CORTICAL AND SUBCORTICAL DISTRIBUTION OF IONOTROPIC PURINERGIC RECEPTOR SUBUNIT TYPE 1 (P2X₁R) IMMUNOREACTIVE NEURONS IN THE RAT FOREBRAIN

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Abstract—Ionotropic purinergic receptors (P2XR) are ATP-gated cationic channels composed of seven known subunits (P2X_{1–7}R) and involved in different functions in neural tissue. Although their presence has been demonstrated in the brain, few studies have investigated their expression pattern. In particular, ionotropic purinergic receptor subunit type 1 (P2X₁R) has been observed in the cerebellum and in brain-stem nuclei. The present study investigates the P2X₁R expression pattern in the rat forebrain using immunohistochemistry. The specificity of the immunolabeling has been verified by Western blotting and *in situ* hybridization methods. P2X₁R immunoreactivity was specifically localized in neurons, dendrites and axons throughout the forebrain. Characteristic differences in the distribution of P2X₁R were observed in different cortical areas. In prefrontal, cingulate and perirhinal cortices, very intense labeling was present in neuronal bodies. In frontal, parietal, temporal and occipital cortices, immunostaining was lighter and mainly found in dendrites and axons. The hippocampal formation was intensely labeled. Labeling was present almost exclusively in dendrites and axons and never in neuronal bodies. The diencephalon was devoid of P2X₁R positive neurons or fibers except for the medial habenular nucleus, which showed very intense P2X₁R immunostaining. Furthermore, two subcortical regions, namely, the nucleus centralis of the amygdala and the bed nucleus of the stria terminalis, showed intense P2X₁R neuronal labeling. Present data indicate that P2X₁R are prevalent in forebrain areas involved in the integration of

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Key words: nucleus centralis of the amygdala, bed nucleus of stria terminalis, limbic cortex, prefrontal cortex, cingulate cortex.

ATP is a signaling molecule that acts through two classes of purinergic receptors (P2R), namely, ionotropic (P2XR) and metabotropic (P2YR) purinergic receptors. P2XR are ionotropic channels involved in fast excitatory neurotransmission, neuromodulatory actions and as sensors of tissue damage (Khakh, 2001; North, 2002; Khakh and North, 2006). Until now, seven distinct P2XR subunits (P2X_{1–7}) have been cloned from mammalian species. P2XR have been identified in virtually all tissues, where their functional role has been demonstrated (Burnstock and Knight, 2004; Khakh and North, 2006). Fewer studies have investigated the distribution and function of P2XR in the CNS; however, some of these have revealed unexpectedly wide, but selective, CNS distribution (Xiang et al., 1998; Kanjhan et al., 1999; Norenberg and Illes, 2000; Yao et al., 2000). Anatomical characterization has provided some information about the expression of the different P2XR subunits. For the ionotropic purinergic receptor subunit type 2 (P2X₂R), which is the one most studied, a widespread pattern has been reported that includes broad sectors of the cerebral cortex, different rhinencephalic structures, many thalamic, hypothalamic and basal ganglia nuclei as well as sensory, motor and integration nuclei of the brainstem and the cerebellar cortex (Kanjhan et al., 1999). Co-localization studies have suggested an important role of the P2X₂R subunit in the regulation of hypothalamic functions (Yao et al., 2003; Florenzano et al., 2006). As to the other subunits, few studies have investigated the topographical and subcellular distribution of P2X_{1–6}R subunits (Loesch and Burnstock, 1998; Yao et al., 2000, 2001; Rubio and Soto, 2001). In experimental lesion models, it has been found that ionotropic purinergic receptor subunit type 1 (P2X₁R) and P2X₂R are up-regulated in neurons after central and peripheral axotomy (Florenzano et al., 2002; Atkinson et al., 2003; Kassa et al., 2007), and a functional interactions with post-axotomy nNOS expression has been suggested (Viscomi et al., 2004; Kassa et al., 2007).

Whether or not the P2X₁R subunit is present in the CNS is still a matter of debate. Indeed, P2X₁R RNA transcripts were demonstrated in the cerebral cortex, hippocampus and striatum (Norenberg and Illes, 2000); P2X₁R protein expression was assessed in the cerebellum

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Abbreviations: BST, bed nucleus of the stria terminalis; BSTLD, bed nucleus of the stria terminalis, lateral division, dorsal part; Ce, nucleus centralis; Cg2, posterior cingulate area; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DG, dentate gyrus; EtC, Entorhinal cortex; fr, fasciculus retroflexus; GFAP, anti-glial fibrillary acid protein; IL, infralimbic; NeuN, neuronal nuclei; PFC, prefrontal cortex; PB, phosphate buffer; PRhC, perirhinal cortex; PrL, prelimbic; P2XR, ionotropic purinergic receptors; P2X₁R, ionotropic purinergic receptor subunit type 1; RSGC, retrosplenial granular cortex; RT, room temperature; S, subiculum.

and in brainstem nuclei (Loesch and Burnstock, 1998; Yao et al., 2000, 2001); and the presence of P2X₁R in pyramidal neurons of the somatosensory cortex was suggested on pharmacological grounds (Pankratov et al., 2002, 2003). Recently a wide debate arose on the specificity of P2X antibodies (Ashour et al., 2006; Anderson and Nedergaard, 2006) and a multi-technical approach has been suggested to support the specificity of immunohistochemical data (Rhodes and Trimmer, 2006). The present study investigates the pattern of expression of the P2X₁R subunit in the forebrain by Western blotting, immunohistochemistry and *in situ* hybridization methods.

