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Methodological guidelines for P2X receptor assays and data interpretation

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P2X receptors (P2XR) are a family of seven cation channels gated by extracellular ATP (eATP). Activation of P2XRs results in diverse cellular responses, including cell signalling, proliferation, differentiation, and death—all critically important in multiple physiological and pathophysiological states. These receptors, therefore, represent therapeutic targets of considerable interest. However, P2XRs, while structurally related, exhibit highly divergent and context-dependent functions. Their spatiotemporal and functional complexity is evident by overlapping expression across multiple cell types that can shift dynamically during physiological processes or disease progression. Furthermore, P2XRs can assemble as homo- or hetero-trimers, with distinct functional properties. These factors complicate definitive identification of a given P2XR responsible for a specific pathophysiological effect. Receptor activity in vivo is transient because of receptor-specific mechanisms and follows eATP breakdown by ectonucleotidases. Any correlation of ATP release with receptor engagement, as assessed in vitro, often does not correspond with the in vivo dynamics. Translation from animal models to humans is complicated by the species-specific pharmacology of some P2XRs, confounded by many animal models in use not fully replicating human P2XR function and regulation in pathology. Furthermore, there are no clinical biomarkers to distinguish incomplete receptor blockade from lack of therapeutic effect. Thus, translation has been very limited. To identify and validate specific P2XR functionalities, future experimental designs should use approaches and assays that can reliably assess receptor involvement, while reducing methodologically flawed findings. We propose guidelines developed in consultation with the purinergic community for consistent and reliable research practices in P2XR studies.

Cell Death and Disease (2026)17:521 ; https://doi.org/10.1038/s41419-026-08730-0

FACTS

- Definitively determining which P2XR subtype mediates a given physiological or pathological effect remains challenging.
- Although heteromeric P2XR differ functionally from homo-trimers, their physiological and pathological relevance is still poorly understood.

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Edited by Professor Ivano Amelio

Received: 18 January 2026 Revised: 15 February 2026 Accepted: 27 March 2026
Published online: 20 April 2026

- Rapid eATP degradation and receptor-specific activation kinetics make it difficult to link ATP release to actual P2XR engagement in vivo.
- Differences between human and rodent P2XR pharmacology, coupled with the scarcity of human-relevant disease models, significantly complicate translational efforts.
- The absence of reliable biomarkers to distinguish insufficient P2XR blockade from genuine therapeutic ineffectiveness hinders clinical progress.

FOREWORD

This paper was conceived in Ferrara, in September 2024, during discussions between Francesco di Virgilio (Fig. 1) and Darek Gorecki on shared concerns regarding the methodological inconsistencies and limitations in experimental designs used to demonstrate P2XR activation across various experimental paradigms. Tragically, three weeks later, Francesco died suddenly in Chengdu, where he had participated in the Neuroglia conference. Francesco was deeply respected and loved by the purinergic community, and this paper is a tribute to him and in recognition of his life's work.

GENERAL CONSIDERATIONS

Seven P2X receptor subunits (P2X1-7R) assembled as homomeric and heteromeric receptors are archetypal ligand (ATP)-gated cationic channels permeable to Na^+, K^+, and Ca^{2+} involved in numerous physiological and pathological processes. These receptors are widely expressed across various cell types, where they play a crucial role in cell signalling [1]. Activation of P2XRs by extracellular ATP (eATP) contributes to diverse cellular functions, including cell proliferation, differentiation, and death. However, their specific roles in these processes vary significantly depending on the cell type and the particular P2XR subtype(s) involved.

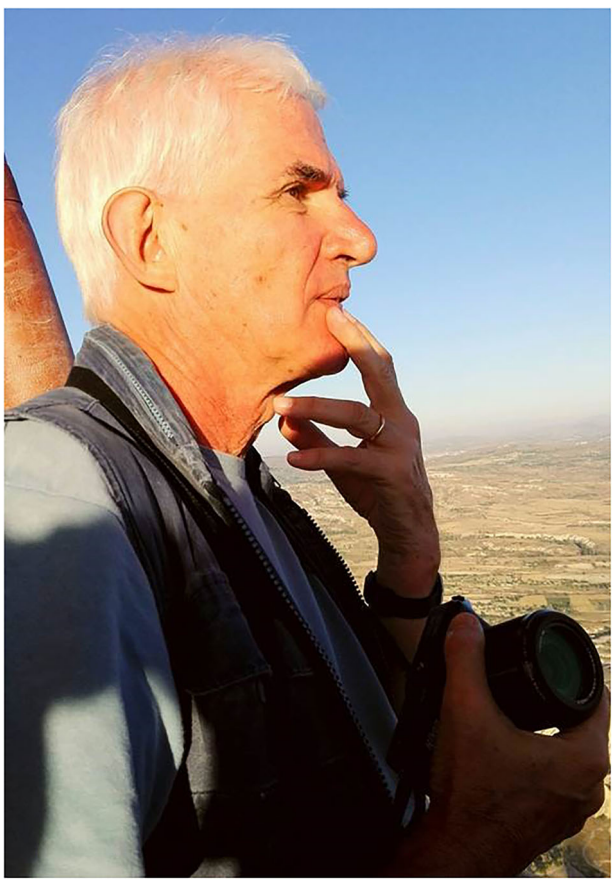

Fig. 1 Francesco Di Virgilio (1954–2024). Finis vitae sed opus pergit.

The outcome of P2XR activation is also highly context-dependent and may lead to contrasting effects—for instance, promoting cell death in one cell type while stimulating proliferation and assisting survival in another. This variability underscores the multifaceted P2XR functions but also highlights the potential of these receptors as therapeutic targets in various human diseases. To this end, a variety of selective pharmacological agonists, antagonists, and biologics have been developed, with new therapeutic agents continually emerging [2]. Several of these agents entered clinical trials, emphasising the potential for P2X receptor-targeted therapies [3, 4]. Yet, many trials reported disappointing results [5, 6], which may partly be due to the incomplete understanding of specific functions of P2XRs.

Given the growing momentum in developing therapeutically relevant drugs targeting P2XRs, it seems important to establish a universally accepted set of criteria to validate the specificity and potency of these agents. It is also helpful to provide the scientific community with reliable recommendations detailing assays that can robustly assess P2XR roles across a variety of experimental paradigms. Such standards would allow researchers to better cross-reference findings between laboratories, without stifling the development of new, improved assays or methods. This approach may also provide newcomers to the field a practical toolkit for assessing core P2XR functions.

We present a contemporary set of guidelines developed in consultation with the wide purinergic community. It is intended for researchers to plan and interpret their experiments, and for reviewers to critically assess experimental approaches involving P2XRs. Adherence to these guidelines should enhance the quality of analysis and facilitate the identification of new P2XR functions and therapeutic applications. As experimental methodologies evolve and new assays emerge, these guidelines will need to be revisited and refined. Nevertheless, these will hopefully serve as a foundation for consistent and reliable research practices in P2XR studies.

Analysing P2XR functions requires several key factors to be considered (Fig. 2), as these receptors exhibit significant functional diversity despite shared characteristics. Specifically, although ATP is the primary agonist for P2XRs, different subtypes have varying response thresholds, spanning the nM to mM range. Furthermore, different P2XRs have distinct desensitisation kinetics; kinetics of receptor-generated ion current and ATP stability are temperature-dependent and pH-sensitive. Therefore, the pH and temperature of the assay medium must be controlled, and agonist application timing optimised. The functional inhibition by extracellular divalent cations must also be considered (see section: *The electrophysiological methods in P2X receptor studies*).

The release of endogenous ATP can be triggered, in a cell-specific manner, by relatively trivial manipulations of cells in culture, and affect the experimental outcome. On the other hand, eATP degradation occurring in the course of the experiment can diminish the P2XRs activation but also produce bioactive breakdown products (e.g., adenosine), activating other receptors, often with opposing functions (see section: *Control for ATP release and degradation*).

Additionally, specific assays may not be applicable across all cell types, tissues, or organisms, with some noticeable species-specific P2XR differences. Primary cell cultures (e.g., neuroglia, immune cells) often express multiple P2XR subtypes whose expression can vary with time in culture and culture conditions. Recombinant expression of specific P2XRs provides more controlled conditions, but such systems should be controlled for the effects of receptor overexpression, altered receptor trafficking, and also ensure the

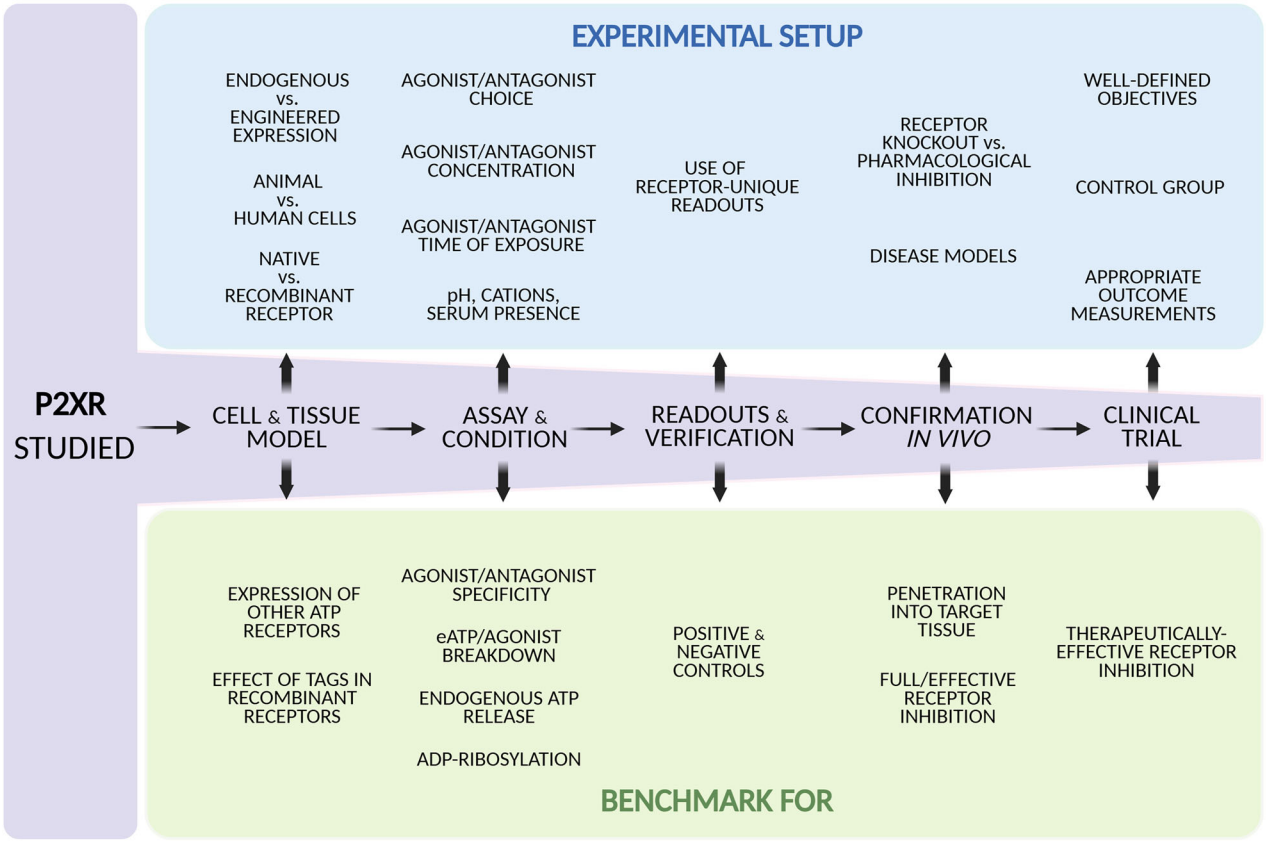

Fig. 2 Experimental framework for P2X receptor studies. Flow diagram summarising key considerations for designing and advancing P2XR studies from in vitro to in vivo in health and disease. Experimental design should integrate selection of cell types and expression systems with controls for receptor functionality and subtype specificity. The choice of agonists and antagonists, including their concentration, exposure time, species selectivity, and susceptibility to enzymatic degradation, must be considered. Breakdown of ATP and related agonists generates metabolites (e.g., ADP, adenosine) that activate other receptors. Assay conditions (e.g., temperature, ion composition, pH) can strongly influence P2XR gating and should be standardised. Measurements should use receptor-specific readouts whenever possible, supported by appropriate positive and negative controls to confirm the specificity of observed effects. Novel findings in vitro should ideally be validated in ex vivo systems (e.g., tissue slices, organoids) and in vivo, including in relevant disease models. In vivo experiments must distinguish between a true absence of biological effect and insufficient receptor activation or inhibition by the pharmacological agents used. Identification and application of biomarkers that confirm adequate modulation of receptor activity are critical for interpreting results in preclinical studies and in clinical trials. All these aspects are explored in depth in the subsequent sections.

appropriate receptor interactome for the physiologically relevant readouts. Moreover, some cell lines can express endogenous receptors.

