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ORIGINAL ARTICLE

Chemical phenotypes of P2X2 purinoreceptor immunoreactive cell bodies in the area postrema

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Abstract Purines such as adenosine 5′-triphosphate (ATP) act as extracellular messengers through specific purinergic receptors. Three different classes of purinergic receptors have been identified and termed P1, P2X, and P2Y. The purinergic receptor subunit P2X2 is a ligand-gated ion channel that is widely expressed by neurons in the CNS. In the brainstem medulla oblongata, the ionotropic P2X2 receptor (P2X2R) is enriched in the area postrema (AP). Two different antisera to P2X2R were used to determine the chemical nature of P2X2R immunoreactive cell bodies in the rat AP, an area lacking a blood–brain barrier. Subcellularly, P2X2R immunoreactivity was located to the periphery of individual cell bodies. The majority of P2X2R-immunoreactive cells were shown to contain tyrosine hydroxylase (TH) (63.5±7.7%) and dopamine β-hydroxylase (61.5±5.1%). Phenylethanolamine *N*-methyltransferase (PNMT)-containing cells were not detected in the AP, supporting a noradrenergic nature of P2X2R cells in the AP. There were no P2X2R-immunoreactive cells in the AP that contained the GABA-synthesizing enzyme glutamic acid decarboxylase 65. Only single vesicular glutamate transporter 2-immunoreactive cell bodies that were not P2X2R-positive were demonstrated in the AP. Some P2X2R-positive cells in the AP were immunoreactive for the neuropeptides substance P and pituitary adenylate cyclase-activating polypeptide, whereas dynorphin-, enkephalin-, or cholecystokinin-positive cells were not P2X2R-immunoreactive. Presence of P2X2R in a majority of noradrenergic cells of the AP implies that ATP may have a regulatory action on neuronal noradrenaline release from the AP, a circumventricular organ with a strategic position enabling interactions between circulating substances and the central nervous system.

Keywords ATP · Brainstem · Dorsal vagal complex · Noradrenaline · Neuropeptide

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Introduction

The purine adenosine-5′-triphosphate (ATP) has been shown to act as a fast synaptic transmitter in the nervous system via purinergic receptors [see 1–3]. Purine and pyrimidine receptors are divided into two classes, termed P1 and P2. P1 receptors are G-protein-coupled receptors (GPCR) that bind adenosine [see 2]. The P2 receptor family is further divided into ionotropic P2X and metabotropic P2Y receptors [see 2, 4]. The P2X receptors contain two transmembrane regions that respond primarily to ATP and are responsible for fast excitatory neurotransmission [see 5]. The P2X receptor family consists of seven subunits (P2X1-P2X7) coded by seven distinct genes, which can combine to form both heteromeric and homomeric receptors [see 5–7]. The P2X2 receptor (P2X2R) has been demonstrated in several regions of the central nervous system [8–11].

In the brainstem, P2X2R is enriched in the dorsal vagal complex (DVC) [8–11]. The DVC is located in the dorsomedial part of the medulla oblongata and consists of the area postrema (AP), nucleus of tractus solitarius (NTS), and dorsal motor nucleus of the vagus (DMX), all structures which are involved in the regulation of autonomic functions. The AP is located on the dorsal surface of the medulla

oblongata at the inferoposterior limit of the fourth ventricle. It is known to be a circumventricular organ with an extremely rich capillary plexus lacking a blood–brain barrier (BBB) [see 12]. The vascular network of the AP consists of fenestrated blood vessels with an absence of tight junctions between endothelial cells that normally form the diffusion barrier between the luminal and the parenchymal surface of the nervous tissue [see 13]. The AP is composed of a small neuron population containing different receptors that can sense signaling factors secreted by peripheral organs [see 12]. Neuronal cell bodies of the AP extend their projections to several multifunctional integrative centers, such as the NTS, dorsal motor nucleus of vagus, nucleus ambiguous, dorsal tegmental nucleus, and lateral parabrachial nucleus [see 14]. The AP receives inputs from the hypothalamic paraventricular and dorsomedial nuclei and from the caudal NTS [14]. The AP represents a chemoreceptor trigger zone for vomiting (emesis) [see 13]. The anatomical position and the lack of an intact BBB makes the AP unique in detecting emetic substances in the blood [see 12]. Activation of the AP is likely to lead to nausea and vomiting through its projection to the neighboring NTS [see 12, 13]. It has been shown that purinergic P2X receptor activation via intraperitoneal injection of a slowly degradable ATP analog, α,β-methyleneATP (α,β-meATP), induces emetic responses in ferrets and *Suncus murinus* (house musk shrews) [15]. Moreover, ATP has been shown to have an excitatory effect on isolated rat area postrema neurons, an effect that can be inhibited by P2X receptor antagonists [16, 17].

In order to clarify the transmitter content of P2X2R-containing cell bodies of the AP, we have performed a series of double-labeling experiments using two different P2X2R antisera combined with antisera to markers for classical transmitters and to several neuropeptides. The aim of the study is to provide results that may help us to understand by which mediators ATP operates after binding to P2X2R in the AP.

Materials and methods

All studies were performed in accordance with guidelines from the Swedish National Board for Laboratory Animals and were approved by the local ethical committee. Male Sprague–Dawley rats ($n=5$), weighing 150–200 g (Scanbur-BK, Stockholm, Sweden) were used. The rats were anaesthetized with a combination of ketamine (75 mg/kg)+medetomidine (1 mg/kg) i.p. and were treated with an injection of colchicine (120 μg in 20 μl 0.9% NaCl; Sigma-Aldrich, St. Louis, MO, USA) via a Hamilton syringe (Hamilton, Reno, NV, USA) into the lateral ventricle (coordinates: 1 mm rostral, 1 mm lateral, and 5 mm ventral of Bregma) using a stereotaxic equipment (Harvard Apparatus, Holliston, MA, USA) 24 h before sacrifice. Colchicine is known to arrest axonal transport, thereby increasing the levels of transmitters, enzymes, and peptides/proteins in the cell soma. Normal (untreated) and colchicine-treated animals were anaesthetized with sodium pentobarbital (40 mg/kg; i.p.) and perfused via the ascending aorta with 50 ml of Ca^{2+}-free Tyrode's solution (37°C) followed by 50 ml of formalin-picric acid fixative (37°C) (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9). Perfusions were thereafter continued for 6 min with ice-cold fixative, as described above. The brains were rapidly dissected out, postfixed in the same fixative for 90 min, and rinsed for at least 24 h in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% Bacitracin (Sigma-Aldrich), and 0.01% sodium azide (Merck, Darmstadt, Germany). The brains were frozen and sections were cut at −20°C (10 μm) in a cryostat (Microm HM 560; Microm International GmbH, Walldorf, Germany) at levels containing the AP (an interval at approximately Bregma levels −13.5 to −14.1). Sections were incubated overnight at 4°C with primary antibodies/antisera (see Table 1 for specifications, sources, and references). For double-labeling, mixtures of primary antibodies/antisera were used. After rinsing the sections in phosphate-buffered saline (PBS; 0.1 M phosphate buffer, pH 7.4) for 30 min, the sections were incubated with: Cy2-conjugated donkey anti-mouse, donkey anti-rabbit, donkey anti-guinea pig or donkey anti-goat secondary antibodies, or Cy3-conjugated donkey anti-rabbit or donkey anti-guinea pig secondary antibodies for 60 min at room temperature (all secondary antibodies were diluted 1:250; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After rinsing in PBS for 30 min, the sections were mounted in 2.5% of 1,4-diazabicyclo[2.2.2] octane (DABCO; Sigma) in glycerol.