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Twelve adult male rats (Wistar, Harlan, Udine, Italy) weighing 200–250 g were used in this study. They were group-housed in standard cages and kept under a 12-h light/dark cycle in a conditioned facility. Food and water were provided *ad libitum*. All experiments were carried out in accordance with the Italian law on the use and care of laboratory animals (DL 116/92) and with the European Communities Council Directive of 24 November 1986 (86/609/EEC). The animals were transcardially perfused under deep anesthesia (Sodium pentobarbital, 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 a 0.1 M pH 7.4 phosphate buffer (PB). Brains were dissected, post-fixed for 2 h at RT and cryoprotected in 30% PB/sucrose at 4 °C. Then, they were frozen with dry ice and cut into 40 µm transverse, sagittal or horizontal sections with a sliding microtome. Three series of transverse sections from the frontal pole to the occipital pole were collected in PB and stored at 4 °C. The first series was Nissl stained with Cresyl Violet for anatomical reference. The second and third series were processed for immunoperoxidase detection, multiple immunofluorescence or fluorescent *in situ* hybridization. We used six animals for immunoperoxidase staining, three animals for multiple immunofluorescence *in situ* hybridization and three animals for Western blotting. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Western blotting

Rats were deeply anesthetized by i.p. injections of sodium pentobarbital (60 mg/kg) and killed by decapitation. The brain was dissected, and the forebrain was taken. The tissues were homogenized, extracted with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS) in PB for 30 min at 4 °C and centrifuged for 10 min at 4 °C (14,000 r.p.m.). The supernatant was collected and sonicated, and the protein content was revealed by the Bradford method. Total protein (100 µg) from each animal was subjected to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were saturated with 5% nonfat dried milk and incubated overnight with the primary antibody at the appropriate dilution (rabbit anti-P2X₁R, 1:200; mouse anti-β-actin, (Chemicon, Temecula, CA, USA) 1:2000). Membranes were then incubated with horseradish peroxidase–conjugated goat anti-rabbit (1:5000) or horse anti-mouse (1:2000) secondary antibodies (Cell Signaling Technology, Beverly, MA, USA). Immunoreactive bands were detected using the enhanced chemiluminescence kit (ECL, Amersham Biosciences, Little Chalfont, UK).

Histology and immunohistochemistry

Immunohistochemical procedures were performed at RT on free-floating sections. PB was used for both chemical dilution and

rinses. All primary antibody solutions were prepared in PB and 0.3% Triton X-100 and incubated overnight at RT. Each incubation step was followed by three 5 min rinses in PB.

For immunoperoxidase staining, sections were treated for 5 min with 0.3% H₂O₂ to block endogenous peroxidase. They were incubated overnight in primary antiserum (polyclonal rabbit anti-P2X₁R, Alomone, Jerusalem, Israel) diluted 1:500, and were then incubated for 2 h in secondary antiserum, diluted 1:200 (biotinylated donkey anti-rabbit, Jackson ImmunoResearch, West Grove, PA, USA). Further, sections were incubated for 1 h in avidin–biotin complex, diluted 1:100 (Vectastain elite, Vector Laboratories, Burlingame, CA, USA). As chromogen, 3,3′ diaminobenzidine 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.

Double immunofluorescence was performed using a solution of rabbit anti-P2X₁R (1:100) and mouse anti-neuronal nuclei (NeuN; 1:100, (Chemicon), lot number 24010098) or rabbit anti-P2X₁R receptor and mouse anti-glial fibrillary acid protein (GFAP) (1:600, Chemicon) or rabbit anti-P2X₁R receptor subunits and mouse anti-OX-42 (1:200; Serotec, Kidlington, Oxford, UK). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a solution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Following incubation with the mix solution of primary antibodies, the sections were incubated for 2 h at RT in a solution of Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson ImmunoResearch Laboratories). Before the last rinse, sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Then they were mounted on gelatin-coated slides and coverslipped in Gel/Mount (Biomedica, Foster City, CA, USA).

Triple fluorescence was performed using sections derived by *in situ* hybridization procedures that were incubated with the anti-P2X₁R (1:100) antibody. Subsequently, they were incubated for 2 h at RT in a solution of Cy3-conjugated mouse anti-digoxigenin and Cy2-conjugated donkey anti-rabbit IgG secondary antibodies (1:100; Jackson ImmunoResearch Laboratories). Before the last rinse, sections were DAPI counterstained (5 min, 1:1000; Sigma). Then they were mounted on gelatin-coated slides and coverslipped in Gel/Mount (Biomedica).

Fluorescence *in situ* hybridization

In situ hybridization experiments were performed using probes in both sense and antisense orientations. The primers used to clone the partial cDNAs from rat total brain RNA were:

P2X₁R forward: 5′-GGA CAG CTC CTT TGT AGT TAT-3′

P2X₁R reverse: 5′-TGG TAG ATG GGT TTG CAG TGC-3′

The cDNA was cloned into the pGemTeasy vector, linearized with *SacI* or with *SacII* and transcribed with T7 or Sp6 polymerases to obtain antisense or sense digoxigenin-labeled cRNA probes, respectively.

