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New frontiers in probing the dynamics of purinergic transmitters *in vivo*

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Abstract

Purinergic transmitters such as adenosine, ADP, ATP, UTP, and UDP-glucose play important roles in a wide range of physiological processes, including the sleep-wake cycle, learning and memory, cardiovascular function, and the immune response. Moreover, impaired purinergic signaling has been implicated in various pathological conditions such as pain, migraine, epilepsy, and drug addiction. Examining the function of purinergic transmission in both health and disease requires direct, sensitive, non-invasive tools for monitoring structurally similar purinergic transmitters; ideally, these tools should have high spatial and temporal resolution in *in vivo* applications. Here, we review the recent progress with respect to the development and application of new methods for detecting purinergic transmitters, focusing on optical tools; in addition, we provide discussion regarding future perspectives.

Keywords

Purinergic transmitters; ATP; Adenosine; Sensors; Imaging

1. Introduction

Purines and pyrimidines are essential components in living cells, where they mediate energy conversion and form the basis for synthesizing nucleic acids. In addition to these intracellular functions, some purines and pyrimidines such as ATP, adenosine, UTP, and UDP-glucose—known collectively as purinergic transmitters—also play a key role in

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Y. L. and Z. W. conceived and wrote the manuscript.

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extracellular signaling via purinergic receptors (Ralevic and Burnstock, 1998). Among these transmitters, adenosine (Ado) and ATP are arguably two of the most thoroughly studied.

As early as 1929, Alan Drury and Albert Szent-Györgyi found that an intravenous injection of Ado induced sleep in animals (Drury and Szent-Györgyi, 1929), providing the first physiological description for this nucleotide. Subsequently, a role for ATP in chemical transmission was identified in sensory nerve endings (Holton and Holton, 1954; Holton, 1959), later giving rise to the concept of purinergic transmission after ATP was identified as the transmitter involved in non-adrenergic, non-cholinergic inhibitory nerves in guinea pig intestinal smooth muscle (Burnstock, 1972).

After decades of research, it is now widely accepted that ATP can function as a neurotransmitter (Pankratov et al., 2006), gliotransmitter (Maienschein et al., 1999; Zhang et al., 2003), or co-transmitter (Burnstock, 2007; Jo and Schlichter, 1999) upon release from secretory vesicles such as synaptic vesicles, glial vesicles, and lysosomes (Zhang et al., 2007) via exocytosis (MacDonald et al., 2006). In addition, ATP can be released from cells via connexin/pannexin hemichannels (Cotrina et al., 1998), anion channels (Sabirov and Okada, 2005) and voltage-gated ion channels (Ma et al., 2018; Taruno et al., 2013). Extracellular Ado is believed to be generated by the breakdown of extracellular nucleotides, particularly ATP and ADP (Chen et al., 2013), via ecto-nucleotidases (Zimmermann et al., 2012), or by the direct efflux of Ado through the so-called equilibrative nucleoside transporters (ENTs) (Lovatt et al., 2012); whether Ado functions as a neurotransmitter released via the exocytosis of synaptic vesicle is currently under debate (Corti et al., 2013; Dale and Frenguelli, 2009). In the extracellular space, Ado and ATP perform distinct functions by activating P1 receptors and P2 receptors, respectively. The P1 receptor family consists of the A_1, A_{2A}, A_{2B}, and A_3 G protein−coupled receptors (GPCRs), whereas P2 receptors include the ionotropic P2X receptor (Khakh and North, 2006) and the G protein−coupled metabotropic P2Y receptor. Both P1 and P2 receptors are widely expressed and have been implicated in a variety of physiological processes (Burnstock, 2008; Chen et al., 2013), including controlling the sleep-wake cycle (Huang et al., 2005; Porkka-Heiskanen et al., 1997), learning and memory (Dias et al., 2013), cardiovascular function (Shryock and Belardinelli, 1997), and the immune response (Eltzschig et al., 2012), as well as pathological processes, including pain (Hasko et al., 2008), epilepsy (Boison, 2012; During and Spencer, 1992; Engel et al., 2016), ischemia–reperfusion (Eltzschig and Eckle, 2011), drug addiction (Dunwiddie, 1999), and neurodegenerative disorders (Schwarzschild et al., 2006).

In addition to the broad effects that Ado and ATP have on various systems, functions of other purinergic transmitters are also identified. For example, ADP plays an important role in platelet aggregation by acting at $P2Y_1$ receptor (Jin et al., 1998); UTP has been found to play a role in nociception in the central (Li et al., 2014; Ren et al., 2016) and enteric (Hockley et al., 2016) nervous systems and is an agonist of P2Y receptors; UDP-glucose may induce inflammatory effects by acting at the $P2Y_{14}$ receptor (Lazarowski and Harden, 2015).

Despite the significant contributions to our understanding of purinergic signaling by many researchers over the past several decades, many key questions remain, including which

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purinergic transmitters are released—and where—under physiological and pathological conditions, how purinergic transmitters are converted from one to another, and details regarding their spatial and temporal release patterns. Answering these questions requires a detection method that provides high spatial and temporal resolution, as well as high molecular precision in order to monitor the dynamics of structurally similar purinergic transmitters, ideally in living animals. Here, we review the technologies that are currently available for detecting purinergic transmitters, including classic non-optical methods and recently developed optical methods. The molecular, pharmacological, and functional profiles of purinergic signaling have been reviewed by other groups (Burnstock, 2008; Chen et al., 2013) and are not discussed here in detail.

