Purinergic Signalling (2016) 12:427–437
DOI 10.1007/s11302-016-9514-7

REVIEW ARTICLE

A critical look at the function of the $P2Y_{11}$ receptor

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Received: 3 September 2015 / Accepted: 17 April 2016 / Published online: 31 May 2016

Abstract The $P2Y_{11}$ receptor is a member of the purinergic receptor family. It has been overlooked, somewhat due to the lack of a *P2ry11* gene orthologue in the murine genome, which prevents the generation of knockout mice, which have been so helpful for defining the roles of other P2Y receptors. Furthermore, some of the studies reported to date have methodological shortcomings, making it difficult to determine the function of $P2Y_{11}$ with certainty. In this review, we discuss the lack of a murine "$P2Y_{11}$-like receptor" and highlight the limitations of the currently available methods used to investigate the $P2Y_{11}$ receptor. These methods include protein recognition with antibodies that show very little specificity, gene expression studies that completely overlook the existence of a fusion transcript between the adjacent *PPAN* gene and *P2RY11*, and agonists/antagonists reported to be specific for the $P2Y_{11}$ receptor but which have not been tested for activity on numerous other adenosine 5′-triphosphate (ATP)-binding receptors. We suggest a set of criteria for evaluating whether a dataset describes effects mediated by the $P2Y_{11}$ receptor. Following these criteria, we conclude that the current evidence suggests a role for $P2Y_{11}$ in immune activation with cell type-specific effects.

Keywords NAD^+ · BzATP · NF157 · NF340 · NF546 · Rat

Electronic supplementary material The online version of this article (doi:10.1007/s11302-016-9514-7) contains supplementary material, which is available to authorized users.

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Introduction

Human purinergic receptors are membrane proteins targeted by various nucleotides to convey intracellular signalling. They are subdivided into P1 and P2 subclasses that bind adenosine and phosphorylated nucleotides, respectively. P1 and P2Y receptors are G protein-coupled receptors, whereas P2X receptors are ligand-gated ion channels. Based on primary ligand affinity and G protein coupling, the P2Y receptors are subdivided into a family of eight receptors: $P2Y_{1,2,4,6,11,12,13,14}$ (reviewed by [1]).

P2RY11 mRNA transcripts were first isolated from human placenta using probes corresponding to partial sequences of third to seventh transmembrane segment of the $P2Y_4$ receptor. The resulting three partial sequences were used to screen a human genomic library for the complete transcript. This resulted in a 1113-base pair (bp) cDNA transcript (AF030335) encoding a 371 amino acid protein sequence (AAB88674.1) [2]. This was later corrected to a 1125-bp transcript (AJ298334) resulting in a 374 amino acid-long protein (CAC29362.1) after it became clear that the first sequence was actually the result of a cDNA transcript arising from intergenic splicing of *P2RY11* and the adjacent gene *PPAN* [3]. Unlike other P2Y receptors, *P2RY11* was interrupted by one intron, and the encoded receptor had much larger second and third extracellular loops than other P2Y subtypes [2]. The $P2Y_{11}$ receptor was found to be activated by adenosine 5′-triphosphate (ATP) and to couple to both phosphoinositide and adenylyl cyclase pathways—a unique feature among the P2Y family.

Nonexistence of a murine *P2RY11* gene orthologue

Transcripts from human *P2RY11* orthologues are present in many other species, including *Xenopus laevis* (AM040941) [4] and dog (NM_001204441) [5–7]. It was questioned

whether canine *P2RY11* was a true orthologue of human *P2RY11*, because the $P2Y_{11}$ protein sequence from dog (NP_001191370) and human (CAC29362.1) has only 70 % amino acid identity and the receptors display strikingly different nucleotide selectivity [6]. On the genetic level, the canine *P2RY11* gene is located in the same synteny as other mammalian species, suggesting that it is indeed an orthologue of the human gene [4] (Fig. 1).

No murine *P2ry11* has yet been cloned, and it is not clear whether rats and mice have a functional $P2Y_{11}$ receptor. Three studies have tried to detect *P2ry11* in murine cells with RT-PCR. Two studies used primers that targeted the human *P2RY11* to explore *P2ry11* in mouse macrophages or rat hippocampus [8, 9]. In the third study primers designed against a claimed rat *P2ry11* sequence were used to test the presence in mouse cells [10]. Only rat hippocampus resulted in a band on agarose gel separation, although blasting the reported primer sequences against the mouse or rat genomes, respectively, also gave no specific result (own observation). Using Ensemble Genome Browser to align the nucleotide sequences surrounding human *P2RY11* with its orthologues from selected mammals, it is evident that no *P2ry11* gene exists at the expected position in rats and mice (Fig. 1). This strongly suggests that the murine genomes do not encode a genuine *P2ry11* gene. Stimulation of murine cells with ATP has been shown to increase cyclic adenosine 3′,5′-monophosphate (cAMP), a phenomenon attributed to $P2Y_{11}$ in human cells [11–16]. The rise in cAMP could arise from secondary effects of ATP acting through other signalling pathways, and the existence of an as yet uncharacterized adenylyl cyclase-coupled receptor sensing ATP cannot be excluded. This unidentified receptor is not predicted to display protein similarity with the human $P2Y_{11}$ receptor (see below).