Use of specific positive and negative controls is an obvious, yet sometimes overlooked, requirement in P2XR assays. The application of appropriate receptor antagonists and corresponding vehicle controls, knockout (KO) cells and organisms, molecular, biochemical, and immunodetection methods confirming the target receptor presence ensures the desired outcome.

The choice of assay/detection methods also requires consideration. Electrophysiology can provide direct ion flux measurements and assess conductance changes, while Ca^{2+} imaging extends the understanding of functional P2XR activation after the opening of the channel (see sections: *The electrophysiological methods in P2X receptor studies and Calcium imaging in P2X receptor studies*). Furthermore, some P2XRs evoke unique functional responses that can be measured (see section: *P2X7 receptor-specific assays*).

Finally, it is important to recognise that a significant portion of data generated in isolated cells maintained in vitro may not accurately reflect the physiological roles of P2XRs in vivo, where the function is often modulated by cell-cell interactions and various physiological and pathological factors. Therefore, in vitro studies must be interpreted with caution and ideally replicated in vivo or in organotypic models to fully understand the functional relevance of P2XR signalling (see sections: *Selection of cellular models and experimental strategies and Assessing P2X receptors* in vivo).

ATP release as a confounding factor, and quantification of eATP

ATP release occurs through multiple pathways, including exocytosis, membrane channels, and passive leakage from damaged cells. While it serves diverse biological roles and can also contribute to pathological conditions, it also needs to be reckoned with when designing any P2XR experiments.

ATP can be released when the cell membrane potential changes, upon agonist or hormone stimulation, hypoxia, or in response to mechanical disturbances such as shear or osmotic stress. The latter phenomena can occur in physiological situations, such as fluid flow in pre-urine in kidney tubule or secreting or distending epithelium [7–9], but also in experimental contexts during perfusion or injection of drugs into a plate reader, or even with pipetting [8, 10]. Therefore, in a specific cell type studied, control experiments with the application of an isotonic physiological solution without any drugs are needed. In cells with high metabolic activity, nutrient (e.g., glucose) concentration in the medium can quickly lead to elevated eATP due to increased intracellular ATP production and release [11]. Therefore, there is a

need to control eATP at the plasma membrane, as this will influence P2XR expression and responses to added ATP analogues and drugs.

As of today, several methods have been developed for eATP quantification. Microelectrode biosensors allow eATP measurements in vivo and provide high accuracy, but require specialised equipment and extensive tissue manipulation, which may cause ATP release [12]. High-performance liquid chromatography (HPLC)-based detection offers precise ATP quantification; at the same time, it requires extensive sample preparation, is unsuitable for in vivo applications, and suffers from low sensitivity, resulting in poor temporal resolution [13].

Fluorescent ATP sensors, such as ATeams, iATPSnFR1.0 [11], ATPOS [14], and GRABATP1.0 [15], utilise fluorescence resonance energy transfer (FRET) or green fluorescent protein (GFP)-based strategies to enable real-time ATP monitoring, including in vivo applications [16, 17]. While fluorescent probes provide high spatial resolution, and visualisation of healthy versus dying cells, highly advanced microscopes and tissue/animal set- ups are required. Tissue penetration by these probes should also be considered [18].

Bioluminescent methods, particularly firefly luciferase-based, remain the most reliable for quantifying eATP [19]. Luciferase-based assays may be "off-line" when ATP is measured in the media isolated from cell cultures or 'on-line' when ATP is measured directly in cell cultures or tissues. Online measurements can be performed by the addition of soluble luciferin-luciferase mixtures to media directly above cells to record ATP release following pharmacological or mechanical stimulation [20]. Alternatively, a luciferase enzyme bound to the cell surface with an anchor protein, e.g., *Staphylococcus* protein A for the spaLUC probe can be used [21], with exogenous luciferin substrate added to the system. In an online assay, luminescence can be rapidly triggered, but the readout may linger after ATP has been degraded, thus providing real-time measurements for ATP release but not its removal. Soluble luciferase-based assays are broadly used in vitro and in biofluids collected by microdialysis [22, 23], but not suitable for in vivo applications [19, 24]. The pmeLUC biosensor, developed by Francesco Di Virgilio's group, overcomes this limitation by anchoring luciferase to the cell membrane [25], thus ensuring precise real-time eATP recording in the immediate cell vicinity and in various disease models, including tumours [26], diabetes [27], inflammation [28], and graft rejection [29]. Additionally, cancer cells can be transfected with the pmeLUC probe and introduced into a host to study eATP dynamics within the tumour microenvironment (TME) [30].

Transgenic pmeLUC mice allow systemic ATP monitoring, aiding studies on ATP-mediated inflammation and neurological disorders [27], and eATP fluctuations in the CNS following cerebral artery occlusion [31, 32]. Continuous advancement of luminescent biosensors may enable the mapping of eATP dynamics throughout the human body. The pmeLUC/nilla recently developed by Di Virgilio and co-workers is a dual-reporter system co-expressing pmeLUC and Renilla luciferases and enables signal normalisation improving accuracy (patent applied for and paper under consideration).

TOOLS USED TO STUDY THE P2X RECEPTORS

Agonists and antagonists used in P2X receptor research

Both nucleotide (Fig. 3) and non-nucleotide antagonists (Fig. 4) for the P2XR have been introduced as research tools [33]. There is a dearth of subtype-selective agonists for use as research tools, but recently, a P2X7R selective antagonist (**[3]**—numbers in square brackets relate to compounds in Figs. 2 and 3) was reported [34]. Many antagonist probes are now a commercially available [35–38].

Exploration of the structure-activity relationships of P2XR ligands has been complicated by the use of non-standard assay methods [36, 39], different pharmacology for hetero- vs. homotrimeric ion channels [33, 40], synthetic challenges [34], the lack (until recently) of structure-based design approaches [38, 40], and the lability of many of the ligands in biological systems [33]. Many of the P2XR antagonists do not compete with ATP, and the elucidation of their allosteric binding enables future ligand discovery [35, 38]. The family of natural products related to ivermectin **[13, 14]** was characterised as P2X4R allosteric enhancers [37]. Their confounding activity at $GABA_A$ receptors has been overcome **[16]**, and a simplified scaffold has been shown to retain the P2X4R-enhancing activity **[15]**.

It is important to scrutinise the selectivity and affinity of a given antagonist to a particular species under study, as many display species variability [33, 35, 36], whereas heteromeric P2XRs (e.g., P2X2/3 R) can show unique species differences compared to homomers. Potency shifts mean rodent doses may not predict human therapeutic doses. Moreover, the pharmacokinetics of a given ligand should be considered, as many of these compounds have short half-lives and lack bioavailability, depending on the route of administration. Much effort has been devoted to the introduction of antagonists that cross the blood-brain barrier (BBB), for probing CNS effects [35]. For example, **[44, 47, 49, 50**, and **52]** readily cross the BBB. The phosphate-containing ligands, mostly P2XR agonists, cannot penetrate the intact BBB and enter the CNS, and their activity in vivo may reflect their conversion to ADP or adenosine, which activate metabotropic P2Y or adenosine receptors.

Antagonists for P2X3R **[29–32]** and P2X7R have progressed to clinical trials for anti-inflammatory **[43**–**45**, **48**] and psychiatric (JNJ-61393215, JNJ-89495120, not shown) therapeutics [41] and as imaging agents **[44**, **50–52]**, [19, 42]. At least one P2X4R antagonist (NC-2600, structure not disclosed) has progressed to the clinical trial stage for chronic pain [35]. P2X3R antagonists, designed for the treatment of chronic cough, have varying degrees of affinity at heteromeric P2X2/P2X3 receptors, which are responsible for side effects, including reduced taste sensation. Ligands for positron emission tomography (PET) imaging in vivo, containing an ^{11}C or ^{18}F isotope (mostly for P2X7R, but also for P2X4R), were characterised [35]. Such tools are important for establishing a pharmacokinetics and pharmacodynamics (PK/PD) relationship for experimental drugs, especially for CNS applications. Unfortunately, various P2X7R antagonists have already failed in clinical trials. One possible reason for the failure may be the many naturally occurring single-nucleotide polymorphisms (SNPs) leading to various missense mutations in the P2X7R that are widespread in human populations [42], but other possibilities should also be considered (see chapter: *Assessing p2x receptors* in vivo)

Modulation of P2X receptors

A key consideration when modulating P2XR activity is the concentration of the agonist. For example, in P2X7R, ATP concentration critically influences the outcome, with low levels promoting pro-survival effects, while high levels can induce cell death [43]. A dose–response experiment is essential to determine the optimal ATP concentration for the desired biological effect and the specific P2XR type.

The duration of agonist exposure is another factor and must be matched to the chosen readout and to the receptor under analysis. Short exposures (seconds) are generally sufficient for assessing ion channel activity, such as Ca^{2+} flux. Longer exposures (minutes) combined with specific readouts identifying the diverse outcomes triggered by different P2XRs (e.g., large pore opening inflammasome activation, proliferation, necroptosis, and receptor shedding for P2X7R [44], can increase the specificity of receptor identification. However, while longer stimulations may be needed, prolonged stimulation can induce cell death, complicating interpretation. Moreover, in the longer-lasting assays, eATP degradation leading to the formation of bioactive metabolites is

