Regional expression of P2Y₄ receptors in the rat central nervous system

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Abstract P2Y receptors are G protein-coupled receptors composed of eight known subunits (P2Y₁, 2, 4, 6, 11, 12, 13, 14), which are involved in different functions in neural tissue. The present study investigates the expression pattern of P2Y₄ receptors in the rat central nervous system (CNS) using immunohistochemistry and in situ hybridization. The specificity of the immunostaining has been verified by preabsorption, Western blot, and combined use of immunohistochemistry and in situ hybridization. Neurons expressing P2Y₄ receptors were distributed widely in the rat CNS. Heavy P2Y₄ receptor immunostaining was observed in the magnocellular neuroendocrine neurons of the hypothalamus, red nucleus, pontine nuclei, mesencephalic trigeminal nucleus, motor trigeminal nucleus, ambiguous nucleus, inferior olive, hypoglossal nucleus, and dorsal motor vagus nucleus. Both neurons and astrocytes express P2Y₄ receptors. P2Y₄ receptor immunostaining signals were mainly confined to cell bodies and dendrites of neurons, suggesting that P2Y₄ receptors are mainly involved in regulating postsynaptic events. In the hypothalamus, all the vasopressin (VP) and oxytocin (OT) neurons and all the orexin A neurons were immunoreac-

tive for P2Y₄ receptors. All the neurons expressing P2Y₄ receptors were found to express *N*-methyl-D-aspartate receptor 1 (NR1). These data suggest that purines and pyrimidines might be involved in regulation of the release of the neuropeptides VP, OT, and orexin in the rat hypothalamus via P2Y₄ receptors. Further, the physiological and pathophysiological functions of the neurons may operate through coupling between P2Y₄ receptors and NR1.

Keywords P2Y₄ receptor · Immunohistochemistry · In situ hybridization · CNS

Introduction

Extracellular purines and pyrimidines act as messengers via purinergic receptors on the plasma membrane. There are two purinergic receptor families: P1 receptors (adenosine receptors, AR) activated by adenosine and P2 receptors activated by ATP, ADP, UTP, and/or UDP. The P2 receptor family includes P2X receptors equivalent to intrinsic calcium-permeable cation channels and metabotropic P2Y receptors that are G protein-coupled receptors [1]. Currently, there are seven cloned P2X (P2X₁ to P2X₇) receptor subunits and at least eight P2Y receptors (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, and P2Y₁₄) [2]. Both purine and pyrimidine nucleotides can modulate a variety of physiological functions by interaction with P2Y receptors [3]. Purine and pyrimidines act as neuromodulators via P2Y receptors and are involved in the physiology and pathophysiology of the nervous system [4]. Determination of the distribution pattern and cell types expressing P2Y receptor subtypes in the central nervous system (CNS) provides important data for functional experi-

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ments. There are many reports about the distribution of P2Y receptors in the CNS [5–11]. However, there are no detailed studies of the distributions of P2Y receptors in the CNS, except for that of P2Y₁ [7]. Human [12, 13], rat [5, 14], and mouse [15, 16] P2Y₄ receptors have been cloned. In the rat, the expression of P2Y₄ mRNA in brain was much higher in neonates than in adults [14]. In situ hybridization in the adult rat brain showed that neuronal P2Y₄ receptor mRNA was present in the ventricular/choroid plexus system and strongly in the pineal gland, as well as in astrocytes [14]. With single cell real-time PCR and Western blot techniques, P2Y₄ receptors were detected in the CA1 and CA3 of rat hippocampus. With RT-PCR, P2Y₄ transcripts were detected in RNA preparations from embryonic rat cortex and in the cultured granular cells of the cerebellum [9]. With a quantitative RT-PCR study, expression of human P2Y₄ receptors was detected in many regions of the human brain, including amygdala, caudate nucleus, cerebellum, cingulate gyrus, globus pallidus, hippocampus, hypothalamus, putamen, striatum, thalamus, and spinal cord [17]. At the present time, there is no detailed description of the distribution of P2Y₄ receptors on different cell types in the CNS. In this study, with single and double-labeling immunohistochemistry, combined use of in situ hybridization and immunohistochemistry, and Western blot analysis, P2Y₄ receptors were found to be expressed widely and to be co-expressed with the classical neuropeptides vasopressin (VP), oxytocin (OT), orexin, and the glutamate receptor *N*-methyl-D-aspartate (NMDA) receptor 1 (NR1) in rat CNS.

Materials and methods

Tissue preparation

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University and associated guidelines on the ethical use of animals. Twelve adult Sprague Dawley rats (250–350 g) were used. The number of animals used and their suffering in this study were minimized. The rats were killed by asphyxiation with CO₂ and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4. The brains were dissected out immediately and immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.2) for 4–6 h. The brains were then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the brains were rapidly frozen by immersion in isopentane at –70°C for 2 min. Coronal sections (20 µm) of the brains were cut with a Leica cryostat (CM1900; Nussloch, Germany) and floated in PBS.

Table 1 Characterization of P2Y₄ receptor-is levels in the rat CNS

Regions	IHC	%
Olfactory bulb		
Mitral cells	+	35
Granular layer	–	0
Tufted cells	+	NC
Anterior olfactory nucleus	++	56
Cerebral cortex		
Agranular insular cortex	++	69
Anterior cingulate cortex	+	45
Ventrolateral orbital cortex	+	74
Piriform cortex	+++	81
Frontal cortex	++	67
Parietal cortex	++	52
Occipital cortex	++	45
Subcortical telencephalon		
Nucleus diagonal band	++	59
Bed nucleus stria terminalis	+	36
Medial septal nucleus	+	47
Lateral septal nucleus	++	32
Islands of Calleja	–	0
Olfactory tubercle	+	34
Accumbens nucleus	+	42
Caudate–putamen	+	1
Globus pallidum	+	5
Substantia innomata	+	23
Medial globus pallidum	++	8
Lateral olfactory tract nucleus	+	34
Anterior cortical amygdaloid nucleus	++	45
Basolateral amygdaloid nucleus	+	58
Central amygdaloid nucleus	++	78
Medial amygdaloid nucleus	++	67
Amygdalohippocampal area	++	51
Hippocampal fields CA1–CA4	++	95
Dentate gyrus	++	95
Epithalamus		
Medial habenular nucleus	+	40
Lateral habenular nucleusa	+	21
Thalamus		
Anterior nuclei	++	67
Anteroventral nucleus	++	72
Laterodorsal nucleus	++	82
Mediodorsal nucleus	++	78
Ventrolateral nucleus	++	73
Ventromedial nucleus	++	87
Reticular nucleus	+	79
Paraventricular nucleus	++	56
Central medial nucleus	+	12
Xiphoid nucleus	+	23
Rhomboid nucleus	+	45
Hypothalamus		

