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GPCR-based sensors for imaging neurochemicals with high sensitivity and specificity

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Abstract

Neurotransmitters and neuromodulators are key neurochemicals that mediate cell-cell communication, maintain the body's homeostasis, and control a wide range of biological processes. Thus, dysregulation of neurochemical signaling is associated with a range of psychiatric disorders and neurological diseases. Understanding the physiological and pathophysiological functions of neurochemicals, particularly in complex biological systems *in vivo*, requires tools that can probe their dynamics with high sensitivity and specificity. Recently, genetically-encoded fluorescent sensors for visualizing specific neurochemicals were developed by coupling neurochemical-sensing G protein-coupled receptors (GPCRs) with a circular-permutated fluorescent protein (cpFP). These GPCR-based sensors can monitor the dynamics of neurochemicals in behaving animals with high spatiotemporal resolution. Here, we review recent progress regarding the development and application of GPCR-based sensors for imaging neurochemicals, and we discuss future perspectives.

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Introduction

Since the discovery of neurotransmitters and neuromodulators in the early 1900s (Loewi 1924; Elliott 1905; Dale & Dudley 1929; Bayliss 1901), extensive studies have been performed in an attempt to understand their physiological functions. Compelling evidence now exists that communication via neurochemical transmission plays a key role in a variety of functions and behaviors ranging from the cellular level to the whole-animal level (Klinkenberg *et al.* 2011; Dale *et al.* 1936; Katz & Miledi 1961; Schultz *et al.* 1997; Carlson 1995). Traditional detection techniques such as microdialysis, electrochemical recording, and electrophysiology are still invasive and have limitations with respect to monitoring a population of neurons with high spatial resolution (Smith *et al.* 1992; Kehr 1992; Mas *et al.* 1995; Adams 1973). The main push forward to better understand the role of neurotransmitters from cellular to whole-animal levels greatly demands new tools with high cell type specificity, physiologically relevant affinity, high molecular specificity, millisecond temporal resolution, and subcellular spatial resolution .

Genetically encoded fluorescent sensors would be an ideal tool for detecting neurochemicals in a highly cell type-specific manner (Liang *et al.* 2015; Lin & Schnitzer 2016). Recently, G protein-coupled receptors (GPCRs) were used as the scaffold for generating genetically encoded sensors, due to their high ligand selectivity and physiologically relevant ligand affinity. To date, GPCR-based sensors have been developed for measuring the *in vivo* dynamics of several neurochemicals; these sensors include the GPCR-activation based acetylcholine (ACh) sensor (GACh sensor) for detecting acetylcholine (Jing *et al.* 2018), the GPCR-Activation Based dopamine (DA) sensor ($GRAB_{DA}$ sensor) (Sun *et al.* 2018)and dLight (Patriarchi *et al.* 2018) sensors for detecting dopamine, and the GPCR-Activation Based norepinephrine (NE) sensor ($GRAB_{NE}$ sensor) for detecting norepinephrine (Feng *et al.* 2019) . When combined with advanced optical imaging techniques, GPCR-based sensors can provide scalable, long-term recordings of specific neurochemicals in a variety of model organisms.

Here, we review the engineering principle, properties, and *in vivo* applications of GPCR-based sensors. Moreover, we propose future directions for developing next-generation GPCR-based sensors with improved performance, negligible downstream signaling, an expanded color spectrum, and a wider range of ligands.

1. GPCRs have unique properties that make them highly suitable as scaffolds for engineering new sensors

Thanks to the development of optical imaging techniques, genetically-encoded fluorescent sensors have become an important tool for recording the *in vivo* dynamics of neurotransmitters and neuromodulators. These sensors were originally designed by combining a ligand-binding protein as the “sensing scaffold” with a circular-permutated fluorescent protein (cpFP) (Baird *et al.* 1999) as the “reporting module”. Previously, prokaryotic periplasmic-binding proteins (PBPs) were used as the scaffold for detecting several neurochemicals, including glutamate (iGluSnFR), GABA (iGABASnFR), and ATP (iATPSnFR), suggesting that this strategy may be applicable for *in vivo* use (Marvin *et al.*

2019; Lobas *et al.* 2019; Marvin *et al.* 2013). However, this design strategy is not feasible in cases which a suitable PBP is not available for the molecule of interest. Therefore, one approach to overcome these limitations associated with using prokaryotic PBPs as the scaffold was to search for a new group of sensing proteins (Wang *et al.* 2018).

