Pergamon

PII: S0306-4522(01)00288-3

Neuroscience Vol. 106, No. 3, pp. 621–631, 2001

0306-4522/01 $20.00+0.00

www.elsevier.com/locate/neuroscience

IMMUNOREACTIVITY TO $P2X_6$ RECEPTORS IN THE RAT HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM: AN ULTRASTRUCTURAL STUDY WITH EXTRAVIDIN AND COLLOIDAL GOLD-SILVER LABELLING

A. LOESCH[a]* and G. BURNSTOCK[b]

[a]Department of Anatomy and Developmental Biology, University College London, Gower Street, London WC1E 6BT, UK

[b]Autonomic Neuroscience Institute, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK

Abstract—The distribution of the purine receptor $P2X_6$ subtype was studied in the rat hypothalamo-neurohypophysial system at the electron microscope level. Receptors were visualised with ExtrAvidin peroxidase conjugate and immunogold-silver pre-embedding immunocytochemistry using a polyclonal antibody against an intracellular domain of the receptor. Application of ExtrAvidin labelling revealed $P2X_6$ receptors in subpopulations of: (i) neurosecretory cell bodies, neurosecretory and non-neurosecretory axons and dendrites of neurones in the paraventricular and supraoptic nuclei; and (ii) pituicytes and neurosecretory axons of the neurohypophysis. Some of the neurosecretory granules observed in the supraoptic and paraventricular nuclei neurone cell bodies, dendrites and axons as well as those in neurohypophysial axons were also positive for the $P2X_6$ receptors. In the paraventricular nucleus, some axons and dendrites of non-neurosecretory neurones positive for $P2X_6$ receptors formed synapses between themselves. Using the immunogold-silver method, the electron-dense particles labelling $P2X_6$ receptors were found in neurosecretory cell bodies of the supraoptic and paraventricular nuclei, in relation to the cytoplasm, endoplasmic reticulum, Golgi complex and neurosecretory granules. The particles indicative of $P2X_6$ receptors were also located in neurosecretory and non-neurosecretory axons including axonal buttons making synapses with $P2X_6$-negative dendrites. In the neurohypophysis, the electron-dense particles were localised in a subpopulation of pituicytes and neurosecretory axons. In neurohypophysial axons, particles were at times seen over the membrane of some neurosecretory granules (immunogold label) or microvesicles (immunoperoxidase label).

We speculate that the $P2X_6$ receptors at the neurohypophysial level may be implicated not only in hormone release from the axon terminals, but also in membrane recycling of the granular vesicles and microvesicles.

Key words: P2X receptors, neurosecretion, electron microscopy, rat.

Extracellular ATP and purinergic P2 receptors play roles in the hypothalamo-neurohypophysial system (Potter and White, 1980; Hiruma and Bourque, 1995; Sperlágh et al., 1998; Loesch et al., 1999; Shibuya et al., 1999). It has been shown that ATP induces a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the hypothalamic neurosecretory neurones (Chen et al., 1994). ATP injected into paraventricular nucleus (PVN) stimulates, via P2 receptors, the release of arginine vasopressin (AVP) from the neurohypophysis (Mori et al., 1992). Evidence has also been presented that multiple P2X receptors are functionally expressed in neurosecretory neurones, at least in those of the supraoptic nucleus (SON) (Shibuya et al., 1999). Earlier studies of SON neurones clearly suggested that a P2 receptor-mediated effect of ATP was an intermediate process in AVP release evoked by central noradrenergic neurones (Day et al., 1993; Buller et al., 1996). ATP has been shown to excite the neurosecretory vasopressin-containing neurones and the effects were prevented by the P2 receptor antagonist suramin (Day et al., 1993). ATP, acting at the P2X receptors, stimulates vasopressin release from SON, but displays characteristics of receptor desensitisation (Kapoor and Sladek, 1999). As regards SON neurones, at least two patterns of desensitisation have been reported for the $[Ca^{2+}]_i$ response to repeated applications of ATP (Shibuya et al., 1999). A recent study by Lemos and Wang (2000) suggests that intracellular/cytoplasmic ATP increases activity of the type II Ca^{2+}-activated K^+ (K_{Ca}) channels in the neurohypophysial axon terminals, whilst extracellular ATP inhibits the type II K_{Ca} current in a dose dependent manner.

Light microscope studies have demonstrated broad distribution of P2X receptors (e.g. $P2X_2$, $P2X_3$ and

*Corresponding author. Tel.: +44-20-7504 2215; fax: +44-20-7679 7349.

E-mail address: a.loesch@ucl.ac.uk (A. Loesch).

Abbreviations: AVP, arginine vasopressin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; K_{Ca}, Ca^{2+}-activated K^+ channels; NA, noradrenaline; NGS, normal goat serum; NHS, normal horse serum; PVN, paraventricular nucleus; SON, supraoptic nucleus; TBS, Tris-buffered saline.

$P2X_4$ receptor subtypes) in the brain including in the hypothalamus and the hypophysis (Kidd et al., 1995; Collo et al., 1996; Soto et al., 1996; Vulchanova et al., 1996; Xiang et al., 1998; Kanjhan et al., 1999). In the hypothalamo-neurohypophysial system, the role for $P2X_2$ receptor and its distribution seem the best determined (Troadec et al., 1998; Xiang et al., 1998; Kapoor and Sladek, 1999; Sperlágh et al., 1999). In one study, the distribution of the $P2X_2$ receptor in the SON, PVN and the neurohypophysis has been examined at the ultrastructural level (Loesch et al., 1999). Some data suggest a role for the $P2X_6$ receptor subtype in the rat hypothalamo-neurohypophysial system since $P2X_6$ receptor mRNA transcript was identified in SON and PVN (Shibuya et al., 1999).

The present study examines for the first time immunoreactivity to $P2X_6$ receptors within the rat hypothalamo-neurohypophysial system at the ultrastructural level and whether the application of two different pre-embedding immunocytochemical methods such as ExtrAvidin and colloidal gold-silver labelling allows more precise detection of the receptors. Discussion of the results concentrates on the comparison of the distribution of $P2X_6$ receptors with that of $P2X_2$ receptors previously described in the neurosecretory system of the rat (Loesch et al., 1999).

EXPERIMENTAL PROCEDURES

Tissue preparation

Principles of good laboratory animal care were followed and animal treatment was in compliance with the UK Animals (Scientific Procedures) Act, 1986, and associated regulations and guidelines. All efforts were made to minimise the number of animals used and their suffering.

Six male adult Sprague–Dawley rats (3–4-month old) were anaesthetised with sodium pentobarbital (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 (TBS) at pH 7.6. Coronal sections of 60–70 μm through the brains at the region of the hypothalamus were cut on a vibratome and collected in TBS. Each section was then cut sagittally with a razorblade through the third ventricle to obtain two sections containing the left or right side of the hypothalamus. Hypophysis containing neurohypophysis was also vibratome sectioned (60–70 μm). From each animal, three to four hypothalamus sections (from the left or right side of the hypothalamus) and two neurohypophysis sections were processed for the pre-embedding electron immunocytochemistry of $P2X_6$ antibody, using the ExtrAvidin peroxidase conjugate method. A similar number of sections from each animal was used in the immunogold-silver labelling technique.

