-adrenergic antagonists, were recorded [93] and are likely to be mediated by ATP (see [94]). Following denervation studies in the rat mesenteric vascular bed, electrical responses similar to those seen during the early stages of development were recorded [95], suggesting that a similar sequence of events occurs during regeneration as takes place during development. P2X receptor subtype mRNA expression in rat mesenteric artery showed high expressions for P2X1 and P2X4 at postnatal day 7, which remained during development until day 360 [96, 97]. A reduction in spontaneous and α_1-adrenoceptor-induced release of ATP from endothelial cells of the rat tail artery occurs with advancing age [98]. The endothelium-dependent relaxant response of the aorta to ATP, mediated by NO, was greatest in 4-week-old rats but declined progressively at 45 and 105 weeks [99].

Oxygen-induced pulmonary vasodilatation is mediated by ATP in newborn lambs; it is attenuated by combined administration of P1 and P2Y receptor antagonists [100]. However, it appears to differ from the mechanism of oxygen-induced pulmonary vasodilatation in the late gestational foetal lamb [101–103]. Infusion of ATP caused a significant decrease in pulmonary vascular resistance in normoxic and hypoxic newborn lambs [104]. Adenosine is also a pulmonary vasodilator in newborn lambs [71].

Skeletal muscle

ATP actions on patched membranes of myoblasts and myotubes cultured from 12-day-old chicken embryos were first demonstrated by Kolb and Wakelam [105]. Using biochemical methods, ATP-induced cation influx was later demonstrated in myotubes prepared from 11-day-old chick embryos and shown to be additive to cholinergic agonist action [106]. Later papers from this group claimed that the myotube P2 receptor triggers phosphoinositide turnover [31, 107] and alters Ca^{2+} influx through dihydropyridine-sensitive channels [108]. ATP has a potent depolarizing action on myotubes derived from pectoral muscle cultured from 11-day chick embryos [109], and its physiological and pharmacological properties have been described in a series of papers [110–114]. The

myotube P2 receptor is not activated by ADP, AMP, adenosine or the non-hydrolyzable ATP analogues α,β-meATP or β,γ-methylene ATP [109]. A single class of ATP-activated ion channel conducts both cations and anions in the myotube [111], and the P2 receptors involved showed marked desensitization [112]. The sensitivity of extracellular ATP has been tested at various stages of development of different muscles [115]. At embryonic day 6, ATP (50–100 μM) elicited vigorous contractions in all the muscles tested, but by embryonic day 17, none of the muscles contracted in response to ATP. The distribution of 5′-nucleotidase during the development of chick striated muscle showed that there is a more restricted distribution in the adult compared to the embryo [116]. A mammalian P2X1 receptor orthologue was identified in embryonic chicken skeletal muscle, perhaps forming heteromultimers with P2X4 and P2X5 receptor subunits [117]. Both P2X5 and P2X6 receptors were present in developing chick skeletal muscles [34, 118].

Purinoceptors were present in mouse C2C12 myotubes [119–123]. P1 receptors activating cAMP were identified as well as $P2Y_2$ receptors, sensitive to ATP and UTP. Occupation of the receptor by ATP or UTP led to formation of IP_3 and release of Ca^{2+} from internal stores as well as from the extracellular space. The responses to ATP of myotubes prepared from E18 mouse embryos from normal and mutant mdg/mdg mice with muscular dysgenesis were studied by Tassin et al. [124]. Using Fura-2 as a probe, they showed that many of the mdg/mdg myotube preparations showed little or no increase in cytoplasmic Ca^{2+} levels. The presence of functional P2X receptors in freshly isolated skeletal muscle cells from prenatal mice has been reported [125]. A punctate staining pattern for P2X7-like receptors was present at postnatal day 1 in mouse motor nerve terminals [126]. It was concluded that P2X7 receptors are expressed by both myelinating Schwann cells and motor neuron terminals, so an autoregulatory role for ATP released by nerve terminals during synaptic transmission was suggested.

P2 receptor expression and responses to ATP change during development of skeletal muscle [34, 115, 127, 128]. P2X5, P2X6 and P2X2 receptors were expressed sequentially with P2X5 and P2X6 receptors associated with the development of the myotube, while P2X2 and $P2Y_1$ receptors appeared to be involved in the formation of the skeletal neuromuscular junction [128–131]. The possibility that extracellular ATP, coreleased with ACh, may serve as a trophic factor during the development of the *Xenopus* neuromuscular junction has been considered [20, 25]. $P2Y_{13}$ receptors regulate phosphate metabolism and fibroblast growth factor-23 secretion during skeletal bone development [132].

Urinary bladder

Expression of P2X1 receptor transcripts was much lower in foetal human bladder than in adult bladder; P2X4 and P2X7 receptors were also expressed in the foetus [133]. The P2 receptor expression shifted from the dome to the body of the bladder with increasing gestation. Obstruction of the foetal male sheep bladder resulted in enlarged, hypocontractile and compliant bladder [134]. However, there was no conclusive evidence for changes in purinergic (or in cholinergic or nitrergic) neurotransmission.

Responses of the rabbit urinary bladder to both parasympathetic cotransmitters ACh and ATP were recognized in newborn animals, but adrenoceptors were poorly expressed until a later stage (see [135]). Newborn bladders generated much greater tension in response to ATP than adult tissue and then declined, while the response to cholinergic agonists did not decline. Rat urinary bladder responses to adenosine (inhibitory) and ATP (excitatory) mediated by P1 and P2X receptors, respectively, occurred at P2, the earliest day studied. In the neonate, adenosine was more potent than in the adult, while ATP potency initially increased with age but then declined; it was highest between P10 and P25. The main pathway for nerve activation of the urinary bladder of newborn mice was cholinergic, with only a small contribution of the purinergic component; this was in contrast to adult bladder, which was equally dependant on cholinergic and purinergic components [136]. These differences were due to changes in ATP release, rather than to changes in receptor function.

In neonates and adults, the rate and pattern of breakdown of ATP and adenosine by ectoenzymes in the rat urinary bladder were identical. This suggested that the marked differences in potency to ATP and adenosine during development were likely to be due to changes in receptor number and/or agonist affinity or efficacy. Small clusters of P2X receptors (about 0.4 μm in diameter) were present on smooth muscle cells at day P1 in rat urinary bladder, although few varicose nerve fibres were present at that time [137]. At P4, many varicose fibres were present and some small clusters of P2X receptors were seen in association with varicosities. Many of the P2X receptor clusters were found adjacent to varicosities of parasympathetic nerve fibres by P21. Increased purinoceptor-mediated contractions were elicited in newborn rat detrusor smooth muscle, which reached adult levels 1 month after birth [138].

Contractile responses of the rat bladder to ATP, released as a cotransmitter from parasympathetic nerves, increased with age [139].

Nervous system

Central nervous system

The emphasis in earlier studies of purines in the CNS was largely about adenosine [7, 140, 141], but since purinergic neurotransmission involving ATP as a transmitter was clearly demonstrated in the medial habenula of the rat brain [142],

many more papers have been appearing concerning P2 receptors in the brain and spinal cord (see [8, 143, 144]).