1.1 The pros and cons of using GPCRs as the sensing scaffold

GPCRs are the largest family of membrane receptors, responsible for "sensing" extracellular chemicals and transducing the resulting signal into an intracellular response. Compared to prokaryotic PBPs, which bind primarily to metabolites, the broad spectrum of GPCR ligands covers most neurotransmitters and neuromodulators, thus providing an ideal natural scaffold for designing a sensor. Importantly, GPCRs have evolved to provide high ligand specificity as well as affinity suitable for detecting the *in vivo* dynamics of specific neurochemicals. Furthermore, although several GPCRs can sense a given neurochemical, they have unique properties; for example, in humans, five subtypes of GPCRs bind dopamine, each with a distinct combination of affinity, pharmacological selectivity and downstream signaling (Grandy *et al.* 1989; Dearry *et al.* 1990; Sunahara *et al.* 1991). This rich natural diversity provides a wealth of opportunities for engineering neurochemical sensors with specific properties and applications.

GPCRs are also an ideal scaffold for engineering neurochemical sensors with high spatiotemporal resolution. For example, the genetically encoded nature of GPCR-based sensors permits their expression in specific cell types using genetic manipulation. With respect to temporal resolution, the conformational change induced by ligand binding and receptor activation occurs on the order of tens of milliseconds (Marcaggi *et al.* 2009; Vilardaga *et al.* 2003; Hoffmann *et al.* 2005). Thus, engineering a GPCR-based sensor based on the receptor's conformational change will inherit millisecond temporal resolution, which is sufficient to reveal the temporal dynamics of neurotransmitter actions in vivo. Comparing with the prokaryotic PBPs as the sensor scaffold, GPCR-based sensors could in principle achieve similar sensitivity and response kinetics, but are better with affinity and selectivity in detecting corresponding neurochemicals, especially in physiological conditions.

Despite these clear advantages, several caveats should be considered when using a GPCR as the scaffold for designing a sensor. First, the assembly, folding, and trafficking of GPCR-based sensors should be considered. Because GPCRs are membrane proteins with an extracellular ligand-binding pocket, it is both essential and challenging to ensure that the GPCR-based sensors are trafficked properly to the cell surface and has the correct membrane topology. Indeed, based on our own experience when screening new sensors, the majority of recombinant GPCR-cpFP proteins have relatively poor trafficking to the plasma membrane and are therefore unable to sense extracellular neurochemicals (Feng *et al.* 2019; Jing *et al.* 2018). Second, post-translational modifications such as phosphorylation and lipid modification can potentially change the sensor's properties, affecting its ability to accurately report extracellular neurochemicals. Lastly, expressing GPCR-based sensors may—at least in principle—alter cellular physiology, as discussed in Section 4. Taken together, these pros and cons should be weighed appropriately and—where needed—solved using rational strategies when engineering GPCR-based sensors.

