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Ultrastructural localisation of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamo-neurohypophysial system

ANDRZEJ LOESCH,[1*] SUHEL MIAH[1] and GEOFFREY BURNSTOCK[2]

[1]*Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK;*
[2]*Autonomic Neuroscience Institute, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK*

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Summary

The distribution of the $P2X_2$ subtype of the purine receptor associated with the extracellular signalling activities of ATP was studied in the rat hypothalamo-neurohypophysial system at the electron microscope level. Receptors were labelled with ExtrAvidin-horseradish peroxidase preembedding immunocytochemistry using a polyclonal antibody against a fragment of an intracellular domain of the receptor. Immunoreactivity to $P2X_2$ receptors was localised in: (i) paraventricular and supraoptic nuclei—in subpopulations of endocrine neurones, neurosecretory and non-neurosecretory axons and dendrites; and (ii) the neurohypophysis—in pituicytes and subpopulation of neurosecretory axons. In both the hypothalamic nuclei examined, labelled asymmetric axo-dendritic synapses were commonly observed. These synapses involved either $P2X_2$-labelled axon terminals (synaptic buttons) and unlabelled dendrites or labelled dendrites and unlabelled axon terminals. Axo-somatic synapses established by $P2X_2$-positive axons on $P2X_2$-positive endocrine cell bodies as well as on $P2X_2$-negative somata were also observed. The functional significance of these findings is discussed.

Introduction

The involvement of adenosine 5′-triphosphate (ATP) and P2 receptors in the regulation of physiological functions of the hypothalamus (and thus hypothalamo-neurohypophysial system) has been known for some time; ATP has been shown to be released from the hypothalamus upon nerve stimulation (Potter & White, 1980; Sperlágh *et al*., 1998a). Application of ATP and its analogues on the endocrine/neurosecretory neurones of the rat supraoptic nucleus (SON) caused P2 receptor-mediated depolarisation (Hiruma & Bourque, 1995), whilst studies with calcium imaging showed that ATP induces a rapid increase in intracellular Ca^{2+} ($[Ca^{2+}]i$) in the neurones (Chen *et al*., 1994). According to Shibuya *et al*. (1999), ionic and Ca^{2+}-signalling mechanisms triggered by ATP may play an important role in the regulation of SON neurosecretory cells. ATP injected into paraventricular nucleus (PVN) can stimulate (via P2 receptors) the release of arginine-vasopressin (VP), which in turn exercises its anti-diuretic effects in the kidney (Mori *et al*., 1992). Similarly, P2 receptor-mediated effects of ATP on SON neurones have been suggested to be an intermediate process in VP release elicited by central noradrenergic neurones (Day *et al*., 1993; Buller *et al*., 1996).

In fetal hypothalamic neurones, ATP, adenosine-5′-0-(3-thiotriphosphate) (ATPγS), and β,γ-imido ATP can induce rapid, suramin-sensitive Ca^{2+} influx with similar potencies, while the effect of α,β-methylene ATP is less than the other compounds (Chen *et al*., 1994). In SON, ATP and α,β-methylene ATP excite the endocrine VP-containing neurones and the effects were inhibited by P2 receptor antagonist suramin (Day *et al*., 1993). In another experiment on rat SON endocrine neurones, it was shown that ATP, α,β-methylene ATP, β,γ-methylene ATP, and 2-methylthio ATP induced depolarisation, which could be inhibited by pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS)—another P2 receptor antagonist (Hiruma & Bourque, 1995).

Messenger RNAs encoding P2 receptors subtypes—$P2X_2$, $P2X_4$ and $P2X_6$—have already been identified in a number of hypothalamic structures (Kidd *et al*., 1995; Collo *et al*., 1996). Immunoreactivity to $P2X_2$ receptors has also been revealed at the light microscope level

* To whom correspondence should be addressed.

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in various regions of the hypothalamus, including the SON and PVN where strong signals from neuronal cell bodies and nerve fibres were observed. (Vulchanova *et al*., 1996; Xiang *et al*., 1998).

The present study concentrates on the distribution of immunoreactivity to $P2X_2$ receptors within the rat hypothalamo-neurohypophysial system at the ultrastructural level, suggesting functional roles in endocrine neurones, axons, dendrites and pituicytes.

Materials and methods

TISSUE PREPARATION

Principles of good laboratory animal care were followed and animal treatment was in compliance with the specific national laws and regulations. Six adult (3- to 4-month old) male Sprague-Dawley rats were anaesthetised with sodium pentobarbitone (60 mg/kg i.p., Sagatal, RMB Animal Health Ltd, Harlow, UK) and perfused through the heart (left ventricle) with fixative containing 4% paraformaldehyde, 0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. The brains were removed and placed in the same fixative for 5 h at 4°C, and then transferred to 4% paraformaldehyde and stored overnight at 4°C. The following day, the brains were rinsed in phosphate buffer for several hours (at 4°C), and then transferred to 0.05 M TRIS-buffered saline at pH 7.6 (TBS). Coronal sections of 60–70 μm through the hypothalamus were cut on a vibratome and collected in TBS. After being washed in TBS, the sections were exposed for 45 min to 0.3% hydrogen peroxide in 33% methanol for blocking endogenous peroxidases, washed in TBS and processed for the pre-embedding electron immunocytochemistry of $P2X_2$ antibody, using the ExtrAvidin peroxidase-conjugate (ABC) methods.

