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Research Report

$P2X_5$ receptors are expressed on neurons containing arginine vasopressin and nitric oxide synthase in the rat hypothalamus

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Abbreviations:
ATP, adenosine 5′-triphosphate
RT-PCR, reverse transcriptase-polymerase chain reaction
PaAP, paraventricular hypothalamic nucleus, anterior parvocellular
PaLM, paraventricular hypothalamic nucleus, lateral magnocellular
PaMP, paraventricular hypothalamic nucleus, medial parvocellular
PaPO, paraventricular hypothalamic nucleus, posterior
PaV, paraventricular hypothalamic nucleus, ventral

ABSTRACT

In this study, the $P2X_5$ receptor was found to be distributed widely in the rat hypothalamus using single and double labeling immunofluorescence and reverse transcriptase-polymerase chain reaction (RT-PCR) methods. The regions of the hypothalamus with the highest expression of $P2X_5$ receptors in neurons are the paraventricular and supraoptic nuclei. The intensity of $P2X_5$ immunofluorescence in neurons of the ventromedial nucleus was low. 70–90% of the neurons in the paraventricular nucleus and 46–58% of neurons in the supraoptic and accessory neurosecretory nuclei show colocalization of $P2X_5$ receptors and arginine vasopressin (AVP). None of the neurons expressing $P2X_5$ receptors shows colocalization with AVP in the suprachiasmatic and ventromedial nuclei. 87–90% of the neurons in the lateral and ventral paraventricular nucleus and 42–56% of the neurons in the accessory neurosecretory, supraoptic and ventromedial nuclei show colocalization of $P2X_5$ receptors with neuronal nitric oxide synthase (nNOS). None of the neurons expressing $P2X_5$ receptors in the suprachiasmatic nucleus shows colocalization with nNOS. These findings provide a morphological basis for possible functional interactions between the purinergic and nitrergic or vasopressinergic neurotransmitter systems.

1. Introduction

Many important functions such as temperature control, feeding, drinking, sexual behavior, defensive reactions and neuroendocrine activities have been shown to be controlled by different nuclei in the hypothalamus (Arancibia et al., 1996; Coote, 1995; Gurin et al., 2003; Kuenzel, 1994; McCann et al., 1994; Swaab et al., 1993). Besides noradrenaline and

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acetylcholine, many other putative neurotransmitters such as serotonin, histamine and neuropeptides have been shown to be involved in the regulation of hypothalamic functions (Palkovits, 1992; Rothwell, 1994; Sakata et al., 1995). Extracellular adenosine 5′-triphosphate (ATP) as a co-transmitter in neurons in the central nervous system has been well documented (Burnstock, 2003). Information about neurons in different nuclei of the hypothalamus that are regulated by extracellular ATP is increasing (Buller et al., 1996; Chen et al., 1994; Day et al., 1993; Furukawa et al., 1994; Gurin et al., 2003; Hiruma and Bourque, 1995; Jo and Role, 2002; Mori et al., 1992; Shibuya et al., 1999; Sorimachi et al., 2001; Vorobjev et al., 2003b). All seven subtypes of P2X receptor mRNA and protein have been detected in various nuclei in the hypothalamus by reverse transcriptase-polymerase chain reaction (RT-PCR) (Kidd et al., 1995; Shibuya et al., 1999; Vorobjev et al., 2003a), in situ hybridization (Kidd et al., 1995; Shibuya et al., 1999) and immunocytochemistry (Atkinson et al., 2004; Loesch and Burnstock, 2001; Loesch et al., 1999; Vulchanova et al., 1996; Xiang et al., 1998; Yao et al., 2003). There has, however, been some controversy about the existence of $P2X_5$ receptors in the hypothalamus. Hybridization experiments showed that there was no $P2X_5$ receptor mRNA in hypothalamus (Collo et al., 1996), and RT-PCR also showed no $P2X_5$ mRNA in the rat supraoptic nucleus. However, single cell RT-PCR showed that about 35% of neurons expressed $P2X_5$ receptor mRNA in the tuberomamillary nucleus of rat hypothalamus, and pharmacological data also showed that functional heteromeric $P2X_{2/5}$ receptors might be present (Vorobjev et al., 2003a). Thus, in the present study, detailed information about the distribution pattern of $P2X_5$ receptors in various nuclei in the hypothalamus has been obtained and their coexistence with arginine vasopressin (AVP) and neuronal nitric oxide synthase (nNOS) using RT-PCR, immunocytochemistry and double immunolabeling methods.