1.2 The principle behind GPCR-based sensors

As discussed above, the most important advantage of using a GPCR as the scaffold for engineering a cpFP-based fluorescent sensor is the conformational change that occurs upon activation by a specific ligand. More than two decades ago, pioneering work by Brian Kobilka and colleagues using the conformation-sensitive dye IANBD directly revealed the ligand-induced conformational change in the β_2AR (Gether *et al.* 1995). More recently, analyzing the crystal structure of several GPCRs, including the β_2AR, M_2R, μOR, and A_{2A}R, provided further insight into the structural changes that occur when the receptor binds its ligand and interacts with its associated G protein (Haga *et al.* 2012; Jaakola *et al.* 2008; Rasmussen *et al.* 2007; Cherezov *et al.* 2007; Huang *et al.* 2015). Specifically, a GPCR has several conformation states, including the inactive, partially active, and full active states, with the largest difference in conformation occurring within transmembrane helices 5 and 6. Ligand binding followed by G protein interaction stabilizes the protein in the active conformation, resulting in downstream signal transduction (Venkatakrishnan *et al.* 2013; Rasmussen *et al.* 2007; Cherezov *et al.* 2007; Rasmussen *et al.* 2011; Gregorio *et al.* 2017; Manglik *et al.* 2015; Scheerer *et al.* 2008) (Figure 1A). Capitalizing on this conformational change, GPCR-based sensors have been engineered in which cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) were fused to the GPCR; activation of the receptor—and thus, the resulting conformational change—gives rise to a change in the FRET (fluorescence resonance energy transfer) signal, which can be detected by fluorescence imaging on CFP and YFP channels (Vilardaga *et al.* 2003; Hoffmann *et al.* 2005) (Figure 1B). Although these FRET-based GPCR sensors have a relatively low signal-to-noise ratio, which limits their use in *in vivo* application, they provided key proof-of-concept that the conformational change upon activation of the GPCR can be exploited for engineering new sensors.

To develop neurochemical sensors that have a high signal-to-noise ratio and are suitable for *in vivo* applications, we recently combined the conformation-sensitive cpFP with GPCRs to generate the GRAB (GPCR Activation-Based) series of sensors for detecting a variety of neurochemicals, including GACh for sensing acetylcholine, $GRAB_{DA}$ for sensing dopamine, and $GRAB_{NE}$ for sensing norepinephrine (Jing *et al.* 2018; Sun *et al.* 2018; Feng *et al.* 2019). Using a similar strategy, Lin Tian's group has developed dLight for sensing dopamine (Patriarchi *et al.* 2018). With GRAB sensors, the cpGFP moiety is inserted within the third intracellular loop of the GPCR; this loop connects transmembrane helices 5 and 6 and undergoes a large conformational change upon ligand binding, inducing a change in fluorescence (Figure 1C). Since most GPCRs share common activation mechanisms, this strategy could in principle be generalized to develop sensors for distinct neurochemicals. Below, we discuss the GPCR-based sensors that have been designed using this new strategy.

Fig. 1. Schematic diagram showing the conformational change induced in three GPCR upon activation, and the general structure of GPCR-based sensors. (A) The structure of rhodopsin, the muscarinic M2 receptor, and the μOR opioid receptor are shown in the inactive (left) and active (right) states. The transmembrane helix 6 (TM6) with the largest conformational change is highlighted in red. Crystal structures are from PDB database (Rhodopsin: 3C9L&3DQB; M2:3UON&4MQS; μOR:4DKL&5C1M) (B) The principle behind FRET-based GPCR sensors, with the receptor fused to the fluorophores YFP and CFP. (C) GPCR-based sensors with the GPCR fused to a single circular-permutated green fluorescent protein (cpGFP). Upon binding its ligand, the conformational change in the GPCR is sensed and reported by either FRET or cpGFP.

2. Overview of GPCR-based genetically encoded sensors

To track the real-time dynamics of specific neurochemicals in a complex biological system, the sensor must have high sensitivity, high specificity, rapid response kinetics, and a negligible effect on cellular physiology. Here, we summarize the various properties of GPCR-based sensors (see Table 1).

2.1 Sensitivity

The sensitivity of a GPCR-based sensor is based on two factors: *i*) the dynamic range of the fluorescence response, and *ii*) the receptor's binding affinity for its ligand. After several iterative rounds of engineering and screening, and focusing primarily on the interface between the GPCR and the cpGFP, today's GPCR-based sensors achieve a >90% change in fluorescence ($\Delta F/F_0$). In addition, the signal-to-noise ratio of the fluorescence response is comparable to highly sensitive electrophysiological recording methods, indicating that the sensor has sufficient sensitivity for reporting the dynamics of neurochemical signaling (Jing *et al.* 2018). With respect to ligand affinity, most GPCR-based sensors retain their core GPCR's affinity, making them suitable for detecting physiological concentrations of neurochemicals. Thus, GPCR-based sensors have sufficiently high sensitivity for monitoring neurochemical signaling at physiologically relevant dynamics; for example, the dopamine sensor $GRAB_{DA}$ is able to report dopamine release from a single dopaminergic fiber induced by minimal electrical stimulation (Sun *et al.* 2018).

