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ORIGINAL ARTICLE

Using antibodies against P2Y and P2X receptors in purinergic signaling research

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Abstract The broad expression pattern of the G protein-coupled P2Y receptors has demonstrated that these receptors are fundamental determinants in many physiological responses, including neuromodulation, vasodilation, inflammation, and cell migration. P2Y receptors couple either G_q or G_i upon activation, thereby activating different signaling pathways. Ionotropic ATP (P2X) receptors bind extracellular nucleotides, a signal which is transduced within the P2X protein complex into a cation channel opening, which usually leads to intracellular calcium concentration elevation. As such, this family of proteins initiates or shapes several cellular processes including synaptic transmission, gene expression, proliferation, migration, and apoptosis. The ever-growing range of applications for antibodies in the last 30 years attests to their major role in medicine and biological research. Antibodies have been used as therapeutic tools in cancer and inflammatory diseases, as diagnostic reagents (flow cytometry, ELISA, and immunohistochemistry, to name a few applications), and in widespread use in biological research, including Western blot, immunoprecipitation, and ELISPOT. In this article, we will showcase several of the advances that scientists around the world have achieved using the line of antibodies developed at Alomone Labs for P2Y and P2X receptors.

Keywords Purinergic signaling · Purinoceptors · P2Y receptors · Antibodies · G protein-coupled receptors · P2X receptors · Ion channels

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P2Y receptors

P2Y receptors can be divided into two main subgroups: the $P2Y_1$ subfamily which comprises $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptor subtypes. They all couple to G_q, thereby activating phospholipase C. They are all activated by various nucleotides, although $P2Y_{11}$ is the sole receptor specifically activated by ATP [1]. Various cellular functions are attributed to these receptors in various tissues and organs such as the brain, autonomic nervous system, epithelia of the airway, gastrointestinal (GI) tract, kidney, eye, vascular tissue, and the immune and inflammatory cells [1]. The second group, the $P2Y_{12}$ subfamily, includes the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, which share little homology with members of the $P2Y_1$ subfamily and are generally known to couple G_i. $P2Y_{12}$ and $P2Y_{13}$ are mostly activated by ADP, while $P2Y_{14}$ is activated by UDP-glucose and other nucleotide sugars. Signaling via the latter is relatively in its early stage of understanding [1]. The $P2Y_{12}$ receptor has an important role in platelets, is highly expressed in the brain, and mediates microglia migration in response to inflammation. $P2Y_{13}$ is also expressed in the brain as well as in the spleen. $P2Y_{14}$ is highly expressed in the brain, placenta, neutrophils,

lymphocytes, epithelial cells from various tissues, and in the GI smooth muscle [1]. As new roles for these receptors are continuously being unraveled and being that most pharmacological tools available to study these receptors are far from specific and mutually affect various receptors, the use of specific antibodies has become essential to study them. Alomone Labs offers specific polyclonal antibodies targeted against all P2Y receptors, enabling the end user to specifically detect the identity of the receptor in question. We would like to emphasize that the use of antibodies is by no means a replacement, but should be used in concert with the pharmacological tools available. This short communication illustrates the purpose and the many advantages using antibodies, specifically Alomone Labs antibodies, have in researching purinergic signaling.

P2Y receptors in the CNS

Although extracellular purines may have both trophic and apoptotic functions in the brain depending on the targeted purine receptor and cell type, little is known about the role of specific purine receptors in neurons [2]. It was shown that both ADP and its stable analogue 2-methylthio-ADP (2MeSADP) induced the upregulation of the cytoprotective protein heme oxygenase-1 in rat cerebellar granule neurons. Selective inhibition of 2MeSADP-responsive receptors $P2Y_1$ and $P2Y_{13}$ and the use of pertussis toxin demonstrated a role of the purinergic $P2Y_{13}$ receptor in this response. Western blot analysis using anti-$P2Y_1$ receptor (#APR-017) and anti-$P2Y_{13}$ receptor (#APR-017) antibodies confirmed those results. These observations reveal a previously unrecognized role in protection against oxidative stress by extracellular purines acting on the metabotropic $P2Y_{13}$ receptor and provide new perspectives for neuroprotective therapies.

Microglial cells are the primary immune effector cells in the brain. Extracellular ATP, e.g., released after brain injury, may initiate microglial activation via the stimulation of purinergic receptors. Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease characterized by the selective loss of lower and upper motoneurons. The pathology is imputable in ~2% of cases to mutations in the ubiquitous enzyme Cu,Zn superoxide dismutase (SOD1). Common theories to explain the pathogenic mechanisms of ALS include the activation of the microglia, responsible for the release of pro-inflammatory factors. The effect of mutant SOD1 on P2 receptor-mediated pro-inflammatory microglial properties was investigated [3]. Primary and immortalized microglial cells from mutant SOD1 mice were used to explore several aspects of activation by purinergic ligands and to analyze the overall effect of such stimulation on the viability of NSC-34 and SH-SY5Y neuronal cell lines. The upregulation of $P2Y_6$ receptors (without changes in $P2Y_2$ and $P2Y_{12}$ receptors) and the downregulation of ATP-hydrolyzing activities in mutant SOD1 microglia were observed using anti-$P2Y_6$ receptor (#APR-006), anti-$P2Y_2$ receptor (#APR-010), and anti-$P2Y_{12}$ receptor antibodies in Western blot analysis. The purinergic activation of the microglia may thus constitute a new route involved in the progression of ALS to be exploited to potentially halt the disease.

The uridine nucleotide-activated $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are widely expressed in the brain and are involved in many CNS processes, including those which malfunction in Alzheimer's disease (AD). However, the status of these receptors in the AD neocortex, as well as their putative roles in the pathogenesis of neuritic plaques and neurofibrillary tangles, remain unclear. In this study, Lai et al. [4] used immunoblotting and immunohistochemistry using anti-$P2Y_2$ receptor, anti-$P2Y_4$ receptor (#APR-006), and anti-$P2Y_6$ receptor antibodies in order to measure $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors, respectively, in two regions of the postmortem neocortex of AD patients and aged controls. $P2Y_2$ immunoreactivity was found to be selectively reduced in the AD parietal cortex, while $P2Y_4$ and $P2Y_6$ levels were unchanged. In contrast, all three receptors were preserved in the occipital cortex, which is known to be minimally affected by AD neuropathology. Furthermore, reductions in parietal $P2Y_2$ immunoreactivity correlated both with neuropathologic scores and markers of synapse loss. These results provide a basis for considering $P2Y_2$ receptor changes as a neurochemical substrate of AD and point toward uridine nucleotide-activated P2Y receptors as novel targets for disease-modifying AD pharmacotherapeutic strategies.

In the CNS, P2 receptors are identified in neurons and glial cells, mediating neuron–neuron, glia–glia, and glia–neuron communication. Amadio et al. [5] qualified in vivo in the adult rat CNS the cellular/subcellular distribution of $P2Y_{12}$ receptor in the cerebral cortex, white matter, and subcortical nuclei (striatum and substantia nigra) by means of electron microscopy, immunofluorescence, confocal, and Western blot analysis using anti-$P2Y_{12}$ receptor (#APR-012) antibody. $P2Y_{12}$ receptor immunoreactivity co-localized only with myelin basic protein and the oligodendrocyte marker RIP in both cell bodies and processes, indicating therefore oligodendrocyte localization. Since in platelets the $P2Y_{12}$ receptor is known to regulate adhesion/activation and thrombus growth/stability, results from the study presented helped speculate by analogy that, in oligodendrocytes, $P2Y_{12}$ receptor signaling might contribute to the migration and adhesion of the glial processes to axons to be myelinated.

Apolloni et al. [6] established the direct toxic role and anticancer activity of UDP in a neuroblastoma cell line SH-

SY5Y and identified the $P2Y_6$ receptor as a novel potential target in anti-tumoral therapies. This work investigated the role of the $P2Y_6$ receptor using anti-$P2Y_6$ receptor antibody in Western blot analysis and immunofluorescence microscopy. The in vitro results contribute to expand the general knowledge about the $P2Y_6$ receptor and to possibly identify its manipulation as a strategy for the understanding and potential relapse of neuroblastoma.

P2Y receptors in the spinal cord

Under normal conditions, pain is associated with electrical activity in small-diameter fibers of dorsal root ganglions (DRG) of the spinal cord. All subtypes of the P2Y receptor family are widely expressed in different parts of the nervous system. The activation of P2Y receptors causes blockade of the N-type calcium channels in DRG cells. This effect may decrease the release of glutamate from DRG terminals in the spinal cord and thereby partly counterbalance the algogenic effect of ATP released from spinal cord nerve terminals upon depolarization and probably from damaged or stressed cells upon pathological conditions [7].

The involvement of P2Y receptors in the modulation of rat spinal neurotransmitters glutamate and noradrenaline release was investigated [7]. The study showed that the inhibitory effect of ATP on glutamate release was mediated by $P2Y_{13}$ and/or by $P2Y_1$ receptors, whereas the inhibition of noradrenaline release was most likely mediated by $P2Y_{13}$ and/or by $P2Y_1$ and $P2Y_{12}$ receptors. In order to explore the cell type-specific distribution of the $P2Y_1$ receptor and to determine whether $P2Y_1$ receptors, involved in the modulation of glutamate release, are expressed on primary afferent nerve terminals or interneurons, immunohistochemical experiments were performed using anti-$P2Y_1$ receptor antibody. Thus, the results indicated that sensory information processing and its modulation by the descending noradrenergic pathway in the spinal cord could be targeted by distinct subtypes of P2Y receptors, providing novel sites of action for potential analgesic compounds.

Microglia in the spinal cord may play an important role in the development and maintenance of neuropathic pain. In spinal microglia, $P2Y_{12}$ is expressed constitutively and is involved in chemotaxis. The activation of p38 mitogen-activated protein kinase (MAPK) occurs in spinal microglia after nerve injury and may be related to the production of cytokines and other mediators, resulting in neuropathic pain. Anti-$P2Y_{12}$ receptor antibody in immunohistochemical analysis of the dorsal horn cells 7 days after nerve injury was used in order to demonstrate that the cells expressing increased $P2Y_{12}$ protein were exclusively microglia [8]. In addition, the data suggested a new mechanism of neuropathic pain in which the increased $P2Y_{12}$ expression works as a gateway in microglia following nerve injury. The activation of this receptor by the released ATP activates the p38 MAPK pathway and thus may play a crucial role in the generation of neuropathic pain.