Sections were post-fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min, then rinsed with 1× PBS (0.1% DEPC treated) for 5 min. Sections were permeabilized with 0.3% Triton X-100 in 1× PBS for 30 min, rinsed with 0.5× SSC (0.1% DEPC treated) for 10 min and incubated in 500 µl of pre-hybridization buffer (2× SSC, 25% formamide, 1% Denhardt's solution, 10% dextran sulfate, 0.5 mg/ml heparin, 0.5 mg/ml *E. coli* tRNA and 0.25 mg/ml of denatured salmon sperm DNA) at 42 °C for 2–3 h. After the prehybridization, 700 ng of digoxigenin-labeled cRNA probe was added to each section. Hybridization was performed overnight at 55 °C. The next day, sections were washed twice with 2× SSC/1 mM EDTA (10 min each), treated with 0.02 µg/ml RNaseA for 30 min and then washed twice with 2× SSC/1 mM EDTA. The stringency wash was performed at 55 °C for 2 h in 0.5× SSC/1 mM EDTA.

Then, sections were washed with $0.5\times$ SSC twice (10 min each at RT). Sections were rinsed three times with $1\times$ PBS and then destined for multiple fluorescence of the P2X₁R protein, as described above.

Controls

The specificity of the P2X₁R primary antiserum was verified by preadsorption with its own specific target peptide (Alomone, Jew-salem, Israel). For Western blotting, preadsorption was performed by mixing the P2X₁R antiserum at the same concentration used for membrane incubation with the peptide ($3\text{ }\mu\text{g/ml}$) for 2 h before incubating the membrane. For immunoperoxidase staining, preadsorption was performed by mixing the antiserum at the same concentration used for immunostaining with the peptide ($10\text{ }\mu\text{g/ml}$) for 1 h before it was used. Adjacent sections were placed in two different wells: one was incubated with P2X₁R primary antiserum, the other with P2X₁R primary antiserum, preadsorbed with the peptide. Sections were then transferred to the same well and incubated with a secondary antibody.

The specificity of the *in situ* hybridization procedures and multiple immunofluorescence was verified by incubating sections with a sense digoxigenin-labeled cRNA probe. After rinses, sections were destined for multiple fluorescence of the P2X₁R protein, as described above.

Data collection and analysis

Boundaries of cortical and subcortical structures were delineated according to Cresyl Violet staining, following Paxinos (Paxinos and Watson, 1997; Paxinos, 2004). Some nuclear subdivisions were not subdivided as such, and instead the name of the whole nucleus was used. Topography and nomenclature were according to Paxinos (Paxinos and Watson, 1997; Paxinos, 2004); habenular subdivisions were identified with reference to Andres et al. (1999). Sections were collected from selected rostrocaudal levels, ranging approximately from $+2.70$ to -6.04 from bregma, with reference to the rat brain atlas of Paxinos and Watson (1997).

The immunoperoxidase material was examined under a light-transmission microscope (Zeiss, Axioskop 2, Jena, Germany) equipped with a CCD camera (ProgRes C10 plus, Zeiss). Double immunofluorescence was examined under a confocal laser scanning microscope (CLSM; Zeiss, LSM 510) equipped with two laser lines: an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm. Triple fluorescent material was examined with a CLSM (Leica, SP5, Wetzlar, Germany) equipped with three laser lines: violet diode emitting at 405 nm, argon emitting at 488 nm and helium/neon emitting at 543 nm. Images were exported in JPEG format, contrast and brightness were adjusted, and final plates were composed with Adobe Illustrator 9 or Corel Draw 9.

RESULTS

Controls

Western Blotting. Western blotting, performed on tissue extracts derived from the rat brain (Fig. 1A), assessed the specificity of the polyclonal P2X₁R antibody. An immunoreactive band was detected at 60 kDa that corresponded to P2X₁R (Fig. 1A, lane 1), as shown by the manufacturer. Preadsorption of the antiserum with the peptide antigen resulted in the absence of the band (Fig. 1A, lane 2), indicating that the antibody detects the appropriate antigen sequence.

Immunohistochemistry. The specificity of the P2X₁R immunoperoxidase staining was verified by a blocking test.

Fig. 1. Control panel; A–C: P2X₁R antibody specificity tests; D–G: *in situ* hybridization specificity test. (A) Western blotting from brain extracts. M, molecular weight marker line (SeeBleu, Invitrogen). Line 1, P2X₁R immunoreactive band is located at 60 kDa. Line 2, preadsorption of the P2X₁R antisera with its peptide antigen resulted in the absence of the band. (B–C) Preadsorption test. P2X₁R immunohistochemistry in adjacent sections of the prefrontal cortex (PFC) using P2X₁R primary antibody alone (B) or after preadsorption with its peptide antigen (C). Note the absence of immunostaining in C. (D–G) Confocal images of *in situ* hybridization with a sense P2X₁R fluorescent riboprobe (E, red) coupled with DAPI histofluorescence (D, blue) and P2X₁R protein immunofluorescence (F, green). Note that P2X₁R protein positive neurons do not show sense mRNA positivity (arrows). Scale bars=100 μm B, G=10 μm .

Preadsorption with the P2X₁R peptide completely abolished the staining in the sections (Fig. 1B, C), confirming the specificity of the antibody for the P2X₁R sequence also when used for immunohistochemistry.

In situ hybridization. *In situ* hybridization performed with a sense probe for P2X₁R mRNA showed a fluorescent signal that was barely detectable above the background (Fig. 1E).

In situ hybridization performed with an antisense probe for P2X₁R mRNA demonstrated P2X₁R mRNA positive cells in the forebrain (Fig. 2C, D). Labeled cells were characterized by a fluorescent rim in the perinuclear cytoplasmic domain and positive cytoplasmic granular structures.