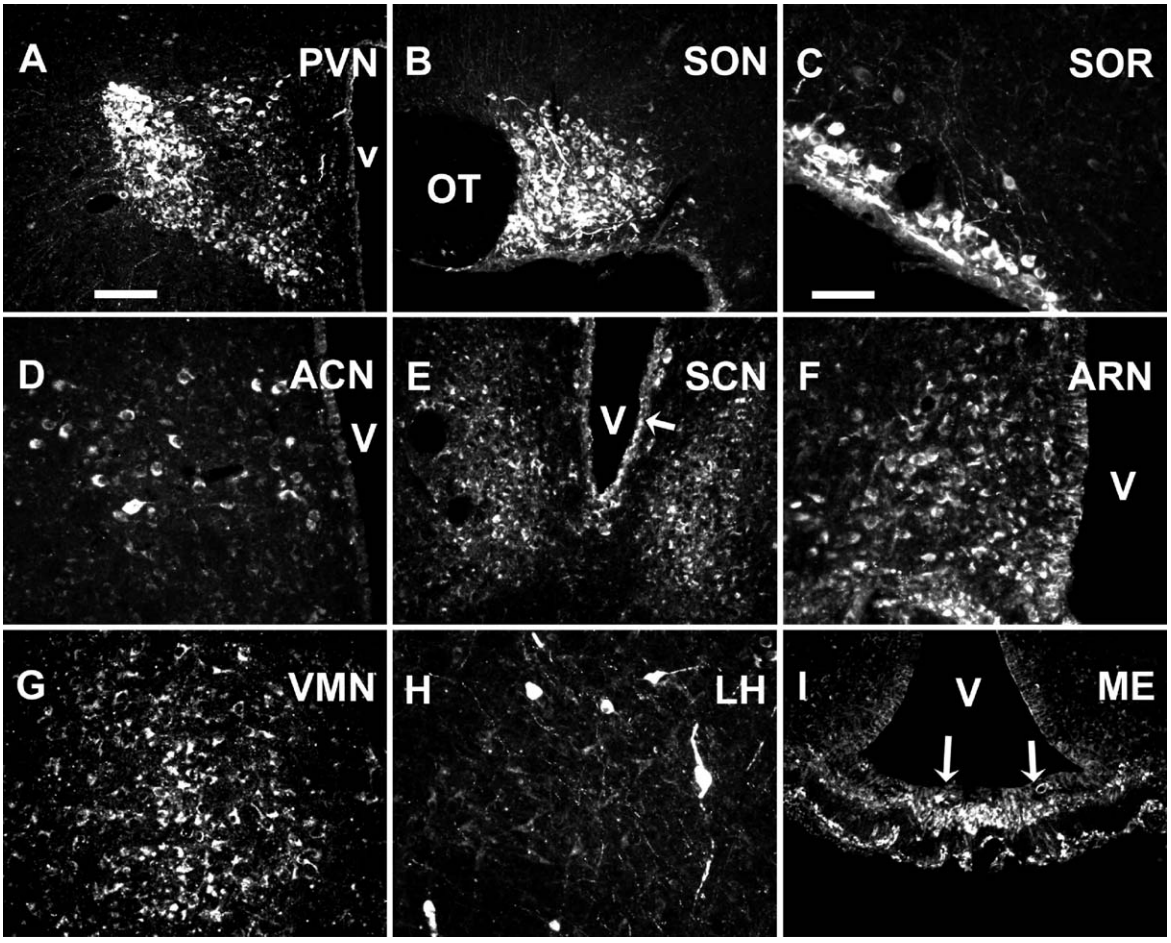

Fig. 1 – $P2X_5$ receptor-ir neurons and fibers in adult rat hypothalamus. (A) $P2X_5$ receptor-ir neurons and fibers in the paraventricular nucleus (PVN) of hypothalamus. (B) $P2X_5$ receptor-ir neurons and fibers in the supraoptic nucleus (SON) of hypothalamus. OT indicates optic tract. (C) $P2X_5$ receptor-ir neurons and fibers in the retrochiasmatic part of supraoptic nucleus (SOR). (D) $P2X_5$ receptor-ir neurons and fibers in the anterior commissural nucleus (ACN). (E) $P2X_5$ receptor-ir neurons and fibers in the suprachiasmatic nucleus (SCN). (F) $P2X_5$ receptor-ir neurons and fibers in the arcuate nucleus (ARN). (G) $P2X_5$ receptor-ir neurons and fibers in the ventromedial nucleus (VMN). (H) $P2X_5$ receptor-ir neurons and fibers in the lateral area of the hypothalamus (LH). (I) $P2X_5$ receptor-ir fibers in the medial eminence (ME). In panels A, D, E and F, V indicates the 3rd ventricle and arrows show positive epithelial cells on the 3rd ventricular wall. Scar bars: A, B = 160 μm, C, D, E, F, G, H, I = 80 μm. In each figure, the dorsal aspect of the nuclei is at the top and the ventral aspect of the nuclei is at the bottom.

2. Results

$P2X_5$ receptor immunoreactivity (ir) was found to be distributed widely in rat hypothalamus. The strongest signals of $P2X_5$-ir were observed in paraventricular and supraoptic nuclei. In the paraventricular nucleus, a high density of $P2X_5$-ir neurons was found in the paraventricular hypothalamic nucleus, lateral magnocellular (PaLM), a medium density of $P2X_5$-ir neurons in the paraventricular hypothalamic nucleus, medial parvocellular (PaMP) and paraventricular hypothalamic nucleus, ventral (PaV) and scattered $P2X_5$-ir neurons in the paraventricular hypothalamic nucleus, posterior (PaPO) and paraventricular hypothalamic nucleus, anterior parvocellular (PaAP) (Figs. 1A, D). In the supraoptic nucleus, a high density of $P2X_5$-ir neurons was demonstrated in both anterior and posterior regions (Figs. 1B, C). Many $P2X_5$-ir neurons were found in the arcuate nucleus, ventromedial and suprachiasmatic nuclei, but the signals of $P2X_5$-ir in those nuclei were lower than that in paraventricular and supraoptic nuclei (Figs. 1E, F, G). There were scattered $P2X_5$-ir neurons in the preoptic area, lateral area, retrochiasmatic area, dorsal area and mammillary nucleus (Fig. 1H). In addition, the epithelium of the third ventriculum was also found to express $P2X_5$-ir signals (Figs. 1A, E, F, I). Many fibers with $P2X_5$-ir were found in the median eminence, lateral area, lateral side of PaLM and dorsal side of supraoptic nucleus (Figs. 1A, B, C, H, I). The results of the RT-PCR studies further showed that $P2X_5$ receptor mRNA was also expressed in the rat hypothalamus (Fig. 2).

Coexistence of $P2X_5$-ir and AVP-ir was found in the paraventricular nucleus, supraoptic nucleus and accessory neurosecretory nuclei. Almost all the AVP-ir neurons were found to express $P2X_5$-ir, although some $P2X_5$-ir neurons did not express AVP-ir (Figs. 3A, B, C, E, F, H). No coexistence of $P2X_5$-ir and AVP-ir was demonstrated in the suprachiasmatic nucleus (Fig. 3G). The fibers with double labeling by $P2X_5$ receptor and AVP antibodies were demonstrated in medial eminence, paraventricular and supraoptic nuclei (Figs. 3C, D, F, H). Coexistence of $P2X_5$-ir and nNOS-ir was found in the paraventricular nucleus, supraoptic nucleus and accessory neurosecretory nuclei, lateral hypothalamic area and ventromedial nucleus (Figs. 4A, B, C, D, E, F, G, H, I). The percentage of coexistences among $P2X_5$-ir, AVP-ir and nNOS-ir in the hypothalamic nuclei are summarized in Tables 1 and 2.

