P2X₂R Purinergic Receptor Subunit mRNA and Protein Are Expressed by All Hypothalamic Hypocretin/Orexin Neurons

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ABSTRACT

Neurophysiologic data suggest that orexin neurons are directly excited by ATP through purinergic receptors (P2XR). Anatomical studies, though reporting P2XR in the hypothalamus, did not describe it in the perifornical hypothalamic area, where orexinergic neurons are located. Here we report the presence of the P2X₂R subunit in the rat perifornical hypothalamus and demonstrate that hypothalamic orexin neurons express the P2X₂R. Double immunohistochemistry showed that virtually all orexin-immunoreactive neurons are also P2X₂R immunoreactive, whereas 80% of P2X₂R-immunoreactive neurons are also orexin positive. Triple-labeling experiments, combining fluorescence in situ hybridization for P2X₂R mRNA and P2X₂R/orexin double immunofluorescence, confirmed these findings. In addition, in situ hybridization demonstrated that P2X₂R mRNA is localized in cellular processes of orexinergic neurons. The present data support neurophysiologic findings on ATP modulation of orexinergic function and provide direct evidence that the entire population of orexin neurons expresses a P2XR subtype, namely, P2X₂R. Thus, purinergic transmission might intervene in modulating key functions known to be controlled by the orexinergic system, such as feeding behavior and arousal. *J. Comp. Neurol.* 498:58–67, 2006. © 2006 Wiley-Liss, Inc.

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Orexin, also known as *hypocretin*, is a recently identified neuropeptide that is attracting attention for its role in the regulation of functions associated with arousal and wakefulness (Saper et al., 2001; Mileykovskiy et al., 2005). These include control of the level of alertness, locomotor activity, and appetite; control of metabolism and energy expenditure; control of the neuroendocrine and autonomic nervous systems; modulation of incoming pain signals; and drug-seeking behavior (Sutcliffe and de Lecea, 2002; Ferguson and Samson, 2003; Sakurai, 2003; Mochizuki and Scammell, 2003; Burdakov, 2004; Scammell and Saper, 2005; Harris et al., 2005). Remarkably, orexin originates from a small group of neurons located in restricted parts of the hypothalamus: the perifornical area (PeF), dorsomedial hypothalamus (DMH), and lateral hypothal-

amus (LH). These neurons project to many parts of the CNS, from neocortex to spinal cord, and in many respects their targets are consistent with the known functions of orexin. Less is known, however, about what controls these

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neurons and which receptors they harbor on their dendrites and soma.

ATP-gated purinergic receptor channels are made up by seven known subunits (P2X₁₋₇R). They are cation-selective channels mediating excitatory synaptic transmission (within milliseconds) and presenting low affinity, in the micromolar range, for ATP. These receptors present almost equal permeability to Na⁺ and K⁺ and substantial calcium permeability (Khakh, 2001; Khakh et al., 2001). P2XR-generated calcium signals, rather than current profiles, mediate the action of these receptors in cellular physiology in many tissues where they are involved in different functions. In the CNS, P2XR are involved in plasticity (Robertson et al., 2001; Florenzano et al., 2006) and neuronal damage (Florenzano et al., 2002; Viscomi et al., 2004) and have been proposed as therapeutic targets in several CNS illnesses (Koles et al., 2005). The P2X₂R is diffusely present in the brain and expressed in central autonomic areas of the brainstem and hypothalamus (Vulchanova et al., 1996; Xiang et al., 1998).