Multiple fluorescence of the P2X₁R protein and mRNA showed that all P2X₁R protein positive cells were also P2X₁R mRNA positive (Fig. 2 filled arrows). On the other side, P2X₁R mRNA positive cells were often not positive for the P2X₁R protein (Fig. 2 arrowheads). However, the tendency of the P2X₁R protein to be located in neuronal processes rather than in the cell body and the almost exclusive cell body location of the P2X₁R mRNA may explain the mismatch between P2X₁R mRNA and P2X₁R protein labeling.

P2X₁R immunostaining pattern

Overall pattern. P2X₁R immunoreactivity appeared localized in neurons, dendrites and axons throughout the forebrain of the rat. P2X₁R immunostaining was distributed in the cytoplasm and was never observed in the nucleus. Based on differences in the immunoperoxidase staining intensity or in the expression within different cell domains, three basic qualitative patterns of immunoreactivity were observed. These basic intensity patterns were: *i*, light staining of axons and dendrites (Fig. 3A, B) *ii*, diffuse staining of the entire cell (Fig. 3C–F), *iii*, intense staining of axons and dendrites (Fig. 3G, H). In addition, neuropil immunostaining was observed in several anatomical regions. These differences in intensity and cellular distribution were apparent mainly when different anatomical regions were compared and were not evident in the same anatomical region. This indicates that the intensity of the expression level and the cellular sites of expression were specific for different anatomical areas and neuronal types.

The neuronal or glial origin of the staining observed was addressed by double NeuN and P2X₁R (Fig. 4A–I) and by double P2X₁R and GFAP (Fig. 4J–L) or OX-42 (data not shown) immunolabeling. In all areas studied, P2X₁R cell body labeling was always associated with NeuN immunofluorescence, and never with GFAP or OX-42 labeling. Taken together data demonstrated the presence of P2X₁R only in neurons.

Throughout the cerebral cortex, a general distribution pattern of P2X₁R immunoreactivity was evident. Immunostained, radially-oriented processes, which could be recognized as apical dendrites extending between layers V and III, were observed (Fig. 3A–F). The neuronal bodies of these apical dendrites were only occasionally stained, identifying them as pyramidal neurons of layer V and, to a lesser degree, of layer VI (Fig. 3C, E). In the absence of somata labeling, confocal imaging of double immunofluorescence for P2X₁R and NeuN confirmed the prevalent layer V pyramidal origin of these processes (Fig. 4). In addition, immunostained axons running toward the deepest layers could often be followed as they entered the white matter. Within this general distribution pattern, different patterns of intensity were observed in different cortical domains. In the isocortex, labeling was characterized by faint staining of the apical and basal dendrites as well as of the axons of layers V–VI pyramidal neurons (Fig. 3A, B; Fig. 4A–C). No clear neuronal body labeling was present (Fig. 3B; Fig. 4B). On the medial and lateral edges of the isocortex, in medial prefrontal and perirhinal areas, corresponding to proiso- and periallo-cortex transitional zones, more intense labeling of neuronal bodies, axons and dendrites was present (Fig. 3C–F, Fig. 4D–F). In the RSGC, neuronal labeling was prevalent in dendrites, with only faint cytoplasmic labeling (Fig. 3G–H; Fig. 4G–I). Intense dendritic and axonal labeling was also present in the hippocampal formation, where no positive neuronal bodies were found (Fig. 5C–F). The piriform cortex was the only cortical structure in which labeling was not detected in either fibers or neuronal bodies. Finally, a striking feature of P2X₁R immunoreactivity was the presence of Golgi-like stained neurons in the nucleus centralis (Ce; Fig. 6C–D) of the amygdala and in the bed nucleus of the stria terminalis (BST; Fig. 6A–B). These two subcortical structures were the only ones that displayed consistent immunoreactive neuronal bodies.

Abbreviations used in the figures

ac	anterior commissure	La	lateral amygdaloid nucleus
BLA	basolateral amygdaloid nucleus, anterior	Lhb	lateral habenula
BMA	basomedial amygdaloid nucleus, anterior	LS	lateral septal nucleus
BSTLD	bed nucleus of the stria terminalis, lateral division, dorsal part	LV	lateral ventricle
BSTLP	bed nucleus of the stria terminalis, lateral division, posterior part	MeA	medial amygdaloid nucleus, anterior part
BSTM	bed nucleus of the stria terminalis, medial division	mHb	medial habenular nucleus
BSTTA	bed nucleus of the stria terminalis, intraamygdalar division	Mol	molecular layer
BSTV	bed nucleus of the stria terminalis, ventral division	opt	optic tract
CeC	nucleus centralis, capsular	Or	oriens stratum
CeL	nucleus centralis, lateral	Py	pyramidal cell layer of the hippocampus
CeM	nucleus centralis, medial	Pol	polymorphic layer
CPu	caudate putamen	PRhC	perirhinal cortex
DG	dentate gyrus	S	subiculum
EtC	entorhinal cortex	Sl	stratum lucidum
fr	fasciculus retroflexus	Sr	stratum radiatum
Grl	granular layer	st	stria terminalis
HiF	hippocampal fissure	TC	temporal cortex
ic	internal capsule	VDB	vertical limb of the diagonal band
		3V	third ventricle

Fig. 2. Confocal images of retrosplenial granular (A, C, E, G) and perirhinal (B, D, F, H) cortices. Triple fluorescence for DAPI (A, B; blue), P2X₁R mRNA (C, D; red) and P2X₁R protein (E, F; green). (A, C, E, G) In retrosplenial granular cortex (RSGC) P2X₁R protein is present in axons and proximal dendrites traceable to P2X₁R mRNA positive neurons (filled arrows). (B, D, F, H). In perirhinal cortex (PRhC) P2X₁R protein, besides axons and proximal dendrites, is also present in cell bodies of mRNA positive neurons (filled arrows). Filled arrows: P2X₁R mRNA and protein positive neurons. Arrowhead: P2X₁R mRNA positive and P2X₁R protein negative neurons. Pial surface on the left side. Scale bars=25 μ m G, H.