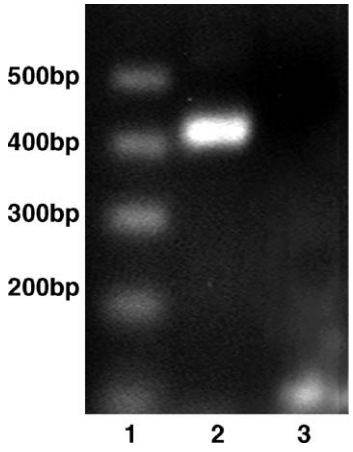

Fig. 2 – Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to investigate the expression of $P2X_5$ receptor transcripts in the hypothalamus of adult rat. Lane 1 shows DNA molecular weight markers, lane 2 is the RT-PCR result from the experimental group and lane 3 is the RT-PCR result of control group after the reverse transcriptase was denatured.

3. Discussion

We used single labeling, double labeling fluorescence immunohistochemistry and RT-PCR to study the distribution of $P2X_5$ receptor protein and mRNA and colocalization of $P2X_5$ receptors with AVP and nNOS in the rat hypothalamus. This study provides the first evidence that $P2X_5$ receptors are widely distributed in the rat hypothalamus and colocalized with AVP and NOS within several nuclei or regions of the rat hypothalamus and hence has provided a substantial neuroanatomical basis for possible functional interactions between the purinergic and nitrergic systems and the purinergic and vasopressinergic systems in rat hypothalamus.

In this study, we found that $P2X_5$ receptor-ir neurons and fibers were distributed widely, but variably, in different regions of the rat hypothalamus. The control experiments with $P2X_5$ antiserum absorbed with $P2X_5$ peptides showed no positive staining. The specificity of the antisera was also verified by previous studies where it was shown to detect the $P2X_5$ receptor subunit, but not the remaining subunits, when expressed in cell lines (Oglesby et al., 1999). RT-PCR confirmed the presence of $P2X_5$ receptor mRNA in the hypothalamus.

Neurons expressing AVP-ir were mainly found in the paraventricular nucleus, supraoptic nucleus, accessory secretory nuclei and suprachiasmatic nuclei, confirming earlier studies (George and Jacobowitz, 1975; Sofroniew and Glasmann, 1981). Our double immunostaining data showed that most of the neurons with AVP-ir in paraventricular nucleus and nearly half of the neurons in supraoptic and accessory secretory nuclei were labeled by the $P2X_5$ receptor antibody; however, no coexistence between $P2X_5$ receptor-ir and AVP-ir was found in the suprachiasmatic or ventromedial nuclei. nNOS-ir neurons were found to be distributed widely in the preoptic region, supraoptic and paraventricular nuclei, lateral hypothalamic area, ventromedial and dorsomedial nuclei, arcuate nucleus and various parts of the mammillary region, confirming earlier studies (Yamada et al., 1996). The present study showed that the nuclei or regions with both nNOS-ir neurons and $P2X_5$ receptor-ir neurons were lateral paraventricular, suprachiasmatic, supraoptic and ventromedial nuclei. The highest percentages of coexistences between $P2X_5$ receptors and nNOS immunolabeling were found in the lateral and ventral paraventricular and ventromedial nuclei. This is the first report that the $P2X_5$ receptor is colocalized with AVP and nNOS in rat hypothalamus nuclei.

Information about interactions between P2X receptors and AVP or NOS systems in the brain is limited (Juranyi et al., 1999; Kapoor and Sladek, 2000; Reiser, 1995). Juranyi et al. (1999) found that, during short-term hypoxia, activation of P2 receptors was responsible for initiating [^{3}H]purine outflow from the rat hippocampal slices. Furthermore, administration of *N*-nitro-L-arginine methyl ester decreased hypoxia evoked