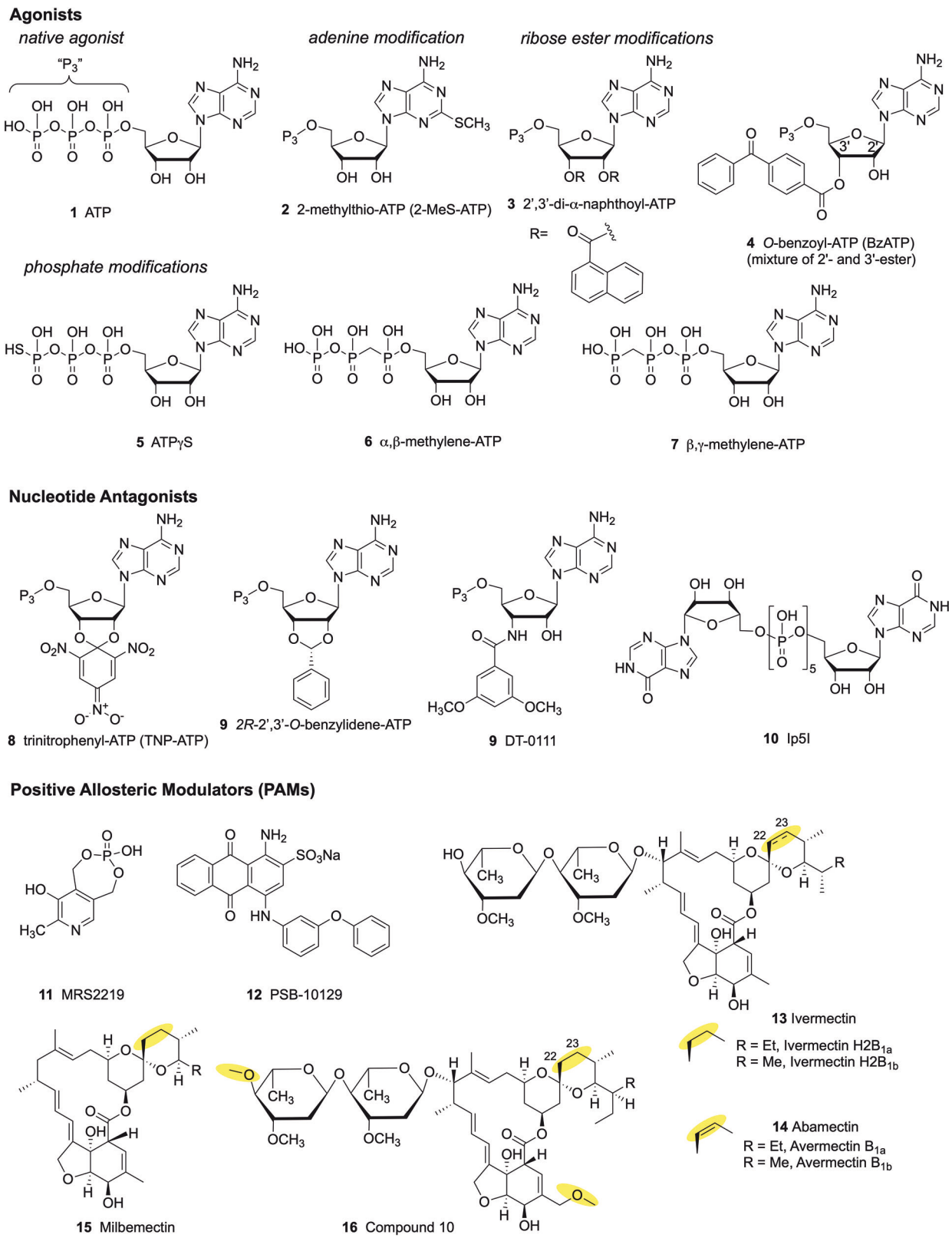

Fig. 3 P2XR nucleotide ligands (agonists and antagonists) and PAMs. The yellow highlights on the PAMs indicate key regions of modification. The compound numbers correspond to those listed in bold in the text. Affinities of selected compounds are as cited [34, 37].

a significant factor (see section *Control for ATP release and degradation*).

Another important consideration is the timing of antagonist application, whether it should be applied before, concurrently with, or after the agonist, and determining the optimal duration of exposure. The answer to these questions depends on the binding site of the antagonist on the P2XR and whether the presence of the natural ligand, ATP, interferes with the conformation of the

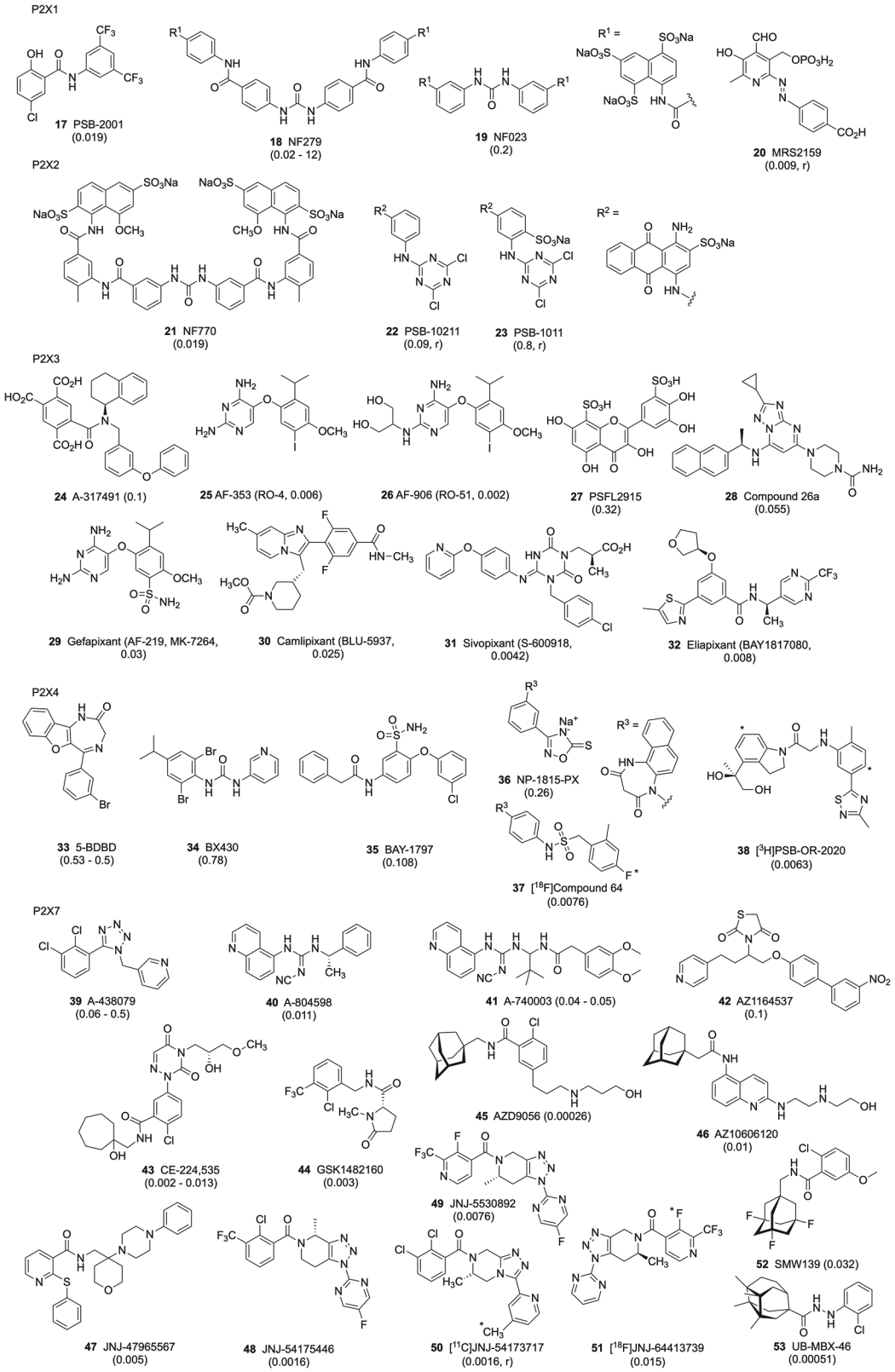

Fig. 4 P2XR non-nucleotide antagonists (allosteric and orthosteric). The compound numbers correspond to those listed in bold in the text. The affinities (μM) [33, 35, 38] at the target P2XR (human, unless noted as rat "*r*") are given in parentheses.

binding site [45]. If so, which is the case for many commonly used P2X7R antagonists (AZ10606120, A438079, or A740003), these should be introduced before the agonist, with a pre-treatment period of 30 min to 1 h. If the binding site is unknown, the recommendation would be to test all three modes of stimulation (pre-, co-, and post-application of the ligand).

One should keep in mind that several P2XR agonists, not only ATP but also some used as more subtype-selective, can activate

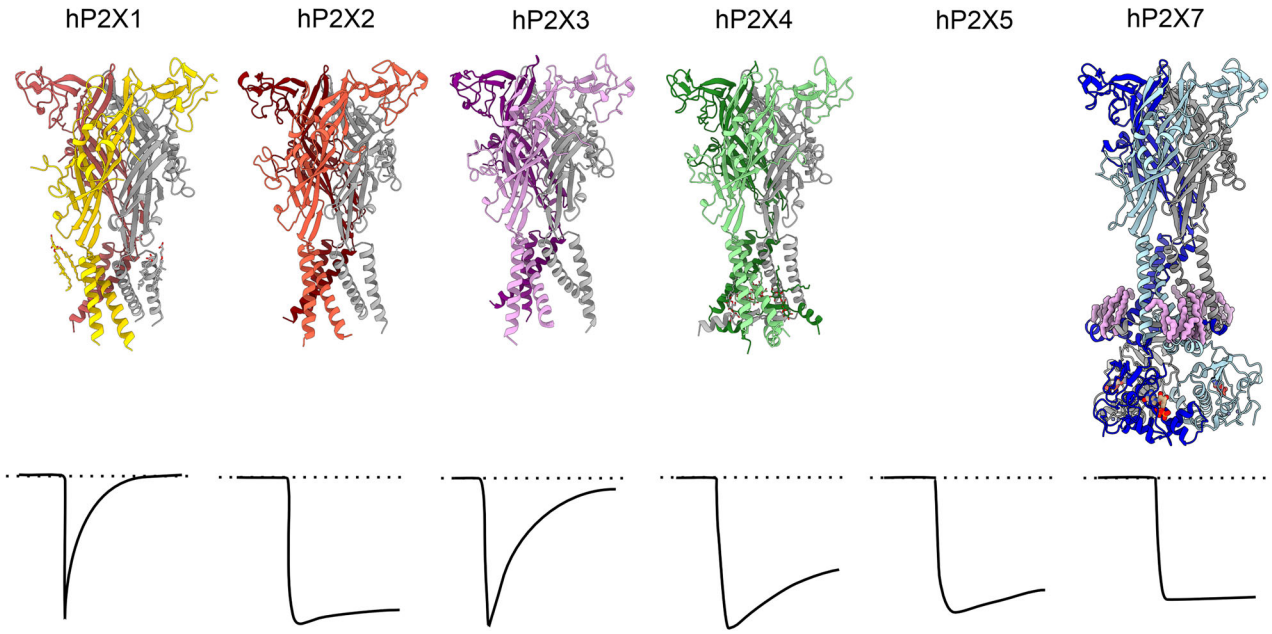

Fig. 5 Architecture of P2X receptor subtypes in the apo closed state conformation. Structures in the apo closed state conformation for hP2X1R (PDB: 9C2A) [54], hP2X2R (PDB: 9DDV) [59], hP2X3R (PDB: 5SVJ) [51], hP2X4R (PDB: 9BQH) [56], and hP2X7R (PDB: 9E3M) [38]. For each model, the protomers are coloured differently, with one protomer always shown in grey. The kinetics of currents mediated by homomeric receptors, once the receptor is activated, are shown underneath. The structure of the P2X5R is not available, while P2X6R subunits cannot homo-oligomerise into a functional P2XR [61].

certain P2Y receptors, and these responses should not be confused with P2XR activation.

Biochemical characterisation of P2X receptors

Recombinant expression of P2XRs using the baculovirus-mediated gene transduction of mammalian cells (BacMam) system can produce enough high-quality receptors for biochemical and structural studies [38, 45–57]. A protocol for expression, detergent solubilisation/reconstitution, and purification of P2XRs is shown as a flowchart in Supplementary Figure 1. P2XRs can be expressed as fusion proteins with an EGFP and octa-histidine affinity tag incorporated at one terminus of each protomer, separated by a protease cleavage sequence to allow removal of both the EGFP and affinity tag before biochemical characterisation of the full trimeric receptor. This approach allows fluorescence size exclusion chromatography (FSEC) to be used for rapid, small-scale ortholog and construct screening as well as identification of optimal purification conditions [57].

The first structural studies of P2XRs, performed with X-ray crystallography on the zebrafish P2X4R (zfP2X4R), the human P2X3R (hP2X3R), the giant panda P2X7R, and the chicken P2X7R, used P2XRs expressed as N-terminal EGFP fusion proteins [45, 48, 49, 51, 58].

