Supportive or detrimental roles of P2Y receptors in brain pathology?—The two faces of P2Y receptors in stroke and neurodegeneration detected in neural cell and in animal model studies

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Abstract This review describing the role of P2Y receptors in neuropathological conditions focuses on obvious differences between results demonstrating either a role in neuroprotection or in neurodegeneration, depending on in vitro and in vivo models. Such critical juxtaposition puts special emphasis on discussions of beneficial and detrimental effects of P2Y receptor agonists and antagonists in these models. The mechanisms reported to underlie the protection in vitro include increased expression of oxidoreductase genes, like carbonyl reductase and thioredoxin reductase; increased expression of inhibitor of apoptosis protein-2; extracellular signal-regulated kinase- and Akt-mediated antiapoptotic signaling; increased expression of Bcl-2 proteins, neurotrophins, neuropeptides, and growth factors; decreased Bax expression; non-amyloidogenic APP shedding; and increased neurite outgrowth in neuronal cells. Animal studies investigating the influence of P2Y receptors in middle cerebral artery occlusion (MCAO) models for stroke prove beneficial effects of P2Y receptor antagonists. In MCAO mice and rats, the application of broad-range P2 receptor antagonists decreased the infarct volume and improved neurological outcome. Moreover, antagonists of the P2Y₁ receptor, one of the most abundant P2Y receptor subtypes in brain tissue, decreased neuronal loss and improved spatial memory in rats after traumatic brain injury (TBI). Currently

available data show a discrepancy between in vitro and in vivo models concerning the benefits of P2Y receptor activation in pathological conditions. In vitro models demonstrate protection by P2Y receptor agonists, but in vivo P2Y receptor activation deteriorates the outcome after MCAO and controlled cortical impact brain injury, a TBI model. To broaden the scope of the review, we additionally discuss publications that demonstrate detrimental effects of P2Y receptor agonists in vitro and publications showing protective effects of agonists in vivo. All these studies help to better understand the significant role of P2Y receptors especially in stroke models and to develop pharmacological strategies for the treatment of stroke.

Keywords Apoptosis · Astrocytes · Brain · Neurons · Neuroinflammation · Neuroprotection · MCAO · P2Y receptor

Abbreviations

2MeSADP	2-Methyl-thio-ADP
AD	Alzheimer's disease
ATPγS	Adenosine 5'-(γ-thio)triphosphate
APP	Amyloid precursor protein
BBB	Blood-brain barrier
ERK	Extracellular-signal-regulated kinase
GFAP	Glial fibrillary acidic protein
HO-1	Heme oxygenase-1
MCAO	Middle cerebral artery occlusion
MCAT	Middle cerebral artery transection
MRI	Magnetic resonance imaging
NGF	Nerve growth factor
OGD	Oxygen glucose deprivation

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PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
PD	Parkinson's disease
PTP	Permeability transition pore
qEEG	Quantitative electroencephalography
ROS	Reactive oxygen species
SOD	Superoxidedismutase
TBI	Traumatic brain injury
TNF α	Tumor necrosis factor α
TTC	2,3,5-Triphenyltetrazolium chloride

Introduction

ATP was discovered many decades ago as the universal cellular energy reservoir for metabolism [1]. About in 1972, ATP was postulated to be a neurotransmitter in the so-called non-adrenergic-non-cholinergic neurotransmission [2]. With rapid development of molecular biological methods, purinergic receptors were cloned in the 1990s and subsequently characterized [3]. As of today, ATP is generally accepted as a transmitter in its own right and a cotransmitter. There are four P1 receptors, seven P2X receptors, and eight P2Y receptors known in the human genome. P2X receptors are ion channels, which support fast synaptic transmission [4]. P1 receptors are G protein-coupled receptors activated by adenosine [5]. The P2Y receptors, on which this review concentrates, are G protein-coupled receptors involved mainly in long-term effects. P2Y₁, P2Y₂, P2Y₄, and P2Y₆ receptors activate phospholipase C and increase the cytosolic Ca²⁺ concentration. The P2Y₁₁ receptor is coupled to G_s and G_q and raises the cAMP level and the Ca²⁺ concentration. P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors inhibit the adenylyl cyclase and decrease the cAMP concentration. Prominent natural agonists of the P2Y₁ receptors are ADP and ATP. The human P2Y₂ receptor is equally activated by ATP and UTP. The P2Y₄ receptor has UTP as agonist, and the P2Y₆ receptor prefers UDP over UTP. The human P2Y₁₁ receptor is mainly activated by ATP. The human P2Y₁₂ and P2Y₁₃ receptors are both activated by ADP. Having ADP-glucose and ADP-galactose as natural agonist makes the human P2Y₁₄ receptor outstanding (for reviews, see [6–9]). Chemical modifications to nucleotides were applied to increase their resistance to hydrolysis. A prominent example is adenosine 5'(γ -thio)triphosphate (ATP γ S). Furthermore, subtype-selectivity was increased, like in 2-methyl-thio-ADP (2MeSADP), which is a highly potent and selective P2Y₁ receptor agonist. Intensive molecular analysis of the binding sites of the P2Y receptors led to the development of a set of non-nucleotide-based subtype-selective agonists and antagonists, which were used to analyze subtype-specific effects [10, 11]. The agonist preference of P2Y receptor subtypes may vary between species, as differences

between humans, mice, and rats have been reported. Comparative analysis of the amino acid sequences of the P2Y receptor family revealed two phylogenetic subgroups. The first group consists of P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ receptors, and the second group consists of P2Y₁₂, P2Y₁₃, and P2Y₁₄ receptors [12].

Degradation of the nucleotide agonists of P2Y receptors after release occurs by ectonucleotidases, a group of enzymes consisting of several families widely distributed in human tissues. Ectonucleotidases, which regulate the activity of nucleotides by dephosphorylation [13], shape the purinergic signal. In human tissue, all eight subtypes of P2Y receptors are found with various functions. Pharmacologically important are P2Y₁ and P2Y₁₂ receptors, which are involved in platelet aggregation, and P2Y₄ receptors, which mediate ion fluxes. Drugs activating the P2Y₁₂ receptor are used for anti-thrombotic therapy and P2Y₄ receptor agonists are used to treat the dry-eye disease [14, 15]. So far, only P2Y₁₂ and P2Y₄ receptor agonists are in clinical use. Besides that, other P2Y receptors are regarded as potential drug targets [16].