Prefrontal and cingulate regions. Differences in labeling intensity were observed on the medial surface of the cerebral hemisphere. In the anterior prefrontal area, including the medial orbital (MO), infralimbic (IL), prelimbic (PrL) and anterior cingulate (Cg1), clear labeling was present in pyramidal neurons (Fig. 3C, D). Most of these neurons were in layer V; fewer were in layer VI. The neuropil staining was light in the supragranular layers, where apical dendrite staining stood out clearly. Conversely, in layers V and VI denser neuropil staining was present, with clearly labeled basal dendrites and axons. Moving caudally into the posterior cingulate area (Cg2), staining intensity gradually weakened although the above-described, general pattern remained. Quite different labeling was seen posterior to Cg2 in the RSGC (Fig. 3G, H). In this latter structure, a band-like distribution of immunoreactivity was seen in layers V and III, separated by an

unstained layer IV. In layer V, immunostaining was mostly confined to the thicker parts of the basal and apical dendrites of the pyramidal neurons, whose unstained neuronal bodies appeared as negative on a darker background (Fig. 3H). This peculiar distribution of immunoreactivity was more evident on double immunofluorescence with P2X₁R and NeuN (Fig. 4G–I). In layer III, a dense mesh of labeled fibers and terminals surrounded the neurons (Fig. 3H; Fig. 4G–I). In the horizontal sections, it could be seen that this fiber mesh was made up of longitudinally organized fibers running parallel to the pial surface (data not shown).

PRhC. In the rhinal sulcus, the general pattern of staining resembled that observed in the PFC, with stronger neuropil staining in the infragranular than in the supragranular layers. Numerous layer V pyramidal neurons were labeled with clear staining of the apical dendrites, which could be followed up to layer III (Fig. 3E, F). The basal dendrites were also clearly labeled, while the neuronal body labeling was somewhat lighter (Fig. 3F). In layer VI, only a few neurons were labeled, while numerous labeled axons could be followed into the subcortical white matter, which was very densely stained (Fig. 3E). This pattern extended throughout all divisions of the insular cortex (IC) and more caudally into the so-called ectorhinal and perirhinal areas.

Frontal, parietal, temporal and occipital cortices. Immunostaining was on average lighter in these isocortical areas than in the PFC and PRhC; also, there was a more even distribution throughout the layers, although background staining was darker. In particular, no clear differences could be found between infragranular and supragranular layers, with neuropil staining that tended to be slightly more intense in layer III and even more intense in layer V (Fig. 3A). On closer inspection at higher magnification, layer V staining was mainly due to labeling of apical dendrites of pyramidal neurons, with only occasional light staining of the neuronal bodies (Fig. 4B). Although axonal labeling was present, it was much less intense than in the PFC and PRhC; therefore, the underlying white matter appeared more lightly stained than in these two latter areas.

Hippocampal formation. Although the hippocampal formation was intensely labeled, staining was seen almost exclusively in dendrites and axons and almost never in neuronal bodies (Fig. 5). The transition between the PRhC and the EtC was abrupt and particularly striking: the dense pyramidal neuronal body staining over a relatively light background of the PRhC suddenly changed into dense fiber staining in layers II to VI of the EtC (Fig. 5B). In this latter structure, although we could not detect immunostaining of the neuronal bodies, the radial organization of the fiber labeling through layers III–VI suggested that this labeling corresponded to a mixture of apical dendrites of pyramidal neurons and axons. At higher magnification, thin-labeled axons and thick-labeled dendrites could be recognized in layer III as well as in layers V and VI (Fig. 5C). In layer II, a relatively dense mesh of fibers could be seen with no evidence of a preferential orientation of processes (Fig. 5C). The white matter underneath the EtC

Fig. 3. P2X₁R immunolabeling in different cortical regions (A, C, E, G) and relative higher magnifications (B, D, F, H). (A, B) Motor cortex. Immunostaining is mainly confined to dendrites in layer IV. (C, D) PrL cortex. Immunopositive neurons can be found in layers V and VI, while layers III to I are essentially devoid of positive neurons. (E, F) PRhC. Note the clear staining of immunopositive neurons in layers V and VI, while layers III to I are essentially devoid of positive neurons. (G, H) RSGC. Immunopositive neurons can be found in layers V and VI, while layers II and IV appear devoid of immunostaining. Note the layer specific arrangement of the staining with apical dendrites in layer V, almost no staining in layer IV and a dense mesh of intensely immunostained processes in layer III. Scale bars=200 μ m A; B=40 μ m; C, E, G=250 μ m; D, F, H=60 μ m.

was densely stained. Coming out of it, labeled axons grouped into thick bundles could be followed into the perforant path traversing the subiculum (S) and heading toward the molecular layer of the dentate gyrus (DG), where they terminated in a very fine mesh of terminal arborizations. Granular cells of the DG were not immunoreactive. No staining was observed in this layer or in the pyramidal cell layer of any of the CA3–CA1 hippocampal fields (Fig. 5B, D). In contrast, a dense mesh of labeled fibers could be seen in the polymorphic layer of the DG and in the strata lucidum, radiatum and oriens of the CA3–CA2 sector of the

hippocampus (Fig. 5E). The densest labeling was in the stratum radiatum; axons could be seen emerging from it and heading toward the CA1 sector. However, it was not possible to verify whether these fibers were Schaffer's collaterals. By contrast, the CA1 sector was devoid of labeling (Fig. 5B). Labeling reappeared in the S, where labeled fibers could be seen between the fiber bundles of the perforant path (Fig. 5D). The density of subicular fibers gradually increased and reached a maximum at the border with the EtC (Fig. 5F). This was also the most densely labeled area of the hippocampal formation. Finally, labeled