2. Non-optical methods for probing purinergic transmitters

2.1 Electrophysiology

Because the binding of ATP to P2X receptors induces an increase in cationic conductance, electrophysiological recording can be used to measure ATP by recording currents through P2X receptors (Fig. 1A1). Using the patch-clamp technique, ATP-induced P2X currents can be recorded in cells expressing endogenous P2X receptors or in “sniffer cells” that express P2X receptors (Hayashi et al., 2004; Zhang et al., 2019). In addition to ionotropic receptors, all purinergic transmitters identified to date also have one or more corresponding metabotropic G protein−coupled receptors (GPCRs), which transduces receptor activation into downstream electrophysiological effects on target cells. For example, electrophysiological studies in guinea pig hippocampal slices revealed that NMDA-induced depression of evoked excitatory postsynaptic currents was abolished by the adenosine A1 receptor antagonist 8-cyclo-pentyltheophylline, suggesting that adenosine is released in an activity-dependent manner (Manzoni et al., 1994). Similarly, the binding of non-adenosine purinergic transmitters to P2Y receptors induces the G protein−mediated opening or closing of downstream ion channels, giving rise to an electrophysiological change that can be recorded in target cells. Although electrophysiological methods are extremely sensitive, they typically have extremely low throughput. Moreover, because electrophysiological methods mainly detect the downstream effects of purinergic transmitters, pharmacology is often required in order to verify molecular specificity. Finally, although electrophysiology can achieve temporal resolution on the order of milliseconds, it does not provide a measure of the transmitter’s release kinetics. Thus, combining electrophysiology with direct detection methods would enable researchers to study both the release of purinergic transmitters and their downstream effects in target cells.

2.2 Microdialysis

A widely used method for directly measuring purinergic transmitters is microdialysis (Fig. 1A2) (Bito et al., 1966; Porkka-Heiskanen et al., 1997), often in combination with high-performance liquid chromatography (HPLC) together with either ultraviolet (UV) absorption detector or mass spectrometry (MS). With this method, a microdialysis probe (~200 μm in diameter), containing physiological solution such as artificial cerebrospinal fluid (aCSF), is first implanted into the brain region(s) of interest (Porkka-Heiskanen et al., 2000), and extracellular small molecules such as purinergic transmitters are collected via passive

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diffusion through the semi-permeable membrane. The dialysates are then separated by HPLC and detected using MS or UV. Microdialysis has several clear advantages compared to other approaches. First, it can be used in living animals, including humans. Second, this approach has extremely high sensitivity for detecting purinergic transmitters (in the nanomolar range). Third, it can be used to detect multiple chemicals simultaneously in one sample. Finally, it can provide the absolute concentrations of multiple chemicals. On the other hand, microdialysis also has several drawbacks. First, the sampling rate is relatively slow, on the order of minutes; this low temporal resolution precludes the possibility of capturing the rapid dynamics of purinergic transmitters. In addition, purinergic transmitters can be metabolized during the lengthy sample preparation. A second disadvantage of microdialysis is that although the microdialysis probe can be quite small, it cannot provide subcellular spatial resolution or even cell-type specificity. Finally, implanting microdialysis probe(s) in the brain can cause tissue damage and/or neuroinflammation, thereby potentially altering the concentration of purinergic transmitters in the local microenvironment (Ohyama et al., 2019).

2.3 Amperometric biosensors

In order to detect purinergic transmitters in real time, researchers have developed a variety of electrochemistry-based methods using electrodes, including enzyme-based amperometric biosensors (Dale, 1998; Dale et al., 2000) and fast-scan cyclic voltammetry (FSCV) using carbon-fiber microelectrodes (Swamy and Venton, 2007). The basic principle of amperometric biosensors (Fig. 1A3) capitalizes on the metabolization of purinergic transmitters to create hydrogen peroxide (H_2O_2) via an enzymatic cascade; the resulting H_2O_2 can then be detected using an amperometer at the microelectrode's surface (Dale and Frenguelli, 2012). For example, to detect ATP, the surface of the microelectrode is coated with the glycerol kinase and glycerol-3-phosphate oxidase (Llaudet et al., 2005). In the presence of ATP, glycerol is phosphorylated by glycerol kinase to produce glycerol-3-phosphate, which is then oxidized by glycerol-3-phosphate oxidase, generating H_2O_2. When glycerol is available at saturated levels, this biosensor provides a final readout that is proportional to the concentration of ATP. A similar strategy has been used to detect adenosine, in this case using three enzymes, adenosine deaminase, nucleotide phosphorylase, and xanthine oxidase (Dale and Frenguelli, 2012). During the enzymatic cascade, adenosine is first converted to inosine, then to hypoxanthine, and finally to xanthine, urate, and H_2O_2.