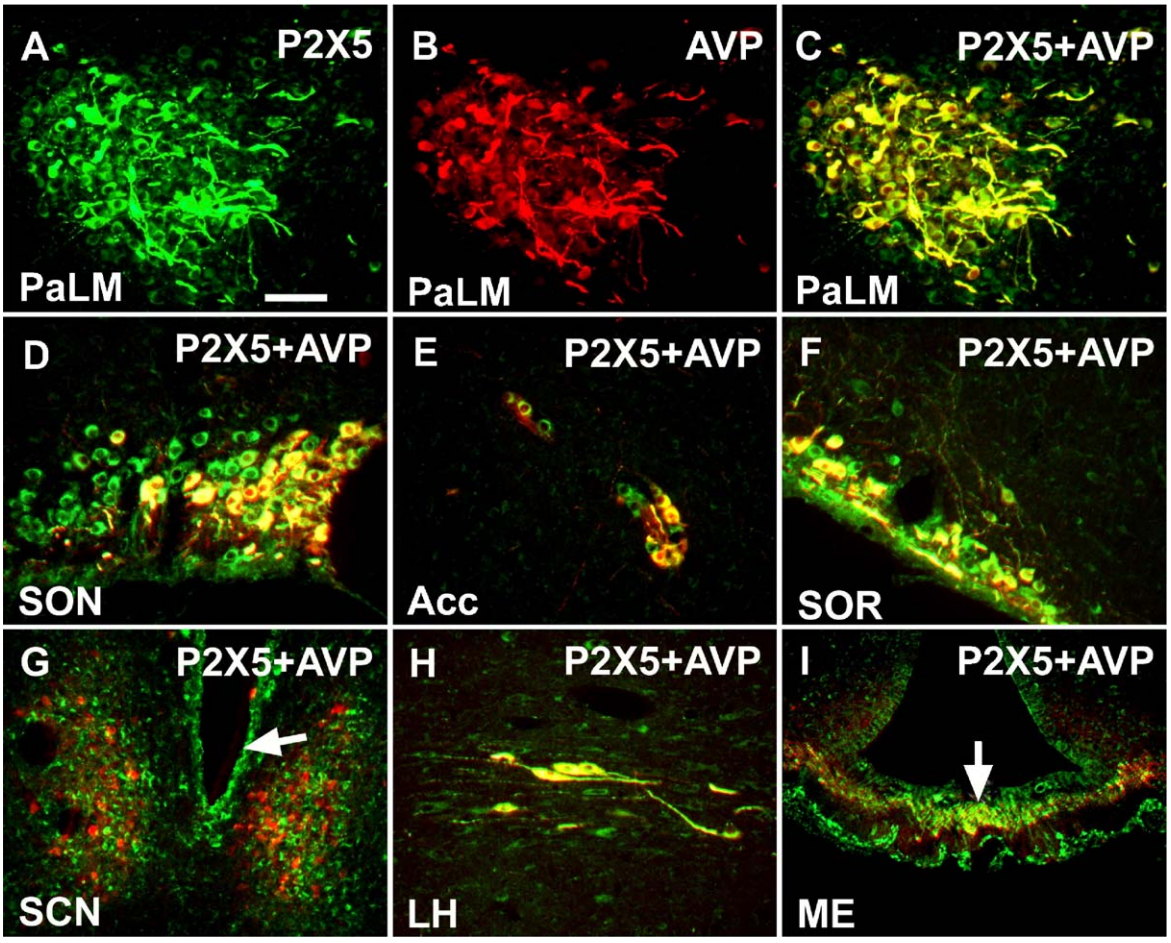

Fig. 3 – Coexistence of $P2X_5$ receptor-ir and AVP in rat hypothalamus. (A) $P2X_5$ receptor-ir neurons and fibers in the paraventricular hypothalamic nucleus, lateral magnocellular area (PaLM; green). (B) AVP-ir neurons and fibers in the PaLM at the same section of panel A (red). (C) Merged image of panels A and B. Note that all the AVP-ir neurons and fibers were colocalized with $P2X_5$ receptor-ir (yellow), but a few $P2X_5$ receptor-ir neurons (green) were not labeled with AVP. (D) Coexistence of $P2X_5$ receptor-ir and AVP-ir in the supraoptic nucleus (SON). Note that nearly all the AVP-ir neurons also expressed $P2X_5$ receptors-ir (yellow), but a number of the $P2X_5$ receptor-ir neurons (green) did not express AVP. (E) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and AVP-ir (red) in the accessory neurosecretory nucleus (Acc). (F) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and AVP (red) in the retrochiasmatic part of supraoptic nucleus (SOR). (G) Colocalization of $P2X_5$ receptor-ir (green) and AVP (red) in suprachiasmatic nucleus (SCN). Note that no coexistence (yellow) was present. (H) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and AVP-ir (red) in paraventricular hypothalamic nucleus, posterior part (PaPO). (I) Coexistence of $P2X_5$ receptor-ir and AVP-ir in the median eminence (ME). Arrow shows fibers that are double-labeled with $P2X_5$ receptor and AVP antibodies, other areas do not show colocalization. Scale bar for all figures = 80 μm. In each figure, the dorsal aspect of the nuclei is at the top and the ventral aspect of the nuclei is at the bottom.

outflow of [^{3}H]purines. These results implied that release of nitric oxide could be a downstream effect of P2 receptor activation and that P2 receptors are involved in the regulation of purine outflow during hypoxic episodes. In the hypothalamus, ATP was shown to be involved in regulation of body temperature and hormone secretion (Gurin et al., 2002, 2003; Kapoor and Sladek, 2000; Mori et al., 1992). Application of ATP to explants of the hypothalamo-neurohypophysial system was shown to evoke an increase in vasopressin release, a response that was attenuated by the P2 receptor antagonist PPADS (Kapoor and Sladek, 2000; Mori et al., 1992). This finding was supported by evidence demonstrating a direct input originating from A1 cells located in the caudal ventrolateral medulla that synapse directly on vasopressin-containing neurons in the supraoptic and paraventricular nuclei and utilize ATP as a co-transmitter (Day et al., 1992, 1993). Recently, Gourine and colleagues showed that ATP acting at P2X receptors (possibly $P2X_3$ and/or $P2X_7$ receptors) played an important role in central mechanisms of body temperature control at various ambient temperatures and during fever (Gourine et al., 2002). These data indicated that, at the hypothalamic level, ATP-induced activation of P2X receptors regulates both hormone secretion and body temperature. The present study is consistent with the possibility of a functional relationship between activation of $P2X_5$ receptors and nitric oxide generation and release of AVP in the hypothalamus, but further support is needed from studies using specific P2 receptor antagonists.