In the human brain, P2Y₁, P2Y₆, P2Y₁₁, P2Y₁₂, and P2Y₁₃ receptors were found on the mRNA level [17]. Moreover, the immunohistochemical analysis showed a localization of the P2Y₁ receptor in neuronal structures in the cerebral cortex, cerebellar cortex, caudate nucleus, putamen, globus pallidus, subthalamic nucleus, red nucleus, hippocampus, and midbrain [18]. Besides neuronal localization, also in astrocytes and oligodendrocytes of the white matter, the presence of P2Y₁ receptors was shown by immunohistochemistry in rats [19]. Additionally, upregulation of P2Y₂ receptor mRNA expression in rat primary cortical neurons after stimulation with IL-1 β was demonstrated [20]. There is a wealth of data concerning the pharmacological relevance of P2Y receptors in stroke, or oxidative stress in neurodegenerative disease.

The aim of this review is to evaluate effects mediated by P2Y receptors in vitro and in vivo, in order to understand the potential role of P2Y receptors as targets for neuroprotection. We analyze in detail the question of why conclusions derived from in vitro models are not fully consistent with conclusions obtained from in vivo models. We critically consider the possibilities for future clinical developments of P2Y receptor-targeting drugs and treatment regimes.

Apoptosis, oxidative stress, and microglia as targets for neuroprotection

Neuroprotection includes pharmaceutical or physical procedures to avoid neuronal cell loss in neurodegenerative diseases, stroke, or traumatic brain injury (TBI). Neuroprotection further includes the protection of glial cells, which maintain and support the functionality and viability of neurons.

Cells may die by apoptosis or necrosis. Necrosis in brain occurs after traumatic injury or in the core of stroke tissue, where occlusions lead to stark limitation in supply of oxygen and glucose. Breakdown of ion gradients causes osmotic swelling and finally permeabilization of cells and subsequent inflammation [21–23]. Apoptosis leads to controlled degradation of cells and avoids further damage in the surrounding area by inflammation. Apoptosis is a hallmark of neurodegenerative diseases and also takes place in the penumbra surrounding the ischemic core in stroke.

Many factors shift the cell fate between apoptosis or survival. A widespread approach for neuroprotection by pharmacological intervention prevents apoptosis. There are two pathways of apoptosis: the extrinsic and the intrinsic pathways with possible cross talk. The extrinsic pathway is mediated by cytokine receptors activated by tumor necrosis factor α (TNF α) leading to activation of caspase 8. The intrinsic pathway involves the permeabilization of the outer mitochondrial membrane and the release of pro-apoptotic factors, like cytochrome C, smac/Diablo, HtrA2, AIF, and Endo G, leading to the formation of the apoptosome and activation of caspase 9. The initiator caspases 8 and 9 activate the effector caspase 3, which cleaves its target proteins leading to degradation of the cytoskeleton and fragmentation of DNA [24–26]. Finally, the cell breaks apart into vesicles which are phagocytosed.

Several factors lead to mitochondria permeabilization. The formation of the mitochondrial permeability transition pore (PTP) triggered by Ca^{2+} overload or reactive oxygen is well documented [27–30]. This might happen by excitotoxicity including excessive glutamate release from depolarized neurons or dead cells, which causes permanent activation of glutamate receptors and persistent increase in the cytosolic Ca^{2+} concentration [31–33]. Pro-apoptotic proteins like Bak and Bax promote the permeabilization of the outer mitochondrial membrane and release pro-apoptotic factors from the mitochondrial intermembrane space. Anti-apoptotic proteins like Bcl-2, Bcl-w, and Bcl-X_L inhibit the pro-apoptotic proteins and support survival [25].

Oxidative stress, the overproduction of reactive oxygen species (ROS), is associated with neurodegenerative diseases and ischemic episodes. Mitochondria are regarded as a major source of ROS, especially complex I and complex III. ROS are side-products of redox reactions [34, 35].

Especially in respiratory chain complexes of mitochondria, but also in oxidoreductases, electrons are partially transferred to oxygen instead of the regular substrate, leading to the formation of superoxide ($\text{O}_2^{\cdot-}$). Superoxide dismutases (SODs) convert superoxide to hydrogen peroxide. Finally, hydrogen peroxide in turn is degraded to water and oxygen by glutathione peroxidase or catalases. In the presence of iron ions, hydrogen peroxide can be converted to highly toxic hydroxyl radicals ($\cdot\text{OH}$) via the Fenton reaction [36].

Excessive ROS production leads to damage of proteins and nucleic acids. Oxidative stress is regarded as a factor contributing to inducement of the PTP and apoptosis [35, 37, 38]. Cells have antioxidant tools to degrade ROS, including SOD, catalase, glutathione peroxidase, and thioredoxins [39–42]. In many cases, it is still under debate whether ROS are the cause or consequence of neurodegenerative diseases.

A major pathological mechanism in neurodegenerative diseases that has gained much attention is the immune activation. The brain features a phenomenon called “immune privilege.” Here, specific immune cells called microglia are responsible for scavenging plaques and infectious agents. In contrast to other tissues, the blood-brain barrier (BBB) largely avoids the infiltration of T cells from capillaries. Moreover, astrocytes and neurons are able to suppress T cell activation and even induce apoptosis in T cells [43]. The reason for this “immune privilege” in the brain is attributed to its sensitivity and restricted regenerative capacity compared to other tissues. However, the adaptive immune response plays a role in stroke or TBI, when BBB might become permeable for T cells [44]. Neurons and astrocytes may upregulate toll-like receptors and release pro-inflammatory cytokines [45]. Consequently, there is a complex interplay of neurons, astrocytes, endothelial cells, microglia, and T cells.

In the past years, an important role of P2Y receptors in microglia emerged. The activation of P2Y₁₂ and P2Y₁ receptors of resting microglia causes their migration along a concentration gradient of ATP/ADP towards the injury. Phagocytosis of injured cells is induced by P2Y₆ receptors activated by UTP/UDP [46–48]. Considering this model, P2Y receptors became a pharmacological target for neuroinflammation.

Reactive astrogliosis is important for the outcome after pathological conditions in the brain. ATP is one of many factors that induce reactive astrogliosis in the injured CNS. This involves proliferation, remodeling, and migration of astrocytes and finally the formation of a glial scar [49]. Theoretically, this will protect and support CNS tissue by formation of a barrier [50]. Otherwise possibly repair processes like axonal outgrowth are inhibited [51]. In neurodegenerative diseases, such as Alzheimer’s disease (AD), a chronic inflammatory response with persistent reactive astrogliosis might contribute to damage [52].