Amperometric biosensors provide sufficient temporal resolution and nanomolar-range sensitivity for detecting ATP and/or adenosine dynamics in different brain regions, including the hippocampus (Wall and Dale, 2013) and cerebellum (Wall and Dale, 2007), and under a variety of physiological and pathological conditions (Dale and Frenguelli, 2009; Gourine et al., 2005). On the other hand, amperometric biosensors have relatively poor molecular specificity and can also detect intermediate products. For example, the adenosine amperometric biosensor is sensitive to both adenosine and its metabolite inosine. Therefore, a control experiment should be performed using a sensor that does not include the adenosine deaminase (Dale and Frenguelli, 2012). When measuring adenosine, the control sensor can be positioned close to the full biosensor in order to distinguish adenosine from similar

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compounds; the adenosine-specific signal can then be calculated by subtracting the control sensor's signal from the full biosensor's signal. Similarly, using an amperometric biosensor to measure ATP has this problem as well. In addition, as with other electrode-based sensors, placing the amperometric biosensors in the brain is invasive and can lead to tissue damage. Finally, amperometric biosensors usually have relatively limited spatial resolution and are unable to detect purinergic transmitter in specific cell types. In principle, if an enzymatic cascade catalyzes the bioconversion of purinergic transmitters other than adenosine or ATP, and if that cascade produces H_2O_2 at levels proportional to the original transmitter's concentration, it should be possible to develop new amperometric biosensors that can detect a diverse number of purinergic transmitters; however, to the best of our knowledge, this method has only been used to detect adenosine and ATP.

2.4 Fast-scan cyclic voltammetry

In addition to amperometry, fast-scan cyclic voltammetry (FSCV) with carbon-fiber microelectrodes (Fig. 1A4) has also been used as an electrochemical method to directly monitor purinergic transmitters, including adenosine (Swamy and Venton, 2007). Traditionally, FSCV has been used to detect monoamine neuromodulators such as dopamine, norepinephrine, and serotonin (Bunin et al., 1998; Park et al., 2011), as monoamines can be readily oxidized, thereby giving rise to signature currents under a specific cyclic voltage scan range. Because adenosine is also an electroactive molecule that undergoes a series of two-electron oxidation steps, it can also be detected using FSCV (Nguyen and Venton, 2015). Compared to amperometric biosensors, FSCV electrodes have similar sensitivity (i.e., in the nanomolar range). Importantly, however, FSCV can achieve temporal resolution on the order of hundreds of milliseconds; thus, FSCV has been used to detect rapid activity-dependent adenosine release (Pajski and Venton, 2010), as well as spontaneous transient adenosine signals (Nguyen et al., 2014) in brain slices. With respect to specificity, adenosine metabolites such as inosine are electrochemically inactive to carbon-fiber electrodes; therefore, FSCV has higher specificity than amperometric biosensors (Nguyen and Venton, 2015). Moreover, although FSCV was successfully used for monitoring adenosine, it was still difficult to expand this method for measuring other purinergic transmitters. Finally, as with other electrode-based methods, FSCV lacks subcellular spatial resolution and cell-type specificity.

3. Optical methods

Compared to the conventional non-optical methods discussed above, optical imaging methods provide direct, sensitive, higher throughput, and long-term monitoring neurotransmitter dynamics in cultured cells, brain slices, and behaving animals *in vivo*. More importantly, the genetically-encoded optical sensor could be targeted to defined celltypes or subcellular compartments to achieve the cell-type specificity and/or subcellular spatial resolution. Compared with the classical electrophysiology recording, optical imaging methods are less invasive and therefore provide repeatable measurements of neurotransmitter dynamics from the same neuronal population. Therefore, optical methods are becoming increasingly popular. Several optical sensors have been developed

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for detecting both intracellular purine metabolites (Table 1) and extracellular purinergic transmitters (Table 2), and are discussed in further detail below.

3.1 Optical sensors for detecting intracellular purine metabolites

Within the cytoplasm, purine metabolites—particularly ATP—serve as a universal and highly important source of cellular energy, and several optical methods have been developed in order to investigate how ATP affects cellular physiology. For example, firefly luciferase—an ATP-consuming enzyme that oxidizes the small-molecule substrate luciferin to produce a bioluminescent product—has been widely used to measure ATP both *in vitro* and *in vivo*. Importantly, luciferase can be expressed in specific target cells or even in subcellular organelles when fused with sequences that target the protein to specific structures such as the mitochondria or synaptic vesicles (Rangaraju et al., 2014); organelle-specific luciferase-based sensors have been discussed in detail elsewhere (Morciano et al., 2017). Unfortunately, luciferase can only be used to detect ATP, not other purine metabolites. Moreover, by detecting ATP, the enzymatic reaction itself consumes ATP, which inevitably leads to changes in cellular metabolism. Recently, Mathew Tantama and colleagues developed a ratiometric bioluminescence resonance energy transfer (BRET) sensor for detecting ATP; this system, called ARSeNL, uses the ATP-independent NanoLuc luciferase and the red fluorescent protein mScarlet as the donor and acceptor, respectively (Min et al., 2019). Using this sensor, they measured changes in energy metabolism both *in vitro* and *in vivo*. Because mammalian cells do not normally express bioluminescent proteins, luminescence-based methods usually have an extremely low background signal (Morciano et al., 2017); on the other hand, bioluminescence-based methods produce low numbers of photons and lack cellular-scale resolution, which can limit its general applicability.