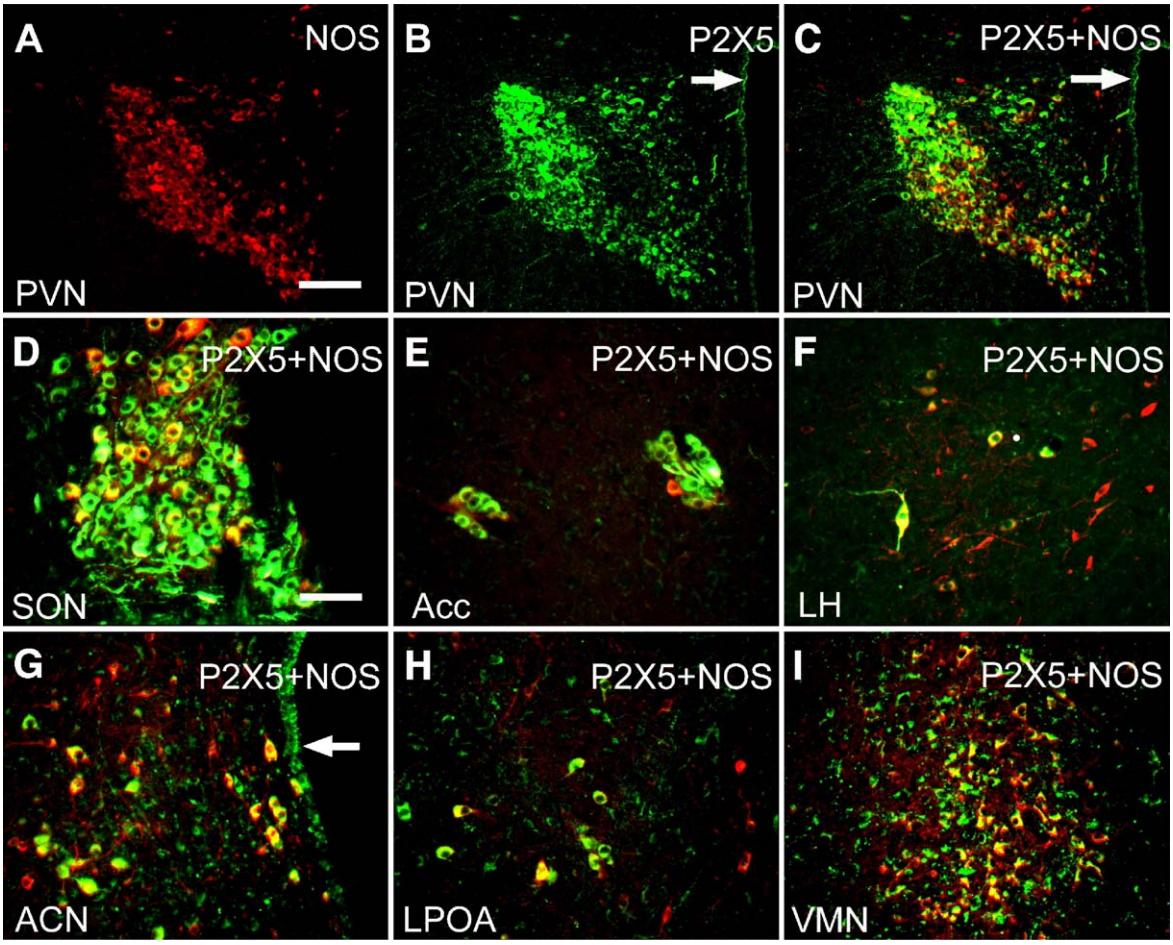

Fig. 4 – Coexistences of $P2X_5$ receptor-ir and nNOS in rat hypothalamus. (A) $P2X_5$ receptor-ir neurons and fibers in the paraventricular hypothalamic nucleus (PVN; green). (B) nNOS-ir neurons and fibers in the PVN at the same section of panel A (red). (C) Merged image of panels A and B, note that most of nNOS-ir neurons and fibers (red) were colocalized with $P2X_5$ receptor-ir (yellow). (D) Coexistences of $P2X_5$ receptor-ir (green) and nNOS-ir (red) neurons in the supraoptic nucleus (SON). Note that nearly all the nNOS-ir neurons also expressed $P2X_5$ receptors-ir (yellow), but a number of $P2X_5$ receptor-ir neurons did not express nNOS. (E) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and nNOS-ir (red) in the accessory neurosecretory nucleus (Acc). (F) Coexistence (yellow) of $P2X_5$ receptor-ir and nNOS-ir in the lateral hypothalamic area (LH). (G) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and nNOS-ir (red) in the area near anterior commissural nucleus (ACN). (H) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and nNOS-ir (red) in the lateral preoptic area (LPOA). (I) Coexistence (yellow) of $P2X_5$ receptor-ir (green) and nNOS-ir (red) in the ventromedial nucleus (VMN). In all figures, arrows indicate positive epithelial cells on the 3rd ventricular wall. Scale bars in panels A, B, C = 160 μm; D to I = 80 μm. In each figure, the dorsal aspect of the nuclei is at the top and the ventral aspect of the nuclei is at the bottom.

In the CNS, populations of nNOS-containing neurons have been demonstrated in several regions of the hypothalamus. Within the paraventricular nucleus, nNOS-immunoreactive neurons were observed in the magnocellular, parvicellular and lateral parvicellular subdivisions. While the largest numbers of nNOS-immunoreactive neurons within the paraventricular nucleus were found in the magnocellular subdivision, the parvicellular subregions also exhibited prominent staining. Additionally, a large number of nNOS-immunoreactive neurons were also detected within the SON (see Yao et al., 2003). NO has been shown to play an important role in regulation of autonomic functions. Previous data have shown that the NO system within the hypothalamus, especially within the paraventricular nucleus, involved in controlling autonomic outflow is altered during heart failure and may contribute to the elevated levels of vasopressin and sympathoexcitation commonly observed in heart failure (Zhang et al., 1998, 2001; Zheng et al., 2005). The present study showed that the $P2X_5$ receptor coexisted with nNOS in the hypothalamic nuclei. These data imply that endogenous ATP could regulate the function of NO neurons in the hypothalamic nuclei through $P2X_5$ receptors and may indirectly control autonomic outflow during heart failure.

The present study showed strong immunostaining for $P2X_5$ receptors in the supraoptic nucleus of the hypothalamus. This was contradictory to previously published data where $P2X_5$ mRNA was not detected (Collo et al., 1996; Shibuya et al., 1999). The $P2X_5$ receptor antiserum used in this study has previously been shown to detect the $P2X_5$ receptor subunit, but not the remaining subunits, when these subunits were expressed in cell lines (Oglesby et al., 1999). No immunoreactivity for $P2X_5$ receptors was observed when the sections were incubated with the antibody

Table 1 – Percentage of neurons in different regions of the rat hypothalamus that express $P2X_5$ receptors and arginine vasopressin (AVP) and those that show double labeling for $P2X_5$ receptors and AVP

Nuclei or region	$P2X_5$+	$P2X_5$+ AVP+	(%) double labeling	$P2X_5$+ AVP–	$P2X_5$– AVP+
PVN					
PaMP	25 ± 5	18 ± 3	72 ± 8	7 ± 4	0
PaLM	78 ± 8	70 ± 6	90 ± 7	8 ± 3	0
PaV	38 ± 6	31 ± 4	81 ± 9	7 ± 5	0
SCN	62 ± 12	0	0	62 ± 12	30 ± 6
SON	66 ± 5	32 ± 6	46 ± 9	34 ± 7	0
ANN	13 ± 4	8 ± 3	58 ± 5	5 ± 2	0
VMN	78 ± 6	0	0	78 ± 6	0