ExtrAvidin labelling

After being washed in TBS, the sections were exposed for 45 min to 0.3% hydrogen peroxide in 33% methanol to block endogenous peroxidases, washed in TBS and processed for immunocytochemistry. The steps of immunoprocedure were generally similar to that previously described (Loesch and Burnstock, 1998). In brief, the main steps included: for blocking non-specific protein binding sites, incubation of sections for 1.5 h with heat-treated 10% normal horse serum (NHS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; distributor: Stratech Scientific Ltd, Luton, UK); incubation for 28 h with a rabbit polyclonal antibody to $P2X_6$ (1.5–3.0 μg antibody/ml of TBS containing 10% NHS and 0.05% thimerosal); incubation for 16 h with a biotin conjugated donkey anti-rabbit IgG (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-Aldrich, Poole, UK) diluted 1:1500 in TBS. After exposure to diaminobenzidine and osmication (1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4), the specimens were dehydrated in a graded series of ethanol and flat embedded in Araldite. Neurohypophysis, anterior SON (and partially the rostrocaudal extent of the nucleus), and the PVN (with subnuclei) were dissected out from flat Araldite sections and mounted on Araldite blocks for ultrasectioning. The ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

Immunogold-silver labelling

After being washed in TBS and prior to immunoprocedure, some vibratome sections were infiltrated for 45 min at room temperature with cryoprotectant consisting of 25% sucrose and 10% glycerol (by volume, in 0.1 M phosphate buffer at pH 7.4). They were placed flat into the mesh holder, to allow excess cryoprotectant to drain off, and then immersed in the cold isopentane (cooled by liquid nitrogen) until they turned white. Sections were next transferred into liquid nitrogen for about 10 s, removed from nitrogen and allowed to thaw in cryoprotectant for a few minutes before being washed in TBS. The freeze-thawed sections and sections not exposed to low temperature but treated with cyanoborohydride coupling buffer at pH 7.5 (Sigma-Aldrich), were both processed for immunocytochemistry at room temperature. The main steps of the procedure included: incubation of sections for 1.5 h with 10% heat-treated normal goat serum (NGS; Nordic Immunology, Tilburg, the Netherlands); incubation for 42 h with a rabbit polyclonal antibody to $P2X_6$ (3 μg antibody/ml of TBS containing 5% NGS and 0.1% sodium azide); washing in TBS; incubation for 16–20 h with a 1-nm gold conjugated goat anti-rabbit IgG (H+L) serum (British Biocell Int., Cardiff, UK) diluted 1:400–1:1000 in TBS containing 0.1% sodium azide; washing in TBS, 0.1 M sodium acetate and/or deionised distilled water; enhancing with silver using a silver enhancing kit (British Biocell Int.). After washing in 0.1 M sodium acetate followed by deionised distilled water, specimens were postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and flat embedded in Araldite. The ultrathin sections were cut from the SON, PVN and the neurohypophysis (as already described for the ExtrAvidin method), stained with uranyl acetate and lead citrate and examined with a JEM-1010 electron microscope.

Controls for immunocytochemistry

The polyclonal antibody to $P2X_6$ used in this study was raised in New Zealand rabbits against specific peptide sequence of $P2X_6$ receptor subtypes and characterised by Roche Bioscience, Palo Alto, CA, USA (Oglesby et al., 1999). In brief, rabbits were inoculated with a 15-amino acid peptide corresponding to 15 receptor-type specific amino acids in the C-terminal region of the $P2X_6$ receptor (intracellular domain): EAGFYWRTKYEEARA, amino acid residues 357–371. IgG fractions were isolated from the immune and preimmune sera following the method of Harboe and Ingild (1973). The protein concentration was determined at 280 nm using an extinction factor of 1.43 for 1 mg/ml. Immunoblotting studies have shown that anti-$P2X_6$ antibody specifically recognised the recombinant $P2X_6$ receptor (a single band at 50 kDa) expressed in CHO-K1 cells (Oglesby et al., 1999); preabsorption of the antibody with an excess of the

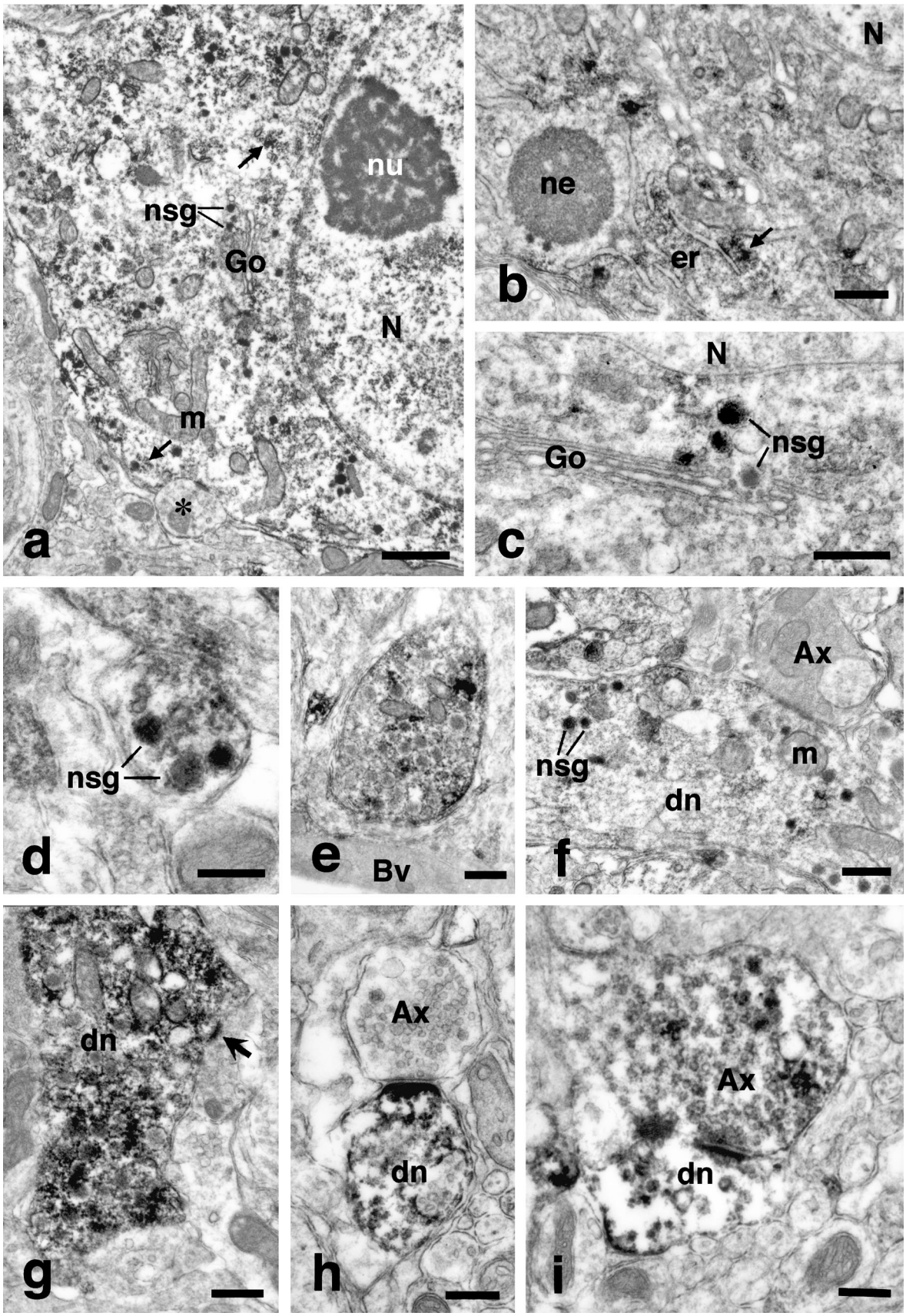

Fig. 1 *(Caption overleaf)*.

synthetic peptide used for the generation of the antibody eliminated the immunoreactivity band. No cross-reaction of the antibody with heterologous receptors ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ receptors) has been observed (Oglesby et al., 1999). In the present study, preabsorption of $P2X_6$ 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 immunolabelling. Furthermore, no immunolabelling was observed when the $P2X_6$ antibody was omitted from the incubation medium and/or replaced with non-immune NHS or NGS and non-immune normal rabbit serum (Nordic Immunology), or when the biotin conjugated donkey anti-rabbit IgG serum or goat IgG:1 nm gold conjugate were omitted from the incubation medium.