Various reports have demonstrated physiological interactions between orexinergic and purinergic systems. The A1 receptors for adenosine, which have been proposed as an endogenous sleep factor, are expressed in orexinergic neurons (Thakkar et al., 2002). Furthermore, it has recently been shown that ATP administration on hypothalamic slices induces a dose-dependent increase in spike frequency of orexin neurons (Wollmann et al., 2005) and DMH neurons (Matsumoto et al., 2004). Thus, physiological and anatomical data suggest the presence of purinergic receptors on orexinergic neurons. In a preliminary report from our group (Florenzano et al., 2003), we showed the presence of the P2X₂R in the PeF where orexin neurons are located. The aim of the present study was therefore to determine whether orexin neurons indeed express the P2X₂R. We performed immunohistochemistry and in situ hybridization to assess the presence of orexin and P2X₂R subunit in the same hypothalamic neurons. Confocal microscopy of multiply labeled sections demonstrated that all neurons expressing orexin also express the P2X₂R mRNA and protein. These data validate the previous hypothesis on the role of ATP in controlling orexinergic activity through a P2XR that contains the P2X₂R subunit.

MATERIALS AND METHODS

Nine adult male rats (Wistar; Harlan Italy) weighing 200–250 g were used in this study. They were group housed in standard cages and kept under a 12-hour light-dark cycle in an air-conditioned facility. All experiments were carried out in accordance with the Italian law on the use and care of laboratory animals (DL 116/92). All animals were transcardially perfused under deep anesthesia (Nembutal, 60 mg/kg, i.p.) with 150 ml of 0.9% saline at room temperature (RT), followed by 200 ml of cold 4% paraformaldehyde in 0.1 M pH 7.4 phosphate buffer (PB). Brains were dissected, postfixed for 2 hours at room temperature, and cryoprotected in 30% sucrose/PB at 4°C. They were then frozen with dry ice and cut into 40-μm transverse sections with a sliding microtome. Different series of transverse sections through the diencephalon were collected in PB and stored at 4°C. These series were used for cresyl violet staining, immunoperoxidase staining, and blocking tests (three rats); single and double

immunofluorescence (three rats); in situ hybridization; and combined in situ hybridization and double immunofluorescence (three rats). For animals destined for in situ hybridization and for multiple labeling combining in situ and immunofluorescence perfusion saline and PB solutions were treated with diethyl pyrocarbonate (DPEC) in all steps until hybridization.

Immunohistochemistry

Primary antibodies and procedures. In the present study, the following primary antibodies were used: 1) a polyclonal rabbit anti-P2X₂R raised against an intracellular C-terminal epitope (residues 457–472; product No. 003; Alomone, Jerusalem, Israel); this antibody was used for all the immunohistochemical procedures; 2) a polyclonal guinea pig anti-P2X₂R raised against an intracellular C-terminal epitope (residues 460–472; catalog No. GP14106; Neuromics, Minneapolis, MN); this antibody was used only as control in single label immunoperoxidase staining; and 3) a polyclonal goat antiorexin-A antibody raised against a peptide mapping at the carboxy terminus (residues 30–80, catalog sc-8070, C-19; Santa Cruz Biotechnology, Santa Cruz, CA). Immunohistochemical procedures as well as immunoperoxidase and double- and triple-labeling fluorescent techniques were performed at RT on free-floating sections. PB/Triton-X 0.3% was used for both antibody dilutions and washes, and every incubation was followed by three washes of 5 minutes each.

Immunoperoxidase staining. For single-labeling immunoperoxidase, sections were treated for 5 minutes with 0.3% H₂O₂ to block endogenous peroxidase and incubated overnight in rabbit anti-P2X₂R (1:500; Alomone), or guinea pig anti-P2X₂R (1:500; Neuromics), or goat antiorexin-A (1:20,000). After incubation with the primary antiserum, sections were incubated for 2 hours in the respective biotinylated secondary antiserum (1:200, donkey anti-rabbit, goat anti-guinea pig, or horse anti-goat; Jackson ImmunoResearch, West Grove, PA). Then, sections were incubated for 1 hour in avidin-biotin complex (1:100; Vectastain Elite; Vector Laboratories, Burlingame, CA). For visualization, the chromogen 3,3'-diaminobenzidine (DAB; 0.05%) with nickel intensification was used. Finally, sections were mounted on chrome-alum-coated slides, air dried, dehydrated with ethanol, cleared in xylene, and coverslipped.