Acute brain inflammation is the result of physical trauma, bacterial meningitis, or primary intracerebral hemorrhage. In this case, microglia and blood-derived immune cells respond by eliminating the invading pathogens, clearing foreign bodies, and then tissue repair and regeneration are initiated [53, 54]. Chronic inflammation is considered as permanent activation of the immune response in the CNS responding to a range of pathological processes involving multiple cell types. Intensive research focuses on the aspects of chronic immune responses in AD, Parkinson’s disease (PD), and stroke in order to support survival and to avoid detrimental effects [55].

Taken together, the neuroprotective intervention includes all procedures which target pathways to avoid apoptosis, dampen the excessive Ca^{2+} entry, limit the inflammatory damage, and decrease oxidative stress, finally conserving the neuronal networks and their functionality as much as possible.

Protective effects of P2Y receptors in vitro in astrocytes

P2Y receptors, in primary neural cells and cell lines, mediate several beneficial effects preventing apoptosis and consequences of oxidative stress. Primary rat astrocytes, which were preincubated with ATP or 2MeSADP for at least 12 h, were protected against hydrogen peroxide-induced cytotoxicity. The protection was mediated via P2Y₁ receptors as demonstrated by application of a specific P2Y₁ receptor antagonist. Furthermore, functional protein biosynthesis, intracellular Ca^{2+} rise, and phospholipase C activity were necessary for this protective effect. Upregulation of oxidoreductase genes like carbonyl reductase, schlafen 4 (SHL4), an enzyme similar to superoxide dismutase 2, and thioredoxin reductase were found to be mediated by the P2Y₁ receptor and responsible for the increased tolerance to hydrogen peroxide [56]. Figure 1 schematically summarizes the protective pathways induced by nucleotides activating P2Y receptors in astrocytes

and 1321N1 astrocytoma cells. In this scheme, pathway ① presents the induction of antioxidant enzymes by P2Y₁ receptors.

Oxygen glucose deprivation (OGD) is a frequently used model to simulate stroke-like conditions in vitro and to analyze its outcome. Exposure to a short period of sublethal OGD was shown in rat astrocytes to increase the tolerance to lethal OGD. Immunohistochemical analysis revealed a transiently increased expression of P2Y₁ and P2Y₂ receptors after sublethal OGD. Blockade of ATP release by heptanol, a hemichannel antagonist, abolished the protective effect of the sublethal OGD. The pathway which provides ischemic tolerance by sublethal OGD involved the phosphorylation of extracellular-signal-regulated kinase (ERK). The protective effect was not observed in the presence of suramin, a broad-range P2 receptor antagonist, and MRS2179 (2'-Deoxy-N⁶-methyladenosine 3',5'-bisphosphate tetrasodium salt), an adenine nucleotide bisphosphate-based P2Y₁ receptor-specific antagonist [57].

Figure 1, pathway ②, illustrates that the activation of P2Y₁ receptors in primary astrocytes or the astrocyte-derived astrocytoma cell line 1321N1 favors survival by activation of ERK.

The P2Y₂ receptor was shown to inhibit trauma-induced apoptosis in 1321N1 astrocytoma cells. Characteristic hallmarks of cell death including DNA fragmentation and increased Bax/Bcl-2 gene expression were investigated after

Fig. 1 Protective pathways induced by nucleotides activating P2Y receptors (P2YR) in astrocytes and 1321N1 astrocytoma cells. The P2Y₁ receptor increases the expression of oxidoreductases to block oxidative stress ①. Antiapoptotic proteins are upregulated via P2Y_{1,2} receptors to block apoptosis ②,③. Activation of P2Y₆ receptor and P2Y₁₂ receptor leads to phosphorylation of ERK and inhibits the apoptotic cell death ④,⑤. P2Y receptors increase the cytosolic Ca^{2+}

concentration. Ca^{2+} induces activity of the tricarboxylic acid cycle which leads to increased amounts of ATP ⑥. Oxidative stress is reduced by ATP which inhibits Fenton reaction and maintains antioxidant defense ⑦. The respective references giving the background information for this scheme are given in the text. Here and in the other figures, hammerhead symbols/arrows signify stimulation/inhibition of a process, or activity or expression level, as explained in details

traumatic injury. The trauma was caused in a model of transient deformation of a flexible membrane. Cells grown on this flexible membrane were stretched by applications of controlled gas pressure pulses in a standardized manner and thus underwent traumatic damage [58]. 1321N1 Astrocytoma cells lack endogenous P2 receptor and serve as a model for P2 receptor-related signaling after transfection with specific P2 receptors. In contrast to the wild-type cells, the P2Y₂ receptor-transfected 1321N1 astrocytoma cells did not show DNA fragmentation or increased Bax/Bcl-2 gene expression after stretch injury. Already the basal nucleotide release was able to mediate this protective effect via P2Y₂ receptors. Activation of ERK 1/2 and Akt in P2Y₂ receptor-transfected 1321N1 astrocytoma was shown after stretch injury by western blot analysis [59]. It was demonstrated earlier that the application of UTP to P2Y₂ receptor-transfected 1321N1 astrocytoma cells activated pro-survival pathways, like up-regulation of Bcl-2, Bcl-x1, neurotrophins, neuropeptides, growth factors, and the downregulation of Bax [60]. In this context, Fig. 1, pathway ③, summarizes how we postulate that P2Y₂ receptors can activate ERK, increase the amount of antiapoptotic proteins, and thereby inhibit apoptosis.

A model for inducing the extrinsic pathway of apoptosis is based on the application of TNF α . The cytokine TNF α is secreted under proinflammatory conditions and may increase cell death in neurodegenerative diseases. The P2Y₆ receptor subtype was shown to inhibit TNF α -induced apoptosis in 1321N1 cells via PKC and phosphorylation of ERK, but without involvement of Akt [61]. We visualize in Fig. 1, pathway ④, the P2Y₆ receptors activating the ERK and supporting survival via ERK.

Another role for the P2Y₆ receptor was demonstrated in microglia, astrocytes, and cortical slices from rats. Application of UDP increased the expression of monocyte chemotactic protein 1 and macrophage inflammatory protein-1 α . The transcription factor nuclear factor of activated T cells was shown to be responsible for the P2Y₆ receptor-induced change of the chemokine expression pattern [62]. Protective effects of monocyte chemotactic protein 1 in mixed cortical cultures from mice against NMDA-induced cytotoxicity have been reported [63]. In conclusion, the P2Y₆ receptor provides a therapeutic target for protection by modulation of the chemokine expression.