P2RY11 or *PPAN-P2RY11*?

The *P2RY11* gene is adjacent to the *PPAN* gene on chromosome 19 in humans. These two genes have been found to form a fusion transcript resulting from the splicing of the human *P2RY11* and *PPAN* genes. The fusion transcript lacks the last two thirds of the final exon in *PPAN* and the first exon in *P2RY11*. Such intergenic splicing is not often observed in mammalian cells, with only a handful of studies showing similar examples [3]. The *PPAN-P2RY11* transcript was tested by northern blot and found to be expressed in all the tissue types examined. It is also upregulated in response to retinoic acid-mediated granulocytic differentiation of HL-60 cells. The fusion transcript is predicted to result in a chimeric protein PPAN-$P2Y_{11}$, with a size of approximately 90 kDa and consisting of most of the $P2Y_{11}$ receptor, including the seven transmembrane loops, linked to the large PPAN protein in an extracellular position. Based on western blot analysis from transfected cells, the relative expression of the fusion protein was found to be much lower than that of the $P2Y_{11}$ receptor itself, suggesting it might be less stable than the $P2Y_{11}$ receptor. This is also reflected in stably transfected CHO-K1 cells, in which the fusion protein generates a lower maximum level of cAMP response to ATP [3].

P2RY11 mRNA shares much of its sequence with the *PPAN-P2RY11* transcript, so they cannot be distinguished by RT-PCR unless primers are designed to recognize only the *P2RY11* transcript. This has been a huge problem in the studies reported so far. To our knowledge, only five articles have been published that investigate *P2RY11* mRNA expression without detecting the fusion transcript [17–21]. All other studies have featured primer sets predicted additionally to recognize the fusion transcript (Table S1). This is a general problem that seems to have been completely overlooked. Use of the wrong sequence to design primers for detecting *P2RY11* mRNA can result in grave errors, as seen, for instance, when primers only target *PPAN* and not *P2RY11* mRNA [22, 23]. Another example is the use of primer sets designed to target only the fusion transcript mRNA, rather than *P2RY11* mRNA [24–26]. Use of such a primer set resulted in a product that was very pronounced in NB4 cells, indicating that the fusion transcript is strongly expressed in these cells. The same cells were also examined by northern blot using probes predicted to

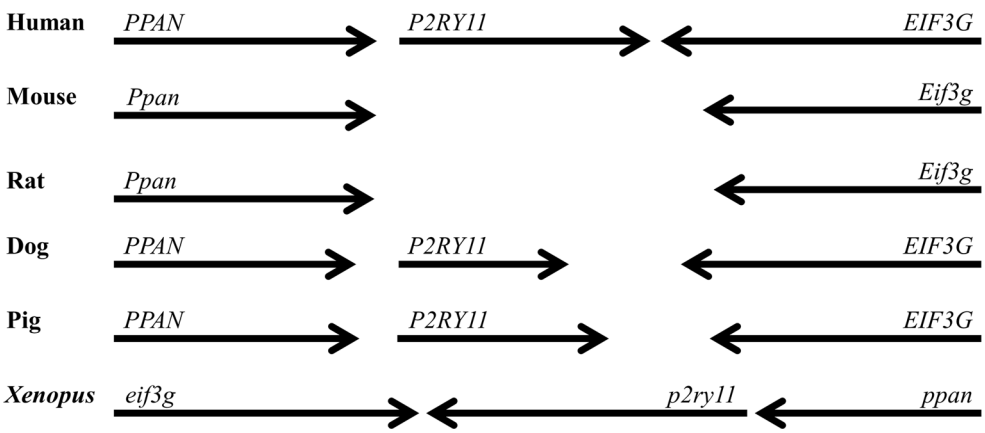

Fig. 1 Genomic alignment showing human and selected other species at the *PPAN*, *P2RY11*, and *EIF3G* genomic synteny. Alignment was based on RefSeq transcript sequences from the Ensembl genome browser (www.ensembl.org)

target both *P2RY11* and *PPAN-P2RY11* mRNA. The fusion transcript was clearly evident on the gel along with *P2RY11* [26]. Alarmingly, this suggests that many studies reporting *P2RY11* mRNA expression might not have measured the correct transcript.

