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P2X receptors are expressed on neurons containing luteinizing hormone-releasing hormone in the mouse hypothalamus

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ABSTRACT

Expression of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors, members of a family of ATP-gated cation channels, on neurons containing luteinizing hormone-releasing hormone (LHRH) in the mouse hypothalamus was studied with double-labeling fluorescence immunohistochemistry. This study demonstrated that different combinations of P2X receptor subunits were expressed on the perikarya and axon terminals of LHRH-producing neurons. It was shown for the first time that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits were expressed on the perikarya of LHRH-producing neurons and $P2X_2$ and $P2X_6$ on their axon terminals. These results suggest that activation of P2X receptors by ATP via different homomeric or heteromeric P2X receptors at both presynaptic and postsynaptic sites could be involved in the regulation of LHRH secretion at the forebrain level.

Extracellular ATP has been identified as an excitatory neurotransmitter, neuromodulator, or humoral factor which acts via P2 purinoceptors [4]. 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 [6]. Numerous lines of evidence indicate that ATP is released as a cotransmitter with other neurotransmitters such as norepinephrine, acetylcholine, nitric oxide and glutamate [5].

Extracellular ATP and P2 purinoceptors play very important roles in the hypothalamo–neurohypophysial system [14,22,28,30,31]. It has been shown that ATP induces a rapid increase in intracellular Ca^{2+} concentration in the hypothalamic neurosecretory neurons [9]. Functional and morphological data showed that ATP, via P2X receptors, was involved in the release of neuropeptides, such as vasopressin and oxytocin, in the hypothalamus [3,10,15,25,30]. Previous data showed that ATP via $P2X_2$ and $P2X_4$, but not via P2Y receptors, stimulated the release of luteinizing hormone-releasing hormone (LHRH) in olfactory placode culture [34]. The olfactory placode is a transient organ in the embryo. LHRH is a very important hormone and is released in pulses at hourly intervals, and this pulsatility is essential for the maintenance of the reproductive function of adult animals [24]. At the present time, there is no data about the expression of P2X on LHRH neurons in adult animals. Thus, in this study, we have examined the colocalization between LHRH and $P2X_{1-6}$ receptors in the mouse brain using double-labeling fluorescence immunohistochemistry.

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Six Kunming strain mice (30–50 g) were used. The mice 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 pH 7.4. 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 coronal sections of the hypothalamus (20 μm in thickness) were cut with a Leica cryostat and floated in PBS.