Fig. 4. Confocal images of double immunofluorescence for NeuN (A, D, G; green) and P2X₁R (B, E, H; red) in different cortical areas and for GFAP (J; green) and P2X₁R (K; red) together with DAPI counterstaining (K; blue). (A–C) Temporal cortex, layer III. P2X₁R immunofluorescence is mainly confined to proximal dendrites and possibly axons. (D–F) PRhC, layer V. P2X₁R immunofluorescence is localized in cytoplasmic, dendritic and possibly axonal domains. (G–I) RSGC. P2X₁R immunofluorescence is confined only to dendrites and possibly axons. Note the network of fibers in lamina III. (J–L) Cingulate cortex, note that all GFAP positive cells are P2X₁R negative. Pial surface on the left side. Scale bars=10 μ m A–F; G–I=35 μ m; J–L=20 μ m.

axons could be seen coming out of the S and heading toward the white matter and the alveus, which were both darkly stained (Fig. 5B).

Extended amygdala. P2X₁R immunoreactive neuronal bodies in subcortical telencephalic regions were found in only two structures, namely, in the BST (Fig. 6A–B) and the amygdala (Fig. 6C–D). Golgi-like immunostained neurons in the BST were selectively found in the dorsal part of the lateral division (BSTLD), where a population of loosely packed, medium size, intensely immunoreactive for P2X₁R, bipolar and multipolar positive neurons displaying an ovoid shape was observed (Fig. 6B). No labeled neurons were seen in the rest of the BST. However, immunopositive varicosities and puncta were detected in the medial division. The central amygdaloid nucleus was the other structure that displayed P2X₁R, Golgi-like, immunostained neu-

rons, and most of its labeling was located in the lateral (CeL) and capsular (CeC) subdivisions (Fig. 6C, D). These neurons, like those of the BSTLD, were medium size, bipolar and multipolar and displayed an ovoid shape (Fig. 6D).

Diencephalon. The thalamus appeared substantially devoid of P2X₁R positive neurons or fibers with the exception of the medial habenular nucleus (mHb), which showed intense P2X₁R immunoreactivity (Fig. 6E). In this structure, labeling was present in the neuropil and fibers, with no evident cell staining. Since the lateral habenular subdivision was not positive, an abrupt change in the staining nicely underlined the medial habenular borders. Although the stria medullaris was not immunoreactive, axonal labeling was present (Fig. 6E) in both the medial and lateral root of the fr. In the hypothalamus, a few scattered immunostained neurons were present in the periventricular nu-

Fig. 5. Nissl staining (A) and P2X₁R immunolabeling (B–F) of the hippocampal formation from horizontal sections. (B) Low magnification of the hippocampal formation. (C) Entorhinal cortex (EtC). (D) Perforant path. (E) CA3 subfield. (F) Border between S and EtC. Scale bars=600 μ m A, B; C, D, E=250 μ m; F=400 μ m.

cleus, beside the third ventricle, and more posteriorly in the central part of the dorsomedial nucleus. Overall, the neuropil staining in the hypothalamus (lateral and medial) was intense with fibers and puncta.

DISCUSSION

The present data demonstrate the selective expression of the P2X₁R subunit in the pyramidal neurons of the neo-cortex in layers V and VI and in two defined amygdaloid structures, namely, BST and Ce. In general, at the cellular level P2X₁R expression tends to be more intense in the basal and apical dendrites of the pyramidal neurons than in the soma. Axonal staining is also frequently found. In the different cortical areas, differences in the intensity and the cellular location of the staining were observed. Particularly evident was the soma staining in the medial prefrontal and perirhinal areas and the almost complete absence of the soma staining in the piriform cortex. These data support the role of P2X₁R at both pre- and post-synaptic sites in regions involved in the integration of cognitive, limbic and autonomic functions.

Methodological considerations

Initial studies indicated the absence or limited expression of the P2X₁R in the CNS. Later, based on immunohisto-

chemical techniques P2X₁R expression was reported in different brain nuclei (Yao et al., 2000, 2001; Florenzano et al., 2002; Viscomi et al., 2004). Different reports questioned the validity of immunohistochemical techniques in detecting P2XR with particular attention to P2X₇R (Anderson and Nedergaard, 2006).

In the present study, we investigated the specificity of the P2X₁R immunolabeling using several approaches; the reliability of our data are supported by different observations. First, the pattern of CNS immunostaining observed is highly specific. P2X₁R are present in neurons and not in glial cells, and are confined to discrete, functionally-related, cortical and subcortical forebrain regions. Second, *in situ* hybridization and immunohistochemistry double labeling consistently demonstrated co-expression of the P2X₁R protein and RNA. Third, the antibodies employed matched all the specificity controls for Western blot and immunohistochemistry.