Unlike bioluminescence-based methods, sensors based on fluorescent proteins can—at least in principle—provide higher spatial and temporal resolution with respect to imaging purinergic transmitters. A fluorescent sensor requires two components: *i*) a binding protein that specifically recognizes purinergic transmitters and generates a conformational change upon ligand binding, and *ii*) a fluorescent component that converts the conformational change into a fluorescent signal. To develop such a sensor, several proteins were covalently coupled to a fluorescent reporter dye that undergoes a change in fluorescence upon binding ATP or ADP, thereby providing a measure of ATP/ADP concentration (Kunzelmann and Webb, 2009, 2010; Vancraenenbroeck and Webb, 2015). Such chemical dye−based methods provide an important tool for quantifying ATP with high sensitivity and high throughput; however, because it can be challenging to target these tools to specific cells, this method is generally limited.

To overcome this limitation, Gary Yellen and colleagues engineered Perceval—a fully genetically encoded fluorescent protein−based sensor for imaging ATP—by inserting circularly permuted monomeric Venus (cpmVenus) into GlnK1, an ATP-binding bacterial protein that regulates ammonia transport (Berg et al., 2009). Like the native GlnK1 protein, Perceval measures the ATP/ADP ratio. However, because Perceval binding is saturated at a relatively low ATP/ADP ratio (< 5:1), its effectiveness is limited in mammalian cells. They therefore increased the sensor's dynamic range so that it can still detect ATP/ADP ratios in

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healthy mammalian cells, which can reach 100:1. This optimized sensor, called PercevalHR (Tantama et al., 2013), produces a stronger signal, thus allowing researchers to measure the ATP/ADP ratio at the single-cell level; moreover, PercevalHR performs well using either one-photon or two-photon excitation. Unfortunately, however, both Perceval and PercevalHR are highly sensitive to pH; therefore, the fluorescence change that derives from the bona fide signal can be difficult to distinguish from an artifact. Moreover, the Perceval-based sensors can only be used to monitor cellular energy levels and would be difficult to use for directly quantifying specific purine metabolites.

To specifically visualize ATP levels within individual living cells, Hiroyuki Noji and colleagues generated a series of sensors based on fluorescence resonance energy transfer (FRET); these sensors, called ATeams, have specific apparent affinities ranging from 7.4 μM to 3.3 mM (Imamura et al., 2009). ATeams contain the epsilon subunit of bacterial F_0F_1-ATP synthase as the ATP-sensing protein "sandwiched" between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). By targeting these sensors to specific subcellular compartments, Noji's group successfully measured ATP levels within the mitochondrial matrix, cytoplasm, and nucleus in cultured HeLa cells. Unfortunately, because FRET efficiency is relatively low, the applications of ATeam are limited, particularly with *in vivo* preparations. In addition to their FRET-based ATeams, Hiroyuki Noji, Hiromi Imamura, and their colleagues developed an excitation ratio−based sensor called QUEEN by inserting circularly permuted enhanced green fluorescent protein (cpEGFP) between two α-helices of the bacterial F_0F_1-ATP synthase epsilon subunit; they then used this sensor to measure ATP levels in *E. coli* cells (Yaginuma et al., 2014). Despite their advantages, both the FRET-based ATeam sensors and the excitation ratio−based QUEEN sensors require customized equipment, have relatively slow imaging rates, and have a lower signal-to-noise ratio compared to single wavelength−based sensors (Lobas et al., 2019).

Recently, Baljit Khakh, Loren Looger, and their colleagues developed a genetically encoded GFP-based single-wavelength ATP sensor called iATPSnFR by inserting circularly permutated super-folder GFP (cpSFGFP) in the epsilon subunit of the *Bacillus PS3* F_0F_1-ATPase (Lobas et al., 2019). This newly developed sensor produces a bright, rapid, specific increase in fluorescence increase upon binding ATP (with peak $\Delta F/F_0$ values of ~3.9 using purified proteins); however, the low affinity of iATPSnFR for ATP (on the order of hundreds of micromolar) might not be sensitive enough for *in vivo* applications, particularly when measuring extracellular ATP.

3.2 Optical sensors for detecting extracellular purinergic transmitters

Several strategies have been used to develop optical sensors for detecting extracellular purinergic transmitters, including: *i*) targeting cytosolic sensors to the plasma membrane, and *ii*) using plasma membrane−localized proteins to engineer new optical sensors. The resulting optical sensors can be based on either bioluminescence or fluorescence, and are discussed in detail below.

3.2.1 Bioluminescence-based sensors—As discussed above, luciferase-based bioluminescent sensors are widely used for measuring ATP (Fig. 1B1). To measure local

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ATP release from activated platelets, George Dubyak and colleagues attached a purified IgG binding domain−tagged luciferase to the surface of cells expressing IgG (Beigi et al., 1999). Similarly, using luciferase either diluted in extracellular solution (Wang et al., 2000) or immobilized to beads (Zhang et al., 2008), Edward Yeung and colleagues successfully measured mechanical stimulation-evoked release of ATP from astrocytes *in vitro*. In addition, by fusing a leader sequence and glycosylphosphatidylinositol (GPI) lipid anchor derived from the folate receptor to luciferase, Francesco Di Virgilio, Paolo Pinton, and their colleagues designed a plasma membrane−targeted luciferase called pmeLUC, which they then used to measure extracellular ATP dynamics both in cultured cells and in living animals injected with pmeLUC-expressing cells (Morciano et al., 2017). Again, although luciferase-based bioluminescent sensors have an extremely low background signal, their output yields relatively low numbers of photons, which reduces their feasibility for imaging purinergic transmitters at the cellular scale. Interestingly, the bioluminescent signal can be amplified by attaching the sensor to a downstream GPCR (Barnea et al., 2008); however, these so-called TANGO assays usually have poor temporal resolution (on the order of minutes to hours), making them less feasible for monitoring purinergic transmitters in real time.