IMMUNOCYTOCHEMISTRY

The steps of ABC immunocytochemistry were generally similar to that previously described (Loesch & Burnstock, 1998). In brief, the main steps included: incubation of sections for 1.5 h with heat-treated 10% normal horse or donkey serum (NHS; Jackson ImmunoResearch Laboratories, West-Grave, Pennsylvania, USA; distributor: Stratech Scientific Ltd, Luton, UK); incubation for 28 h with a rabbit polyclonal antibody to $P2X_2$ (1.5–3.0 μg of antibody/ml of TBS containing 10% NHS and 0.05% thimerosal); incubation for 16 h with a biotin-conjugated donkey-antirabbit immunoglobulin G(H + L) serum (Jackson ImmunoResearch Laboratories) diluted 1 : 500 in TBS containing 1% NHS and 0.05% thimerosal; incubation for 4 h with ExtrAvidin-horseradish peroxidase conjugate (Sigma) diluted 1 : 1500 in TBS. After exposure to diaminobenzidine and osmication, the specimens were dehydrated and embedded in Araldite. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

CONTROLS FOR IMMUNOCYTOCHEMISTRY

The rabbit polyclonal antibody to $P2X_2$ used in this study was generated against unique peptide sequences of $P2X_2$ receptor subtypes and characterised by Roche Bioscience, Palo Alto, USA (see Chan *et al*., 1998; Xiang *et al*., 1998). In brief, the immunogen used was a synthetic peptide representing a sequence of 15 receptor-type specific amino acids located in the C-terminal part of the receptor (intracellular domain): QQDSTSTDPKGLAQL, amino acids residues 458–472. The peptide was covalently linked to keyhole limpet haemocyanin (KLH). The antibody was raised in New Zealand rabbits by multiple monthly injections with the immunogen (performed by Research Genetics, Inc., Huntsville, AL, USA). IgG fractions were isolated from the immune sera and from pre-immune controls using chromatography on DEAE Affigel-Blue (Bio-Rad, Hemel Hempstead, UK) or following the method of Harboe & Inglid (1973). The protein concentration was determined at 280 nm using an extinction factor of 1.43 for 1 mg/ml. The specificity of the antibody was verified by Western blot analysis of membrane preparations from recombinant CHO-K1 cells expressing the cloned $P2X_2$ receptor. Immunoblotting studies have shown that anti-$P2X_2$ antibody specifically recognises the recombinant $P2X_2$ receptor expressed in CHO-K1 cells (62 kDa band); preabsorption of the antibody with an excess of the synthetic peptide used for the generation of the antibody eliminated immunoreactivity/band (see Xiang *et al*., 1998). No cross-reaction of the antibody with expressed $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors was also shown (Xiang *et al*., 1998). In the present study, preabsorption of $P2X_2$ antibody with the antigen (synthetic peptide used for the generation of the antibody) at a concentration of 5 μg/ml of diluted antibody (5 μg peptide: 3 μg antibody) was sufficient to abolish immunostaining. Furthermore, no immunolabelling was observed when the $P2X_2$ antibody was omitted from the incubation medium and/or replaced with non-immune NHS and non-immune normal rabbit serum (Nordic Immunology, Tilberg, The Netherlands), or when the biotin-conjugated donkey-antirabbit IgG serum was omitted from the incubation medium.

Results

$P2X_2$ receptor immunoreactivity was found in the rat hypothalamo-neurohypophysial system. Both SON and the magnocellular part of PVN hypothalamic nuclei examined displayed labelled somata and numerous neuronal processes—axons and dendrites (Figs 1 and 2); the neurohypophysis displayed labelled pituicytes and neurosecretory axons (Fig. 3).

SON AND PVN

Throughout the SON and PVN magnocellular part, endocrine soma positive and negative for $P2X_2$ receptors were observed as were positive and negative dendrites and axons (Figs 1 and 2). The staining intensity of the soma varied considerably, with moderately stained cells predominating (Fig. 1a–c). Immunoprecipitate was primarily associated with the cytoplasm (matrix), particularly in the vicinity of the cisterns of the granular endoplasmic reticulum (Fig. 1a–c). The membranes (cytosolic site) of mitochondria were also labelled (Fig. 1c). Most of the granular vesicles (neurosecretory granules) observed in $P2X_2$-labelled somata were immuno-negative (Fig. 1b). Cell bodies