Blocking tests. The specificity of the primary antisera was verified by preabsorption with their own specific target peptides in single-labeling immunoperoxidase-stained sections. In addition, to exclude the possibility of a cross-reaction between the P2X₂R antiserum and the orexin protein and between the orexin antiserum and the P2X₂R protein, preabsorptions were also done between the P2X₂R antiserum (Alomone) and the orexin peptide (Bachem, Bubendorf, Switzerland) and between the orexin antiserum and the P2X₂R peptide (Alomone). The preabsorptions were done by mixing the antisera, at the same concentrations as above, with the peptides (30 μg/ml for orexin and 50 μg/ml for P2X₂R peptides) for 1 hour before to incubate the sections. The specificity of the P2X₂R distribution pattern was verified by comparing sections in single-labeling immunoperoxidase reacted with P2X₂R antibodies from Alomone and Neuromics.

Double labeling. Double immunofluorescence was performed with a cocktail solution of goat antiorexin antibody (1:400) and rabbit anti-P2X₂R antibody (1:100;

Alomone). After overnight incubation, the sections were incubated for 2 hours in a cocktail of Cy2-conjugated donkey anti-goat and Cy3-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson ImmunoResearch). Sections were then mounted on gelatin-coated slides and coverslipped in Gel Mount (Biomedica, Foster City, CA).

In situ hybridization

A fragment of the rat purinergic P2X₂R mRNA was amplified by polymerase chain reaction (PCR). The forward primer (nt 1,461–1,480; 5'-TCCTCACTAACTACAGACC-3') and the reverse primer (nt 1,721–1,740; 5'-CCCTGAATACTGTATGAGAG-3') were designed to amplify a fragment of the 3'UTR of the P2X₂R mRNA (accession No. NM_053656). The 279-bp product was gel purified and cloned into the pGEM-T-EasyVector System I (Promega, Madison, WI) and sequenced. The construct was linearized with SacI or SacII to synthesize the digoxigenin-labeled sense or antisense probes with the SP6 or T7 RNA polymerase, respectively. Transcriptions were performed with the *in vitro* MAXIScript kit (Ambion, Austin, TX).

In situ hybridization with the digoxigenin-UTP probes was performed in free-floating permeabilized sections. The sections were incubated with Sigma prehybridization buffer (30 minutes), then hybridized overnight at 56°C in hybridization buffer (Sigma, St. Louis, MO) containing 500 ng of the denatured antisense or sense probes. After the hybridization, the sections were washed in 2× SSC buffer, RNase-treated (10 µg/ml) for 30 minutes at 37°C, and washed twice in 0.5× SSC buffer at 56°C for 30 minutes. The probe was then visualized by immunofluorescence by a TRITC-conjugated sheep antidigoxigenin antibody (1:100; Roche, Mannheim, Germany). The same procedure was performed with sense probes that failed to give any signals.

Triple labeling was carried out by first incubating the sections labeled for P2X₂R-mRNA with a cocktail solution of the primary antibodies antiorexin (1:400) and anti-P2X₂R (1:100; Alomone). After overnight incubation, sections were incubated for 2 hours in a cocktail of Cy5-conjugated donkey anti-goat, Cy2-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson ImmunoResearch) and TRITC-conjugated sheep antidigoxigenin antibody (1:100; Roche). Sections were mounted on gelatin-coated slides and coverslipped in Gel Mount.

Qualitative and quantitative analysis

The immunoperoxidase material was examined with a light transmission microscope (Zeiss Axioskop 2) equipped with a video camera. Double-immunofluorescence and triple-fluorescence labeling was examined with a confocal laser scanning microscope (CLSM; Zeiss LSM 510) equipped with an argon laser emitting at 488 nm, a helium/neon laser emitting at 543 nm, and a helium/neon laser emitting at 633 nm. In some acquisitions, to give evidence of axons and dendrites located on focal planes far from the cell bodies, we generated projections from a z-stack series of images acquisition of optical sections at increments of 1.04 µm in the z-axis, ×100 magnification, 0.7 scan zoom, and digitized at 1,024 × 1,024 pixels.