Adenosine receptors appeared early and reached higher adult levels in the brains (most notably in the cerebellum) of mice pups chronically exposed in utero to caffeine [145]. A study of the distribution of adenosine-binding sites in the cat visual cortex showed changes in the laminar binding pattern during postnatal development [146]. Adenosine receptors were detected in the rat forebrain in young neonates, preceding N-protein coupling [147]. In a study of the developing guinea pig brain, A_1 receptors were present from E19 and it was claimed that in cerebellum, but not in forebrain, postnatal coupling of adenosine A_1 receptors to associated G proteins is much more extensive than in the prenatal period [148].The developmental properties of adenosine A_{2A} receptors differ from those of A_1 receptors during postnatal development of rat striatum. A_{2A} receptor binding sites were low at birth (about 3 % of adult levels) and then increased mostly between birth and 5 days and then again from 15 days to adulthood [149]. In contrast, expression of A_1 receptor mRNA in the brain was first described at gestation day 14. It was restricted to portions of neuroepithelium caudate putamen, piriform cortex, hypoglossal nucleus and ventral horn of spinal cord. By gestational age 17, A_1 receptor expression pattern in the brain was similar to those observed in adults. The development of adenosine uptake sites in the guinea pig brain has been described. A_1 receptors are widely distributed at birth (about 10 % of adult levels) and then increase gradually until adulthood, with a peak during the second week of postnatal life [150–152]. The ratio of adenosine (A_{2A}) receptors to dopamine D_2 receptors in the rat striatum increases with age, involving both presynaptic and postsynaptic mechanisms [153]. A decrease in striatal A_2 receptor mRNA expression has been demonstrated with in situ hybridization histochemistry in rat striatum between 3 and 24 months, but it was suggested that this may be related to neuronal loss over the same period [154]. Postnatal changes in expression of A_{2A} receptors have been described in various brain regions [155]. It was suggested that postnatal changes in adenosine receptors may explain age-dependent differences in stimulatory caffeine effects and protection against seizures throughout development. Further, since A_{2A} receptors show a codistribution with D_2 receptors throughout development, they also speculate that caffeine may partly exert its actions via dopamine receptors. Wide distribution of $P2Y_1$ receptors was reported in the 1-day-old chick brain [156]. Age-dependent changes in presynaptic neuromodulation via A_1 receptors have been described in hippocampus of mouse [157] and rat [158, 159]. There is a down-regulation and reduced responsiveness to presynaptic A_1 receptors modulating ACh release during postnatal development and ageing. A_1 receptor down-regulation occurred in foetal brain after caffeine or theophylline treatment of pregnant rats [160]. Down-regulation and reduced responsiveness to presynaptic A_1 receptors modulating ACh release in the hippocampus during postnatal development and ageing were reported [161].

Caffeine decreased the incidence of neonatal respiratory disturbances, perhaps reflecting the early dominance of the adenosinergic system in the brainstem [162]. The developmental properties of adenosine A_{2A} receptors differed from those of A_1 receptors during postnatal development of rat striatum. A_{2A} receptor binding sites were low at birth (about 3 % of adult levels) and increased mostly between birth and 5 days and then again from 15 days to adulthood. The ratio of adenosine A_{2A} receptors to dopamine D_2 receptors in the rat striatum increased with age, at both presynaptic and postsynaptic sites. A_1 and A_{2A} receptors played a major role during the development of the rat uncrossed retinotectal pathways [163].

Rhythmic movements were regulated by purinergic transmitters in frog embryos [164]. ATP was released during swimming that activated P2Y receptors to cause an increase in the excitability of the spinal motor circuits by inhibiting voltage-gated K^+ currents via a $P2Y_1$ receptor [164, 165]. Adenosine, resulting from the breakdown of ATP, acted on P1 receptors to reduce the voltage-gated Ca^{2+} currents lowering excitability of the motor circuits to oppose the actions of ATP [166]. It was suggested that a gradually changing balance between ATP and adenosine underlies the rundown of the motor pattern for swimming in *Xenopus*. In an earlier study, Dale [167] presented evidence to suggest that delayed production of adenosine underlies temporal modulation of swimming in the frog embryo and is likely to result from feed-forward inhibition of the 5′-ectonucleotidase in the spinal cord. A homologue of apyrase was identified in *Xenopus* during early development [168]. Subsequent comprehensive studies have shown that the NTPDase, NPPase gene families and CD73 are expressed at all stages of development in *Xenopus* [26, 169, 170].

ATP [171] and adenosine [172] modulate the activity of inspiratory neurons in the brain stem of neonatal rats (see [7]). The activity of neurons in the rostral ventrolateral medulla and the respiratory motor output were depressed by adenosine, with a more pronounced decrease in respiratory activity in younger animals. During the first 2 weeks of postnatal development, ATP excitation of glutamate inspiratory drive to mouse hypoglossal neurons remained constant. A secondary inhibitory response occurred, mediated by adenosine acting via A_1 receptors after enzymatic breakdown of extracellular ATP [173]. Adenosine, acting on A_1 receptors after breakdown of ATP, evoked a secondary inhibitory response. It has been claimed that adenosine plays a central role in modulating ventilation in the newborn piglet and is involved in the diphasic ventilatory responses to hypoxia. R-PIA, an adenosine receptor agonist, caused a decrease in ventilation which was blocked by theophylline indicating mediation by P1 receptors [174]. R-PIA caused a considerably more pronounced effect

in 1- to 3-day-old animals than in 8-day-old animals and was shown to bind with higher affinity in the brains from newborn animals compared to older animals. The authors suggested that this might explain the potent therapeutic effect of the adenosine antagonist, theophylline, on recurrent apnea in preterm infants. In studies of anesthetized newborn piglets, it was concluded that adenosine contributes to ventilatory depression caused by hypoxia [175, 176]. In another investigation of the role of adenosine in the hypoxic ventilatory response of the newborn piglet, the authors concluded that adenosine plays a central role in modulating ventilation in the newborn piglet and is involved in the biphasic ventilatory responses to hypoxia [177]. It has been claimed that the inspiratory output of XII motor neurons that control the airways in mammals is potentiated by $P2Y_1$ receptors in neonatal rat in vitro [178].

Responses of sympathetic preganglionic neurons in a neonatal rat brain stem–spinal cord preparation were evoked by ATP and adenosine. In rat locus coeruleus neurons, P2 receptors first appear to be functional soon after birth, increasing thereafter to reach maturity in animals older than 18 days [179]. α,β-MeATP was strongly active on glycinergic presynaptic nerve terminals projecting to rat substantia gelatinosa neurons at P28–30, perhaps contributing to the fine control of the pain signal in spinal cord dorsal horn neurons [180]. Excitatory synapses, mediated by both glutamate and ATP in rat superficial dorsal horn, were functional from the first postnatal days [181]. P2X3 receptors on motoneurons of the nucleus ambiguus were down-regulated during the first two postnatal weeks, indicating their involvement in the control of oesophageal motor activity in early development [182].

Extracellular ATP facilitated the release of dopamine via P2 receptor activation in parts of the mesolimbic system, including organotypic slice cocultures of the ventral tegmentum, substantia nigra and prefrontal cortex [183]. These authors showed a time-, region- and cell type-dependent in vitro and in vivo expression pattern of different P2 receptor types in the dopaminergic systems, suggesting an important role of purinergic signalling in development and growth. Both ATP and adenosine have been shown to modulate the activity of inspiratory neurons in the brain stem of neonatal rats (see [7]). Adenosine depressed both the activity of neurons in the rostral division of the ventrolateral medulla and the respiratory motor output, with a more pronounced decrease in respiratory activity in younger animals. ATP excitation of glutamate inspiratory drive to mouse hypoglossal neurons remained constant during the first 2 weeks of postnatal development. A secondary inhibitory response was due to adenosine acting on A_1 receptors after breakdown of ATP. ATP and adenosine mediate responses of sympathetic preganglionic neurons in a neonatal rat brain stem–spinal cord preparation. Activation of $P2Y_1$ receptors in the glomerulus stimulated neuronal network activity in the developing olfactory bulb [184].