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

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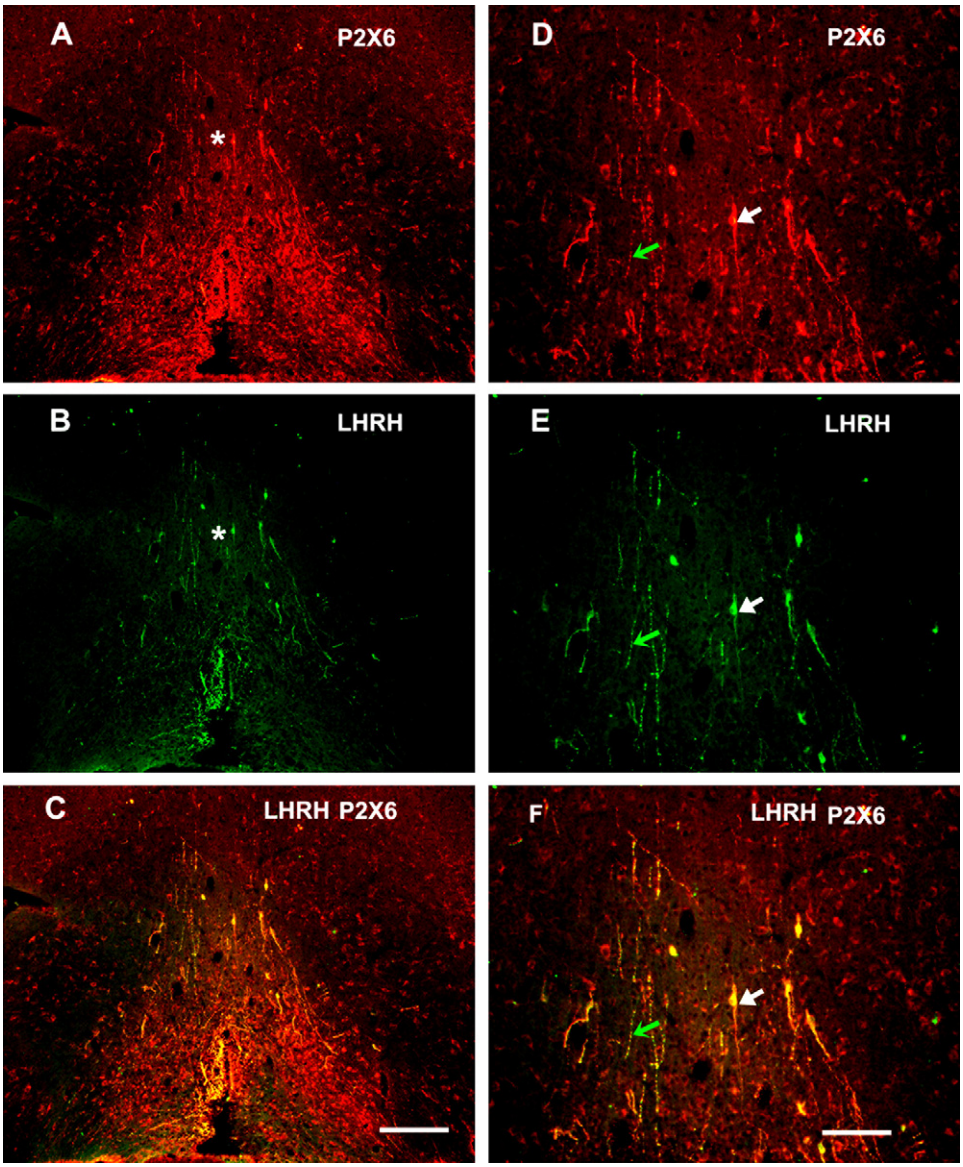

Fig. 1. Colocalization of $P2X_6$ receptor-ir and LHRH-ir in the area around the OVLT of mouse forebrain. (A) $P2X_6$ receptor-ir neurons and fibers (red). (B) LHRH-ir neurons and fibers (green) from the same section of (A). (C) The merged image from (A) and (B), note that all the LHRH-ir neurons (green) are colocalised with $P2X_6$ receptor-ir (red). Double-labeled neurons and fibers are yellow in color and the majority of the $P2X_6$ receptor-ir cells are only labeled with the red color. (D) A high magnification of the area indicated by a star in (A). (E) A high magnification of the area indicated by a star in (B). (F) The merged image from (D) and (E). Note that all the LHRH-ir neurons (green) and fibers are colocalised with $P2X_6$ receptor-ir (red). A white or green arrow indicates double labeled neurons or fibers in (D)–(F), respectively. Scale bar in (A)–(C) = 200 μm, in (D)–(F) = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Oglesby et al [27], no cross-reactivity was observed with other P2X antisera.

Simultaneous detection of two antigens by immunostaining usually requires primary antibodies from two different species. A novel double-labeling immunostaining method for immunodetection of two independent antigens has been described [33]. 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 signals 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 [40,41]. 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 LHRH antibody (rabbit polyclone, Santa Cruz), diluted 1:2000 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 Laboratories) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in extravidin peroxidase (Sigma) diluted 1:1000 in PBS for 30 min at room temperature. The LHRH immunoreactivity was visualized by the TSA Fluorescein system (NEL701, NEN, USA). After visualization the sections were incubated with the second primary antibodies of $P2X_{1-6}$ diluted 1:200 (rabbit anti-rat IgG, Roche) in the antiserum dilution solution overnight at 4 °C. Subsequently the sections were incubated with Cy3 conjugated donkey-anti-rabbit (Jackson ImmunoResearch Laboratories) 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.