The quantitative analysis of single, double, and triple immunofluorescence-labeled neurons was performed on digital images acquired through CLSM by using a ×10 objective at a 0.7 zoom factor. Qualitative and quantita-

tive observations were limited to the caudal half of the hypothalamus (2 to 4 mm behind Bregma) in particular focusing on PeF, LH, and DMH areas (Paxinos and Watson, 1994; Paxinos, 2004). Labeled cells were identified based on the cellular profile.

Three animals were used for P2X₂R/orexin double-labeling quantitative analyses. For each animal, all labeled cells in 10 sections were counted. Quantitative analyses in triple-labeled material was performed in three animals by counting all labeled cells in two sections. Double- and triple-labeled neurons were analyzed off-line through a CLSM proprietary image analysis program (Zeiss, LSM 2.3). Two or three digital images of the same optical section (one for each laser channel: green, red, and blue) were acquired, digitally merged, and used for cell counting. The features of immunolabeled neurons were analyzed by zooming on the cells and by serially excluding each channel (green, red, or blue) better to appreciate cellular labeling. Morphometric measures were obtained by using the image-analysis tools in the CLSM proprietary image-analysis program (Zeiss LSM 2.3). Plates were generated with adjustment of the contrast and brightness of digital images (Corel Draw 9).

RESULTS

P2X₂R immunostaining of the diencephalon with anti-P2X₂R rabbit polyclonal antibodies resulted in the labeling of cell bodies and fibers in restricted regions. In general, the P2X₂R pattern observed was similar to that previously described, with labeled cells in the paraventricular, arcuate, tuberomammillary, and supraoptic nuclei (Vulchanova et al., 1996; Xiang et al., 1998; Yao et al., 2003). In addition, in our material, a conspicuous group of P2X₂R-positive neurons was observed in the dorsal part of the tuberal hypothalamus, centered on the PeF, immediately above the fornix (Fig. 1A). Labeled cells extended laterally into the LH and medially into the DMH. In the DMH, fewer neurons were stained compared with the PeF. In the material reacted with the anti-P2X₂R antibody raised in guinea pig from Neuromics, although the general pattern of hypothalamic staining was similar to that previously reported (Vulchanova et al., 1996; Xiang et al., 1998; Yao et al., 2003), positive neurons were not present in PeF, LH, or DMH areas (data not shown).

To verify the specificity of the P2X₂R rabbit antibodies, as well of the orexin antibodies, we performed several blocking tests. No blocking tests for the guinea pig P2X₂R antibodies were performed. Preabsorption with the P2X₂R peptide completely abolished all staining (Fig. 1E). The specificity of the P2X₂R signal at the protein level was further investigated by performing an mRNA *in situ* hybridization for the P2X₂R receptor (Fig. 2A). Also in this case, P2X₂R mRNA was found in restricted regions of the hypothalamus, but, most importantly, it was also present in PeF, LH, and DMH, areas where P2X₂R-positive neurons were revealed by immunohistochemistry. As shown in Figure 2A, the P2X₂R mRNA signal was not limited to the cell bodies but was also high in fibers and possibly in terminals. The P2X₂R mRNA signal was almost absent when the sense P2X₂R mRNA probe was used (Fig. 2D).

In sections reacted with antiorexin antibody, orexin-positive neurons were also found in the hypothalamus centered on the PeF and extending into the LH laterally and the DMH medially. The distribution of orexin neurons

Fig. 1. Immunoperoxidase staining of hypothalamic sections through the perifornical area reacted for the P2X₂R subunit (A,C,E) or orexin (B,D,F). C–F: Blocking tests. In C and F, sections preabsorbed with the orexin peptide, and, in D and E, sections preabsorbed with the P2X₂R peptide. f, Fornix. Scale bars = 60 μ m in A,B; 400 μ m in C–F.

in PeF, LH, and DMH presented a striking similarity to that of the P2X₂R-labeled cells, suggesting that the two proteins could be expressed by the same cells. This can be appreciated by comparing sections immunostained for orexin or for P2X₂R (Fig. 1).