$P2Y_{11}$ antibodies lack specificity

RT-PCR is a very sensitive method for studying gene expression that enables very small amounts of mRNAs to be amplified that might not play a significant role in the tissue. Several examples indicate that *P2RY11* mRNA detection with RT-PCR is not associated with the expression of functional $P2Y_{11}$ receptor. For instance, NT-2 cells were confirmed to express *P2RY11* mRNA [27, 28], but ATP was not found to produce an increase in the amount of cAMP [28]. $CD4^+$ T lymphocytes express the gene for $P2Y_{11}$ [18], but two $P2Y_{11}$ agonists have no effect on cAMP accumulation [29]. Prostate cancer cells express *P2RY11* transcripts, yet the pharmacological profile suggests that no functional $P2Y_{11}$ receptor is present on these cells [30]. It is possible that these discrepancies are due to the very low level of protein expression from the *P2RY11* mRNA or because the receptor does not translocate to the surface of these cells under normal circumstances.

Proof of protein expression largely depends on antibody detection. Table S2 provides an overview of the antibodies used to detect the $P2Y_{11}$ receptor. Most antibodies developed against $P2Y_{11}$ receptor target the C-terminus. When C-terminal sequences were compared with other P2Y G_q-coupled receptors, they were found to share the sequence motif SE-QXK/RSE [31], suggesting that this part of the receptor is not a good choice for specific $P2Y_{11}$ receptor detection. It is worth noting that the C-terminal is also part of the PPAN-$P2Y_{11}$ chimeric protein, so antibodies against this epitope will not distinguish the two proteins when used in immunocytochemistry or immunohistochemistry. C-terminal-targeting antibodies will discriminate $P2Y_{11}$ and PPAN-$P2Y_{11}$ when used for western blot, since they have different predicted sizes, of 40 and 90 kDa, respectively.

Most reports of $P2Y_{11}$ receptor protein expression come from studies employing a polyclonal rabbit antibody #APR-015. This antibody recognizes the $P2Y_{11}$ receptor C-terminal residue 357–373 (NATAAPKPSEPQSRELS). When used for western blot, the #APR-015 antibody resulted in bands of 33–60 kDa, all of which were stated to be monomeric $P2Y_{11}$. In one study, western blot of placental tissue protein extracts resulted in bands of 50, 60, 100, 150, and 200 kDa, which were interpreted as being the result of multimeric $P2Y_{11}$ receptor assembly [32]. Multimerization of G protein-coupled receptors including purinergic receptors has been widely observed [33], and, indeed, $P2Y_{11}$ is known to form a heterodimer with $P2Y_1$, as described below [34]. Nevertheless, multimerization is not known to result in covalent binding of receptors, and complete denaturation of the proteins as part of the western blot procedure should remove the larger bands. Hence, it is not clear what caused the bands seen in the human placental tissue. The 50-kDa band from placental control tissue believed to be $P2Y_{11}$ was compared with homogenate from pancreatic islets with a smaller band size of 45 kDa that were also claimed to be $P2Y_{11}$ [32]. In most studies, specificity of the signal from the #APR-015 antibody was tested by blocking with the control peptide antigen [11, 35–37]. In the case of the placental control tissue, the signal was still evident at 50 kDa after blocking with the immunogenic peptide. Together, these findings indicate that the #APR-015 antibody is not specific. This is consistent with results obtained in our own laboratory.

The #APR-015 antibody has been used to detect $P2Y_{11}$ in many other species. In murines, this antibody has led to the detection of what has been named the "$P2Y_{11}$-like receptor" [11, 16, 38–40]. Other antibodies have also been used to examine the existence of the murine $P2Y_{11}$. The $P2Y_{11}$ antibody #AB9590 targeting the human $P2Y_{11}$ C-terminus detects a large, 75-kDa band on western blot, which is believed to be $P2Y_{11}$ on the basis of the results of experiments using rat tissue [41, 42], and it has been claimed that another antibody with an unknown epitope confirms $P2Y_{11}$ receptor expression in rat neutrophils with a 40-kDa signal [43].

Even if there is no gene in murines located at the expected genetic synteny, the gene might have translocated and maintained its ability to produce a functionally active protein. A protein blast search using UniProt for the human $P2Y_{11}$ receptor protein sequence found the murine $P2Y_1$ receptor to be the closest murine protein, with a 32 % sequence similarity, i.e., the same degree of similarity that human $P2Y_{11}$ shares with human $P2Y_1$ (Table S3). This shows that murines do not express a protein resembling human $P2Y_{11}$ and calls very sharply into question the use of the term "$P2Y_{11}$-like receptor." It also casts doubt on the specificity of the antibodies used to detect $P2Y_{11}$ receptor protein reported in the literature.