In another study, the expression and function in nociception of $P2Y_1$ and $P2Y_{12}$ using their respective Alomone Labs antibodies via immunohistochemistry analysis was examined in sensory neurons [9]. The results demonstrated that nociception sensitivity is modulated by the integration and balance of nucleotide signaling through G_q- and G_i-coupled P2Y receptors which are altered in response to inflammatory injury. G_i-coupled P2Y receptors are broadly expressed in peripheral sensory neurons, inhibit nociceptive signaling, and reduce behavioral hyperalgesia in vivo and thus represent potential targets for the development of novel analgesic drugs. On the other hand, $P2Y_1$, a G_q-coupled ADP receptor, is required for the full expression of inflammatory hyperalgesia.

P2Y receptors in the ear

Purinergic signaling is inherently important to the hearing organ and involves signal transduction via metabotropic G protein-coupled P2Y receptors. Many of the principal elements of the purinergic signaling complex have been well characterized in the cochlea, forming the basis of paracrine and autocrine communication systems in this organ. The results presented below reinforce the significance of extracellular nucleotide and nucleoside signaling to hearing.

Huang et al. [10] used anti-$P2Y_2$ receptor, anti-$P2Y_4$ receptor, anti-$P2Y_6$ receptor, and anti-$P2Y_{12}$ receptor antibodies in order to evaluate their expression during development of the rat cochlea using immunohistochemistry analysis. The key findings of this study involved the dynamic interplay between the expressions of the closely related P2Y receptors mentioned above within the highly differentiated cochlear tissue. None of the receptors exhibited significant expression at the embryonic period as P2Y receptor expression is pronounced in the developing CNS. In the cochlea, neuronal P2Y receptor expression did not commence until day 18 of the embryonic period. During maturation of the hearing organ, P2Y receptor expression became increasingly compartmentalized even to a subcellular level in the case of the sensory hair cells and some supporting cells.

The distribution of NTPDase5 and NTPDase6, two intracellular members of the membrane-bound ectonucleoside triphosphate diphosphohydrolases (E-NTPDase) family, in the inner ear was investigated [11]. Their expression was linked to the regulation of P2 receptor signaling in the adult rat cochlea. By using anti-$P2Y_6$ receptor and anti-$P2Y_{14}$

receptor (#APR-018) antibodies in immunofluorescence studies, it was found that noise-induced upregulation of $P2Y_6$ receptors in the spiral ganglion neurons further supports the involvement of NTPDase5 in regulating P2Y receptor signaling. Noise stress also induced $P2Y_{14}$ receptor expression in the root processes of the outer sulcus cells, but this was not associated with the localization of the E-NTPDases.

P2Y receptors in the eye

In the postnatal and mature retina, many processes are controlled by the action of nucleotides. Their effects are partly mediated via the activation of metabotropic P2Y receptors. However, little is known about the developmental regulation and cellular localization of P2Y receptor subtypes in the retina.

Immunohistochemical and neurophysiological methods [12] demonstrated that purinergic signaling via $P2Y_1$ and $P2Y_4$ receptors might contribute to differentiation processes in the postnatal retina. Immunostaining adult rat retina for $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors showed that $P2Y_4$ expression overlapped with the Müller cell marker, cellular retinaldehyde-binding protein (CRALBP; Fig. 1). $P2Y_4$ was expressed predominantly in Müller cell end-feet (Fig. 1b). Punctuate structures, forming a small band in the inner plexiform layer (IPL), potentially representing synapses, showed an intense staining for $P2Y_4$, too. $P2Y_2$ (Fig. 1a) and $P2Y_6$ (Fig. 1c) expression was largely restricted to retinal neurons such as ganglion cells and cells of the inner nuclear layer (INL). Immunohistochemical staining using anti-$P2Y_4$ receptor antibody showed indisputable expression of $P2Y_4$, detectable only after the third postnatal week (P20).

Intense neuronal activity in the sensory retina is associated with a volume increase of neuronal cells and a decrease in the osmolarity of the extracellular space fluid. Wurm et al. [13] further showed the existence of an endogenous purinergic mechanism that prevented hypo-osmotic swelling of retinal glial (Müller) cells in mice. They suggested that autocrine/paracrine activation of purinergic receptors via UTP and enzymes is crucially involved in the regulation of the glial cell volume. By using anti-$P2Y_4$ receptor antibody in immunohistochemistry experiments, they showed that $P2Y_4$ immunolabeling was localized to the somata of retinal neurons, but not glial cells, while in other retinal layers, UTP may be more directly involved in the regulation of glial cell volume, as indicated by the co-localization of $P2Y_4$ labeling and CRALBP (Fig. 1d).

The expression of various P2Y receptor subtypes has been demonstrated in the retina, but the localization of P2Y receptors and their role in retinal signaling remain unclear. The major findings of the study conducted by Ward et al. [14] are that $P2Y_4$ receptors are heavily expressed within the inner retina, particularly within the dendrites and axon terminals of rod bipolar cells and in cells postsynaptic to cone bipolar cells, as demonstrated in Western blot and immunocytochemistry methods using anti-$P2Y_4$ receptor antibody. Moreover, altering neural activity with light altered $P2Y_4$ expression within rod bipolar cell axon terminals, and injection of the agonist UTP caused a substantial reduction in the amplitude of the rod and cone post-receptoral response (PII), indicating further evidence for purines in retinal processing.

Detachment of the neural retina from the pigment epithelium causes, in addition to photoreceptor deconstruction and neuronal cell remodeling, an activation of glial cells. It has been suggested that gliosis contributes to the impaired recovery of vision after reattachment surgery that may involve both formerly detached and non-detached retinal areas. Müller and microglial cell reactivity was monitored in a porcine model of rhegmatogenous retinal detachment to determine whether gliosis is present in detached and non-detached retinal areas. The results demonstrated that distinct symptoms of Müller cell gliosis were not only restricted to the detached retinal areas but occurred also in non-detached retinal regions of the surgical eyes. Furthermore, among physiological changes, the immunoreactivities for $P2Y_1$ (Fig. 2, left panel) and $P2Y_2$ (Fig. 2, middle panel), but not $P2Y_4$ (Fig. 2, right panel) receptor proteins, apparently increased only in detached areas [15].

Extracellular ATP has been shown to mobilize intracellular calcium in cultured ovine lens epithelial cells and in human lens epithelium, suggesting a role for purines in the modulation of lens transparency. The expression profiles of P2Y receptor isoforms were characterized throughout the rat lens at both the molecular and the functional levels [16]. The epithelium does not express $P2Y_1$ and $P2Y_2$, but the underlying fiber cells, which differentiate from the epithelial cells, exhibit strong membranous labeling. $P2Y_1$ expression extended deeper into the lens compared with $P2Y_2$, and its expression co-localized with Cx50 gap junction plaques, while $P2Y_2$ did not. Immunohistochemistry using isoform-specific anti-$P2Y_4$ receptor and anti-$P2Y_6$ receptor antibodies indicated that $P2Y_4$ and $P2Y_6$ receptors were observed in both epithelial cells and fiber cells, but the labeling was predominantly cytoplasmic in nature. The inability of UTP and UDP to mobilize intracellular calcium in the epithelium and the predominantly cytoplasmic location of $P2Y_4$ and $P2Y_6$ suggest that these receptors may represent an inactive pool of receptors that may be activated under non-physiological conditions. In contrast, their results indicated that $P2Y_1$ and $P2Y_2$ are functionally active in fiber

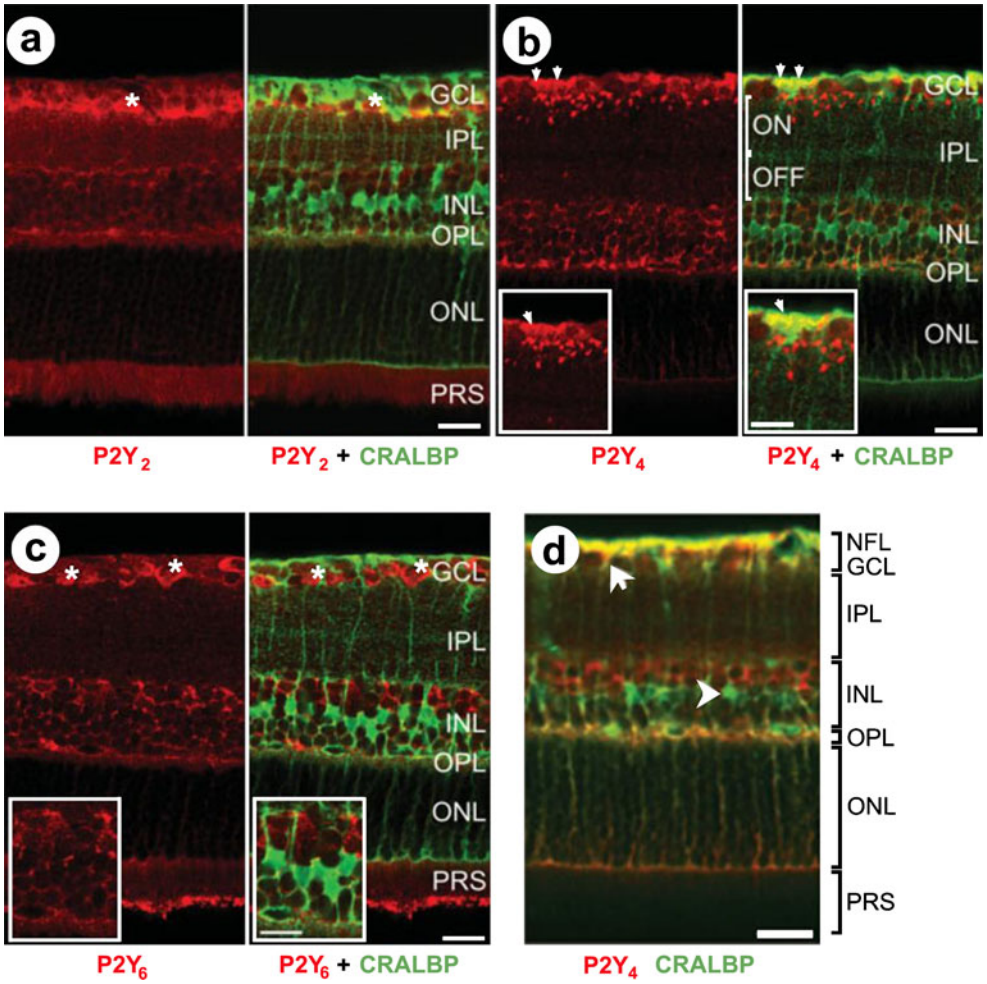

Fig. 1 Expression of P2Y receptors in Müller cells of the adult rat and mouse retinal slices. Immunohistochemical staining was accomplished using anti-$P2Y_2$ receptor (#APR-010) and anti-$P2Y_6$ receptor (#APR-011) antibodies. **a** No distinct immunoreactivity for the $P2Y_2$ receptor (*red*) was observed in Müller cells. However, ganglion cells (*asterisk*) and structures within the inner plexiform layer (*IPL*), inner nuclear layer (*INL*), and outer plexiform layer (*OPL*) display faint staining. **b** $P2Y_4$ receptor (*red*) is highly expressed in Müller cell end-feet (*arrows*), as detected using anti-$P2Y_4$ receptor antibody (#APR-006). Additionally, a band of globular structures, probably representing synapse of bipolar cells in the ON-sublamina (*ON*) of the IPL, is stained for this receptor. **c** The staining pattern of the $P2Y_6$ receptor (*red*) is similar to that of $P2Y_2$. The insets in (**b**) and (**c**) show the ganglion cell layer (*GCL*) and the INL, respectively, at higher magnification. *ONL* outer nuclear layer, *OFF* OFF-sublamina of the IPL, *PRS* photoreceptor segments. *Scale bars*, 20 μm; *inset scale bars*, 10 μm. **d** Immunohistochemical staining was carried out using anti-$P2Y_4$ receptor antibody. The slices were co-stained against the glial cell marker cellular retinaldehyde-binding protein (*CRALBP*); co-labeling yielded a yellow-orange merge signal. *Arrows*, Müller cell end-feet; *arrowheads*, Müller cell somata. *GCL* ganglion cell layer, *IPL* inner plexiform layer, *NFL* nerve fiber layer, *ONL* outer nuclear layer, *PRS*, photoreceptor segments. *Scale bars*, 20 μm. **a**–**c** Adapted from reference 12 with permission of John Wiley and Sons. **d** Adapted from reference 13 with permission of Wiley-Blackwell