RESULTS

Application of ExtrAvidin (Figs. 1 and 2) and immunogold-silver (Figs. 3 and 4) labelling techniques revealed immunoreactivity for $P2X_6$ receptors in the rat hypothalamo-neurohypophysial system; subpopulations of cell bodies, axons and dendrites of the SON and the magnocellular part of PVN were labelled, as were the pituicytes and neurosecretory axons of the neurohypophysis. Sections treated with low temperature or cyanoborohydride buffer prior to application of the immunogold labelling procedure displayed similar $P2X_6$ receptor localisation, although cyanoborohydride treatment resulted in poorer preservation of the ultrastructural details, e.g. a number of neurosecretory granules observed in the axon or dendrite profiles frequently did not display an electron-dense granular core (see results and electron micrographs).

ExtrAvidin immunolabelling

SON and PVN. With ExtrAvidin labelling of the SON and PVN magnocellular part, neurosecretory cell bodies, dendrites and axons, both positive and negative structures for $P2X_6$ receptors were observed (Fig. 1). In the labelled neurosecretory cell bodies, immunoprecipitate was found throughout the cytoplasm (Fig. 1a), most profoundly as a cluster pattern adjacent to the granular endoplasmic reticulum (Fig. 1b). Granular vesicles (neurosecretory granules, 100–200 nm), which were present in the cell bodies, were immunolabelled; some granules appeared to be unlabelled. The labelled and unlabelled granular vesicles were clearly seen in the vicinity of the Golgi complex (Fig. 1c). Axons and dendrites of a neurosecretory nature were observed in SON and PVN, with some displaying immunoreactivity for $P2X_6$ receptors (Fig. 1d–g). $P2X_6$ receptor-positive neurosecretory axons (Fig. 1d, e) and dendrites (Fig. 1f, g) contained a mixture of labelled and unlabelled granular vesicles of neurosecretory type (120–200 nm). Although distinguishing between the neurosecretory axons and dendrites was at times difficult, the profiles containing endoplasmic reticulum, ribosomes or multivesicular bodies and receiving synaptic input (see Fig. 1f) were thought to be dendrites. Some axon terminals dominating in small agranular vesicles made synapses with $P2X_6$-positive dendrite profiles (Fig. 1h). In the PVN, $P2X_6$-positive axon terminals synapsing on $P2X_6$-positive non-neurosecretory dendrites were occasionally seen (Fig. 1i). There was no evidence in the present study that SON and PVN $P2X_6$-positive neurosecretory cell bodies, their dendrites or axons received $P2X_6$-positive synaptic input. The labelled neurosecretory cells commonly received synaptic input from $P2X_6$-negative axons (Fig. 1a, f).

Neurohypophysis. The neurohypophysis displayed both neurohypophysial axons and pituicytes immunoreactive and negative for the $P2X_6$ receptors (Fig. 2). The labelled neurosecretory axons were scattered among unlabelled ones (Fig. 2a). The unlabelled axons dominated in the neurohypophysis. The $P2X_6$-positive axon terminals observed in the vascular region contained both labelled neurosecretory granules (Fig. 2b) and microvesicles (Fig. 2c). For the labelled microvesicles, the immunoprecipitate was associated with the vesicle membrane. In labelled pituicytes, the immunoprecipitate was primarily confined to the cytoplasm and endoplasmic reticulum; some cells were more intensely labelled than others (Fig. 2d, e). In the apposition to $P2X_6$-positive pituicytes, axons unlabelled and labelled for $P2X_6$ receptors could be seen (Fig. 2d, e).

Immunogold-silver labelling

SON and PVN. With pre-embedding immunogold-silver labelling, electron-dense particles were detected in a subpopulation of neural profiles of the SON and PVN. At low microscope power, the particles found in the labelled neurosecretory cell bodies were seen in the cytoplasm (Fig. 3a). At higher magnification, however, the

Fig. 1. PVN (a, c, d, f, h, i) and SON (b, e, g) labelled for $P2X_6$ receptors by the ExtrAvidin method. (a) The labelled neurosecretory cell body shows immunoprecipitate (black, small arrows) localised in various regions of the cytoplasm. Note the unlabelled axon (asterisk) synapsing on the cell body. (b) A fragment of neurosecretory cell demonstrates clusters of immunoprecipitate (arrow) associated with the granular endoplasmic reticulum. (c) In the vicinity of the Golgi complex, note three labelled and one unlabelled neurosecretory granules. (d) An immunoreactive axon displays labelled neurosecretory granules (110–140 nm). (e) An immunoreactive axon near a blood vessel is filled with unlabelled and labelled neurosecretory granules. (f) An immunoreactive dendrite contains a few labelled neurosecretory granules; an unlabelled axon terminal synapsing on the dendrite is also present. (g) A dendrite with labelled and unlabelled neurosecretory granules. An arrow points to an axo-dendritic synapse. (h) An unlabelled axon terminal containing agranular and a few granular vesicles, forms an asymmetric synapse with a $P2X_6$ positive dendrite; note the intense immunodeposit over the postsynaptic density. (i) A $P2X_6$-positive axon terminal mostly with spherical agranular vesicles forms an asymmetric synapse with a $P2X_6$-positive dendrite. N, nucleus; nu, nucleolus; nsg, neurosecretory granules; Go, Golgi complex; m, mitochondrion; er, endoplasmic reticulum; ne, nematosome; Bv, blood vessel; dn, dendrite; Ax, axon terminal. Scale bars = 1 μm (a), 0.5 μm (b, c, e, f, g), 0.25 μm (d, h, i).