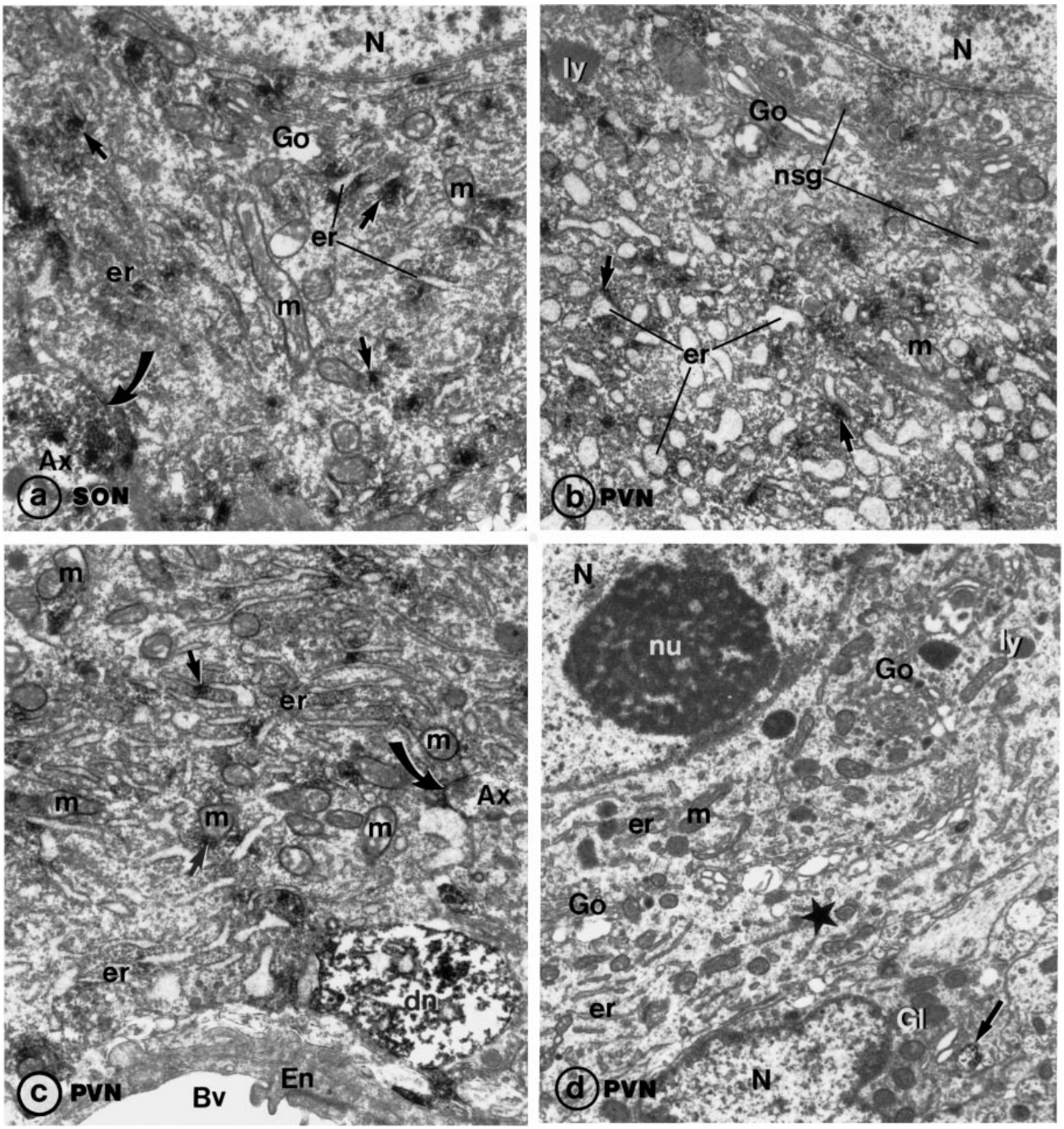
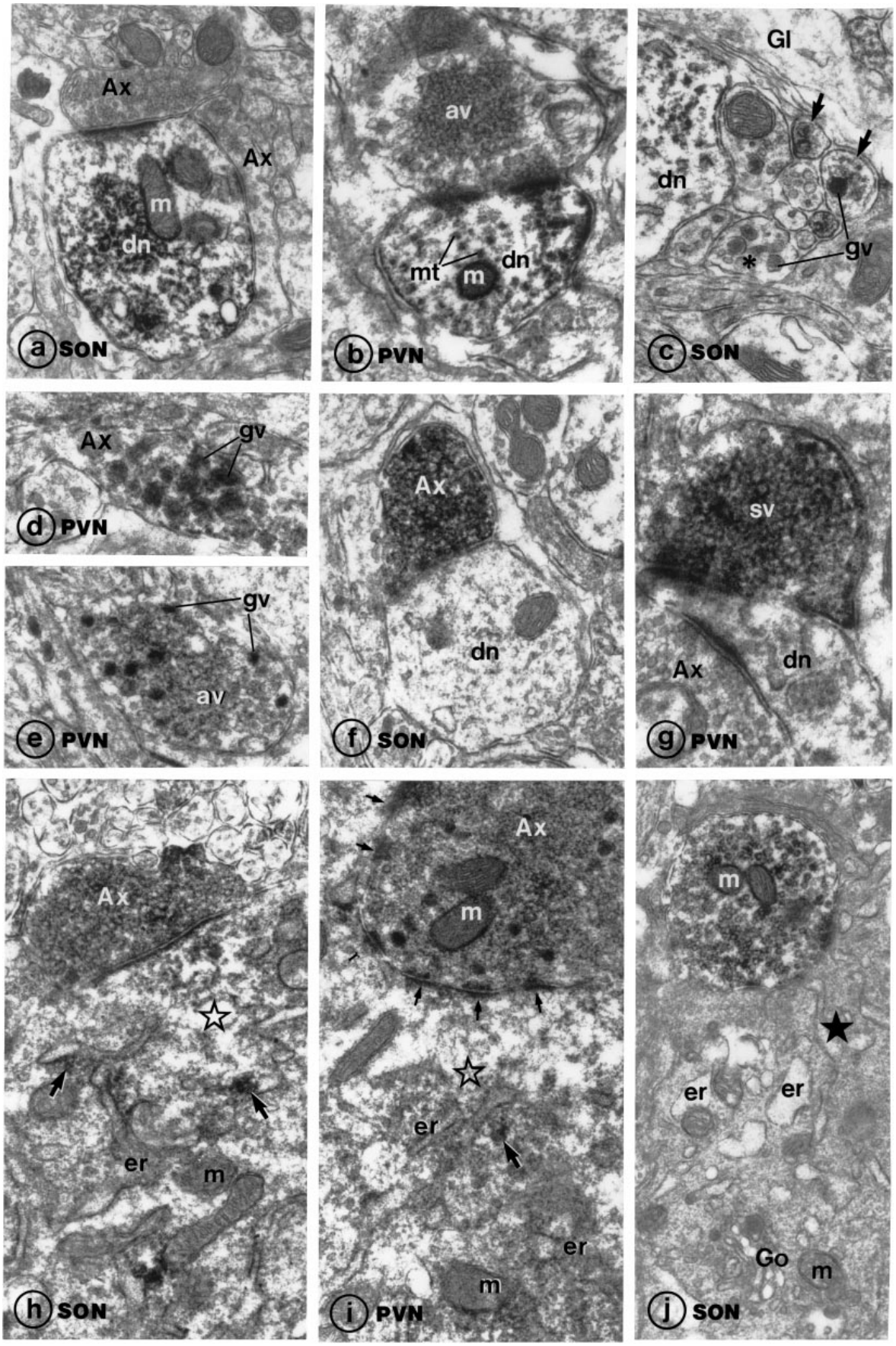