Table 1 (continued)

Regions	IHC	%
Suprachiasmatic nucleus	++	NC
Medial preoptic nucleus	+	43
Medial preoptic area	++	54
Supraoptic nucleus	+++	96
Paraventricular nucleus	+++	52
Ventromedial nucleus	+	73
Arcuate nucleus	++	87
Tuberomammillary nucleus	+++	92
Midbrain		
Zona incerta	+	NC
Lateral geniculate nucleus	+	54
Medial geniculate nucleus	+	48
Substantia nigra zona compacta	++	46
Substantia nigra zona reticulate	+	67
Supramammillary nucleus	++	42
Ventral tegmental area	+	65
Oculomotor nucleus	++	43
Edinger–Westphal nucleus	+	34
Central gray	+/-	NC
Superior colliculus	+	23
Inferior colliculus	+	34
Interpeduncular nucleus	+	39
Trochlear nucleus	+	54
Pedunculo pontine tegmental nucleus	+	68
Hindbrain		
Pontine nuclei	+++	87
Raphe dorsalis	+	85
Mesencephalic trigeminal nucleus	+++	99
Dorsomedial tegmental area	+	63
Locus ceruleus	+	46
Subceruleus nucleus	+	34
Barrington's nucleus	+	54
Pontine reticular nucleus, ventral part	++	76
Pontine reticular nucleus, caudal part	+	78
Red nucleus	+++	89
Motor trigeminal nucleus	+++	83
Superior olivary nucleus	+	67
Principal sensory trigeminal nucleus	+	88
Spinal trigeminal nucleus	+	54
Paragigantocellular nucleus	++	56
Abducens nucleus	+++	85
Facial nucleus	++	90
Accessory facial nucleus	++	80
Ambiguous nucleus	++	86
Inferior olive	+++	89
Hypoglossal nucleus	+++	91
Dorsal motor vagus nucleus	+++	93
Solitary tract nucleus	+	70
Lateral reticular nucleus	+	88

Table 1 (continued)

Regions	IHC	%
Curate fasciculus	++	67
Area postrema	++	53
Cerebellum		
Purkinje cells	+	45
Granular layer	–	NC
Cerebellar nucleus	++	87
Spinal cord		
Dorsal horn	++	56
Ventral horn	++	78

IHC immunohistochemistry, % the percentage of P2Y₄ receptor-ir neurons against the total number of neurons immunostained with NeuN antibody in the individual nuclei of the rat CNS, *NC* not counted

The density of P2Y₄ receptor-is was scored as: absent (–), weak (+), moderate (++), and heavy (+++)

Immunohistochemistry

Immunohistochemistry for localization of P2Y₄ receptors was performed using rabbit polyclonal antibody against a unique peptide sequence of rat P2Y₄ receptor provided by Alomone Lab, Israel. The peptide sequence of the P2Y₄ receptor is of amino acid sequence 337–350 (HEESISR-WADTHQD; Accession CAA72241).

Endogenous peroxidase was blocked by 3% H₂O₂ 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 P2Y₄ receptor antibody, diluted 1:600 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in ExtrAvidin peroxidase (Sigma Chemical Co., Poole, UK) diluted 1:1,000 in PBS for 1 h. P2Y₄ receptor immunoreactivity (-ir) was visualized by a freshly prepared color reaction mixture solution containing 0.05% 3,3'-diaminobenzidine, 0.05 M sodium phosphate, 0.004% NH₄Cl, 0.2% glucose, 0.04% nickel ammonium sulfate, and 0.1% glucose oxidase. All incubations were held at room temperature and separated by 3–5 min washes in PBS. Finally, the sections were then mounted on slides, dehydrated and cleared in xylene, and embedded. The density of the P2Y₄ receptor protein immunostaining signals was scored as follows: absent (–), weak (+), moderate (++), and heavy (+++) [18].

Double-labeling immunofluorescence

The following protocol was used for double immunofluorescence staining with the two primary antibodies from the same hosts. 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 [19]. 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 [20]. 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% NHS, 0.2% Triton X-100 in PBS for 30 min, followed by incubation with P2Y₄ antibodies diluted in antibody dilution solution (10% NHS, 0.2% Triton X-100, and 0.4% sodium azide in PBS) overnight at 4°C.

Fig. 1 Specificity tests. **a, b** P2Y₄ receptor antibody specificity tests. **c, d** In situ hybridization specificity test. P2Y₄ receptor immunostaining (-is) in sections of the supraoptic nucleus of the hypothalamus using P2Y₄ receptor primary antibody alone (**a**) or after preabsorption with its peptide antigen (**b**). Note the absence of immunostaining in **b**. P2Y₄ receptor mRNA hybridization signal in sections of the supraoptic nucleus of the hypothalamus using antisense (**c**) or sense (**d**) digoxigenin-labeled cRNA probes. Note the absence of hybridization signals in **d**. Scale bars=200 μ m in **a** and **b** and 100 μ m in **c** and **d**. **e** Western blotting from brain extracts. *M* molecular weight marker. Lane 1 P2Y₄ receptor immunoreactive band is located at about 43 kDa. Lane 2 preabsorption of the P2Y₄ receptor antisera with its peptide antigen, which resulted in the absence of the band

Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in extravidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. The P2Y₄ immunoreactivity was visualized by the TSA Cy3 system (NEL704A, NEN, USA). After visualization, the sections were incubated with the second primary antibodies of glial filament acid protein (GFAP, a marker for astrocytes, mouse monoclonal antibody from Chemicon), Iba-1 (a marker for microglia, Goat polyclone from Abcam), myelin basic protein (MBP, a marker for oligodendrocytes, mouse monoclonal from Chemicon), NeuN (a neuron marker, mouse monoclonal from Chemicon), NR1 (goat polyclone from Santa Cruz), orexin A (rabbit polyclone from Abcam), VP (rabbit polyclone from Abcam), and OT (rabbit polyclone from Abcam) diluted in the antiserum dilution solution overnight at 4°C. Subsequently, the sections were incubated with FITC-conjugated donkey anti-rabbit or mouse or goat (Jackson ImmunoResearch) diluted 1:200 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 counterstained with 5 µg/ml Hoechst 33342.

