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ORIGINAL PAPER

P2X receptors are differentially expressed on vasopressin- and oxytocin-containing neurons in the supraoptic and paraventricular nuclei of rat hypothalamus

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Abstract In the present study, the distribution of P2X receptor protein and colocalization of P2X receptors with vasopressin and oxytocin in the supraoptic and paraventricular nuclei of rat hypothalamus was studied using double-labeling fluorescence immunohistochemistry. The results showed that vasopressin-containing neurons expressed $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor and oxytocin-containing neurons expressed $P2X_2$, $P2X_4$ and $P2X_5$ receptors in the supraoptic nucleus. In the paraventricular nucleus, vasopressin-containing neurons expressed $P2X_4$, $P2X_5$ and $P2X_6$ receptors, while oxytocin-containing neurons expressed $P2X_4$ receptors. This study provides the first evidence that P2X receptor subunits are differentially expressed on vasopressin- and oxytocin-containing neurons in the supraoptic and paraventricular nuclei, and hence, provides a substantial neuroanatomical basis for possible functional interactions between the purinergic and vasopressinergic systems, and the purinergic and oxytocinergic systems in the rat hypothalamus.

Keywords P2X receptor · Vasopressin · Oxytocin · Colocalization · Supraoptic nucleus · Paraventricular nucleus · Hypothalamus · Rat

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Introduction

Extracellular ATP has been identified as an excitatory neurotransmitter, neuromodulator, or humoral factor which acts via P2 purinoceptors (Burnstock 2006). P2 purinoceptors belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. Currently, seven P2X receptor subtypes ($P2X_{1–7}$) and eight P2Y receptor subtypes ($P2Y_{1,2,4,6,11–14}$) are recognized (Burnstock 2007a). Numerous lines of evidence indicate that ATP is released as a cotransmitter with other neurotransmitters such as norepinephrine, acetylcholine, nitric oxide and glutamate (Burnstock 2007b).

Extracellular ATP and P2 purinoceptors play very important roles in the hypothalamo-neurohypophysial system (Hiruma and Bourque 1995; Loesch et al. 1999; Potter and White 1980; Shibuya et al. 1999; Sperlágh et al. 1999). It has been shown that ATP induces a rapid increase in intracellular Ca^{2+} concentration in the hypothalamic neurosecretory neurons (Chen et al. 1994). ATP injected into paraventricular nucleus (PVN) stimulates the release of vasopressin (VP) from the neurohypophysis via P2 receptors (Mori et al. 1992). Evidence has also been presented that multiple P2X receptors are functionally expressed in neurosecretory neurons, at least in those of the supraoptic nucleus (SON) (Shibuya et al. 1999). Earlier

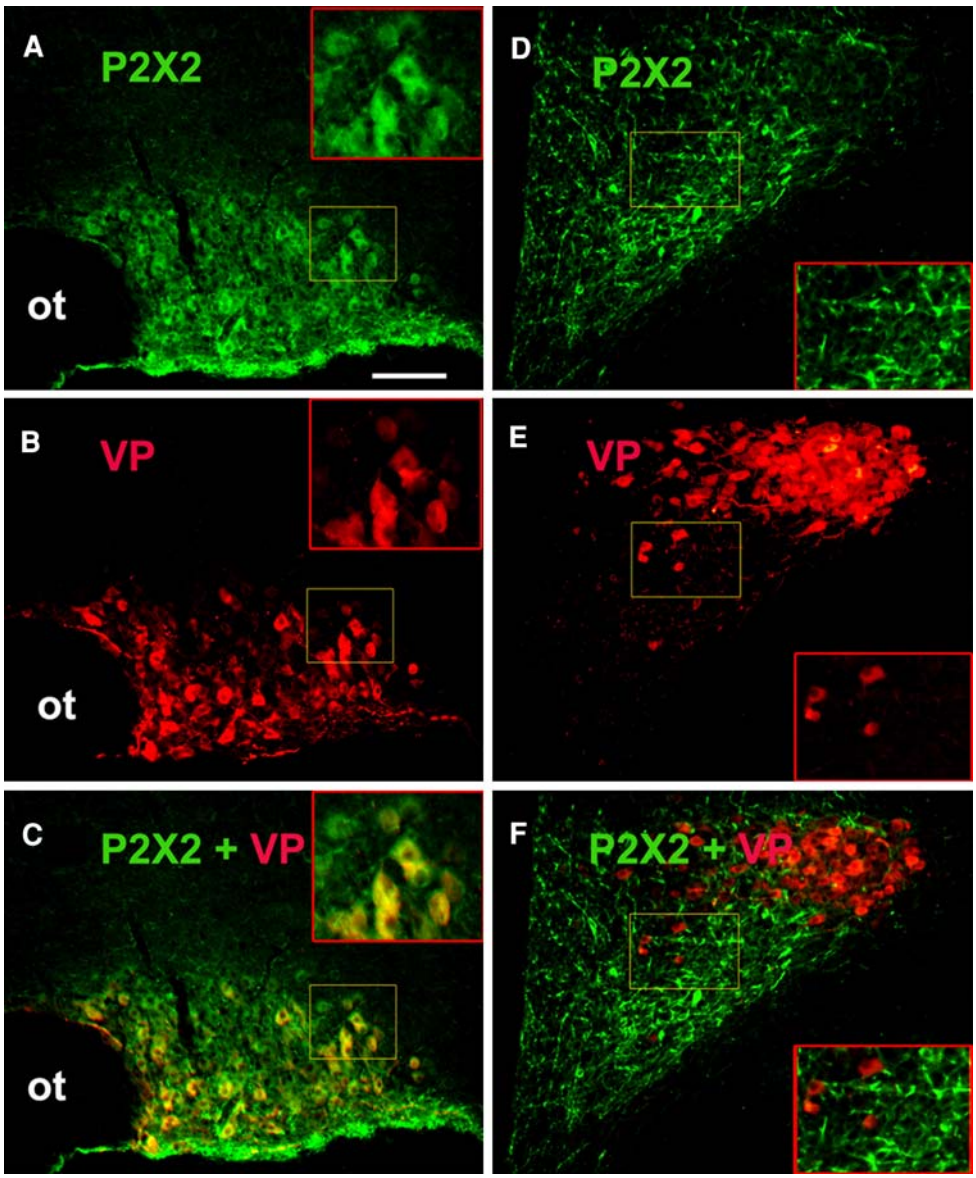

Fig. 1 Colocalization of $P2X_2$ receptor-ir and VP-ir in the SON and PVN of rat hypothalamus. **a** $P2X_2$ receptor-ir neurons and fibers (*green*) in the SON. **b** VP-ir neurons and fibers (*red*) in the SON from the same section of **a**, note that VP-ir neurons are mainly in the ventral part of the SON. **c** Merged image from **a** and **b**, note that all the VP-ir neurons (*red*) are colocalized with $P2X_2$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and some of the $P2X_2$ receptor-ir cells are only labeled with the *green* color. **d** $P2X_2$ receptor-ir neurons and fibers in the PVN. **e** VP-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that no coexistence of VP-ir and $P2X_2$ receptor-ir was found. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. Scale bar in **a–f** = 100 μm

studies of SON neurons suggested that a P2 receptor-mediated effect of ATP was an intermediate process in VP release evoked by central noradrenergic neurons (Day et al. 1993; Buller et al. 1996). ATP has been shown to excite the neurosecretory VP-containing neurons and the effects were prevented by the P2 receptor antagonist, suramin (Day et al. 1993). Purinergic and adrenergic agonists synergize in stimulating VP and oxytocin (OT) release (Kapoor and Sladek 2000).