Fig. 1. Cell bodies of neurosecretory neurones of endocrine somata of the supraoptic nucleus (SON) (a) and paraventricular nucleus (PVN) (b–d) labelled for P2X$_2$ receptors. (a) A fragment of the labelled cell body displaying immunoprecipitate (distinctive black stain—small arrows) localised predominantly in the cytoplasm associated with endoplasmic reticulum (er). A P2X$_2$-positive axon (Ax) containing synaptic vesicles can be seen adjacent to the soma (curved arrow). N, nucleus; Go, Golgi complex; m, mitochondria. × 189 00. (b) Labelled soma containing mostly 'swollen' short cisterns of endoplasmic reticulum; note unevenly distributed clusters of immunoprecipitate. nsg, unlabelled neurosecretory granules; ly, lysosome. × 20 500. (c) Note immunoprecipitate in association with elongated cisterns of endoplasmic reticulum and some mitochondria (label at cytoplasmic site of the membrane). Curved arrow indicates asymmetric axo-somatic synapse made by an unlabelled axon terminal. dn, P2X$_2$-positive dendrite; Bv, blood vessel; En, endothelium. × 22 800. (d) A P2X$_2$-negative endocrine soma (star) and a glial cell (Gl) showing no immunoprecipitate. One P2X$_2$-positive nerve fibre profile can also be seen (arrow). nu, nucleolus. × 13 700.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of endocrine neurones negative for $P2X_2$ (Fig. 1d) were similar to those observed in the immunocytochemical control preparations (data not shown). Examples of the nerve fibres—dendrites and axons, positive for $P2X_2$ are shown in Fig. 2. $P2X_2$-labelled dendrites were commonly present in both nuclei examined. They formed asymmetrical synapses with unlabelled axon profiles with small agranular vesicles (Fig. 2a and b). None of the granular vesicles/neurosecretory granules ($\sim$200 nm) of obvious magnocellular type were labelled in $P2X_2$-labelled dendrites neither within PVN (magnocellular part) nor SON. Some axons contained $P2X_2$-labelled granular vesicles of various sizes (Fig. 2c–e). Axon profiles negative for $P2X_2$ but containing granular vesicles were also seen (Fig. 2c). Axons positive for $P2X_2$ were frequently seen adjacent to unlabelled dendrites or forming asymmetrical synaptic contacts with them (Fig. 2f and g). Fewer $P2X_2$-positive axons were seen establishing synapses with the cell body of endocrine neurones (Fig. 2h–j). No $P2X_2$-positive neurosecretory axons were involved in synaptic contacts with other labelled axons or dendrites. The unequivocal identification of the shape of the synaptic vesicles was difficult in the present study due to the obscuring effect of immunoprecipitate. Therefore, it is not clear whether some of the labelled buttons contained e.g. flattened vesicles and were involved in establishing synaptic contacts observed in the present study.