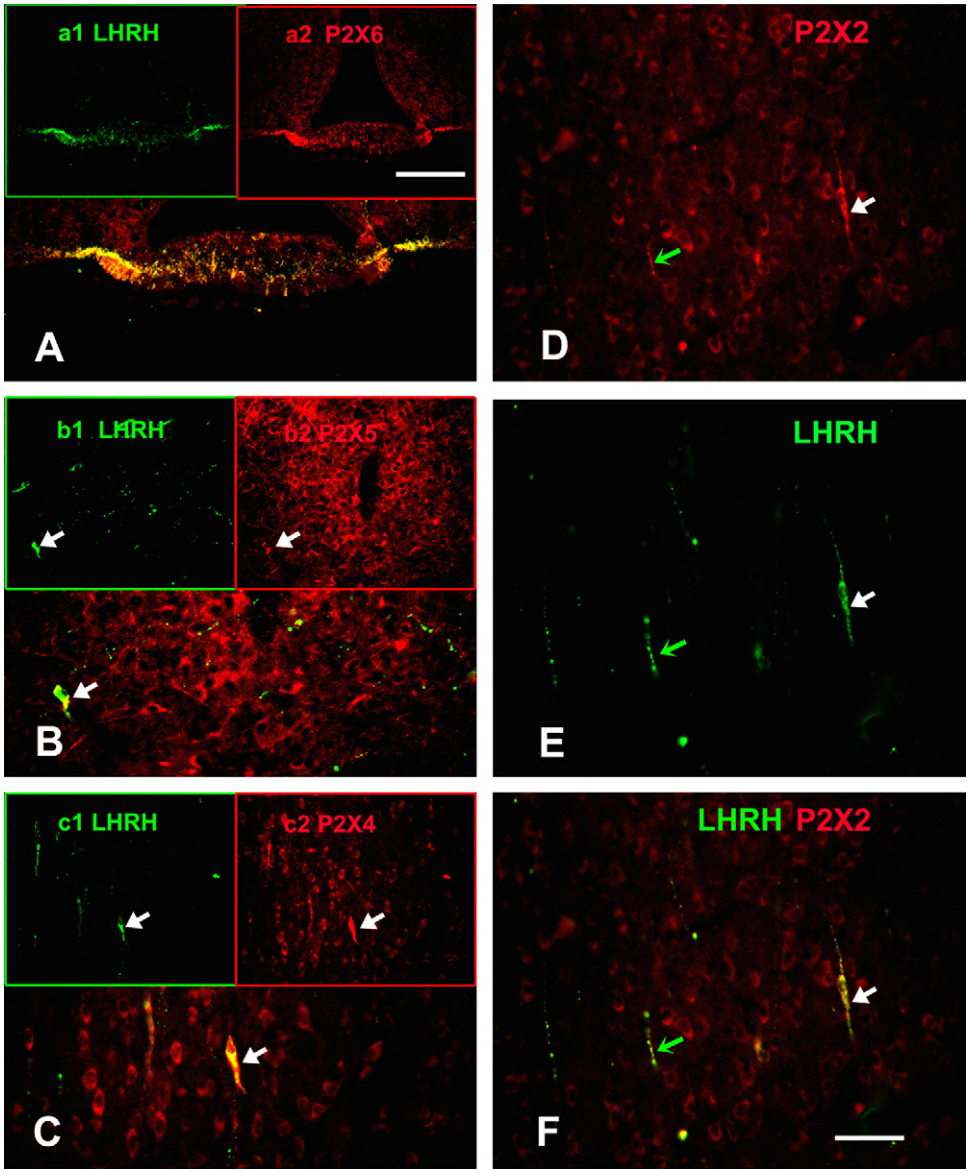

Fig. 2. Colocalization of P2X receptor-ir and LHRH-ir in the mouse forebrain. (A) Colocalization of $P2X_6$ receptor-ir and LHRH-ir in the median eminence. (Aa1) LHRH-ir fibers in the median eminence (green). (Aa2) $P2X_6$ receptor-ir fibers and neurons in the median eminence and arcuate nucleus of the hypothalamus. (A) The merged image from (Aa1) and (Aa2). Note that all the LHRH-ir fibers are overlapped with $P2X_6$ receptor-ir fibers, but $P2X_6$ receptor-ir cells in the arcuate nucleus are not overlapped with LHRH-ir fibers. (B) Colocalization of $P2X_5$ receptor-ir and LHRH-ir neurons and fibers in the nucleus of the horizontal limb of the diagonal band. (Bb1) LHRH-ir neurons and fibers (green). (Bb2) $P2X_5$ receptor-ir fibers and neurons. (B) The merged image from (Bb1) and (Bb2). Note that all the LHRH-ir neurons are overlapped with $P2X_5$ receptor-ir neurons, but LHRH-ir fibers are not overlapped with $P2X_5$-ir fibers. (C) Colocalization of $P2X_4$ receptor-ir neurons and LHRH-ir neurons and fibers in the medial septal nucleus. (Cc1) LHRH-ir neurons and fibers. (Cc2) $P2X_4$ receptor-ir neurons. (C) The merged image from (Cc1) and (Cc2). Note that a LHRH-ir neuron is clearly overlapped with a $P2X_4$ receptor-ir neuron. (D–F) Colocalization of $P2X_2$ receptor-ir and LHRH-ir neurons and fibers in the nucleus of the vertical limb of the diagonal band. (D) $P2X_2$ receptor neurons and fibers. (E) LHRH-ir neurons and fibers. (F) The merged image from (D) and (E). Note that all the LHRH-ir neurons are overlapped with $P2X_2$ receptor-ir neurons, but many $P2X_2$ receptor-ir cells are not colocalised with LHRH-ir. A white or green arrow indicates double labeled neurons or fibers in (D)–(F), respectively. Scale bar in (A)–(F) = 100 μm, in (Bb1), (Bb2), (Cc1), and (Cc2) = 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

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 were synthesized by Roche Bioscience, Palo Alto. No staining was observed in those specimens incubated with the antibody solutions re-absorbed with P2X peptides.

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 using Adobe-Photoshop. The focal plane on the microscope was not adjusted whilst determining whether a particular cell colocalised both P2X receptors and LHRH. 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 colocalise both P2X receptor and LHRH.