By introducing mutations at critical residues in the receptor—either near the ligand-binding pocket or the site of interaction with the G protein—the sensor's affinity can be further optimized in order to increase sensitivity. For example, mutating the threonine at position 205 in the dopamine D2 receptor (T205M) increases ligand affinity (Sung *et al.* 2016), and introducing this mutation into the $GRAB_{DA}$ sensor increases its affinity for dopamine by an order of magnitude (from ~130 nM to ~10 nM) without affecting the sensor's peak response (Sun *et al.* 2018). Therefore, developing sensors that span a wide range of affinities will provide a panel of highly sensitive sensors for use in a range of applications.

2.2 Selectivity

GPCR-based sensors retain their receptor's high ligand selectivity, as the reporter molecules are located primarily on the intracellular domains, largely sparing the extracellular ligand-binding pocket (see Figure 1). Thus, the GACh sensor, which is based on the muscarinic acetylcholine receptor, has a robust response to acetylcholine but is relatively insensitive to nicotine and other neurotransmitters and neuromodulators (Jing *et al.* 2018). GPCR-based sensors are even able to discriminate between structurally similar neurochemicals; for example, although the catecholamines dopamine and norepinephrine are structurally similar, the GPCR-based dopamine sensors $GRAB_{DA}$ and dLight are 10-fold to 30-fold more sensitive at detecting dopamine than norepinephrine and are insensitive to other neurochemicals (Sun *et al.* 2018; Patriarchi *et al.* 2018). To discriminate ever further between these two molecules, we engineered and characterized a $GRAB_{NE}$ sensor based on the α2A adrenergic receptor (Feng *et al.* 2019); this $GRAB_{NE}$ sensor is >300-fold more sensitive at detecting norepinephrine compared to dopamine, and the sensor's range for detecting dopamine greatly exceeds the physiological concentration of dopamine, making it an ideal sensor for reporting the dynamics of norepinephrine with high specificity. Given the high selectively for their respective

ligands, we believe that the dopamine and norepinephrine sensors can be combined in order to dissect the dynamics of both molecules *in vivo*, with extremely high precision.

In addition to retaining selectivity for their endogenous ligand, GPCR-based sensors also retain the receptor's sensitivity for pharmacological compounds. For example, the dopamine sensors $GRAB_{DA}$ and dLight, which are based on the D_2R and D_1R receptor subtypes, respectively, respond selectively to their subtype-specific agonists and antagonists (Sun *et al.* 2018; Patriarchi *et al.* 2018).

2.3 Kinetics

Thanks to the rapid activation kinetics of GPCRs (Marcaggi *et al.* 2009; Vilardaga *et al.* 2003; Hoffmann *et al.* 2005), GPCR-based sensors provide high temporal resolution, with rise-time kinetics (τ_{ON}) on the order of tens to hundreds of milliseconds. Although the temporal resolution of GPCR-based sensors does not reach the level of directly recording currents through ligand-gated ion channels, it is still considerably faster than other methods for measuring GPCR activation, which typically rely on measuring downstream signaling. By simultaneously recording fluorescence and electrophysiology, we found that the rise-time kinetics for the fluorescence signal in GACh and $GRAB_{NE}$ are significantly faster than measuring the current response downstream of endogenous muscarinic and adrenergic receptors (Jing *et al.* 2018; Feng *et al.* 2019).