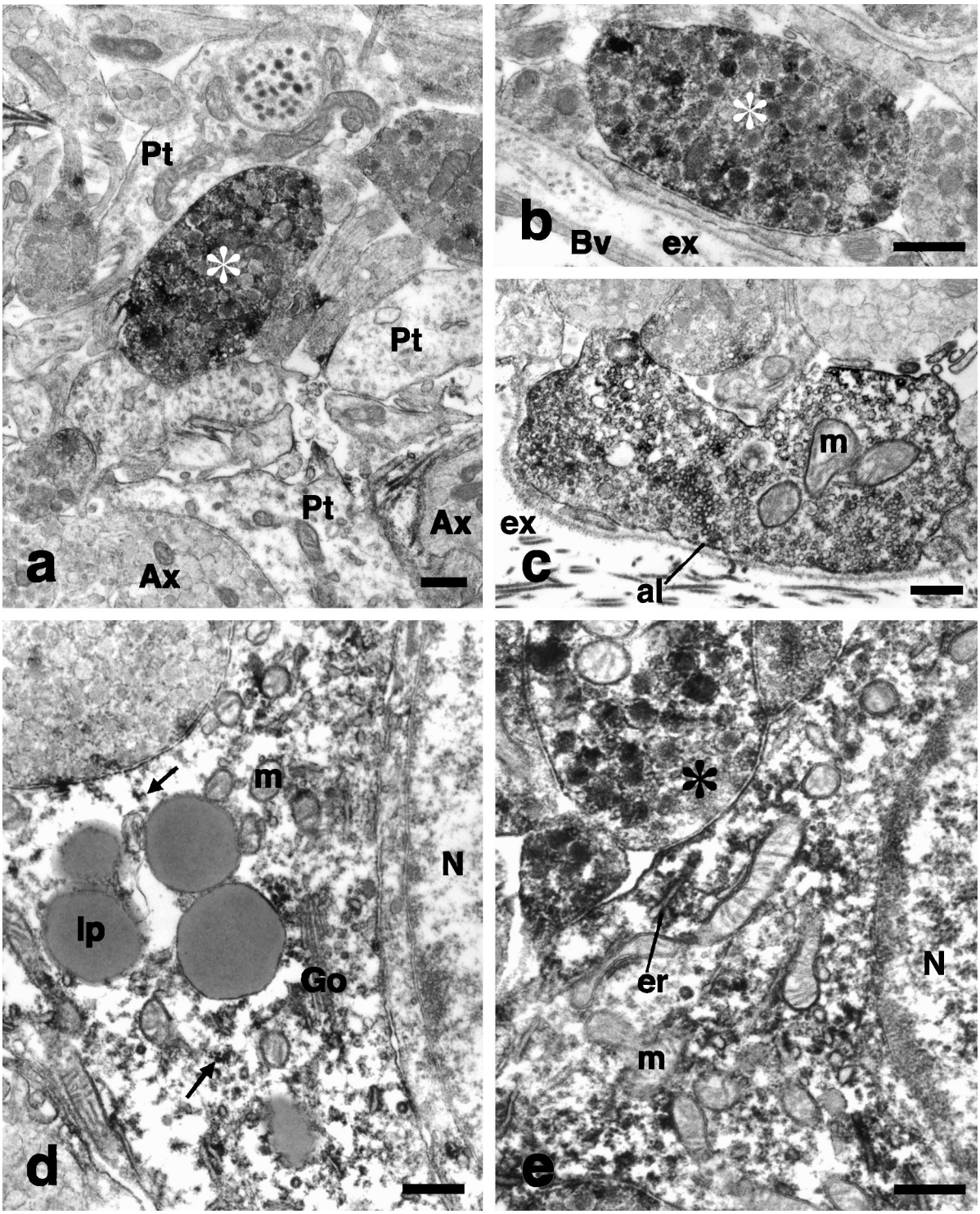

Fig. 2. Neurohypophysis labelled for P2X$_6$ receptors by the ExtrAvidin method. (a) A P2X$_6$-positive axon containing neurosecretory granules is indicated (asterisk); unlabelled axons and pituicytes are also present. (b) Neurosecretory axon terminal near blood vessel is rich in P2X$_6$-positive neurosecretory granules. (c) Note the abundance of labelled microvesicles in an axon terminal abutting the perivascular area; the axolemma and outer membrane of mitochondria are also labelled. (d) The cytoplasm of a P2X$_6$-positive pituicyte shows small clusters of immunoprecipitate (arrows). (e) Note intense immunolabelling of both the pituicyte and the adjacent neurosecretory axon (asterisk) and granular endoplasmic reticulum with associated immunoprecipitate. Ax, axon; Pt, pituicyte; Bv, blood vessel; ex, extracellular matrix; al, axolemma; m, mitochondrion; Go, Golgi complex; lp, lipid droplet; N, nucleus of pituicyte; er, granular endoplasmic reticulum. Scale bars = 0.5 μm (a–e).

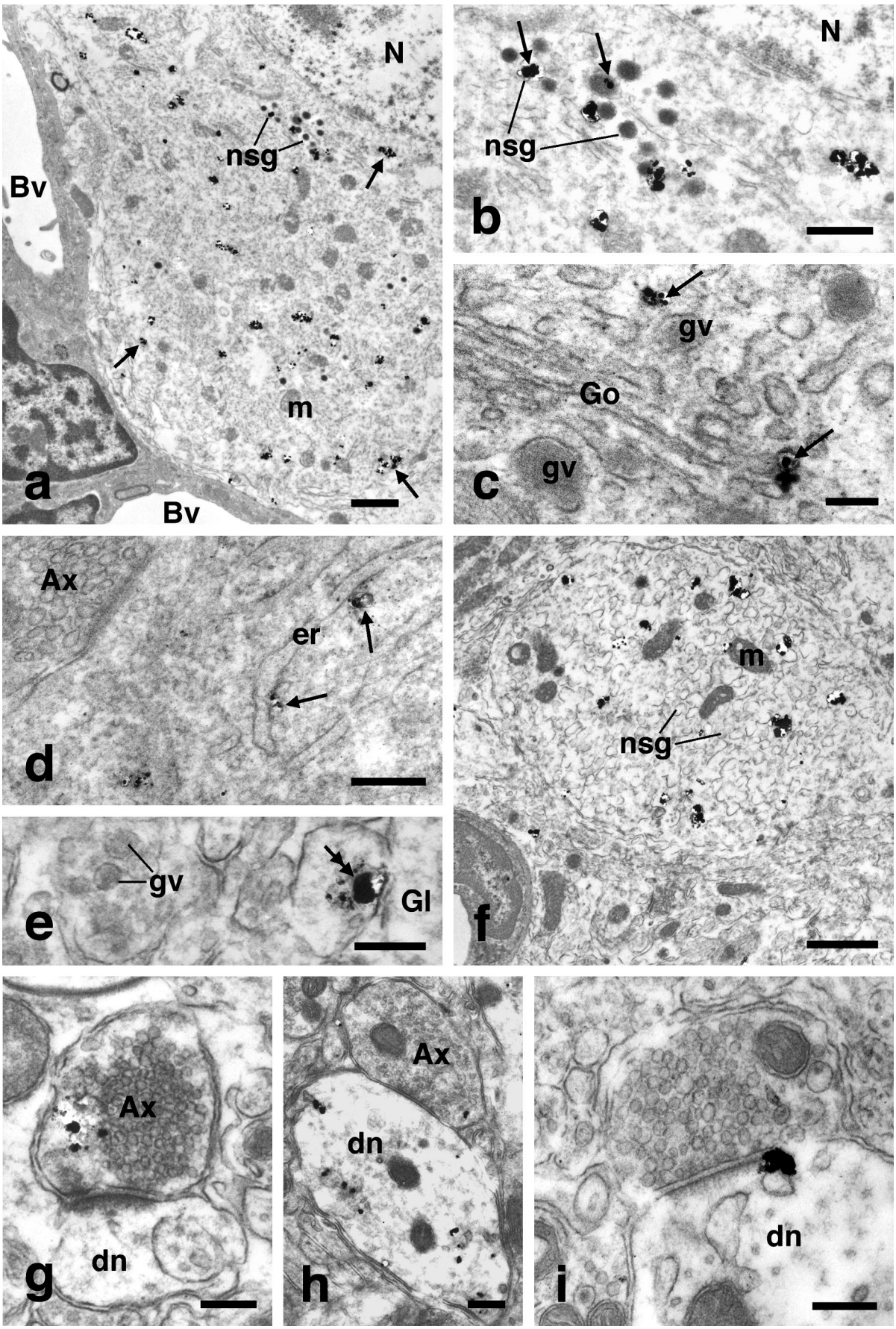
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Fig. 3.