VP and OT are mainly synthesized in the SON and PVN of the hypothalamus (George and Jacobowitz 1975; Swaab et al. 1975; Sofroniew and Glasmann 1981). Previous studies have demonstrated the distribution of P2X receptors in the SON and PVN of the hypothalamus (Collo et al. 1996; Kanjhan et al. 1999; Kidd et al. 1995; Loesch and Burnstock 2001; Loesch et al. 1999; Soto et al. 1996; Vulchanova et al. 1996; Xiang et al. 1998, 2006a; Cham et al. 2006). However, none of the studies were designed to distinguish different P2 receptor subtypes on VP- and OT-containing neurons. In the present study, we have examined the expression of $P2X_{1-6}$ receptors and their colocalization with VP or OT in the SON and PVN of the rat hypothalamus using double-labeling fluorescence immunohistochemistry.

Materials and methods

Tissue preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second

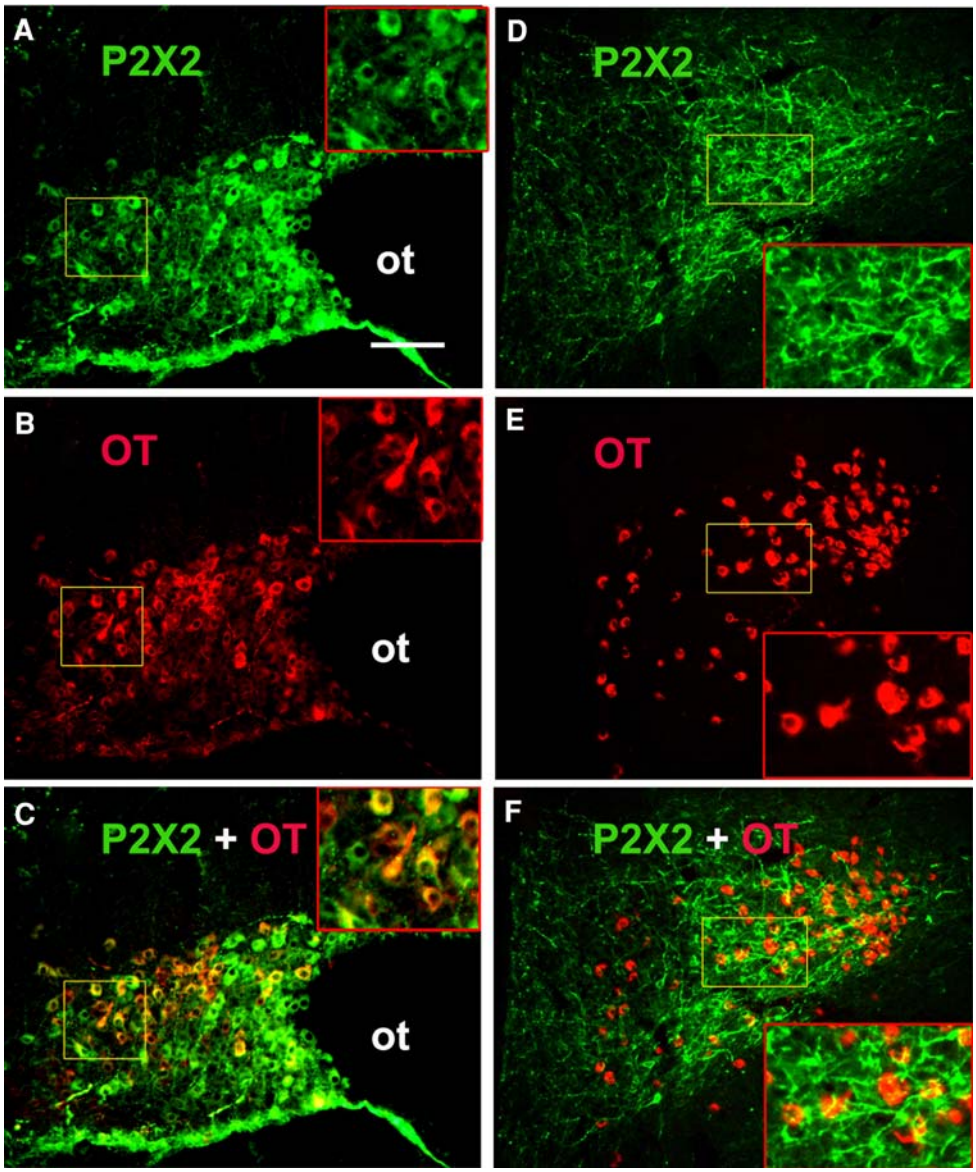

Fig. 2 Colocalization of $P2X_2$ receptor-ir and OT-ir in the SON and PVN of rat hypothalamus. **a** $P2X_2$ receptor-ir neurons and fibers in the SON. **b** OT-ir neurons and fibers in the SON from the same section of **a**, note that OT-ir neurons are mainly in the dorsal part of the SON. **c** Merged image from **a** and **b**, note that all the OT-ir neurons (*red*) are colocalized with $P2X_2$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and some of the $P2X_2$ receptor-ir cells are only labeled with the *green* color. **d** $P2X_2$ receptor-ir neurons and fibers in the PVN. e.OT-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that little coexistence of OT-ir and $P2X_2$ receptor-ir was found. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a**–**f** = 100 μm

Military Medical University. Six adult Wistar rats (250–300 g) were used. The rats were killed by asphyxiation with CO_2 and perfused through the aorta with a 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/L phosphate buffer pH7.4. The brains were removed and the hypothalamus was dissected out immediately and immersed in 4% paraformaldehyde in 0.1-M phosphate-buffered saline (PBS, pH 7.2) for 2–4 h. The hypothalamus blocks were then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the hypothalamus blocks were rapidly frozen and the coronal sections of the hypothalamus (20 μm in thickness) were cut with a Leica (Heerbrugg, Switzerland) cryostat and floated in PBS.

Double-labeling fluorescence immunohistochemistry

Immunohistochemistry for $P2X_{1-6}$ receptors was performed using rabbit polyclonal antibodies against the unique peptide sequences of the P2X receptor subtypes provided by Roche Bioscience, Palo Alto, CA. The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO K1 cells expressing the cloned P2X receptors. As previously reported by Oglesby et al., no cross-reactivity is observed with other P2X antisera (Oglesby et al. 1999).

Simultaneous detection of two antigens by immunostaining usually requires primary antibodies from two different species. A novel double-labeling immunostaining