Control experiments

Control experiments were carried out with P2Y₄ antiserum preabsorbed with P2Y₄ receptor peptide at a concentration of 25 µg/ml. The amino acid sequence for this peptide is 337–350 (HEES ISRWADTHQD). Sense digoxigenin-labeled cRNA probe was used as a negative control of in situ hybridization.

Combined use of immunohistochemistry and in situ hybridization

In order to confirm the specificity of the P2Y₄ receptor antibody, the combination use of immunohistochemistry and

Fig. 2 Distribution of P2Y₄ receptor-is and mRNA signals in the piriform cortex (**a**, **b**, **c**), choroid plexus of the lateral ventricle (**d**) and CA1 region of the hippocampus (**e**), and supra-optic nucleus of hypothalamus (**f**). **a** Localization of P2Y₄ receptor mRNA. **b** This shows P2Y₄ receptor-is at the same field of **a**. **c** The merged image of **a** and **b**. **d**, **e**, **f** These shows the merged images of P2Y₄ receptor-is (green) and mRNA signals (red) in the choroid plexus of the lateral ventricle (**d**), CA1 region of the hippocampus (**e**), and supraoptic nucleus of the hypothalamus (**f**). Note that all the cell bodies expressing P2Y₄ receptor protein also express mRNA. All scale bars=100 µm

in situ hybridization histochemistry was used. The following is the protocol for this combination method. In situ hybridization was first carried out. Antisense and sense digoxigenin-labeled cRNA probes were synthesized with a DIG-RNA labeling Kit (Roche Boehringer Mannheim) using linearized templates of pSPT18 inserted by rP2Y₄ cDNA 1573–1856 (GenBank Y14705), using a previously described protocol [21]. Briefly, floating rat brain sections were washed 3×5 min in 0.1 mol/l PBS, in 0.1 mol/l glycine/PBS, and in 0.4% Triton X-100/PBS for 10 min each. The sections were then incubated in protease K (1 µg/ml) in PBS for 30 min at 37°C. The activity of protease K was stopped by fixation in 4% paraformaldehyde for 5 min, followed by 2×3 min washes in PBS to remove fixative from the sections. The sections were incubated in 0.25% acetic diaminobenzanhydride with

0.1 mol/l triethanolamine (pH 8.0) for 10 min at room temperature, followed by washing in 0.6 mol/l sodium chloride and 0.06 mol/l sodium citrate (2×SSC) for 10 min. Digoxigenin-labeled cRNA (0.5×µg/ml) of either antisense or sense probes was added to hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 mol/l NaCl, 1×Denhardt's solution, 0.05 mol/l Tris-HCl (pH 8.0), 1 mmol/l EDTA, and 250 µg/ml *Escherichia coli* tRNA (RNase-free). Hybridization was carried out for 16 h at 56°C in a hybridization oven. The sections were washed in 4×SSC for 20 min at 37°C, followed by incubation in 2×SSC containing 20 µg/ml RNase A (Sigma) for 30 min at 37°C to digest the RNA probes that did not hybridize with the targeted RNA. The sections were further washed in 1×SSC and 0.2×SSC at 37°C for 20 min, respectively. After post-hybridization

Fig. 3 Distribution of P2Y₄ receptor-is in olfactory bulb and primary olfactory cortex. **a** P2Y₄ receptor-is in olfactory bulb. **b** High magnification of the area enclosed by a rectangle in **a**. Note the weak to moderate P2Y₄ receptor-is in the mitral cell layer, external plexiform layer, and glomerular layer of the olfactory bulb. **c** P2Y₄ receptor-is in the accessory olfactory bulb. **d** High magnification of the area of the AOD enclosed by a rectangle (accessory olfactory, dorsal part) in **c**. **e** P2Y₄ receptor-is in the piriform cortex. **f** High magnification of the area enclosed by a rectangle in **e**. Scale bars= 500 µm in **c**; 200 µm in **a** and **e**; and 100 µm in **b**, **d**, and **f**

wash, endogenous peroxidase was blocked by 3% H₂O₂ in PBS for 30 min. The sections were pre-incubated in 10% NHS, 0.2% Triton X-100 in PBS for 30 min followed by incubation with sheep anti-digoxigenin antibody (Roche Boehringer Mannheim), diluted 1:500 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-sheep IgG (Jackson) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in ExtrAvidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. The P2Y₄ mRNA hybridization signal was visualized by the TSA (tyramide signal amplification) Cy3 system (NEL704A, NEN, USA). After visualization, the sections were incubated with the P2Y₄ receptor antibody diluted 1:500 in the

antiserum dilution solution overnight at 4°C. Subsequently, the sections were incubated with FITC-conjugated donkey anti-rabbit (Jackson) diluted 1:100 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3×10 min washes in PBS. Finally, the sections were then mounted on slides and embedded in 50% glycerol PBS.

For Western blot analysis, the rats were deeply anesthetized by sodium pentobarbital (60 mg/kg) and killed by decapitation. Brains were rapidly removed and lysed with 20 mM Tris-HCl buffer, pH 8.0, containing 1% NP-40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% β-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase and phosphatase inhibitors (Sigma). Protein concentration was determined by the BCA protein assay method using bovine serum albumin (BSA) as standard.