The percentage of neurons in different regions of the rat hypothalamus that express $P2X_5$ receptors and arginine vasopressin (AVP) and those that show double labeling for $P2X_5$ receptors and AVP. $P2X_5$+, $P2X_5$ receptor-ir neurons; $P2X_5$+ AVP+, $P2X_5$ receptor-ir neurons also expressing AVP-ir; $P2X_5$+ AVP–, $P2X_5$ receptor-ir neurons not expressing AVP-ir; $P2X_5$– AVP +, AVP-ir neurons not expressing $P2X_5$ receptor-ir; (%) double labeling, the percentage of $P2X_5$+ AVP+ neurons. PVN, paraventricular nucleus; PaMP, paraventricular hypothalamic nucleus, medial parvocellular part; PaLM paraventricular hypothalamic nucleus, lateral magnocellular part; PaV, paraventricular hypothalamic nucleus, ventral part; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; ANN, accessory neurosecretory nuclei; VMN: ventromedial nucleus.

solutions pre-absorbed with the $P2X_5$ receptor peptide. This suggests that the immunoreactivity for $P2X_5$ receptors seen in this study in the hypothalamus is specific, although non-specific immunostaining cannot be definitively excluded. The discrepancy in $P2X_5$ receptor staining from this study and the previously reported absence of mRNA for $P2X_5$ receptors may be due to the age of the animals. In this study, adult animals were used, whereas in the other two studies (Collo et al., 1996; Shibuya et al., 1999), young animals of 2–4 weeks were used.

In conclusion, the present study has demonstrated that $P2X_5$ receptors are distributed widely in the rat hypothalamus. Significant numbers of AVP-containing neurons in supraoptic nucleus, paraventricular nucleus with accessory neurosecretory nuclei expressed the $P2X_5$ receptor. Furthermore, significant numbers of nNOS-containing neurons in supraoptic nucleus, paraventricular nucleus, accessory neurosecretory nuclei and ventromedial nucleus were labeled by the $P2X_5$ receptor antibody. These findings provide a morphological basis for possible functional interactions between the purinergic and nitrergic or vasopressinergic neurotransmitter systems. Such interactions may be important in regulation of hormone secretion and body temperature at the hypothalamic level.

4. Experimental procedures

4.1. *Tissue preparation*

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with Home Office (UK) regulations covering Schedule One Procedures and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. All protocols were approved by the local animal ethics committee. Six adult Wistar rats (250–300 g) were used. The rats were killed by asphyxiation with CO_2 and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4. The brains were removed, and the hypothalamus was microdissected out whole immediately and immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 24 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 by immersion in isopentane at –70 °C for 2 min. Coronal sections (30 μm) of the hypothalamus were cut with a Leica cryostat (Leica, Heerbrugg, Switzerland) and floated in PBS.

4.2. *Immunohistochemistry*

Immunohistochemistry for $P2X_5$ receptors was performed using rabbit polyclonal antibodies against a unique peptide sequence of $P2X_5$ receptors provided by Roche Palo Alto (CA, USA). The immunogens used for the production of the polyclonal $P2X_5$ receptor antibody were synthetic peptides corresponding to the carboxyl terminal of the cloned rat $P2X_5$ receptor, covalently linked to keyhole limpet hemocyanin. The peptide sequences of the $P2X_5$ receptor are of amino acid sequence 437–452 (RENAIVNVKQSQILH). The polyclonal antibody was raised by multiple monthly injections of New Zealand White rabbits with the corresponding peptides (prepared by Research Genetics, Huntsville, AL). The $P2X_5$ receptor antiserum used in this study has previously been shown to detect the $P2X_5$ receptor subunit, but not the remaining subunits, when these subunits were expressed in cell lines (Oglesby et al., 1999). As previously reported, no cross-reactivity is observed with other P2X receptor antisera (Oglesby et al., 1999).

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 $P2X_5$ receptor antibody, diluted 1:1500 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 ImmunoResearch Lab, West Grove, PA) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in ExtrAvidin peroxidase (Sigma Chemical Co., Poole, UK) diluted 1:1000 in PBS for 30 min at room temperature. The $P2X_5$ immunoreactivity was visualized by the TSA (tyramide signal amplification) Fluorescein system (NEL701, NEN, USA). After visualization, the sections for single immunostaining were mounted, the sections for double immunostaining were incubated with the second primary antibodies of AVP (Chemicon Int., CA, USA) diluted 1:10000 and nNOS (sheep anti rat, Chemicon Int.) diluted 1:1000 in the antiserum dilution solution overnight at 4 °C. Subsequently, the sections were incubated with Cy3-conjugated donkey-anti-sheep (Jackson) diluted 1:300 in antiserum dilution solution for 1 h at room temperature. All the

Table 2 – Percentage of neurons in different regions of the rat hypothalamus that express $P2X_5$ receptors and neuronal nitric oxide synthase (nNOS) and those that show double labeling for $P2X_5$ receptors and nNOS

Nuclei or regions	$P2X_5$+	$P2X_5$+ nNOS+	(%) double labeling	$P2X_5$+ nNOS–	$P2X_5$– nNOS+
PVN					
PaMP	28 ± 4	12 ± 3	31 ± 5	16 ± 5	7 ± 2
PaLM	75 ± 6	66 ± 5	87 ± 7	9 ± 4	9 ± 4
PaV	37 ± 5	34 ± 3	90 ± 8	3 ± 2	8 ± 3
SCN	58 ± 10	0	0	58 ± 10	6 ± 2
SON	70 ± 6	34 ± 5	48 ± 8	36 ± 7	0
ANN	15 ± 3	6 ± 3	42 ± 5	9 ± 4	0
VMN	0 ± 4	40 ± 6	56 ± 8	30 ± 8	28 ± 9

Percentage of neurons in different regions of the rat hypothalamus that express $P2X_5$ receptors and neuronal nitric oxide synthase (nNOS) and those that show double labeling for $P2X_5$ receptors and nNOS. $P2X_5$+, $P2X_5$ receptor-ir neurons; $P2X_5$+ nNOS+, $P2X_5$ receptor-ir neurons also expressing nNOS-ir; $P2X_5$+ nNOS–, $P2X_5$ receptor-ir neurons not expressing nNOS-ir; $P2X_5$– nNOS+, nNOS-ir neurons not expressing $P2X_5$ receptor-ir; (%) double labeling, the percentage of $P2X_5$+ nNOS+ neurons. PVN, paraventricular nucleus; PaMP, paraventricular hypothalamic nucleus, medial parvocellular part; PaLM, paraventricular hypothalamic nucleus, lateral magnocellular part; PaV, paraventricular hypothalamic nucleus, ventral part; SCN, suprachiasmatic nucleus; SON, supraoptic nucleus; ANN, accessory neurosecretory nuclei; VMN, ventromedial nucleus.

incubations and reactions were separated by 3 × 10 min washes in PBS.