For comparison, double-labeling with guinea pig and rabbit P2X2R antisera was performed using the tyramide signal amplification (TSA) (Perkin Elmer, Shelton CT, USA) technique. Sections were air dried for 2 h in room temperature, quenched with 1% H_2O_2 in PBS for 15 min, preincubated with PBS for 20 min, and incubated with guinea pig P2X2R antiserum overnight. On the second day, sections were washed in TNT buffer (Perkin Elmer) for 30 min, incubated with TNB buffer (0.10 M Tris–HCl, 0.15 M NaCl, 0.5% BMP; Perkin Elmer) for 30 min, incubated with horse radish peroxidase (HRP)-conjugated donkey secondary antibody (diluted 1:250 in TNB buffer, Jackson ImmunoResearch), washed in TNT buffer (0.1 M Tris–HCl, 0.15 M NaCl, 0.05% Tween-20; Perkin Elmer) for 30 min, incubated with TSA amplification reagent (diluted 1:100 for fluorescein or 1:200 for Cy3 in amplification diluent (Perkin Elmer) and washed in TNT buffer for 15 min. Before incubation with rabbit P2X2R antiserum, sections were quenched again with H_2O_2, and the protocol repeated for the second primary and secondary

Table 1 Specifications on primary antibodies/antisera

Immunogen	Host species	Dilution	Code/source/reference
P2X2R	Rabbit	1:5,000	APR-003; Alomone Labs, Jerusalem, Israel; [see ref. 18]
P2X2R	Guinea pig	1:7,000	GP14106; Neuromics, Edina, MN, USA; [see ref. 18]
TH	Rabbit	1:800	[see ref. 19]
DBH	Mouse	1:500	Clone 4 F10.2; Chemicon, Temecula, CA, USA
PNMT	Rabbit	1:400	31 M; [see ref. 20]
GAD65	Mouse	1:1,000	MAB351; Millipore, Temecula, CA, USA
VGLUT2	Guinea pig	1:500	[see ref. 21]
Substance P	Mouse	1:25	sc-58591; Santa Cruz Biotechnology, Santa Cruz, CA, USA
PACAP	Mouse	1:20	MabJHH1; [see ref. 22]
Dynorphin	Guinea pig	1:4,000	GP10110; Neuromics, Edina, MN, USA
Enkephalin	Mouse	1:400	MAB350; Millipore, Temecula, CA, USA
CCK	Rabbit	1:800	CCK 26–33; R18; [see ref. 23]

antibodies. After the final washing with TNT buffer on the third day, slides were mounted in DABCO. Sections were examined in a BioRad RadiancePlus confocal laser scanning system (Bio-Rad Laboratories, Hercules, CA, USA). The excitation wavelength was 488 nm for Cy2- and 543 nm for Cy3-induced fluorescence. Images were acquired in sequential mode to avoid potential "bleed-through" of fluorescence.

For triple-labeling, a mixture of guinea-pig P2X2R, rabbit antiserum to tyrosine hydroxylase (TH) and mouse monoclonal antibodies to dopamine β-hydroxylase (DBH) were used. After rinsing the sections in phosphate-buffered saline (PBS; 0.1 M phosphate buffer, pH 7.4) for 30 min, the sections were incubated with: Cy2-conjugated donkey anti-guinea pig, Cy3-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-mouse secondary antibodies for 60 min at room temperature (all secondary antibodies were diluted 1:250; Jackson ImmunoResearch Laboratories). After rinsing in PBS for 30 min, the sections were mounted as previously described.

Sections double-labeled with different P2X2R antisera were examined in a Nikon Microphot-SA microscope and images were collected using a Nikon DXM1200c camera combined with the NIS Elements F 3.0 software (Nikon, Tokyo, Japan). Sections triple labeled with P2X2, TH, and DBH antisera/antibodies were collected using a Nikon Digital Sight DS-Qi1 camera combined with a Digital Sight DS-U2 control unit. Images were processed using Adobe Photoshop CS2 (Adobe, Inc., San Jose, CA, USA).

To evaluate antisera specificity, rabbit P2X2R antiserum APR-003 was preabsorbed with immunogen peptide (amino acid sequence: SQQDSTSTDPKGLAQL) corresponding to residues 457–472 of rat P2X2R (Swiss-Prot accession number P49653) and guinea pig P2X2R antiserum GP14106 was preabsorbed with immunogen peptide (amino acid sequences: DSTSTDPKGLAQL) corresponding to residues 460–472 of rat P2X2R (Swiss-Prot accession number P49653**)** at a concentration of 10^{-5} M for 2 h in a shaker at room temperature. Adjacent sections were incubated with preabsorbed or non-preabsorbed guinea pig or rabbit P2X2R antiserum.

For quantification of P2X2R/TH and P2X2R/DBH double-immunoreactive neurons, double-labeled cells were counted and compared to the total number of P2X2R-positive cells in images of immunolabeled cross-sections. Six sections per animal (n=4) were counted. For statistical analysis, one-way analysis of variance (ANOVA) followed by Tukey post-test was used. PrismGraph software (GraphPad Software, Inc., San Diego, CA, USA) was used to perform the statistical analysis.

Results

Incubation with rabbit antiserum APR-003 or guinea pig antiserum GP14106 to the P2X2R antisera revealed strong P2X2R immunoreactivity in the AP and NTS (Fig. 1a, c) of colchicine-treated rats (n=5). Particularly strong P2X2R immunoreactivity was observed in the zone between the AP and NTS, forming a V-shaped structure (*funiculus separans*) (Fig. 1a, c). A few P2X2R-immunoreactive cell bodies and scattered P2X2R-immunoreactive fibers were seen in the DMX (Fig. 1a, c). Other areas of the medulla oblongata at the level of the AP were devoid of P2X2R immunoreactivity (Fig. 1a, c; data not shown). In the AP, P2X2R immunoreactivity was demonstrated in both cell bodies and cellular extensions (Figs. 2c, d; 3d, g; 4d; 5b; 7c; 8c; 9c; and 10a, c, e). In the NTS, there was a very high density of P2X2R-immunoreactive fibers without clear evidence for labeling of cell bodies (Figs. 1a, c; 2a, b; 3a; 4a; 5a; 6a; 7a; and 8a).