Intense immunolabelling of P2X3 receptors in the postnatal (P7 and P14), but not adult, rat brain was reported [185]. Staining was restricted to the hindbrain, in particular, the mesencephalic trigeminal nucleus, the superior and inferior olive, the intermediate reticular zone, the spinal trigeminal tract and the prepositus hypoglossal nucleus at E16. P2X7 receptor mRNA was detected in the E19 rat by in situ hybridization in brain ependymal, but not neurons [186]. Human foetal astrocytes expressed low levels of P2X7 receptor mRNA and protein in primary cultures [187]. P2X7 receptors mediated caspase-8/9/3-dependent apoptosis in developing rat primary cortical neurons [188].

Green fluorescent protein-tagged P2X2 receptor studies of embryonic hippocampal neurons led to the claim that ATP application can lead to changes in dendritic morphology and receptor distribution [189]. P2X2 receptors were identified on Purkinje neurons in the neonatal cerebellum, mRNAs for P2X1-4 and P2X6 subunits were identified in the cerebellum during the first postnatal week, and coexpression of two units in Purkinje cells demonstrated with patch clamping [190].

Purinergic signalling in precursor cells, neuroglial progenitors and differentiating neurons during neurogenesis of embryonic rat neocortex was reported [191]. Neuroglial progenitors from the ventricular and subventricular zones showed increase in $[Ca^{2+}]_i$ in response to ATP. A detailed study of the expression pattern for P2X3 receptors in embryonic neurogenesis was published [192]. P2X3 receptors first appeared in the hindbrain neural tube and sensory ganglia at E11–11.5. At E14.5, P2X3 receptors appeared in the optic tract, nucleus tractus solitarius and mesencephalic trigeminal nucleus. However, P2X3 immunoreactivity was down-regulated in early postnatal brain stem.

P2X receptor expression changed during postnatal development of the rat cerebellum [193]. All P2X receptor subtypes were expressed (except P2X3) on Purkinje cells and deep cerebellar nuclei at P3. At P7, there was upregulation of P2X5 and P2X6 receptors, and microglial cells expressed P2X1 and P2X7 receptor immunoreactivity. Dendritic trees of Purkinje cells were intensely labelled for P2X1-7 receptors (except for P2X3) at P14. P2X4 receptors were expressed on microglia and P2X5 receptors on granular cells. At P21 and P66, the P2X receptors were down-regulated on Purkinje cells and deep cerebellar nuclei, but P2X5 receptors on granular cells were upregulated. Endogenous release of ATP started to enhance synaptic activity in rat Purkinje neurons by the end of the second postnatal week [194].

The sequential expression of P2X receptor subtypes during embryonic rat brain development revealed that P2X3 receptors appeared first at E11, P2X2 and P2X7 receptors at E14, while P2X4, P2X5 and P2X6 receptors did not appear until birth and P2X1 receptors even later [195]. ATP inhibited motor axon outgrowth during early embryonic neurogenesis,

probably via the P2X3 receptor, and it was suggested that P2X7 receptors were involved in programmed cell death during embryogenesis. At E9.5, P2X3 receptors were shown to be present in the hindbrain, midbrain, diencephalon and forebrain neuroectoderm of mouse brain and, at E10.5, in the marginal layer of diencephalon, midbrain and hindbrain [196]. Spontaneous and evoked postsynaptic currents in embryonic chick hypothalamus appeared to arise from the concurrent activation of both γ-aminobutyric acid (GABA) and P2X receptors [197].

ATP acting via P2X and P2Y receptors contributed to modulate network-driven giant depolarizing potentials in the rat hippocampus during early stages of postnatal development [198]. ATP, via both P2X and P2Y receptors, appeared to shape hippocampal connectivity during postnatal development [199]. Activation of $P2Y_1$ receptors increased the frequency of $GABA_A$-mediated spontaneous postsynaptic current in CA3 principal neurons in early postnatal (P1–P6) rat hippocampus [200]. In vitro studies of sensorimotor cortical neurons from 14-day-old and 30-day-old rats have shown that Ca^{2+} release could be evoked by ATP indicating the presence of P2Y receptors [201]. Almost all P14 neurons appeared to possess such receptors, whereas only about one third of neurons from P30 rats responded to ATP, suggesting that substantial changes in signalling mechanisms occur in neocortical neurons in the third to fourth week of postnatal development.

Changes in the distribution of the ectoenzymes involved in the breakdown of ATP and adenosine in the brain during foetal and neonatal development have been reported [7, 202]. Ecto-5′-nucleotidase was redistributed during development of the cat visual cortex. Ecto-5′-nucleotidase levels were low at 30 and 35 days of gestation of foetal guinea pigs but increased rapidly during the 40- to 60-day period. In contrast, adenosine deaminase was present at 30 days of gestation and was maintained at the same level until 60 days. Adenosine deaminase-staining neurons were observed in the olfactory cortex of rat embryos as early as E15. Ca^{2+}–ATPase in the rat spinal cord during embryonic development was intensely active in the roof and floor plates, but not in the basal and lateral plates at E12, suggesting a role for Ca^{2+}–ATPase in early differentiation of neuroepithelial cells. ATP induced a rise in $[Ca^{2+}]_i$ in embryonic spinal cord astrocytes. P1 and P2 receptor involvement in the proliferation of human foetal cortical astrocytes was reported. Postnatal development of ATPase, ADPase and ATP diphosphohydrolase activity in the cerebral cortex has also been studied [203, 204]. The activities increased steadily from birth, reaching maximum values at 21 days of age. A marked increase in activity of ecto-5′-nucleotidase was also seen in rat olfactory bulb during neonatal development [205]. During early postnatal life, the enzyme was found within synapses in the brain, but a glial pattern of expression dominated in the adult, with the exception of the olfactory bulb where both glial and synaptic staining was present in mature adults. Ecto-NTPDase2 was expressed transiently at E18 in astrocytic cells in the outer molecular layer of the dentate gyrus and in cerebellar white matter [206]. Postnatal development of ecto-nucleotidase activity in the cerebral cortex increased steadily from birth, reaching maximum values at 21 days of age. Increased activity of ecto-5′-nucleotidase was also seen in rat olfactory bulb during neonatal development. Ecto-5′-nucleotidase was present first in immature Purkinje cells at birth and increased throughout postnatal development. In contrast, alkaline phosphatase activity did not appear on the granular neurons in the developing cerebellum until day 7 postnatal. Ecto-ATPase, ecto-ADPase and ecto-5′-nucleotidase activities were shown to change in relation to age in synaptosomes of rat spinal cord [207]. In synaptic plasma membranes isolated from rat cerebral cortex, NTPDase1 activity increased from birth to day 30, after which it declined [208]. In general, ATP and ADP hydrolysis decreased in older animals.

It is known that neonatal hypothyroidism leads to abnormal development of the CNS. Hypothyroidism changes adenine nucleotide hydrolysis by ecto-5′-nucleotidase activities in synaptosomes from hippocampus and cerebral cortex in rats in different phases of postnatal development [209]. Neonatal hypothyroidism enhances the metabolism of adenine nucleotides in astrocyte cultures from rat brain [210]. Interactions between thyroid hormones and adenosine receptor-mediated responses during brain development are discussed in a review by Ahmed et al. [211]. Leukaemia inhibitory factor is required for normal development of hippocampal astrocytes, a process that is regulated by spontaneous release of ATP from neurons [212].