The suggestion that the two proteins might be colocalized in the same neuronal population was then tested by double immunofluorescence for P2X₂R and orexin and by coupling this double immunofluorescence with in situ hybridization fluorescence for mRNA P2X₂R. Confocal microscopy of double immunofluorescence for orexin and P2X₂R showed a very high degree of colocalization in cell bodies and varicose processes (Fig. 2G–I). Indeed, almost all orexin-positive cells were also P2X₂R positive, with only a small population of P2X₂R-positive cells not expressing orexin. This small population of P2X₂R-positive/orexin-negative cells was evenly distributed and intermingled with the population of double-labeled cells (Fig. 2I). Quantitative analyses performed on PeF, DMH, and LH

areas are reported in Table 1 and confirmed the qualitative observations; i.e., virtually all orexin-positive cells were P2X₂R positive, whereas between 75% and 80% of P2X₂R-positive cells were orexin positive.

The possibility of cross-reactivity between the antisera and the orexin or P2X₂R epitopes was ruled out by blocking tests. Preabsorption of each antisera with the two peptides was performed before running the immunohistochemical reaction. P2X₂R immunoreactivity was not reduced by preabsorption with the orexin peptide (Fig. 1C), although it was absent after preabsorption with the P2X₂R peptide (Fig. 1E). Similarly, orexin immunoreactivity was not reduced by preabsorption with the P2X₂R peptide (Fig. 1D) but was absent after preabsorption with the orexin peptide (Fig. 1F). The capacity of orexin neurons to express P2X₂R was further tested by coupling double immunofluorescence for P2X₂R and orexin with in situ hybridization fluorescence for P2X₂R mRNA. As expected, in the examined areas, almost all orexin-positive

Figure 2

P2X₂R EXPRESSION IN HYPOCRETIN/OREXIN NEURONS

TABLE 1A. Double Labelling: Number of Cells in 10 Sections Throughout the PeF, DMH, and LH Areas

	Total cells	Prot	Orx	Prot/Orx	Percentage DL on total cells	Percentage DL on Orx	Percentage DL on Prot
Rat 1	474	88	18	368	77.64	95.34	80.70
Rat 2	466	108	9	349	74.89	97.49	76.37
Rat 3	455	110	13	332	72.97	96.23	75.11
Mean	465.00	102.00	13.33	349.67	75.17	96.35	77.39
SD	9.54	12.17	4.51	18.01	2.35	1.08	2.93

¹Prot, P2X₂R protein; RNA, P2X₂R mRNA; Orx, orexin protein.

TABLE 1B. Triple Labelling: Number of Cells in Two Sections Throughout the PeF, DMH, and LH Areas

	Total cells	Single labelling			Double labelling			Triple labelling	
		Prot	RNA	Orx	Prot/RNA	Prot/Orx	RNA/Orx	Prot/RNA/Orx	Percentage Prot/RNA/Orx
Rat 1	60	2	2	0	2	4	1	49	81.67
Rat 2	109	3	1	0	1	3	1	100	91.74
Rat 3	108	3	0	0	2	1	1	101	93.52
Mean	92.33	2.67	1.00	0.00	1.67	2.67	1.00	83.33	88.98
SD	28.01	0.58	1.00	0.00	0.58	1.53	0.00	29.74	6.39

¹Prot, P2X₂R protein; RNA, P2X₂R mRNA; Orx, orexin protein.

cells were found to express both the P2X₂R protein and the P2X₂R mRNA (Table 1, Figs. 3, 4).