Fig. 4 Distribution of P2Y₄ receptor-is in the motor cortex (**a**, **b**, **c**) and agranular insular cortex (**d**, **e**, **f**). **a** P2Y₄ receptor-is in the motor cortex. **b**, **c** These show high magnification of the areas enclosed by rectangles *a1* and *a2* in layers II–III and layer V of **a**, respectively. An apical dendrite of a pyramidal neuron in layer V of **b**, and a glial-like cell with weak P2Y₄ receptor-is in layer I are indicated by an arrow. **d** P2Y₄ receptor-is in the agranular insular cortex. **e**, **f** These show high magnification of the areas enclosed by rectangles *d1* and *d2* in the molecular layer and V layer in **d**. Note that an arrowhead shows the endplate of an astrocyte with P2Y₄ receptor-is and an arrow shows astrocyte-like cells with P2Y₄ receptor-is. Scale bars=500 μm in **a** and **d** and 100 μm in **b**, **c**, **e**, and **f**

One hundred micrograms of protein samples from brains was loaded per lane, separated by SDS-PAGE (12% polyacrylamide gels), and then was electrotransferred onto nitrocellulose membranes. The membranes were blocked with 10% non-fat dry milk in Tris-buffered saline for 2 h and incubated overnight at 4°C with P2Y₄ receptor antibody diluted 1:600 in 2% BSA in PBS. The membranes were then incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:5,000 in 2% BSA in PBS for 1 h at room temperature. The color development was performed with 400 µg/ml Nitro-Blue Tetrazolium, 200 µg/ml 5-bromo-4-chloro-3-indolyl phosphate, and 100 µg/ml levamisole in TSM2 (0.1 mol/l Tris-HCl buffer, pH 9.5, 0.1 mol/l NaCl, and 0.05 mol/l MgCl₂) in the dark. Bands were scanned using a densitometer (GS-700; Bio-Rad Laboratories).

Photomicroscopy and data analysis

Images were taken with a Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop 5.0, USA).

The number of neurons with P2Y₄ receptor immunostaining (-is) and NeuN-is was counted unilaterally throughout the caudorostral extent of the respective nuclei, as defined by the atlas of Paxinos and Franklin [22]. Data for each of the nuclei analyzed were obtained from each of the rats used. Three to five sections from each animal were used, and the average percentage of P2Y₄ receptor-is against NeuN-is in neurons in the individual regions/nuclei was calculated. The numbers presented in Table 1 represent

Fig. 5 Distribution of P2Y₄ receptor-is in the nucleus of the vertical and horizontal limb of the diagonal band (**a**, **b**), dorsal part of lateral septal nucleus (**c**), caudate-putamen (**d**), and amygdaloid complexes (**e**, **f**). **b** High magnification of the area enclosed by a rectangle in the diagonal band of **a**. **f** High magnification of the *la* nucleus enclosed by a rectangle in **e**. *la* lateral amygdaloid nucleus, *en* entorhinal cortex, *cc* corpus callosum, *LV* lateral ventricle, *VOLT* vascular organ of the lamina terminalis. Scale bars= 200 µm in **a** and **e** and 100 µm in **b**, **c**, **d**, and **f**

the average percentage of immunopositive cells observed unilaterally in the individual regions/nuclei.

Results

P2Y₄ receptor-is was demonstrated in a wide-ranging expression pattern for this subunit throughout the CNS. P2Y₄ receptor-is was heavy in many regions, such as magnocellular neuroendocrine neurons of the hypothalamus, red nucleus, pontine nuclei, mesencephalic trigeminal nucleus, motor trigeminal nucleus, ambiguous nucleus, inferior olive, hypoglossal nucleus, and dorsal motor vagus nucleus. The P2Y₄ receptor-is signals were mainly confined to the cell body and dendrite of the neuron, implying that P2Y₄ receptors are mainly involved in regulating postsyn-

aptic events. This staining was blocked in control sections exposed to the P2Y₄ receptor antisera, preabsorbed with its specific peptide (Fig. 1a, b). Western blotting, performed on tissue extracts derived from the rat/hypothalamus, assessed the specificity of the polyclonal P2Y₄ receptor antibody. An immunoreactive band was detected at about 43 kDa (Fig. 1e, lane 1). Preabsorption of the antiserum with the peptide antigen resulted in the absence of the band (Fig. 1e, lane 2), indicating that the antibody detects the appropriate antigen sequence. Sections which were incubated in antisense digoxigenin-labeled cRNA probe showed specific hybridization signals in the cytoplasm, while the sections which were incubated in sense digoxigenin-labeled cRNA probe, showed no hybridization signal (Fig. 1c, d). Combined methods of immunohistochemistry and in situ hybridization histochemistry showed that almost all the neurons with P2Y₄ receptor-

Fig. 6 Distribution of P2Y₄ receptor-is in CA1–CA4 fields and DG granule cells of the hippocampus and thalamus (d, e, f). **b, c** These show high magnification of the areas enclosed by rectangles *a1* and *a2* of the CA1 and DG regions in **a**, respectively. An arrow indicates a glial-like cell with weak P2Y₄ receptor-is in **c**. **e, f, g** These show high magnification of the areas enclosed by rectangles *d1*, *d2*, and *d3* in **d**. *LP* lateral posterior thalamic nucleus, *MD* mediodorsal thalamic nucleus, *VP* ventral posterior thalamic nucleus. Scale bars=500 μ m in **a** and **d** and 100 μ m in **b, c, e**, and **f**

is were also found to express P2Y₄ mRNA, as shown in the supraoptic nucleus, primary somatosensory cortex, hippocampus, and choroid plexus of the fourth ventricle (Fig. 2). These data from the combined use of immunohistochemistry and in situ hybridization histochemistry further confirmed the specificity of the P2Y₄ receptor antibody.

Olfactory bulb

The main olfactory bulb, anterior olfactory nucleus, and primary olfactory cortex showed P2Y₄ receptor-is at low to high levels (Fig. 3). Scattered positive cells, with weak to moderate immunostaining, were detected in the external plexiform layer, mitral cell layer, and internal granular cell

layer (Fig. 3a, b). Moderate to heavy immunostaining was detected in the anterior olfactory nucleus (Fig. 3c, d). Heavy immunostaining was detected in layer II of the piriform cortex (Fig. 3e, f). Scattered small glial-like cells were detected in layer I (Fig. 3e).