NEUROHYPOPHYSIS

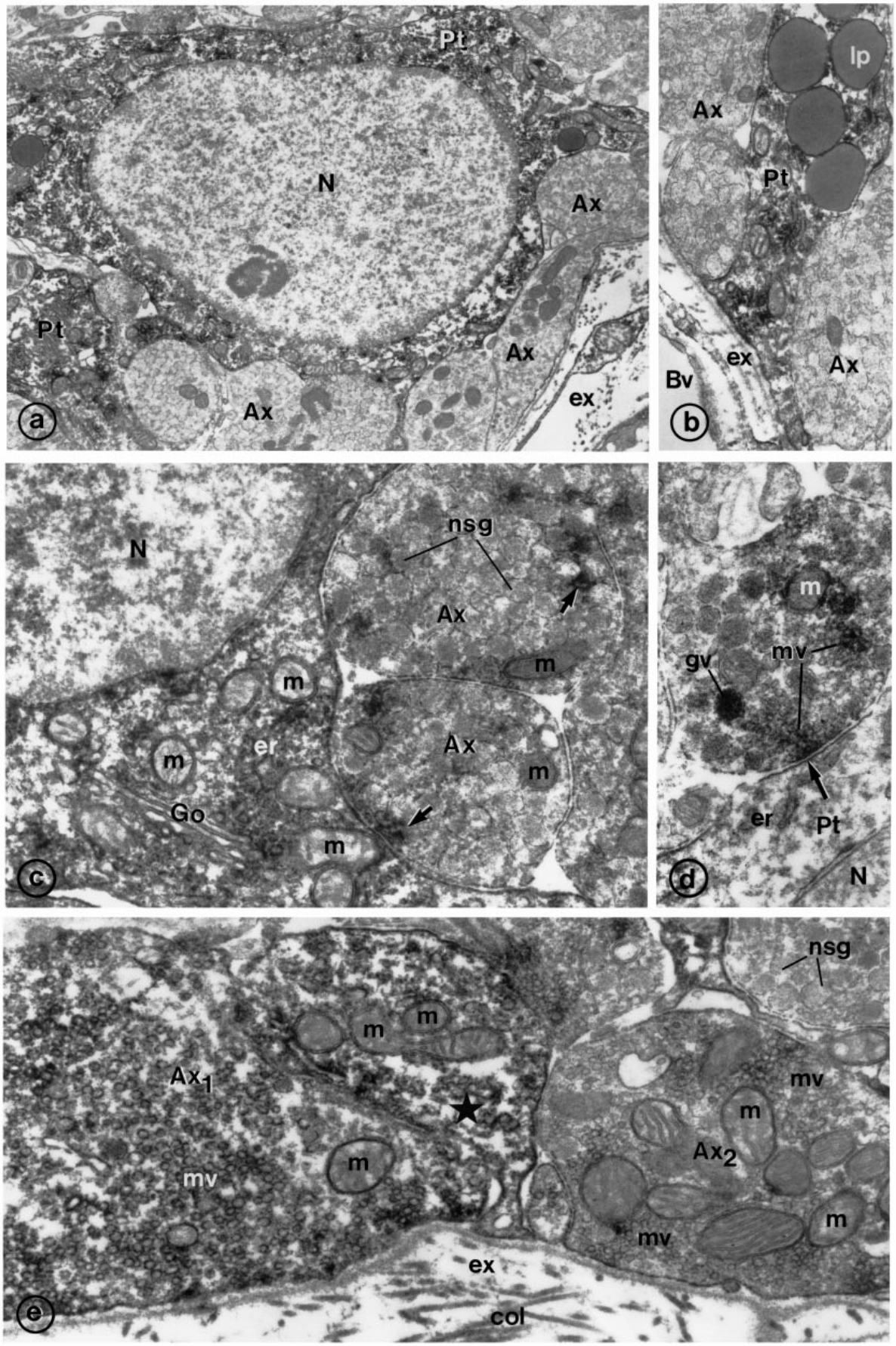
Immunoreactivity to $P2X_2$ was dominant in the pituicytes including their processes localised in perivascular region (Fig. 3a and b); most of the cells observed in the neurohypophysis were immuno-positive. Not all $P2X_2$-positive pituicytes were intensely labelled and immunoprecipitate was not always equally distributed throughout the cell. Within these cells and their perivascular processes, the immunoprecipitate was associated with the cytoplasm and the membranes of intracellular organelles, mostly of the nucleolemma, endoplasmic reticulum and mitochondria (Fig. 3c). Like in the SON and PVN, both $P2X_2$-positive and $P2X_2$-negative axons were observed throughout the neurohypophysis. However, axons either immunonegative for $P2X_2$ or displaying weak immunoreactivity seemed dominant (Fig. 3a–c). Some axon profiles contained intensely labelled granular vesicles (neurosecretory granules?) and/or microvesicles seen amongst unlabelled granules; such axons were at times establishing synaptoid contacts with pituicytes (Fig. 3d). Intensely stained microvesicles due to the $P2X_2$-label association with the membranes of the vesicles were particularly visible in axon terminals that appeared to be scarce or free of neurosecretory granules at the perivascular region (Fig. 3e).