On the other hand, the fluorescence decay kinetics (τ_{OFF}) of GPCR-based sensors range from approximately 100 ms to 2000 ms. Taken together, the kinetics of GPCR-based sensors are similar to—or in some cases, faster than—the kinetics of native metabotropic GPCRs, making these sensors an ideal tool for reliably measuring the dynamics of neurochemical signals.

Table. 1. The performance of currently available GPCR sensors.

Sensor	Scaffold	Peak response ($\Delta F/F_0$)	Ligand affinity	Rise-time kinetics (τ_{ON})	Decay-time kinetics (τ_{OFF})	References
GACh	M3 receptor	90%	1 μM	200 ms	800 ms	Jing M, *et al, Nature Biotech*, 2018
$GRAB_{DA1m}$	D2 receptor	90%	130 nM	60 ms	700 ms	Sun FM, *et al, Cell*, 2018
$GRAB_{DA1h}$	D2 receptor	90%	10 nM	140 ms	2500 ms	Sun FM, *et al, Cell*, 2018
dLight1.1	D1 receptor	230%	330 nM	ND	ND	Patriarchi, T, *et al, Science*, 2018
dLight1.2	D1 receptor	340%	770 nM	10 ms	90 ms	Patriarchi, T, *et al, Science*, 2018
$GRAB_{NE1m}$	α2A receptor	230%	930 nM	70 ms	750 ms	Feng JS, *et al, Neuron*, 2019
$GRAB_{NE1h}$	α2A receptor	130%	83 nM	30 ms	2000 ms	Feng JS, *et al, Neuron*, 2019

ND, not determined. Most of these properties are from measurements *in vitro* in cultured cells expressing sensors, except kinetics of dLight1.1 and dLight1.2 are from slice preparation.

2.4 Influence on cellular physiology

As mentioned above, GPCRs play an important role in cellular physiology by transducing extracellular signals (i.e., ligand binding) to intracellular downstream pathways. Therefore, expressing GPCR-based sensors should ideally have little effect on normal cellular physiology. In principle, GPCR-based sensors could affect cellular physiology by activating downstream signaling processes or by competing with endogenous receptors for ligand binding. With respect to downstream signaling, we systematically tested two major pathways downstream of GPCRs, the G protein-dependent pathway and the arrestin-dependent pathway. Generally speaking, all GPCR-based sensors have significantly reduced—or even negligible—coupling with these downstream pathways, possibly due to steric hindrance from the bulky cpGFP that occupies the region where the G protein and arrestin proteins bind the receptor. Moreover, the third intracellular loop, which is important for downstream signaling, was truncated during the engineering process, possibly contributing to the decreased signal coupling in GPCR-based sensors.

Whether a GPCR-based sensor competes with endogenous GPCRs for ligand binding depends largely on the sensor's ligand affinity and expression level. As mentioned above, most GPCR-based sensors have a similar ligand affinity as their corresponding endogenous receptor, and the sensor's affinity can be further tuned by mutagenesis. Using electrophysiology and calcium imaging of neuronal activity, we found that expressing GPCR-based sensors does not affect the physiological properties of endogenous receptors with respect to sensing neurochemicals or mediating synaptic transmission (Jing *et al.* 2018; Sun *et al.* 2018; Feng *et al.* 2019) (Figure 2). We have also shown that chronic expression of the GPCR-based sensors did not cause observable changes on cellular properties including electrophysiological signals and calcium responses (Jing *et al.* 2018; Sun *et al.* 2018; Feng *et al.* 2019). Ideally, by increasing the sensor's fluorescence response and optimizing the sensor's ligand affinity, one can then optimize the sensor's expression level in order to obtain a high signal-to-noise recording of neurochemicals while minimizing the effect on cellular physiology.

Fig. 2. GPCR-based sensors do not alter cellular physiology. (A) Cells expressing the acetylcholine sensor GACh2.0 have similar ACh-induced currents compared to control (Ctrl) neurons in cultured brain slices. (B) Expressing GACh2.0 in the *Drosophila* antennal lobe does not affect the odorant-evoked calcium response measured using the genetically encoded calcium indicator RGECO. Scale bar, 10μm. Figures are modified from original research paper(Jing *et al.* 2018).