Cerebral cortex

P2Y₄ receptor-is was observed throughout the cerebral cortex (Table 1). Generally, the levels of P2Y₄ receptor-is were higher in layers II, III, and V, although P2Y₄ receptor-is neurons were also detected in layers I, IV, and VI, as shown in the primary motor cortex (Fig. 4a, b, c). The P2Y₄ receptor-is signals were mainly detected in the cytoplasm of

Fig. 7 Distribution of P2Y₄ receptor (a–f) in the hypothalamus. **a** P2Y₄ receptor-is is found in the supraoptic nucleus (SON) and lateral hypothalamus. **b** P2Y₄ receptor-is is found in the paraventricular nucleus of the hypothalamus (PVN). **c** Immunostaining in the arcuate nucleus (Arc), ventral medial nucleus, lateral area, dorsomedial nucleus, and ependymal cell of the third ventricle (3v). **d** P2Y₄ receptor-is is found in the medi-odorsal area, perifornix nucleus (PeF), and lateral area. **e** P2Y₄ receptor-is is found in the ventral tuberomammillary nucleus (VTM). **f** High magnification of the VTM in **e** enclosed in a rectangle. Scale bars=200 μ m in **c**, **d** and **e**; 100 μ m in **a** and **b**; and 50 μ m in **f**

the neurons (Fig. 4b). Weakly immunostained glial-like cells were detected in layer I (Fig. 4c, e). In the agranular insular cortex, the intensity of P2Y₄ receptor-is was higher than that in the superior layers (Fig. 4d, e, f).

Subcortical telencephalon

Weak to moderate immunostaining of neurons was observed in the vertical and horizontal parts of the diagonal band, lateral septal nucleus, substantia innomata, lateral olfactory tract nucleus, olfactory tubercle, bed nucleus stria terminalis, and accumbens nucleus (Fig. 5a, b, c). Scattered neurons were also observed in the globus pallidus and caudate–putamen (Fig. 5d). The amygdaloid complex showed moderate to heavy P2Y₄ receptor-is (Fig. 5e, f, Table 1).

Fig. 8 Distribution of P2Y₄ receptor-is in the substantia nigra of the midbrain (a–c) and the hindbrain at the level of the ventral cochlear nucleus, anterior part (d, e, f). **a** Low power image of immunostaining in neurons of the midbrain. **b** P2Y₄ receptor-is in the substantia nigra, compact part (SNc) from the area enclosed by rectangle *a1* in **a**. **c** P2Y₄ receptor-is in the substantia nigra, reticular part (SNr) from the area enclosed by rectangle *a2* in **a**. Note, an arrow indicates an astrocyte endplate around a blood vessel. **d** P2Y₄ receptor-is was found in the mesencephalic trigeminal nucleus (Mes5), locus coeruleus (LC), and posterodorsal tegmental nucleus (PDTg). Note that heavy P2Y₄ receptor-is was detected in large neurons of Mes5. **e** P2Y₄ receptor-is was found in the spinal trigeminal nucleus (sp5). **f** P2Y₄ receptor-is was found in the pontine reticular nucleus, caudal part (PnC). Scale bars=500 μ m in **a** and 100 μ m in **b**, **c**, **d**, **e**, and **f**

Pyramidal cells of hippocampal fields CA1–CA4 and the granule cells of the dentate gyrus showed moderate immunostaining for the P2Y₄ receptor (Fig. 6a, b, c). Neurons in the hilus of dentate gyrus were also labeled with heavy immunostaining, mainly confined to cell bodies and apical dendrites. Small glial-like cells with weak P2Y₄ receptor-is were also detected in the synaptic region and dendrite region of the dentate gyrus (Fig. 7c).

Epithalamus and thalamus

The medial habenular nucleus and lateral habenular nucleus showed weak immunostaining for P2Y₄ receptors in the cell body of neurons (Fig. 6a). Thalamic nuclei showed weak to moderate immunostaining for P2Y₄ receptors

(Fig. 6e, f, g, Table 1). P2Y₄ receptor immunostaining was confined to cell bodies.

Hypothalamus

Weak to heavy P2Y₄ receptor-is was detected throughout the hypothalamus (Table 1). Heavy immunostaining was detected in the magnocellular neuroendocrine neurons, including supraoptic nucleus and its retrochiasmatic part, paraventricular nuclei, accessory neurosecretory nuclei, and tuberomammillary nucleus (Fig. 7a, b, e, f). Scattered neurons with heavy P2Y₄ receptor-is were observed in the perifornical nucleus, dorsal medial area, and lateral area

(Fig. 7d). The ependymal cells of the third ventricle were also heavily immunostained (Fig. 7c). Moderate P2Y₄ receptor-is was detected in the preoptic area, anterior area, and arcuate nucleus (Fig. 7c).

Midbrain

Midbrain neurons of the substantia nigra showed moderate P2Y₄ receptor-is (Fig. 8a, b, c). Weak to moderate P2Y₄ receptor-is was detected in many regions of the midbrain, as shown in Table 1. In neurons of the red nucleus, scattered neurons heavily immunostained with P2Y₄ receptors were observed. Central gray neurons showed weak or no immunostaining.

Fig. 9 Distribution of P2Y₄ receptor-is in the hindbrain at the posterior level (a–d) and the cerebellum (e, f). **a** Low power image of immunostaining in the transverse plane. P2Y₄ receptor-is was found in the cuneate nucleus (*cu*), dorsal motor nucleus of vagus (*10*), hypoglossal nucleus (*12*), nucleus of the solitary tract (*sol*), spinal trigeminal nucleus, interpolar part (*sp5i*), lateral reticular nucleus (*LRT*), raphe obscurus nucleus (*ROB*), and parvocellular reticular nucleus (*PCRt*). **b, c, d** These show high magnification of the regions enclosed by rectangles *a1*, *a2*, and *a3* in **a**. **e** P2Y₄ receptor-is was found in Purkinje cells and Bergmann cells in the Purkinje cell layer. Weak immunostaining in the cell bodies of Purkinje cells was observed, such as indicated by an arrow in **f**, and heavy immunostaining in the cell body of Bergmann cells was observed, such as indicated by an arrowhead in **f**. *py* pyramidal tract. Scale bars=500 μ m in **a**; 200 μ m in **e**; and 100 μ m in **b, c, d**, and **f**

Hindbrain

P2Y₄ receptor-is was widely distributed throughout the rat hindbrain (Table 1). Heavy P2Y₄ receptor-is was detected in the pontine nuclei, mesencephalic trigeminal nucleus, motor trigeminal nucleus, ambiguous nucleus, inferior olive, hypoglossal nucleus, and dorsal motor vagus nucleus, (Fig. 8d, e, f). Moderate P2Y₄ receptor-is was also detected in many hindbrain regions (Fig. 9a, b, c, d; Table 1).