In general, incubation with rabbit P2X2R antiserum APR-003 resulted in a stronger staining as compared to staining obtained with the guinea pig P2X2R antiserum (Fig. 1a, c). In order to enhance the sensitivity of the guinea

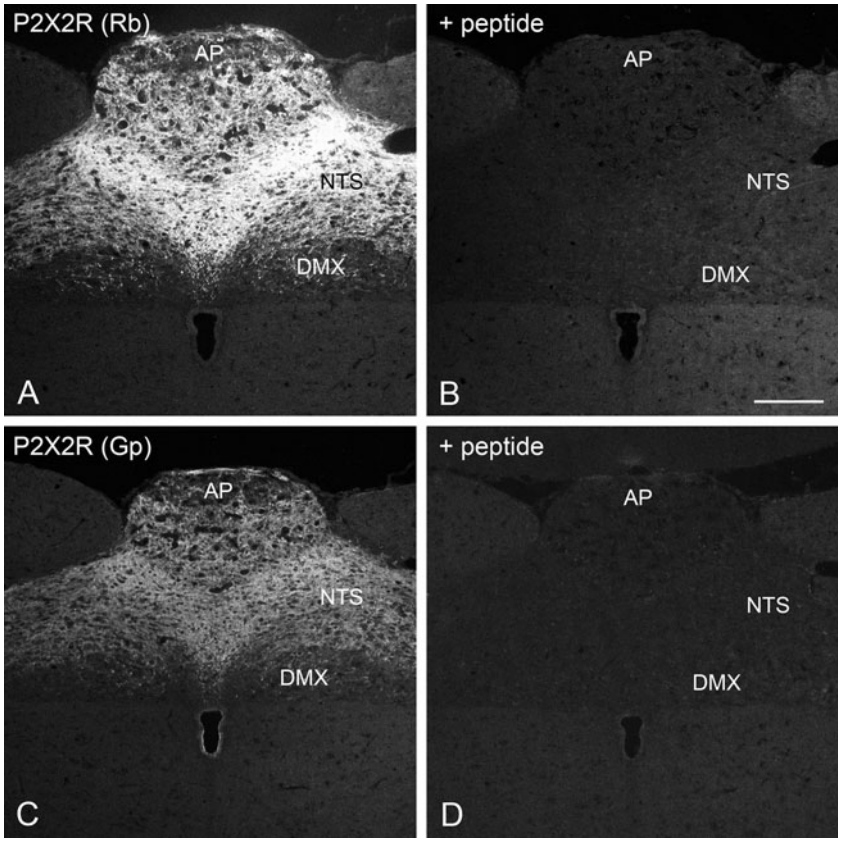

Fig. 1 **a**–**d** Images of two semi-adjacent sections of the rat area postrema (AP) after incubation with rabbit (Rb) antiserum APR-003 (**a**) or guinea pig (Gp) antiserum GP14106 (**c**) generated to the P2X2 receptor (P2X2R) and rabbit or guinea pig P2X2R antiserum preabsorbed with P2X2R immunogen peptide at a concentration of 10^{-5} M (**b**, **d**). P2X2R gives rise to dense immunostaining in the entire AP. Very strong P2X2 immuno-reactivity is seen in the dorsal aspect of the nucleus tractus solitarius (NTS), whereas weaker staining is observed in the ventral part of the NTS and the dorsal motor nucleus of the vagus (DMX) (**a**, **c**). Preabsorption of rabbit or guinea pig P2X2R antisera with immunogen peptide results in a total disappearance of P2X2R immunoreactivity in the AP, NTS and DMX (compare **a**, **c** with **b**, **d**, respectively). Scale bar=200 μm

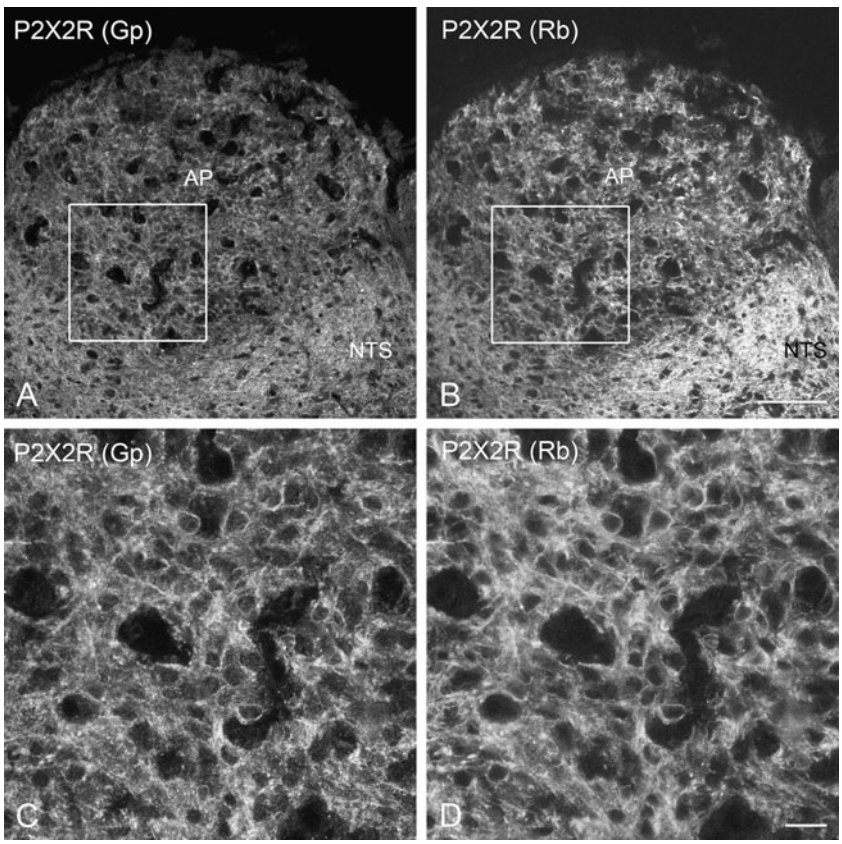

Fig. 2 **a**–**d** Images of a section of the rat area postrema (AP) after double-labeling with guinea pig (Gp) antiserum GP14106 (**a**, **c**) and rabbit (Rb) antiserum APR-003 (**b**, **d**) to the P2X2 receptor (P2X2R). *Rectangles* in **a** and **b** indicate higher magnification as shown in **c** and **d**. Both antisera give rise to identical staining patterns. *DMX* dorsal motor nucleus of the vagus, *NTS* nucleus tractus solitarius. Scale bars=100 μm (**a**, **b**); 20 μm (**c**, **d**)