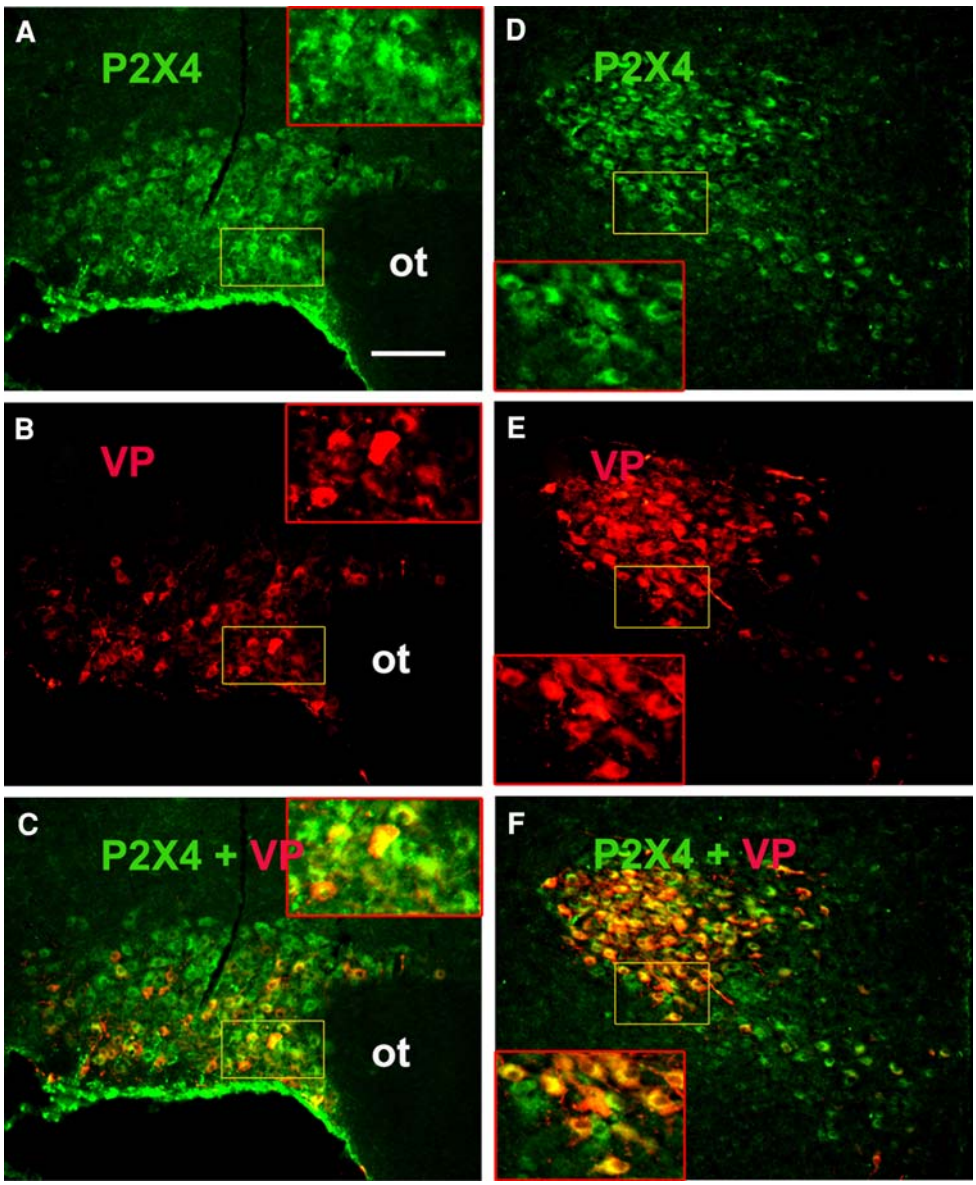

Fig. 3 Colocalization of $P2X_2$ receptor-ir and NeuN-ir in the PVN of rat hypothalamus. **a** $P2X_2$ receptor-ir neurons and fibers in the PVN. **b** NeuN-ir in the PVN from the same section of **a**. **c** A high power figure from an area indicated by a *star* in **a**, *arrows* indicate the $P2X_2$ receptor-ir cell bodies. **d** A high power figure from an area indicated by a star in **b**, *arrows* indicate the NeuN positive cell nuclei, which are the nuclei of $P2X_2$ receptor-ir cells in the same position in **d**. **e** The same area of **d** and **e** counter-stained by DAPI, *arrows* indicate the nuclei, which are the nuclei of NeuN positive cell and $P2X_2$ receptor-ir cells in the same position in **d** and **e**. **f** Merged image from **c**, **d** and **e**, note that all the $P2X_2$ receptor-ir cells are also labeled by the NeuN antibody, the *arrows* indicate the cells double-labeled with $P2X_2$ receptor and NeuN antibodies and counter-stained with DAPI, in the PVN. Also note that the *pink nuclei* are those of neurons and the *blue nuclei* are those of non-neurons. *Scale bar* = 100 μm in a, b, = 50 μm in **c**, **d**, **e**, **f**

method for immunodetection of two independent antigens has been described (Teramoto et al. 1998). The principle of the method is that the first antigen is detected by the first primary antibody that is diluted so extensively that it cannot be detected with conventional methods; a highly sensitive tyramide signal amplification (TSA) system is used to identify this antibody; the second antigen is stained with the secondary primary antibody and detected by conventional immunostaining. We have used this double-labeling protocol of fluorescence immunohistochemistry successfully (Xiang and Burnstock 2005; Xiang et al. 2006b). The following protocol was modified from this protocol. Endogenous peroxidase was blocked by 1% H_2O_2 in PBS for 30 min. The sections were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with P2X antibody, diluted 1:2,000 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, the sections were incubated with biotinylated donkey-anti-rabbit IgG (Jackson Immuno-Research Laboratories, West Grove, PA, USA) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in extravidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. The P2X immunoreactivity was visualized by the TSA Fluorescein system (NEL701, NEN, USA). After visualization the sections were incubated with the second primary antibodies of VP diluted 1:5,000 or OT (rabbit anti-rat IgG, Abcam) diluted 1:5,000 or GFAP or NeuN diluted 1:200 (mouse anti-rat IgG, Chemicon) in the antiserum dilution solution overnight at 4°C. Subsequently the sections were incubated with Cy3 conjugated donkey-anti-rabbit or