Only one non-commercial C-terminal antibody has been used to detect $P2Y_{11}$ and PPAN-$P2Y_{11}$ in a transfected cell system [3]. No other $P2Y_{11}$-targeting antibody has undergone similar validation before use. This antibody recognized 90 kDa PPAN-$P2Y_{11}$, three bands of around 45 kDa on western blot reported to be $P2Y_{11}$ with different degrees of glycosylation, and gave no signal from cells with the empty vector. The first exon of $P2Y_{11}$ was predicted to encode a potential N-glycosylation site that could give rise to the different-sized bands [3]. In another study, the $P2Y_{11}$ protein was tagged with a short protein sequence from human influenza hemagglutinin (HA) and expressed in dog epithelial cells following detection of the HA tag by western blot using an anti-HA antibody. This resulted in a signal of around 46 kDa [44]. The HA tag itself

has a predicted size of only approximately 1 kDa, again suggesting the size of $P2Y_{11}$ to be around 45 kDa.

$P2Y_{11}$ internalization and dimerization

Detection of $P2Y_{11}$ protein with an expected band size on western blot is not final proof of the existence of a functional $P2Y_{11}$ receptor on the cell surface. Results of studies of the cellular trafficking and localization of the $P2Y_{11}$ receptor are ambiguous and difficult to interpret. A lack of pharmacological desensitization of the $P2Y_{11}$ receptor has been observed in several cell systems [45, 46]. In 1321N1, astrocytoma cells expressing $P2Y_{11}$-eGFP the receptor do not show ligand-activated endocytosis. When co-expressing its close homologue, the $P2Y_1$ receptor, in 1321N1 cells, $P2Y_{11}$ undergoes ligand-activated internalization as visualized by confocal microscopy. Using co-pull-down, co-immunoprecipitation, and FRET, $P2Y_{11}$ hetero-oligomerizes with $P2Y_1$ [34]. HEK-293 cells in contrast to 1321N1 cells express $P2Y_1$ endogenously [27], and $P2Y_{11}$ receptor was found to internalize following stimulation with 100 μM 3′-*O*-(4-benzoyl)benzoyl-ATP (BzATP)—a stable ATP analogue in HEK-293 cells. It was therefore suggested that $P2Y_1$ was necessary for internalizing $P2Y_{11}$ [47]. However, an earlier study showed that 100 μM BzATP did not cause internalization of wild-type $P2Y_{11}$ in transfected HEK-293 cells [34]. Further evidence that $P2Y_{11}$ internalization is not entirely dependent on $P2Y_1$ co-expression was found in HEK-293 cells transfected with a C-terminal YFP-tagged $P2Y_{11}$. In this system, stimulation with 100 μM ATP did not induce internalization. Instead, it was induced by co-expression of G protein receptor kinase 2 [48]. Together, these studies show that surface expression of the $P2Y_{11}$ receptor is dependent on many factors, and further work is needed to fully understand how this is regulated. There is also a slow desensitization of the $P2Y_6$ receptor resulting from the lack of specific C-terminal agonist-induced phosphorylation [49]. Hence, $P2Y_{11}$ receptor internalization studies performed on a C-terminal fluorescent conjugate might sterically mask residues that are essential for internalization and that would explain the ambiguous results.

The pharmacological profile of $P2Y_{11}$

The rank order of potency for a series of ATP-derived nucleotides on $P2Y_{11}$ receptor activation was found to be AR-C67085 > BzATP > adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), adenosine-5′-(α-thio)-triphosphate (ATPαS) > dATP > adenosine 5′-*O*-2-thiodiphosphate (ADPβS) > 2-methylthio-adenosine 5′-triphosphate (2-meSATP). These compounds all work in the micromolar range [45, 50]. 2-Propylthio-ATP-αB B-isomer and 2-propylthio-ATP-αB, β-γ-dichloromethylene B-isomer act in the nanomolar range on $P2Y_{11}$ and are up to 87-fold stronger than ATP [51]. One paper also suggested that $P2Y_{11}$ might have some constitutive activity [3].

Triphosphate nucleotides including uracil, guanine, cytosine, and thymine 5′-triphosphates (UTP, GTP, CTP, TTP) and uridine 5′-diphosphate (UDP) were originally shown to be inactive in 1321N1 and CHO-K1 cells stably expressing $P2Y_{11}$ [2, 3, 45]. In 2003, UTP and ATP were both found to be increased cytosolic Ca^{2+} concentration with similar EC_{50} and maximal responses in transfected 1321N1 cells [52], and UTP has also been suggested to act on the murine "$P2Y_{11}$-like receptor" [53]. Although UTP did not lead to increased inositol 1,4,5-trisphosphate (IP_3) production, as occurred with ATP, the increase in intracellular Ca^{2+} led the authors to suggest that UTP acted as a Ca^{2+}-mobilizing agonist via $P2Y_{11}$ [52]. This has recently been refuted by studies using stably expressing 1321N1 cells treated with ATP and UTP measuring intracellular Ca^{2+} and IP_3 formation, which showed that UTP was neither a biased agonist nor an antagonist at the human $P2Y_{11}$ receptor [54, 55].