The intracellular distribution of P2X₂R protein and mRNA signals presented some peculiar aspects. Immunoperoxidase staining showed P2X₂R protein expression to be moderate to intense, with an uneven labeling of the soma and proximal processes but not of the nucleus (Fig. 5). The P2X₂R protein pattern of expression presented a gross granular appearance in discrete cytoplasmic regions, especially on the major cellular axis around the nucleus. In confocal images of multiply labeled sections (Figs. 2A–C, 5B–G), the granules were positive for both P2X₂R protein and mRNA, suggesting a colocalization in the same intracellular structures. In the outer part of the cytoplasm as well in dendrites, the granularity appeared more delicate, possibly indicating a vesicular storage. In the neuropil, varicose processes, resembling axonal structures, presented high positivity for orexin, P2X₂R protein, and P2X₂R mRNA (Fig. 5E–G). The staining appeared grossly granular. Morphometric analysis showed that the size of positive varicosities ranged between 0.5 and 2.0 μ m, considerably larger than the granules observed in the outer part of the soma and proximal dendrites, which ranged between 0.2 and 0.5 μ m.

DISCUSSION

The present data demonstrate that P2X₂R are present in virtually all orexin neurons of PeF, LH, and DMH areas

of the dorsal tubular hypothalamus, providing anatomical evidence to support the hypothesized ATP modulation of orexin neurons through P2X₂R. The presence of purinergic receptors on orexin neurons is in line with other observations. About 30% of orexin neurons express the adenosine receptor A1, a purinergic receptor (P1), and a key regulator in the control of sleep-wakefulness (Thakkar et al., 2002). P2XR have also been implicated in thermoregulation (Gurin et al., 2003), as have orexin neurons (Monda et al., 2003). Possibly the most compelling evidence derives from electrophysiological experiments on LH and DMH orexin neurons, which show 1) that ATP application can increase orexin cell activity in a dose-dependent manner independently of synaptic input, suggestive of postsynaptic ion channel opening (Matsumoto et al., 2004; Wollmann et al., 2005), and 2) P2XR-dependent responses, consistent with the presence of P2X₂R (Jo and Role, 2002; Matsumoto et al., 2004; Wollmann et al., 2005).

Previous studies addressed the distribution of purinergic receptors in the hypothalamus by immunohistochemistry and failed to reveal P2X₂R-positive neurons in the dorsal tubular hypothalamus (Vulchanova et al., 1996; Xiang et al., 1998; Kanjhan et al., 1999), where orexin neurons are located (Peyron et al., 1998; Nambu et al., 1999). Indeed, antibodies used in previous studies (Vulchanova et al., 1996; Xiang et al., 1998; Yao et al., 2003), such as one of the anti-P2X₂R antibodies used in the present study (namely, the guinea pig antibody), did not evidence P2X₂R-positive cells in PeF, LH, or DMH areas. This is the first time that P2X₂R-positive neurons have been identified in these latter areas. A possible explanation for the difference from previous reports resides in the expression of different splice variants of the P2X₂R subunit as well as in detection methods (Stojilkovic et al., 2005). Indeed, it has been reported that some of these splice variants present different neuronal distributions at the mRNA level (Simon et al., 1997; Schadlich et al., 2001). The use of a fluorescent riboprobe in the 3'UTR region of the mRNA allowed us to recognize all the splice variants. Finally, differences from previous reports in the P2X₂R hypothalamic labeling could be due to the specific-

Fig. 2. Confocal images through PeF and LH areas. **A–C:** In situ P2X₂R mRNA hybridization with the antisense oligonucleotide coupled with P2X₂R protein immunofluorescence. Projection of 14 confocal images. A: P2X₂R mRNA. B: P2X₂R protein. C: Merge. Note the accumulation of P2X₂R mRNA and P2X₂R protein in dendrites (arrowheads), fibers, and varicosities (arrows). **D–F:** In situ P2X₂R mRNA hybridization with the sense oligonucleotide coupled with orexin immunofluorescence. D: P2X₂R mRNA. E: Orexin. F: Merge. **G–I:** Double immunofluorescence for P2X₂R protein and orexin. G: P2X₂R protein. H: Orexin. I: Merge. Note that some P2X₂R labeled cells do not stain for orexin (arrows). f, Fornix. Scale bars = 10 μ m in A–C; 50 μ m in D–F; 30 μ m in G–I.