LHRH-immunoreactive (-ir) neurons were mainly found in the area around the organum vasculosum of the lamina terminalis (OVLT). Labeled neurons, in general, appeared elongated and bipolar or unipolar (Fig. 1B and C). LHRH-ir fibers were mainly found in the OVLT and the median eminence (ME). The LHRH-ir fibers resembled strings of pearls that could be followed for up to several millimeters (Fig. 2Aa1).

It was observed that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor antibodies labeled different subpopulations of neurons to different

intensities in the area around the OVLT, no signal was observed with $P2X_1$ and $P2X_3$ receptor antibodies in this area. In the ME and OVLT, where LHRH-ir fibers were found abundantly, only $P2X_6$ receptor antibody labeled fibers, which resembled LHRH-ir fibers (Figs. 1A and 2Aa2).

When using double-labeling fluorescence immunohistochemistry, it was observed that all the LHRH-ir neurons were also positive for $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors, although the immunostaining intensity was different. The order of immunostaining intensity with different antibodies was $P2X_6 > P2X_4 > P2X_2 > P2X_5$. All the LHRH-ir fibers were also strongly labeled by $P2X_6$ antibody (Figs. 1C, F, and 2A). Some LHRH-ir fibers, especially these nearby the perikarya, were labeled by $P2X_2$ antibody (Fig. 2F), but LHRH-ir fibers in the ME and OVLT were not labeled by $P2X_2$ receptor antibody. Almost no LHRH-ir fibers were labeled by $P2X_4$ or $P2X_5$ receptor antibodies (Fig. 2B and C).

We used double-labeling fluorescence immunohistochemistry to study the colocalization of P2X receptor protein with LHRH-ir neurons of the mouse forebrain. This study provides the first evidence that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors are expressed on LHRH-containing neurons and hence has provided a substantial neuroanatomical basis for possible functional interactions between the purinergic and the LHRH system in the mouse forebrain.

Neurons expressing LHRH were mainly found in the areas around the OVLT, and LHRH-ir fibers were mainly found in the OVLT and ME; this confirms earlier studies [12]. Our data from double-labeling fluorescence immunohistochemistry showed that $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits are expressed on the perikarya of LHRH-containing neurons and $P2X_2$ and $P2X_6$ receptor subunits on the LHRH-ir fibers. These results indicate that ATP may affect the physiological functions of LHRH-containing neurons at presynaptic sites via $P2X_2$ and $P2X_6$ receptors and postsynaptic sites via $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptors in the mouse forebrain.