4.3. *Photomicroscopy and data analysis*

Images of the immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica) attached to a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany). Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged by using Adobe Photoshop 5.0. The focal plane on the microscope was not adjusted while determining whether a particular cell colocalized both $P2X_5$ and nNOS or AVP. 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 colocalize both $P2X_5$ receptors and nNOS or AVP. The number of immunopositive neurons was counted unilaterally throughout the caudorostral extent of the respective nuclei as defined by the atlas of Paxinos and Watson (1986). Data for each of the nuclei analyzed were obtained from each of the rats used. Three to five sections from each animal were used, and the average number in one section was calculated. The numbers presented in Tables 1 and 2 represent the average number of immunopositive cells observed unilaterally per section ± SEM (Yao et al., 2003).

Control experiments were carried out with $P2X_5$ antiserum pre-absorbed with $P2X_5$ receptor peptide at a concentration of 25 μg/ml. The amino acid sequence for this peptide is: 437–452 (RENAIVNVKQSQILH), synthesized by Roche Palo Alto. No staining was observed in those specimens incubated with the antibody solutions pre-absorbed with $P2X_5$ receptor peptides. Controls for double labeling consisted of the secondary primary antibodies being replaced by 10% NHS. No red staining for the secondary primary antibody was observed for controls indicating no cross-reaction between secondary antibodies.

4.4. *RT-PCR*

Three Wistar rats (250–300 g) were used for the RT-PCR study. Three copies of total RNAs were extracted from hypothalami using the SV Total RNA Isolation System (Promega, WI, USA). RT-PCR was performed using Ready-to-Go RT-PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, UK). Reverse transcription was performed using the Moloney murine leukemia virus reverse transcriptase. Primer sequences for $P2X_5$ receptors (Shibuya et al., 1999) were used for amplification reactions, as reported previously. The sense sequence used was GCCGAAAGCTTCACCATTTCCATAA, and the antisense sequence used was CCTACGGCATCCGCTTTGATGTGATAG. Reverse transcription and cDNA amplification for $P2X_5$ receptors were carried out with a thermal cycler (Hybaid, UK) in a two-step protocol using Ready-To-Go RT-PCR Beads (Amersham). Every sample was further treated with Amplification Grade DNase I (Sigma) to remove any residual DNA present that could generate false-positive results. Briefly, 1 μg of total RNA was reverse transcribed using the pd(T)12–18 as the first-strand primer at 42 °C for 30 min, and the enzyme was denatured at 95 °C for 5 min. The sequence-specific primers were then added to the reaction mixtures, and the PCR cycling parameters were 95 °C for 45 s, 58 °C for 1 min, 72 °C for 1 min for 35 cycles followed by a further stage of 10-min extension at 72 °C. The resulting PCR products were resolved in a 1.5% agarose gel containing ethidium bromide and observed under ultraviolet illumination. RT-PCR results for the hypothalamus were confirmed by repetition with three separate RNA samples. Control experiments were conducted by denaturing the reverse transcriptase (95 °C for 10 min) before the RT-PCR reaction. These experiments demonstrated that, on denaturation of the reverse transcriptase, no $P2X_5$ receptor cDNA could be detected (Fig. 2).

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REFERENCES

Arancibia, S., Rage, R., Astier, H., Tapia-Arancibia, L., 1996. Neuroendocrine and autonomous mechanisms underlying thermoregulation in cold environment. Neuroendocrinology 64, 257–267.

Atkinson, L., Batten, T.F., Moores, T.S., Varoqui, H., Erickson, J.D., Deuchars, J., 2004. Differential co-localisation of the $P2X_7$ receptor subunit with vesicular glutamate transporters VGLUT1 and VGLUT2 in rat CNS. Neuroscience 123, 761–768.

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.

Burnstock, G., 2003. Purinergic receptors in the nervous system. In: Schwiebert, E.M. (Ed.), Current Topics in Membranes.