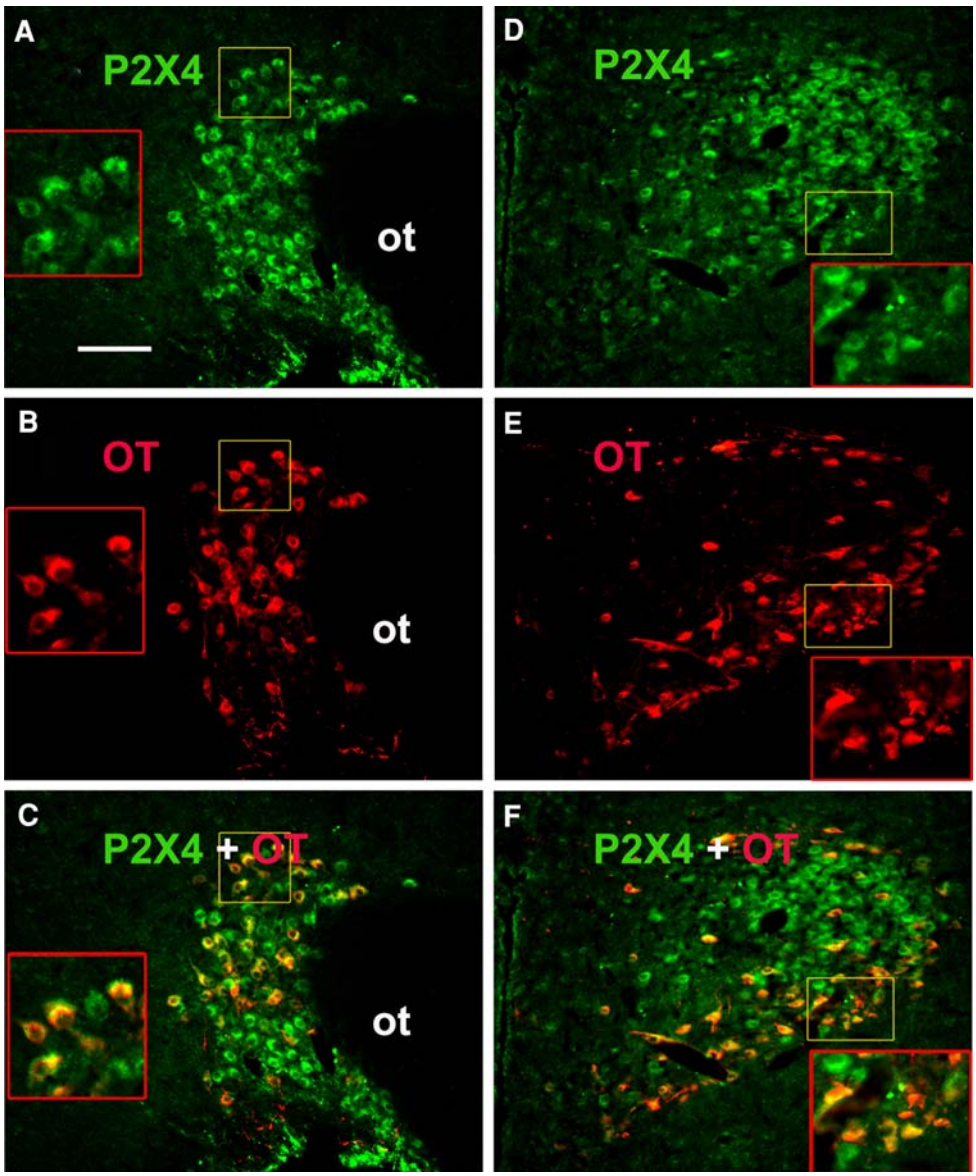

Fig. 4 Colocalization of $P2X_4$ receptor-ir and VP-ir in the SON and PVN of rat hypothalamus. **a** $P2X_4$ receptor-ir neurons and fibers (*green*) in both ventral and dorsal parts of the SON. **b** VP-ir neurons and fibers (*red*) in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that nearly all the VP-ir neurons (*red*) are colocalized with $P2X_4$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and many of the $P2X_4$ receptor-ir cells are only labeled with the *green* color in the dorsal part of the SON. **d** $P2X_4$ receptor-ir neurons and fibers in the PVN. **e** VP-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that nearly all the VP-ir neurons were colocalized (*yellow*) with $P2X_4$ receptor-ir and some of the $P2X_4$ receptor neurons are only labeled with the *green* color. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a–f** = 100 μm

mouse (Jackson ImmunoResearch) diluted 1:400 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3 × 10–min washes in PBS. Some sections were counter-stained with 5 μg/ml Hoechst 33342.

Control experiments

The control experiments were carried out with P2X antiserum-absorbed with P2X peptides at a concentration of 25 μg/ml. The amino acid sequences of these peptides are synthesized by Roche Bioscience, Palo Alto. No staining was observed in those specimens incubated with the antibody solutions pre-absorbed with P2X peptides (Fig. 8).

Photomicroscopy and data analysis

Images were taken with the Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop). The two-channel readings for green and red fluorescence were merged by using Adobe Photoshop. The focal plane on the microscope was not adjusted whilst determining whether a particular cell colocalized both P2X receptors and VP or OT. Only neurons that demonstrated the same morphology, orientation and position when viewed under the two different filters (in the same focal plane) for the detection of Cy3 and FITC were deemed to co-localize both P2X receptor and VP or OT. The number of immunopositive neurons was

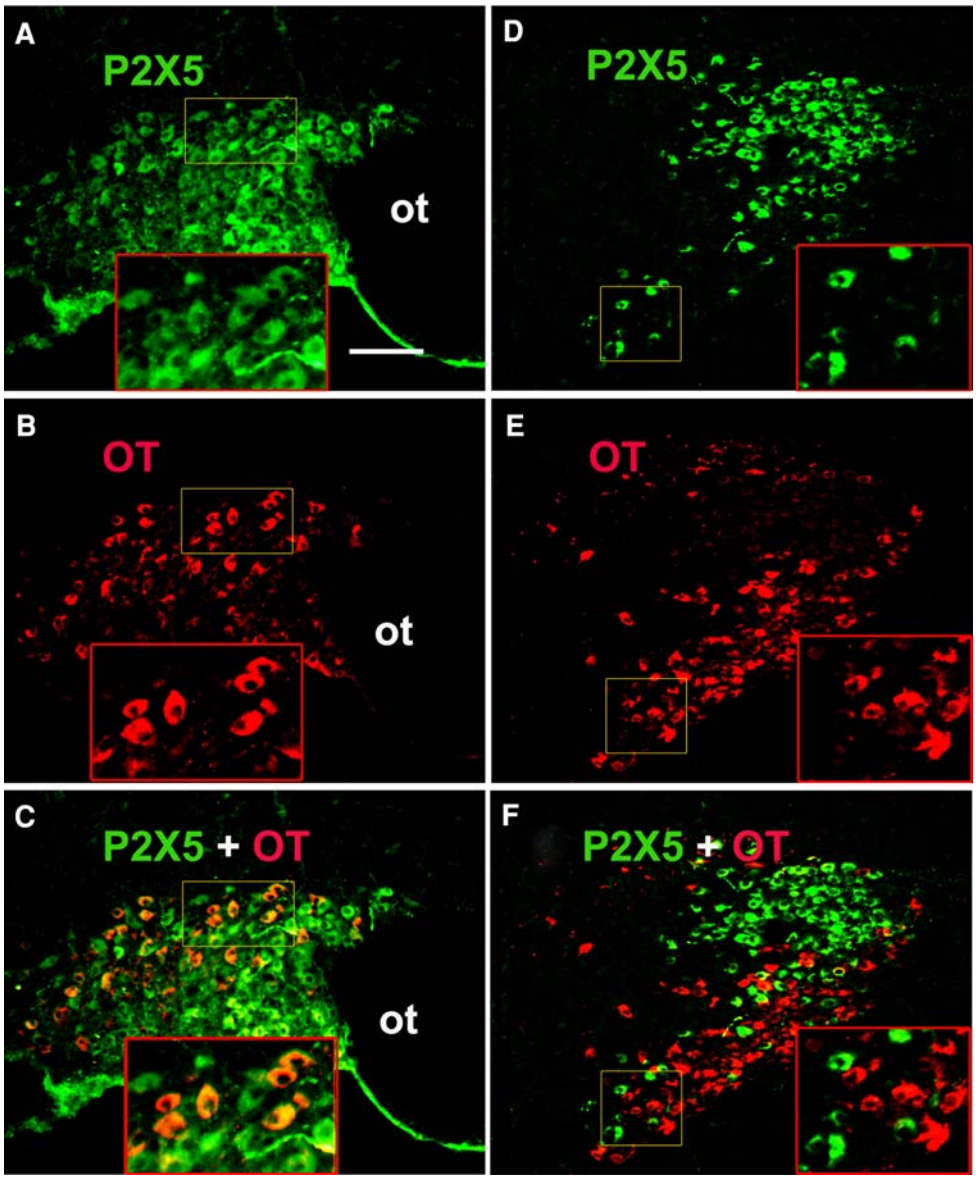

Fig. 5 Colocalization of $P2X_4$ receptor-ir and OT-ir in the SON and PVN of rat hypothalamus. **a** $P2X_4$ receptor-ir neurons and fibers in the SON. **b** OT-ir neurons and fibers in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that nearly all the OT-ir neurons (*red*) are colocalized with $P2X_4$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and some of the $P2X_4$ receptor-ir cells are only labeled with the *green* color. **d** $P2X_4$ receptor-ir neurons and fibers in the PVN. **e** OT-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that nearly all the OT-ir neurons are colocalized (*yellow*) with $P2X_4$ receptor-ir, mainly in the PaMP and perhaps all of the $P2X_4$ receptor-ir neurons are only labeled with the *green* color in the PaLM. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a**–**f** = 100 μm

counted unilaterally throughout the caudo-rostral extent of the respective nuclei as defined by the atlas by Paxinos and Watson (1986). Data for each of the nuclei analyzed were obtained from each of the rats used. Five sections from each animal were used, and the average number and percentage of positive cells in one section was calculated. The numbers presented in the tables represent the average numbers and percentages of immunopositive cells observed unilaterally per section ± SEM (Yao et al. 2003; Xiang et al. 2006b).