Cerebellum

Weak to moderate P2Y₄ receptor-is was detected in Purkinje cells. Moderate to heavy immunostaining was also detected in small cells with a long apical process, which appeared to be Bergmann astrocytes (Fig. 9e, f). No clear P2Y₄ receptor-is was observed in granule cells. Deep cerebellar nuclei exhibited moderate P2Y₄ receptor-is.

Spinal cord

Moderate to heavy P2Y₄ receptor-is was observed in neurons and neuropil of the spinal gray matter at the cervical, thoracic, lumbar, and sacral levels, as shown for the lumbar spinal cord (Fig. 10a, b, c, d). Immunostaining was evident in many

motoneurons in the ventral horn of the spinal cord (Fig. 10a, d). In the white matter, numerous small cells with weak to moderate P2Y₄ receptor-is were also detected (Fig. 10c).

The density of P2Y₄ receptor-is in the rat CNS was scored and summarized in Table 1.

Identification of the cell types expressing P2Y₄ receptors

In order to identify the type of cells expressing P2Y₄ receptor in the CNS, a double-labeling immunofluorescence technique was used. The results showed that the majority of the cells expressing P2Y₄ receptor-ir also expressed NeuN (a marker for neurons), as shown in Fig. 11a, b, c, d, implying that P2Y₄ receptors were mainly expressed in neurons in the CNS. The P2Y₄ receptor immunoreactive cells were found not to express Iba-1 (a microglial marker) or MBP (a marker for oligodendrocytes), as shown in the primary motor cortex and agranular insular cortex (Fig. 11e, f). In the external plexiform layer, glomerulus layer of olfactory bulb, cerebral cortex, especially molecular layer, hippocampus, cerebellum, and white matter of spinal cord, the glial-like cells expressing P2Y₄ receptor were found to express GFAP (a marker for astrocytes), as shown in the olfactory bulb and cerebellar cortex (Fig. 12a, b, c, d). The method of combining in situ hybridization and immunofluorescence also confirmed that GFAP immunoreactive astrocytes expressed P2Y₄ receptor mRNA, as shown in the optic chiasm (Fig. 12e, f).

Fig. 10 Distribution of P2Y₄ receptor-is (a–d) in the thoracic spinal cord. **a** Low power image of P2Y₄ receptor-is in the spinal cord. Note the moderate immunostaining in the gray matter (**a**, **b**, **d**). **b**, **c**, **d** These show high magnification of the areas enclosed by rectangles **a1**, **a2**, and **a3**. Scale bars=500 μ m in **a** and 100 μ m in **b**, **c**, and **d**

Fig. 11 Identification of cells expressing P2Y₄ receptors. **a** P2Y₄ receptor-is in the IV and V layers of the motor cortex. **b** NeuN-is in the same field of **a**. **c** Merged image of **a** and **b**. Note that the majority of the cells with P2Y₄ receptor-is was also labeled by NeuN antibody as indicated by an *arrow*, although some structures with P2Y₄ receptor-is around the blood vessel, such as indicated by an *arrowhead*, was not labeled by the NeuN antibody. **d** High magnification of the area enclosed by a rectangle in **c**. **e** P2Y₄ receptor and MBP-is in the piriform cortex. Note that no coexistence was observed. **f** P2Y₄ receptor and Iba-1-is in the piriform cortex. Note that no coexistence was observed. All the *scale bars*=100 μ m

Coexistence of P2Y₄ receptors with AVP, OT, orexin, and NR1

Heavy immunostaining of P2Y₄ receptors was detected in the magnocellular endocrine neurons of rat hypothalamus, which implies that AVP- and OT-expressing neurons may express P2Y₄ receptors. Double-labeling immunofluorescence technique was used to investigate this. The results showed that almost all AVP and OT-expressing neurons in the supraoptic nucleus, paraventricular nucleus, and accessory neurosecretory nucleus were also immunoreactive for P2Y₄ receptors, as shown for the supraoptic nucleus (Fig. 13a, b). Heavy P2Y₄ receptor immunostained neurons in the dorsal medial area, perifornical nucleus, and lateral area of the hypothalamus were found to express orexin A. All the orexin A immunoreactive neurons were seen to express P2Y₄ receptors (Fig. 13c, d). NR1-is was widely detected in the rat CNS, as shown in the primary motor cortex, supraoptic nucleus, and substantia nigra (Fig. 14b,

d, f). Double immunofluorescence showed that almost all the neurons expressing P2Y₄ receptors also expressed NR1, as shown in the regions mentioned above (Fig. 14a–f).

Discussion

This is the first extensive study undertaken to investigate the distribution of P2Y₄ receptor protein in the CNS using immunohistochemistry and in situ hybridization histochemistry. These data provide clear evidence for an extensive expression of P2Y₄ receptors in the CNS. The specificity of the antibody for the detection of the P2Y₄ receptor protein used in this study was confirmed by control experiments, including Western blot and experiments where the P2Y₄ antibodies were preabsorbed with its own peptide or omitting the primary antibody. No staining was observed, indicating that the specificity of the antibody used is very good. The fact that the neurons with P2Y₄ receptor immunostaining

Fig. 12 Identification of cells expressing P2Y₄ receptors. **a** P2Y₄ receptor-is in the olfactory bulb. **b** The merged image of P2Y₄ receptor (red) and GFAP (green)-is in the same field of **a**. Note that the majority of the cells with GFAP-is was also labeled by the P2Y₄ receptor antibody, such as indicated by an arrow. **c** P2Y₄ receptor-is in the cerebellar cortex. Note that weak to moderate P2Y₄ receptor-is was detected in Purkinje cell bodies. Around the Purkinje cells were many small cells with heavy P2Y₄ receptor-is were also detected, such as indicated by an arrow. **d** The merged image of P2Y₄ receptor (red) and GFAP (green)-is in the same field of **c**. Note that the majority of

the small glial-like cells with P2Y₄ receptor-ir was also labeled by the GFAP antibody, such as indicated by an arrow. **e** P2Y₄ receptor mRNA hybridization signals in the optic chiasm. Note that weak, but clear, P2Y₄ receptor mRNA hybridization signals were detected in some small cells, such as the three indicated by arrows. **f** Merged image of P2Y₄ receptor mRNA hybridization signals (red) and GFAP (green)-is in the same field of **e**. Note that the majority of the small glial-like cells with P2Y₄ receptor immunoreactive mRNA hybridization signals was also labeled by the GFAP antibody, such as the three indicated by arrows. All scale bars=100 μm

were also found to express P2Y₄ mRNA further confirmed the specificity of the antibody and also confirm the specificity of in situ hybridization histochemistry for P2Y₄ receptor mRNA.