3.2.2 Fluorescence-based sensors—Compared to bioluminescence-based sensors, fluorescence-based sensors have significantly higher brightness, which enables researchers to visualize purinergic transmitters with optical precision and/or millisecond temporal resolution. Therefore, fluorescence-based sensors are often used to analyze molecules and events that occur with rapid dynamics at a subcellular level using both *in vitro* and *in vivo* preparations. Here, we discuss the recent progress in fluorescence-based sensors for measuring purinergic transmitters, including cell-based sensors, FRET-based sensors, and single-wavelength sensors.

3.2.2.1 Cell-based sensors: Several cell-based sensors—also referred to as “sniffer” cells (Fig. 1B2)—have been developed for monitoring purinergic transmitters. With this method, a specific cell surface receptor that recognizes the purinergic transmitter of interest is expressed in the sniffer cells, which are then placed in close proximity to the target cells. For example, the Ca^{2+} dye Fluo-4-AM was loaded in the mouse glioma cell line GL261, which express high-sensitivity purinergic receptors, and these cells were then placed on the surface of a mouse brain slice and used to indirectly image ATP release; the Ca^{2+} signal measured in the sniffer cells was due specifically to the release of ATP, as it was blocked by the purinergic receptor blocker Reactive Blue-2 (Haas et al., 2006). A similar strategy was used to detect changes in extracellular adenosine by placing A_1R-expressing and Fura-2 loaded HEK293T cells in the brain slice (Yamashiro et al., 2017). Although cell-based sensors can report specific purinergic transmitters with high sensitivity, they are not suitable for detecting the release of purinergic transmitters with high cell-type specificity or subcellular resolution. In addition, the sniffer cells would need to be implanted in specific tissues when used in living animals, making this approach potentially too invasive for use in *in vivo* applications.

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3.2.2.2 FRET-based sensors: In principle, the above-mentioned limitations can be overcome using a genetically encoded probe, which allows researchers to monitor neuromodulator dynamics in specific cell types with subcellular spatial resolution. One such example is FRET-based sensors. For example, Martin Lohse and colleagues developed a CFP/FlAsH-based FRET sensor using the adenosine 2A receptor ($A_{2A}R$) as the backbone (Hoffmann et al., 2005). This sensor, which has similar affinity for $A_{2A}R$ agonists compared to the wild-type $A_{2A}R$, produces an ~10% change in the FRET ratio in response to a saturated concentration of adenosine. Similarly, Baljit Khakh and colleagues used the ATP receptor $P2X_2$ as the detecting module in their FRET sensor $P2X_2$-Cam for imaging ATP; specifically, they fused the $P2X_2$ receptor with a YC3.1 tag, which contains CFP and YFP separated by the Ca^{2+} sensor Cameleon (Richler et al., 2008). The Cameleon domain undergoes a conformational change upon binding Ca^{2+}, changing the relative positions of the CFP and YFP moieties, thereby causing an increase in the FRET ratio. The authors found that $P2X_2$-Cam has an ~37% change in the FRET ratio in response to a saturated concentration of ATP, with an EC_{50} of approximately 13 μM. Unfortunately, because FRET-based sensors have a relatively low signal-to-noise ratio, they are less suitable for use in *in vivo* applications. Indeed, although $P2X_2$-Cam was used in live zebrafish to measure the application of ATP-induced ratiometric changes, it has not been used to detect endogenous ATP release *in vivo*. In addition to using plasma membrane−localized $A_{2A}R$ or $P2X_2$ receptors to engineer optical sensors, cytosolic sensors could also be targeted to the plasma membrane for probing extracellular purinergic transmitters. For example, Mathew Tantama and colleagues re-engineered the FRETbased ATeam ATP sensor to be expressed on the cell surface, and the new ecAT3.10 sensor (Fig. 1B3) enabled probing ATP release in cultured cells but not *in vivo* (Conley et al., 2017).

3.2.2.3 Single-wavelength sensors: Compared to FRET-based sensors, single-wavelength sensors have a higher signal-to-noise ratio and are therefore more suitable for measuring endogenous neuromodulator dynamics *in vivo*. For example, in order to image extracellular ATP, the genetically encoded single-wavelength iATPSnFR (Fig. 1B4) was displayed at the extracellular side of the plasma membrane (Lobas et al., 2019). Despite their advantages, however, sensors based on bacterial binding proteins—including iATPSnFR—have relatively low ligand affinity, which limits their application, particularly with respect to *in vivo* preparations. Although the sensor's affinity could theoretically be improved through molecular engineering, this can be highly time-consuming. Moreover, although this strategy has been used to develop ATP sensors, to the best of our knowledge it has not been used to develop sensors for other purinergic transmitters.