A protective role of the P2Y₁₂ receptor was shown in 1321N1 astrocytoma cells where it inhibited the TNF α -induced apoptosis by phosphorylation of ERK1/2 [64]. Pathway ⑤ in Fig. 1 demonstrates the anti-apoptotic action of the P2Y₁₂ receptor via ERK1/2.

However, there are also reports of detrimental P2Y receptor-mediated effects in vitro. In 1321N1 astrocytoma cells expressing the P2Y₁ receptor, incubation with 2MeSADP caused increased activity of caspase 3. The

increase of this apoptosis marker was mediated by the stress-activated protein kinase family [65].

Besides the nucleotide-induced P2Y receptor-related protective effects, nucleotides might also act positively as chemical antioxidants. This process involves the chelation of iron ions by nucleotides, thereby inhibiting the Fenton reaction which produces highly toxic hydroxyl radicals from hydrogen peroxide. As shown in cell-free systems, nucleotide analogues concentration-dependently inhibit the Fenton reaction. Primary cortical neurons from rats were protected by nucleotide analogues from iron-induced oxidative stress [66]. The inhibition of the Fenton reaction is a pharmacological target to reduce oxidative stress. Generally, all P2Y receptors that raise intracellular Ca²⁺ induce the tricarboxylic acid cycle and increase the ATP production. An increased amount of ATP generally supports the maintenance of the ion homeostasis and the antioxidant defense, and inhibits the Fenton reaction. In Fig. 1, pathway ⑥ shows how increased Ca²⁺ elevates the ATP level to inhibit the Fenton reaction. The paradigm of the Fenton reaction as a target for neuroprotection by pharmacologically designed nucleotides is given by pathway ⑦ in Fig. 1.

Protective effects of P2Y receptors in vitro in neurons

Little is known about the possible protective effects of P2Y receptor activation in neurons. An indirect protection of neurons by astrocytes via purinergic signaling was described. Nucleotide-conditioned astrocytes may protect neurons against hydrogen peroxide via interleukin-6 release. In this study, the P2Y₁ receptor-specific agonist 2MeSADP was used. The pathway of interleukin-6-mediated protection remains unsolved, but induction of antioxidative proteins has been suggested [67]. Figure 2 schematically summarizes the mechanisms by which P2Y receptor signaling in neurons counteracts oxidative stress. In this scheme, pathway ① shows the indirect protection of neurons via astrocytic P2Y₁ receptors.

A direct protective function of the P2Y₂ receptor was demonstrated in mouse primary cortical neurons. Under inflammatory conditions, the P2Y₂ receptor is upregulated by interleukin-1 β . Microscopic imaging demonstrated that activation of the P2Y₂ receptor in mouse primary cortical neurons by UTP increased neurite outgrowth. This effect was regulated by phosphorylation of the actin-depolymerizing factor cofilin. Neurite extension is regarded as a protective pathway, which maintains neuronal networks or even regenerates damaged tissue after stroke or in neurodegenerative diseases, like AD, PD, and multiple sclerosis [68]. Induction of neurite outgrowth by the P2Y₂ receptor in neurons is illustrated in Fig. 2, pathway ②.

The balance between cell survival and cell death is critically regulated by trophic and apoptotic factors. Obstruction of

Fig. 2 P2Y receptor (P2YR) signaling in neurons counteracts oxidative stress and Alzheimer's pathology. Astrocytes release interleukin-6 after P2Y₁ receptor activation. Interleukin-6 protects neurons from oxidative stress by an unknown pathway involving the IL-6 receptor ①. Activation of P2Y₂ receptors induces protective neurite outgrowth ②. P2Y₂ receptors inhibit apoptosis via ERK ③. P2Y₂ receptors induce ADAM10/17 activity to cleave APP to the soluble non amyloidogenic sAPP ④. The P2Y₁₃ receptor protects against oxidative stress by upregulation of HO-1 via Nrf-2 ⑤. Activation of the P2Y₁₃ receptor leads to activation of ERK and blocks apoptosis ⑥

brain vessels by blood clots causes OGD and depletion of trophic factors, which induces apoptosis. Cultured cells are starved of serum to mimic the condition of apoptosis induced by trophic factor deprivation. A role in apoptosis was suggested for the P2Y₂ receptor in PC12 cells and dorsal root ganglion neurons. A period of 12 h of serum starvation in PC12 cells leads to DNA fragmentation and caspase 3 activation. These events are features of apoptosis, which were shown to be inhibited by treatment with both NGF (nerve growth factor) and the P2Y receptor agonist ATP γ S. Silencing-RNA experiments to knock down the P2Y₂ receptor revealed that the P2Y₂ receptor subtype is responsible for the ATP γ S-mediated protection. Application of inhibitors of ERK, U0126, and Akt, LY294002 showed that inhibition of apoptosis by ATP γ S involved ERK and Akt, but not TrkA which is part of the NGF-mediated antiapoptotic signaling. In dorsal root ganglion neurons from wild-type but not from P2Y₂^{-/-} knockout mice, the P2Y receptor agonist ATP γ S inhibited serum starvation-induced apoptosis [69]. Pathway

③ in Fig. 2 represents the antiapoptotic action of P2Y₂ receptors in neurons by activation of ERK and Akt.

Ischemic conditions can be mimicked chemically by application of an inhibitor of complex IV, KCN, which leads to cell death. Such experiments were carried out with rat primary cortical neurons and showed that preincubation with ATP for 3 to 8 h before the 3-h KCN treatment significantly increased the viability of the cells, when measured at 12 h after the insult. The application of the P2 receptor antagonist suramin blocked the protection from KCN treatment. Furthermore, the protection by ATP was absent in neurons from a mutant mouse model, which did not express the transcription cofactor LMO4. The incubation with ATP upregulated the expression of the inhibitor of apoptosis protein 2. Upregulation of LMO4 was mediated by CREB and ERK [70].