However, in each of these initial expression constructs, the P2XR was truncated to various extents at both the N- and C-termini to facilitate crystallisation. The truncation, in some cases, resulted in receptors with altered gating or even non-functional receptors. Subsequent single particle cryogenic electron microscopy (cryo-EM) studies of full-length wild-type human P2X1R (hP2X1R), human P2X2R (hP2X2R), human P2X4R (hP2X4R), rat P2X7R (rP2X7R), mouse P2X7R, and human P2X7R (hP2X7R) each used a C-terminal fusion protein [38, 52–56, 59]. Dodecyl-β-D-maltopyranoside (C12M), with or without the addition of cholesterol hemisuccinate (CHS), can be utilised for successful detergent reconstitution. The fusion protein is initially isolated with immobilised-metal affinity chromatography (IMAC) and then digested with protease to remove all tags. The final purification step involves separating the trimeric P2XR from the tags and protease with size-exclusion chromatography (SEC) to make the protein suitable for biochemical and/or structural studies (Supplementary Figure 1). The architecture of P2X receptor subtypes in the apo closed state conformation is shown in Fig. 5.

The purification and isolation of P2XRs in an unbound, apo state conformation is complicated by the binding of endogenous ATP released during cell lysis [51, 54, 56, 59]. It is worth noting that this problem is subtype-specific. The P2X7R has the lowest affinity for ATP and does not desensitise; as such, isolating an unliganded, apo state conformation for this subtype was not an issue [38, 52, 53, 55], For each of the desensitising P2XR subtypes, however, standard purification strategies resulted in the isolation of receptors in an ATP-bound, desensitised state conformation, hampering the biochemical and structural analysis of these subtypes in apo closed or ligand-bound inhibited state conformations [46, 51, 54, 56, 59]. To overcome this complication, the purification protocol for the hP2X3R was modified to include extensive dialysis of harvested cell membranes before detergent solubilisation to remove the bound ATP [51]. This protocol was further refined/optimised to isolate the hP2X1R in the apo closed state conformation [54]. This method prevents endogenous ATP from altering conformational states and can be applied to the purification of other P2XR subtypes, such as the hP2X2R [59].

Several ligand-binding assays using purified P2XRs enable the characterisation and quantification of small-molecule/receptor interactions. For example, radioactive filter binding and scintillation proximity assays (SPA) have been used to directly measure the low-nanomolar affinity of radiolabelled ATP (^{3}H-ATP) for detergent-solubilized zfP2X4R and hP2X3R, respectively [51, 58]. SPA has also been applied to evaluate competitive antagonist binding to detergent-solubilized hP2X3R by quantifying inhibition or displacement of ^{3}H-ATP binding [51]. In contrast, these radioligand-based approaches have been unsuccessful for P2X7R, likely due to its substantially lower affinity for ATP. To address this limitation bio-layer interferometry (BLI) has recently been employed to measure both the binding kinetics and equilibrium affinity of ATP to purified, detergent-solubilized rP2X7R and hP2X7R [38, 53, 55, 60].

These studies provide direct measurements of the dissociation constant (K_D) and confirm that P2X7R exhibits lower affinity for ATP than other P2XR subtypes [38, 55].

The major advantage of BLI is the possibility of studying the interactions of ligands to receptors without requiring radioactive compounds. While radioactive ATP is readily available and relatively inexpensive, it can only be used to assess the binding of competitive antagonists through displacement assays. The synthesis of radioactive analogues for ligands that bind outside the orthosteric ATP-binding pocket is not easily accomplished. BLI, on the other hand, can be used to evaluate the binding of allosteric antagonists, as was successfully performed for the binding of methyl blue to rP2X7R [53]. It should be noted, though, that measuring the binding of small molecules to the P2X7R is at the edge of detectability for this method (for example, the binding of three ~500 Da molecules of ATP to a P2X7R that is >200 kDa in size), and BLI might not be ideally suited for hydrophobic ligands.

The electrophysiological methods in P2X receptor studies

Electrophysiological techniques used to study P2XRs, as well as to identify novel subtype-selective agonists and antagonists for clinical applications, include two-electrode voltage-clamp and whole-cell patch-clamp recordings. The pharmaceutical industry leverages high-throughput automated patch-clamp approaches, which enable simultaneous recording from multiple cells [62].

The patch-clamp technique combined with targeted mutagenesis revealed the general characteristics of homo or hetero-trimeric P2XRs (e.g., P2X1/5R, P2X2/3R), the subunit structure–function correlations [63], ATP binding pocket arrangement [64], and agonist/antagonist specificity.

As frog oocytes and HEK293 cells do not express endogenous P2XRs, all subtypes of recombinant P2XRs, and their modifications, can be expressed in these cells following transfection with specific constructs [63]. Patch-clamp recordings demonstrated that the recombinant and native receptors respond identically to ATP. However, it has to be remembered that recombinant receptors expressed in heterologous systems are unlikely to have their native interactome involved in downstream signalling.

Thus, the ion current recordings can reveal molecular events immediately coupled to receptor activation (inward fluxes of Na^+/Ca^{2+} and outward flux of K^+). The Ca^{2+} imaging further extends our understanding of P2XRs' function by supplying information on the secondary increase of $[Ca^{2+}]_i$ upon opening of the receptor-channel (see section: *Calcium imaging in P2X receptor studies*).

Primary immune cells and either cells isolated from the CNS and peripheral nervous system or brain slices or cells in vivo can be subjected to patch-clamp recordings. The single cells and brain slices can be used acutely or can be kept in culture for a couple of days. Cell culture has the disadvantage that it creates artificial conditions, which may result in a loss or modification of receptor expression.

Construction of agonist concentration–response curves and the study of agonist-antagonist interactions is feasible using submerged organ baths. The bath perfusion has, however, serious drawbacks, because the final drug concentration builds up slowly, and measurements can be made only in the equilibrium state. More advantageous is the pressure application of agonists at single drug concentrations from micropipettes, whereas fast local superfusion systems are optimal, because these allow for the rapid change of minute quantities of drug concentrations around the cell [65, 66]. However, in many cells, fast superfusion or injection can cause ATP release, and this needs to be controlled.

Filling of cells with a fluorescent dye, such as lucifer yellow, helps cell identification. Co-staining using lucifer yellow with post-hoc immunohistochemistry with one of the neuronal, astrocytic, oligodendrocyte, or microglial markers ensures cell-type identification.

It is also worth noting the unique properties of specific P2XRs subtypes, such as rapid desensitisation of P2X1Rs and P2X3Rs [63], agonist sensitivity where P2X7R requires micromolar-millimolar range, considerably higher than P2X1-6 receptors, or the ability to open the large pore on long-lasting or repetitive agonist application (see chapter: *P2X7 RECEPTOR-SPECIFIC ASSAYS*). The functional inhibition by extracellular divalent cations (Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}) is also a consideration [67], where e.g., P2X7R currents can be amplified by the low divalent cationic conditions in the bath solution [68].

Use of structural analogues of ATP with increased specificity for particular subtypes can help distinguish receptors even further (see section: *Agonists and antagonists used in P2X receptor research*).

Calcium imaging in P2X receptor studies

Recording changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is used in studies of P2XR function and pharmacology. This method is advantageous for cells growing in suspension or primary cells that are not easily transfected or difficult to patch, such as myeloblasts [69]. Several fluorescent dyes, with specific properties, are being used, including Fura-2 (ratiometric, reduces background noise), Fluo-4 (single-wavelength, easy for high-throughput screening), and GCaMP (genetically encoded Ca^{2+} indicators for cell-specific live imaging). Ratiometric $[Ca^{2+}]_i$ measurements are based on the monitoring of changes in the Fura-2 emission ratio at 340/380 nm excitation wavelengths, allowing estimation of the actual $[Ca^{2+}]_i$ [70]. The protocol was developed for cells maintained in suspension, but it can be used for most adherent cell types following their detachment. However, some cells need to adhere to the substrate to show P2XR-dependent Ca^{2+} fluxes. In such cases, it is still possible to use Fura-2 by growing the cells on coverslips at an adequate density, loading them with the dye while still on this support, and mounting the coverslips in a fluorimeter cuvette for measurements [71–73].

Fura-2 signals can be imaged in cells and tissues using microscopes with appropriate light sources (e.g., monochromators, UV lasers) and means of changing fast between the two excitation wavelengths used to monitor calcium signals [74–76]. One of the Fura-2 advantages is that monitoring of individual wavelengths and a ratio can indicate diffusion of the fluorophore out of the cell due to pore formation [77].

When studying responses in cells transfected with a specific P2XR, co-transfection with GFP and Fura-2 imaging allows simultaneous monitoring of adjacent untransfected cells, which do not express the receptor but still take up Fura-2, thereby serving as an internal control [78].

Other techniques are based on live single-cell confocal microscopy with specialised Ca^{2+} sensors. Microplate readers or flow cytometry can be used to assess P2XR-dependent $[Ca^{2+}]_i$ fluctuations in a large number of cells [70]. Fluo-2 AM, albeit not allowing for quantification of the calcium entry, has been used to detect P2XR-activated $[Ca^{2+}]_i$ dynamics in primary cells difficult to culture, such as osteoclasts [79] or peripheral neurons [80].

A series of P2X-based eATP sensors was recently developed (see section: *ATP release as a confounding factor, and quantification of eATP*) [81]. However, these sensors depend on cell transfection or genetic engineering of animals for their expression, and therefore, not for measuring naturally occurring P2XR activity.

Several factors must be considered when performing P2XR activity assays with Ca^{2+} sensors. The agonists available are generally not specific for a single subtype. Therefore, to prove the activation of a specific receptor, it is advisable to use controls with selective antagonists whenever available. For P2X4R, for which a selective agonist is not currently available, its effects can be evaluated by potentiating the ATP activity with a selective positive

allosteric modulator, such as ivermectin [82, 83] (see section: *Agonists and antagonists used in P2X receptor research*).

Even when proceeding with the transfection of a single subtype in cells not expressing any P2XR, such as the widely used HEK293 cell line, one should keep in mind that the purinergic agonists can activate some P2Y receptors. P2Y are G protein-coupled receptors that trigger Ca^{2+} release from the endoplasmic reticulum as well as store-operated Ca^{2+} entry, thus causing $[Ca^{2+}]_i$ increases. P2Y-activated Ca^{2+} transients generally have a distinctive shape, as $[Ca^{2+}]_i$ rises and descends rapidly, so their appearance is different from that of the sustained entry measured for non-desensitising P2XRs. However, to completely exclude a P2Y-dependent component in the $[Ca^{2+}]_i$ rise activated by the agonist applied, control experiments with no Ca^{2+} in the measurement buffer, and with either EDTA or BAPTA addition to completely chelate extracellular Ca^{2+}, can be performed. Under these conditions, any $[Ca^{2+}]_i$ rise following the administration of an agonist denotes that Ca^{2+} is released from intracellular stores and, therefore, has a P2Y-dependent effect. For more details on the experimental settings, see [43, 84–86].

P2X7 RECEPTOR-SPECIFIC ASSAYS

The P2X7R functions as an ion channel, yet it is distinctive in its low sensitivity to eATP, its near-complete absence of desensitisation [52] and its ability to engage additional signalling and effector mechanisms. Some of these, such as large pore formation, oligomerization domain, leucine-rich repeat receptor, pyrin-domain containing-protein 3 (NLRP3) inflammasome activation, and extracellular vesicle release generally occur upon prolonged stimulation with high eATP concentrations. Low eATP seems to induce signals independent of the channel function [87]. These differences presumably involve the complex interactome (reviewed in ref. [88]), tethered to the unique P2X7R C-terminus. While not yet fully understood, such ion flux-independent responses should be considered when unusual effects of P2X7R activation are identified [89].

P2X7 receptor and pore formation

One of the differential physiological functions of P2X7R activation when compared to the other P2XRs is the formation of 'large' pores (LP) in the plasma membrane. By 'large' it is denoted a pore size bigger than the ion channel gate of P2X7R (6.4 Å) up to 900 Da [90].

P2X7R-induced dye uptake has been determined in different cell models and is used as a method to identify P2X7R function. Both the formation and functional significance of the P2X7R LP can be cell-type-specific and also altered in disease states. Cells expressing endogenous P2X7Rs (such as macrophages) or cells expressing it ectopically (HEK293 or oocytes) have all been used to demonstrate P2X7R-induced LP formation [91].