Many other nucleotide compounds target the $P2Y_{11}$ receptor, including diadenosine polyphosphate Ap2A and its isomers P18 and P24 [56, 57]. Nicotinamide adenine dinucleotide (NAD^+) is also capable of activating $P2Y_{11}$ and causing an increase in intracellular Ca^{2+}, IP_3, and cAMP in $P2Y_{11}$-transfected 1321N1 cells [58, 59]. Human mesenchymal stem cells and neutrophils are activated by NAD^+ presumably through $P2Y_{11}$ [60, 61]. In these studies, specificity of the response was determined by inhibiting NAD^+-signalling with G_s and protein kinase A (PKA) inhibitor, NF157 antagonist, and *P2RY11* knockdown.

Initially, similar potencies were found for the adenylyl cyclase and phosphate inositol pathways [2]. These experiments were carried out in two cell lines, and reinvestigating the two signalling pathways in both cell types revealed a cell-specific difference in ATP potency, with the IP_3 signalling being 15-fold that of the cAMP signalling [62]. Blocking the separate G protein pathways in HL-60 cells revealed that IP_3 and cAMP signalling function independently [46]. This means that the parental cell line is extremely important when using transfected cellular systems to evaluate the pharmacological profile of the $P2Y_{11}$ receptor, because it may give rise to considerable differences in agonist potencies and efficacies.

Most pharmacological $P2Y_{11}$ receptor studies have been based on transfection studies with fluorescently tagged or non-tagged $P2Y_{11}$. C-terminal eGFP-tagged human and canine $P2Y_{11}$ receptors both showed similar signalling properties to the respective non-tagged receptors [6, 51]. Nearly all vectors expressing human $P2Y_{11}$ described in published studies were created from the initial *P2RY11* sequence arising

from the fusion transcript (AF030335) [2, 34, 45, 47, 48, 52, 54, 56, 58, 59, 62–67]. The sequence difference results in a slightly altered N-terminal of the $P2Y_{11}$ protein from its rightful MAANVSGAK to MDRGAK that originates from the transgenic splicing with *PPAN*. The biological activity of the two different receptor constructs was shown to be similar when comparing Ca^{2+} signalling after stimulation with various ATP analogues [55]. Even though this minor change in amino acid sequence in the N-terminal did not appear to affect signalling, it might still be very important in the internalization studies performed using the original transcript from the splice variant.

G protein-coupled receptors are known to homo- and hetero-oligomerize. $P2Y_{11}$ hetero-oligomerizes with $P2Y_1$. This dimerization changes the ligand selectivity of the $P2Y_{11}$ receptor and serves to fine-tune the signalling [33]. The $P2Y_{11}$ antagonist 8,8′-(carbonylbis(imino-3,1-phenylene-carbonylimino-(4-fluoro-3,1-phenylene)-carbonylimino))bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt (NF157) is unable to inhibit the effect of BzATP on $P2Y_{11}$ in transfected HEK-293 cells that endogenously express $P2Y_1$. Inhibition is effective in 1321N1 cells that do not express $P2Y_1$ [34]. This information suggests that it might be important to determine the presence of $P2Y_1$ when evaluating previous and future $P2Y_{11}$ pharmacological data.

Selective $P2Y_{11}$ activation and inhibition

There are reports of several non-selective P2 inhibitors being used in the characterization of the $P2Y_{11}$ receptor (Table 1). The first antagonist reported to display some $P2Y_{11}$ selectivity was NF157. This antagonist was tested for $P2Y_{11}$ selectivity and showed partial selectivity over $P2Y_{1,2}$ and P2X2,3,4,7 but not towards P2X1 [79]. Another more specific $P2Y_{11}$ antagonist 4,4′-(carbonylbis(imino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(naphthalene-2,6-disulfonic acid) tetrasodium salt (NF340) had four times as much antagonistic potency as NF157 in a Ca^{2+}-based assay and ten times the potency in a cAMP assay. This compound was evaluated and reported to display $P2Y_{11}$ selectivity over $P2Y_{1,2,4,6,12}$ and P2X1,2,2-3 [55].

None of the endogenous ligands reported acts specifically on $P2Y_{11}$, making the investigation of the physiological role of the receptor challenging. Only one compound is currently available as a specific $P2Y_{11}$ agonist: 4,4′-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-α,α′-diphosphonic acid) tetrasodium salt (NF546) (Table 1). This compound was evaluated for specificity over the same set of purinergic receptors as NF340 and proved to be a quite selective $P2Y_{11}$ agonist, although it also activates $P2Y_2$, $P2Y_6$, and $P2Y_{12}$ at higher doses.