cells and that their differential subcellular expression patterns suggest they may regulate distinct processes in the lens under steady-state conditions.

P2Y receptors in the heart, cardiovascular and vascular systems

Purinergic signaling has been shown to be important in the cardiac and cardiovascular systems [17]. Presented are a number of studies examining the functions of these receptors in these vital systems.

Cardiac fibroblasts are a major cell type in the heart. They have an important role in the functional and structural aspects of the organ by secreting growth factors, cytokines, and components of the extracellular matrix. During myocardial infarction, ATP and ADP are released [18]. In addition, various P2Y subtypes have been detected in rat cardiac fibroblasts [19], and various studies indicate that these receptors may play a role in cardiac function and may be involved in the pathological processes of cardiac diseases [20]. Cardiac fibroblasts are differentiated into myofibroblasts which seem to play an important role in myocardial infarction [21]. The differentiation of cardiac

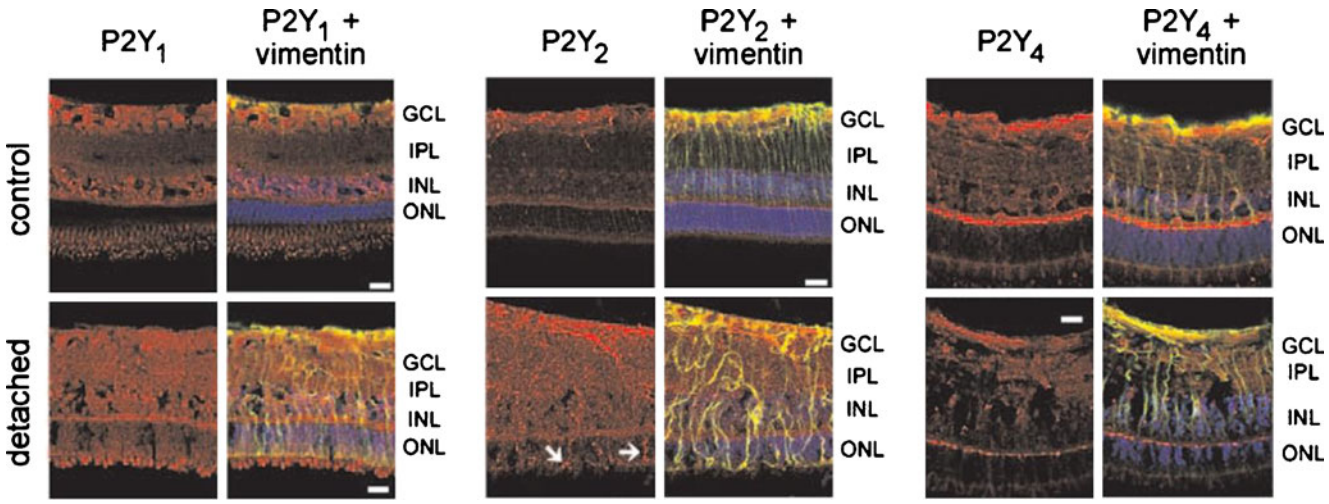

Fig. 2 Differential expression of P2Y receptors in glial following retinal detachment. Immunohistochemical staining of control retinas and from detached areas of porcine retinas 7 days after surgery was done using anti-$P2Y_1$ receptor (#APR-009), anti-$P2Y_2$ receptor (#APR-010), and anti-$P2Y_4$ receptor (#APR-006) antibodies (*red*). Immunoreactivities for $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptor proteins (*red*) coincide with that of intermediate filament constituents vimentin (*green* and *yellow* for merging signal). *Arrows*, vimentin-positive fibers in the outer retina that were also stained for $P2Y_2$ protein. *GCL* ganglion cell layer, *INL* inner nuclear layer, *IPL* inner plexiform layer, *NFL* nerve fiber layer, *ONL* outer nuclear layer. *Scale bars*, 20 μm. Adapted from reference 15 with permission of Association for Research in Vision and Ophthalmology

fibroblast to myofibroblasts leads to the expression of discoidin domain receptor 2 and α-smooth muscle actin. Therefore, this cell type can be distinguished in part by the expression of these proteins. Using a panel of Alomone Labs anti-P2Y receptor antibodies, it was demonstrated that $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{13}$ are all expressed by myofibroblasts and that all except for $P2Y_{13}$ are also functional, as demonstrated by the use of pharmacological agents [20]. Overall, the study shows that the expression of different P2Y receptors which are stimulated by different agonists suggests a complex physiological role for these nucleotide receptors in regulating cardiac myofibroblast function in heart disease.

In the blood, integrins play an important role in mediating the interaction between monocytes and epithelial cells [22, 23]. In addition, integrins can also interact with other proteins, leading to the activation of various signaling pathways, many of which have an impact on many cellular events [24]. The $\alpha_V\beta_3$ integrin influences cell signaling via a plethora of receptors, including $P2Y_2$ [25–27]. However, the biological outcome of the interaction between $P2Y_2$ and $\alpha_V\beta_3$ integrin is not understood. The activation of $P2Y_2$ receptors which are endogenously expressed in U037 cells (monocytes) caused clustering of α_V integrins, thereby activating a signaling cascade involving the ERK1/2 MAPK pathway and cell migration [24]. The downregulation of $P2Y_2$ expression via shRNA abolished α_V integrin expression and concomitantly downstream signaling, as demonstrated by Western blot analysis which showed that both $P2Y_2$ and α_V integrin expression were downregulated following treatment with their respective shRNA, but that α_V integrin shRNA had no effect on the expression of $P2Y_2$ expression. The ability of $P2Y_2$ to activate cell motility via integrin activation may have important implications in inflammatory processes and the treatment of inflammation per se [24].

P2Y receptors in the gastrointestinal tract and its surroundings

Purinoceptors play an important role in the regulation of the GI tract motility. Reports show that P2 receptors modulate relaxation of the GI tract [28], while others demonstrate that they participate in the contraction [29]. These contradicting results suggest that the contracting/relaxing activities of purinergic receptors may be developmentally regulated. Different approaches were used in order to verify this hypothesis. Alomone Labs anti-$P2Y_1$, $P2Y_2$, $P2Y_4$ receptor antibodies were used to show that mostly $P2Y_1$ and $P2Y_2$ receptors are expressed, and the use of pharmacological tools showed that $P2Y_1$ mediates the relaxant response in the GI tract from the first week after birth, whereas the contractile activities observed shortly after birth decrease. The transition from contraction to relaxation properties of $P2Y_1$ may be related to the cells which express the receptors or to a change in the coupling to G proteins [28].

The nature of purinocetors was studied in the rat distal colon since the neurotransmission input is usually non-adrenergic and non-cholinergic [30]. Using Alomone Labs anti-P2Y receptor antibodies in immunocytochemical studies showed that various P2Y receptors are expressed in different

regions of the distal colon (Fig. 3). Immunostaining was specific since the pre-incubation of each antibody with its respective peptide antigen abolished all immunoreactivity. $P2Y_1$ was co-localized with α-smooth actin, indicating that it is expressed in vascular and enteric smooth muscle cells (Fig. 3a). It also displayed a muscular localization, although its expression did not overlap that of S-100. $P2Y_2$ was detected in myenteric neurons (Fig. 3b), $P2Y_4$ was detected in enteric glial cells (Fig. 3c), $P2Y_6$ was shown to be expressed in vascular and enteric smooth muscle cells (Fig. 3d), $P2Y_{11}$ in enteric glial cells (Fig. 3e), and $P2Y_{12}$ was detected in enteric nerves (Fig. 3f). The study further showed that more than one P2 receptor subtype is involved in purinergic relaxation of the circular muscle of the rat distal colon.