LP formation can be studied employing different plasma membrane-impermeant cationic fluorescent dyes, including Yo-Pro-1 iodide and ethidium bromide. Generally, to stabilise the fluorescent signal, these dyes need to be added 10-15 min before ATP-mediated opening of the P2X7R. Also, use of a physiological buffer [92] or a sucrose-based saline solution [69, 93, 94] rather than cell culture media or PBS is preferable for optimal response, and serum in the assay buffer dramatically reduces the potency of ATP effects (see section: *Impact of serum in the assay buffer*). Dye uptake usually starts 5-10 min after ATP stimulation and lasts for ~30 min [95]. However, in some studies, shorter dye preincubation times were sufficient, and responses were evoked within seconds [96]. Dye uptake can also be assessed in cells in suspension using a fluorimeter with stirred cuvettes, and in slices [97].

Anionic dyes, such as lucifer yellow, can also be used, but seem to present different uptake properties depending on the cell type employed, e.g., being captured by macrophages, but not by HEK293 cells expressing P2X7R [97, 98]. In muscle cells but not in macrophages, autophagy is needed for LP formation and molecules entering through the pore can be targeted to phagophores [99].

Analysis of P2X7R structure, both in apo and ATP-bound states, was unable to identify a receptor state with a dilated pore [52]. Therefore, it is still an open question whether the P2X7R ion channel dilates over time of activation and forms an initial conduit for LP formation that can then be amplified with the activation of accessory proteins. Several proteins have indeed been linked to P2X7R LP formation, including pannexin-1 and gasdermin D. The expression of these proteins will condition the dye uptake found in different cellular models.

When analysing LP formation in lipopolysaccharide (LPS)-primed macrophages, it needs to be considered that P2X7Rs also trigger the NLRP3 inflammasome (see section: *NLRP3 inflammasome activation*); activation of caspase-1 will lead to gasdermin D oligomerization in the plasma membrane and the formation of pores [100]. These pores allow the dyes to pass and could interfere with the identification of P2X7R LP induction. Thus, specific inhibitors of NLRP3 (e.g., MCC950) need to be used to eliminate the contribution of gasdermin D to dye uptake.

NLRP3 inflammasome activation

When eATP is studied as a pro-inflammatory molecule, classical experimentation relies on the treatment of macrophages with mM concentrations to trigger the P2X7R-induced nucleotide-binding NLRP3 inflammasome. These treatments are usually acute (around 30 min), and the use of a physiological buffer [92] without serum is recommended to obtain the best responses (see section: *Impact of serum in the assay buffer*).

Before application of ATP, macrophages are primed with compounds that activate NF-κB, with various concentrations of bacterial lipopolysaccharide (LPS) being most commonly used, but Pam3CysSerLys4, a synthetic triacylated lipopeptide (Pam3CSK4), is also utilised [101]. The concentrations and time of this stimulation may need optimisation for specific cells and experimental designs. Recombinant cytokines (IFNγ, TNFα) could also be applied, alone or in combination with LPS, but it should be noted that proteins produced in bacterial systems may have LPS contamination.

After priming and before ATP addition, macrophages should be washed thoroughly to remove cytokines produced as a consequence of priming, as well as to remove non-adherent cells. Critical morphological changes can be observed when the P2X7R is triggered for 30 min in primed macrophages, as the plasma membrane of these cells becomes permeable due to pyroptosis, and cells acquire a rounded phenotype with a granular cytoplasm [102, 103].

One of the common measures of the pro-inflammatory actions of eATP in primed macrophages is the release of IL-1ß, since NLRP3 inflammasome induction activates caspase-1, and this enzyme maturates IL-1ß, favouring its release [104]. ELISA for IL-1ß is a fast and quantifiable method, but since IL-1ß is produced as an inactive pro-cytokine during the priming step, an ELISA that mainly recognises mature IL-1ß is highly recommended for the correct interpretation. Alternatively, the proteins in cell supernatants can be concentrated, and detection of mature IL-1ß, active fragments of caspase-1, or Nt-fragment of gasdermin D can be used as markers of inflammasome activation [105].

The outcome of inflammasome activation may vary depending on the cell type. Mouse primary peritoneal macrophages are more responsive to P2X7R activation than mouse bone marrow-derived macrophages, which are widely used in most of the studies [106]. Human primary macrophages derived from monocytes respond to the classical protocol of LPS and ATP stimulation, but due to difficulties in obtaining and variability issues, the human myeloid cell line THP-1 is widely used as a

substitute [107]. However, THP-1 requires a differentiation step with phorbol esters (i.e., phorbol 12-myristate 13-acetate, PMA) or IFNγ before the priming step. Various protocols to differentiate THP-1 (in terms of time and concentration of PMA or IFNγ) can produce different, sometimes ill-reproducible results. One verified protocol is described in [108].

For human blood monocytes, ATP stimulation can be done in diluted whole blood or in purified monocytes after LPS priming. Since blood cells are non-adherent, the priming media is not washed, and ATP is added in the presence of LPS stimulation. IL-1ß release needs to be measured by ELISA, as very little of the cytokine is produced. Alternatively, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain (CARD)) oligomerization can be measured with flow cytometry [109, 110]. This protocol allows for a monocyte-specific assessment of ATP-induced NLRP3 inflammasome formation, and this measure could be used as a standardised method to investigate the immunocompetence of myeloid cells in patients [111].

Additional determinants of inflammatory processes triggered downstream of P2X7R activation in macrophages include inflammatory lipid production (prostaglandin or thromboxane), CD14 shedding, metalloprotease activation, or release of extracellular vesicles [112–114].

Stimulation of extracellular vesicle release upon P2X7 receptor activation

The release of various types of vesicles from cells mediates the transfer of biomolecules during physiological and pathological processes. The P2X7R-dependent mechanism of extracellular vesicles (EV) release was demonstrated in immune, CNS, and peripheral nervous system [115–117], and in cancer cells [118]. These EVs can carry pro-inflammatory cytokines, miRNA, ATP, and mitochondria [118].

When investigating EVs released upon P2X7R activation, it is important to eliminate external vesicle sources that would interfere with the analysis. Thus, cells to be used in the assay can be grown in a complete culture medium, but since serum-supplemented culture medium contains EVs, once the cells reach the desired density, the medium should be removed, and the cells washed carefully to minimise stress. Cells should be exposed to agonists in either serum-free medium or filtered physiological solutions [23, 116, 119]. P2X7R-dependent vesicular release can be induced either by ATP concentrations ranging from 1 to 3 mM or by BzATP in concentrations between 100 and 300 µM.

Release begins within minutes, and a 15–30 min incubation period is sufficient to collect enough EVs for subsequent characterisation. The cellular debris can be removed from the supernatant with gentle centrifugation, and the vesicles can then be pelleted at high speed, depending on their subtype. The vesicle pellet should be washed to remove residual agonists and re-centrifuged. To preserve vesicle integrity, all steps should be done at 4 °C, and vesicles stored at −80 °C. Repeated freeze-thaw cycles may reduce their quality [120].

Given that P2X7Rs are not the only player involved in the vesicular release process, and that their activation can change their content, the important control step is to collect vesicles from unstimulated cells. These two vesicle populations can be compared in terms of number, size, bioactive content, and biological effects in vitro and in vivo. The characterisation of EVs released upon P2X7R stimulation typically involves confocal and electron microscopy, as well as Nanosight or Videodrop technologies, to assess vesicle size and mitochondrial content. Their cellular origins can be further explored using Western blotting with markers from distinct subcellular compartments [117, 121, 122]. However, this approach remains limited by the current lack of specific markers that reliably distinguish individual EV subclasses or identify vesicles derived from particular subcellular sites. Moreover, the continuous refinement of EV classification and the emergence of newly defined subclasses add a layer of complexity that must be considered [116, 117].

P2X7 receptor responses, cell adhesion and phagocytosis

During the phagocytosis process, macrophage P2X7Rs are internalised and degraded, and it takes 24–48 h for the cell to replenish its pool at the cell membrane. Moreover, the process of plate adhesion is a form of frustrated phagocytosis. Thus, when conducting P2X7R assays in macrophages, it is advised that any functional analyses, such as permeabilization, Ca^{2+} mobilisation measurements, or IL-1β secretion are performed 24–48 hours after the adhesion step to obtain accurate measurements [123].

Likewise, purinergic signalling analysis during macrophage infection with intracellular parasites requires specific considerations. Some of these infections involve phagocytosis of the parasite. Therefore, any measurements of the infection impact should be performed at least 24 hours after the infection step. However, this is relevant only when infecting macrophages with intracellular bacteria *Chlamydia trachomatis*, or the protozoan *Leishmania amazonensis* [124–126]. This precaution is not necessary when the macrophage infection is caused by the protozoan *Toxoplasma gondii*, since this intracellular parasite actively enters the cells and is not phagocytosed [127].

MODULATORS AFFECTING P2X RECEPTOR SIGNALLING

P2XR function is determined not only by eATP availability but also by eATP flux resulting from both tonic/pulsative release mechanisms and rapid hydrolysis by ectonucleotidases. Function and signalling are also impacted by the surrounding ionic milieu [128, 129], the lipid composition of the membrane [130, 131] and the presence of serum components that can modulate receptor activity.

Control for ATP release and degradation

In purinergic signalling, equally important to receptor activation by eATP in an autocrine or paracrine manner are the other components of cellular purine turnover cascade, which include: (i) the release of ATP; (ii) catabolism and inactivation of eATP and other related compounds via a network of purine-converting ectoenzymes; (iii) binding of nucleotide-derived adenosine to G-protein coupled adenosine receptors; and finally, (iv) scavenging of extracellular adenosine by ecto-adenosine deaminase or cellular adenosine uptake through the equilibrative nucleoside transporters (e.g., ENT1/SLC29A1) and concentrative nucleoside transporters (CNTs/SLC28) (Fig. 6). This ATP release and degradation should be taken into consideration when designing the experiments. Specifically, activation of P2X7R and most other P2XRs usually occurs at eATP concentrations within the low millimolar or sub-millimolar range that significantly exceeds the nanomolar ATP levels reported for extracellular fluids and interstitial space [120]. This apparent discrepancy can be explained by focal ATP release and the ability of the cells to retain high concentrations of eATP in the pericellular space without significant nucleotide convection into the bulk milieu [132, 133]. Such relatively high concentrations of eATP at the outer aspect of the plasma membrane are detected in pancreatic beta cells responding to high glucose levels. Simultaneous imaging of eATP and intracellular ATP sensor indicates close coordination between cell metabolism and ATP release [11].

The presence of a complex network of membrane-bound and soluble nucleotide-inactivating and ATP-regenerating enzymes adds another level of complexity to the experimental design, as these regulatory mechanisms govern the duration and magnitude of purinergic responses. Furthermore, cellular adenosine uptake by ENT or CNT, and its intracellular metabolism are expected to have an equally important yet understudied role in activation of adenosine receptor-independent metabolic and epigenetic functions [134–136].