gold-silver particles were detected in the close vicinity of granular vesicles (neurosecretory granules); some particles were seen over the granular vesicles (Fig. 3b). The particles were also associated with the cisterns of the Golgi complex (Fig. 3c) and the cytoplasmic surface of granular endoplasmic reticulum (Fig. 3d). Both the non-secretory and neurosecretory axon profiles also displayed gold-silver particles (Fig. 3e–g); axon profiles containing vesicles of 200–300 nm were thought to be of the neurosecretory type (Fig. 3f). $P2X_6$-positive axon terminals of a non-secretory type containing small agranular synaptic vesicles displayed labelling particles; these axons occasionally formed synapses with unlabelled dendrites (Fig. 3g). Other axon terminals were unlabelled and made synapses with $P2X_6$-labelled dendrites (Fig. 3h), in which the label could also be seen localised at the postsynaptic density (Fig. 3i). In the present study, no labelling was observed in the axons synapsing on neurosecretory cell bodies (Fig. 3d).

Neurohypophysis. Immunogold-silver particles were found within subpopulations of neurosecretory axons and pituicytes (Fig. 4); some axons and pituicytes were unlabelled. In the labelled axons, the particles appeared to be localised among the neurosecretory granules. The axons examined at the higher magnification revealed that some of the particles were located over the membrane of the neurosecretory granules (Fig. 4a). Most of the microvesicles observed in the axon terminals abutting the blood vessel seemed unlabelled. However, some labelling particles were located near axon terminal membrane/axolemma, where microvesicles and polymorphic vesicles were also present (Fig. 4b). In the labelled pituicytes, the label for $P2X_6$ receptors was displayed throughout the cytoplasm (Fig. 4c), at times in association with endoplasmic reticulum (Fig. 4d). Axon profiles bordering on labelled pituicytes frequently showed a scarcity or lack of labelling of neurosecretory granules or microvesicles. (Fig. 4d).

DISCUSSION

The present ultrastructural study involving two immunocytochemical techniques describes the presence of $P2X_6$ receptors within the rat hypothalamo-neurohypophysial system. The data therefore extend previous immunocytochemical and/or *in situ* hybridisation studies demonstrating the presence of P2X receptors at the light microscope level (Collo et al., 1996; Vulchanova et al., 1996; Xiang et al., 1998).

The combination of the pre-embedding immunocytochemical techniques used in our study has provided some new information. It was apparent that more antigenic sites were labelled with the ExtrAvidin method than with colloidal gold-silver immunocytochemistry. This is not surprising however, because with the immunogold labelling, the 1-nm gold–IgG conjugate is of a greater size than the IgG that is applied in the ExtrAvidin method, and thus the gold–IgG conjugate has more restricted access to the antigenic sites of the tissue (fewer $P2X_6$ receptors were labelled). None the less, there was a clear overlap of the labelling that indicates the antigenic sites by the two techniques, e.g. the relation of the label to the endoplasmic reticulum, Golgi complex and neurosecretory granules in the neurosecretory cell bodies. The labelling of the neurosecretory granules in the neurosecretory axon and the cytoplasmic localisation of label in the pituicytes was also evident by both immunoprocedures. However, no labelling of microvesicles was observed with the immunogold method, whereas the ExtrAvidin method labelled the membrane of the vesicles. Taking into account the differences of the two immunoprocedures, it is possible that the immunogold-silver particles primarily labelled the sites rich in antigens, e.g. large clusters of $P2X_6$ receptors. In both immunoprocedures, free immunolabel was detected in the cytoplasm and in association with some intracellular organelles and structures, e.g. endoplasmic reticulum. At this stage, we do not know why there is 'internalisation' of immunoreactivity. It is likely that $P2X_6$ receptors function in co-assembly with other P2X receptor subunits in certain intracellular structures, some of which can be the sites of the receptor synthesis, e.g. the endoplasmic reticulum (both $P2X_{2/6}$ and $P2X_{4/6}$ heteromultimers have been documented as functional P2X receptors). Whether the presence of 'free' immunolabelling in the cytoplasm has anything to do with the transportation of the receptor remains speculative. Internalisation (of immunolabelling) of P2X receptors has already been observed at the ultrastructural level in other regions of the CNS (Lê et al., 1998b).

Some interesting issues are raised from the present study. For example, it is not clear why only a subpopu-

Fig. 3. PVN (a–c, e–g) and SON (d, h, i) labelled for $P2X_6$ receptors with immunogold-silver method. (a) The neurosecretory cell body positive for $P2X_6$ receptors shows the gold-silver dense particles located primarily in the cytoplasm (arrows). (b) Higher magnification of the fragment of the cell presented in panel a shows a relation of immunolabel (arrows) to some neurosecretory granules. (c) Highly magnified Golgi complex of $P2X_6$ positive neurosecretory neurone demonstrates the immunoparticles in association with the vesicle/cistern components; unlabelled granular vesicles are also seen. (d) A fragment of $P2X_6$-positive neurosecretory neurone displays immunolabel at the granular endoplasmic reticulum. An unlabelled axon making an axosomatic synapse is also seen. (e) Note one axon displaying $P2X_6$ immunolabel probably obscuring a granular vesicle (double-headed arrow); another axon shows unlabelled granular vesicles. (f) $P2X_6$ immunolabel is present in a large axon packed with neurosecretory granules whose dense cores were lost during the procedures. (g) $P2X_6$-labelled axon terminal forms an asymmetric synapse with an unlabelled dendrite. (h) An unlabelled axon terminal (only one labelling particle can be seen) forms a symmetric synapse with a $P2X_6$-positive dendrite. (i) In the labelled dendrite note the immunolabel (probably resulting from the silver enhancement of a few gold particles) located at the postsynaptic density. N, nucleus; nsg, neurosecretory granule; m, mitochondrion; Bv, small blood vessel; Go, Golgi complex; gv, granular vesicle; er, endoplasmic reticulum; Ax, axon; Gl, glia; dn, dendrite. Scale bars = 1 μm (a, f), 0.5 μm (b), 10 nm (c), 0.2 μm (d, e, g, i), 0.25 μm (h).