Molecular cloning of P2X receptor subunits indicates that a subunit is composed of a 379–472-amino acid protein, in which the N and C terminals are in the cytoplasm connected by two-transmembrane-spanning segments and a large extracellular loop [26,29]. Because a single two-transmembrane subunit alone cannot form an ion channel, it is speculated that a receptor is assembled with multiple subunits [16,26]. P2X receptor subunits are able to form homo- 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 [6,13,16]. Thus $P2X_2$, $P2X_4$, $P2X_5$ and $P2X_6$ receptor subunits expressed on LHRH-containing neurons could assemble into homomeric $P2X_2$, $P2X_4$ or $P2X_5$ channels or heteromeric $P2X_{2/6}$ and $P2X_{4/6}$ channels; $P2X_2$ and $P2X_6$ receptor subunits expressed on LHRH-containing fibers could assemble into homomeric $P2X_2$ and heteromeric $P2X_{2/6}$. Further studies using specific antagonists are required for unequivocal support of these possible functional combinations of P2X receptor multimers on LHRH-ir perikarya of the neurons and LHRH-ir fibers in the mouse forebrain.

ATP release from healthy cells is a physiological mechanism [2]. ATP is released from both peripheral and central neurons, but also from many non-neuronal cell types, such as astrocytes. The cytoplasm of most neurons contains 2–5 mM ATP and higher concentrations of ATP (up to 100 mM) are stored in synaptic vesicles [5]. P2X purinoceptors are a family of ATP-gated cation channels with permeabilities to, for example, calcium, sodium and potassium [26]. These channels are selectively permeable to cations ($p_{Ca^{2+}}$ approximately two fold to five fold greater than p_{Na^+} and p_{K^+}) [11]. 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 [9,32,34].

Although the data about interactions between P2X receptors and the LHRH system is limited, there is still some evidence in vitro to suggest that these interactions may exist within the forebrain. A hypothalamic role has been suggested for extracellular ATP to facilitate copper uptake and copper stimulation of the release of LHRH from the ME, via an interaction with purinergic receptors [1]. ATP releases LHRH from isolated hypothalamic granules [8]. LHRH is released from the hypothalamus in pulses at normal reproductive function. Studies of an in vivo culture preparation of LHRH neurons derived from embryonic olfactory placode show that ATP stimulates LHRH release, probably via $P2X_2$ and $P2X_4$ receptor subtypes, and may be involved in synchronization of Ca^{2+} oscillations that appear to underlie the pulsatile release of LHRH [34]. The authors also speculate that glial cells expressing $P2Y_1$ and $P2Y_2$ receptors may also participate in this process.

The character of LHRH neuron activity is synchronization. But LHRH neurons are widely scattered in the preoptic area of the hypothalamus [12,31], and there are few synaptic interactions or gap-junctional interactions among them [39]. Thus, it is difficult to understand how LHRH neurons function synchronously. Previous functional and morphological data showed that LHRH neurons were innervated by axons containing β-endorphin [21], neuropeptide Y [36], GABA [19], glutamate [18], tyrosine-hydroxylase [20], substance P [35], corticotropin releasing factor [23], 5-HT [17], vasoactive intestinal polypeptide [38] and acetylcholine [37]. ATP is a primitive signaling molecule that has been retained as a co-neurotransmitter with other neurotransmitters in both the peripheral and central nervous systems. Acetylcholine, GABA, catecholamine, substance P, neuropeptide Y and vasoactive intestinal polypeptide have been shown to be co-neurotransmitters with ATP [7]. Astrocytes have been reported to express P2X and P2Y purinoceptors [6]. It is therefore hypothesized that ATP, released as a cotransmitter from these terminals, may regulate the activities of LHRH neurons via P2X receptors. ATP can also induce spontaneous intracellular Ca^{2+} oscillations of astrocytes via their P2X or P2Y receptors, subsequently the oscillated astrocytes release their gliotransmitters, such as ATP and glutamate, to act at LHRH neurons and result in Ca^{2+} oscillations and synchronization of LHRH neurons.

In conclusion, the present study demonstrates that different combinations of P2X receptor subunits are expressed on the perikarya and axon terminals of LHRH-ir neurons in the mouse hypothalamus. Activation by ATP of different homomeric or heteromeric P2X receptors at both presynaptic and postsynaptic sites could regulate the synchronization and LHRH release of LHRH-ir neurons. These findings provide the morphological basis for possible functional interactions between the purinergic and LHRH neuron systems. Such interactions may be important in the regulation of LHRH secretion at the forebrain level.

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