3. *In vivo* applications for GPCR-based sensors

Given the highly complex morphological and physiological properties of neurons, the ability to monitor the dynamics of specific neurochemicals at the single-cell level, in real time, would provide valuable insights in the function of these chemicals with respect to regulating neuronal activity and behavior. Because they are genetically encoded, GPCR-based sensors such as GRAB and dLight can be expressed in specific cell types in specific brain regions, and they have the sensitivity and ligand specificity needed to report the endogenous dynamics of specific neurochemicals.

By combining genetic manipulation with optical recording, it is now possible to use GPCR-based sensors to measure neurochemical release *in vivo* in a wide range of awake, freely behaving animals.

For example, transgenic *Drosophila* expressing $GRAB_{DA}$ sensors have used to probe the compartment-specific release of dopamine in response to various physiologically relevant stimuli in the olfactory center mushroom body. Similarly, the GACh sensor has been used to report odorant-evoked acetylcholine release in specific glomeruli within the *Drosophila* antennal lobe, providing evidence that these sensors can be used to monitor spatially restricted neurotransmitter release (Figure 3A). In transgenic zebrafish, both $GRAB_{DA}$ and $GRAB_{NE}$ were used to measure neurotransmitter release evoked by a visual looming stimulation, even down to the single-cell level (Figure 3B). In freely behaving mice, GPCR-based sensors have been used to report sensory stimulation–evoked acetylcholine release and behavior-related dopamine and norepinephrine release using fiber photometry recording and *in vivo* two-photon imaging (Figure 3C and D) (Jing *et al.* 2018; Sun *et al.* 2018; Patriarchi *et al.* 2018; Dong *et al.* 2019; de Jong *et al.* 2019; Corre *et al.* 2018; Feng *et al.* 2019; Mohebi *et al.* 2019). In principle, the GPCR-based sensors could also be used together with mini-scopes to achieve real-time imaging of neurochemicals in free-behaving animals. In addition, $GRAB_{DA}$ sensors have been used successfully in zebra finches (songbirds) in order to measure the contribution of dopaminergic modulation in vocal learning (Figure 3E) (Tanaka *et al.* 2018).

Fig. 3. *In vivo* applications of GPCR-based sensors for studying behavior in a variety of animal models and preparations. (A) Two-photon imaging was used to measure acetylcholine release evoked by odorant application in transgenic *Drosophila* (left), with high spatial resolution in the antennal lobe area of GH146-Gal4:UAS-GACh2.0 flies. The images at the right show the fluorescence response following application of mineral oil (as a control) and two concentrations of the odorant isoamyl acetate (IA). (B) *In vivo* single-cell confocal imaging of a transgenic (HuC:$GRAB_{NE1m}$) zebrafish was used to measure noradrenergic activity before, during, and after a visual looming stimulus. (C) Imaging of task-related dopaminergic activity was measured at the single-cell level using dLight1.2 expressed in layer 2/3 of the M1 cortex in mice. (D) Fiber photometry recordings of the $GRAB_{DA}$ sensor was used to monitor dopamine release in the nucleus accumbens (NAc) of a freely moving male mouse during mounting and intromission. (E) The $GRAB_{DA1h}$ sensor was used to record an increase in dopamine levels in juvenile male birds in response to a live tutor song. Scale bars, 10μm in (A) and (B), 50μm in (C). Figures are modified from original research paper(Jing *et al.* 2018; Sun *et al.* 2018; Tanaka *et al.* 2018; Feng *et al.* 2019; Patriarchi *et al.* 2018) with permission from the publisher.