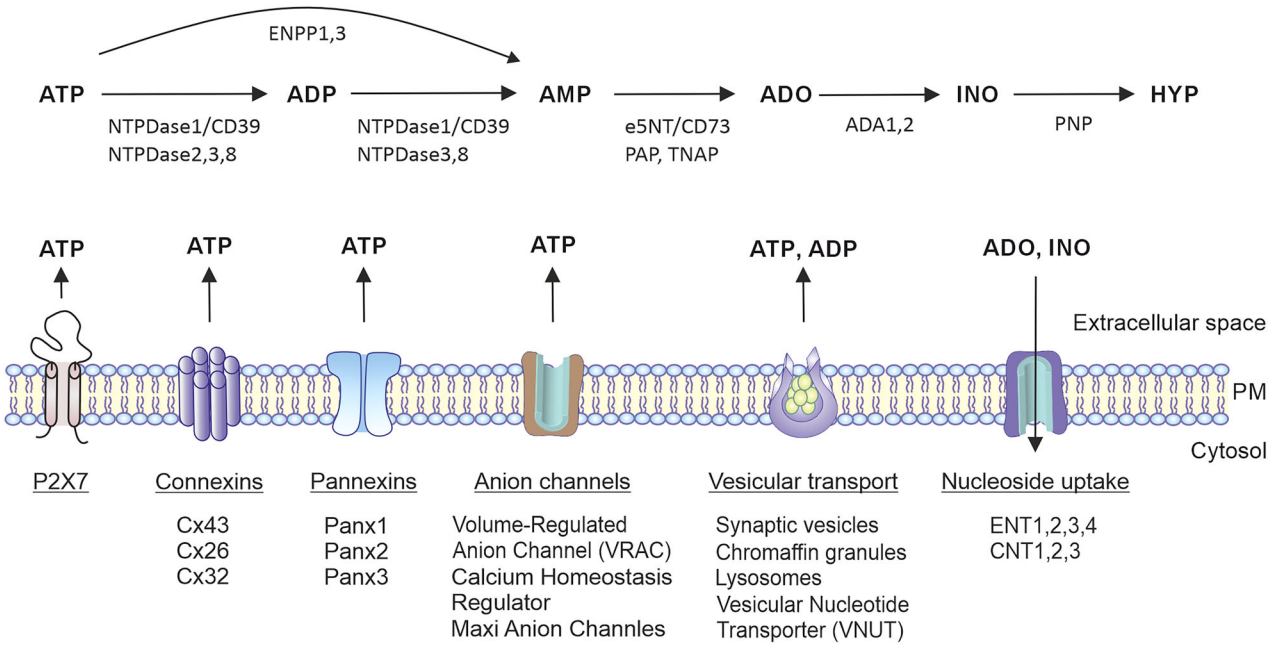

Fig. 6 Pathways of cellular ATP release and degradation to other purinergic agonists. Along with massive nucleotide leakage following cell damage, ATP is released through several, often cell-type–specific, non-lytic pathways including vesicular exocytosis, ATP diffusion through plasmalemmal channels such as P2X7R (P2X7), connexin/pannexin hemichannels, and anion channels. Once in the extracellular space, ATP is rapidly degraded. This breakdown shapes the duration and magnitude of ATP signalling and shifts the balance toward its metabolites, which act as agonists at other purinergic receptors. These mechanisms need to be taken into consideration during designing the experiments and data interpretation. The extracellular purine-inactivating pathways include: ecto-nucleoside triphosphate diphosphohydrolase-1 (NTPDase1, also known as CD39), other members of this family, NTPDase2, NTPDase3, and NTPDase8, ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1), ecto-5′-nucleotidase (e5NT, also known as CD73), adenosine deaminase (ADA), and purine nucleoside phosphorylase (PNP). For clarity, only the most important purine-inactivating ectoenzymes are shown. Extracellular adenosine (ADO) can be transported into the cells via equilibrative (ENT) or concentrative (CNT) nucleoside transporters, followed by intracellular metabolism. Cx connexin, HYP hypoxanthine, INO inosine, Panx pannexin, PM plasma membrane.

Overall, it is important to consider ATP release and extra- and intra-cellular purine metabolism when studying the biological functions of P2XRs. These are especially relevant, but can be overlooked, under experimental settings of long-term treatment of the cells or tissues with supraphysiological concentrations of exogenous ATP.

Impact of serum in the assay buffer

Most cell types used to explore purinergic signalling require a complex mix of nutrients, hormones, adhesion molecules, and growth factors in their growth media. These are introduced by the addition of a serum. Foetal bovine serum (FBS) is the most commonly used supplement, human serum is employed where humanised conditions are desired, and sera of other species (e.g., horse) are used for specific applications. Synthetic serum replacements are utilised mostly in cell culture experiments requiring the elimination of animal-derived products [137].

However, the inclusion of serum in the assay may impact results significantly. FBS contains bioactive ATP [22, 138], where the broad range reflects the significant FBS batch-to-batch variability. Thus, cells grown in serum-supplemented media are exposed to varying degrees of purinergic stimulation even before any experimental manipulations. This should be considered in the assay design.

Serum also contains soluble and catalytically active forms of NTPDase1/CD39, ecto-nucleotide pyrophosphatase/phosphodiesterase-1 (ENPP1), adenylate kinase-1, and other purine-converting enzymes [139]. Therefore, additional heat-inactivation of serum samples may be useful for excluding the possibility of undesirable hydrolysis and metabolism of ATP and other purinergic agonists in the assay medium. Furthermore, FBS and horse serum contain active matrix metalloprotease 2 (MMP2) that can cleave the P2X7R on the cell surface. Physiologically, sustained activation of P2X7Rs triggers the release of active MMP2, which, in a feedback loop mechanism cleaves the receptor (amongst other proteins), thus halting its function. This mechanism appears to be universal, operating in cells as diverse as macrophages, dystrophic myoblasts, human tumour cells, and P2X7R-transfected HEK293 cells [140].

Significant MMP2 activity is detected in various sera, and heat inactivation fails to eliminate this activity. As a result, serum concentrations of 5% or higher in the assay buffer have been shown to inhibit P2X7R ion channel function and large pore formation in different cell types [138]. On the other hand, tissue inhibitors of metalloproteinases, especially tissue inhibitor of metalloproteinases 2 (TIMP-2) present in the blood, may inhibit MMP-2 activity, which could explain why P2X7R function can still be measured in whole blood [141].

Consequently, serum should be avoided, or synthetic serum replacements should be considered for specific experiments. Alternatively, MMP2 inhibitors have been shown to effectively restore P2X7R functions. On the other hand, in in vitro studies investigating cells from organs with permeable capillaries, inclusion of blood or serum in the receptor assays may mimic the conditions in vivo.

SELECTION OF CELLULAR MODELS AND EXPERIMENTAL STRATEGIES

Cells used in in vitro assays and the ex vivo systems: practical considerations

Native primary cells often express multiple P2XR subtypes, and these can vary under physiological and pathological conditions. This heterogeneity can make the isolation of the contributions of individual P2XR challenging.

Recombinant expression systems, where a target P2XR is overexpressed in a heterologous cell line, allow investigations of precise receptor properties, such as ion permeability and kinetics under well-defined settings [142]. Yet, certain commonly used cell lines can endogenously express some purinergic receptors, especially P2Y receptor subtypes [143] and these background signals can complicate data interpretation.

Therefore, it is essential to verify receptor expression profiles in experimental systems, especially novel ones, through RT-PCR, Western blotting, or functional assays to ensure accurate attribution of responses to the intended P2XR. Moreover, for heterologous models to produce physiologically relevant data, the impact of overexpression, potentially altered receptor trafficking, and the cellular interactome must be carefully considered, as receptor density and the absence of the native membrane composition, intracellular signalling partners, and regulatory proteins can influence receptor functions significantly. Depending on the expression vector used and/or the presence and localisation of a tag (whether N- or C-terminal), or sometimes the location of the mutated site in a recombinant receptor, the expression levels, the localisation, and the functionality of P2XR can be affected. Therefore, it is recommended to characterise the receptor presence, and its correct localisation at the cell membrane before any kind of measurements [144].

This precaution is also mandatory when working with established cell lines that can express multiple variants of P2XRs. Specifically, in the case of P2X7R, specific variants that retain the ion channel activity but lack LP formation have been identified [145, 146]. Among those, a P2X7B splice variant present only in humans has been associated with multiple phenotypes, including cell proliferation [93, 147], cancer progression, metastasis, and therapy resistance [69, 94, 116, 117, 144, 148, 149]. Similarly, the nfP2X7R variant was attributed a role in cancer cell proliferation [146], and clinical trials with an antibody specifically targeting this isoform are ongoing [150, 151]. It is therefore important to characterise all the variants expressed by a cell line, which can be achieved using Western blot analysis probed with antibodies that recognise the receptor variants.

Monocultures of neurons and oligodendrocytes, prepared following standard techniques, are used to evaluate the impact of P2XR agonists and antagonists in e.g., paradigms of stroke [152–155]. While primary cortical neurons and oligodendrocytes are relatively easy to handle in culture, they have limitations when studying purinergic receptors. For example, neurons derived from late embryonic stages require sufficient maturation (at least three weeks in culture) to express P2X7R [152]. In contrast, brain oligodendrocytes are typically obtained through a lengthy procedure (2–3 weeks), often resulting in cultures with some astrocyte contamination. It is also important to consider whether prolonged culture conditions might not artifactually induce the expression of these P2XRs [156]. Alternatively, optic nerve oligodendrocyte cultures mature rapidly (within 4–5 days in vitro) but yield relatively few cells, most of which decline after a week [157].

P2X receptors in neurogenesis models

P2XRs emerge in early neuronal development and are implicated in neural phenotype determination as well as in neurodegenerative processes [157]. These processes can be modelled using neural differentiation of neural stem cells. Embryos harvested from timed pregnant rats (E12.5–E14.5) or mice (E12.5) can be used for the isolation of embryonic telencephalon [158]. The telencephalons are dissociated, and cells are plated into non-adherent grade dishes for suspension culture and neurosphere formation for 5–10 days in culture medium supplemented with growth factors. Then, neurospheres are collected and replated into laminin/poly-L-lysine-coated dishes for differentiation induction into neurons, astrocytes, and oligodendrocytes. Alternatively, postnatal day 19 (P19) mouse pluripotent cells can be used for neuronal differentiation [158].

The precautions outlined in the sections: *Impact of serum in the assay buffer and P2X7 receptor responses, cell adhesion and phagocytosis*, should be carefully considered.

Differentiated neurospheres should be assayed for neural maturation together with P2XR expression by immunostaining and quantitative image analysis. For that, fixed neurospheres can be immunostained for neuronal β-tubulin, astrocytic glial fibrillary acid protein (GFAP), and oligodendrocyte Gal-C expression [158]. For P2XR immunodetection, the use of primary subunit-selective antibodies of a well-known specificity is important [159]. For quantitative imaging, slides scanned using epifluorescence or confocal microscope can then be subjected to segmentation imaging analysis [160]. 4′,6-Diamidino-2-2phenylindole (DAPI) staining is used for automatic cell nuclei recognition, followed by the analysis of the cells positive for the respective neural antigens and purinergic receptor subtypes. Negative controls in the absence of primary antibodies are needed to determine the threshold. If available, neurospheres from P2XR KO mice can be used to confirm the specificity of the antibody signal.

A lung tissue slice model for P2X receptor analyses

The precision-cut lung slices (PCLS) can serve as a physiological model that bridges the gap between in vitro cell culture and in vivo animal models [161]. The lung tissue can be collected from mice or humans following lung transplantation surgery. PCLS should retain lung architecture, the extracellular matrix composition, and the differentiated state of all cell populations, including immune cells [162]. A low-melting agarose solution is injected into the trachea, lung inflation is carefully monitored, cold PBS is poured over the lung to solidify the agarose, and then sliced using a vibratome. The sliced lung tissue can be cultured for hours or even a few days in standard culture medium. This approach allows comparing different treatments on P2XRs expression and/or activity, as a single lobe can yield multiple slices, and each slice provides a sufficient number of cells for analysis by flow cytometry, Western blot, or immunohistochemistry. Additionally, this approach enables the use of both healthy and diseased lungs, providing a strong translational link into clinical applications.

ASSESSING P2X RECEPTORS IN VIVO

Studying P2XRs in vivo is crucial for bridging the gap between molecular mechanisms and their physiological and pathological effects within the whole organism. Unlike in vitro systems, in vivo studies preserve native cell-cell and cell-extracellular matrix interactions, physiological ion gradients, and the presence or absence of direct contact with serum components (see section: *Impact of serum in the assay buffer*), all of which influence P2X receptor function. The dynamic regulation of ATP release and degradation by ectonucleotidases, which significantly alter receptor activation patterns, can only be accurately assessed in vivo.