Peripheral nervous system

The neural ectoderm folds to form the neural tube during early embryological development. Cells in the neural crest migrate into the mesoderm and then differentiate and mature to become glial cells and neurons. Some form primary afferent neurons of the dorsal root ganglia (DRG), while others form postganglionic neurons of the sympathetic and parasympathetic ganglia. Other cells form the enteric nervous system and adrenomedullary chromaffin cells. The sensory neurons of nodose, petrosal and trigeminal ganglia are derived partly or entirely from the neural placodes. Adenosine inhibited neurite outgrowth of chick sympathetic neurons taken from 11-day chick embryos and killed by apoptosis about 80 % of sympathetic nerves supported by growth factor over the next 2 days in culture [213]. Specific A_1 or A_2 agonists were not neurotoxic. The toxic effects of adenosine were not antagonized by aminophylline but were prevented by an adenosine transporter or adenosine deaminase inhibitor, suggesting an intracellular site of action for the toxic effects of adenosine. It was concluded that adenosine and its breakdown enzymes

play an important role in the regulation of growth and development of sympathetic neurons. In follow-up experiments, the authors suggested that adenosine induced apoptosis by inhibiting mRNA and protein synthesis [214].

Responses to ATP were described in ciliary neurons acutely dissociated from embryonic chick ciliary ganglia taken at day 14 [215]. The relative potency of agonists in producing transient inward currents with patch recording is ATP > 2-methylthioATP > ADP; neither adenosine, AMP nor α,β-meATP is effective, but suramin is an antagonist. The authors suggest that the P2 receptor subtype involved might be P2Y, but in view of subsequent knowledge about the functional properties of cloned subtypes of the P2 receptor family, it seems more likely to belong to the P2X receptor family. ATP-evoked currents in cultured DRG neurons from rat embryos modulate spontaneous glutamate release via P2X2 and P2X3 receptors [216]. Early expression of P2X3 receptors in prenatal human DRG neurons has been reported [217].

P2X receptors on cultured embryonic DRG neurons mediate the release of substance P [218]. P2X3 receptors were expressed on most neurons in embryonic mouse trigeminal ganglia and DRG. This was in contrast to adult ganglia, which do not express P2X3 receptors on large-diameter neurons [192, 196, 219]. Most sensory neurons in mouse DRG, trigeminal and nodose ganglia expressed P2X3 receptors at E14. However, after birth, there was a decline to about 50 % of neurons showing positive staining [219]. Isolectin B_4 (IB_4)-positive neurons in sensory ganglia appeared at birth. They increased to about 50 % by P14, when they mostly expressed P2X3 receptors. Peptide release from neurons in embryonic DRG is augmented by ATP via P2Y receptors [220].

Rat superior cervical ganglia (SCG) sympathetic neurons were responsive to ATP and α,β-meATP at E18, birth and during the early postnatal period, via P2X2/3 heteromultimer receptors; these responses were reduced in mature rats [221]. This change in P2X receptor expression occurs when synaptogenesis is taking place in the SCG, suggesting a role for purinergic signalling in this process. During early postnatal life, IB_4-binding DRG neurons (that express P2X3 receptors) switch from nerve growth factor to glial cell-derived neurotrophic factor dependence [222]. P2 receptors modulated NA release from chick sympathetic neurons cultured from 12-day-old embryos [223, 224]. This suggested that both a facilitatory P2 receptor and an inhibitory receptor were involved. Cultured mice and rat paravertebral sympathetic neurons from the first few days after birth appeared to express different purinoceptors [225]. Neurons from both species responded via P2Y receptors activated by UTP to cause depolarization and NA release.

Ninety-three percent of the tyrosine hydroxylase-negative (parasympathetic) neurons in the rat pelvic ganglion expressed P2X2 receptors in both young and old rats [226]. P2X7 receptors in satellite glial cells were shown to mediate functional expression of P2X3 receptors in immature DRG neurons [227].

Retina

There are many studies of purinergic signalling in the retina of adult mammals, but relatively few reports about embryonic retina. Spontaneous waves of excitation in the developing rabbit retina were detected at E22, and involvement of purinergic receptor activation in these waves was investigated [228]. Adenosine has been implicated in chick retinal development. A_1 receptors were suggested to have different functions in the embryonic retina [229].

ATP-induced rise in intracellular Ca^{2+} was mediated by $P2Y_2$ or $P2Y_4$ receptors in embryonic chick neural retina, and there was a decline of ATP-induced rise in intracellular Ca^{2+} just prior to synaptogenesis (see [7]). Large Ca^{2+} signals in chick retinal cells were triggered at E3 by ATP and UTP, acting via P2Y receptors [230]. Simultaneously, ATP, ADP and UTP stimulated proliferation of retinal cells via $P2Y_1$ and $P2Y_4$ receptors and multiple intracellular signalling cascades [231]. Autocrine or paracrine release of ATP has been claimed to be involved in the regulation of DNA synthesis in the neural retina at early embryonic stages [232]. Suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid inhibited the ATP-induced increase in [^{3}H]thymidine incorporation in retinal cultures from E3. The change in Ca^{2+} signalling mediated by P_{2u} (i.e. $P2Y_2$ or $P2Y_4$) receptors during development may be involved in the differentiation of neuroepithelial cells or progenitor cells into neurons. P2 receptors appear to mediate the regulation of retinal progenitor cell proliferation at early embryonic stages, perhaps in collaboration with growth factors [232]. Proliferation of both bipolar and Müller cells in early developing chick retina at E6–8 is probably mediated by $P2Y_1$ receptors [233]. ATP induced proliferation of cultured retinal cells [233]. ATP evoked increase in $[Ca^{2+}]_i$ in radial Müller cells in the postnatal rabbit retina [234].

ATP signalling associated with Ca^{2+} waves is present in both retinal pigment epithelium [235, 236] and in the developing neural retina [230, 237]. Activation of P2 receptors in the chick embryo retina evoked an increase in $[Ca^{2+}]_i$ and controls the rate of division of progenitor cells [231, 236]. Pearson et al. [236] showed that the ATP release in the retinal pigment epithelium came about through the spontaneous gating of connexin43 hemichannels. Blockade of these hemichannels with a connexin mimetic peptide not only greatly reduced ATP release but also greatly reduced the proliferation of progenitor cells in the adjacent neural retina.

Guanine nucleotides block agonist-driven Ca^{2+} influx in chick embryo retinal explants [238]. RT-PCR studies revealed changes in the expression of P2X3 and P2X5 receptor mRNA

at different postnatal stages, P23 to P210, of pigmented retinas [239]. Also, P2X7 mRNA showed positive identification in the retina of postnatal rats (P23–P210) [240]. Data has been presented that suggests that purinergic signalling via $P2Y_1$ and $P2Y_4$ receptors on Müller cells may contribute to differentiation in the postnatal rat retina [241].

Inner ear

P2X2 receptor mRNA expression was present in the precursors of the cells bordering the cochlear endolymphatic compartment at E12 and in spinal and vestibular ganglia during embryonic development of the rat inner ear [242]. P2X2 receptor mRNA was not expressed in inner and outer hair cells until after P10 through P12, concomitant with the onset of hearing. These data are in accordance with the roles of the P2X2 receptor both in the process of labyrinthine development and in the regulation of auditory and vestibular sensory transduction. P2X1 receptors provide the signal transduction pathway for development of afferent and efferent innervation of the sensory hair cells and cochlea morphogenesis [243]. Using confocal immunofluorescence, P2X3 receptor expression has been characterized in the mouse cochlea from E16 [244]. Spiral ganglion neuron cell bodies and peripheral neurites projecting to the inner and outer hair cells were labelled for P2X3 receptor protein from E18 to P6. They were diminished around P6 and were no longer present at the onset of hearing (around P11). This suggests a role for P2X3 receptor-mediated purinergic signalling in cochlea synaptic reorganization and establishment of neurotransmission that occurs just prior to the onset of hearing function [245]. Brain-derived neurotrophic factor-mediated development of spiral ganglion neurons was shown to be inhibited by P2X3 and P2X2/3 receptor-mediated signalling in neonatal rat cochlea [246].