Results

$P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor immunoreactivity(-ir) was found throughout the caudo-rostral extent of the SON and PVN of the rat hypothalamus using the antibodies for the $P2X_{1–6}$ receptor subunits. $P2X_2$ receptor-ir was found in the SON, including the SOR (supraoptic nucleus, retrochiasmatic part). $P2X_2$ receptor-ir cells were found to be distributed in both the ventral and dorsal part of the SON. The immunostaining intensity of some positive cells was higher than that of others. Coexistence of $P2X_2$ receptor-ir and VP-ir or $P2X_2$ receptor-ir and OT-ir was found in the SON. Almost all the VP-ir or OT-ir cells were found to express $P2X_2$ receptor-ir, although some $P2X_2$ receptor-ir cells did not express VP-ir or OT-ir, respectively (Figs. 1, 2). $P2X_2$ receptor-ir cells were also found in the whole PVN, especially in the PaMP (paraventricular hypothalamic nucleus, medial parvicellular part) and PaPo (paraventricular hypothalamic nucleus, posterior part). No coexistence of $P2X_2$ receptor-ir and VP-ir or $P2X_2$

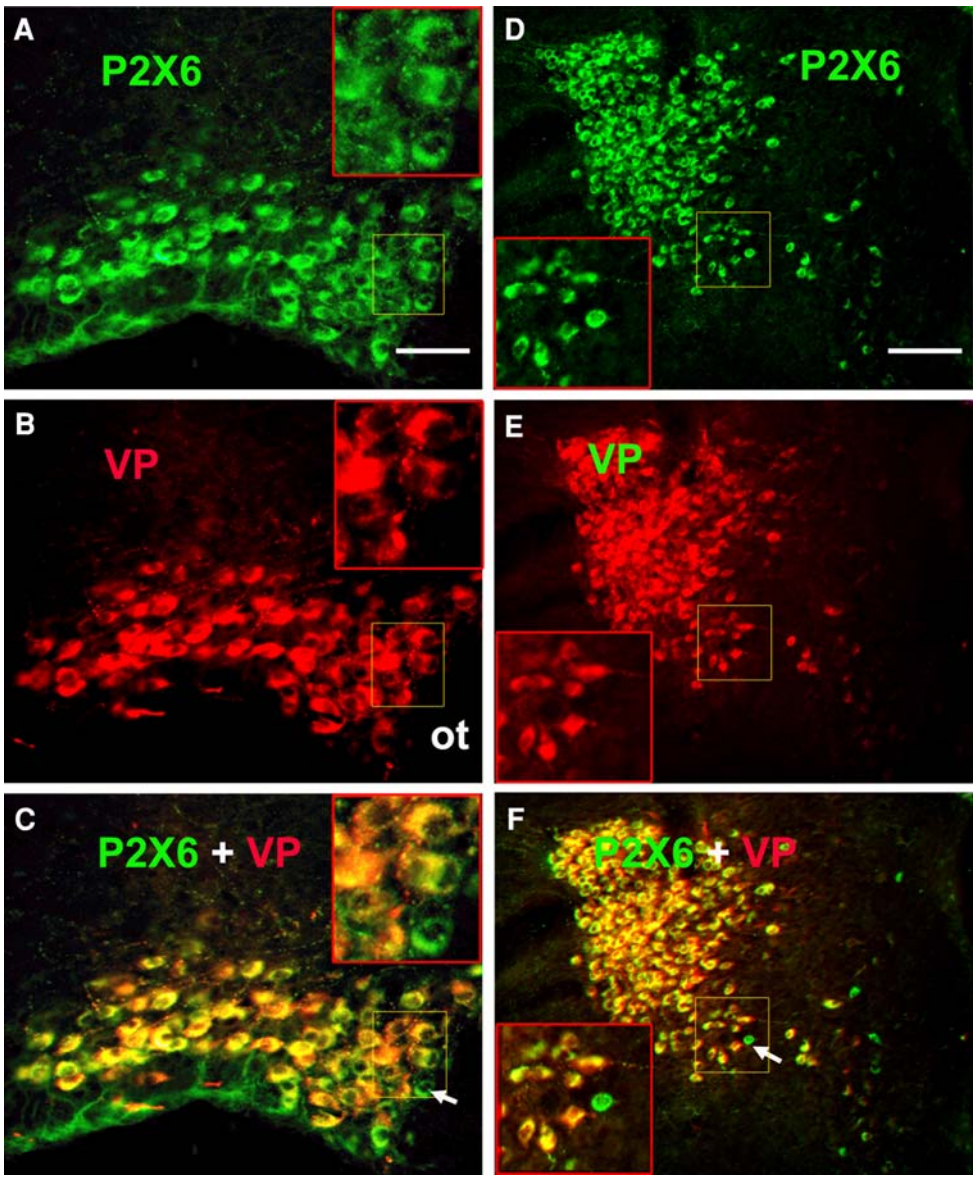

Fig. 6 Colocalization of $P2X_5$ receptor-ir and OT-ir in the SON and PVN of rat hypothalamus. **a** $P2X_5$ receptor-ir neurons and fibers in the SON. **b** OT-ir neurons and fibers in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that perhaps all the OT-ir neurons (*red*) are colocalized with $P2X_5$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and some of the $P2X_5$ receptor-ir cells are only labeled with the *green* color in the ventral part of the SON. **d** $P2X_5$ receptor-ir neurons and fibers in the PVN. **e** OT-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that no coexistence of OT-ir and $P2X_5$ receptor-ir was observed. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a–f** = 100 μm

receptor-ir and OT-ir was demonstrated in the PVN (Figs. 1, 2). In order to identify whether these $P2X_2$ receptor-ir cells were neurons or not, NeuN antibody (a neuron marker) was used. The results showed that all the $P2X_2$ receptor-ir cells were also labeled by the NeuN antibody (Fig. 3). $P2X_4$ receptor-ir was found in the SON and PVN. In the SON, $P2X_4$ receptor-ir cells were found in both the ventral and dorsal part of the SON. $P2X_4$ receptor-ir was also found in the caudo-rostral extent of the PVN. Coexistence of $P2X_4$ receptor-ir and VP-ir or $P2X_4$ receptor-ir and OT-ir was found in the SON and PVN. Almost all the VP-ir or OT-ir cells were found to express $P2X_4$ receptor-ir, although some of the $P2X_4$ receptor-ir cells did not express VP-ir or OT-ir (Figs. 4, 5). We have previously reported the distribution of $P2X_5$ receptor-ir cells and its coexistence with VP-ir in the hypothalamus (Xiang et al. 2006a). In the present study, coexistence of $P2X_5$ receptor-ir and OT-ir was found in the SON, but not in the PVN (Fig. 6). $P2X_6$ receptor-ir was found in the SON, mainly in the ventral part. Almost all the $P2X_6$ receptor-ir cells were also found to express VP-ir, although some glial-like cells in the ventral margin did not express VP-ir (Fig. 7). Those $P2X_6$ receptor-ir glial-like cells in the ventral margin of the SON were identified as astrocytes, as they were found to express GFAP (Fig. 9). $P2X_6$ receptor-ir was found in the whole PVN, especially in the PaLM (paraventricular hypothalamic nucleus, lateral magnocellular part) and PaMP. Almost all the $P2X_6$ receptor-ir cells were also found to express VP-ir in this nucleus (Fig. 7). No coexistence of $P2X_6$ receptor-ir and OT-ir was found in both the SON and PVN (Fig. 8). No $P2X_1$ or $P2X_3$ receptor-ir was found in the SON and PVN (Fig. 9). The