For instance, purinergic responses in cancer illustrate the complex interplay between ATP release, specific purinergic receptor activation on various cells, and eATP metabolism with its signalling consequences (Fig. 7). For example, recent studies have demonstrated an interplay between P2X7R, ectonucleotidases and ADORA in cancer progression [117, 163, 164].

Likewise, neuroinflammation involves a complex interplay of P2X7R and P2X4R on microglia and astrocytes, whose functional properties evolve throughout the inflammatory process. However, P2XRs on immune cells also play a role in this response, particularly when these cells gain access to the brain. Similarly, the roles of P2X2R in respiratory regulation and P2X3R in chronic cough can only be fully understood within the physiological context of a living system.

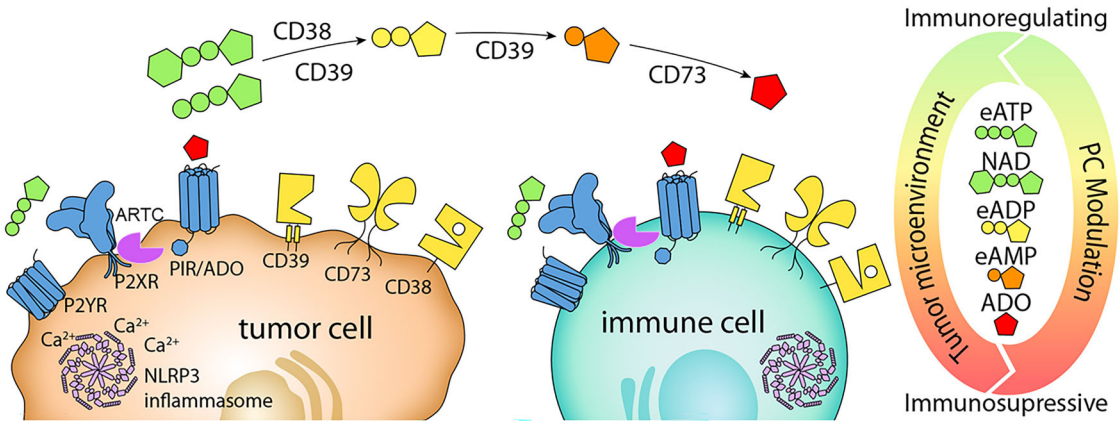

Fig. 7 Purinergic signalling in tumours and in antitumor immune responses. The abundance of eATP is a prominent characteristic of the tumour microenvironment (TME). Within the TME, eATP acts either as an immunoregulator or an immunosuppressor, depending on the purinergic components (e.g., receptors and ectonucleotidases) and associated proteins expressed by the tumour and TME cells, and by posttranslational modification, e.g., ADP-ribosylation of P2X7, carried out by species-specific ADP-ribosyltransferases. Abbreviations used: eATP, extracellular ATP. ADO, adenosine, PC, purinergic actors, P2X, purinoreceptor of the P2 family, P1R, purinoreceptor of the P1 family (adenosine receptor), ARTC, ADP-ribosyltransferase, Inflammasome complex (NLRP3, ASC, PYCARD, CASP1). Figure Dr Julia Hambach, University Medical Center Hamburg-Eppendorf, Germany.

Beyond mechanistic insights, in vivo studies are essential for therapeutic targeting and preclinical validation of novel P2XR agonists and antagonists at physiologically relevant drug concentrations.

Experimental strategies for investigating P2X receptors in vivo

In vivo studies typically involve pharmacological approaches, genetic models, or a combination of both.

Pharmacological methods utilise P2XR antagonists, which can acutely or chronically block receptor function. JNJ-47965567, a selective P2X7R antagonist, has been shown to reduce mania-like behaviour in the amphetamine-induced hyperactivity [165, 166] and depressive-like behaviour [167]. A-317491, a P2X3R antagonist, effectively reduced chronic and inflammatory pain in rodent models [168, 169], while 5-BDBD, a P2X4R inhibitor, alleviated neuropathic pain [170] and implicated microglia-neuronal signalling in nitroglycerine-induced migraine model [171]. AZ10707120 and A740003 were shown to significantly reduce cancer growth and dissemination in several murine models [116, 172, 173].

Importantly, several considerations such as drug species-specificity [36, 144, 174], hetero-trimer involvement, BBB permeability, off-target effects, and dose selection are crucial when using the pharmacological agents [33].

Drug repurposing approach offers several advantages in drug development, including cost reduction and accelerating treatment availability for various disorders, and is especially important in rare diseases that lack dedicated drug development programmes due to limited commercial incentives. For example, AZT (Zidovudine), a nucleoside reverse transcriptase inhibitor, has been shown to block P2X7R function and alleviate disease progression in the mouse model of Duchenne muscular dystrophy [175].

Genetic approaches include KO models (reviewed in [176]. P2X7R KO mice have been widely used to study neuropathic pain [177], depression [167, 178], neuroinflammation [179], and exocrine gland secretion [180]. Notably, C57BL6 and DBA2 mice commonly used experimentally express an allelic gene variant that results in compromised functionality of the expressed P2X7R, specifically impacting the ability to form pores [181]. Hypofunctional receptor expression can modulate disease phenotypes in mouse models, potentially mitigating or exacerbating observed outcomes.

Several reporter mice have been used to try and identify the cell type-specific expression and localisation of P2X7Rs in the brain. This includes a mouse model which expresses soluble GFP under the mouse *P2rx7* promoter [182], and a reporter mouse with P2X7R C-terminally fused to EGFP [183], Of note, follow-up studies have shown that the reporter mouse expressing soluble GFP also shows increased expression of P2X4Rs and P2X7Rs. Thus, results from this mouse model should be considered with caution [184].

P2X3R KO mice have been used to examine chronic pain [185, 186] and visceral hypersensitivity [187], while P2X4R KO mice help investigate their role in neuropathic pain [170, 188] and affective disorders [189].

The cell type-specific effects of P2X receptor signalling in vivo—lessons from P2X7R

Different actions involve cell type-specific expression of P2XRs, of which the P2X7Rs have been implicated in numerous seemingly opposing functions, such as pro-inflammatory and pro-apoptotic roles [190–192] vs. promoting cell differentiation, proliferation and cellular survival [84, 193, 194]. While P2X7R expression in immune cells and neuroglia is widely acknowledged, some controversy still exists regarding its presence or absence in neurons [183, 195–197]. In addition to the complex post-transcriptional and post-translational regulation of P2X7Rs [88], different splice variants present different affinities to ATP and downstream signalling profiles [198], possibly adding to cell type-specific differences in P2X7R function. It is therefore important to consider this diversity in experimental paradigms.

P2X7R antagonists reached clinical trials [199, 200], but results were unsatisfactory [41]. Challenges in translating pre-clinical data into the clinic [201–203] may derive from species-specific differences (e.g., rodents vs humans), including possible differences in the cell type-specific expression pattern and downstream signalling landscape. Moreover, when using antagonists in CNS disease models, drug BBB penetration might be an issue, with differential effects in areas with intact and disrupted BBB [201, 202, 204]. Importantly, opposing effects of P2X7R activation on different cell types may dilute results, leading to erroneous/misleading conclusions. For example, while P2X7R deletion in microglia led to a milder seizure and epilepsy phenotype, P2X7R deletion in neurons increased seizure severity [197]. Of note, no effects had been observed in full P2X7R KO mice, with anti- and pro-convulsant functions possibly outweighing each other. Cell type-specific effects may also be the cause of opposing findings using different disease models (e.g., Alzheimer's disease [205], epilepsy [206] or cancer [201]). Moreover, the impact of P2XR

stimulation on certain cell types on other cells (e.g., P2X7R-mediated microglia activation affecting neuronal functioning) should be factored into the experimental design.

Therefore, a cell type-specific deletion of P2XR may provide a more detailed characterisation of the role of this receptor in health and disease. Mouse models, including LoxP mice from the European Conditional Mouse Mutagenesis (EUCOMM) Program and mice expressing floxed humanised P2X7 receptors, are now available [207]. Importantly, dissection of the cell type-specific function of P2XRs at different stages during development or disease progression may allow the development of cell type-specific treatment strategies involving adeno-associated viruses [197, 208] or targeted nanoparticles [209] but also identify ideal treatment windows. While cell-type-specific KO mice are an invaluable tool to establish the cell-type-specific contributions there are certain aspects that need to be taken into consideration. Several Cre lines have been shown to 'leak' into other cell types. For example, Cre lines using the *Cx3CR1* promoter to target microglia may also target non-microglial cells such as macrophages, astrocytes, or neurons [210–212]. Cre lines using the *Thy-1* promoter also target T-cells [213], which show a high expression of P2X7Rs [210]. Therefore, expression profiles of Cre lines should be carefully characterised, ensuring P2XR KO is achieved only in the desired cell line. Further, studies using conditional models should ensure that Cre-activating factors, such as tamoxifen [214], do not interfere with the disease model or impact on P2XR expression. P2XR expression in floxed mice may be altered, sometimes in a cell type-specific manner [176]. Thus, careful considerations should be taken when choosing the right controls.

Behavioural studies to assess P2X receptor functions

The P2XR family plays crucial roles in pain signalling, neuroinflammation, mood regulation, and cognitive processes. Understanding their function in vivo requires well-designed behavioural studies combined with pharmacological approaches and genetically modified animal models.

Behavioural tests should align with the specific P2XR function under investigation. Pain sensitivity can be assessed using von Frey filaments (mechanical pain), the hot plate test (thermal pain), and complete Freund's adjuvant test (inflammatory pain) [215]. Emotional conditions can be evaluated along the behavioural despair (FST, TST), learned helplessness [216] and anhedonia aspects, open field test (psychomotor agitation (EPM, LDB), and social interaction test (sociability) [217]. Cognitive functions, such as memory and learning, may be examined using the Morris water maze and passive avoidance tests [218]. Exocrine gland function can easily be assessed by the collection of pancreatic juice, saliva, and tears [180].

To ensure robust and reproducible results, baseline behaviour should be recorded before drug administration, as individual variability in locomotion, anxiety, or pain thresholds may markedly influence treatment outcomes. Doses should be selected based on prior pharmacokinetic and pharmacodynamic studies to optimise receptor engagement without inducing off-target effects or toxicity [166, 219, 220]. The timing of behavioural assessments relative to drug exposure should also be carefully standardised, considering both the onset and duration of action. Randomisation, blinding of experimenters, and inclusion of both sexes are essential to minimise bias and increase translational value [221, 222]. Furthermore, detailed reporting of environmental conditions, such as housing, circadian phase of testing, and prior handling, is critical, as P2XR activity can be influenced by stress, immune state, and metabolic factors [223, 224]. Combining behavioural tests with electrophysiology, imaging, and molecular analyses enhances mechanistic understanding and supports the identification of causal links between receptor function and behavioural phenotypes.

Constraints of experimental disease models in exploring P2X receptor involvement

The roles of P2XRs in stroke and multiple sclerosis have been investigated with intensity. In stroke research, middle cerebral artery occlusion (MCAO) models are widely used to assess their contribution and therapeutic potential ([152] reviewed in ref. [225]). P2X4R and P2X7R have been implicated in reactive microgliosis, neuroinflammation, and ischaemic lesion expansion, whereas P2X1R and P2X3R may contribute to vascular and sensory components of ischaemic brain injury [226, 227]. However, the inherent variability of MCAO models in the extent of ischaemic damage, due to differences in occlusion technique, collateral circulation, and postoperative care, can obscure subtle pharmacological effects. Incomplete representation of human stroke pathophysiology in aged patients or those with comorbidities (e.g., hypertension, diabetes), where P2XR expression and function can shift, is another limitation. Therefore, it is important to standardise the procedure before evaluating the therapeutic potential of subtype-selective ligands.