P2X7 receptor immunolabelling was observed in the primary auditory neurons of the spiral ganglion from E18 to adult and in the fibres innervating the sensory inner and outer hair cells from birth to adult [247]. The authors speculated that P2X7 receptors may be involved in signal transduction and modulation as well as in regulating cell death during development and in pathological conditions. It was reported that $P2Y_4$ and P2X2 receptors were coexpressed and contributed to the regulation of short circuit currents in rat cochlear neonatal outer sulcus cells [248]. They showed further that $P2Y_4$ receptor expression rapidly declined postnatally and reached near adult levels on postnatal day 14. In a later paper, the expression of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ receptors was identified in the rat cochlea during development [249]. Beginning in the late embryonic period, P2Y receptors were found in the cells lining the cochlea partition associated with the electrochemical environment that provides the driving force for sound transduction. Early postnatal $P2Y_2$ and $P2Y_4$ protein expression in the greater epithelial ridge supported the view that initiation and regulation of spontaneous activity in the hair cells prior to hearing onset are mediated by purinergic signalling.

Evidence was presented to suggest that P2X3 receptors mediate vestibular synaptic augmentation in the inner ear of chicken embryos [250]. They showed further that P2Y receptor ($P2Y_1$, $P2Y_2$ and $P2Y_6$) mRNA was present during the early stage (E15) rather than the later stage (E21) of the development of the vestibular system. It was suggested that temporal changes in P2Y receptor expression during development might be involved in the establishment of the endolymphatic ion composition needed for vestibular and auditory transduction. A transient structure, Köllicker's organ, consisting of epithelial cells that lie adjacent to the hair cells, appears during the development of the cochlea. Spontaneous electrical potentials in these cells were mediated by spontaneous release of ATP via connexin hemichannels to act via P2X and P2Y receptors [251, 252]. These cells exhibit spontaneous Ca^{2+} waves activated by ATP [253] and may allow the establishment of the tonotopic organization of the cochlea and spiral ganglion cell innervation, although this is disputed [254]. Changes in expression of the ectonucleotidases, NTPDase5 and NTPDase6, and uridine diphosphate-activated $P2Y_6$ receptors were observed during rat cochlea development [255].

In the developed cochlea, hemichannel-mediated ATP release may be important in regulating the electromotility of the outer hair cells [256]. This electromotility is an active amplifier of mechanical movement of the basilar membrane and determines the sensitivity of the cochlea to sound stimulation. A review by Mammano [254] summarizes the current understanding of the roles of ATP in cochlear development and function and the possible roles of connexin hemichannels. Interestingly, mutations or deletions of connexins, in particular, Cx26 [257] and Cx30, cause sensorineural hearing loss, suggesting that ATP release via these hemichannels may have fundamentally important roles in the development and maintenance of a functional cochlea [254, 258].

Gastrointestinal tract

Non-adrenergic, non-cholinergic (NANC) nerve-mediated responses were observed before birth in mouse and rabbit small intestine [259]. Quinacrine fluorescence, which is a marker for high levels of vesicle-bound ATP, was observed before birth in enteric neurons of rabbit ileum and stomach, 3 days before catecholamine fluorescence was detected in enteric nerves [260]. In the guinea pig taenia coli, the purinergic NANC inhibitory system appeared at 8 weeks of gestation, while cholinergic excitatory transmission was not present until birth [261].

P2X3 receptor immunoreactivity in the myenteric plexus of the embryonic rat stomach was present on both extrinsic and

intrinsic nerves [262]. The extrinsic sensory nerve fibres first expressed P2X3 receptors as early as E12, which extended rapidly on to the whole stomach by E14. However, the intrinsic enteric neuron cell bodies slowing positive P2X receptor immunoreactivity did not appear until birth (P1), peaked by P14 and then decreased in maturing animals. P2X3 and P2X5 receptors are present in large numbers of myenteric neurons in newborn guinea pigs compared to adults, whereas P2X5 receptor mRNA is found more frequently in adults [263]. Intraganglionic laminar nerve endings and intramuscular arrays were seen first postnatally at P1 and P7, respectively [262].

Several studies of postnatal developmental changes in purinergic signalling in the small intestine have been reported. ATP and ADP produced contractile responses in rat duodenal segments at P1 [264]. This response increased with age to day 7, followed by a decrease, and was non-existent by day 21. The relaxant responses to ATP and ADP, however, appeared at day 21 and continued to increase up to day 70 and probably later. Responses to adenosine or AMP were not elicited until day 14.

A_{2B} receptors in the longitudinal muscle of the rat duodenum were present at day 15, while A_1 receptors did not appear until after day 20; both receptor subtypes mediated relaxation. A_{2B} receptors in the muscularis mucosa mediated contraction from day 10. P2Y receptors mediated relaxation of the longitudinal muscle at day 25, while in the muscularis mucosa, P2Y receptors mediated contraction. After day 20, contractions were mediated by both P2X and P2Y receptors. The longitudinal muscle of the rat colon relaxed via A_{2B} and P2Y receptors, while the muscularis mucosa contracted via A_1 and probably $P2Y_2$ or $P2Y_4$ receptors [264].

In the mouse gastrointestinal tract, combined pharmacological and immunohistochemical studies showed that contraction was mediated by $P2Y_1$ receptors from P3 to P8 [265]. However, relaxation of longitudinal muscle throughout the gastrointestinal tract from day 12 onwards was via $P2Y_1$ receptors located both on smooth muscle and on a subpopulation of myenteric neurons. P1, $P2Y_2$ and/or $P2Y_4$ receptors and were also present on intestinal smooth muscles. The relaxant response during postnatal development was mediated by $P2Y_1$ receptors that gradually appeared along the length of the gastrointestinal tract, in the stomach from day 3, from day 6 in the duodenum, day 8 in the ileum and day 12 in the colon. The shift from contraction to relaxation occurred 1 week before weaning, suggesting that this may contribute to the changes that take place in the gut when the food compositions change from maternal milk to solid food.

Adenosine deaminase was localized predominantly on the keratinized squamous epithelium that lines the mucosal layer of the oesophagus, forestomach and the simple columnar epithelium of proximal small intestine of the mouse gastrointestinal tract. The levels of adenosine deaminase in these tissues were low at birth but reached very high levels within the first 2 weeks of postnatal life [266]. There was strong immunostaining of P2X4 receptors on the epithelial cells of proximal and distal newborn rat gut, P2X6 receptor immunostaining in capillary vessels in proximal newborn gut and differences in the amounts of P2X4 and P2X6 receptor expression in newborn compared to adult proximal and distal intestine [267].

Lung

The development of purinergic signalling in the lung described in early papers has been reviewed [7]. Increase in $[Ca^{2+}]_i$ signals in rat foetal lung epithelial cells was evoked by ATP and UTP. In epithelia explanted from foetal rat lung, receptors to adenosine, ATP and UTP were located on apical membranes throughout the lung, while basolateral receptors for these agonists were functional later in gestation in distal lung and in trachea. P2X7 mRNA was detected in E19 rat embryos by in situ hybridization in bronchial epithelium. ATP increased surfactant secretion as early as day 1 in newborn rats, but the effect of UTP did not appear until 4 days after birth. Adenosine analogues interrupted foetal breathing movements, but apnea was not produced in newborn lambs. Adenosine, ATP and ADP evoked oxygen-induced pulmonary vasodilatation in foetal lambs, probably via both A_{2A} and P2Y receptors [268]. P2X3 receptors are expressed on vagal sensory nerve terminals in rat lung where they make contact with neuroepithelial bodies (NEBs) a few days before birth [269]. This is consistent with the function of NEBs as oxygen sensors before the carotid body O_2 sensory system is fully developed at about 2 weeks after birth. Adenosine played a central role in modulating ventilation in the newborn piglet and was involved in the biphasic ventilatory responses to hypoxia.