AD is a widespread neurodegenerative disease featuring increased amyloid β release and formation of senile plaques leading to gradual dementia. A possible role for P2Y₂ receptors in the treatment of AD was first suggested, when in 1321N1 astrocytoma cells the activation of P2Y₂ receptors was shown to increase the release of the non-toxic soluble amyloid precursor protein (sAPP α) [71]. Generally, the non-amyloidogenic cleavage of APP to the sAPP α is regarded as a potential mechanism to reduce the risk of AD. The idea was pursued, when rat primary cortical neurons were treated with interleukin-1 β , a proinflammatory cytokine that caused an increase in P2Y₂ receptor mRNA expression. Treatment of rat primary cortical neurons with UTP led to increased sAPP α secretion by the α -secretase ADAM10/17, which was not dependent on G_{q/11} activity [20]. In a later work, which was also carried out in N2A cells, α -secretase activity was shown to be increased by the P2Y₂ receptor [72]. The TgCRND8 mouse model of AD, which was used in [73], studies animals containing Swedish and Indiana mutations in the APP gene leading to AD pathology. The work showed increased expression of the P2Y₂ receptor mRNA in the brain of TgCRND8 animals within 10 and 25 weeks of age compared to littermate controls. TgCRND8 mice with heterozygous or homozygous deletion of the P2Y₂ receptor had a survival time after birth of less than 4 and 12 weeks, respectively. Additionally, heterozygous deletion of the P2Y₂ receptor in TgCRND8 mice increased the amyloid β plaque load and decreased the expression of the microglial marker CD11b. This study strongly indicates a neuroprotective role of the P2Y₂ receptor in the removal of amyloid β plaques via microglial cells [73].

Besides the avoidance of amyloid β deposit formation, the clearance of amyloid β deposits by microglia could significantly contribute to protection in AD. Treatment of primary mouse microglia with oligomeric and fibrillar amyloid β solutions caused ATP release and increased

P2Y₂ receptor expression. Activation of the P2Y₂ receptor with ATP or UTP increased amyloid β uptake of microglial cells from wild-type mice but not from P2Y₂ receptor^{−/−} mice. UTP also enhanced the amyloid β degradation in wild-type mice [74]. The possible involvement of the P2Y₂ receptor in preventing the AD pathology is included in Fig. 2 as pathway ④.

A protective role was proposed for the P2Y₁₃ receptor in cerebellar granule neurons. Incubation with 2MeSADP, an ADP analogue and agonist of P2Y receptors, induced the expression of the cytoprotective protein heme oxygenase-1 (HO-1). The transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) was critical for the activation of HO-1. Preincubation with 2MeSADP attenuated the hydrogen peroxide-induced cytotoxicity, but protection did not occur in the presence of MRS2211 (2-[(2-chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonoxy)methyl]-4-pyridinecarboxaldehyde disodium salt), which is a specific non-nucleotide P2Y₁₃ receptor antagonist. These results suggest that the P2Y₁₃ receptor is useful to counteract oxidative stress in neurons [75]. Antioxidant signaling in neurons by P2Y₁₃ receptors via Nrf2 and HO-1 is presented in Fig. 2, pathway ⑤. Another protective action of the P2Y₁₃ receptor in neurons was shown in cerebellar granule neurons from rats. Cells were treated with 2MeSADP for 2 h before addition of excitotoxic glutamate (100 μ M). Significant protection was observed by live/dead staining and shown to be mediated by phosphorylation of Erk1/2. This antiapoptotic action of the P2Y₁₃ receptor in neurons is depicted in Fig. 2 in pathway ⑥ [76].

In mixed neuronal/glial cultures, it was shown that lipopolysaccharide- or lipoteichoic acid-induced neuronal loss was prevented by MRS2578 (1-(3-isothiocyanatophenyl)-3-[4-[(3 isothiocyanatophenyl)carbamothioylamino]butyl]thiourea), a P2Y₆ receptor antagonist or by apyrase, which degrades natural P2Y receptor agonists. Treatment with the P2Y₆ receptor agonist induced phagocytosis of neurons. The viability of the cultures was assessed by propidium iodide staining and, moreover, morphological changes were determined [77]. In this study, protection of neurons by P2Y receptor antagonism has been found. Protection occurs by inhibition of microglial P2Y₆ receptor-mediated phagocytosis of neurons, but not by activation of neuronal P2Y receptors.

The neuroprotective potential of microglial P2Y₁₂ receptors was also investigated [78]. Increased neurotoxicity after OGD in neurons-astrocytes cocultures by addition of microglia was mitigated by P2Y₁₂ receptor knockdown [78]. Neuronal viability was determined by detection of microtubule-associated protein-2. This study strongly suggests a role of microglial P2Y₁₂ receptors in neuroprotection.

Protective effects of P2Y receptors in vivo in animal models of stroke and traumatic brain injury

In vivo experiments provide the opportunity to test whether agents have protective effects in the context of the whole organism to better understand diseases and find treatments for human diseases. By genetic engineering, mice or rats are generated that feature the pathophysiology of neurodegenerative diseases, like AD or PD. Animals may also be exposed to standardized procedures to mimic stroke or TBI. Present research concerning P2Y receptors in pathophysiology in animal models focuses on stroke and TBI. Stroke is a major cause of death in the modern civilization, but its clinical treatment is largely restricted to thrombolysis by plasminogen activator. Yet there is no neuroprotective pharmaceutical intervention which precludes the neurodegeneration after stroke. This urgent need induced intensive research in this field.

A very well-established animal model of stroke is the middle cerebral artery occlusion (MCAO) usually accomplished with mice or rats. Different MCAO models differ by duration of interrupting the blood flow, ranging from transient to permanent or the method of introducing the occlusion. The latter can be caused by the vasoconstrictive peptide endothelin-1, clot injection, surgical filaments, or transection followed by electrocauterization or ligation.

Several studies investigated the role of P2 receptors in MCAO models. The influence of suramin, a general P2 receptor antagonist, on the infarct size was analyzed in mice subjected to focal cerebral ischemia [79]. In this model, focal brain ischemia was induced by middle cerebral artery transection (MCAT) and bilateral occlusion of the common carotid arteries. Six hours after ischemia, the neurological status was measured by motor tests and the infarct volume was analyzed by microtubule-associated protein 2 immunostaining. The rats which received 30 min before MCAT an intravenous injection of suramin (100 mg/kg) showed an improved neurological score and a decreased infarct volume. The estimated end concentration in the brain after 100 mg/kg intravenous injection of suramin is 10 μ M. At a concentration of 10 μ M, mainly P2X receptors but not P2Y receptors are inhibited, and the blockade of P2X receptors may inhibit excessive intracellular Ca²⁺ rise leading to apoptosis. At higher concentrations, that are >100 μ M, interaction of suramin with P2Y receptors is possible. Interestingly, the group of animals pretreated with 250 mg/kg did not show a decreased infarct volume. This loss of protection could be due to inhibition of beneficially acting P2Y receptors at this concentration. The study investigated the outcome with mice at 6 h after onset of MCAT, which is a relatively short term [79].