Discussion

The present electron microscope study describes the presence of $P2X_2$ receptors associated with subpopulations of cell bodies of endocrine neurones, axon profiles and dendrites in the rat hypothalamic SON and PVN nuclei, as well as pituicytes and some neurosecretory axons in the neurohypophysis. These data are consistent with the view that extracellular ATP acting via $P2X_2$ receptors has physiological roles in the hypothalamo-neurohypophysial tract of the rat, and extend the previous immunocytochemical and/or *in situ* hybridisation studies at light microscope level, which have described a wide distribution of $P2X_2$ receptor in the rat hypothalamus (Collo *et al*., 1996; Vulchanova *et al*., 1996; Xiang *et al*., 1998).

In their study on $P2X_2$ receptor distribution in rat hypothalamus, Xiang *et al*. (1998) demonstrated intense labelling of nerve fibres (axons and dendrites) as well as cell bodies in the SON and PVN nuclei including magnocellular and parvocellular parts of PVN; fewer immuno-positive nerve fibres were noted

Fig. 2. Neuropil of supraoptic nucleus (SON) (a, c, f, h) and paraventricular nucleus (PVN) (b, d, e, g, i, j) labelled for $P2X_2$ receptors. (a) $P2X_2$-labelled dendrite (dn) with two asymmetrically synapsing unlabelled axons (Ax). m, mitochondria. $\times$ 28 500. (b) Note an unlabelled axon with closely packed together small spherical agranular synaptic vesicles (av) establishing asymmetrical synapse with a labelled dendrite. mt, microtubules. $\times$ 52 000. (c) A group of unmyelinated small axons close to labelled dendrite; some axons are positive for $P2X_2$ (arrows) and display a presence of labelled granular vesicles (gv; 110–120 nm); an axon containing $P2X_2$-negative granular vesicles can also be seen (asterisk). Gl, process of a glial cell. $\times$ 29 000. (d) $P2X_2$-positive axon displaying numerous labelled granular vesicles (neurosecretory granules?) (100–130 nm). $\times$ 54 000. (e) In $P2X_2$-positive axon note predomination of agranular vesicles over granular vesicles. $\times$ 36 700. (f) $P2X_2$-labelled axon profile adjacent to unlabelled dendrite; also note that obscuring effect of immunoprecipitate in the labelled axon prevents clear identification of synaptic vesicles. $\times$ 43 000. (g) One $P2X_2$-positive and one $P2X_2$-negative axon form asymmetrical synapses with an unlabelled dendrite. In labelled axon note closely packed synaptic vesicles (sv). $\times$ 56 000. (h) Note asymmetrical axo-somatic synapse established by $P2X_2$-positive axon terminal on $P2X_2$-positive endocrine soma (star); arrows point to immunoprecipitate. er, granular endoplasmic reticulum. $\times$ 35 000. (i) A fragment of a large $P2X_2$-positive axon terminal containing many small agranular synaptic vesicles and a few granular vesicles (70–80 nm) contacts symmetrically with the $P2X_2$-positive soma; at the pre- and post- synaptic membranes note symmetrically dispersed $P2X_2$-labelled dense material (small arrows). $\times$ 45 000. (j) An axon profile filled with $P2X_2$-labelled agranular synaptic vesicles (a label mostly stains the membrane of the vesicles) is establishing asymmetrical synapse with a $P2X_2$-negative endocrine soma (black star). Go, Golgi complex. $\times$ 26 600.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in the SON. However, due to the limited resolution of light microscopy, no interrelationship between a high density nerve fibre network (and positive neurones) in the endocrine hypothalamus displaying $P2X_2$-immunoreactivity could be analysed in detail (Xiang *et al*., 1998). In the present study, 'moderate' $P2X_2$-synaptic input to the cell bodies of endocrine neurones was noted. However, $P2X_2$-positive axon profiles passing close to the soma but not establishing synapses, were seen frequently, suggesting that these were directing elsewhere. Many axodendritic synaptic contacts were observed either between $P2X_2$-labelled axon terminals and immuno-negative dendrites or more frequently between immuno-negative axon terminals and $P2X_2$-labelled dendrites. No evidence for involvement of $P2X_2$-immunoreactive neurosecretory axons in establishing synapses with other $P2X_2$ immuno-positive neuronal elements was observed. The morphological variability of $P2X_2$-labelled axon profiles observed in the present study, some of which were making synaptic contacts seem important for ATP regulation of endocrine neurones as does the richness of labelled dendrites contacting unlabelled nerve profiles. These data also suggest that $P2X_2$ receptors may have physiological actions both at presynaptic and postsynaptic sites. It is likely that the diversity of the labelling of pre- and post-synaptic neuronal membranes of axons, dendrites and/or somata observed in the present study expresses the differences in the density of the distribution of $P2X_2$ receptors in these cells, suggesting functional differences.