Adenosine has a regulatory role in lung surfactant secretion in the newborn rabbit [270]. Ventilation-induced increase in secretion of lung surfactant was inhibited by P1 receptor antagonists. ATP stimulation of surfactant in type II cells in adult rats is mediated by both a $P2Y_2$ receptor coupled to phospholipase C and a receptor coupled to adenylate cyclase; UTP also activated the $P2Y_2$ receptor but did not stimulate cAMP formation. ATP increased cAMP formation in newborns but did not promote phospholipase D activation until day 4. Thus, the adenylate cyclase-coupled ATP signalling mechanism is functional early in development, but the $P2Y_2$ receptor pathway is not.

Vas deferens

Rats are not sexually active until about 10 weeks, so that changes in purinergic signalling in the developing vas deferens might occur later than in the gut. ATP and NA are cotransmitters in the sympathetic nerves supplying the vas

deferens (see [271]). EJPs produced by ATP in response to sympathetic nerve stimulation of the vas deferens were not observed in mice less than 18 days postnatal [272]. At 3 weeks postnatal (the earliest time studied), the responses of the rat vas deferens to field stimulation with single or trains of pulses lacked the adrenergic component, but the non-adrenergic component was present [273]. Responses to ATP first appeared at day 15 and then increased with age [264].

Adenosine, acting via prejunctional A_1 receptors, inhibited sympathetic neurotransmission in the rat vas deferens when nerve-mediated contractions were first seen at day 15, but its potency decreased with age. Inhibitory postjunctional A_2-like receptors and prejunctional A_1 receptors were present from days 10 to 15, respectively. Postjunctional excitatory A_1 receptors did not appear until after day 20. Stimulation of the hypogastric nerve produced monophasic contractions of the vas deferens in 2-week-old guinea pigs. Nerve-evoked contractions of the circular muscle layer of the guinea pig vas deferens decreased with increasing age, apparently due to purinergic postjunctional rather than prejunctional mechanisms. Postnatal androgen exposure of hypogonadal mice that are deprived of androgens after birth resulted in inhibition of purinergic EJPs in the vas deferens in adult mice, and P2X1 receptors could not be demonstrated with immunofluorescence [274].

Other organs

Chondrocytes, isolated from the cephalic region of day 19 chick embryo sterna, release nucleotides into the extracellular milieu, although they are rapidly degraded; it is claimed that they are involved in both chondrocyte maturation and matrix mineralization [275]. Extracellular ATP modulates $[Ca^{2+}]_i$ in retinoic acid-treated chondrocytes isolated the cephalic portion of day 14 chick embryonic sterna [276]. They speculate that immature chondrocytes may generate adenine nucleotides that then act in a paracrinal manner on chondrocytes that are at a later stage of maturation. Rapid deamination of adenosine in cultures of foetal mouse calvarial bones was shown and taken to account, at least in part, for the failure to observe effects of adenosine in bone metabolism in culture [277]. Cells of osteogenic and chondrogenic lineage derived from foetal metatarsal bones were exposed to ATP^{4-}; cells of haemopoietic origin were permeabilized and killed, while cells of non-haemopoietic origin (e.g. osteoblasts, chondrocytes) were insensitive to ATP^{4-} and survived [278]. This system allows the study of the properties and functions of osteogenic or chondrogenic cells without interference by the presence of cells of haemopoietic origin. ATP pyrophosphohydrolase has been purified and partially characterized from foetal bovine epiphyseal cartilage of patients with chondrocalcinosis [279]. The most prominent location of P2X7 receptor mRNA in E19 rat embryos is the bone marrow, but bone marrow cells from mouse femur also showed strong immunoreactivity for P2X receptors [186].

In the liver, the hepatic ATP/ADP ratio showed significant decrease during the first day of postnatal life in starved newborn rats, correlating directly to the gluconeogenic flux [280].

Enzymes for the breakdown of extracellular purines were described in the developing rat testis, but full metabolic involvement in terms of Mg^{2+} ATPase and 5′-nucleotidase was not achieved until 45 days postnatal [281].

In rat kidney, intrarenal adenosine is a physiological regulator of glomerular filtration rate and renal blood flow and appears to play a key role in the hypoxaemia-induced renal insufficiency in newborn rats [282]. Adenosine is involved in the regulation of the action of insulin on rat fat cell metabolism during postnatal development and ageing [283].

ATP and ADP and, to a lesser extent, AMP and adenosine increased insulin secretion from the isolated perfused newborn dog pancreas [284].

$P2Y_2$ receptors are strongly expressed on NA-containing adrenal chromaffin cells, but very little on adrenaline-containing cells in mature rats [285]. In contrast, in newborn rats, $P2Y_2$ receptors are expressed equally on both NA and adrenaline-containing cells. A loss of $P2Y_2$ receptor expression on both NA- and adrenaline-containing cells occurs in the adrenal gland of old (22 month) rats compared to newborn animals. It was suggested that ATP, acting via $P2Y_2$ receptors, may influence the phenotypic expression of chromaffin cells into NA- or adrenaline-containing cells during early development.

Differential coupling of $P2Y_1$ receptors to $G\alpha_{14}$ and $G\alpha_{q/11}$ proteins during the development of rat salivary (submandibular) gland has been described, with two bands (42 and 52 kDa) present in 1-week-old rats, but only the 42 kDa band was detected in the submandibular gland cells of 4- to 6-week-old rats [286, 287].

In the skin, Merkel cells appear in the epidermis of planum nasale of rat foetuses from the 16th day of intrauterine development and nerve fibres form close associations with them by day 20 [288]. Since it is known that Merkel cells contain high levels of peptide-bound ATP and are in close association with sensory fibres expressing P2X3 receptors (see [289]), this is of interest.

Figure 7 summarizes the expression of purinoceptors during postnatal development of duodenum, colon, vas deferens and urinary bladder.

Stem cells

Pluripotent stem cells (embryonic carcinoma and embryonic stem cells) have features of early embryonic cells [290, 291]. They are capable of differentiating into the three primary germ layers of the embryo. Embryonic stem cells are present in

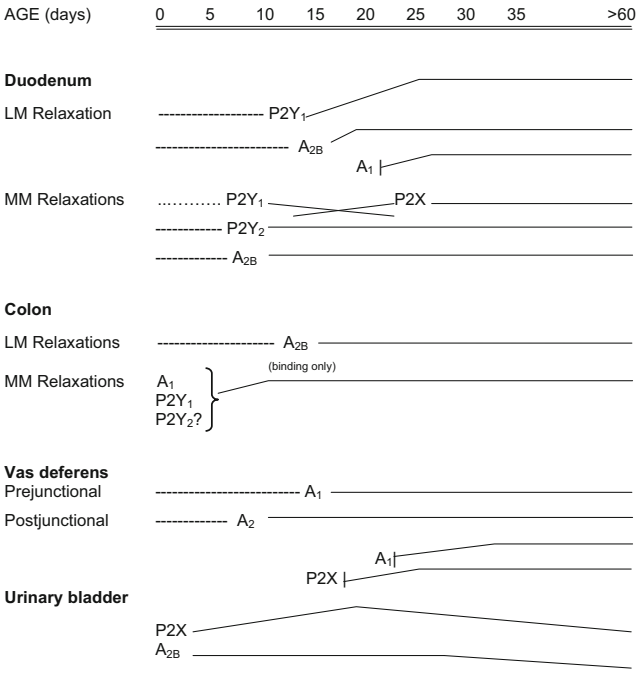

Fig. 7 Diagram summarizing the development of functional responses mediated by purine receptors in the rat duodenum, colon, urinary bladder and vas deferens. The *dashed lines* represent ages at which it was not possible to study functional responses, and the *solid lines* show when responses were observed, with the slope of the line indicating whether a response, in general, increased or decreased with age (reproduced with permission from [264])

foetal as well as in adult tissue. The importance of purinergic signalling in stem cell biology, including regulation of proliferation, differentiation and cell death, has been revealed [9, 292–294].