Another study investigated the long-term effects of pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) on recovery from MCAO in mice [80]. The MCAO was caused by electrocauterization of the middle cerebral

artery. PPADS was administered with an implanted osmotic pump 15 min prior to the MCAO and until day 7 after the injury. After MCAO, the clinical outcome was investigated by behavioral tests, magnetic resonance imaging (MRI), quantitative electroencephalography (qEEG), and histological analysis at different days until the maximum of 28 days after MCAO. The functional recovery of the PPADS-treated group compared to the control group was improved as monitored by qEEG. Mice treated with PPADS showed improved motor abilities in rotarod and beam walk tests after MCAO. Investigation of the infarct volume by MRI showed significant reduction in the PPADS-treated animals compared to the control group from day 1 until day 7, but not after 28 days. Generally, the study suggests that early inhibition of P2 receptors could be beneficial for the motoric and functional recovery after stroke [80].

The exact mechanism how broad range P2Y receptor antagonists, like suramin or PPADS, reduce infarct size and improve motor and functional recovery is unknown. Figure 3 summarizes the frequently proposed mechanisms of protection. In pathway ①, inhibition of P2Y receptors on astrocytes could reduce the glutamate release and thereby decrease the excitotoxic cell death of neurons. In pathway ②, inhibition of P2Y receptors could reduce the TNF α release from astrocytes avoiding the extrinsic pathway of apoptosis in neurons. Ca²⁺-induced ATP release from astrocytes which exacerbates the aforementioned toxic effects could be blocked by general P2Y receptor agonists in pathway ③.

It has been described that increased intracellular Ca²⁺ in astrocytes leads to release of proinflammatory cytokines, glutamate, and ATP that exacerbates the release by further stimulation of intracellular Ca²⁺ rise [81–86]. Especially under

pathological conditions, this contributes to cellular damage, as can be seen in the model systems.

Another hypothesis concerning the mechanism of protection in MCAO models could be the inhibition of P2Y receptors in microglia. Activation followed by migration and phagocytosis of target cells by microglia is controlled by P2Y₁₂ and P2Y₆ receptors. ATP and ADP leak from cells to activate the P2Y₁₂ receptors on microglia and the ensuing P2Y₁₂ receptor activity contributes to shift of cellular phenotype and migration towards the damaged cells. Phagocytosis of damaged cells is stimulated by UDP activating the P2Y₆ receptors on microglia. Microglial expression of the P2Y₁₂ receptor is decreased and expression of the P2Y₆ receptor is increased in this process [46, 87, 88]. This immunological response is necessary for the restoration of tissue integrity. However, viable or salvageable cells may become victims of phagocytosis exacerbating damage under pathological condition. This might explain the reduced infarct volume after MCAO in P2Y receptor antagonist-treated animals.

Protective effects of P2Y receptor antagonist on microglia in animal models are illustrated in the summary in Fig. 4.

Indeed, a few studies prove the neuroprotective potential of microglial P2Y₆ and P2Y₁₂ receptors in animal models. Microglial P2Y₆ receptor-related protection was observed in vivo when rats showed decreased neuronal loss as a response to lipopolysaccharide injection into the striatum by application of the P2Y₆ receptor antagonist MRS2578. Neuronal loss was determined with confocal microscopy by immunohistochemical staining of sections using NeuN antibodies [77]. Another study focused on the function of the microglial P2Y₁₂ receptors during ischemia [78]. Here, ischemia was induced by bilateral carotid artery occlusion. P2Y₁₂

Fig. 3 Neuroprotection by P2Y receptor (P2YR) antagonists in animal models—hypothesized signaling pathways. P2Y receptor antagonists inhibit release of glutamate, proinflammatory cytokines, and ATP, and thereby block apoptosis. Astrocytes release glutamate ①,

proinflammatory cytokines ②, and ATP ③ after P2Y₁/P2Y₂ receptors activation. Glutamate and ATP contribute via glutamate receptors and P2Y₁/P2Y₂ receptors to further increase of cytosolic Ca²⁺ concentration in neurons. Ca²⁺ overload leads to apoptosis in neurons ④

Fig. 4 P2Y receptor (P2YR) receptors antagonists acting on microglia in animal models—hypothetical mechanisms of action. ATP activates P2Y₁₂ and P2Y₁ receptors on microglia cells and contributes to cellular activation. Activated microglia release TNF α , which might cause apoptosis in neurons and astrocytes by the extrinsic pathway. After binding of UDP, the P2Y₆ receptors cause activated microglia to initiate phagocytosis. There is a homeostatic balance between detrimental phagocytosis of potentially salvageable astrocytes and neurons and beneficial removal of apoptotic debris. Activation of microglia and execution of phagocytosis could be inhibited by P2Y receptor antagonists

receptor expression knockdown mice or animals treated with the P2Y₁₂ receptor antagonist clopidogrel suffered less neuronal injury by bilateral carotid artery occlusion [78].

A P2Y₁ receptor-specific study was performed with P2Y₁ receptor knockout (KO) mice focusing on cognitive functions and the neuroinflammatory response after MCAO [89]. Here, transient MCAO was induced by insertion of a nylon filament with an infarction time of 45 min. One week after MCAO, a contextual fear conditioning test was performed with wild-type and P2Y₁ receptor KO mice. The wild-type mice showed significantly reduced cognitive ability after MCAO compared to the sham-operated wild-type group, whereas the P2Y₁ receptor KO mice showed no decline after MCAO compared to the sham-operated P2Y₁ receptor KO mice group. There was no difference in the sensory motor deficits after MCAO between wild-type and P2Y₁ receptor KO mice compared to respective sham-operated control group. Improved cognitive outcome in wild-type mice after MCAO could be achieved by application of a P2Y₁ receptor-specific antagonist MRS2500 (4-[2-iodo-6-(methylamino)-9H-purin-9-yl]-2-(phosphonoxy)bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt). Further neuroinflammatory cellular change of the hippocampus was analyzed by immunohistochemical staining. Brain sections were stained with labeled antibodies to mark activated astrocytes (anti-GFAP) and activated microglia (anti-Iba1). Only in the CA2/3 region, the glial fibrillary acidic protein (GFAP) expression in P2Y₁ receptor KO MCAO mice was suppressed significantly as compared to the sham-operated control group. However,

in all hippocampal regions, the expression of Iba1 was significantly reduced in the P2Y₁ receptor KO MCAO mice at 3 weeks and 2 months after MCAO [89].