For multiple sclerosis research, experimental autoimmune encephalomyelitis (EAE) remains a standard immune-driven demyelination model and has been used in demonstrating P2X7R involvement in oligodendrocyte death, axonal damage, and neuroinflammation [152]. However, EAE primarily models T cell-mediated pathology. Complementary non-immune demyelination paradigms, such as cuprizone and lysolecithin exposure, can reveal P2XR-dependent mechanisms intrinsic to oligodendroglia, astrocytes, and microglia (e.g., ATP-driven P2X4R-mediated remyelination, P2X7R-triggered cytotoxicity) [228–231]. Combining these models allows differentiation of immune- versus glia-intrinsic roles of P2XR subtypes, thus improving translational relevance.

Models exploiting specific receptor functionalities should offer a more specific insight into the disease mechanism. For example, a protocol detecting P2X7 receptor activation in vivo in the lungs of mice, based on this receptor's ability to form a large pore, has been developed. The method involves intranasal administration of TOPRO™-3, a non-permeant DNA-intercalating dye, followed by fluorescence measurement using flow cytometry [232]. eATP enhances TO-PRO™-3 fluorescence mainly in lung immune cells in a P2X7R-dependent manner.

RECEPTOR MODULATION AND ENGINEERING APPROACHES

The low sensitivity of P2X7Rs for ATP can be modulated from the extracellular side of the plasma membrane by other proteins, by posttranslational modification, e.g., ADP-ribosylation of arginine residues, and/or nanobodies and antibodies [233–236].

Gating P2X7 receptors with ADP-ribosylation of extracellular arginine residues

Gating of P2X7Rs can be modulated by ADP-ribosylation of extracellular arginines [237–239]. This NAD-dependent reaction is catalysed by cell surface ADP-ribosyltransferases ARTC1 and ARTC2 (CD296) (Supplementary Fig. 2) [240–242]. Humans carry a pseudogene for ARTC2, but the orthologue ARTC1 has been reported to ADP-ribosylate P2X7R in human cells [243–245]. The reaction requires co-expression of P2X7Rs and ARTC on the same cell surface, as by mouse immune cells or some tumour cells [245, 246]. For a particular N-terminal variant of mouse P2X7R (P2X7k), ADP-ribosylation at R125 suffices to trigger receptor activation and gating [247]. ADP-ribosylation of other mouse P2X7R variants or human P2X7Rs does not, by itself, gate the receptors, but reduces the threshold for their gating by ATP [240, 248, 249]. Here, ADP-ribosylation resembles the effects of P2X7Rs -potentiating antibodies and nanobodies (see section: *Using antibodies and nanobodies to modulate ATP-mediated gating of P2X7 receptors*).

Notably, the submicromolar quantities of NAD released during routine isolation of primary cells from mouse spleen, liver, or kidney, suffice to induce chronic activation and death of T cell subsets that co-express P2X7k and ARTC2.2, i.e., regulatory T cells (Tregs), tissue resident memory T cells (Trm), and natural killer T (NKT) cells [239, 246, 249, 250].

This process can be controlled by the addition of ARTC2-blocking nanobodies (commercially available) to the medium of a cell culture 10 minutes before harvesting [251]. In mice, injection of the nanobodies intravenously or intraperitoneally [250] or the adeno-associated virus (AAV) vector-driven expression of ARTC2-blocking nanobody in vivo [252] are effective.

Using antibodies and nanobodies to modulate ATP-mediated gating of P2X7 receptors

A set of P2X7R-specific antibodies and nanobodies from immunised mice, rabbits, and llamas is now available [183, 235, 236, 253–256] (Supplementary Fig. 3). Many of these bind the native receptor without affecting its function. Some, however, effectively potentiate or block the gating of P2X7Rs by ATP [236].

Blocking antibodies inhibit or prevent ATP-induced gating of P2X7Rs. None of the potentiating antibodies or nanobodies selected so far induces gating of P2X7Rs on their own, but they markedly reduce the threshold for gating of P2X7Rs by ATP [236]. Thus, nanobodies and antibodies are useful tools, not only to detect P2X7Rs on cells ex vivo and in vivo, but also to modulate their function in experimental settings [235, 236, 255].

P2X7R-specific nanobodies are stable soluble binding modules that can easily be fused to other nanobodies, the Fc-domain of any antibody isotype, or even the capsid of AAV vectors (Supplementary Fig. 3) [257, 258]. All these constructs can be secreted at high yield by transfected HEK293 cells, and then the product can simply be added to a P2X7R cell assay before or during treatment with ATP.

In mice, nanobodies and heavy chain antibodies can reach P2X7Rs on the surface of parenchymal immune cells in the spleen, liver, and kidney within 10 min after intravenous or intraperitoneal injection [236, 249]. In contrast, targeting P2X7Rs on microglia or immune cells in the brain, requires either stereotactic injection into the cerebrospinal fluid or continued endogenous production in the periphery, e.g., upon intramuscular administration of AAV vectors coding for the selected nanobody [259], as described later.

Application of AAV vectors coding for selected nanobody-based biologics to modulate P2X7 receptor function in vivo

Based on their properties, nanobodies are a useful novel tool to validate the contribution of P2X7Rs in the pathophysiology of several diseases [31, 239, 255, 259–262]. To avoid repeated nanobody injections, AAV vectors encoding these biologics have been developed to induce their systemic in vivo production (Supplementary Fig. 4). Using this approach, termed AAVnano, a systemic production of anti-P2X7R nanobody-based biologics following a single intramuscular vector-injection can result in serum concentrations ranging from 10 to 40 µg/ml within 14 to 21 days post-transduction, increasing to about 100 µg/ml 30–60 days later [260–262]. Inhibitory or potentiating effects of nanobody-based biologics start to be detectable 7–14 days post AAVnano administration, reaching their maximum effect at around 21 days, and persisting until at least day 120 [252, 260–262].

This AAVnano approach has been used to evaluate the functional contribution of P2X7Rs in several disease animal models, including acute as well as chronic colitis, autoimmune diseases, and several cancer models. The penetrance of the P2X7R blocking nanobody across the blood-brain barrier to reach P2X7R at the surface of microglial cells was evaluated using either direct intracerebroventricular delivery of the recombinant nanobody, its systemic injection, or following the AAVnano strategy, i.e., upon intramuscular administration of AAV vectors coding for the same nanobody-based biologics [259]. Interestingly, 21 days post AAVnano peripheral administration, 80% of the P2X7Rs on the surface of microglial cells were bound with the nanobody-based biologics, which resulted in significant P2X7R inhibition [259].

Manipulation of P2X receptors by optical control techniques

Given the broad activity of ATP across all P2XRs and most P2Y receptors [263], conventional approaches may lack the specificity to resolve receptor subtype functions in vivo. Emerging optical control strategies, including optopharmacology and optogenetics, enable high-resolution, spatiotemporal modulation of P2XR activity, facilitating precise functional dissection.

Optopharmacology uses light-sensitive molecules or their derivatives to modulate the activity of native receptors. ATP can be linked covalently to a protecting group, such as 4,5-dimethoxy-2-nitrobenzyl (DMNB), 4,5-dimethoxy-2-nitrophenyl (DMNPE), and α-carboxy-2-nitrobenzyl (CNB). These caged compounds remain inactive until exposed to UV light, which releases ATP within milliseconds but in a controlled manner, activating P2XRs in the targeted region [264–267]. A new class of caged ATP derivatives combines photocaging and enhanced lipophilic modifications, enabling more rapid ATP release and passive cell uptake [268]. These compounds are favoured for studying the intracellular P2X pathways, such as that of P2X4 in lysosomes [269] and P2X7 receptors in mitochondria [191].

The concentration of ATP released by a single light pulse can be adjusted to account for the P2XR sensitivity to the agonist (e.g., low for P2X7 receptors). Furthermore, caged receptor-specific or selective agonists or antagonists could be used, as demonstrated for P2Y1/P2Y12 [270] and A_{2A} [271] receptors.

Irreversibility is a drawback of the caged compounds approach [268]. In pursuit of reversible manipulation, photoswitchable ligands are being designed, allowing P2XR channel opening or closing under different wavelengths [272]. Another approach exploited the light- sensitive cis–trans isomerization of the azobenzene, with azobenzene-linked maleimide bound to a cysteine residue introduced into the transmembrane domain, allowing P2X2R, P2X3R, and heteromeric P2X2R/P2X3R receptors channel opening and closing in response to 440 nm or 360 nm wavelengths, respectively [273–275].

This latter approach combines optopharmacological tools with optogenetics, which involves the genetic modification of cells to express proteins whose functions can be controlled by light [276]. Whilst photoswitchable ligands targeting P2XRs have so far been explored in vitro only, optogenetics allows for the precise activation or inhibition of genetically engineered receptors in living tissues [277]. Albeit not yet used for P2XRs, the opsin opto-A_{2A} receptors have demonstrated the potential of this approach in mouse brains [278].

Additionally, chemogenetics that uses engineered receptors designed to respond to specific, novel ligands can be implemented in parallel with optogenetic techniques [279]. A key distinction between optogenetics and chemogenetics is the temporal control over cellular activity. Optogenetics allows for real-time activation or inhibition of P2XRs with millisecond precision, while chemogenetics induces slower responses that last from minutes to hours. Spatial control is also more precise with optogenetics, as light can be delivered to specific regions using fibre-optic cables. In contrast, chemogenetics relies on the viral expression pattern and Cre-driver lines, which may influence the spatial resolution of receptor activation [280].

Optical control tools' unique challenges include off-target effects and phototoxicity with caged compounds, and compensatory responses and potential functional alterations related to receptor engineering [277, 281]. The selection of optogenetic viral

vectors for cell-specific targeting has been covered elsewhere [282], and most studies indicated that at least 3 weeks are necessary to achieve stable viral expression.

CONCLUSIONS

P2X receptor research has advanced significantly in the past two decades, yet specific challenges have delayed translation into actual therapies. We propose that future experimental designs should consider the following challenges, inclusive of: (i) Complex and overlapping receptor expression patterns on multiple cell types making cell populations responsible for a given physiological or pathological effect in vivo difficult to identify unequivocally; (ii) Dynamic regulation and plasticity, where P2XR expression and function changes during physiological process or throughout disease progression; (iii) P2XR forming homo- or hetero-trimers with distinct pharmacology, so results from homomeric studies may not reflect physiological signalling; (iv) A relative paucity of highly selective ligands that can reach the target tissue (e.g. permeate BBB), and the species-specific pharmacology that may contribute to failed translation of animal studies, further exacerbated by animal models and biochemical reactions (e.g., ribosylation) not fully replicating human disease in the context of P2XR regulation; (v) Receptor stability measured in vitro not corresponding with the in vivo conditions, where P2XR activity may be a lot more transient; (vi) eATP breakdown by ectonucleotidases making direct correlation of ATP release with receptor engagement difficult to standardise. These guidelines aim to support experimental design and reliable interpretation of results to improve reproducibility and facilitate translation into clinical applications.

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ACKNOWLEDGEMENTS

This article is based upon work from PRESTO COST Action CA21130, supported by COST (European Cooperation in Science and Technology) www.cost.eu; www.p2xcost.eu. HU and RCS research on purinergic signaling is supported by the National Institute of Science and Technology (INCT) for Purinergic Signaling: Challenges for 21st Century Health, which is funded by the National Council for Scientific and Technological Development (CNPq, grant No. 409156/2024-8) and the São Paulo Research Foundation (FAPESP, grant No. 2025/26861-0).

AUTHOR CONTRIBUTIONS

DCG conceived the article. All authors contributed to the writing of the manuscript. DCG wrote the final version, with constructive input from AV. All authors approved the submitted version of the article.

COMPETING INTERESTS

SCR is a scientific founder of Purinomia Biotech Inc and has provided consultancy services for eGenesis and Merck Pharma; his interests are reviewed and managed by Beth Israel Deaconess Medical Center in accordance with conflict-of-interest policies at Harvard Medical School. All other authors declare no conflicts of interest.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-026-08730-0.

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