Given that the $P2Y_{11}$ amino acid residues involved in NF340 and NF546 binding largely overlap with binding of ATP [55], there is a possibility that NF340 and NF546 bind to other ATP-binding receptors. NF546 and NF340 are currently the two compounds known to display the highest selectivity for $P2Y_{11}$ over a range of other purinergic receptors. The specificity has only been tested for a subset of P2 receptors, so nothing is known about the effect of these compounds on signalling through, for example, P2X4 and P2X7.

Table 1 Compounds and their effects on $P2Y_{11}$ and purinoceptor signalling

$P2Y_{11}$ agonists and antagonists					
Compound	CAS no.	$P2Y_{11}$	P2Y	P2X	Refs.
PPADS	149017-66-3	–	Non-selective	Non-selective	[45, 50, 68–71]
Suramin	129-46-4	Antagonist	Non-selective	Non-selective	[45, 50, 71–74]
RB2	12236-82-7	Antagonist	Non-selective	Non-selective	[45, 75]
Reactive red	17804-49-8	Antagonist	Non-selective	Non-selective	[16, 67, 76]
AMPS	93839-85-1	Agonist/antagonist	Non-selective	Non-selective	[50, 74, 77, 78]
NF157	104869-26-3	Antagonist	Weak antagonists at $P2Y_{1,2}$	Not selective over P2X1 Weak antagonist at P2X2,3,4,7	[59, 79]
NF340	202982-98-7	Antagonist	Selective over $P2Y_{1,2,4,6,12}$	Selective over P2X1,2,2-3	[55]
Iantherans	–	Agonists	Partial agonists at $P2Y_{1,2}$	NA	[80]
Ap2A and its isomers P18 and P24	85065-24-3	Agonists	NA	P18: P2X7 antagonist P24: P2X7 agonist	[14, 81]
NF546	1006028-37-0	Agonist	Weaker agonist for $P2Y_{2,6,12}$	Selective over P2X1,2,2-3	[55]

AMPS adenosine 5′-thiomonophosphate, *Ap2A* diadenosine diphosphate, *NA* not available, *NF157* 8,8′-(carbonylbis(imino-3,1-phenylene-carbonylimino-(4-fluoro-3,1-phenylene)-carbonylimino))bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt, *NF340* 4,4′-(carbonylbis(imino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(naphthalene-2,6-disulfonic acid) tetrasodium salt, *NF546* 4,4′-(carbonylbis(imino-3,1-phenylene-carbonylimino-3,1-(4-methyl-phenylene)-carbonylimino))-bis(1,3-xylene-α,α′-diphosphonic acid) tetrasodium salt, *PPADS* pyridoxal phosphate-6-azophenyl-2′,4′-disulfonic acid, *RB2* reactive blue 2

Consequently, published experimental studies using NF340 and NF546 for physiological $P2Y_{11}$ characterization should continue to be assessed with caution, as it has not been proved that these compounds are specific to $P2Y_{11}$ over all other ATP receptors. One example is that of the diadenosine diphosphate isomer P18, which both antagonizes P2X7 and activates $P2Y_{11}$ [14, 81], demonstrating the need to test possible $P2Y_{11}$ agonistic and antagonistic compounds on all ATP-binding P2 receptors before drawing any conclusions about specificity.

It is also worth noting that NF157, NF340, and NF546 have all been used to study the murine "$P2Y_{11}$-like receptor" that shows many of the same properties as seen in humans [14, 41–43, 82–84]. Assuming that no $P2Y_{11}$ receptor exists in murines, these compounds must have other mechanisms by which they interfere with cell signalling. If that is indeed the case, it is very likely that the compounds will also have effects in human cells not mediated by the $P2Y_{11}$ receptor. On the other hand, should NF157, NF340, and NF546 target an as yet unidentified murine receptor, then this receptor would not be predicted to share homology with the human $P2Y_{11}$ receptor (Table S3), in which case the specificity of these compounds would be questionable.

Pinpointing an effect of signalling via the $P2Y_{11}$ receptor is difficult with the currently available pharmacological tools. For instance, $P2Y_{11}$ is the only ATP receptor known to mediate a rise in intracellular cAMP, which many experimental studies consider to be proof of $P2Y_{11}$ receptor signalling in response to ATP. This cannot be considered valid proof of $P2Y_{11}$ receptor activation, since ATP might also act through $P2Y_2$ to stimulate cellular release of arachidonic acid, which can act in an autocrine fashion after conversion to prostaglandins and result in a rise in intracellular cAMP through prostaglandin receptors [85]. This is also evidenced by the observation that stimulation with ATP results in significant cAMP increases in various cell types in rats and mice [11–16, 86]. This shows that the mechanism for an increase in intracellular cAMP following ATP stimulation is not by itself proof of $P2Y_{11}$ receptor activation.