Support for the specificity of CNS P2X₁R expression is provided by studies demonstrating P2X₁R mediated transmission in the same CNS areas in which we showed P2X₁R immunostaining. Pyramidal neurons of the somatosensory cortex present P2X₁R in dendrites and axons (Fig. 3), and fast excitatory postsynaptic current matching the P2X₁R characteristics were identified in the same neurons (Pankratov et al., 2002, 2003). Furthermore, in the medial

Fig. 6. P2X₁R immunolabeling in subcortical regions. (A, B) Coronal section at the level of the anterior commissure. Note the intense neuronal labeling in the BST. (B) Inset of A. (C, D) Coronal section at the level of the amygdalar complex. Note the selective staining of the Ce. (D) inset of C. (E, F) Habenular complex. Note the intensity of P2X₁R immunostaining in the medial habenula and fasciculus retroflexus (fr). (F) Inset of E. Scale bars=400 μ m A, C; B, D=50 μ m; E=200 μ m; F=30 μ m.

habenula, where we observed intense dendritic P2X₁R expression (Fig. 6), early studies reported P2 mediated excitation (Edwards et al., 1992; Robertson et al., 1999) corresponding to the later described P2X₁R kinetic profile (North, 2002).

Anatomofunctional considerations

Within the general framework of extensive P2X₁R expression in the forebrain, different major points of interest can be underlined. In PFC and PRhC areas, P2X₁R expression is present in the neurons of layers V–VI, depicting the cell profile with soma as well as dendritic and axonal labeling. Although cortical labeling is still present outside these areas in the same classes of neurons, it is confined to axons and proximal dendrites. Efferent neurons in layers V and VI are glutamatergic, mainly directed to subcortical structures, and they make only a minor contribution to cortico-cortical

connections. This suggests a role for P2X₁R in controlling the information output from the cortex. Furthermore, PFC and PRhC, which are considered the rat homologues of the human PFC, display differences in intensity when compared with the remaining subdivisions of the isocortex, suggesting prevalent purinergic control over the output of the former areas.

At the subcortical level, the main point of interest is the solitary presence of P2X₁R positive neurons in the BST and Ce. These nuclei share reciprocal connections, and it has been suggested that they are part of a single anatomical entity commonly referred to as the “extended amygdala” (Alheid, 2003). In particular, BST would represent the rostralateral end and Ce, the caudolateral end of the central division of the extended amygdala. This concept relies upon similarities between Ce and BST in terms of their extrinsic and intrinsic connectivity, cell morphology and

neurochemistry. Concerning connectivity, BST and Ce receive and send extrinsic projections from almost the same areas. They receive a major contribution from the entorhinal, agranular insular, IL and perirhinal cortices and from the parabrachial nuclei, caudal medulla and nucleus of the tractus solitarius. BST and Ce appear to project mainly to hypothalamic nuclei, brain stem nuclei and, to a lesser extent, to thalamic territories. The BSTL and Ce share reciprocal connections mainly via the stria terminalis, and GABA has been suggested as the major neurotransmitter for both BST and Ce. In addition, several neuropeptides have been identified in both neurons and fibers of BST and Ce. Our results indicate that ATP can be another excitatory neurotransmitter used in this circuit and they underline the neurochemical homogeneity of these two nuclei, thus providing support for the functional identity of the BST and Ce. Moderate to high immunoreactivity has been shown for P2X₁₋₆R subunits in the nucleus of the tractus solitarius (Yao et al., 2000, 2001), and functional responses mediated by the P2X₁R have been found. This nucleus is the primary visceral relay station in the brainstem, which in turn issues projections to the amygdala and other structures. In addition, all of the cortices that project to the BST and the Ce appear particularly enriched in the P2X₁R (present study) and other P2XR subunits (Norenberg and Illes, 2000). Thus, a circuit from the higher cognitive to the lower autonomic centers appears to be enriched in P2XR subunits, particularly in the P2X₁R subunit.

The hippocampal formation and the medial habenula display high immunostaining of dendritic processes, axons and neuropil. In the hippocampal formation, all regions except the CA1 display P2X₁R intensely labeled fibers. The habenular complex is a phylogenetically old structure (Andres et al., 1999) where, according to Nauta (1974), limbic and striatal mechanisms intermix directly. The dorsal diencephalic conduction system found in the habenular complex is an anatomical relay between the limbic forebrain and the midbrain (Sutherland, 1982) and has been involved in complex behaviors, psychosis syndromes and schizophrenia (Ellison, 1994). Despite the high potentiality of this relay structure in the crosstalk between different regions of the limbic system, the habenula still appears to be a neglected structure. The habenula receives afferents mainly from the septum through the stria medullaris and sends efferences through the fr (Nauta, 1974) to the interpeduncular nucleus, ventral tegmental area, raphe and substantia nigra. The habenular cytological organization has only been studied recently (Andres et al., 1999; Geisler et al., 2003) and is characterized by a highly complex subnuclear parcellation with differences in the expressions of neuroactive molecules. In our material, P2X₁R expression was selectively confined to the medial habenula, which displayed homogeneous staining throughout the rostro-caudal extension. The stria medullaris did not show any particular staining, suggesting that the P2X₁R receptor is targeted mainly to postsynaptic locations in the medial habenula, while the fr was highly stained, indicating that in the projection areas the receptor may be targeted to pre-synaptic locations.