The duodenum secretes HCO_3^- which is in part responsible for protecting the upper GI tract from gastric acid. Intestinal alkaline phosphatase (IAP) expressed along the brush border has been shown to work at alkaline pH and has an ATPase activity, although its exact function is unknown. What is known, however, is that increased secretion of HCO_3^- by extracellular ATP enhances the enzyme's activity, suggesting a negative feedback loop. The authors show that ATP is indeed a substrate for IAP activity [31]. In addition, the inhibition of IAP leads to the release of ATP, which activates purinergic receptors in the

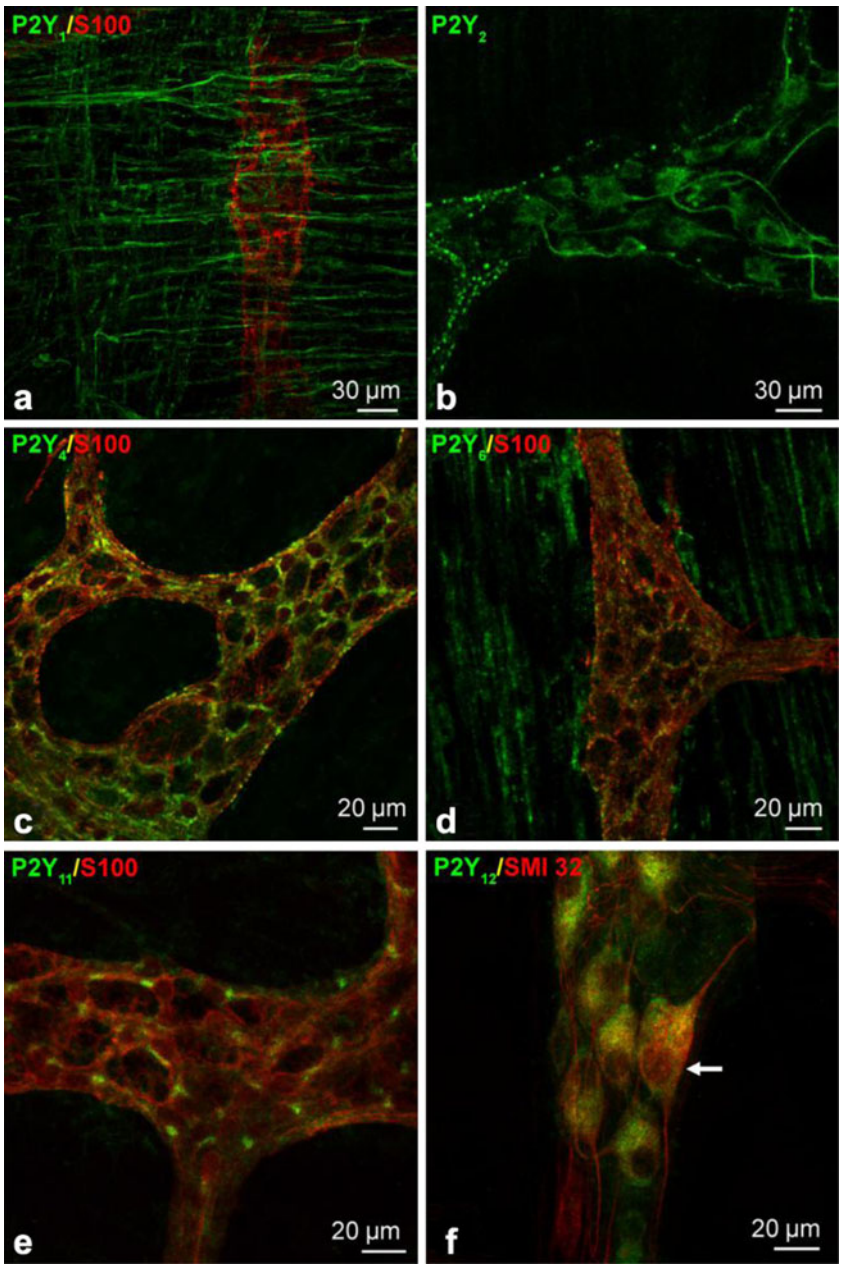

Fig. 3 Expression of P2Y receptors in rat distal colon. All P2Y immunohistochemical staining was done using Alomone Labs anti-P2Y receptor antibodies. **a** Double immunolabeling reveals that $P2Y_1$ is expressed in enteric smooth muscle cells and S100 in enteric glial cells. **b** In the myenteric plexus, $P2Y_2$ is detected in neurons. **c** $P2Y_4$ is detected in enteric glial cells. **d** $P2Y_6$ is expressed in vascular and enteric smooth muscle cells. **e** $P2Y_{11}$ is detected in enteric glial cells and $P2Y_{12}$ is expressed in enteric nerves (**f**). Adapted from reference 30 with permission of Elsevier

duodenum and, subsequently, the secretion of HCO_3^-, which in turn activates IAP, thereby decreasing ATP concentrations and P2Y signaling. Immunofluorescence studies using anti-$P2Y_1$ receptor, anti-$P2Y_2$ receptor, anti-$P2Y_4$ receptor, and anti-$P2Y_6$ receptor antibodies showed that $P2Y_1$ receptors are expressed on the brush border membranes of the duodenum (Fig. 4a). $P2Y_2$ and $P2Y_4$ expression were not observed (Fig. 4b, c), while that of $P2Y_6$ had a diffuse pattern of expression (Fig. 4d). The receptors were also pharmacologically characterized such that the activation of $P2Y_1$ with ATP triggered an increase in HCO_3^- secretion, which was reduced by the addition of $P2Y_1$ antagonists.

P2Y receptors in other tissues and cells

Clara-like cells are epithelial cells present in the intra pulmonary airways of various animal species and reside near pulmonary neuroepithelial bodies (NEBs). They demonstrate pluripotent stem cell-like features and are mainly involved in airway epithelial regeneration following lung injury [32–34]. NEBs are clusters of pulmonary neuroendocrine cells and have been suggested to have a role in various physiological processes in the lungs, with a significant role in airway oxygen sensing [35, 36]. Purinergic signaling in NEBs has only been indirectly demonstrated by ATP accumulation in secretory vesicles of rat NEBs [37]. In order to understand the possible cross talk between NEBs and Clara-like cells, the hypothesis that ATP acts as a paracrine transmitter in the NEB microenvironment was tested. Calcium imaging, pharmacology, and immunohistochemistry (using Alomone Labs antibodies) were all employed to identify and localize purinoceptors [33]. Clara-like cells were shown to express functional $P2Y_2$ receptors. The study further shows that the two cell types are indeed coupled by the release of ATP by NEB cells and the subsequent activation of purinoceptors ($P2Y_2$) localized on the membranes of Clara-like cells. This signaling pathway may be essential for normal airway function, for the regeneration of airway epithelial following injury, and also perhaps for the pathogenesis of small cell carcinomas [33].

P2Y receptors in disease states

Hepatocytes express receptors for high-density lipoprotein (HDL) which are responsible for carrying cholesterol from peripheral tissues to the liver for elimination. Studies show that HDL levels are inversely proportional to the risk of developing coronary artery disease [38]. In hepatocytes, HDL undergoes endocytosis in a $P2Y_{13}$-dependent manner [38–40]. The signaling pathway leading from $P2Y_{13}$ activation to the internalization of HDL was examined. The study shows that $P2Y_{13}$ stimulation specifically induced RhoA activation and its effector ROCK I, leading to HDL endocytosis through cytoskeleton reorganization. $P2Y_{13}$ silencing via shRNA, which was demonstrated by both a decrease in mRNA levels and protein levels (as shown by Western blot analysis using anti-$P2Y_{13}$ receptor antibody), prevented the membrane translocation of ROCK I induced by known $P2Y_{13}$ agonists. Deciphering new molecular targets involved in HDL endocytosis may be important for

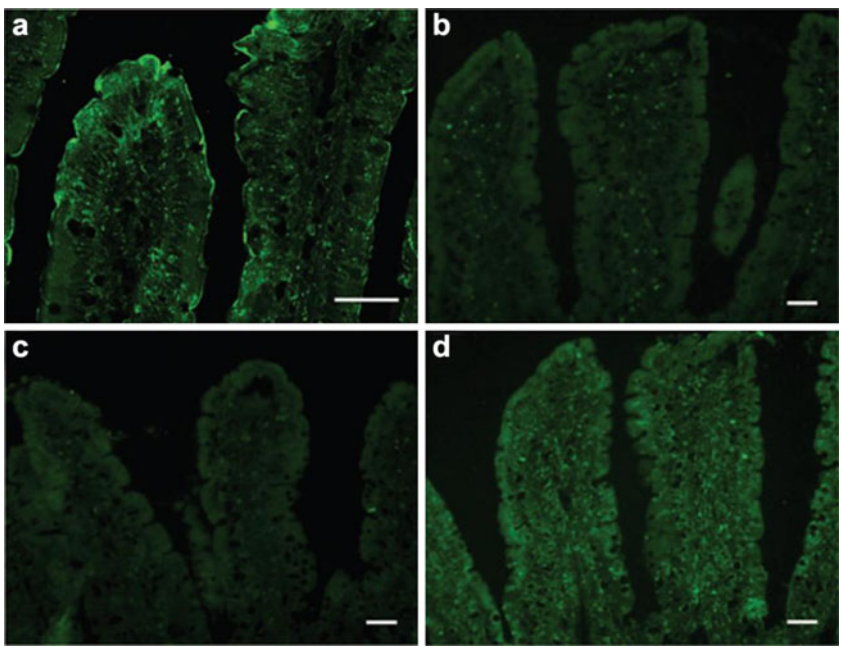

Fig. 4 Expression of P2Y receptors in rat duodenal epithelium. Immunohistochemical staining was done using anti-$P2Y_1$ receptor antibody (#APR-009), anti-$P2Y_2$ receptor antibody (#APR-010), and anti-$P2Y_6$ receptor antibody (#APR-011). **a** $P2Y_1$ is expressed along the brush border membranes of villous cells. $P2Y_2$ and $P2Y_4$ receptors are not expressed (**b**, **c**). **d** $P2Y_6$ receptor is diffusely expressed in the villous cells and interstitium. Adapted from reference 31 with permission of John Wiley and Sons

the development of drugs in order to enhance the reverse cholesterol transport [38].

Low-intensity pulsed ultrasound (LIPUS) is a noninvasive technique used to stimulate the healing process of bone fractures. The only known molecular event involved in LIPUS treatment is the release of purines from osteoblasts. P2 receptors were pharmacologically detected in osteoblasts. Activation was monitored by calcium mobilization which was abolished upon the addition of inhibitors of endoplasmic reticulum calcium release. Although all P2Y receptor subtypes tested were detected in indirect flow cytometry experiments using their respective Alomone Labs antibodies (Fig. 5), $P2Y_1$ seems to have a prominent role upon LIPUS treatment as inhibitors of this receptor abolished the proliferation of osteoblasts [41].

Melanoma is a tumor originating from melanocyte. These tumors express $P2Y_1$ and $P2Y_2$ receptors, among others [42]. Immunocompromised mice were injected with melanoma cells, and the role of purinergic agonists on tumor size and development was examined. Following ATP administration, the tumors had a necrotic appearance. Immunohistochemistry studies using Alomone Labs antibodies showed that the expression of $P2Y_1$ increased in the areas adjacent to the necrotic region, strongly suggesting that ATP can be used as a treatment for melanoma.

Achondroplasia is a pathology causing dwarfism. It is caused by a mutation in the fibroblast growth factor receptor 3 (FGFR3) gene, leading to the constitutive activation of the FGFR3 signaling pathway. In addition, the mutant form of the receptor is unable to mobilize calcium, suggesting that calcium mobilization (or lack of) plays a role in the development of the pathology. P2Y receptors have been described in chondrocytes (cells in the endochondral bone growth in mammals) [43]; however, their presence and function in achondroplastic chondrocytes have been examined in the presented study [44]. Using confocal microscopy, immunoreactivity against $P2Y_1$, $P2Y_2$, $P2Y_6$, and $P2Y_{11}$ was detected using Alomone Labs antibodies, while that of $P2Y_4$ was not present in achondroplastic chondrocytes. The results were also confirmed by Western blot analysis. Treating these cells with dinucleotide polyphosphates gave rise to an increase in intracellular calcium concentrations. This effect was eliminated (to various extents) by applying different P2 antagonists. Treating chondrocytes with FGF9, the natural ligand of FGFR3, promoted calcium mobilization which was not observed when treating achondroplastic condrocytes with the same ligand, due to the constitutively active FGFR3 signaling cascade. However, when treating these cells with dinucleotide polyphosphates prior to FGF9 treatment, the calcium mobilization was restored by a mechanism that is yet still not understood. The study suggests that the activation of P2Y receptors may be a useful pharmacological tool to reset the modified biochemical process observed in achondroplasia.