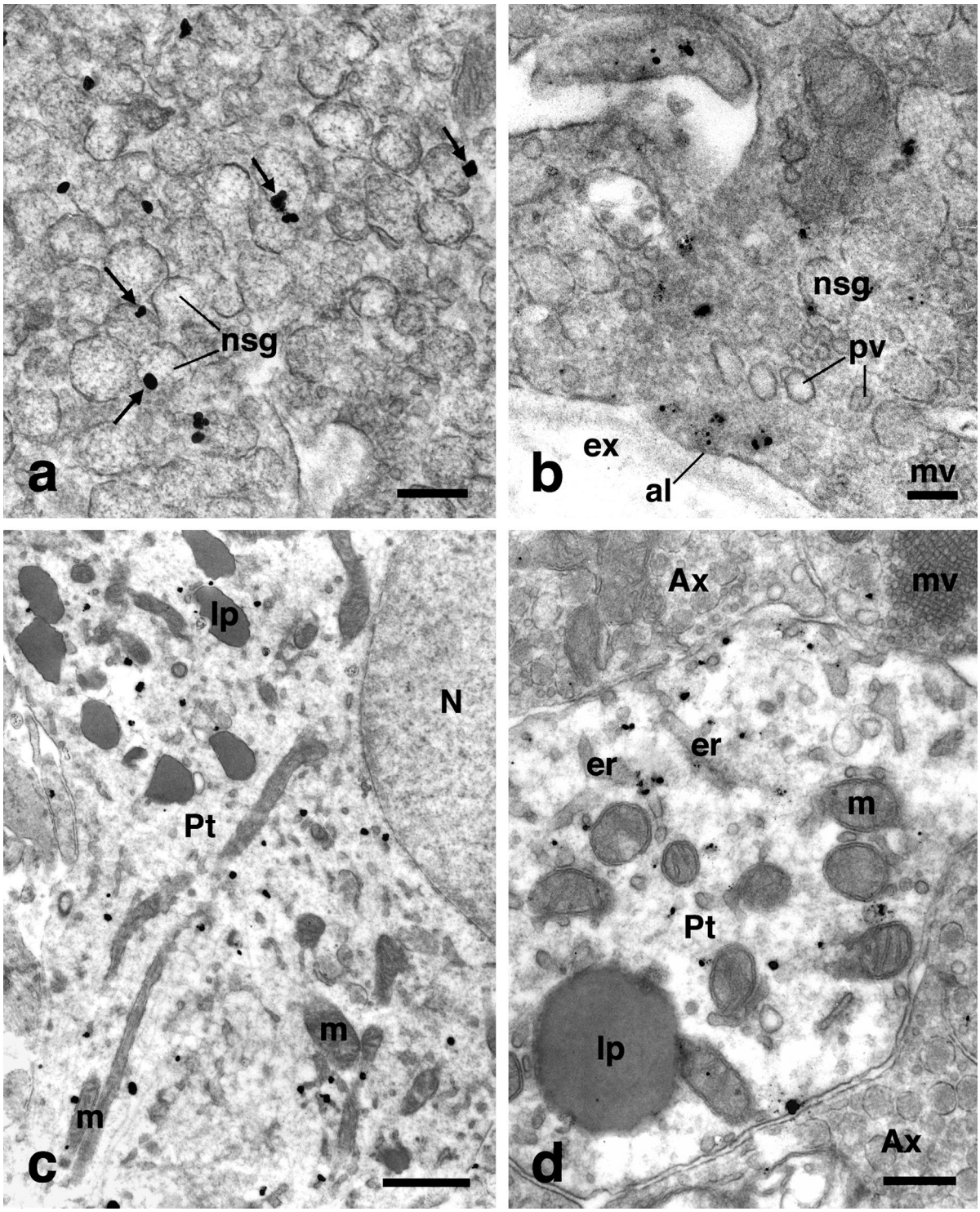

Fig. 4. Neurohypophysis labelled for $P2X_6$ receptors with the immunogold-silver method. (a) A fragment of neurohypophysial axon with neurosecretory granules; note some gold-silver particles located over the granule membrane (arrows). The cores of the granules were lost during the procedures. (b) In axon terminal abutting perivascular region, note the labelled particles are close to the terminal axolemma; near the labelling sites, microvesicles, neurosecretory granules or polymorphic vesicles are also seen. (c) $P2X_6$-labelled pituicyte displays cytoplasmic location of the labelling particles. (d) A process of labelled pituicyte surrounded by unlabelled neurosecretory axons; some labelled particles are associated with endoplasmic reticulum. Note unlabelled microvesicles in an adjacent axon profile. nsg, neurosecretory granule; al, axolemma; mv, microvesicle; pv, polymorphic vesicle; ex, extracellular perivascular space; N, nucleus; m, mitochondrion; lp, lipid droplet; Ax, neurosecretory axon; er, endoplasmic reticulum; pt, pituicyte. Scale bars = 0.2 μm (a), 100 nm (b), 1 μm (c), 0.5 μm (d).

lation of neurosecretory granules, particularly observed with the ExtrAvidin method, display immunoreactivity for $P2X_6$ receptors and why other granules remained immunonegative. A similar finding has been reported for the $P2X_2$ receptor localisation in neurosecretory granules (Loesch et al., 1999). The present study also demonstrated labelled neurosecretory granules in $P2X_6$-positive dendrites, whilst our previous study of the SON and PVN detected only unlabelled granules in $P2X_2$-positive dendrites (Loesch et al., 1999). The meaning of such a discrepancy is not known at this stage. The neurosecretory granules of the hypothalamo-neurohypophysial system are known to co-store ATP with oxytocin and AVP (Gratzl et al., 1980; Zimmermann, 1994) and an increase in intra-axonal Ca^{2+} stimulates the release of these substances to extra-axonal space/circulation (Troadec et al., 1998; Sperlágh et al., 1999). Whether the positive immunolabelling of the neurosecretory granules can be related to the presence of ATP in the intragranular pool remains speculative. The present results suggest that labelled neurosecretory granules may contain P2X receptors ($P2X_6$ receptors).

Another issue concerns the diverse immunoreactivity of microvesicles; these appeared to be positively labelled (the vesicle membrane) by the ExtrAvidin method but remained mostly unlabelled by the immunogold technique. It seems reasonable to look for some explanation of this phenomenon in terms of the techniques/methodology applied, as has already been discussed. However, some axon terminals abutting the perivascular region displayed immunogold-silver labelling in the proximity of the terminal axolemma, where microvesicles were also present. This suggests that at least some parts/fragments of the microvesicles and/or neurosecretory granules express $P2X_6$ receptors, e.g. the membrane structures – the vesicle/granule remnants following hormone release (membrane recycling; see Broadwell et al., 1984). It is also likely that microvesicles of the neurosecretory axon terminals are involved in storage of Ca^{2+} at concentrations suitable for post-stimulus recovery (Nordmann and Chevallier, 1980). In this case, the expression of P2X receptors by microvesicles may vary from axon to axon depending on the axon's physiological state. Combined electrophysiological and immunocytochemical studies, e.g. on isolated neurohypophysial axons, would help to answer such questions. Whether the labelling of the microvesicles by the ExtrAvidin method has anything to do with the recovery of the axon terminal following the release of a neurohypophysial hormone is not known, nor is it known whether microvesicles examined contained Ca^{2+}.