Another argument advanced to confirm $P2Y_{11}$ receptor activation arises from the use of BzATP targeting $P2Y_{11}$ and P2X7. BzATP stimulation leads to an increase in intracellular Ca^{2+} via ion flux from the extracellular space in the case of P2X7 or from intracellular stores in the case of $P2Y_{11}$. An increase in intracellular Ca^{2+} following BzATP stimulation carried out in Ca^{2+}-free medium is often concluded to occur via $P2Y_{11}$. Such interpretations might be oversimplified and the observation possibly does not even involve $P2Y_{11}$. Studies exploring the effects of purinergic signalling on various endpoints suggesting activation of $P2Y_{11}$ are listed in Table S4, including the attempts to confirm $P2Y_{11}$ receptor involvement.

P2RY11 knockdown: a note of caution

The lack of specific agonists/antagonists challenges the examinations of the specific physiological role of the $P2Y_{11}$ receptor. Methods to knock down *P2RY11* mRNA expression provide a good supplemental technique for studying $P2Y_{11}$ receptor function. However, the RNA silencing sequences are often not provided, making it difficult to replicate and evaluate the specificity of the RNA silencing sequence used. In these cases, it is not possible to distinguish the effect of *P2RY11* and *PPAN-P2RY11* (Table S1). Also, *P2RY11* RNA silencing efficiencies have most often been verified using questionable RT-PCR and antibody detection, as described above. Hence, the results from these studies must be interpreted with some caution.

Identification of genuine effects mediated by $P2Y_{11}$

Due to the lack of effective detection and functional methods, the physiological role of the $P2Y_{11}$ remains unclear. To assess the physiological effects most likely to be mediated by the $P2Y_{11}$ receptor, we propose a set of criteria that can be used to identify the studies that are most likely to have targeted $P2Y_{11}$ function. First, experiments had to be carried out in a non-murine species using tissue or cells shown to express *P2RY11* or *PPAN-P2RY11* mRNA or protein by western blot with the correct band size of around 40 ± 10 kDa (Table 2, expression criteria). Tissue, cell types, and cell lines that fulfill the expression criteria are listed in Table S5.

Table 2 Expression and functionality criteria, respectively, used to select physiological effects reported in literature that were most likely mediated by the $P2Y_{11}$ receptor

Expression criteria
1. Non-murine species
2. Specific *P2RY11/PPAN-P2RY11* PCR primers or WB with 40 ± 10 kDa band

Functionality criteria
1. Use tissue/cells that fulfill the expression criteria
2. At least two of the following:
   - Use of NF546/NF157/NF340
   - *P2RY11* RNA interference
   - Negative in test for other P2 receptors including $P2Y_1$ and/or $P2X_7$

Only tissue and cells from a non-murine species using PCR primers specific to *P2RY11*/*PPAN-P2RY11* or detecting protein on western blot (WB) with a size in the expected range of 40 ± 10 kDa were considered to express the $P2Y_{11}$ receptor. Studies that used tissue or cells that fulfilled the expression criteria and applied at least two of three different approaches to prove the activation of the $P2Y_{11}$ receptor were considered most likely to describe an effect mediated by the $P2Y_{11}$ receptor. Many studies have investigated the role of the $P2Y_{11}$ receptor but have not fulfilled these criteria [11–16, 18, 21, 24–26, 36, 41–43, 55, 56, 61, 67, 74, 82, 83, 86–122]

As discussed previously, all currently available methods used to investigate the role of the $P2Y_{11}$ receptor have limitations. Thus, our criteria for evaluating the activation of the $P2Y_{11}$ receptor require it to have been proved by at least two of three approaches for it to be considered truly mediated by $P2Y_{11}$ (Table 2, functionality criteria). These approaches were (1) the use of pharmacological compounds with proven specificity for $P2Y_{11}$ over most other P2 receptors (currently NF546, NF157, and NF340), (2) *P2RY11* RNA interference, and (3) tests for activation of other P2 receptors with specific focus on $P2Y_1$ that share the greatest homology with the $P2Y_{11}$ receptor and P2X7, which is also activated by BzATP. None of these three approaches is considered independently valid for the reasons discussed above. When used in combination in tissue shown in the same or another study to express the $P2Y_{11}$ receptor, we consider this a reliable way of identifying effects that are truly mediated by the $P2Y_{11}$ receptor with the currently available methods.