To further investigate the relationship between neuromodulation and physiological function, monitoring of neuromodulators must be combined with methods to control activity, for example optogenetics and chemogenetics. In this respect, it is interesting to note that the dLight sensor was recently combined with optogenetics or chemogenetics in order to study dopaminergic modulation in mice (Augustine *et al.* 2019; Patriarchi *et al.* 2018). Similarly, we successfully combined optogenetics with real-time monitoring of dopaminergic activity using $GRAB_{DA}$ sensors (Figure 4)(Sun *et al.* 2018). Thus, combining these robust methods can provide a powerful new set of tools for studying neuromodulation. Using several GPCR-based sensors that specifically report different neurochemicals, it will likely be possible to dissect in detail whether the same group of neurons or different groups of neurons receive distinct forms of neuromodulation, as well as how this functional heterogeneity in terms of receiving neuromodulation affects their function within the neural circuit.

Fig. 4. $GRAB_{DA}$ can detect both the release and modulation of dopamine evoked by optogenetic stimulation. The red-activated excitatory opsin C1V1 was expressed in the substantia nigra pars compacta (SNc) and used to optically activate dopaminergic neurons, while $GRAB_{DA1m}$ was expressed in the striatum (Str) and used to measure dopamine. The $GRAB_{DA1m}$

fluorescence signal was prolonged by application of the dopamine transporter blocker methylphenidate and blocked by application of the D_2R-specific antagonist Etic. tdTomato was co-expressed with $GRAB_{DA1m}$ and imaged simultaneously in order to rule out any possible artifacts. Figures are modified from original research paper(Sun *et al.* 2018) with permission from the publisher.

4. Future directions and perspectives for GPCR-based sensors

The broad applicability of GPCR-based sensors makes them well suited for monitoring *in vivo* neurochemical activity with millisecond temporal resolution. Importantly, these sensors can be optimized further, and the variety of GPCR-based sensors can be expanded in order to sense a wider range of physiologically relevant molecules with negligible effects on the cell's normal physiological processes.

4.1 Optimizing performance

In principle, existing GPCR-based sensors can be improved by increasing their dynamic range, tuning their ligand affinity and response kinetics, and further reducing downstream coupling. The availability of the crystal structure of GPCRs provides important information regarding the residues that are critical for ligand binding and conformational changes, which can help facilitate the rational design and optimization of GPCR-based sensors. Inserting mutations near the domain responsible for maintaining the receptor in the inactive state could alter the ligand binding–induced conformational change and/or ligand affinity (Ballesteros *et al.* 2001; Shapiro *et al.* 2002), possibly affecting the performance of the resulting GPCR-based sensor. Moreover, obtaining the crystal structure of GPCR-based sensors would be helpful for optimizing their performance using systematic and targeted mutagenesis. To minimize potential downstream coupling, we developed a strategy to prevent binding of the endogenous G protein to the GPCR-based sensor while retaining the full fluorescence signal (Figure 5A). This strategy involves attaching a G protein-derived "mini-G protein" or peptide (Carpenter & Tate 2016; Hamm *et al.* 1988) to the C-terminus of the sensor, thereby suppressing activation of endogenous G proteins through competitive binding (Wu *et al.* 2019).

4.2 Expanding the color spectrum

Current GPCR-based sensors include a single fluorescent protein, which prevents the simultaneous measurement of multiple cellular events. Moreover, the excitation and emission spectra of GFP-based proteins have relatively shallow penetration, making them less suitable for *in vivo* imaging of deeper structures. By replacing the cpGFP moiety with cpRFP, red-wavelength calcium and voltage sensors have been developed (Dana *et al.* 2016; Zhao *et al.* 2011; Akerboom *et al.* 2013; Abdelfattah *et al.* 2016). In principle, a GPCR-based sensor containing cpRFP could be engineered (Figure 5B). In the future, the color spectrum of GPCR sensors can be expanded, which will not only allow the simultaneous measurement of neurochemical signals and calcium/voltage signals, but also the sensors to be used simultaneously with other optics-based actuators such as channelrhodopsins and other optically controlled molecules such as photoswitchable kinases (Zhou *et al.* 2017), thus providing a comprehensive functional map of neurochemical activity.