There is no evidence from the present study for $P2X_6$-containing synaptic input to the $P2X_6$-positive neurosecretory cell bodies of both the SON and PVN. In contrast, it has previously been reported that $P2X_2$-positive axons establish synaptic contacts with $P2X_2$-positive neurosecretory neurones in these nuclei, as also do the axo-dendritic synapses involving $P2X_2$-positive axon terminals (Loesch et al., 1999). As yet, it is not known whether $P2X_6$ and $P2X_2$ receptors are associated with the same or a different population of neurosecretory neurones, whether the receptors are colocalised and/or whether they appear as heteromultimeric structures of P2X receptor subtypes. Colocalisation studies (application of double or even triple immunolabelling) would clearly help to answer these questions. Lack of $P2X_6$-positive synaptic input to the $P2X_6$-positive neurosecretory neurones of the SON and PVN in the present study suggests that at least some of the $P2X_6$-positive axons differ from those displaying $P2X_2$ receptors (Loesch et al., 1999). Yet, the site of origin of both the $P2X_2$- and $P2X_6$-positive axons of non-neurosecretory type in the SON and PVN is not clear. The hypothalamic neurosecretory neurones receive synaptic inputs from noradrenaline (NA)-, GABA- and glutamate-containing neurones (see Armstrong, 1995; Buller et al., 1996; Sperlágh et al., 1998; Theodosis et al., 1998; El Majdoubi et al., 2000). It is thus possible that some of these axons also express the P2X receptors. Good candidates for colocalisation of $P2X_6$ receptors are the NA-containing nerve fibres arising from the A1 group of noradrenergic neurones in the caudal brainstem; these neurones may mediate the responses of vasopressin-containing neurones and be the native sources of ATP released in the SON (Buller et al., 1996). According to Kapoor and Sladek (1999), vasopressin-containing neurones of the SON receive direct excitatory input from the caudal ventral lateral medulla, where ATP in A1 neurones may act as the primary excitatory neurotransmitter. Interestingly, a study of A1 NA-containing neurones of the caudal medulla, which provide a direct excitatory input to the SON vasopressin-containing neurones, was one of the first demonstrations of a specific physiological role for central purinergic neurones (Day et al., 1993). Hormone release experiments have clearly demonstrated the coexistence of NA and ATP in the hypothalamic nerve terminals (Sperlágh et al., 1998). During stimulation of neurohypophysial hormone release, e.g. oxytocin, where changes in the intracellular Ca^{2+} store occur, increased synaptic input into hypothalamic neurosecretory neurones has been demonstrated for the GABA- and glutamate-containing nerve fibres (see Theodosis et al., 1998). Since the present study did not detect $P2X_6$ synaptic input to $P2X_6$-positive neurosecretory cells, these receptors seem to operate mostly at postsynaptic sites at the SON and PVN level. This is unlike the $P2X_2$ receptor situation, where both the pre- and postsynaptic locations of the receptor have been detected in the hypothalamo-neurohypophysial system (Loesch et al., 1999).

As in the previous ultrastructural study of the $P2X_2$ receptors (Loesch et al., 1999), pituicytes immunoreactive for $P2X_6$ receptors were apparent in the present study. It was also clear that not all pituicytes expressed $P2X_6$ receptors. The presence of $P2X_6$-positive pituicytes suggests a relationship of these cells with the extracellular action of ATP, and mobilisation of intracellular ions via $P2X_6$ receptors. This is in concert with the possible participation of pituicytes in the ion control of the neurohypophysis and consequently the release of neurohypophysial hormones (Stoeckel et al., 1975; Shaw and Morris, 1980). Depolarisation by K^+ results in the

uptake of Ca^{2+} by pituicytes (Shaw and Dyball, 1984). It has also been shown that vasopressin induces/mobilises $[Ca^{2+}]_i$ in pituicytes (Hatton et al., 1992; Boersma et al., 1993b). Pituicytes, which are commonly related to glial cells (glial fibrillary acidic protein-positive astrocytes), are known to contain vasopressin and oxytocin themselves and thus together with vasopressin- and oxytocin-containing neurohypophysial axons are involved in the release of vasopressin and oxytocin (Boersma et al., 1993a). In fact, the dynamic interaction between the neurohypophysial axons and pituicytes (Hatton, 1990) may lead to auto- and/or 'cross-regulation' of vasopressin and oxytocin release (Boersma et al., 1993a). Whether the pattern of localisation of $P2X_6$ receptors (e.g. their internalisation) in pituicytes relates to the endocrine activity of the hypothalamo-neurohypophysial system is not known at this stage. Pituicytes are not the only glial cells that display P2X receptors. Astrocytes from the rat cerebellum have been reported to display $P2X_1$ receptors (Loesch and Burnstock, 1998). Application of extracellular ATP to astrocytes has been shown to stimulate an increase in $[Ca^{2+}]_i$ in these cells via activation of P2 receptors; as a consequence, the ATP-evoked calcium signal mediates the protein phosphorylation system in these cells (Neary et al., 1991). Glial cells seem to be involved in certain feedback mechanisms. They reply to neuronal activity by increasing their $[Ca^{2+}]_i$ which subsequently stimulates the release of agents from the glia themselves to modulate the neuronal activity (Araque et al., 1999).

The exact role of the $P2X_6$ receptor in the hypothalamo-neurohypophysial system is not yet clear. In some other cell systems, e.g. human embryonic kidney cells (HEK293) transfected with $P2X_6$ cDNA, the homomeric $P2X_6$ receptor is unable to function on its own – a so-called 'false' or 'silent' receptor (Soto et al., 1996; Torres et al., 1999; see King et al., 2000). However, the $P2X_6$ receptor can function in hetero-oligomeric association with other P2X receptor subunits (Torres et al., 1999). The best known co-assembly of the $P2X_6$ receptor is probably with $P2X_2$ and $P2X_4$ subunits in *Xenopus* oocytes (Lê et al., 1998a; King et al., 2000). It is likely that $P2X_6$ receptor subunits act in a regulatory role within a P2X receptor heteromeric structure, modifying the receptor function (Torres et al., 1999). As regards the hypothalamo-neurohypophysial system of the rat, our own preliminary electron immunocytochemical data suggest that both $P2X_6$ and $P2X_2$ receptors can be colocalised in some neural and pituicyte cells (unpublished observations, A. Loesch).

CONCLUSION

The results of the present study have demonstrated that $P2X_6$ receptors are present within the rat hypothalamo-neurohypophysial system and that they are mostly located postsynaptically on neurosecretory neurones.

Acknowledgements—The authors wish to thank Mr R. Jordan for editorial assistance. The support of Roche Bioscience (Palo Alto, CA, USA) is gratefully acknowledged.

REFERENCES

Araque, A., Parpura, V., Sanzgiri, R.P., Haydon, P.G., 1999. Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci. 22, 208–215.

Armstrong, W.E., 1995. Morphological and electrophysiological classification of hypothalamic supraoptic neurons. Prog. Neurobiol. 47, 291–339.

Boersma, C.J., Sonnemans, M.A., Van Leeuwen, F.M., 1993a. Immunoelectron microscopic demonstration of oxytocin and vasopressin in pituicytes and in nerve terminals forming synaptoid contacts with pituicytes in the rat neural lobe. Brain Res. 611, 117–129.

Boersma, C.J., Van Leeuwen, F.W., O'Brien, W.G., Law, G.J., Mason, W.T., Bicknell, R.J., 1993b. Dynorphin 1–17 delays the vasopressin induced mobilization of intracellular calcium in cultured astrocytes from the rat neural lobe. J. Neuroendocrinol. 5, 583–590.

Broadwell, R.D., Catalde, A.M., Balin, B.J., 1984. Further studies of the secretory process in hypothalamo-neurohypophysial neurons: an analysis using immunocytochemistry, wheat germ agglutinin-peroxidase, and native peroxidase. J. Comp. Neurol. 228, 155–167.

Buller, K.M., Khanna, S., Sibbald, J.R., Day, T.A., 1996. Central noradrenergic neurons signal via ATP to elicit vasopressin responses to haemorrhage. Neuroscience 73, 637–642.

Chen, Z.P., Levy, A., Lightman, S.L., 1994. Activation of specific ATP receptors induces a rapid increase intracellular calcium ions in rat hypothalamic neurons. Brain Res. 641, 249–256.

Collo, G., North, R.A., Kawashima, E., Merlo-Pich, E., Neidhart, S., Surprenant, A., Buell, G., 1996. Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. J. Neurosci. 16, 2495–2507.

Day, T.A., Sibbald, J.R., Khanna, S., 1993. ATP mediates an excitatory noradrenergic neuron input to supraoptic vasopressin cells. Brain Res. 607, 341–344.