Using double immunofluorescence labeling, and in situ hybridization and immunofluorescence, the present study showed that P2Y₄ receptors were expressed in astrocytes of some regions in the rat CNS, which was consistent with the earlier study of the cloning and distribution of P2Y₄ receptors in rat CNS [14]; cultured astrocytes expressing P2Y₄ receptors were established by the methods of RT-PCR

and calcium imaging [11] and astrocytes expressing P2Y₄ receptor protein was confirmed by immunofluorescence [23].

The distribution of P2Y₄ receptor expression within the CNS shown here considerably extends previous distribution studies, which reported P2Y₄ receptor protein or mRNA in several populations of neurons in different regions, including: rat spinal cord [24]; the ventricular/choroid plexus system [14]; CA1 and CA3 cells of rat hippocampus; embryonic rat cortex [9]; and human amygdala, caudate nucleus, cerebellum, cingulate gyrus, globus pallidus, hippocampus, hypothalamus, locus coeruleus, medial frontal

Fig. 13 Coexistences of **a** P2Y₄ receptors (red) and VP (green), and **b** P2Y₄ receptors and OT (green) in the SON, and **c**, **d** P2Y₄ receptors (red) and orexin A (green) in the dorsomedial area, perifornic nucleus and lateral area of the hypothalamus. **a1** P2Y₄ receptor-is. **a2** VP-is. **a** Merged image of **a1** and **a2**. Note that all the VP neurons were almost all labeled by the P2Y₄ receptor antibody, although some P2Y₄ receptor immunoreactive neurons in the dorsal part of the SON were not labeled by the VP antibody. **b1** P2Y₄ receptor-is. **b2** OT-is. **b** Merged image of **b1** and **b2**. Note that almost all the OT neurons were

labeled by the P2Y₄ receptor antibody, although some P2Y₄ receptor immunoreactive neurons in the ventral part of the SON were not labeled by the OT antibody. **c** P2Y₄ receptor-is. **d** Merged image of **c** and orexin A-is in the same field of **c**. Note that all the orexin A neurons were labeled by the P2Y₄ receptor antibody, although some P2Y₄ receptor immunoreactive neurons were not labeled by the orexin A antibody, such as indicated by a red arrow. A yellow arrow indicates a doubled-labeled neuron. Scale bars=333 μm in **a1**, **a2**, **b1**, and **b2** and 100 μm in **a**, **b**, **c**, and **d**

gyrus, medulla oblongata, nucleus accumbens, parahippocampal gyrus, putamen, striatum, substantia nigra, superior frontal gyrus, thalamus, and spinal cord [17].

The present study showed heavy P2Y₄ receptor-ir in the ependymal cells of the ventricles and the choroid plexus in the rat cerebrospinal fluid system by both immunohistochemistry and in situ hybridization for protein and mRNA. This result was consistent with a previous report, which showed that the highest levels of P2Y₄ receptor transcripts in adult rat brain sections was in the ventricular system [14]. The previous reports gave some clues about the possible functions of P2Y₄ receptors expressed at high levels in the ventricular system. It was reported that regulation of K⁺ secretion across strial marginal cell epithelium occurred via P2Y₄ receptors at the apical membrane [25]. It was also shown that P2Y₄ receptors fully mediate the chloride secretory response to UTP in both small and large intestines, except at the basolateral side of the jejunum, where both P2Y₂ and P2Y₄ receptors are involved [26]. It was suggested

that the physiological role of P2Y₄ receptors in Reissner's membrane was likely to regulate Na⁺ homeostasis in the endolymph [27]. Stimulation of P2Y₂ and P2Y₄ receptors led to K⁺ secretion [28], while UTP- and ATP-induced chloride secretory responses observed in wild-type mice were abolished in P2Y₄-null mice [29]. Collectively, these data clearly show that P2Y₄ receptors are involved in electrolyte balance of different tissues. Therefore, it is suggested that P2Y₄ receptors in the ventricular system may be involved in this function as well, though further experiments are needed to confirm this.

Extracellular ATP and P2 purinoceptors play very important roles in the hypothalamo-neurohypophysial system [30–32]. It was shown that ATP induced a rapid increase in intracellular Ca²⁺ concentrations in hypothalamic neurosecretory neurons [33]. ATP injected into the paraventricular nucleus stimulated the release of VP from the neurohypophysis via P2 receptors [34]. Evidence was also presented that multiple P2X receptors are expressed in neurosecretory

Fig. 14 Coexistence of P2Y₄ receptors (*red*) and NR1 (*green*) in the **a, b** motor cortex, **c, d** SON, and **e, f** substantia nigra (SN). **a, c, e** These are P2Y₄ receptor-is in the motor cortex, SON, and SN, respectively. **b, d, f** These are the merged images of P2Y₄ receptors (*red*) and NR1 (*green*)-is in the same field of **a, c**, and **e**, respectively. Note that most of the P2Y₄ receptor immunoreactive cells were also labeled by the NR1 antibody, although a minority of the neurons were only labeled by the NR1 antibody, such as those indicated by *arrows*. All scale bars=100 μ m

neurons [18, 20, 31, 35–38]. Earlier studies of supraoptic nucleus (SON) neurons suggested that a P2 receptor-mediated effect of ATP was an intermediate process in VP release evoked by central noradrenergic neurons [39]. ATP has been shown to excite the neurosecretory VP-containing neurons and the effects were prevented by the P2 receptor antagonist, suramin [39]. Purinergic and adrenergic agonists synergize in stimulating VP and OT release [40]. ATP induced both calcium influx and release of calcium from intracellular stores in SON neurons [41]. Calcium influx reflects activation of P2X receptors and voltage-gated calcium channels, while Ca^{2+} release reflects activation of P2Y receptors. The P2Y₁ receptor subtype is predominantly responsible for ATP-induced release of calcium from intracellular stores, as indicated by the ability of a P2Y₁ receptor-specific antagonist (MRS2179), to eliminate the