4. Future perspectives

Despite significant advances over the past few decades, current methods for detecting purinergic transmitters are still limited in several respects, including poor spatial and/or temporal resolution, insufficient sensitivity, and/or low specificity; thus, a method that satisfies all of these criteria is needed. Yet the question remains: how to engineer a sensor that can be used to monitor structurally similar purinergic transmitters with high specificity and sensitivity? In fact, nature has already provided us with an important clue: in mammals,

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all purinergic transmitters identified to date bind at least one G protein−coupled receptor. Thus, GPCRs provide an excellent scaffold for developing tools to detect purinergic transmitters. In this respect, a promising new strategy is to generate single-fluorophore GPCR Activation-Based (GRAB) sensors (Jing et al., 2019); indeed, this strategy has been used successfully to engineer sensors for detecting acetylcholine (Fig. 2A) (Jing et al., 2018), dopamine (Patriarchi et al., 2018; Sun et al., 2018), and norepinephrine (Feng et al., 2019). Importantly, these sensors have a wide dynamic range, rapid kinetics, single-cell resolution, and—most importantly—exquisite selectivity (Fig. 2B). In addition, most GRAB sensors have negligible coupling with their GPCR's downstream signaling pathways, and have no detectable effects on cellular physiology (Wu et al., 2019). These sensors allow researchers to monitor neurotransmitter dynamics *in vivo* in a variety of organisms, including flies, fish, birds (Tanaka et al., 2018), and mice (Fig. 2C).

Given the wide variety of chemical-sensing GPCRs expressed in the nervous system, this strategy can likely be used to develop sensors for detecting a wide range of molecules, including purinergic transmitters (Fig. 2D). Moreover, by attaching fluorescent proteins with different excitation/emission profiles, multiple sensors with non-overlapping spectra can be developed and then used in combination to study the complex interplay between different purinergic transmitters. In this respect, the use of simultaneous multi-color imaging to measure the release of multiple purinergic transmitters in real time will provide important insights into how these purinergic transmitters function in the brain and other tissues in both health and disease.

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Highlights

- Purinergic transmitters play critical roles in physiological and pathological processes.
- Current methods for purinergic transmitter detection are thoroughly summarized.
- New optical sensors are promising for monitoring purinergic transmitters *in vivo*.

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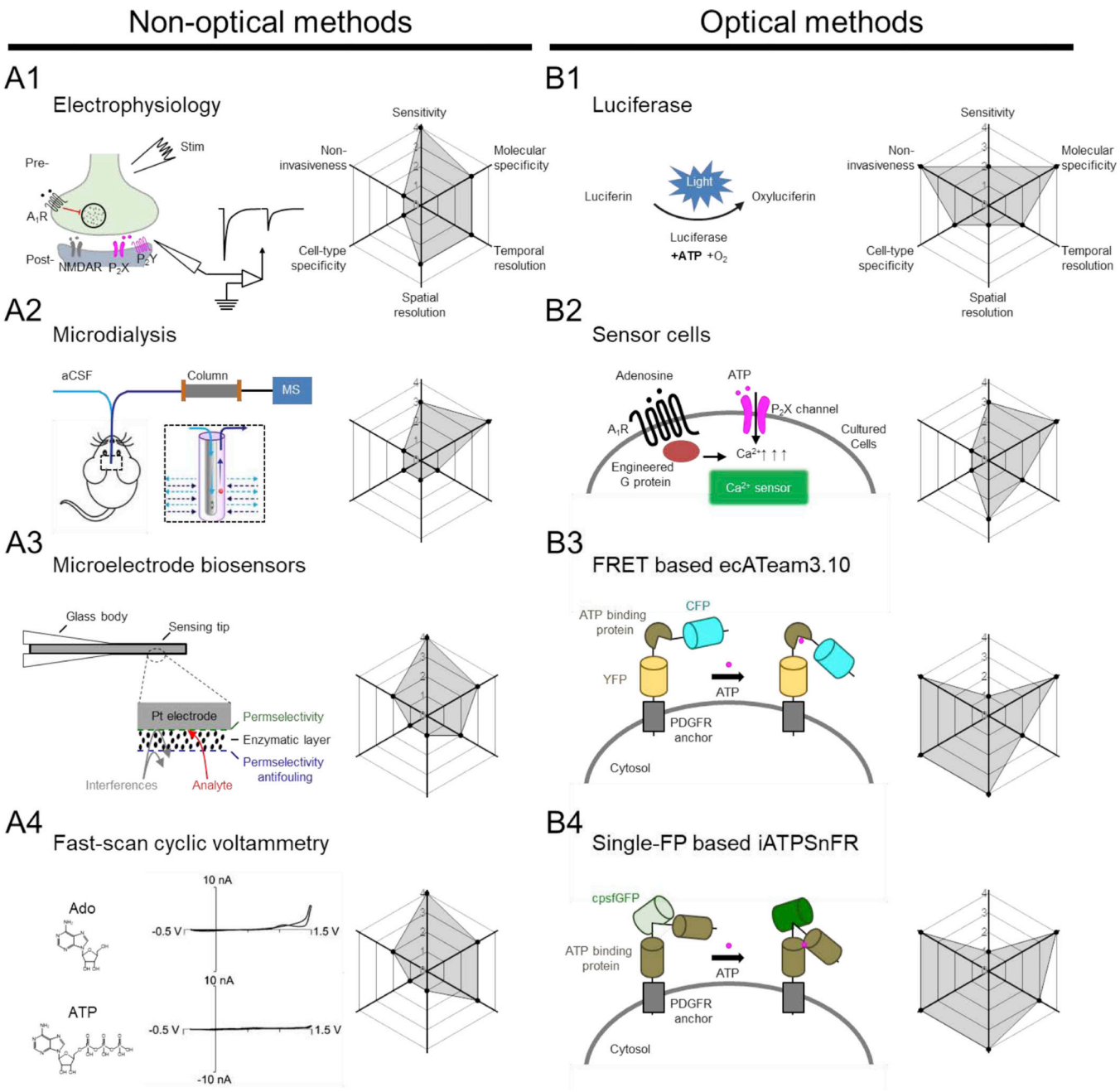