4.3 Subcellular targeting

It has been suggested that subcellular application of neurochemicals might activate localized receptor pools that function as separate computation units. In principle, genetically-encoded GPCR-based sensors could be targeted to specific subcellular compartments within the neuron, for example, the axon or postsynaptic spines. Additional engineering steps would be required to target the sensor to a specific subcellular structure, for example by fusing the sensor with a subcellular targeting motif similar to the strategy used for calcium sensors (Broussard *et al.* 2018). Comparing the dynamics of neurochemicals in different compartments within a single neuron—ideally in combination with a calcium and/or voltage indicator in order to monitor their downstream effects—will likely reveal new insights in neuromodulation.

4.4 Expanding the detection range

An exciting new direction for designing future GPCR-based sensors is to expand the repertoire of sensors to include additional neurochemicals (Figure 5C). This will open new avenues in the field of neuroscience, as most neurochemicals—particularly lipids and neuropeptides—currently lack a suitable method for studying their physiological properties distribution *in vivo*. From a mechanistic perspective, the currently known GPCR structures suggest that most GPCRs—particularly those in the rhodopsin family—have a highly conserved activation mechanism, with the largest conformational change occurring in transmembrane helices 5 and 6 (see Figure 1A). Indeed, Lin Tian's group successfully used the strategy for designing dLight sensors using other GPCRs, including the β1, β2, and α2 adrenergic receptors, the κ- and μ-type opioid receptors, the 5-hydroxytryptamine (serotonin) receptor 2A, and the melatonin type 2 receptor (Patriarchi *et al.* 2018). In addition, we developed a series of GRAB sensors selective for acetylcholine, dopamine, and norepinephrine, supporting the notion that this strategy can also be expanded to develop a wide range of new GPCR-based sensors. Of course, each new sensor will require extensive protein engineering to optimize its performance *in vivo*. Nevertheless, we believe that the introduction of a wide family of GPCR-based sensors will greatly benefit the entire neuroscience community (Figure 5D).

Fig. 5. Overview of future directions for developing GPCR-based sensors. (A) Downstream signaling via GPCR-based sensors can be prevented by fusing a mini-G protein or G protein–derived peptide to the C-terminal domain, serving as a competitive inhibitor of endogenous G proteins. (B) The color spectrum of GPCR-based sensors can be expanded by replacing cpGFP with another fluorescent protein, for example, cpRFP. (C) Phylogenetic tree covering the family of neurochemical-activated GPCRs, including their respective ligands. (D) In principle, GPCR-based sensors can be developed and optimized for detecting a wide range of neurochemicals. Abbreviations: 5HT, 5-hydroxytryptamine (serotonin); ACh, acetylcholine; Ado, adenosine; CCK, cholecystokinin; DA, dopamine; eCB, endocannabinoid; Epi; epinephrine; GABA, γ-aminobutyric acid; His, histamine; MT, Melatonin; NE, norepinephrine; NPY, neuropeptide Y; NT, neurotensin; OP, opioid; OT, oxytocin; S1P, sphingosine-1-phosphate; SST, somatostatin (somatotropin release inhibiting factor); VIP, vasoactive intestinal peptide.

List of abbreviations

ACh: acetylcholine
ATP: adenosine triphosphate
A_2AR: adenosine receptor A2a
β_2AR: β_2 adrenergic receptor
cpFP: circular-permutated fluorescent protein
CFP: cyan fluorescent protein
DA: dopamine
FRET: Foster resonance energy transfer
GPCR: G-Protein-Coupled receptor
GACh: GPCR-activation based acetylcholine sensor
$GRAB_{DA}$: GPCR-activation based dopamine sensor
$GRAB_{NE}$: GPCR-activation based norepinephrine sensor
GABA: γ-aminobutyric acid
M_2R: muscarinic acetylcholine receptor 2
NE: norepinephrine
PBP: periplasmic binding protein
TM: transmembrane helix
μOR: μ opioid receptor
YFP: yellow fluorescent protein

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--Human subjects --
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Competing interests

M.J. and Y.L. have filed patent applications, the value of which could be affected by this publication.

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