ity of the antibodies used, as observed in the present study with one of the two P2X₂R antibodies tested. The preabsorption experiments and in situ hybridization for P2X₂R mRNA performed support the specificity of the P2X₂R labeling in orexin neurons.

The possibility that orexin cells may express other P2XR subunits as part of heteromeric P2XR channels appears likely. Nevertheless, preliminary immunohistochemical experiments testing P2X_{1,4,7} subtypes in the orexin hypothalamic region have not resulted in significant labeling (data not shown). However, caution is warranted in that, as already observed with the P2X₂R subtype, immunohistochemistry can provide false-negative results. Heteromeric or homomeric assembly of different P2XR subunits modulates the receptor functionality, and this issue could be of particular interest in understanding

the cellular and molecular features that shape the functions of orexinergic neurons. Indeed, these neurons can sustain a fast firing rate (Burdakov, 2004) that does not depend on sodium- or calcium voltage-dependent currents (Eggermann et al., 2003). P2XR are voltage-independent calcium channels, which can sustain a rise in calcium under different conditions from those needed in voltage-dependent calcium signaling pathways (Robertson et al., 2001). In fact, the homomeric P2X₂R presents the highest calcium permeability and longest desensitization time among the various subunits (North, 2002). Thus, the physiological properties of P2X₂R are compatible with some interesting aspects of the electrical activity of orexinergic neurons.

The P2X₂R protein and mRNA subcellular expression pattern presents some interesting features that could be related to the receptor trafficking and synaptic plasticity. In our material, the P2X₂R protein and mRNA colocalized in intracellular granules of various sizes located in the soma and cellular processes as well as in distal axonal branches. At the protein level, this subcellular pattern of expression of the receptor seems to reflect mainly the main sites of trafficking, then the final sites of expression, the cellular membrane. A possible explanation is that immunocytochemical methods detect more efficiently the sites presenting a higher antigen concentration, such as the trafficking sites. In support of this hypothesis are light and electron microscopy observations on P2X₂R (Loesch et al., 1999) as well as on other ligand-gated receptors, such as glutamate receptors (Petralia and Wenthold, 1992; Petralia et al., 1994; Tachibana et al., 1994). Indeed, earlier investigations performed on AMPA and NMDA receptors (Petralia and Wenthold, 1992; Petralia et al., 1994; Tachibana et al., 1994) have shown that the subcellular distribution pattern reflects mainly the sites of receptor protein synthesis, modification, and transport. For the P2X₂R, electron microscopy investigations in the hypothalamic-neurohypophyseal system (Loesch et al., 1999) have shown receptor expression in the cell body primarily associated with the cytoplasm, granular endoplasmic reticulum, mitochondria, and neurosecretory granules. In the same

Fig. 3. Confocal images of triple labeling in PeF, DMH, and LH areas obtained by coupling in situ P2X₂R mRNA hybridization and P2X₂R/orexin double immunohistochemistry. **A,B:** Merge. **C:** P2X₂R mRNA (red). **D:** P2X₂R protein (green). **E:** Orexin (blue). **f:** Fornix. Scale bars = 300 μ m in A; 40 μ m in B; 10 μ m in C–E.

Fig. 4. Histogram of cell counts of orexin and P2X₂R protein immunofluorescence and mRNA in situ hybridization. Triple/total cells: percentage of triple-labeled cells on the total number of labeled cells. Triple/Orx cells: percentage of orexin neurons expressing also P2X₂R protein and mRNA. P2X₂RmRNA/protein: percentage of P2X₂R protein-positive neurons also positive for P2X₂R mRNA.