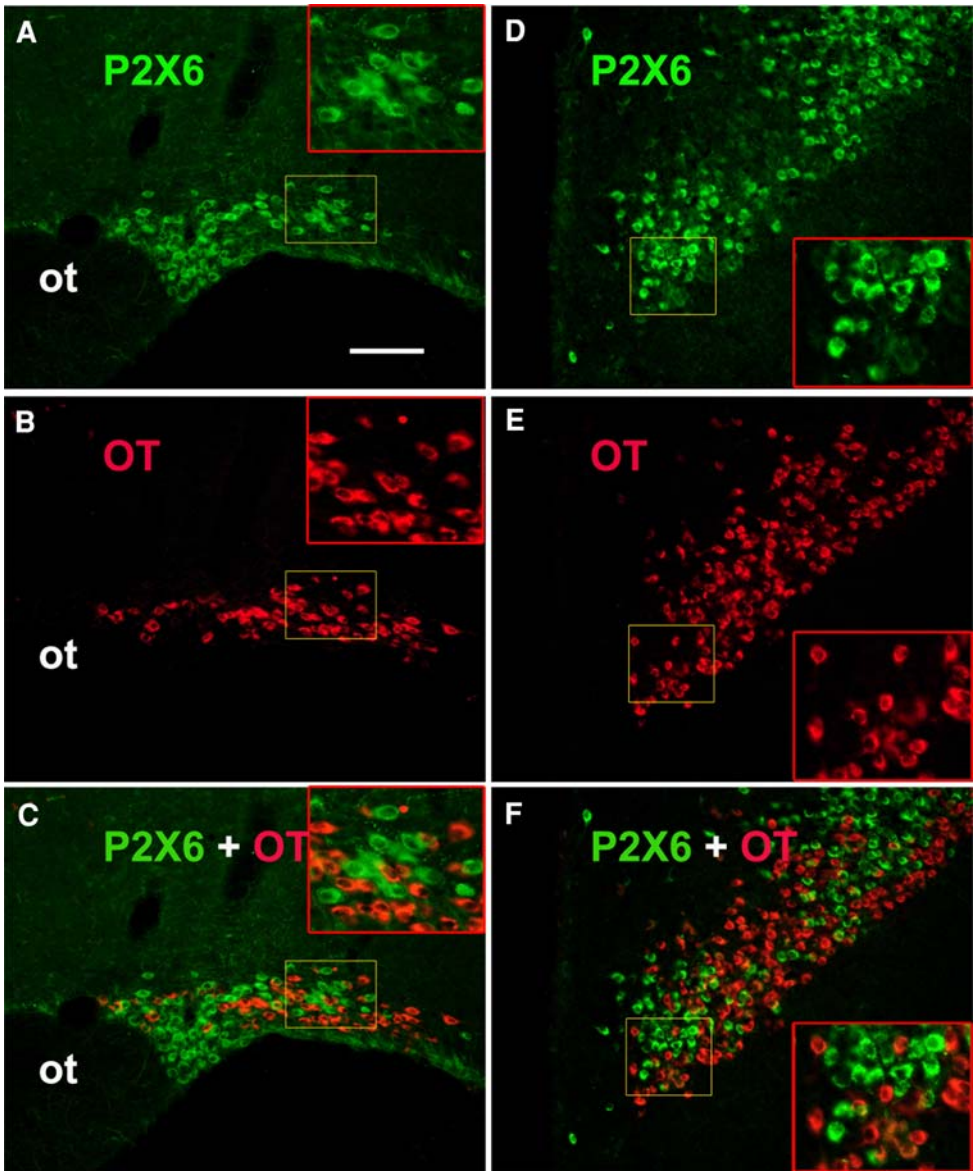

Fig. 7 Colocalization of $P2X_6$ receptor-ir and VP-ir in the SON and PVN of rat hypothalamus. **a** $P2X_6$ receptor-ir neurons and fibers (*green*) mainly in the ventral part of the SON. **b** VP-ir neurons and fibers (*red*) in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that all the VP-ir neurons (*red*) are colocalized with $P2X_6$ receptor-ir (*green*). Double-labeled neurons are *yellow* in color and some glial-like cells with $P2X_4$ receptor-ir in the ventral margin are only labeled with the *green* color. A single labeled neuron with $P2X_6$ receptor-ir is indicated by an *arrow*. **d** $P2X_6$ receptor-ir neurons in the PVN. **e** VP-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that all the VP-ir neurons are colocalized (*yellow*) with $P2X_6$ receptor-ir and a few of the $P2X_6$ receptor neurons only labeled with the *green* color were also observed. An *arrow* indicates a single labeled neuron with $P2X_6$ receptor-ir. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a**–**c** = 50 μm, in **d**–**f** = 100 μm

number of cells that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus are summarized in Tables 1, 2, 3, 4, 5, 6, 7.

Discussion

We used double-labeling fluorescence immunohistochemistry to study the distribution of P2X receptor protein and colocalization of P2X receptors with VP or OT in the SON and PVN of the rat hypothalamus. This study provides the first evidence that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors are differentially expressed on VP- and OT-containing neurons in the SON and PVN of rat hypothalamus; and hence, has provided a substantial neuroanatomical basis for possible functional interactions between the purinergic and the vasopressinergic system, and also for interactions between the purinergic and oxytocinergic systems in rat hypothalamus.

Neurons expressing VP-ir and OT-ir were mainly found in the SON and PVN of rat hypothalamus, confirming earlier studies (George and Jacobowitz 1975; Swaab et al. 1975; Sofroniew and Glasmann 1981). Our data from double-labeling fluorescence immunohistochemistry showed that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits are differentially expressed on VP- and OT-containing neurons of the SON and PVN. VP-containing neurons expressed $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors and OT-containing neurons expressed $P2X_2$, $P2X_4$ and $P2X_5$ receptors in the SON. In the PVN, VP-containing neurons