Fig. 1. Overview of select non-optical and optical methods for detecting purinergic transmitters.

In each panel, the principle (with example data, where indicated) behind for each method is shown at the left; the corresponding radar graph summarizing the method's performance index (including sensitivity, molecular specificity, cell-type, non-invasiveness, spatial resolution, and temporal resolution) is shown at the right, with arbitrary units ranging from 0 to 4. Abbreviations: aCSF, artificial cerebrospinal fluid; Ado, adenosine; ATP, adenosine triphosphate; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; IgK, light chain kappa; MS, mass spectrometry; NMDAR, *N*-methyl-D-aspartate receptor; PDGFR, platelet-derived growth factor receptor; YFP, yellow fluorescent protein.

Schematic shown in (A3) adapted from (Dale and Frenguelli, 2012); data shown in (A4) adapted from (Swamy and Venton, 2007); schematic shown in (B3) adapted from (Conley et al., 2017); schematic shown in (B4) adapted from (Lobas et al., 2019).

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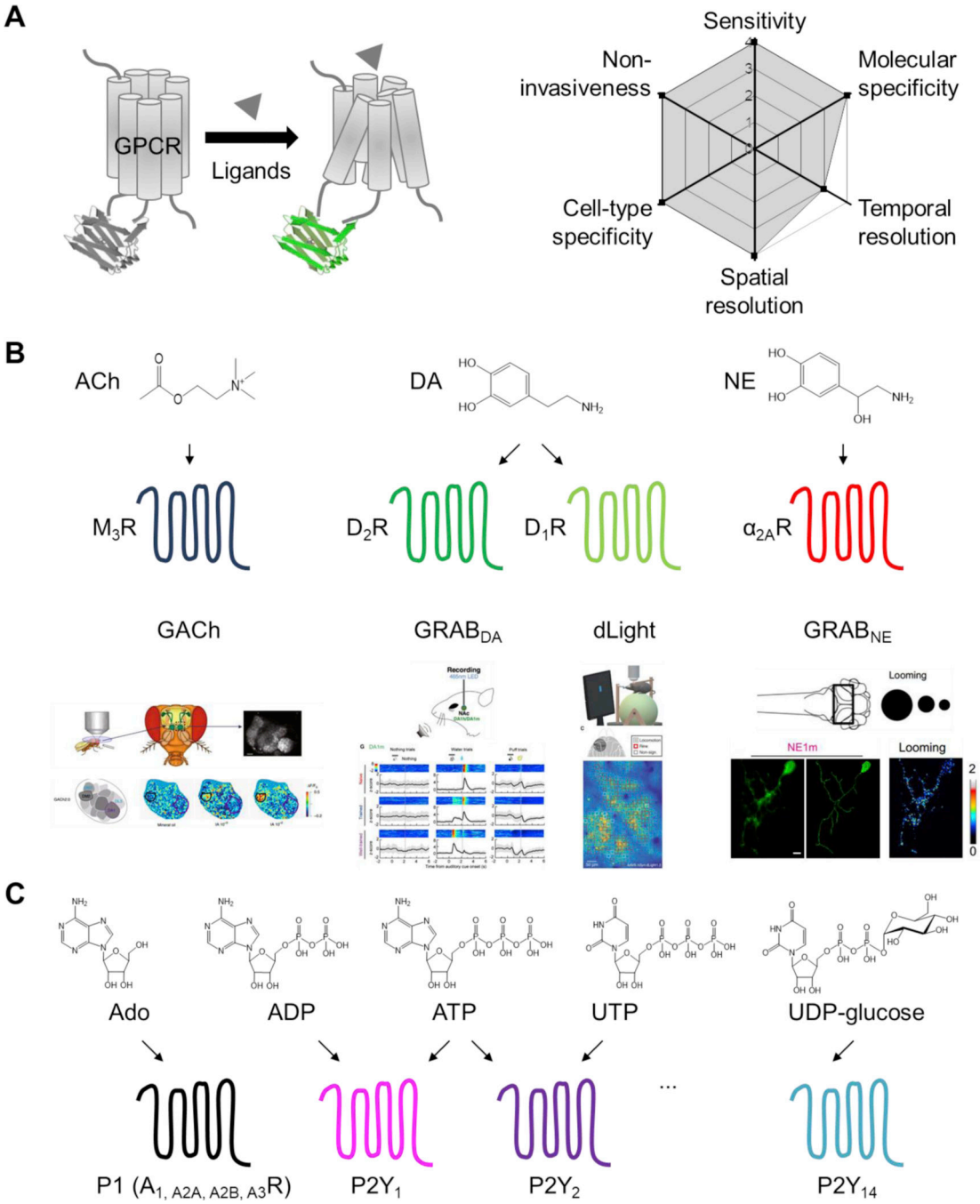