In the next section, we discuss the many uses of our antibodies directed against ionotropic P2X receptors.

P2X receptors

The P2X receptors are a family of ligand-gated, nonselective cation channels that open in response to extracellular ATP. In vertebrates, the family comprises seven members termed $P2X_1$–$P2X_7$ that share 40–50% sequence homology. The P2X receptors have a topology that includes

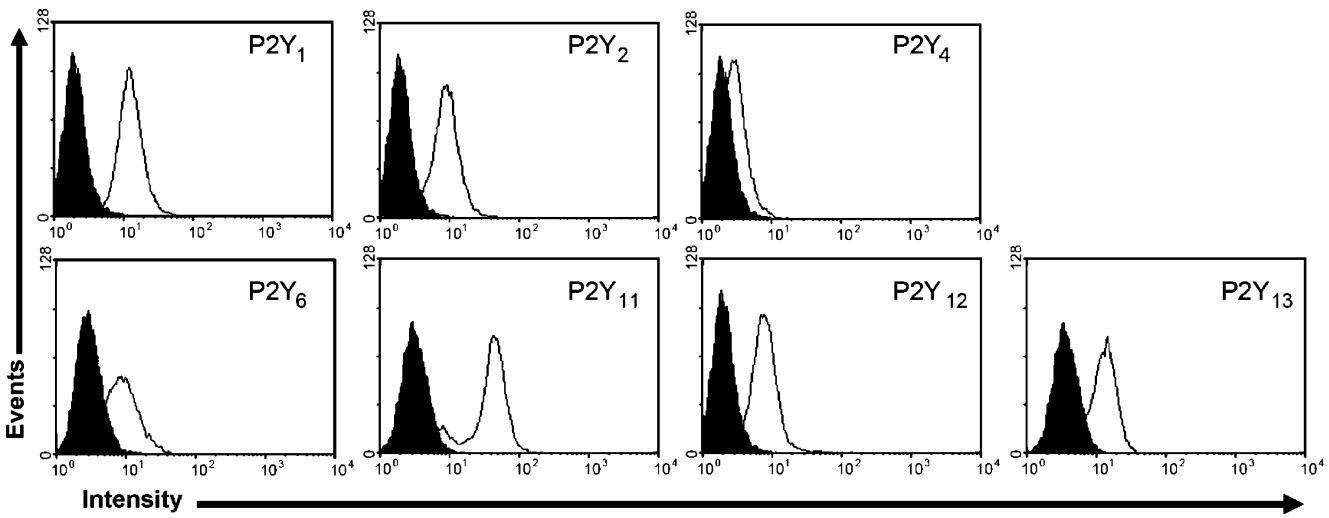

Fig. 5 Expression of P2Y receptors in osteoblasts. Using flow cytometry analysis, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$ receptors were detected in osteoblasts using their respective Alomone Labs antibodies (*open histograms*). Incubating the antibody with the control peptide antigen was used as a negative control (*filled histograms*). Adapted from reference 41 with permission of Elsevier

two transmembrane domains, a large glycosylated extracellular domain, and intracellular N- and C-termini. Their functional subunit is a trimer, and they can form either a homomeric unit (with the exception of $P2X_6$) or heterotrimers with other P2X subunits which display properties that differ from the corresponding homomeric form. The P2X receptors play key roles in various physiological processes such as nerve transmission, pain sensation, and immune response [45], but open questions, with important physiological and pharmaceutical implications, still exist in this expanding field of research [46].

Alomone Labs invests great efforts in the development of antibodies that are tailored to the needs of the research community. Our antibody development process begins with a deep understanding of the structure, biology, and functionality of each and any one of the receptors and channels against which we prepare the antibodies. Our antibodies are prepared using a peptide chosen from the sequence of each one of the channels that is thoroughly examined to preclude cross-reactivity first of all with other members of the close channel family and then with other known non-related proteins. Our antibodies are always affinity purified in order to provide the best possible specific antibody content and avoid as much as possible unwanted non-relevant antibodies in the final antibody preparation. In addition, our antibodies are always rigorously tested in a Western blot assay in the appropriate target tissues to confirm functionality.

Below, we have summarized for each P2X isoform recent literature citing the use of Alomone Labs anti-P2X receptor antibodies and the contribution of these works to our understanding of the roles played by P2X receptor channels in several physiological and pathophysiological processes. Due to space constrictions, only a handful of these contributions will be acknowledged, and we regret any inconvenience that this may arise.

$P2X_1$

$P2X_1$ is relatively widely expressed with prominent expression in platelets, megakaryocytes, smooth muscles, and vas deferens.

$P2X_1$ is the sole P2X receptor present in platelets, and the activation of the receptor generates an increase in intracellular Ca^{2+}, which in turn leads to the secretion of secretory granules and shape change [45, 47]. Indeed, using Alomone Labs anti-$P2X_1$ receptor antibody (#APR-001), Vial et al. [48] showed the co-localization of $P2X_1$ with CD41, a known marker of platelets and megakaryocytes, in samples obtained from bone marrow and spleen (Fig. 6, top panels). $P2X_1$ platelet localization in human platelets was confirmed later by several groups using the anti-$P2X_1$ receptor antibody [49, 50].

It is now widely recognized that $P2X_1$ has a key role in platelet function. For instance, it has been shown that the

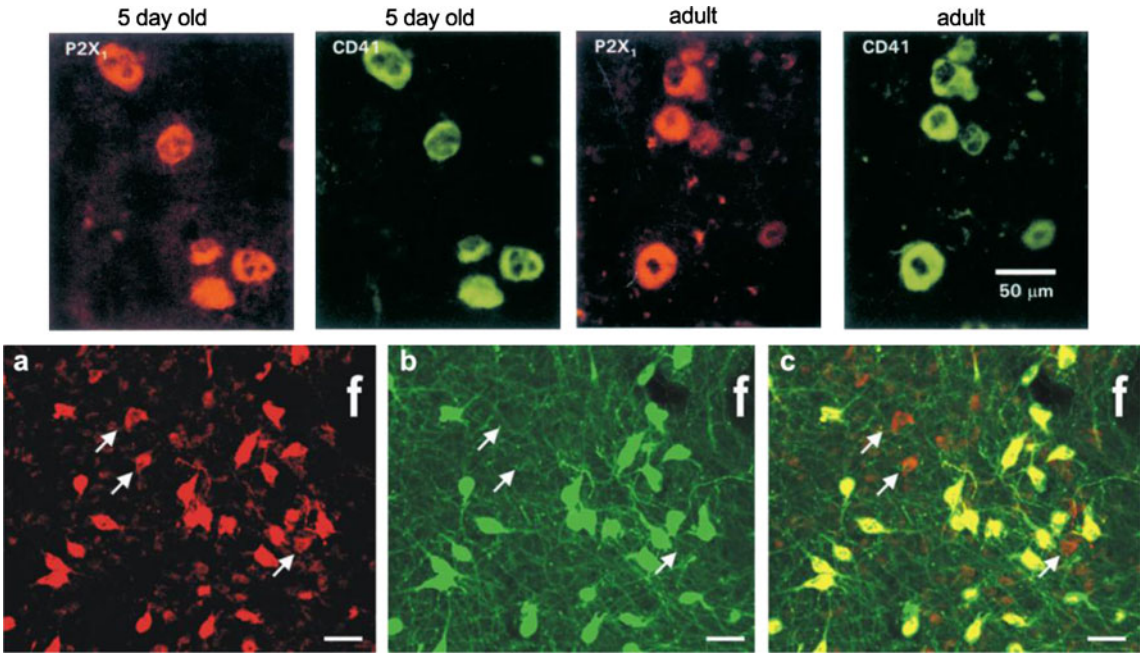

Fig. 6 Co-localization of $P2X_1$ and CD41 in bone marrow (*top panel*) and double immunofluorescence for $P2X_2R$ channel and orexin in hypothalamic neurons (*bottom panel*). *Top panel*, $P2X_1$ receptor immunoreactivity (*red*, #APR-001) is co-localized with CD41 (*green*). Similar levels of immunoreactivity were detected in tissues from 5-day-old (*left two images*) and adult mice (*right two images*). *Bottom panel*, $P2X_2R$ channel staining (*red*, #APR-003) (*a*); orexin staining (*green*) (*b*); merge (*c*). Note that some $P2X_2R$-labeled cells do not stain for orexin (*arrows*). *f* fornix. *Top panels*; adapted from reference 48 with permission of John Wiley and Sons (*a–c*). Adapted from reference 55 with permission of John Wiley and Sons

activation of $P2X_1$ by ATP can synergize with low levels of known platelet activators, such as thrombin and collagen, to increase platelet aggregation. Indeed, the importance of $P2X_1$ activation in platelets seems particularly important under conditions of high shear stress, suggesting that $P2X_1$ is an attractive antithrombotic target [47].

$P2X_1$ receptors have a key role as neurotransmitter receptors at sympathetically innervated smooth muscle cells in urinary bladder, vascular smooth muscle cells, and skeletal muscle, among others. Indeed, the localization of $P2X_1$ in rat pulmonary artery smooth muscle cells and human skeletal muscle cells was demonstrated using anti-$P2X_1$ receptor antibody [51, 52].

The localization of $P2X_1$ receptor in afferent arteriole vascular smooth muscle cells is of particular importance as it demonstrates a critical role for this receptor in renal autoregulation, a process by which the kidney is able to maintain constant renal blood flow and glomerular filtration rate over changes in arterial pressure. Zhao et al. [53] nicely showed $P2X_1$ immunoreactivity on the vascular smooth muscle cells of microdissected renal interlobular artery and afferent arteriole using anti-$P2X_1$ receptor antibody.

$P2X_2$

The $P2X_2$ channel is mostly expressed in the central and peripheral nervous system with predominant expression in dorsal ganglia neurons, mesenteric ganglia neurons, taste buds, and astrocytes and plays a key role in synaptic transmission, pain, and taste perception.