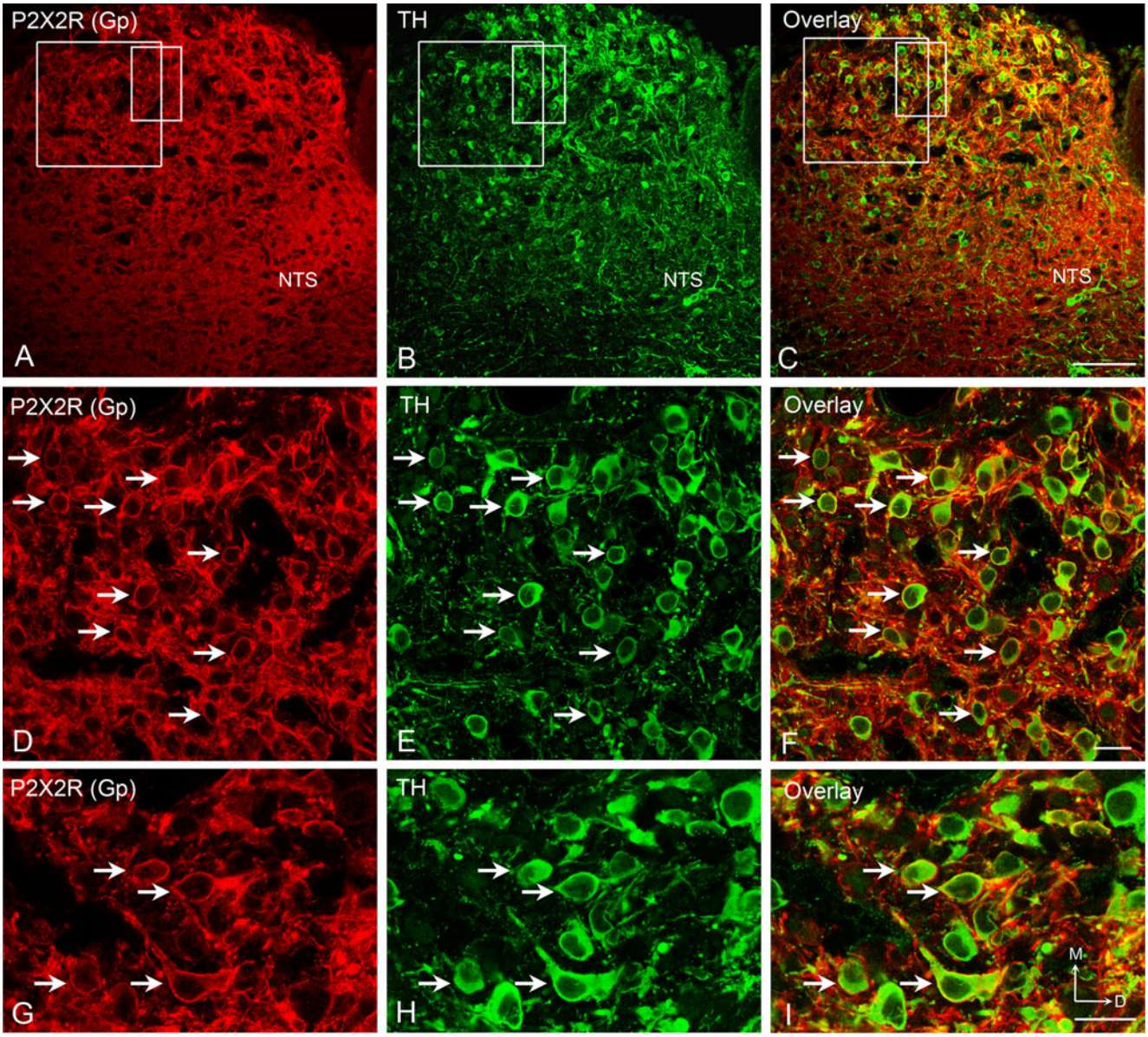

Fig. 3 **a**–**i** Images of sections of the rat area postrema (AP) incubated with guinea pig (Gp) antiserum to the P2X2 receptor (P2X2R) (**a**, **d**, **g**) (*red*) and rabbit antibodies to the catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) (**b**, **e**, **h**) (*green*). Higher magnifications seen in **d**–**f** and **g**–**i** are indicated by boxes in **a**–**c**. Overlay of P2X2R and TH immunoreactivity is shown in images **c**, **f**, and **i**. **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. **b** Many TH-immunoreactive cell bodies are seen in all part of the AP. **d**–**i** Higher magnifications reveal that virtually all P2X2R-positive cell bodies in the AP also contain TH immunoreactivity (compare *arrows*). **d**, **g** High magnification of double-labeled cells in the AP illustrates a preferential subcellular localization of P2X2R immunoreactivity to the periphery of the cell body, most likely representing labeling of the plasma membrane, whereas TH immunoreactivity is present in the cytoplasm. *NTS* nucleus tractus solitarius, *D* dorsal, *M* medial. Scale bars=100 μm (**a**–**c**); 20 μm (**d**–**i**)

pig P2X2R antiserum, TSA was used in some experiments. The use of TSA resulted in an increased sensitivity and intensity of the staining, but at the expense of a lower cellular resolution. In individual cell bodies, the two different P2X2R antisera revealed that P2X2R immunoreactivity was predominantly localized to the periphery, most likely representing labeling of the plasma membrane (Figs. 2c, d; 3d, g; 4d; 5b; 7c; 8c; 9a,c; and 10a,c,e).

Incubation with rabbit P2X2R antiserum APR-003 or guinea pig P2X2R antiserum GP14106 that had been preabsorbed with P2X2R blocking peptide (10^{-5} M) did not show any immunoreactivity as compared to adjacent sections incubated with antisera alone (Fig. 1a–d). Incubation of the same section with rabbit antiserum APR-003 and guinea pig antiserum GP14106 revealed that both antisera stained identical cell populations and neuronal structures as