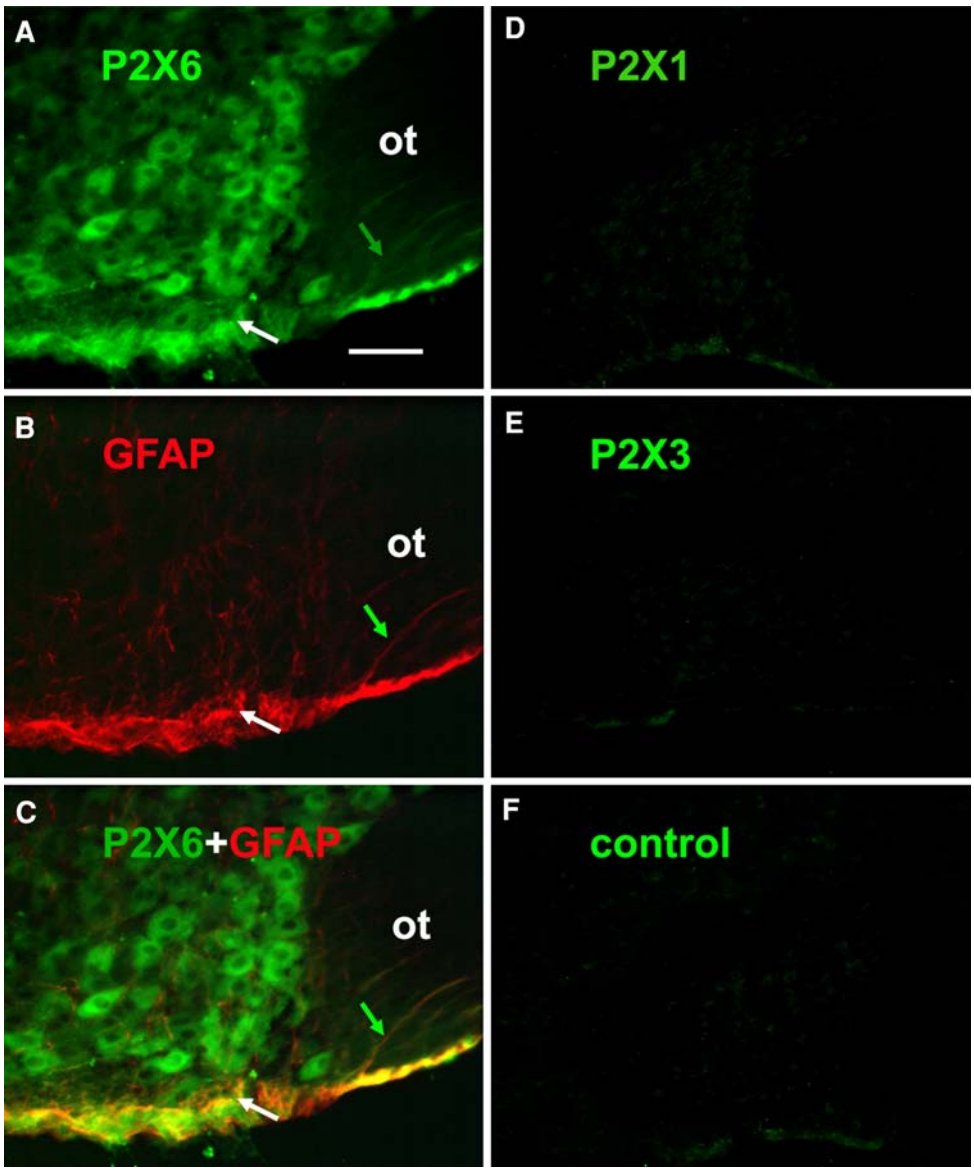

Fig. 8 Colocalization of $P2X_6$ receptor-ir and OT-ir in the SON and PVN of rat hypothalamus. **a** $P2X_6$ receptor-ir neurons and fibers in the SON. **b** OT-ir neurons and fibers in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that no coexistence of OT-ir and $P2X_4$ receptor-ir was observed. **d** $P2X_6$ receptor-ir neurons and fibers in the PVN. **e** OT-ir neurons and fibers in the PVN. **f** Merged image from **d** and **e**, note that no coexistence of OT-ir and $P2X_4$ receptor-ir was observed. *ot* optic tract. The images with *red frames* are magnified two times from the images with *yellow frames* in **a**, **b**, **c**, **d**, **e**, **f**. *Scale bar* in **a**–**f** = 100 μm

expressed $P2X_4$, $P2X_5$ and $P2X_6$, but not $P2X_2$ receptors and OT-containing neurons expressed $P2X_4$, but not $P2X_2$, $P2X_5$ or $P2X_6$ receptors, although $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors were expressed in this nucleus. These results indicate that ATP may affect the physiological functions of VP- and OT-containing neurons via different subunits of homomeric or heteromeric P2X receptors in these two nuclei, respectively.

P2X receptor subunits are able to form homomeric and heteromeric multimers as the functional receptor channels. Homomeric $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ channels and heteromeric $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{1/4}$, $P2X_{2/3}$, $P2X_{2/6}$, $P2X_{4/6}$ and $P2X_{4/7}$ receptor channels have been characterized following heterologous expression (North 2002; Burnstock 2007a; Guo et al. 2007). Thus, $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits expressed on VP-containing neurons of the SON could assemble into homomeric $P2X_2$, $P2X_4$ or $P2X_5$ channels or heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels; $P2X_2$, $P2X_4$ and $P2X_5$ receptor subunits expressed on OT-containing neurons could assemble into only homomeric $P2X_2$, $P2X_4$ and $P2X_5$ channels; $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits expressed on VP-containing neurons of the PVN could assemble into homomeric $P2X_4$ and $P2X_5$ channels, or heteromeric $P2X_{4/6}$; $P2X_4$ receptor subunits expressed on OT-containing neurons could only assemble into homomeric $P2X_4$ channels. The differential P2X receptor subunits expressed in VP- and OT-containing neurons in SON and PVN are summarized in Table 8. Further studies using specific antagonists are required for unequivocal support of these possible functional combinations of P2X receptor multimers on VP-ir and OT-ir neurons in these two nuclei.

Table 1 $P2X_2$ receptor and VP

Nuclei	$P2X_2$+	$P2X_2$+ VP+	Double-labeling (%)	$P2X_2$+ VP−	$P2X_2$− VP+
PVN	65 ± 11	0	0	65 ± 11	63 ± 10
SON	76 ± 12	42 ± 8	55 ± 11	34 ± 7	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Table 2 $P2X_2$ receptor and OT

Nuclei	$P2X_2$+	$P2X_2$+ OT+	Double-labeling (%)	$P2X_2$+ OT−	$P2X_2$− OT+
PVN	71 ± 15	0	0	71 ± 15	75 ± 13
SON	68 ± 9	33 ± 5	48 ± 7	35 ± 9	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Table 3 $P2X_4$ receptor and VP

Nuclei	$P2X_4$+	$P2X_4$+ VP+	Double-labeling (%)	$P2X_4$+ VP−	$P2X_4$− VP+
PVN	72 ± 12	56 ± 8	78 ± 11	16 ± 9	0
SON	59 ± 10	34 ± 9	59 ± 17	25 ± 8	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Table 4 $P2X_4$ receptor and OT

Nuclei	$P2X_4$+	$P2X_4$+ OT+	Double-labeling (%)	$P2X_4$+ OT−	$P2X_4$− OT+
PVN	89 ± 15	53 ± 13	59 ± 15	36 ± 10	0
SON	74 ± 11	35 ± 6	47 ± 8	39 ± 9	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

VP and OT are two vital neuropeptides in the hypothalamus, involved in a series of important physiological functions (Buijs et al. 1983). Although the data about interactions between P2X receptors and the VP or OT systems are limited, there is still some evidence to suggest that these interactions may exist within the hypothalamus. ATP was found to be involved in regulation of body temperature and hormone secretion in the hypothalamus (Mori et al. 1992; Gourine et al. 2002; Kapoor and Sladek 2000). Application of ATP to explants of the hypothalamo-neurohypophyseal

Table 5 $P2X_5$ receptor and OT

Nuclei	$P2X_5$+	$P2X_5$+ OT+	Double-labeling (%)	$P2X_5$+ OT−	$P2X_5$− OT+
PVN	81 ± 17	0	0 81 ± 17	47 ± 17	
SON	61 ± 12	31 ± 8	51 ± 13	30 ± 10	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Table 6 $P2X_6$ receptor and VP

Nuclei	$P2X_6$+	$P2X_6$+ VP+	Double-labeling (%)	$P2X_6$+ VP−	$P2X_6$− VP+
PVN	76 ± 15	73 ± 3	96 ± 4	3 ± 2	0
SON	63 ± 9	61 ± 2	97 ± 3	2 ± 1	0

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

Table 7 $P2X_6$ receptor and OT

Nuclei	$P2X_6$+	$P2X_6$+ OT+	Double-labeling (%)	$P2X_6$+ OT−	$P2X_6$− OT+
PVN	81 ± 15	0	0	85 ± 15	69 ± 20
SON	46 ± 8	0	0	46 ± 8	39 ± 10

Table shows the number of neurons that express P2X receptor subunits and VP or OT and the percentage of those that show double-labeling for P2X receptor subunits and VP or OT in the SON and PVN of the rat hypothalamus

$P2X_2$+, $P2X_2$, receptor-ir neurons; $P2X_2$+ VP+, $P2X_2$ receptor-ir neurons also expressing VP-ir; $P2X_2$+ AVP−, $P2X_2$ receptor-ir neurons not expressing VP-ir; $P2X_2$− VP+, VP-ir neurons not expressing $P2X_2$ receptor-ir; (%) double-labeling, the percentage of $P2X_2$+ VP+ neurons. *PVN* paraventricular nucleus; *SON* supraoptic nucleus

system was shown to evoke an increase in VP release, a response that was attenuated by the P2 purinoceptor antagonist, pyridoxalphosphate-6-azonphenyl-2′,4′-disulphonic acid (Mori et al. 1992; Kapoor and Sladek 2000). This finding was supported by the evidence demonstrating a direct input originating from A1 cells located in the caudal ventrolateral medulla in which synapse directly on VP-containing neurons in the SON and PVN and utilize ATP as a cotransmitter (Day et al. 1992, 1993).