Nine articles fulfilled the functionality criteria. Several of these studies report $P2Y_{11}$ activation in various immune cells to result in a pro-inflammatory response. The effects include a lower rate of CX_3CL-mediated endothelial killing and migration in natural killer (NK) cells [123], delayed apoptosis in neutrophils [124], and increased chemotaxis in granulocytes [58, 59]. In mesenchymal stem cells, NAD^+ stimulation of $P2Y_{11}$ resulted in cytokine release and chemotaxis [60]. $P2Y_{11}$ facilitated skin repair by the release of interleukin-6 (IL-6) in keratinocytes following IFNγ-induced ATP stimulation [125] and in LXA_4-treated bronchial cystic fibrotic epithelium $P2Y_{11}$ promoted proliferation, migration, and wound repair [20]. IL-6 and other cytokines were also released following LPS-induced ATP release and $P2Y_{11}$ activation in THP-1 macrophages as a pro-inflammatory response [84]. This suggests that $P2Y_{11}$ helps mediate the response to immune triggers during inflammation in immune cells. One article describes $P2Y_{11}$ exercising an immuno-suppressive role in monocyte-derived dendritic cells by decreasing the release of cytokines such as IL-6 and IL-12 following LPS-induced ATP release [22]. This suggests that the $P2Y_{11}$ receptor acts in a cell type-specific manner and that a pro- or anti-inflammatory response might depend on many other factors, such as the immune trigger or the subset of other ATP-sensing receptors present on the cell.

It is important to note when deducing the physiological role of the $P2Y_{11}$ receptor as a meta-analysis from the available literature is that this is a self-fuelling system. The data produced are based on evidence from previous findings. Hence, the role of the $P2Y_{11}$ receptor as an immunomodulatory receptor does not rule out the possibility that it contributes to other important effects and merely reflects the data available.

P2RY11 polymorphisms—a hint of $P2Y_{11}$ receptor functions

The activity of $P2Y_{11}$ as an immune-regulatory receptor has been reasserted by reports of *P2RY11* single nucleotide polymorphisms (SNPs) associated with human disorders with immunological pathogenesis. A *P2RY11* A87T polymorphism (rs3745601) increases the odds of acute myocardial infarction (AMI) and is associated with a higher level of blood C-reactive protein [126]. $P2Y_{11}$ receptors carrying the mutation have reduced Ca^{2+} and cAMP signalling properties [47], implying that less $P2Y_{11}$ signalling was associated with AMI. Another *P2RY11* polymorphism (rs2305795) is associated with the sleep disorder narcolepsy [18, 127]. It is unclear whether this has a functional effect on the pathogenesis or is merely the result of linkage disequilibrium between another associated polymorphism located in the neighboring gene, *EIF3G* (rs3826784) [128]. The *P2RY11* rs2305795 polymorphism is located in the 3′-untranslated region that usually plays a role in regulating transcription. Concordantly, the level of gene expression of *P2RY11* is lower with the narcolepsy-associated genotype in $CD8^+$ T lymphocytes and NK lymphocytes and is correlated with cell viability [18]. Growing evidence indicates that the cellular immune system plays a role in cardiovascular disease (reviewed by [129]) and narcolepsy (reviewed by [130]). This indirectly supports the hypothesis that $P2Y_{11}$ plays a role in immune regulation.

It is possible that the $P2Y_{11}$ receptor is involved in other immunopathological conditions. Many genome-wide association studies do not include probes for detecting polymorphisms in this gene, which means that the genetic associations of this receptor in immunogenic diseases largely remain to be discovered. Including probes for detecting disease-associated polymorphisms in *P2RY11* could expand the range of diseases associated with variations in the *P2RY11* gene. Such knowledge would provide several pointers for the direction of future research.

Conclusion

The purinergic $P2Y_{11}$ receptor senses ATP and NAD^+ released into the extracellular environment. This review provides a critical summary of the research into $P2Y_{11}$ receptor expression and function. Overall, investigations are often incomplete or ambiguous and all too often based solely on pharmacological speculations. They are further compromised by the fact that murines most probably do not have a true orthologue to the human *P2RY11*, since bioinformatic tools do not predict a similar a genetic sequence, mRNA transcript, or protein to the human $P2Y_{11}$ receptor in murines. Gene expression studies of human *P2RY11* have disregarded the existence of the fusion transcript *PPAN-P2RY11* when

designing primers and therefore might not have measured the correct transcript accurately. Additionally, protein detection with antibodies lacks specificity since the band size observed with western blot varies considerably. Further, some $P2Y_{11}$ antibodies detect an epitope present in murines. The agonists and antagonists with reported selectivity for the $P2Y_{11}$ receptor have not been tested for reactivity towards several other ATP receptors. To address these challenges, we have proposed a set of criteria that can be used when evaluating the evidence regarding the function of $P2Y_{11}$. Using these criteria, research to date suggests a role for $P2Y_{11}$ in immune activation with cell type-specific effects.

Acknowledgments Financial support was provided as a Lundbeck Fellowship awarded to Birgitte Rahbek Kornum by the Lundbeck Foundation.

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