Indeed, using anti-$P2X_2$ receptor antibody (#APR-003), Ohta et al. [54] showed the localization of $P2X_2$ in rat cultured myenteric neurons, implying that purinergic signaling is involved in the regulation of GI functions. Similarly, Florenzano et al. [55] demonstrated the co-localization of $P2X_2$ with orexin in hypothalamic neurons, suggesting that $P2X_2$ may be involved in arousal and wakefulness (Fig. 7a–c).

The involvement of $P2X_2$ in taste transduction was demonstrated by showing the localization of $P2X_2$ in taste buds using Alomone Labs' respective antibody [56]. $P2X_2$ forms functional heteromers with $P2X_3$ channels, and the $P2X_2/P2X_3$ unit has been described as a major player in pain transmission (see also below). Using anti-$P2X_2$ receptor antibody, the presence of the receptor was demonstrated in rat dissociated DRG neurons from normal and inflamed animals [57].

$P2X_3$

$P2X_3$ and $P2X_2/P2X_3$ receptors are selectively localized on sensory neurons in trigeminal, nodose, and DRG. $P2X_3$ receptors are now recognized as major players in mediating the primary sensory effects of ATP and, as such, are of major importance in nociception and mechanosensory transduction. Hence, $P2X_3$ and $P2X_2/P2X_3$ represent unique targets for novel analgesic agents that function as receptor antagonists [58].

Current theories predict that chronic pain is brought about by a gain in the expression and function of sensory nociceptors that enhance their signal flow to the brain to ensure the long-lasting nature of this process. In this regard, both $P2X_3$ and $P2X_2/P2X_3$ receptors were found to be modulated by several other receptors and ion channels known to be involved in pain perception. For instance, a selective, slow upregulation of nociceptive $P2X_3$ receptors on trigeminal neurons by the neuropeptide calcitonin gene-related peptide, a known endogenous pain mediator, was demonstrated using anti-$P2X_3$ receptor antibody (#APR-016) [59]. Similarly, using anti-$P2X_3$ receptor and anti-$P2X_2$ receptor antibodies, it was shown that the neutralization of endogenous nerve growth factor, another key pain transmission molecule, decreased $P2X_3$ receptor-mediated currents and enhanced the expression of $P2X_2$ subunits by $P2X_3$-immunoreactive neurons [60].

In addition, using Alomone Labs antibodies in immunohistochemical studies, Banerjee et al. [61] showed that $P2X_3$ exhibited a significant upregulation both in DRGs and in nodose ganglia of rats having chronic esophagitis, suggesting that the upregulation of $P2X_3$ receptors in sensory neurons following inflammation may contribute to altered sensation from the esophagus (Fig. 7a).

$P2X_4$, $P2X_5$, and $P2X_6$

$P2X_4$ is widely expressed in many neuronal and non-neuronal tissues. Localization studies at both protein and mRNA levels have demonstrated a wide expression pattern for the $P2X_4$ channel: rat brain, kidney, lung, heart, spinal cord, and more were shown to express the receptor [62]. Although its role in most of these tissues remains elusive, due to the lack of highly specific antagonist, recent studies suggest an important role for $P2X_4$ in chronic inflammation and neuropathic pain by spinal cord microglia. Following spinal cord injury or peripheral nerve injury, the upregulation of $P2X_4$ receptor in activated microglia was detected. Partial nerve injury (PNI) induced a restricted de novo expression of $P2X_4$ by activated microglia. Immunohistochemical staining, comparing $P2X_4$ expression in the spinal cord of sham-operated mice versus PNI mice using the specific anti-$P2X_4$ receptor antibody (#APR-002), indicated that sham-operated mice expressed low, barely detectable levels of $P2X_4$, while PNI mice, 10 days following the injury, expressed significant levels of $P2X_4$ (Fig. 7b, c) [63]. It seems that the activation of the microglia following nerve injury is not mediated by $P2X_4$ since the activation of

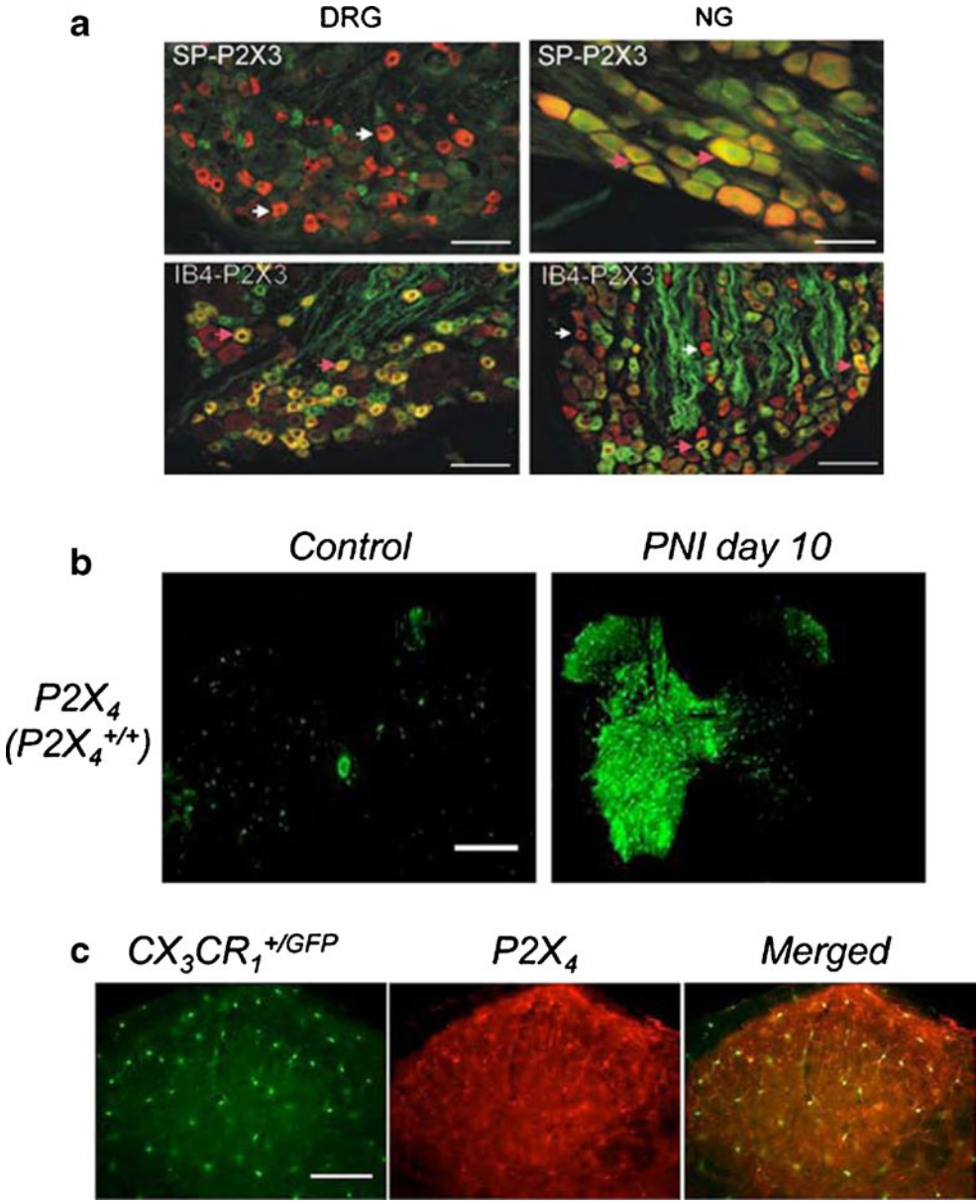

Fig. 7 Distribution pattern of $P2X_3$ immunoreactivity in peptidergic and non-peptidergic small-diameter c-fibers in dorsal root ganglion (*DRG*) and nodose ganglion (*NG*) neurons. Expression of $P2X_4$ in activated spinal microglia following peripheral nerve injury (*PNI*). **a** DRG sections were stained with $P2X_3$ (*red*, #APR-016) and substance P (*SP*, *green*) antibodies (*upper panel*) or with $P2X_3$ (*red*) and isolectin-B4 (*IB4*, *green*) antibodies (*lower panel*). *Pink arrows* indicate $P2X_3$-positive cells with IB4 or SP immunostaining, and *white arrows* indicate $P2X_3$-positive cells without IB4 or SP binding. **b** Immunohistochemical staining comparing $P2X_4$ expression in sham and injured mice using anti-$P2X_4$ receptor antibody (#APR-002, *green*). $P2X_4$ expression was barely detectable in sham animals, whereas 10 days post-PNI, it was upregulated ipsilateral to the lesion. *Scale bar*, 500 μm. **c** $P2X_4$ expression (*red*) in activated microglia following peripheral nerve injury is co-localized with microglia-specific eGFP fluorescence in $CX3CR1^{+/GFP}$ mice 10 days post-PNI. *Scale bar*, 100 μm. **a** Adapted from reference 61 with permission of Springer. **b**–**c** Adapted from reference 63 with permission of Society for Neuroscience

microglia in nerve injured $P2X_4^{-/-}$ mice was not affected [63]. However, in $P2X_4^{-/-}$ mice with spared nerve injury (neuropathic pain model), tactile allodynia was significantly reduced compared with wild-type mice, indicating a possible role for $P2X_4$ in chronic pain [63].

Apart from their importance as a tool in proteomics studies by identifying protein expression, tissue distribution, and more, antibodies can also be used as a tool for determining structural and functional interaction. Assembly and trafficking of the $P2X_4$ and $P2X_7$ channels were demonstrated in microglia cells prepared from cerebral cortices of newborn rats using anti-$P2X_4$ receptor and anti-$P2X_7$ receptor (#APR-004) antibodies [64]. Using cross-linking agents, biotinylation technique, Blue native PAGE, and specific antibodies, it was demonstrated that in primary cultures of rat microglia, $P2X_4$ and $P2X_7$ receptors preferentially assemble as homotrimers [64]. Analysis of $P2X_4$ and $P2X_7$ expression on the plasma membrane of primary microglia cells indicated that $P2X_4$ resides predominantly within intracellular compartments, while $P2X_7$ showed a prominent expression at the plasma membrane. The activation of the microglia with lipopolysaccharide increased the stability of $P2X_4$ expression on the plasma membrane within 3 h post-stimuli followed by a modest increase in the overall expression of the channel, as demonstrated by Western blot analysis.

The use of subunit-specific antibodies enables the detection of the different P2X subunits in tissues as well as in primary cultures or cell lines. In rat cultured cortical astroglia, immunohistochemical studies demonstrated that

glial fibrillary acidic protein (GFAP)-positive astroglia express multiple P2X receptors, predominantly the $P2X_4$, $P2X_6$, and $P2X_7$ (using anti-$P2X_4$ receptor, anti-$P2X_6$ receptor (#APR-013), and anti-$P2X_7$ receptor antibodies) as well as P2Y receptors. Although expressing both P2X and P2Y receptors, it was demonstrated that $P2Y_1$ and $P2Y_2$ receptors are involved in calcium signaling in response to extracellular nucleotides, thereby playing a role in cell-to-cell signaling (Fig. 8) [65].