We have no information about the origin of the $P2X_2$-labelled fibres forming the synapses on the cell bodies of endocrine neurones. In the hypothalamus, the endocrine neurones receive synaptic inputs from noradrenaline (NA)-, γ-aminobutyric acid (GABA)- and glutamate-containing neurones/nerve terminals (see Buller *et al*., 1996; Sperlágh *et al*., 1998a; Theodosis *et al*., 1998). NA-containing nerve fibres arising from the A1 group of noradrenergic neurones in the caudal brainstem, which is believed to mediate the responses of VP-containing neurones in SON, are also assumed to be the native sources of ATP released in SON (Buller *et al*., 1996). The co-existence of NA and ATP in the nerve terminals in hypothalamus has been supported by neurotransmitter release experiments (Sperlágh *et al*., 1998a). However, ATP and NA may be released from separate populations of synaptic vesicles as has been suggested, for example, in sympathetic nerves (Todorov *et al*., 1996). An increase in GABAergic and glutamatergic synaptic inputs to hypothalamic endocrine neurones has been demonstrated during stimulation of hormone release e.g. oxytocin (OT; L) (see Theodosis *et al*., 1998).

Previous studies on the hypothalamic SON and/or PVN revealed the presence of mRNA transcripts not only for $P2X_2$ but also for $P2X_3$, $P2X_4$, $P2X_6$ and $P2X_7$ receptors (Collo *et al*., 1996; Shibuya *et al*., 1999). Signals from mRNAs for $P2X_2$, $P2X_4$ and $P2X_6$ receptors appeared to be stronger in SON than PVN (Collo *et al*., 1996) and mRNAs for $P2X_3$, $P2X_4$ and $P2X_7$ were predominant (Shibuya *et al*., 1999). As yet, functional and pharmacological studies on the hypothalamic neurones have not revealed a dominant subtype of P2X receptor (Day *et al*., 1993; Chen *et al*., 1994; Hiruma & Bourque, 1995). It seems likely that hypothalamic endocrine neurones express heteromultimers of P2X receptor subtypes. It has namely been shown that even application of high concentrations of ATP antagonists such as suramin (1–10 mM) or PPADS (100 μM) did not entirely inhibit the ATP-induced excitatory responses of SON neurones, suggesting the involvement of suramin- and PPADS-resistant P2X receptor subtypes—possibly $P2X_4$ and $P2X_6$ (Bo *et al*., 1995; Hiruma & Bourque, 1995). In fact, little is known about the expression of functional multisubunit complexes of P2X receptors in general.

The results of the present study clearly show that not all axons in the neurohypophysis displayed immunoreactivity to $P2X_2$ receptors. However, the $P2X_2$ receptor-labelling was seen within some granular vesicles (neurosecretory granules?) in a subpopulation of axons and in association with the membrane of microvesicles and plasmalemma in axon terminals that generally appeared to be free of labelled neurosecretory granules. Whether there was an association between the labelling of neurosecretory granules for $P2X_2$ receptors and the content of ATP is open to speculation, as is the significance of the labelling of microvesicles and their membrane in perivascular axon terminals in particular. ATP is known to be co-stored

Fig. 3. Neurohypophysis labelled for $P2X_2$ receptors. (a) Neurohypophysis demonstrating $P2X_2$-labelled pituicytes (Pt) and numerous $P2X_2$-negative neurosecretory axons (Ax). N, nucleus; ex, perivascular extracellular matrix. $\times$ 10 800. (b) $P2X_2$-positive pituicyte process (Pt) abutting the perivascular area. Bv, blood vessel; lp, lipid droplets. $\times$ 21 000. (c) Higher magnification example of $P2X_2$-positive pituicyte with Golgi complex (Go), mitochondria (m) and endoplasmic reticulum (er); also note immunolabelling (arrows) in adjacent axons. nsg, neurosecretory granules $\times$ 35 000. (d) Note clusters of microvesicles (mv) within $P2X_2$-positive axon making synaptoid contact (arrow) on 'lightly' labelled pituicyte (Pt); at least one granular vesicle (gv; neurosecretory granule?) showing positive labelling for $P2X_2$ can be seen. $\times$ 43 000. (e). Fragment of perivascular region of neurohypophysis showing one $P2X_2$-positive (Ax_1) and one $P2X_2$-negative/partialy stained (Ax_2) axon terminals; a star indicates a $P2X_2$-positive cell process that is difficult to identify (an axon, pituicyte?). Note abundance of labelled microvesicles in Ax_1 terminal; label is attached to the axolemma and the membrane of the vesicles. $\times$ 31 500.