Besides permanent MCAO models, the transient MCAO model provides a tool to investigate transient ischemic events, which resemble the clinical pathogenesis from stroke development to thrombolysis. The influence of P2Y₁ receptor agonists and antagonists on the outcome of transient MCAO in mice was investigated [90]. Here, the carotid artery of mice was reversibly occluded by insertion of a nylon monofilament for 30 or 60 min. The infarct size was determined by 2,3,5-triphenyltetrazolium (TTC) staining at 72 h after transient MCAO. TTC is converted by the activity of mitochondrial dehydrogenases and therefore changes color. The application of the methanocarba ring-based P2Y₁ receptor agonist MRS2365 ([[(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt) increased the infarct volume in the mice exposed to 30 min transient MCAO, but not in the mice exposed to 60 min transient MCAO. The P2Y₁ receptor antagonist MRS 2179 decreased the infarct volume, when it was applied in 3 pulses with 12 h intervals. MRS2179 was more effective in decreasing the infarct volume, when it was applied at 0, 12, and 24 h after transient MCAO as compared to the later applications at 36, 48, and 60 h after transient MCAO. The motor function of the mice was assessed by rotarod test which records the latency to fall from a rotarod. The mice treated with MRS 2179 showed improved motoric function compared to the untreated mice, as demonstrated by increased latencies to fall in the rotarod test. Immunostaining with Iba1 and OX42, a microglial marker, showed lower intensity in the group treated with MRS 2179 implicating that microglial activation and/or macrophage accumulation was inhibited. The investigations of the involved pathways showed that MRS2179 lowered the amount of p-RelA, a subunit of NF κ B, and that the inhibition of NF κ B with ammonium pyrrolidine dithiocarbamate mimicked the effects of MRS2179 concerning the infarct size and motor functions after stroke. Taken together, this study suggests that P2Y₁ receptor activation by MRS2365 initiates the NF κ B pathway enhancing the inflammatory response and increasing the infarct size after stroke. Blocking the P2Y₁ receptor by MRS2179 attenuates the inflammatory response and decreases the infarct size [90].

The mechanism of protection by P2Y₁ receptor-specific antagonists in MCAO models is unknown. Possible hypotheses are similar to those explaining the protective effects of broad range P2Y receptor antagonist, which we discussed above in Fig. 3. These effects on the inflammatory response cannot be explained by inhibition of P2Y₆ and P2Y₁₂ receptors on microglia, but by induction of release of TNF α and glutamate from astrocytes. TNF α is a proinflammatory cytokine which might contribute to enhanced inflammatory

responses and cell death. Glutamate release exacerbates excitotoxicity and leads to degeneration of neurons.

A stroke model different from MCAO uses the injection of Rose Bengal into the mice and the illumination of arteries on the cortical surface with a green laser at 543 nm causing the formation of clots in these vessels [91]. Here, the infarct size was evaluated with three different methods. The fluorescently labeled CD40 antibody was injected 2, 3, or 4 days after photo-thrombotic clotting and *in vivo* images were recorded. CD40 is a protein from the TNF receptor family and an important marker for necrosis during ischemia. TTC staining was performed on brain sections of mice at days 1 and 5 after photo-thrombotic clotting. Transgenic mice expressing GFP-GFAP were used for experiments. The infarction border was visualized by confocal imaging as absence of GFP fluorescence at day 1 or 2 after stroke. This also allows monitoring the degree of swelling in astrocytes. All methods showed that a tail vein injection of 2MeSADP (0.1 ml, 100 μ M) significantly reduced the infarct size after stroke and decreased the swelling of astrocytes in the border area of the ischemic core. Application of Ruthenium 360, which is a specific blocker of mitochondrial Ca^{2+} uptake, inhibited the 2MeSADP-mediated protective effect. The protection by 2MeSADP is attributed to the increase of intracellular Ca^{2+} and stimulation of the mitochondrial matrix dehydrogenases. The enhanced mitochondrial metabolism helps to maintain the ion homeostasis and avoids swelling and cell death in astrocytes. An important characteristic of the Rose Bengal photo-thrombosis model is that it produces much smaller ischemic volumes in comparison to MCAO models. The former resemble silent strokes. The results from the Rose Bengal photo-thrombosis model are extraordinary as it represents the only *in vivo* stroke model that demonstrates benefits of P2Y receptor activation [91].

The Rose Bengal photo-thrombosis model was also applied to transgenic mice expressing yellow fluorescent protein in neurons and green fluorescent protein in astrocytes [92]. Confocal imaging at 24 h after photo-thrombosis revealed that both infarct size and neuronal loss were reduced by injection of 2MeSADP directly after onset of photo-thrombosis. Investigation of dendrites showed beading at 3 h after onset of photo-thrombosis. Beading is a morphological hallmark of early ischemic damage. Injection of 2MeSADP at 3 h after photo-thrombosis could reverse beading of dendrites within 3 h. Mitochondrial polarization during ischemia was estimated by tetra-methyl rhodamine methyl ester staining. This method showed that depolarization that occurred after 3 h of ischemia could be reversed by injection of 2MeSADP. Mitochondrial repolarization after photo-thrombosis did not occur in inositol-(1,4,5) trisphosphate receptor-type2 knockout mice [92]. These results underline that P2Y receptors and the inositol trisphosphate- Ca^{2+} signaling pathway might play an important role in neuroprotection in mild strokes. This study underlines the significance of the photo-thrombosis model in

demonstrating beneficial effects of P2Y receptor agonists. The interpretation of these exceptional findings will be given in the context of our conclusions below.

Similar to stroke, the TBI after accidents is a common cause of death and disability. A study investigated the role of P2 receptor antagonists in TBI [93]. A controlled cortical impact was applied to the head of mice with a pneumatic impactor. MRS2179, a P2Y₁ receptor antagonist, was administered with an osmotic minipump, which was implanted 3 days prior to the controlled cortical impact. At days 3 or 7 after the controlled cortical impact, hippocampal slices of the CA3 region were investigated with cresyl violet staining to determine the neuronal loss. Groups treated with MRS2179 showed significantly reduced neuronal loss after controlled cortical impact. In the same study, cognitive functions were tested in the Morris water maze at 3 or 7 days after controlled cortical impact. The Morris water maze uses a pool with turbid water which has a safe platform close to the surface. Marks on the wall of the pool allow orientation. Swim speed and latency time are recorded for mice to find the safe platform, after learning its position. Animals treated with MRS2179 showed reduced latency but similar peak swim speed compared to the control group. This reduced latency may be attributed to improved cognition in the MRS2179-treated group of animals. The mechanism of protection is unclear, but reduced release of glutamate may inhibit excitotoxicity. Blocking hippocampal P2Y₁ receptors might lead to an enhancement of synaptic signaling which might trigger pro-survival pathways and antioxidant mechanism [93].