ATP-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular $[\text{Ca}^{2+}]_i$ [41]. Furthermore, the P2Y₁ receptor agonist, 2-methylthio-ADP induced large increases in $[\text{Ca}^{2+}]_i$ in all SON neurons tested, while UTP or UDP only induced small increases in $[\text{Ca}^{2+}]_i$ in a minority of SON neurons [41]. This data implies that P2Y₄ receptors in the VP and OT neurons play a role to limit the release of VP and OT from magnocellular neuroendocrine neurons of the rat hypothalamus. The fact that high level expression of P2Y₄ receptors was detected in those neuroendocrine neurons in the present study suggests that P2Y₄ receptors should have a role in regulating the functions of neuroendocrine neurons. Experiments are needed to clarify this issue.

Histaminergic neurons in the tuberomammillary nucleus (TMN) of the posterior hypothalamus send projections through the whole brain [42, 43] controlling arousal and

attention [44, 45]. They are tonically active during waking and this activity is suppressed during sleep [45, 46]. A homeostatic theory of sleep suggests that adenosine accumulation during waking increases sleep drive, while high ATP levels support waking [47]. Previous data shows that single TMN neurons express variable P2X receptors, and the major subtype involved (by pharmacological evidence) is the P2X₂ receptor [48]. P2Y₁ and P2Y₄ receptors, which are involved in physiological activities of neurons in the TMN of the posterior hypothalamus, were also observed [49]. In this study, heavy immunostaining for P2Y₄ receptors was detected in the TMN, which supports the possibility that P2Y₄ receptors play a role in the neuron activity of the TMN.

The lateral hypothalamus harbors a variety of functionally distinct neuron populations. One of these cell groups, the orexin neurons [50], has gained considerable attention because their loss seems to be a causative factor for narcolepsy [51]. Orexin has also been postulated to play a crucial role in energy homeostasis and feeding behavior [50, 52]. Previous data suggested that purinergic receptors may be involved in regulation of these orexin neurons. Orexin neurons in the dorsal medial area, perifornical nucleus, and lateral area of the rat hypothalamus were shown to express P2X₂ receptor mRNA and protein [53]. Pharmacological data also showed that orexin cells in the hypothalamus were excited directly by extracellular ATP, acting via P2X receptors, presumably P2X₂ receptors [54]. To our knowledge, there is no information about P2 receptors on orexin neurons in the hypothalamus. Therefore, the present study presents morphological evidence for the first time, raising the possibility that purines and pyrimidines act on orexin neurons via P2Y₄ receptors.

There are increasing reports that show that coupling of P2Y receptors to neuronal ion channels is an important way to regulate the functions of neurons [3, 55–57]. The coupling between P2Y receptors and ion channels, such as P2Y₁ and P2Y₂ and N-type Ca²⁺ and P2Y₆ and N-type Ca²⁺/M-type K⁺ have been demonstrated in sympathetic neurons [55]. The coupling of P2Y₄ and NR1 was also shown in cultured cerebellar granular neurons [57].

The present study showed that P2Y₄ receptors were expressed in the majority of neurons in the rat CNS, and almost all the P2Y₄ receptor immunoreactive neurons were also immunoreactive for NR1. Glutamate is an important excitatory neurotransmitter. Glutamate receptor activation during injury of the nervous system can provoke waves of release of the neurotransmitters glutamate, aspartate, and ATP that leads to further NR1 and P2 receptor activation [58, 59]. This results in intracellular Ca²⁺ overloading, free radical production, proteolytic damage, and apoptotic cascades. It has been suggested that there is a relationship between glutamatergic and purinergic signals in several

pathological states [9, 60, 61], establishing both the inhibitory action of P2 receptor antagonists on glutamatergic transmission [60, 62], and the release of glutamate mediated by ATP through P2Y receptors [63]. A hypothesis for more direct interactions between NR1 and P2Y receptors has been put forward, which is supported by electrophysiological studies [56, 64] and by the indirect demonstration that certain P2Y receptors can modulate the function of several ion channels through a PDZ domain at the C-terminal [65]. The coupling of P2Y₄ receptors and NR1 in rat CNS needs to be confirmed by functional studies.

In summary, this study provides details of the distribution pattern of the P2Y₄ receptor subtype in the adult rat CNS using P2Y₄ receptor-specific antisera and riboprobe-based in situ hybridization. The distribution of P2Y₄ receptor mRNA expression matches well that of P2Y₄ receptor protein. P2Y₄ receptor expressing neurons are distributed widely in the rat CNS. Heavy P2Y₄ receptor immunostaining was observed in the magnocellular neuroendocrine neurons of the hypothalamus, red nucleus, pontine nuclei, mesencephalic trigeminal nucleus, motor trigeminal nucleus, ambiguous nucleus, inferior olive, hypoglossal nucleus, and dorsal motor vagus nucleus. P2Y₄ receptor immunostaining was mainly confined to the cell body and dendrite of the neuron, implying that P2Y₄ receptors are mainly involved in regulating postsynaptic events. The cell types expressing P2Y₄ receptors are neurons and astrocytes. In the hypothalamus, heavy P2Y₄ receptor immunostaining was detected on magnocellular neuroendocrine neurons. All VP and OT neurons were immunoreactive for P2Y₄ receptors. All orexin A neurons were also immunoreactive for P2Y₄ receptors. All neurons expressing P2Y₄ receptors were found to express NR1. These data imply that purines and pyrimidines may be involved in regulation of the release of the neuropeptides VP, OT, and orexin in rat hypothalamus via P2Y₄ receptors, and the physiological and pathophysiological functions of neurons via coupling between P2Y₄ receptors and NR1.

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