in neurosecretory granules of neurohypophysial axons (Gratzl *et al*., 1980; Zimmermann, 1994). Recent studies on the release properties of neurohypophysial axons revealed that application of extracellular ATP evoked a dose-dependent increase in $[Ca^{2+}]i$ in about 40% of the axons (isolated) and triggered significant VP release (Troadec *et al*., 1998). These studies also showed that ATP can be co-released with OT from one population of axon terminals to act as a paracrine-autocrine messenger to (i) stimulate the entry of Ca^{2+} to other neighbouring axon terminals via a $P2X_2$ receptor and subsequently (ii) stimulate the secretion of VP from those terminals. These functions therefore raise the possibility of stimulation of the release of neurohypophysial hormones by the mechanisms independent of depolarisation-evoked secretion (Sperlágh *et al*., 1998b; Troadec *et al*., 1998). The finding of the present study of $P2X_2$ labelling in subpopulations of intact axons in the neurohypophysis supports the observations by Troadec *et al*. (1998) of subpopulations of isolated neurohypophysial axons displaying ATP-evoked $[Ca^{2+}]i$ increase. Interestingly, it has been suggested that microvesicles of neurosecretory axons can store Ca^{2+} at concentrations suitable for post-stimulus recovery (Nordmann & Chevallier, 1980).

A major finding in the present study concerns the immuno-reactivity of pituicytes for $P2X_2$ receptors, including the labelling of their processes in relation to blood vessels—the sites of neuropeptide release from the neurosecretory axon terminals into the circulation. This may suggest the involvement of pituicytes in the mobilisation of intracellular ions, e.g. Ca^{2+}, via $P2X_2$ receptors. In fact, the possibility of participation of pituicytes in Ca^{2+} trafficking through their cytoplasm; the control of the ionic environment of the neurohypophysial axons and therefore of the neurohypophysial hormone release were suggested some time ago (see Stoeckel *et al*., 1975; Shaw & Morris, 1980). It is likely that pituicytes are the sites of Ca^{2+} uptake caused by K^+ depolarisation (Shaw & Dyball, 1984). Rat pituicytes in culture have been shown to respond to VP with a mobilisation of Ca^{2+} from intracellular stores (Hatton *et al*., 1992; Boersma *et al*., 1993b). Furthermore, electron-immunocytochemical studies of rat neurohypophysis showing immunoreactivity to VP and OT in pituicytes and revealing the existence of synaptoid contacts between these cells and VP- and OT-containing neurosecretory axon terminals suggest that pituicytes participate in auto- and/or 'cross-regulation' of VP and OT release (Boersma *et al*., 1993a). Pituicytes are commonly recognised as being glial cells—neurohypophysial astrocytes involved in the regulation of the neurosecretory phenomenon and displaying dynamic interactions with neurosecretory axons (see Hatton, 1990). It is interesting to note that some astrocytes from other region of the CNS, e.g. molecular layer of rat cerebellum displayed immunoreactivity to $P2X_1$ receptors (Loesch & Burnstock, 1998); the distribution of immunoreactivity, however, differed from that of $P2X_2$ receptors observed in the pituicytes. Studies of rat cerebral cortical astrocytes in primary culture have demonstrated that extracellular ATP stimulates increase in $[Ca^{2+}]i$ via activation of P2 receptors and consequently mediates protein phosphorylation system in these cells (Neary *et al*., 1991). Recent evidence shows that glial cells respond to neuronal activity with an increase of their $[Ca^{2+}]i$, which triggers the release of agents/transmitters from the glia themselves and, in turn, causes a feedback regulation of neuronal activity; thus a modulatory function for these cells has been suggested (Araque *et al*., 1999).

Concerning the pituitary gland, the role of extracellular ATP and related receptors (e.g. P2U receptors, now considered to include $P2Y_2$ and $P2Y_7$, and P2X receptors including $P2X_2$) have been primarily associated with the endocrine function of the anterior part of the gland—the adenohypophysis (e.g. Chen *et al*., 1995; Vulchanova *et al*., 1996; Koshimizu *et al*., 1998). For example, P2U receptors have been demonstrated to mediate the release of luteinizing hormone from gonadotrophes (Chen *et al*., 1995), whereas the mRNAs for $P2X_2$ and the involvement of $P2X_2$ receptors in controlling cationic (Ca^{2+}) influx have been observed in somatotrophs (Koshimizu *et al*., 1998). Vulchanova and colleagues (1996) revealed immunoreactivity to $P2X_2$ receptors in scattered cells of the anterior pituitary.

In conclusion, the results of the present study have demonstrated that $P2X_2$ receptors are present within the rat hypothalamo-neurohypophysial system. There is evidence to suggest that these receptors are involved in the regulation of this neuroendocrine system and in the role of pituicytes in this process.

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