The hypothesized mechanisms of protection in TBI models are quite similar to mechanism of protection discussed for the MCAO models, as presented above in Fig. 3.

Conclusions and future perspectives

There seems to be a conflict concerning reports describing a protective role of P2Y receptors either in *in vitro* or *in vivo* brain damage models. This includes models for oxidative stress, apoptosis, TBI, and MCAO. P2Y receptor activation *in vitro* is protective by antioxidant and antiapoptotic actions. Improved outcome in models of MCAO and TBI after P2Y receptor blocking has been observed in *in vivo* studies. This appears to be contradictory, but one has to keep in mind that those cellular models and animal models differ strongly from a methodological point of view. Cellular models are well suitable for investigation of pathways connected to receptors, mRNA, and protein levels or the influence of toxic agents. However, their significance for description of the complex interplay of events during diseases in a whole organism is limited.

In contrast to cellular models, animal models imply crosstalk of different cell types, including astrocytes, neurons,

and microglia. The role of microglia and blood-derived immune cells during neuroinflammation is completely absent in cellular models, but contributes crucially to the pathogenesis of neurodegenerative disease, stroke, and TBI.

Microglial P2Y₆ and P2Y₁₂ receptors were shown to be targets for neuroprotection in vivo and in neural/microglial mixed cultures. Medication with P2Y₆ and P2Y₁₂ receptor antagonists to avoid phagocytosis of salvageable cells is a promising pharmaceutical application for stroke and also for neurodegenerative diseases displaying chronic inflammation. Application of specific synthetic P2Y₆ and P2Y₁₂ receptor agonists or antagonists might help to induce beneficial immunomodulation.

Another important factor which distinguishes in vitro models from the in vivo MCAO models concerning P2Y receptors is that the cells in vitro are often preincubated with nucleotides and have sufficient amounts of oxygen and substrates during that period. This enables adaptation mechanisms like upregulation of antioxidant enzymes and antiapoptotic proteins. The MCAO model provides no opportunity for such adaptation because treatment takes place after MCAO and oxygen and substrates are instantaneously limited. Here, treatment with P2Y receptor agonists worsens the outcome by increased cytosolic Ca²⁺ concentration, enhanced glutamate release, and exacerbated neuroinflammation.

This design of the in vivo studies in which preincubation protocols are not tested resembles the clinical practice. In this context, the study using the photo-thrombosis-induced infarct model seems to support the idea that adaptation requires oxygen and substrates. This is the only study which shows that P2Y receptor agonists improve the outcome in a stroke model. The study uses a model resembling a silent stroke with small infarct volume and less pronounced limitation of oxygen and substrates. Also early application of 2MeSADP was more beneficial than application after a 24-h delay. Generally, the photo-thrombosis-induced infarct model leaves a time window for adaptation mechanisms that are enhanced by P2Y receptor activation. The inflammation process is less severe and the amount of salvageable brain tissue is higher as compared to MCAO models. This suggests that the application of P2Y₁ receptor agonists in mild cases of stroke could improve the outcome.

The studies using a MCAO model prove beneficial effects of P2Y receptor antagonists and harmful effects of P2Y₁ receptor agonists concerning the size of the infarct and the motor abilities. These studies suggest that during MCAO the P2Y₁ receptor enhances the inflammatory response and increases the infarct size in the brain during the first days after the incident. A pronounced and instant limitation of oxygen and substrates in the MCAO model leads to massive cell death and release of nucleotides, which makes further P2Y receptor agonist treatment ineffective. However, the longest period of time between MCAO and determination of the infarct size

was 28 days after MCAO. At 28 days after MCAO, there was no difference between the untreated and the P2Y receptor antagonist-treated group. To draw further conclusions for clinical use, it is necessary to investigate the long-term outcome of stroke models using P2Y receptor agonists or antagonists beyond 28 days after MCAO.

Yet, in the P2Y receptor family, only the P2Y₁ receptor was specifically targeted for investigations in the context of stroke. Other P2Y receptor subtypes could also be considered as potential drug targets. A pretreatment regime shows protective effects in cellular models. It would be interesting to investigate a pretreatment regime with P2Y receptor agonists in a MCAO model. The currently available data suggest that after severe stroke or TBI, a treatment with P2Y₁ receptor antagonist may improve the outcome, whereas during mild stroke a treatment with P2Y₁ receptor agonists might be beneficial. Treatment of risk groups with P2Y receptor agonists as preventive measure against stroke should be investigated. For pharmacological use, the design of P2Y receptor agonists and antagonists, which specifically target the brain by passing the BBB, is necessary. The potential pharmaceuticals have to be chemically stable to provide sufficient bioavailability. Negative interference with other P2Y receptor-dependent processes, like blood coagulation, has to be ruled out. P2Y receptors need to be specifically targeted.

Interaction of P2Y receptors with other types of P2Y receptors or membrane proteins needs to be considered in this process. It is also noteworthy to mention that both in primary cell cultures and in vivo models, respectively, different subtypes of P2Y receptors are expressed. Finally, P2Y receptors may form homo- or heterooligomers with each other or interact with other receptors in neurons and glia cells [94]. We have previously reported heterooligomerization of P2Y₁₁ receptors specifically with P2Y₁ receptors that controls the internalization and ligand selectivity of the P2Y₁₁ receptor [95, 96]. In addition, adenosine A1 receptors and P2Y₁ receptors form multimeric complexes in astrocytes [97]. Additionally interactions with P2XRs were reported in primary rat cerebellar astrocytes [98]. From that point of view even if one P2Y receptor subtype might be predominantly responsible for the observed effects, the contribution of other P2Y receptor subtypes or interaction partners has to be clarified.

Our analysis and conclusions will certainly motivate substantial further research concerning the role of purinergic signaling during stroke and neurodegenerative conditions based on the design of specific P2Y receptor ligands for pharmaceutical use.

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