The P19 murine embryonal carcinoma cell line has been used for studying mechanisms of early neurogenesis [295]. Cultured P19 cells treated with retinoic acid form tridimensional cell aggregates, which resemble the blastula stage of embryonic development [296–298]. Differentiation into neuronal cells occurs by day 8, while glial cells predominate in the culture on day 14. Purinergic receptor activity was shown to be essential for proliferation and the progress of neurogenesis [299]. Differentiating P19 cells at the neural progenitor stage exposed to blockers of P2Y and P2X receptor-mediated calcium signal transduction led to inhibition of proliferation.

$P2Y_2$ receptors potentiate proliferation of embryonic stem cells [300]. Early in embryonic development, P2X receptors also appear. Neuronal progenitors, isolated from midbrain of E10.5 mice, possess all components of the purinergic signalling system. mRNA for adenosine, P2X and P2Y receptors and ectonucleotidases are expressed, and stimulation of purinoceptors elicited neuronal formation [301]. P2X3, P2X4, $P2Y_1$ and $P2Y_4$ receptor expression was high in embryonic P19 cells but then decreased following induction of differentiation [302]. P2X4/6 and P2X2/6 heteromultimer receptors may also be expressed on P19 cells. P2X6 receptor subtype expression increased during prenatal and postnatal mouse brain development [303]. $P2Y_1$ receptors are the major subtype involved in regulating cell proliferation and differentiation, less so by the $P2Y_2$ receptor subtype and with only a minor role for P2X4 receptors [299]. Chronic inhibition of $P2Y_1$ and, possibly, P2X2 receptor activity induced differentiation of P19 cells, resulting in loss of NMDA receptor activity in neuronal-differentiated cells, whereas blockade of $P2Y_2$ and, possibly, P2X2 receptors resulted in inhibition of cholinergic receptor responses.

Isolated neural stem cells from foetal brain or neurogenic areas of adult brain proliferate as free-floating spherical expansions, called neurospheres, expressing the neural progenitor marker nestin [304]. Removal of growth factors led cells of the outer layers of the neurosphere to migrate and differentiate into three major neural phenotypes, neurons, astrocytes and oligodendrocytes [305, 306]. Neurospheres from foetal rat brain expressed P2X2–7, as well as $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors [307]. Proliferation of human neural stem cells cultured from telecephalon tissues from a 15-week gestational age embryo was induced by ATP, and activation of P2 receptors released $[Ca^{2+}]_i$ from thapsigargin-sensitive intracellular stores [308], indicating mediation via P2Y receptors.

Evidence was presented to identify a novel role for P2X7 receptors in control of mouse embryonic stem cells, showing that they can mediate a pro-survival or pro-death signal depending on the mode of activation [309]. P2X7 receptor expression and activity are upregulated in mouse embryonic stem cells and modulate proliferation and suppress differentiation [310]. Expression and regulation of the ATP-binding cassette transporter, ABCG2, in human embryonic stem cells have been reported [311].

P2Y receptors are involved in regulation of embryonic and postnatal neurogenesis. P2Y and muscarinic ACh receptors appear to be the first functionally active receptors, present after gastrulation [29]. P2Y receptors, especially the $P2Y_1$ subtype, were widely expressed in the embryonic rat brain as early as day 11 [312]. A marked decrease in mRNA to $P2Y_1$ receptors and upregulation of mRNA for $P2Y_2$ receptors were observed on freshly isolated astrocytes during development of rat hippocampus [313]. P2Y receptor proteins were strongly expressed transiently in structures that were likely to be involved in functions specific to embryonic development. $P2Y_4$ receptors disappeared from the brain stem and ventral spinal cord postnatally. Calcium waves propagate through radial glial cells in the proliferative central ventricular zone, requiring both $P2Y_1$ receptors and connexin hemichannels [314].

Subventricular zone-derived neurospheres express two ecto-nucleotidases, NTPDase2 and alkaline phosphatase, which are markers for subsets of progenitor cells in the developing and adult mouse brain, supporting the notion that

purinergic signalling contributes to embryonic, postnatal and adult neurogenesis [315]. There is widespread mRNA expression for ectonucleotide pyrophosphatase/phosphodiesterase 1-3 (E-NPP1-3) in different rat brain regions during development and ageing [316]. There was an increase in both ATP and ADP hydrolysis by ecto-NTPDases 1–3 in synaptic plasma membranes isolated from rats in the first and second months of life and then decreased in adult life [317].

Hypothalamic tanycytes comprise a third neurogenic cell population in the adult brain capable of generating new neurons in the hypothalamus [318–320]. They can also release ATP, particularly in response to glucose, which can act at $P2Y_1$ receptors to trigger Ca^{2+} waves [321, 322]. Like other neural stem cells in the brain, tanycytes also express NTPDase2 [201]. The role of ATP in tanycyte proliferation and differentiation has not, so far, been examined. Given that tanycytes have several similarities to radial glial cells, which are regulated by ATP signalling [314], an investigation of the actions of ATP would seem warranted [322].

Ageing

Some studies of changes in purinergic signalling in ageing have been published. Two populations of binding sites for adenosine, corresponding to A_1 and A_2 receptors, were described in the brain of both young and old rats, but both the binding sites were greater in old rats [323]. Adenosinergic inhibition of synaptic potentials was significantly enhanced in hippocampal slices from aged rats contributing to age-related decline in synaptic efficacy [324]. Activation by endogenous adenosine via A_1 receptors mediated synaptic plasticity (both long-term potentiation and long-term depression), and this was maintained in aged rats [325]. Caffeine has been used to counteract age-related cognitive decline. For example, acute treatment with caffeine was claimed to reverse the impairment of olfactory discrimination and short-term social memory in ageing rats [326]. In layer 5 of cortex, synapses exhibit age-dependent changes in their integrative properties. For young rats (<P14), the probability of transmitter release is high and the synapses exhibit depression with repeated high-frequency stimulation [327, 328]. However, these synapses in older rats (>P30) exhibit a much lower release probability and change from being depressed during high-frequency stimulation to being facilitated. Kerr et al. [329] have shown that this developmental change in the integrative properties of these synapses can be explained by a developmental change in the extracellular concentration of adenosine acting via the A_1 receptor. At young ages, adenosine levels are low, but increase with age. The presynaptic action of adenosine on the A_1 receptor causes inhibition of transmitter release and shifts the synapses to a low probability release mode, which is then capable of facilitation during high-frequency trains of stimuli.