El Majdoubi, M., Poulain, D.A., Theodosis, D.T., 2000. Activity-dependent morphological synaptic plasticity in an adult neurosecretory system: magnocellular oxytocin neurons of the hypothalamus. Biochem. Cell Biol. 78, 317–327.

Gratzl, M., Torp-Pedersen, C., Dartt, D.A., Treiman, M., Thorn, N.A., 1980. Isolation and characterization of secretory granules from bovine neurohypophyses. Hoppe Seylers Z. Physiol. Chem. 361, 1615–1628.

Harboe, N., Ingild, A., 1973. Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand. J. Immunol. 1 (Suppl.), 161–164.

Hatton, G.I., 1990. Emerging concepts of structure-function dynamics in adult brain; the hypothalamo-neurohypophysial system. Prog. Neurobiol. 34, 437–504.

Hatton, G.I., Bunting, R., Mason, W., 1992. Arginine vasopressin mobilises intracellular calcium via V_1-receptor action in astrocytes (pituicytes) cultured from adult neural lobes. Brain Res. 588, 75–83.

Hiruma, H., Bourque, C.W., 1995. P_2 purinoceptor-mediated depolarization of rat supraoptic neurosecretory cells *in vitro*. J. Physiol. 489, 805–811.

Kanjhan, R.R., Housley, G.D., Burton, L.D., Christie, D.L., Kippenberger, A., Thorne, P.R., Luo, L., Ryan, A.F., 1999. Distribution of the $P2X_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system. J. Comp. Neurol. 407, 11–32.

Kapoor, J.R., Sladek, C.D., 1999. Role of ATP in the regulation of vasopressin release. FASEB J. 13, A1061.

Kidd, E.J., Grahames, C., Simon, J., Michel, A., Barnard, E.A., Humphrey, P.P.A., 1995. Localization of P2X-purinoceptor transcripts in the rat nervous system. Mol. Pharmacol. 48, 569–573.
King, B.F., Townsend-Nicholson, A., Wildman, S.S., Thomas, T., Spyer, K.M., Burnstock, G., 2000. Coexpression of rat $P2X_2$ and $P2X_6$ subunits in *Xenopus* oocyte. J. Neurosci. 20, 4871–4877.
Lê, K.-T., Babinski, K., Séguéla, P., 1998a. Central P2X4 and P2X6 channels subunits coassemble into a novel heteromeric ATP receptor. J. Neurosci. 18, 7152–7159.
Lê, K.T., Villeneuve, P., Ramjaun, A.R., McPherson, P.S., Beaudet, A., Séguéla, P., 1998b. Sensory presynaptic and widespread somatodendritic immunolocalization of central ionotropic P2X ATP receptors. Neuroscience 83, 177–190.
Lemos, J.R., Wang, G., 2000. Excitatory versus inhibitory modulation by ATP of neurohypophysial terminal activity in the rat. Exp. Physiol. 85S, 67S–74S.
Loesch, A., Burnstock, G., 1998. Electron-immunocytochemical localization of $P2X_1$ receptors in the rat cerebellum. Cell Tissue Res. 294, 253–260.
Loesch, A., Miah, S., Burnstock, G., 1999. Ultrastructural localisation of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamo-neurohypophysial system. J. Neurocytol. 28, 495–504.
Mori, M., Tsushima, H., Matsuda, T., 1992. Antidiuretic effects of purinoceptor agonists injected into the hypothalamic paraventricular nucleus of water-loaded, ethanol-anesthetized rats. Neuropharmacology 31, 585–592.
Neary, J.T., Laskey, R., Van Breemen, C., Blicharska, J., Norenberg, L.O., Norenberg, M.D., 1991. ATP-evoked calcium signal stimulates protein phosphorylation/dephosphorylation in astrocytes. Brain Res. 566, 89–94.
Nordmann, J.J., Chevallier, J., 1980. The role of microvesicles in buffering $[Ca^{2+}]_i$ in the neurohypophysis. Nature 287, 54–56.
Oglesby, I.B., Lachnit, W.G., Burnstock, G., Ford, A.P.D.W., 1999. Subunit specificity of polyclonal antisera to the carboxy terminal regions of the P2X receptors, $P2X_1$ through $P2X_7$. Drug Dev. Res. 47, 189–195.
Potter, P., White, T.D., 1980. Release of adenosine 5′-triphosphate from synaptosomes from different regions of the brain. Neuroscience 5, 1351–1356.
Shaw, F.D., Dyball, R.E., 1984. The relationship between calcium uptake and hormone release in the isolated neurohypophysis. A reassessment. Neuroendocrinology 38, 504–510.
Shaw, F.D., Morris, J.F., 1980. Calcium localization in the rat neurohypophysis. Nature 287, 56–58.
Shibuya, I., Tanaka, K., Hattori, Y., Uezono, Y., Harayama, N., Noguchi, J., Ueta, Y., Izumi, F., Yamashita, H., 1999. Evidence that multiple $P2X_2$ purinoceptors are functionally expressed in rat supraoptic neurones. J. Physiol. 514, 351–367.
Soto, F., Garcia-Guzman, M., Karschin, C., Stuhmer, W., 1996. Cloning and tissue distribution of a novel P2XX receptor from rat brain. Biochem. Biophys. Res. Commun. 223, 456–460.
Sperlágh, B., Mergl, Z., Jurányi, Z., Vizi, E.S., Makara, G.B., 1999. Local regulation of vasopressin and oxytocin secretion by extracellular ATP in the isolated posterior lobe of the rat hypophysis. J. Endocrinol. 160, 343–360.
Sperlágh, B., Sershen, H., Lajtha, A., Vizi, E.S., 1998. Co-release of endogenous ATP and [^{3}H]noradrenaline from rat hypothalamic slices: origin and modulation by α_2-adrenoceptors. Neuroscience 82, 511–520.
Stoeckel, M.E., Hindelang-Gertner, C., Dellmann, H.-D., Porte, A., Stutinsky, F., 1975. Subcellular localization of calcium in the mouse hypophysis. I. Calcium distribution in the adeno- and neurohypophysis under normal conditions. Cell Tissue Res. 157, 307–322.
Theodosis, D.T., El Majdoubi, M., Pierre, K., Poulain, D.A., 1998. Factors governing activity-dependent structural plasticity of the hypothalamoneurohypophysial system. Cell Mol. Neurobiol. 18, 285–298.
Torres, G.E., Egan, T.M., Voigt, M.M., 1999. Hetero-oligomeric assembly of P2X receptor subunits. J. Biol. Chem. 274, 6653–6659.
Troadec, J.D., Thirion, S., Nicaise, G., Lemos, J.R., Dayanith, G., 1998. ATP-evoked increases in $[Ca^{2+}]_i$ and peptide release from rat isolated neurohypophysial terminals via a P_{2X2} purinoceptor. J. Physiol. 511, 89–103.
Vulchanova, L., Arvidsson, U., Riedl, M., Wang, J., Buell, G., Surprenant, A., North, R.A., Elde, R., 1996. Differential distribution of two ATP-gated ion channels (P2X receptors) determined by immunocytochemistry. Proc. Natl. Acad. Sci. USA 93, 8063–8067.
Xiang, Z., Bo, X., Oglesby, I.B., Ford, A.P.D.W., Burnstock, G., 1998. Localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamus. Brain Res. 813, 390–397.
Zimmermann, H., 1994. Signalling via ATP in the nervous system. Trends Neurosci. 17, 420–426.

(*Accepted* 28 *June* 2001)