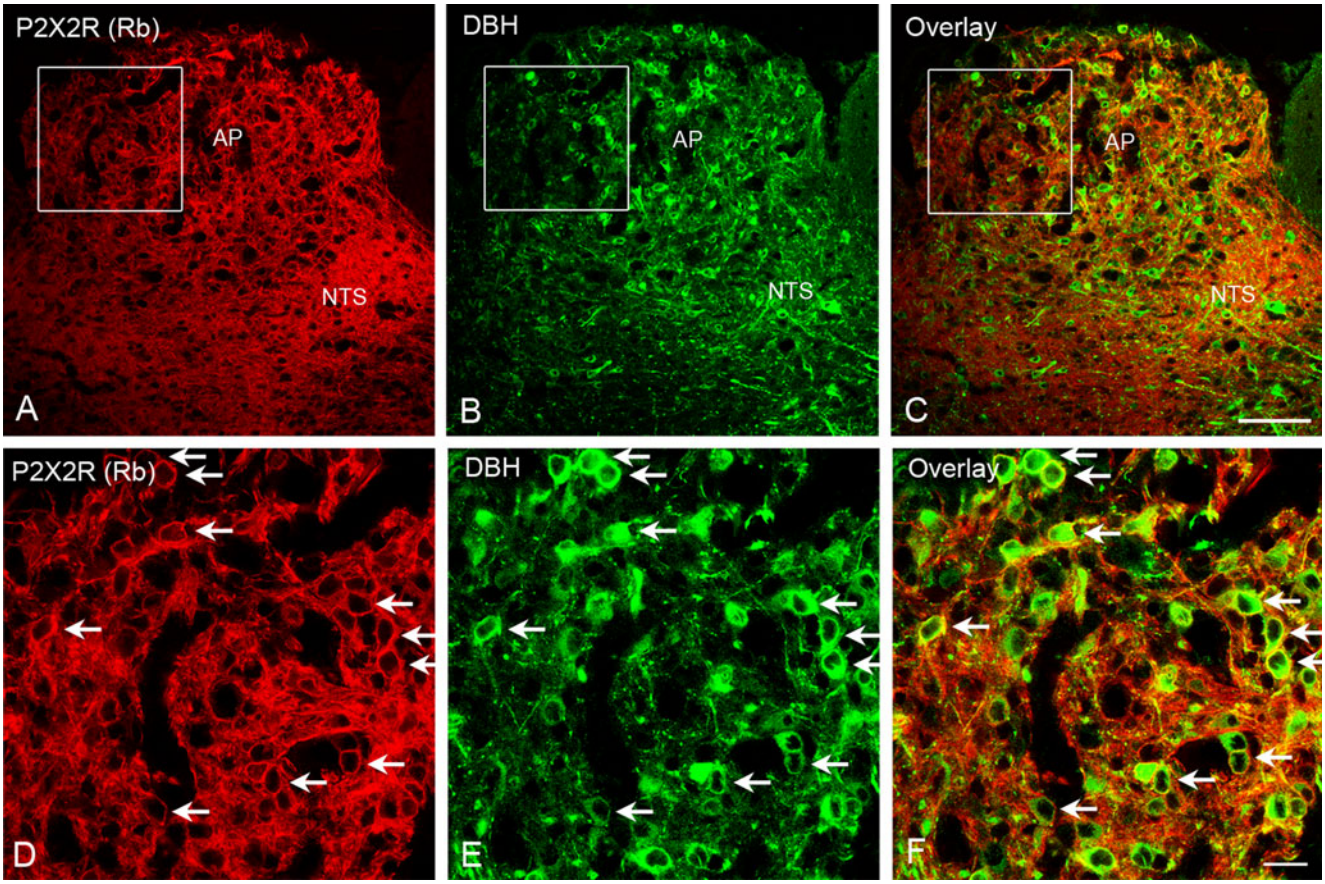

Fig. 4 **a**–**f** Images of sections of the rat area postrema (AP) incubated with rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**, **d**) (*red*) and mouse monoclonal antibodies to dopamine-β-hydroxylase (DBH), the enzyme required for synthesis of noradrenaline and adrenaline (**b**, **e**) (*green*). Higher magnifications as seen in **d**–**f** are indicated by boxes in **a**–**c**. Overlay of P2X2R and DBH immunoreactivity is shown in images **c** and **f**. **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. **b** DBH-immunoreactive cell bodies are seen throughout the AP. Higher magnifications reveal that most P2X2R-positive cell bodies in the AP also contain DBH immunoreactivity (compare arrows in **d**–**f**). High magnification of double-labeled cells in the AP illustrates a preferential subcellular localization of P2X2R immunoreactivity to the periphery of the cell body, most likely representing the plasma membrane (**d**), whereas DBH immunoreactivity is present in the cytoplasm (**e**). *NTS* nucleus tractus solitarius. Scale bars =100 μm (**a**–**c**); 20 μm (**d**–**f**)

well as gave identical staining at the subcellular level, which further supported the specificity of the two different P2X2R antisera (Fig. 2a–d).

To investigate the chemical phenotypes of P2X2R-immunoreactive neurons in the AP, a series of double-labeling experiments were conducted using rabbit P2X2R antiserum APR-003 and guinea pig P2X2R antiserum GP14106 in combination with antisera to markers for classical transmitters and neuropeptides. In the AP, double-labeling with guinea pig antiserum to P2X2R and rabbit antiserum to the catecholamine synthesizing enzyme tyrosine hydroxylase (TH) showed that the majority of P2X2R-positive cell bodies also contained TH (Fig. 3a–i). Similarly, double-labeling with rabbit antiserum to P2X2R and mouse monoclonal antibodies to the noradrenaline- and adrenaline-synthesizing enzyme DBH revealed that the majority of P2X2R-positive cell bodies also contained DBH (Fig. 4a–f). Triple-labeling, combining guinea pig antiserum to P2X2R with rabbit TH antiserum and mouse monoclonal DBH antibodies showed that P2X2R-positive neurons contained TH and DBH immunoreactivity (Fig. 5a–f). Quantification revealed that 63.5±7.7% (mean±S.E.; n=4) and 61.5±5.1% (mean±S.E.; n=4) of P2X2R-positive cells contained TH and DBH, respectively. In contrast, no cells containing immunoreactivity for the adrenaline-synthesizing enzyme phenylethanolamine-*N*-methyltransferase (PNMT) were demonstrated in the AP, whereas PNMT-positive cells were seen in the NTS (Fig. 6b). These results support the presence of noradrenaline-, but not adrenaline-containing cells, in the AP.

P2X2R antiserum combined with antiserum to the GABA-synthesizing enzyme glutamic acid decarboxylase 65 (GAD65) did not reveal any colocalization. Instead, the P2X2R- and GAD65-immunoreactive cell bodies were seen to represent distinct entities (Fig. 7a–d). Using an antiserum to the vesicular glutamate transporter 2 (VGLUT2), a marker for glutamatergic neurons, mainly nerve terminals,

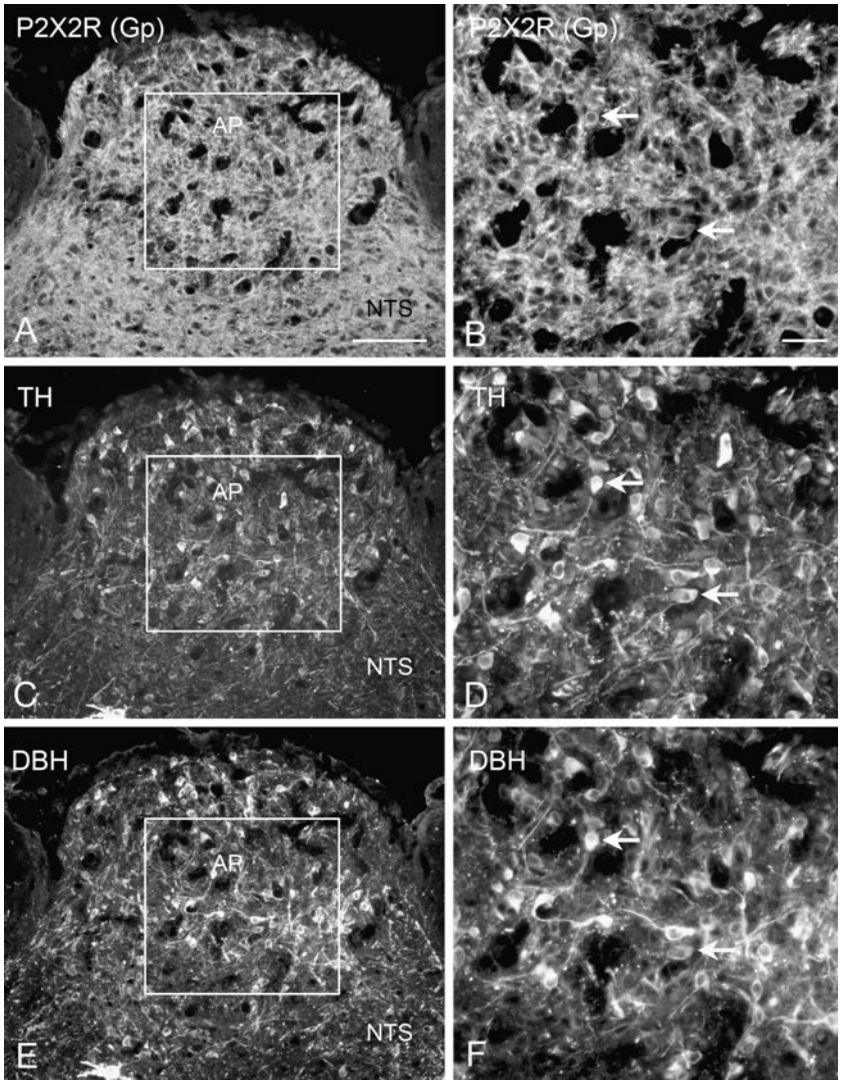

Fig. 5 **a**–**f** Images of a section of the rat area postrema (AP) incubated with guinea pig (Gp) antiserum to the P2X2 receptor (P2X2R) (**a**, **b**), rabbit antibodies to tyrosine hydroxylase (TH) (**c**, **d**) and mouse monoclonal antibodies to dopamine-β-hydroxylase (DBH) (**e**, **f**). Higher magnifications as shown in **b**, **d**, and **f** are indicated by *boxes* in **a**, **c**, and **e**, respectively. **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. Many cell bodies containing TH (**c**) and DBH (**e**) immunoreactivity seen in different parts of the AP. Higher magnifications reveal that P2X2R-immunoreactive cell bodies contain TH and DBH immunoreactivity (see *large arrows* in **b**, **d**, and **f**). *NTS* nucleus tractus solitarius. Scale bars=100 μm (**a**, **c** and **e**); 20 μm (**b**, **d** and **f**)