Table 8 VP- and OT-containing neurons express differential P2X receptor subunits in PVN and SON

Nuclei	VP-containing neurons	OT-containing neurons
SON	$P2X_2$, $P2X_4$, $P2X_5$, $P2X_6$	$P2X_2$, $P2X_4$, $P2X_5$
PCRM	$P2X_2/P2X_6$, $P2X_4/P2X_6$	–
PVN	$P2X_4$, $P2X_5$, $P2X_6$	$P2X_4$
PCRM	$P2X_4/P2X_6$	–

SON supraoptic nucleus, *PCRM* possible combinations of receptor multimers, *PVN* paraventricular nucleus

P2X purinoceptors are a family of ATP-gated cation channels with permeabilities to, for example, calcium, sodium and potassium (North 2002). These channels are selectively permeable to cations (p_{Ca}^{2+} approximately twofold–fivefold greater than p_{Na}^{+} and p_{K}^{+}) (Gever et al. 2006). Calcium is a vital second messenger and plays an equally important role in practically every cell type and controls many physiological functions, such as the release of neuropeptides and neurotransmitters. This second messenger induced by ATP via P2X purinoceptors was reported to be involved in the release of neuropeptides in the hypothalamic neurohypophysial system (Chen et al.

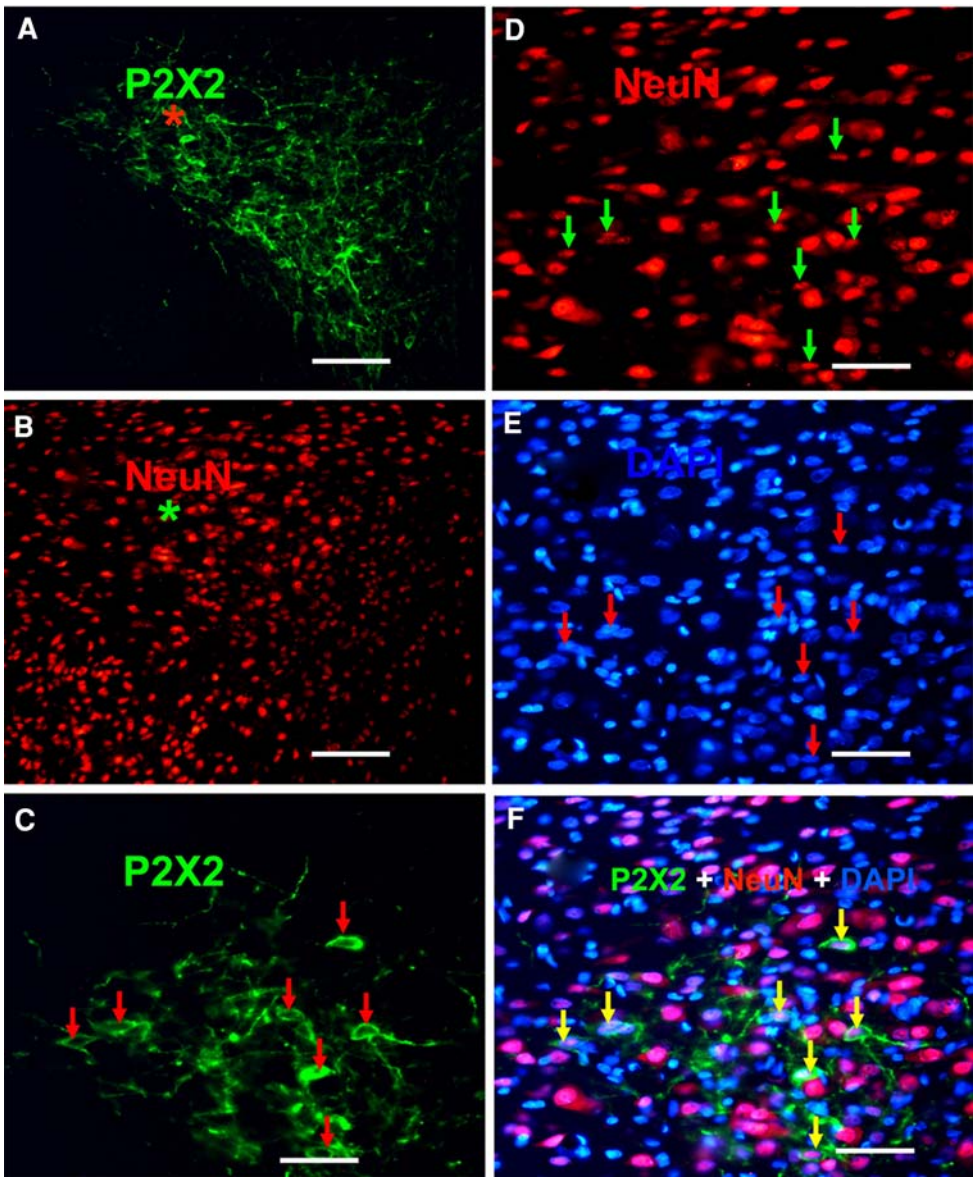

Fig. 9 Distribution of $P2X_6$ receptor-ir and GFAP-ir in the SON of rat hypothalamus (**a**, **b**, **c**) are $P2X_1$ and $P2X_3$ receptor-ir in the SON (**d**, **e**) and control experiment (**f**). **a** $P2X_6$ receptor-ir cells and fibers in the SON. **b** GFAP-ir cells and fibers in the SON from the same section of **a**. **c** Merged image from **a** and **b**, note that coexistence of GFAP-ir and $P2X_2$ receptor-ir was observed in the ventral margin of the SON. *Arrows* indicate the double-labeled cells and fibers in the ventral margin of the SON. **d** Absence of $P2X_1$ receptor-ir in the SON. **e** Absence of $P2X_3$ receptor-ir in the SON. **f** Absence of $P2X_6$ receptor-ir in the SON, note that almost no $P2X_6$ receptor-ir was observed. *Scale bar* in **a–f** = 50 μm

1994; Troadec et al. 1998). Chen et al. (1994) has shown that ATP can induce a rapid increase in intracellular Ca^{2+} concentration in the hypothalamic neurosecretory neurons. Troadec et al. (1998) showed that the Ca^{2+} increase and VP release were induced by ATP in single isolated rat neurohypophysial nerve terminals using fura-2 imaging and specific radioimmunoassay. They further confirmed that the observed Ca^{2+} increases were elicited by Ca^{2+} entry through a P2X channel receptor, rather than via a voltage-dependent Ca^{2+} channel. These data imply that activation of different homomeric or heteromeric P2X receptor channels on VP and OT neurons in the SON and PVN could elicit Ca^{2+} increases.

In conclusion, the present study demonstrates that P2X receptor subunits are differentially expressed on VP- and OT-containing neurons in the SON and PVN of the rat hypothalamus. Activation of P2X receptors by ATP via different homomeric or heteromeric P2X receptors could stimulate release of VP and OT from the neurons in the SON and PVN neurons. These findings provide the morphological basis for possible functional interactions between the purinergic and vasopressinergic or oxytocinergic neurotransmitter systems. Such interactions may be important in regulation of hormone secretion at the hypothalamic level.

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