Fig. 2. A proposed toolbox for detecting purinergic transmitters

(A) The principle behind our proposed GPCR Activation-Based (GRAB) sensors (left) for detecting transmitters, with the corresponding theoretical performance index (right). (B) A similar GRAB strategy was successfully used for *in vivo* monitoring of acetylcholine (ACh), dopamine (DA), and norepinephrine (NE) in behaving animals. (C) A proposed toolbox of GRAB sensors for monitoring various purinergic transmitters; the structure of

each transmitter is shown at the top. Data shown in (B) adapted from (Jing et al., 2018), (Sun et al., 2018), (Patriarchi et al., 2018), and (Feng et al., 2019).

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Table 1.

Overview of optical sensors for detecting intracellular purine metabolites

Sensor	Ligand	Binding protein / module	Optical reporter	ΔF/F_0 or FRET/ BRET ratio	Kd or EC50	Kinetics	*In vivo* application	Ref.
Luciferase	ATP	No	Bioluminescence	N.D.	N.D.	N.D.	N.D.	(DeLuca and McElroy, 1974)
Syn-ATP	Presynaptic ATP	No	Bioluminescence	~0.25	2.3 mM	N.D.	N.D.	(Rangaraju et al., 2014)
ParM-based sensor	ADP	Bacterial actin-like ParM	Chemical dyes	>3.5/~15	0.45/~30 μM	0.65/9.5×10^4 $M^{-1}s^{-1}$ (τ_{on}),2.9 s^{-1} (τ_{off})	N.D.	(Kunzelmann and Webb, 2009)
FRET-Based chemosensor	Nucleoside polyphosphates	Binuclear zinc complex	Chemical dyes	~33	0.1–1 μM	N.D.	N.D.	(Kurishita et al., 2010)
Perceval and PercevalHR	Cytosolic ATP/ADP ratio	Bacterial GlnKI	cpmVenus	KR*: 0.5/3.5	N.D.	N.D.	N.D.	(Berg et al., 2009; Tantama et al., 2013)
ATeam	Cytosolic ATP	Bacterial F_0F_1-ATP synthase	CFP/YFP	~1.5	7.4 μM-3.3 mM	~17μM/S (τ_{on}), 98ms (τ_{off})	N.D.	(Imamura et al., 2009)
ARSeNL	ATP	Bacterial F_0F_1-ATP synthase	mScarlet/NanoLuc	~5	~1.1 mM	N.D.	Mice	(Min et al., 2019)
QUEEN	Cytosolic ATP	Bacterial F_0F_1-ATP synthase	cpEGFP	<4 (excitation ratio)	7 μM	N.D.	N.D.	(Yaginuma et al., 2014)
iATPSnFR	ATP	Bacterial F_0F_1-ATP synthase	cpSFGFP	~3.0 (?)	138 μM, 350 μM	< 5s (τ_{on}), >1s (τ_{off})	N.D.	(Lobas et al., 2019)

K_R* represents a half-maximal signal change, which was used to quantify the ability of Perceval sensors to report ATP/ADP ratio. N.D., not determined.

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Table 2.

Overview of optical sensors for detecting extracellular purinergic transmitters

Sensor	Ligand	Binding protein / module	Optical reporter	$\Delta F/F_0$ or FRET/BRET ratio	Kd or EC_{50}	Kinetics	*In vivo* application	Ref.
pmeLUC	ATP	No	Bioluminescence	~8	> 10 μM	N.D.	Mice	(Morciano et al., 2017)
TANGO Assay	GPCR ligands	GPCRs	Reporter gene	>10	~ 1 nM	>8 h	N.D.	(Barnea et al., 2008)
Sniffer cells	Adenosine, ATP, or ADP	A_1R, P2X, or P2Y	Ca^{2+} sensor	~1.5 (?) for Ado	8 0μM (Ado)	N.D.	N.D.	(Yamashiro et al., 2017)
ecATeam3.10	ATP	Bacterial F_0F_1- ATP synthase	CFP/YFP	~0.27	~11 mM	~13–172 s (τ_{on})	N.D.	(Conley et al., 2017)
$A_{2A}R$-based FlAsH	Adenosine	$^{A}2A^{R}$	CFP/FlAsH	~0.1	Similar to WT $A_{2A}R$ (?)	66–88 ms (τ_{on})	N.D.	(Hoffmann et al., 2005)
$P2X_2$-Cam	ATP	$P2X_2$	CFP/YFP	~0.37	~14 μM	~6 s (decay)	Zebrafish	(Richler et al., 2008)
iATPSnFR	ATP	Bacterial F_0F_1- ATP synthase	cpSFGFP	~1.0	350 μM	<5 s (τ_{on}), >1 s (τ_{off})	N.D.	(Lobas et al., 2019)

N.D., not determined.

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