Extracellular levels of adenosine in the striatum were not affected by age, although there were differences in the mechanisms of adenosine release and metabolism. For example, erythro-2-(hydroxy-3-nonyl) adenine, an adenosine deaminase inhibitor, increased adenosine levels in the striatum of young, but not old, rats [330]. Maintenance of a constant extracellular adenosine level in the ageing brain may be a homeostatic mechanism. In old striatum, levels of A_2 receptor mRNA and A_2 receptor binding sites were reduced by 32 and 20 %, respectively [154]. A_1 and A_{2A} receptors both played a role in controlling motor activity in rats [153]. 2-[4-(2-p-Carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680), an A_{2A} receptor agonist, significantly increased spontaneous outflow of glutamate and aspartate in young, but not in old rats [331]. In contrast, there was increased spontaneous outflow of GABA in old, but not young rats exposed to CGS21680 [332]. A_1 and A_{2A} receptor binding was modified in aged striatum, hippocampus and cortex of the rat [333].

Modulation of cortical ACh release via P1 receptors was decreased in ageing rats [334], and a decrease in A_1 receptor gene expression was described in mouse cerebral cortex of aged rats [335]. Reduced efficiency of A_1 receptors to modulate synaptic transmission in the hippocampus of aged rats has been reported [336]. Down-regulation of adenosinergic function may underlie the enhanced excitability seen in hippocampal neurons of the CA1 subregion of aged animals [159]. Cross talk between A_1 and A_{2A} receptors in the hippocampus and cortex has been reported and A_{2A} receptors shown to control A_1 receptor function via PKC, but not PKA, in young adult, but not aged rats [337]. Adenosinergic activities seem to be unaffected by ageing in the cerebellum and substantia nigra. Neuron–glial synaptic currents were mediated by P2X as well as glutamate receptors. In the ageing mouse brain, the density of P2X receptors is reduced as is their role in glial synaptic current generation [338].

Ischaemic tolerance is impaired in aged heart. RT-PCR analysis showed age-related decline of A_3 receptors and induction of A_{2B} receptors in ageing mouse myocardium, changes that were mimicked by ischaemia in young, but not old, hearts [339]. They showed further that in aged hearts, ischaemia selectively reduced A_1 receptor levels. An increase in density of A_1 receptors in rabbit heart in old age in contrast to the diminished β-adrenergic responsiveness has been reported in the senescent heart [340]. There are conflicting reports about changes in purinergic signalling in the vascular system during old age. However, this may be explained by the variation in expression of P2 receptor subtypes in different vessels in different species and in different pathophysiological conditions (see [341]). Age-related changes in the relative importance of NA and ATP as mediators of the contractile responses of the rat tail artery to sympathetic nerve stimulation have been reported. The ATP component was dominant in young rats but declined with age [342]. ATP appeared to be the sole mediator of the sympathetic

contractile response in the tail artery in some young rats. However, there was a shift from purinergic to adrenergic signalling in old age, which was reflected by the responses to ATP and α,β-meATP, as well as the expression of P2X receptors [343].

Expression of $P2Y_1$ and $P2Y_2$ receptors and contractile responses to 2-methythio ADP and UTP were also decreased with age. It was speculated that the dramatic reduction in expression of P2 receptors in the rat tail artery during development and ageing was related to the role of the tail artery in temperature regulation. Constriction of rat mesenteric arteries by ATP decreased with age [344]. ATP is a cotransmitter with NA in sympathetic nerves supplying blood vessels in young human skin; NA, however, becomes the dominant neurotransmitter in old age [345]. Cerebral microvessels in old rats showed reduced ATPase activity, perhaps contributing to the blood–brain barrier function changes found in old rats [346]. Ageing is associated with impaired ability to modulate sympathetic vasoconstrictor activity and reduced exercise hyperaemia. ATP-induced vasodilation was low in the sedentary elderly, and interstitial ATP content during exercise and $P2Y_2$ receptor expression were higher in the active elderly compared to the sedentary elderly [347].

In the corpus cavernosum of rats, ageing was accompanied by a decrease in P2X1, but an increase in P2X7 receptors, and there was increased expression of $P2Y_4$ receptors in the bladder [348]. It was suggested that these changes in purinergic signalling probably contribute to the development of erectile dysfunction and higher detrusor activation in ageing rats.

Contractile responses of the aged rat bladder to ATP were significantly greater than those of the young bladder, compared to little change in the responses to ACh or KCl [349]. The purinergic component of nerve-mediated contractions of the human bladder was also increased with age, due largely to increased release of ATP [350]. The response of the bladder to α,β-meATP increased with age [351], but P2X1 and P2X3 receptor mRNA did not change with age. In aged male mice, there was an increase in bladder voiding, augmented P2X3 receptor-mediated afferent nerve firing during bladder filling and an increase in urothelial activation by ATP [352]. The authors speculated that this may reflect the increase in overactive bladder in ageing humans. In old guinea pigs, the purinergic component of sympathetic cotransmission was dominant in seminal vesicles [353]. P2X4 receptor expression increased in pancreatic islets in old age, but $P2Y_1$ receptor expression was lost [354].

An increase in density of A_1 receptors in rabbit heart in old age has been claimed [340] in contrast to the diminished β-adrenergic responsiveness in the senescent heart [355]. In the immature (25 days old) rat heart, adenosine was found to have little role in the modulation of contractile responsiveness via activation of either A_1 or A_2 receptors, but enhanced antiadrenergic and stimulatory functions of adenosine via A_1 and A_2 receptor activation were present in the heart of mature (79 days old) rats [356]. Reduced adenosine A_1 receptor and G_α protein coupling in rat ventricular myocardium during ageing has been reported [357]. Age-related decline in β-adrenergic and adenosine A_1 receptor function in the rat heart is attenuated by dietary restriction [358].

Age-related changes in P2 receptor mRNA have been described in rat arteries [359]. P2X1 receptor mRNA was reduced in basilar artery in 19-month compared to 2-month-old rats, while $P2Y_1$ and $P2Y_2$ receptor mRNA increased. mRNA for P2X receptors was described in postnatal rat mesenteric arteries [360]. P2X1 and P2X4 receptors were most strongly expressed, P2X2 and P2X7 receptors less so, while P2X3 and P2X5 receptors were only weakly expressed and no expression of P2X6 receptors; no differences in expression were seen between 7 and 28 days postnatal.

Summary

Several interesting generalizations emerge from the analysis in this review.

1. Purinergic signalling is present from the earliest developmental stages (before classical adrenergic signalling) and usually declines with maturation; sometimes, this involves reduction in potency of purines and pyrimidines, and sometimes, particular purinoceptor subtypes are no longer expressed. In a few cases, purinergic signalling has been demonstrated to control major developmental processes.
2. There are examples where a similar sequence of events occurs during regeneration after trauma to adult tissues as occurs during development, e.g. skeletal muscle and mesenteric arteries.
3. During postnatal development, responses to nucleosides and nucleotides increase or decrease with age depending on the physiological demands of the particular system involved.
4. Both P1 and P2 receptors are present early in development of many systems, but there are a few examples where the earliest effects of ATP are mediated via adenylate cyclase, and only later in receptor activation does the IP_3 second messenger system for P2Y receptor transduction come into operation (e.g. type III pneumocytes in lung). Fast P2X purinergic signalling may appear a little later in development compared to P1 and P2Y receptors, except perhaps in urinary bladder.

There are some compelling pioneering studies that implicate extracellular purines and pyrimidines in embryonic and stem cell functions, but the roles of purinergic signalling in development are still in its infancy. ATP is an ancient signalling molecule utilized early in evolution and has been retained

as a very successful communicating molecule for the activities of most cells in the body (see [361–363]). This supports the view that it would have also been utilized in the complex mechanisms involved in embryonic development and regeneration, in keeping with the general principle that 'ontogeny repeats phylogeny'.

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