Lacking specific antagonists to the $P2X_4$ channel, $P2X_4^{-/-}$ knockout (KO) mice were used to elucidate the role of $P2X_4$ in hippocampal synaptic transmission. KO of $P2X_4$ was assessed by Western blot analysis [66]. It was demonstrated that $P2X_4$ in wild-type animals affects long-term potentiation, an effect which was abolished in the KO animals [66].

In a study, testing the effect of different external ionic environments on calcium changes and the expression of P2X purinergic receptors in control and cystic fibrosis airway epithelial cells, it was demonstrated that CFBE41o− airway epithelial cells express $P2X_4$, $P2X_5$, as well as $P2X_6$ by immunohistochemical studies using anti-$P2X_4$ receptor,

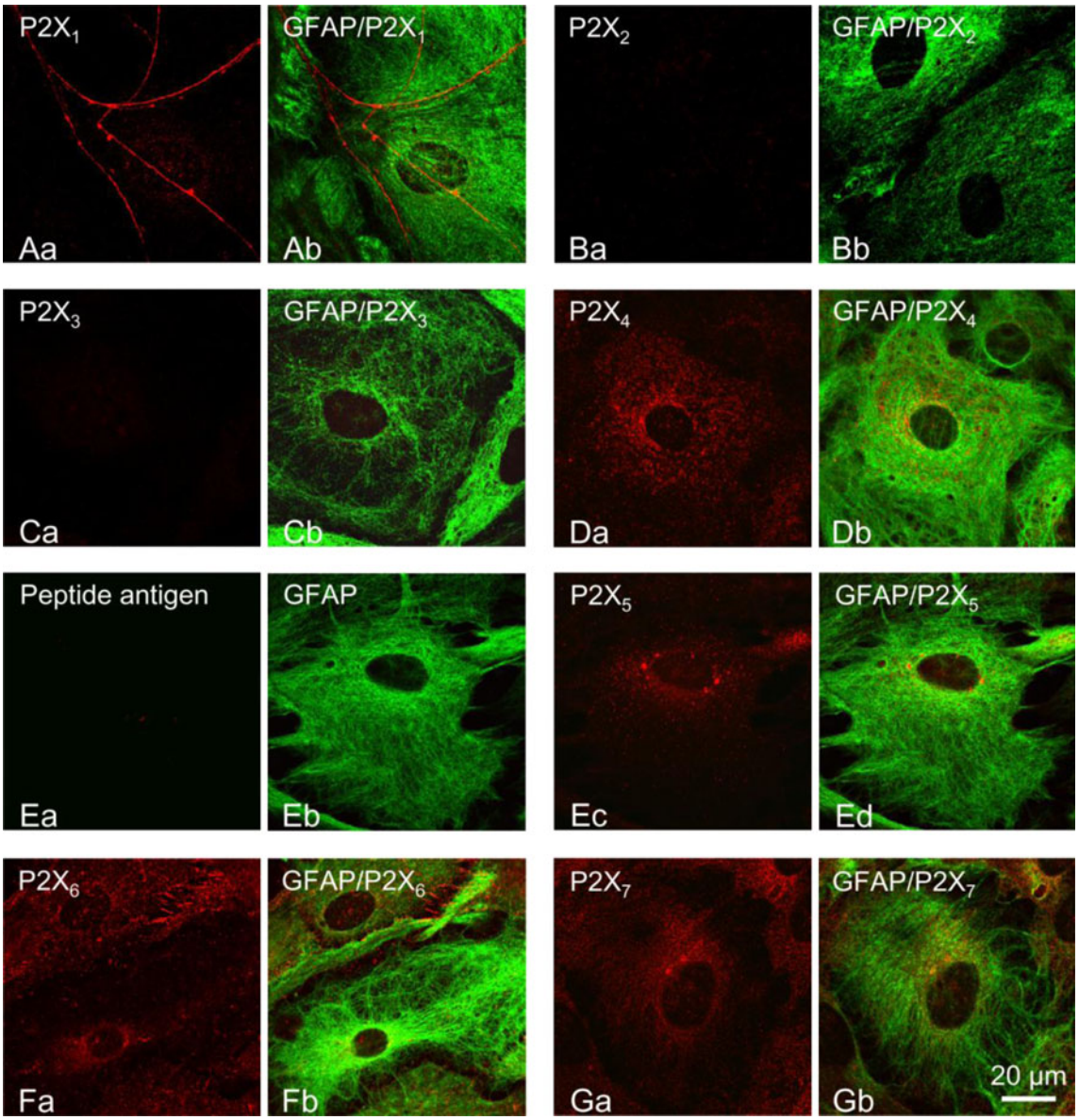

Fig. 8 Localization of P2X receptor subunits on astroglia. Localization of P2X receptors in astroglia was characterized by double immunofluorescence staining of $P2X_1$ (*A*), $P2X_2$ (*B*), $P2X_3$ (*C*), $P2X_4$ (*D*), $P2X_5$ (*E*), $P2X_6$ (*F*), $P2X_7$ (*G*) using their respective Alomone Labs antibodies (Cy3, *red*) and GFAP (Cy2, *yellow green*). Strong staining for the $P2X_1$ receptor was detected only in axon-like fibers (*A*). No clear immunoreactivity was observed for $P2X_2$ and $P2X_3$ receptor subtypes on astroglia (*B*, *C*). $P2X_4$, $P2X_5$, $P2X_6$, $P2X_7$ receptors were found to be expressed (*Da*, *Ec*, *Fa*, *Ga*). As representative control images are the anti-$P2X_5$ receptor antibody (#APR-005) pre-incubated with its control peptide antigen (*Ea*) and GFAP fluorescence alone (*Ea–d*). Adapted from reference 65 with permission of Elsevier

anti-$P2X_5$ receptor (#APR-005), and anti-$P2X_6$ receptor antibodies [67].

$P2X_7$

Once cloned from rat brain, $P2X_7$ was described as the P2Z receptor which is responsible for the ATP-dependent lysis of macrophages through the formation of membrane pores permeable to large molecules and could function also in fast synaptic transmission as a calcium-permeable cation channel [68].

$P2X_7$ channels are reported to be functionally expressed in several types of lymphocytes in which they mediate responses to high extracellular ATP, especially programmed cell death and cytokine secretion. In addition, $P2X_7$ channels are expressed in macrophages and the related brain microglia, where they mediate activation and proliferation and therefore are central players in immunogenic responses to inflammation. $P2X_7$ are probably expressed in neurons where they function both as classical ligand-gated ion channels and as mediators of programmed cell death in response to environmental signals.

The central role played by $P2X_7$ channels in responses of the immune system have placed it as a promising drug candidate to treat immune system-derived conditions. In such a case, the exact expression pattern and the exact function of the receptor in each tissue are necessary for drug development as a risk assessment. This is the point where specific antibodies, specific pharmacology, and KO techniques are valuable experimental tools.

Alomone Labs has generated two anti-$P2X_7$ receptor antibodies, one intracellular (#APR-004 and its ATTO-550 fluorescent-labeled version, #APR-004-AO), targeting the $P2X_7$ C-terminal, which is unique among the family of P2X receptors, and a different antibody, anti-$P2X_7$ receptor (extracellular) targeting an epitope on the extracellular moiety of the channel (#APR-008 and its FITC-conjugated version, #APR-008-F). The design of two different antibodies was of particular importance as the $P2X_7$ channel is encoded by a multi-exon gene and therefore different splice variants of the protein may exist.

Both antibodies recognize in Western blot analysis and immunocytochemistry $P2X_7$-transfected cells versus the untransfected counterpart in several different systems such as HEK cells, *Xenopus* oocytes, etc. [69–74].

The lack of $P2X_7$ expression in KO mice was confirmed using Alomone Labs antibodies in Western blot or immunohistochemistry analyses in the following tissues: salivary gland [73], lung [73], tymocytes [75], peritoneal macrophages [76], bone marrow-derived macrophages [64], microglia [64], astroglia [77], retina [78], ependymal cells along the lateral ventricle of the mouse brain [79], and calvarial osteoblasts (skull bone cells) [80].

In several other tissues, $P2X_7$ immunoreactivity was seen also in KO mice; these include: brain [73, 76] (and see early discussion in [81]), splenic follicles and lymph node [82], and spleen [73]. However, some of these observations were accompanied by the discovery of a novel $P2X_7$ splice variant which was not knocked out by the KO procedure and its mRNA was detected in many tissues including brain [73] or by the notion that lymphocytes from KO mice exhibit enhanced $P2X_7$ responses [82].

$P2X_7$ in the immune system

In mouse T lymphocytes, $P2X_7$ expression was assessed by anti-$P2X_7$ receptor (extracellular) antibody in immunohistochemical studies on splenic follicles and by Western blot analysis of lymph node [82]. It is worth mentioning that in that particular study, Taylor et al. discovered an enhanced $P2X_7$ activity in lymphocytes from KO mice, which was accompanied by the detection of $P2X_7$ expression in these gene-targeted mice. $P2X_7$ was also detected differentially by Western blot analysis in spleens of wild-type versus KO mice [73], which also demonstrated that a functional $P2X_7$ splice variant with an alternative transmembrane domain 1 escaping gene inactivation in $P2X_7$ KO mice.

In mouse tymocytes, $P2X_7$ receptors mediate ATP-dependent non-selective pore formation and cell death. The receptor expression in these cells (from two mouse strains) was confirmed by Western blot analysis with the intracellular antibody and a comparison to parallel cells from $P2X_7$ KO mice in which expression was not detected [75]. The main pathway of P2X7R-mediated thymocyte death was necrosis/lysis rather than apoptosis.

Using anti-$P2X_7$ receptor and anti-$P2X_7$ receptor (extracellular) antibodies, it was shown by Western blot analysis that the expression of $P2X_7$ receptors in peritoneal macrophages from control mice is totally absent in similar preparations from KO mice [76]. By using the intracellular antibody in FACS analysis in the same cell type, the expression of the channel was demonstrated. Regarding modulation of the receptor's activity in relation to cell death, it was shown that nucleoside triphosphate diphosphohydrolase 1 (NTPDase1)/CD39 (which is the dominant ectonucleotidase expressed by murine peritoneal macrophages) protects peritoneal macrophages from ATP-induced death via the suppression of $P2X_7$ activation by high extracellular ATP [83]. Co-immunoprecipitation experiments in these cells demonstrated that following activation with a "danger signal," Biglycan, a ubiquitous leucine-rich repeat proteoglycan of the extracellular matrix, $P2X_7$ receptors interact with TLR2/4 in wild-type but not in KO mice to activate interleukin 1β secretion [84].