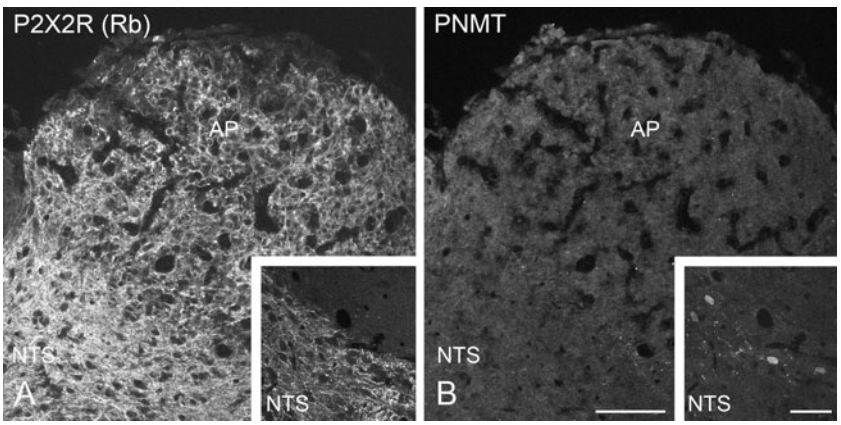

Fig. 6 **a**, **b** Images of sections of the rat area postrema (AP) incubated with rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**) and guinea pig (Gp) antiserum to the adrenaline-synthesizing enzyme phenylethanolamine *N*-methyltransferase (PNMT) (**b**). **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. **b** No PNMT-immunoreactive cell bodies are seen throughout the AP. Higher magnification reveal that PNMT-immunoreactive cell bodies are present in the NTS. *NTS* nucleus tractus solitarius. Scale bars=100 μm (**a**, **b**); 20 μm (*boxes* in **a**, **b**)

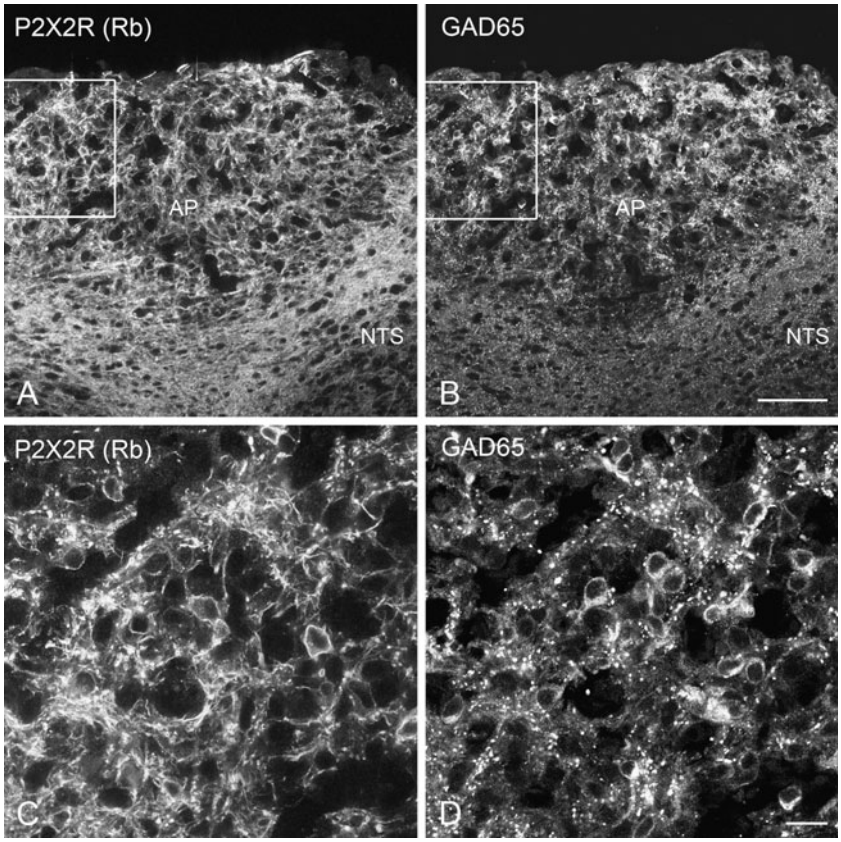

Fig. 7 **a**–**d** Images of a section of the rat area postrema (AP) after double-labeling with rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**, **c**) and mouse monoclonal antibodies to the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) (**b**, **d**). **c**, **d** Higher magnifications are indicated by boxes in **a** and **b**, respectively. **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. **b** GAD-immunoreactive cell bodies and nerve terminals are detected throughout the AP. **c**, **d** Higher magnification reveals that P2X2R- and GAD-positive neurons represent two distinct cell populations. *DMX* dorsal motor nucleus of the vagus, *NTS* nucleus tractus solitarius; Scale bars=100 μm (**a**, **b**); 20 μm (**c**, **d**)