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REFERENCES

- Alheid GF (2003) Extended amygdala and basal forebrain. *Ann N Y Acad Sci* 985:185–205.
- Anderson CM, Nedergaard M (2006) Emerging challenges of assigning P2X₇ receptor function and immunoreactivity in neurons. *Trends Neurosci* 29:257–262.
- Andres KH, von Düring M, Veh RW (1999) Subnuclear organization of the rat habenular complexes. *J Comp Neurol* 407:130–150.
- Ashour F, Atterbury-Thomas M, Deuchars J, Evans RJ (2006) An evaluation of antibody detection of the P2X₁ receptor subunit in the CNS of wild type and P2X₁-knockout mice. *Neurosci Lett* 397:120–125.
- Atkinson L, Shigetomi E, Kato F, Deuchars J (2003) Differential increases in P2X receptor levels in rat vagal efferent neurones following a vagal nerve section. *Brain Res* 977:112–118.
- Burnstock G, Knight GE (2004) Cellular distribution and functions of P₂ receptor subtypes in different systems. *Int Rev Cytol* 240:31–304.
- Edwards FA, Gibb AJ, Colquhoun D (1992) ATP receptor-mediated synaptic currents in the central nervous system. *Nature* 359:144–147.
- Ellison G (1994) Stimulant-induced psychosis, the dopamine theory of schizophrenia, and the habenula. *Brain Res Brain Res Rev* 19:223–239.
- Florenzano F, Viscomi MT, Cavaliere F, Volonté C, Molinari M (2002) Cerebellar lesion up-regulates P2X₁ and P2X₂ purinergic receptors in precerebellar nuclei. *Neuroscience* 115:425–434.
- Florenzano F, Viscomi MT, Mercaldo V, Longone P, Bernardi G, Bagni C, Molinari M, Carrive P (2006) P2X₂R purinergic receptor subunit mRNA and protein are expressed by all hypothalamic hypocretin/orexin neurons. *J Comp Neurol* 498:58–67.
- Geisler S, Andres KH, Veh RW (2003) Morphologic and cytochemical criteria for the identification and delineation of individual subnuclei within the lateral habenular complex of the rat. *J Comp Neurol* 458:78–97.
- Kanjhan R, Housley GD, Burton LD, Christie DL, Kippenberger A, Thorne PR, Luo L, Ryan AF (1999) Distribution of the P2X₂ receptor subunit of the ATP-gated ion channels in the rat central nervous system. *J Comp Neurol* 407:11–32.
- Kassa RM, Bentivoglio M, Mariotti R (2007) Changes in the expression of P2X₁ and P2X₂ purinergic receptors in facial motoneurons after nerve lesions in rodents and correlation with motoneuron degeneration. *Neurobiol Dis* 25:121–133.
- Khakh BS (2001) Molecular physiology of P2X receptors and ATP signalling at synapses. *Nat Rev Neurosci* 2:165–174.
- Khakh BS, North RA (2006) P2X receptors as cell-surface ATP sensors in health and disease. *Nature* 442:527–532.
- Loesch A, Burnstock G (1998) Electron-immunocytochemical localization of P2X₁ receptors in the rat cerebellum. *Cell Tissue Res* 294:253–260.
- Nauta HJ (1974) Evidence of a pallidohabenular pathway in the cat. *J Comp Neurol* 156:19–27.
- Norenberg W, Illes P (2000) Neuronal P2X receptors: localisation and functional properties. *Naunyn Schmiedeberg Arch Pharmacol* 362:324–339.
- North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067.
- Pankratov Y, Lalo U, Krishtal O, Verkhratsky A (2002) Ionotropic P2X purinoreceptors mediate synaptic transmission in rat pyramidal neurones of layer II/III of somato-sensory cortex. *J Physiol* 542:529–536.

- Pankratov Y, Lalo U, Krishtal O, Verkhratsky A (2003) P2X receptor-mediated excitatory synaptic currents in somatosensory cortex. *Mol Cell Neurosci* 24:842–849.
- Paxinos G, Watson C (1997). The rat brain in stereotaxic coordinates, Third ed. New York: Academic Press.
- Paxinos G (2004). The rat nervous system, Third edition. Oxford: Elsevier Academic Press.
- Rhodes KJ, Trimmer JS (2006) Antibodies as valuable neuroscience research tools versus reagents of mass distraction. *J Neurosci* 26:8017–8020.
- Robertson SJ, Burnashev N, Edwards FA (1999) Ca^{2+} permeability and kinetics of glutamate receptors in rat medial habenula neurones: implications for purinergic transmission in this nucleus. *J Physiol* 518 (Pt 2):539–549.
- Rubio ME, Soto F (2001) Distinct localization of P2X receptors at excitatory postsynaptic specializations. *J Neurosci* 21:641–653.
- Sutherland RJ (1982) The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neurosci Biobehav Rev* 6:1–13.
- Viscomi MT, Florenzano F, Conversi D, Bernardi G, Molinari M (2004) Axotomy dependent purinergic and nitrergic co-expression. *Neuroscience* 123:393–404.
- Xiang Z, Bo X, Oglesby I, Ford A, Burnstock G (1998) Localization of ATP-gated P2X2 receptor immunoreactivity in the rat hypothalamus. *Brain Res* 813:390–397.
- Yao ST, Barden JA, Finkelstein DI, Bennett MR, Lawrence AJ (2000) Comparative study on the distribution patterns of P2X(1)-P2X(6) receptor immunoreactivity in the brainstem of the rat and the common marmoset (*Callithrix jacchus*): association with catecholamine cell groups. *J Comp Neurol* 427:485–507.
- Yao ST, Barden JA, Lawrence AJ (2001) On the immunohistochemical distribution of ionotropic P2X receptors in the nucleus tractus solitarius of the rat. *Neuroscience* 108:673–685.
- Yao ST, Gourine AV, Spyer KM, Barden JA, Lawrence AJ (2003) Localisation of P2X2 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.

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