In experiments that ruled out the expression of $P2X_7$ in human neutrophils, the mouse macrophage cell line J774

was used as a positive control, and indeed, $P2X_7$ expression was demonstrated by Western blot analysis with both the intracellular and extracellular antibodies. In addition, immunocytochemistry with anti-$P2X_7$ receptor (extracellular)-FITC demonstrated P2X7 membrane expression in living J744 cells [72].

$P2X_7$ in the CNS

Although $P2X_7$ is clearly expressed in brain tissue, the question whether its expression is restricted to non-neuronal cells is a matter of ongoing debate (see below).

In ependymal cells along the lateral ventricle of the mouse brain, $P2X_7$ immunoreactivity was observed in brain slices, which was absent in KO mice. Using gold labeling and electron micrographs, it was shown that $P2X_7$ is expressed in the plasma membrane of an ependymal cell specifically localized on the surface of the cilia and microvilli of these barrier cells [79].

Western blot analysis of mouse microglial cells (as well as in macrophages) showed that $P2X_7$ is highly expressed in control, but not in KO mice preparations. In addition, using immunoprecipitation methods, it was revealed that homotrimers of $P2X_7$ were able to co-immunoprecipitate with $P2X_4$, suggesting that an interaction occurs between rather than within receptor complexes. $P2X_7$ receptors were predominantly at the cell surface, whereas $P2X_4$ receptors were predominantly intracellular [64].

Several splice variants of $P2X_7$ were discovered and characterized to a certain extent, but in most cases, their differential functions, physiological roles, and localization are still a matter of research. However, the availability of two antibodies directed against different domains of the channel may serve as a tool for detecting the differential localization of these splice isoforms. Western blot analysis of lysates from astrocytes in cultures originating from either the cortex or the hippocampus revealed the possible presence of different isoforms of the $P2X_7$ receptor in astrocytes cultured from distinct brain regions. Anti-$P2X_7$ receptor antibody detected the presence of a protein band of the predicted molecular weight of 66 kDa in both hippocampal and cortical astrocytes. The presence of a band corresponding to the full-length receptor in both astrocyte cultures was confirmed by the anti-$P2X_7$ receptor (extracellular) antibody. This antibody recognized in the hippocampal, but not in cortical cultures, a major band of about 27 kDa, likely corresponding to a cytoplasmic tail-deleted variant of the receptor, given it was not detected by the anti-$P2X_7$ receptor antibody [85].

In cortex–striatum–subventricular zone organotypic cultures, anti-$P2X_7$ receptor antibody in immunocytochemistry studies was used to show that $P2X_7$ expression is upregulated and that its staining is co-localized with microglial marker OX42 following 40 min of oxygen and glucose deprivation (OGD) [86]. This upregulation contributes to microglia activation and the following neuronal damage. A similar upregulation was evaluated in the mouse N9 microglial cell line, as seen in Western blot, following OGD [86].

By performing immunohistochemistry on spinal cord preparations and Western blot analysis on spinal cord microglial cell lysates of rats receiving morphine, $P2X_7$ immunoreactivity was co-localized with the microglial marker OX42, but not the astrocytic marker GFAP, in the spinal cord. The protein level of spinal P2X7R was upregulated after chronic exposure to morphine and contributed to the generation of tolerance (Fig. 9) [87].

Whether the $P2X_7$ receptor is expressed in neurons remains controversial. One of the main reasons for this ongoing debate arises from the fact that $P2X_7$ immunore-

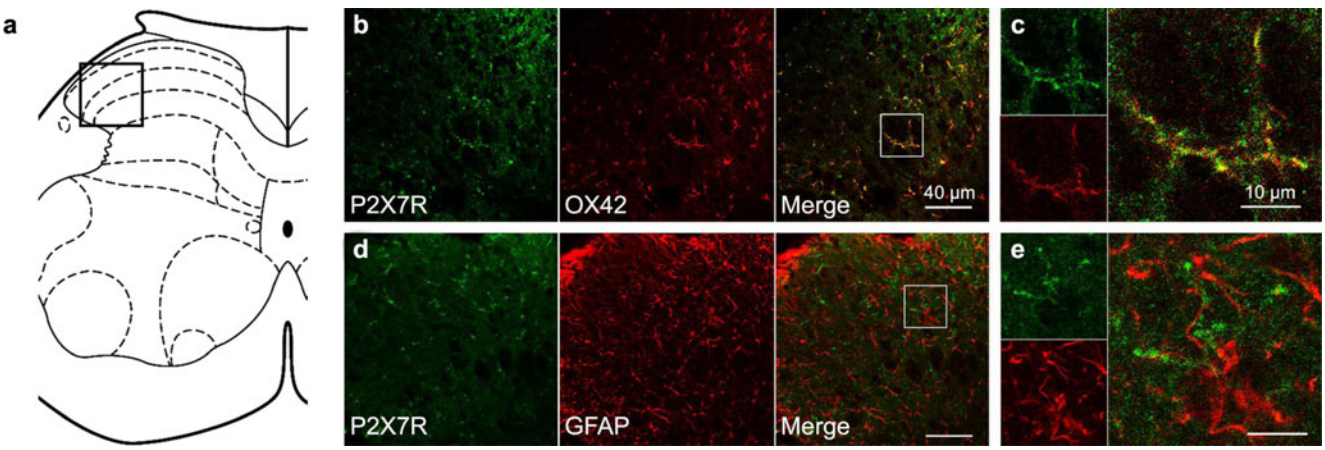

Fig. 9 Double immunostaining of P2X7R and cell-specific markers in morphine-tolerated rats. **a** Schematic diagram of the spinal cord. *Black open square* (**a**) marks the corresponding scope of confocal images (**b**, **d**) on the spinal cord section. *White open squares* (**b**, **d**) mark the corresponding scope of amplified images (**c**, **e**) on the confocal images. **b**, **c** Double immunostaining of P2X7R and the microglial marker OX42. **d**, **e** Double immunostaining of P2X7R and the astrocytic marker GFAP. *Scale bars*, 40 μm (**b**, **d**) and 10 μm (**c**, **e**). Adapted from reference 87 with permission of Society for Neuroscience

activity is maintained in brain preparations originating from $P2X_7$ KO mice. For example, two bands of 65 and 77 kDa are detected in Western blot analysis using anti-$P2X_7$ receptor antibody in the wild-type mouse whole brain, which is maintained in brains from KO animals, while the single 77-kDa band that is detected in peritoneal macrophages of wild-type animals is abolished by the KO [76]. In addition, $P2X_7$ immunoreactivity which is detected on cerebellar granule cells from wild-type mice in culture is seen also in such cells from KO animals.

$P2X_7$ is expressed in the developing rat brain from embryonic day 14 and might be involved in programmed cell death during embryogenesis. Immunoreactivity of the $P2X_7$ receptor protein in the developing brain was detected in the lateral hypothalamus at E14, the lateral olfactory tract and optic tract and in the cerebellum, midbrain, and medulla at E18 [88].

$P2X_7$ was also detected by Western blot analysis in rat primary cortical neurons in culture, which were confirmed as such by positive staining with monoclonal antibody against the neuronal marker NeuN. The $P2X_7$ positive signal was reduced once the cells were treated with a specific antisense oligonucleotide. Functional studies with specific pharmacology have suggested that extracellular ATP mediates neuronal apoptosis through the activation of the $P2X_7$ receptor and its downstream signaling pathways involving JNK1, ERK, and caspases 8/9/3 [71].

$P2X_7$ expression and role in other tissues

The expression of $P2X_7$ was confirmed in calvarial osteoblasts (skull bone cells) in Western blot analysis using anti-$P2X_7$ receptor (extracellular) antibody, a signal which was absent in the same cells from KO mice. In addition, $P2X_7$ was expressed in MC3T3-E1 osteoblasts and MLO-Y4 osteocytes where it is activated following mechanical load, leading to prostaglandin release from these cells and the subsequent bone formation [80]. $P2X_7$ expression was also confirmed in synoviocytes from rheumatoid arthritis patients by Western blot analysis. Pharmacological activation of $P2X_7$ in these joint cells leads to increased IL-6 secretion, suggesting a role for $P2X_7$ in a pro-inflammatory pathway [69].

$P2X_7$ was also reported to be expressed in skeletal muscle cells: In mouse myotubes in culture, $P2X_7$ immunoreactivity was confirmed by immunocytochemistry and Western blot analyses; it was shown that the $P2X_7$ signal is significantly reduced following specific siRNA treatment. Combining these results with functional studies, it is suggested that the activation of ionotropic $P2X_4$, $P2X_5$, and $P2X_7$ and metabotropic $P2Y_1$ and $P2Y_4$ receptors participates in forming the calcium transients of multinucleated myotubes [89].

In pancreatic cancer and chronic pancreatitis patients, some evidence for $P2X_7$ upregulation is observed in comparison to normal pancreas. This upregulation was statistically significant in RT-PCR experiments, but not in comparative Western blot or immunohistochemical analyses. A detailed examination of immunohistochemistry experiments demonstrated that most of the $P2X_7$ immunoreactivity arises from staining of immune system-infiltrating cells and was absent from parenchymal cells [90]. In the insulinoma cell line INS-1e, $P2X_7$ expression was detected by Western blot analysis (which was in correlation to mRNA levels), which increased following $P2X_7$ agonist application. However, $P2X_7$ receptors are probably not involved in insulin secretion or apoptosis induction in these cells [91].

Recently, a novel $P2X_7$ splice variant ($P2X_7k$) with an alternative first transmembrane domain was discovered. Interestingly, this splice variant escapes gene inactivation in KO mice, and its expression in different tissues (including brain) of both wild-type and $P2X_7$ KO mice was confirmed using specific RT-PCR primers. In addition, at the protein level, its expression was confirmed using Western blot analysis in spleen, salivary gland, and lung from wild-type mice as well as in the spleen of KO mice [73].

Ionotropic and matabotropic purinoceptors have indeed proven to be most important in various biochemical and physiological processes, as pointed out in the above examples. Importantly, the pharmacological tools that have been used to unravel these important receptors are nowadays being coupled to antibodies. Indeed, the above examples have also strongly shown that purinoceptor-targeted small molecules and antibodies are intertwined in revealing the localization and function of these receptors. Commercially available antibodies provided by Alomone Labs are undeniably valuable tools in understanding and deciphering the specific purinergic receptor subtype involved, a task that most pharmacological tools has yet to show. The anti-P2Y receptor and the anti-P2X receptor antibodies enable the detection of the various receptors via Western blot analysis, immunohistochemistry, immunocytochemistry, as well as indirect or direct flow cytometry.

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