Fig. 5. High magnification of P2X₂R protein and mRNA labeled cells in the LH. **A:** Immunoperoxidase single labeling for P2X₂R protein of a cell showing the granular distribution of the staining in the soma and dendrites (arrows) and in varicose processes (arrow-

heads). **B,E:** P2X₂R mRNA. **C,F:** P2X₂R protein. **D,G:** Merge. Note the colocalization of P2X₂R protein and mRNA signals around the nucleus (D), proximal dendrites (D), and varicose processes (G). Scale bars = 12 μ m in A; 8 μ m in B–D; 6 μ m in E–G.

study, P2X₂R expression in dendrites was confined to postsynaptic specializations, microtubules, mitochondria, and granules, whereas P2X₂R expression in axons was in synaptic contacts and in granular and in agranular vesicles of different sizes. For our material, confocal microscopy showed that varicose processes are positive for orexin

and P2X₂R protein and mRNA, indicating a colocalization at presynaptic levels. For P2X₂R, this staining pattern has been previously described only for the nucleus tractus solitarius (Yao et al., 2001). For orexin, the presence of a high number of positive varicose axons and terminals throughout the brain is a very common finding (Nambu et

al., 1999). Although we did not perform an electron microscopy investigation, and our study could not distinguish between varicose axons and terminals, the presence of both orexin and P2X₂R in varicosities suggests the possibilities of functional interactions between the purinergic and the orexinergic systems at presynaptic levels.

The present study also shows, for the first time, localization of the P2X₂R mRNA in dendrites and axons. Dendritic/axonal mRNA localization and local translation are two important mechanisms to regulate gene expression and synaptic plasticity (Martin, 2004). mRNAs encoding olfactory marker proteins have been localized in axons of the olfactory bulb (Job and Eberwine, 2001), and mRNAs encoding for receptors such as GluR1 and GlyR have been detected in hippocampal dendrites (Racca et al., 1997; Kacharmina et al., 2000). So far, except for vasopressin and oxytocin, very few mRNAs encoding for neuropeptides have been detected in axons of hypothalamic neurons (Mohr and Richter, 2004). The present data extend these findings to the purinergic receptor system in the hypothalamic region. Thus, ATP-mediated excitation may act independently in different synaptic compartments of the same neurons where the receptor is synthesized locally.

In conclusion, the convergence of physiological (Wollmann et al., 2005) and anatomical (present study) data indicates that orexinergic hypothalamic neurons may be excited by ATP via activation of P2X₂R. P2X₂R-mediated ATP signaling must have a crucial role to play in the modulation of orexin neurons, insofar as all the orexin-containing neurons were found to express this receptor. The significance of this finding is not yet clear. One explanation is that ATP is coreleased in synaptic vesicles with several other neurotransmitters and can modulate their release (Cunha and Ribeiro, 2000; Khakh, 2001). Among these neurotransmitters, acetylcholine and noradrenaline are known to exert direct actions on orexin neurons (Burdakov, 2004; Bayer et al., 2005). Thus, ATP could exert two types of actions on orexin neurons: one direct through P2X₂R activation and one indirect through modulation of neurotransmitters known to act on orexin neurons. Alternatively, ATP-signaling is crucial for neuron–glia communication and for calcium homeostasis (Brookes et al., 2004). Different pathological conditions, such as head trauma or hypoxia, can induce dramatic increases of ATP concentrations (Ciccarelli et al., 2001) with subsequent activation of P2X₂R-expressing cells. Because P2X₂R potentiates responses at a lower pH, such as during brain edema or ischemia, it has been stressed that orexin cells expressing P2X₂R may represent the link between focal hypothalamic lesion and influences on the arousal system (Matsumoto et al., 2004; Wollmann et al., 2005). Thus, identification of the receptor subtype responsible for the ATP modulation of the orexin system may represent a key factor in the development of specific drugs able to modulate the complex functions mediated by orexin neurons.

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