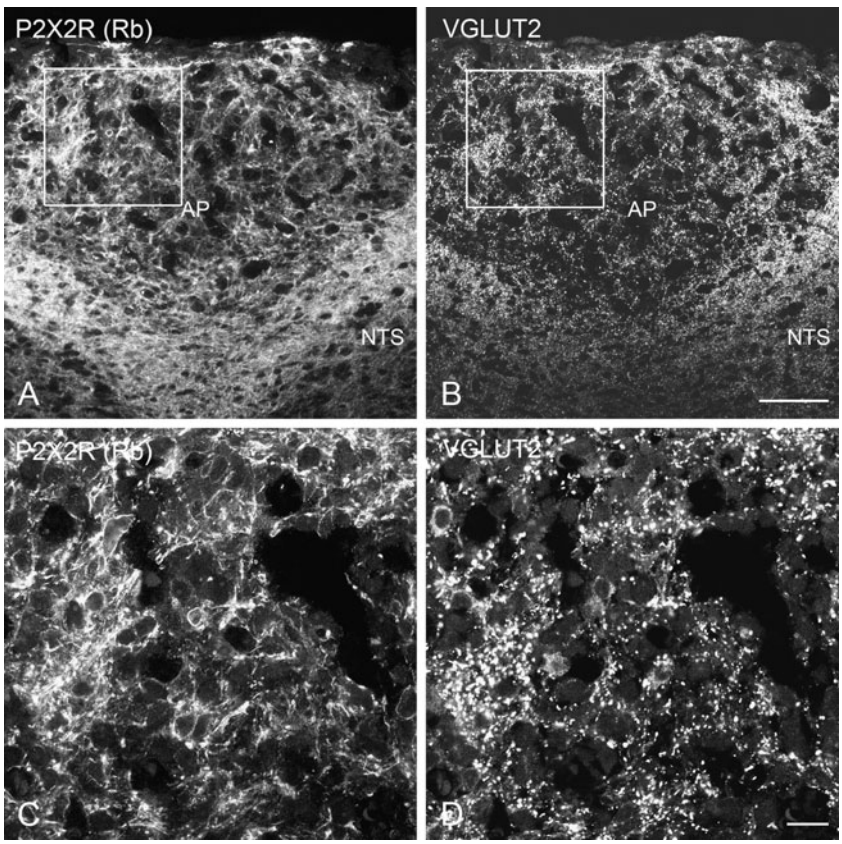

Fig. 8 **a**–**d** Images of a section of the rat area postrema (AP) after double-labeling with rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**, **c**) and guinea pig antibodies to the vesicular glutamate transporter 2 (VGLUT2) (**b**, **d**), a marker for glutamatergic neurons. **c**, **d** Higher magnifications are indicated by *boxes* in **a** and **b**, respectively. **a** P2X2R-immunoreactive cell bodies and neural extensions are detected throughout the AP. **b** VGLUT2-immunoreactive cell bodies and nerve terminals are detected throughout the AP. **c, d** Higher magnification reveal no double-positive cells. *DMX* dorsal motor nucleus of the vagus, *NTS* nucleus tractus solitarius. Scale bars=100 μm (**a**, **b**); 20 μm (**c**, **d**)

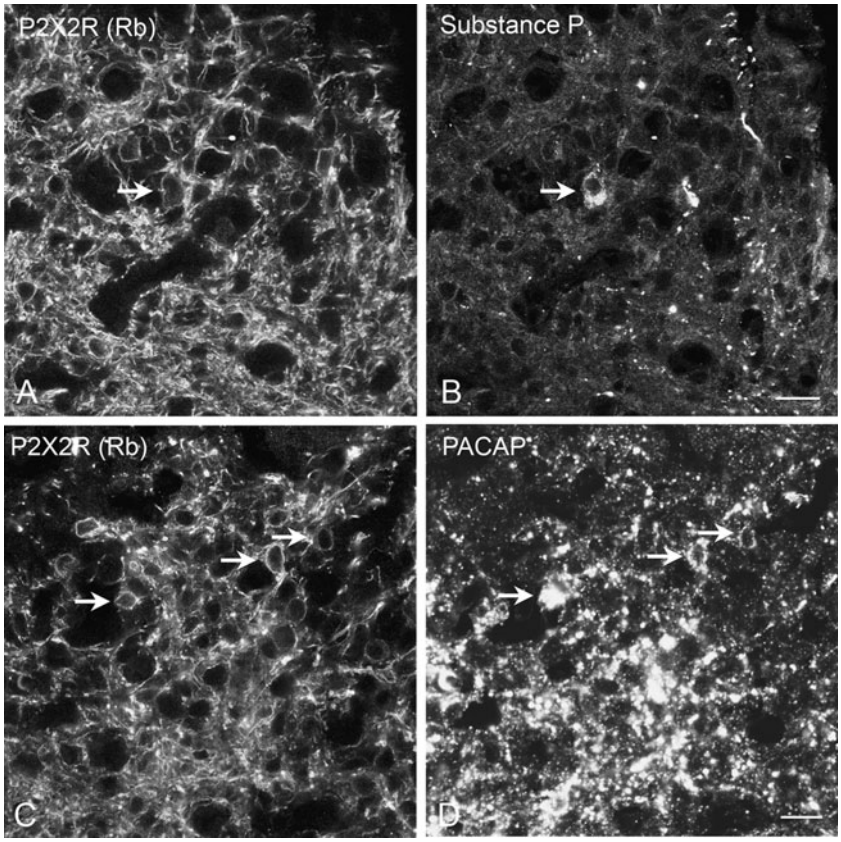

Fig. 9 **a**–**d** Images of a section of the rat area postrema (AP) after double-labeling with rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**, **c**) and mouse monoclonal antibodies to substance P (**b**) or pituitary adenylate cyclase-activating polypeptide (PACAP). Tyramide signal amplification (TSA) was used to enhance staining with PACAP antibodies. Single P2X2R-positive neurons also contain substance P (compare *arrows* in **a** and **b**) or PACAP (compare *arrows* in **c** and **d**). Scale bars=20 μm

only a few labeled cell bodies could be detected even after colchicine treatment (see "Discussion"). Such VGLUT2-immunoreactive cell bodies were not seen to contain P2X2R immunoreactivity (Fig. 8a–d).

Using antisera to several neuropeptides, it was seen that a few P2X2R-immunoreactive cell bodies in the AP contained immunoreactivity for substance P or PACAP (Fig. 9a–d), but not for dynorphin, enkephalin, or cholecystokinin (CCK) (Fig. 10a–f).

Discussion

The present study shows that the P2X2R purinoreceptor protein is highly enriched in the AP, NTS and to a lesser extent also in the DMX, whereas other regions of the medulla oblongata at the level of the AP are virtually devoid of P2X2R immunoreactivity. These results are in agreement with previously published results on the localization of P2X2R mRNA [10, 24, 25] and P2X2R protein [8–11]. Whereas the presence of P2X2R mRNA and protein in cell bodies of the NTS has been described [8, 26], others have seen P2X2R immunoreactive cell bodies in the AP and DMX, but not the NTS [9]. The latter results are in agreement with our results. However, it is important to point out that the high density of P2X2R-immunoreactive structures in the NTS, made it difficult to discriminate between P2X2R immunoreactivity confined to nerve fibers vs. the plasma membrane in cell bodies. It has been shown that P2X2R-immunoreactivity in the NTS is present in neuronal fibers as revealed by electron microscopy [9].

The specificity of the immunoreaction obtained with the rabbit (APR-003) and guinea-pig (GP14106) antisera to P2X2R was supported by preabsorption experiments and the demonstration of P2X2R immunoreactivity in the same cell bodies and fibers using double-labeling as well as an identical labeling and subcellular localization combining the rabbit and guinea pig P2X2R antisera. The specificity of the antisera used in the present study was also supported by earlier published results on the specificity of the two used antisera employing the same antisera in the hypothalamus [18]. Furthermore, others have reported a similar distribution of P2X2R-immunoreactive cell bodies and fibers in the AP using the same antiserum (APR-003) [9].