Purinergic Receptors and Signaling, vol. 54. Academic Press, San Diego, pp. 307–368.
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.
Coote, J.H., 1995. Cardiovascular function of the paraventricular nucleus of the hypothalamus. Biol. Signals 4, 142–149.
Day, T.A., Sibbald, J.R., Smith, D.W., 1992. A1 neurons and excitatory amino acid receptors in rat caudal medulla mediate vagal excitation of supraoptic vasopressin cells. Brain Res. 594, 244–252.
Day, T.A., Sibbald, J.R., Khana, S., 1993. ATP mediates an excitatory noradrenergic neuron input to supraoptic vasopressin cells. Brain Res. 607, 341–344.
Furukawa, K., Ishibashi, H., Akaike, N., 1994. ATP-induced inward current in neurons freshly dissociated from the tuberomammillary nucleus. J. Neurophysiol. 71, 868–873.
George, J.M., Jacobowitz, D.M., 1975. Localization of vasopressin in discrete areas of the rat hypothalamus. Brain Res. 93, 363–366.
Gourine, A.V., Melenchuk, E.V., Poputnikov, D.M., Gourine, V.N., Spyer, K.M., 2002. Involvement of purinergic signalling in central mechanisms of body temperature regulation in rats. Br. J. Pharmacol. 135, 2047–2055.
Gurin, V.N., Gurin, A.V., Melenchuk, E.V., Spyer, K.M., 2002. Effect of activation and blockade of central P2X receptors on body temperature. Ross. Fiziol. Zh. Im. I.M. Sechenova 88, 731–740.
Gurin, V.N., Gurin, A.V., Melenchuk, E.V., Spyer, K.M., 2003. The effects of activation and blockade of central P2X receptors on body temperature. Neurosci. Behav. Physiol. 33, 845–851.
Hiruma, H., Bourque, C.W., 1995. P2 purinoceptor-mediated depolarization of rat supraoptic neurosecretory cells in vitro. J. Physiol. 489, 805–811.
Jo, Y.H., Role, L.W., 2002. Coordinate release of ATP and GABA at in vitro synapses of lateral hypothalamic neurons. J. Neurosci. 22, 4794–4804.
Juranyi, Z., Sperlagh, B., Vizi, E.S., 1999. Involvement of P2 purinoceptors and the nitric oxide pathway in [^{3}H]purine outflow evoked by short-term hypoxia and hypoglycemia in rat hippocampal slices. Brain Res. 823, 183–190.
Kapoor, J.R., Sladek, C.D., 2000. Purinergic and adrenergic agonists synergize in stimulating vasopressin and oxytocin release. J. Neurosci. 20, 8868–8875.
Kidd, E.J., Grahames, C.B., Simon, J., Michel, A.D., Barnard, E.A., Humphrey, P.P., 1995. Localization of P2X purinoceptor transcripts in the rat nervous system. Mol. Pharmacol. 48, 569–573.
Kuenzel, W.J., 1994. Central neuroanatomical systems involved in the regulation of food intake in birds and mammals. J. Nutr. 124, 1355S–1370S.
Loesch, A., Burnstock, G., 2001. Immunoreactivity to $P2X_6$ receptors in the rat hypothalamo-neurohypophysial system: an ultrastructural study with extravidin and colloidal gold–silver labeling. Neuroscience 106, 621–631.
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.
McCann, S.M., Gutkowska, J., Franci, C.R., Favaretto, A.L., Antunes-Rodrigues, J., 1994. Hypothalamic control of water and salt intake and excretion. Braz. J. Med. Biol. Res. 27, 865–884.
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.
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 P2X receptors $P2X_1$ through $P2X_7$. Drug Dev. Res. 47, 189–195.
Palkovits, M., 1992. Peptidergic neurotransmitters in the endocrine hypothalamus. Ciba Found. Symp. 168, 3–10.
Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, 2nd ed. Academic Press, New York.
Reiser, G., 1995. Ca^{2+}- and nitric oxide-dependent stimulation of cyclic GMP synthesis in neuronal cell line induced by P2-purinergic/pyrimidinergic receptor. J. Neurochem. 64, 61–68.
Rothwell, N.J., 1994. CNS regulation of thermogenesis. Crit. Rev. Neurobiol. 8, 1–10.
Sakata, T., Kang, M., Kurokawa, M., Yoshimatsu, H., 1995. Hypothalamic neuronal histamine modulates adaptive behavior and thermogenesis in response to endogenous pyrogen. Obes. Res. 3, 707S–712S.
Shibuya, I., Tanaka, K., Hattori, Y., Uezono, Y., Harayama, N., Noguchi, J., Ueta, Y., Izumi, F., Yamashita, H., 1999. Evidence that multiple P2X purinoceptors are functionally expressed in rat supraoptic neurones. J. Physiol. 514, 351–367.
Sofroniew, M.V., Glasmann, W., 1981. Golgi-like immunoperoxidase staining of hypothalamic magnocellular neurons that contain vasopressin, oxytocin or neurophysin in the rat. Neuroscience 6, 619–643.
Sorimachi, M., Ishibashi, H., Moritoyo, T., Akaike, N., 2001. Excitatory effect of ATP on acutely dissociated ventromedial hypothalamic neurons of the rat. Neuroscience 105, 393–401.
Swaab, D.F., Hofman, M.A., Lucassen, P.J., Purba, J.S., Raadsheer, F. C., Van de Nes, J.A., 1993. Functional neuroanatomy and neuropathology of the human hypothalamus. Anat. Embryol. (Berl.) 187, 317–330.
Vorobjev, V.S., Sharonova, I.N., Haas, H.L., Sergeeva, O.A., 2003a. Expression and function of P2X purinoceptors in rat histaminergic neurons. Br. J. Pharmacol. 138, 1013–1019.
Vorobjev, V.S., Sharonova, I.N., Sergeeva, O.A., Haas, H.L., 2003b. Modulation of ATP-induced currents by zinc in acutely isolated hypothalamic neurons of the rat. Br J. Pharmacol. 139, 919–926.
Vulchanova, L., Arvidsson, U., Riedl, M., Wang, J., Buell, G., Surprenant, A., North, R.A., 1996. Differential distribution of two ATP-gated ion channels P2X receptors determined by immunocytochemistry. Proc. Natl. Acad. Sci. U. S. A. 93, 8063–8067.
Xiang, Z., Bo, X., Oglesby, I., Ford, A., Burnstock, G., 1998. Localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamus. Brain Res. 813, 390–397.
Yamada, K., Emson, P., Hokfelt, T., 1996. Immunohistochemical mapping of nitric oxide synthase in the rat hypothalamus and colocalization with neuropeptides. J. Chem. Neuroanat. 10, 295–316.
Yao, S.T., Gourine, A.V., Spyer, M.K., Barden, J.A., Lawrence, A.J., 2003. Localisation of $P2X_2$ receptor subunit immunoreactivity on nitric oxide synthase expressing neurones in the brain stem and hypothalamus of the rat: a fluorescence immunohistochemical study. Neuroscience 121, 411–419.
Zhang, K., Zucker, I.H., Patel, K.P., 1998. Altered number of diaphorase (NOS) positive neurons in the hypothalamus of rats with heart failure. Brain Res. 786, 219–225.
Zhang, K., Li, Y.F., Patel, K.P., 2001. Blunted nitric oxide-mediated inhibition of renal nerve discharge within PVN of rats with heart failure. Am. J. Physiol.: Heart Circ. Physiol. 281, H995–H1004.
Zheng, H., Li, Y.F., Cornish, K.G., Zucker, I.H., Patel, K.P., 2005. Exercise training improves endogenous nitric oxide mechanisms within the paraventricular nucleus in rats with heart failure. Am. J. Physiol.: Heart Circ. Physiol. 288, H2332–H2341.