Our analysis of the chemical nature of the high number of P2X2R-immunoreactive cell bodies in the AP revealed that the majority of P2X2R-containing cell bodies in the AP have a noradrenergic nature [27, 28], since they contained TH and DBH, but not PNMT. The noradrenergic AP neurons send dense projections to the NTS and the lateral parabrachial nucleus, both regions associated with regulation of feeding behavior [14, 29, 30]. The noradrenergic cells of the AP have been shown to be involved in the hypophagic action of amylin

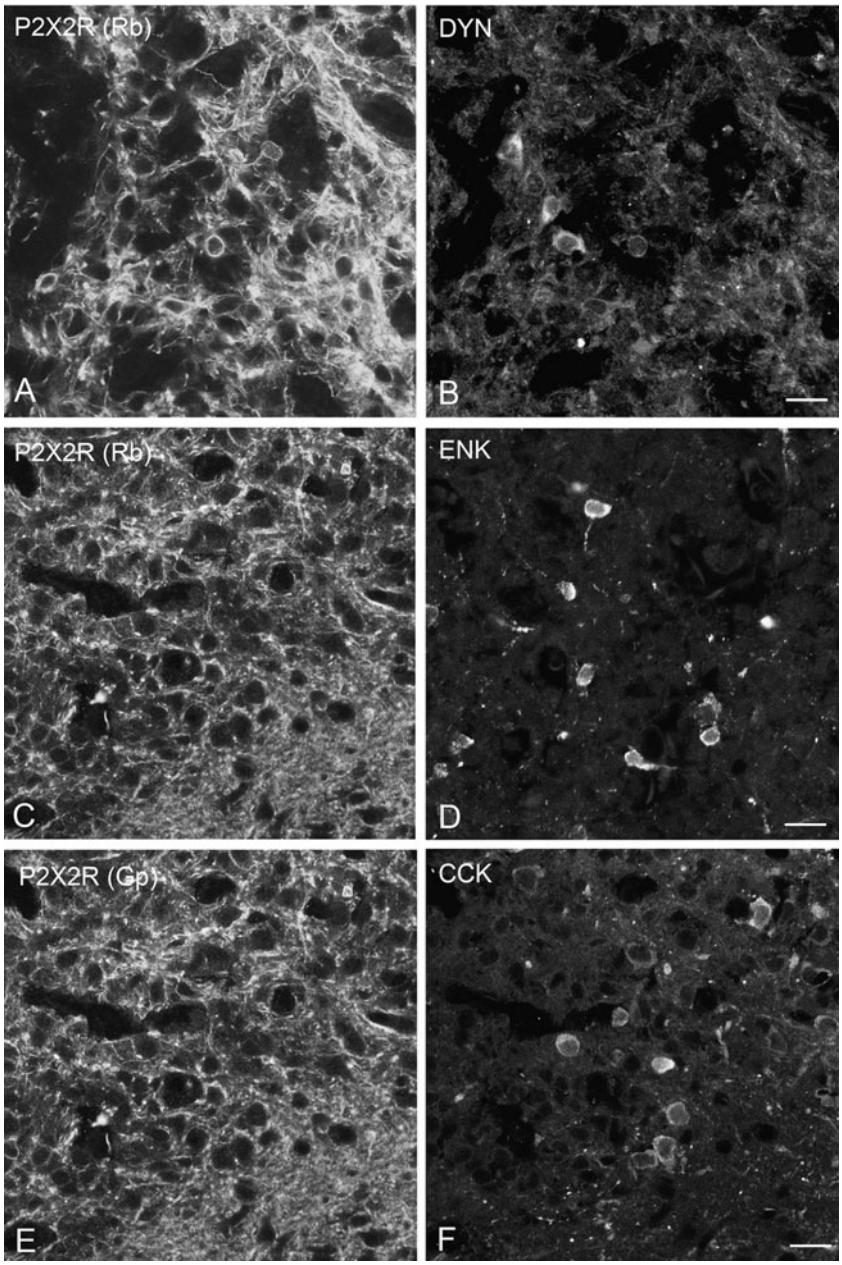

Fig. 10 **a**–**f** Images of a section of the rat area postrema (AP) after combining rabbit (Rb) antiserum to the P2X2 receptor (P2X2R) (**a**, **c**) with guinea pig (antiserum to dynorphin (DYN) (**b**) or mouse monoclonal antibodies to enkephalin (ENK) (**d**) or combining guinea pig (Gp) antiserum to P2X2R (**e**) with rabbit antiserum to cholecystokinin (CCK) (**f**). There is no evidence for the presence of P2X2R immunoreactivity in DYN-, ENK- or CCK-immunoreactive cell bodies. Scale bars=20 μm

[31]. Lesioning noradrenaline-containing neurons in the AP using a saporin-conjugated antibody against DBH, has shown an unresponsiveness to amylin's hypophagic action [31]. Whether the ATP acting on P2X2R-containing neurons in the AP originates from ATP-containing nerve fibers innervating the AP, such as the hypothalamic paraventricular and dorsomedial nuclei or reciprocal connections from the NTS or lateral parabrachial nucleus remains to be shown. Since the AP contains a network of TH- and DBH-containing neuronal fiber network, it is also possible that the AP neurons themselves release ATP and that the P2X2R receptors are presynaptic and serve as autoreceptors. Recently, a vesicular nucleotide transporter (VNUT or SLC17A9) mediating neuronal ATP release has been identified [32] and used for the anatomical identification of VNUT-immunoreactive neurons [33]. A cellular marker for VNUT may be used to clarify whether the ATP releasing neurons are the same ones that have P2X2R. Such a marker may be used to clarify whether the P2X2R in the AP neurons may represent autoreceptors. The possibility that ATP acting on P2X2R in the AP originates from the circulation may also be considered.

Earlier studies have shown that P2X2R-immunoreactive cell bodies of the AP are glutamatergic, but not GABAergic [9]. The authors used primary antibodies to glutamate and GABA, whereas we have used the glutamatergic marker VGLUT2 and the GABAergic marker GAD65. It is important to point out that glutamate is the precursor for GABA synthesis and that antibodies to glutamate itself or to the

plasma membrane-bound glutamate transporter are not ultimate markers for glutamatergic neurons. Instead, the protein that transports glutamate into synaptic vesicles represents a unique marker for glutamatergic neurons. However, since VGLUT2 is a protein located to the membrane of synaptic vesicles, immunostaining obtained with VGLUT2 antisera is primarily located to nerve terminals. Therefore, antisera to VGLUT2 are not optimally suitable for the demonstration of glutamatergic cell bodies. Even after intraventricular administration of colchicine, a treatment that results in accumulation of proteins and peptides in the cell soma, only few glutamatergic neuronal cell bodies are generally visualized using antisera to VGLUTs [see e.g. 34]. Therefore, it is likely that we only have visualized a small subpopulation of glutamatergic cell bodies in the AP and that the total number of cell bodies with a glutamatergic nature is far higher than demonstrated in the present study. That the current immunohistochemical analysis does not visualize all VGLUT2-containing cell bodies in the AP is supported by the demonstration of many VGLUT2 mRNA-containing cells in the AP, which are TH-immunoreactive [35].

Apart from containing the classical transmitters noradrenaline and glutamate, but not GABA, the neuronal cell bodies of the AP also express several neuropeptides, including enkephalin [36], cholecystokinin (CCK) [37], and substance P (SP) [38]. Our results showed that the P2X2R-immunoreactive cell bodies in the AP contained the neuropeptides substance P and PACAP, but not the opioid peptides dynorphin and enkephalin or CCK.

In conclusion, this study shows that the majority of AP neurons contain the classical transmitters noradrenaline, suggesting that ATP after binding to P2X2R may affect noradrenaline release from AP neurons. In addition, the P2X2R neurons of the AP contain the neuropeptides substance P and PACAP, but not dynorphin, enkephalin, or CCK. The origin for the ATP acting on P2X2R-containing neurons in the AP remains to be established.

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