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Lighting up the brain: genetically encoded fluorescent sensors for imaging neurotransmitters and neuromodulators

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Measuring the precise dynamics of specific neurotransmitters and neuromodulators in the brain is essential for understanding how information is transmitted and processed. Thanks to the development and optimization of various genetically encoded sensors, we are approaching the stage in which a few key neurotransmitters/neuromodulators can be imaged with high cell specificity and good signal-to-noise ratio. Here, we summarize recent progress regarding these sensors, focusing on their design principles, properties, potential applications, and current limitations. We also highlight the G protein-coupled receptor (GPCR) scaffold as a promising platform that may enable the scalable development of the next generation of sensors, enabling the rapid, sensitive, and specific detection of a large repertoire of neurotransmitters/neuromodulators *in vivo* at cellular or even subcellular resolution.

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Introduction

In the mammalian central nervous system, several billions of interconnected neurons control a wide range of key physiological processes, from basic sensation and motor control to higher brain cognitive functions such as memory, decision-making, and self-awareness. The communication between neurons is mediated predominantly via a specialized structure called the synapse, in which neurotransmitters are enriched in synaptic vesicles at the presynaptic terminal; these transmitters are released into the synaptic cleft when action potentials invade the terminal, thereby activating or inhibiting the postsynaptic neuron. Classic neurotransmitters, such as glutamate and GABA, by generally activating ionotropic receptors, mediate the extremely fast, spatially confined, point-to-point synaptic transmission. Neuromodulators such as dopamine and neuropeptides, typically by acting on metabotropic G protein-coupled receptors (GPCRs) to initiate downstream signal cascades, are thought to mediate neurotransmission, at least in part by a relatively slow, long-range, diffuse form, called volume transmission. The exact mode of action for neurotransmitters/neuromodulators is complex, because they are capable of activating both ionotropic receptors and GPCRs, with multiple isoforms that have distinct affinities and/or diverse intracellular effectors. Not surprisingly, dysregulation of neurotransmitter and/or neuromodulator release has been linked to many neurological disorders, including depression, schizophrenia, dementia, and neurodegenerative diseases [1–3].

Given these essential functions that neurotransmitters and neuromodulators play in the brain, the ability to monitor their dynamics is critical for understanding their regulation and activity at the molecular, cellular, and circuit levels. However, tracking specific neurotransmitters with high precision is extremely challenging due to the complex nature of the central nervous system with respect to its anatomical and chemical features. Each individual neuron is essentially a functionally isolated unit with distinct intrinsic physiological properties, as well as a unique molecular signature; thus, neurons respond heterogeneously to stimuli. Moreover, billions of neurons in the brain are connected both anatomically and functionally by excitatory and inhibitory processes mediated by fast synaptic transmission and/or slow volume transmission. As a consequence of this connectivity, neurotransmitter levels are regulated by individual neurons even in subcellular compartments, as well as by complicated circuits across different brain regions. This configuration demands the highly specific detection of neurotransmitters and neuromodulators, with high temporal and spatial resolution that can match the dynamics of the central nervous system. On the other hand, each neurotransmitter/neuromodulator has a unique set of chemical properties, ranging from amino acids to monoamines, purines, and peptides. This creates an obvious biological dilemma: any general detection method would need to cover the entire spectrum of chemically diverse transmitters, while still retaining the specific ability to discriminate between different transmitters.

To overcome this challenge, in recent decades several pioneering research tools have been developed and refined for measuring the dynamics of neurotransmitters and neuromodulators. These tools are designed to sense various features of neurotransmission, and they vary with respect to their detection sensitivity and specificity, temporal and spatial resolution, cell specificity and invasiveness. Here, we provide an overview of these sensors' design principles, properties, and current limitations, focusing on genetically encoded fluorescent sensors, which are ideally suited to track the precise dynamics of neurotransmitters and neuromodulators.

Detecting neurotransmitters/ neuromodulators using optical imaging

Compared with traditional methods such as microdialysis (Figure 1a), current recording, and electrochemical detection (Figure 1b), optical imaging is widely accepted as a non-invasive, high-throughput method for tracking specific molecules, building upon the foundation of fluorescent probes. Unlike tools that directly measure the concentration of neurotransmitters/neuromodulators, synthetic FM dyes [4], quantum dots [5], fluorescent false neurotransmitters (FFNs) [6], and pH-sensitive fluorescent proteins [7–9] have all been used to image the exocytosis of synaptic vesicles, which indirectly reflects the release of neurotransmitters. Despite their advantages and widespread usage, however, these methods either lack molecular specificity or have relatively low temporal resolution due to vesicle recycling.

To directly measure the dynamics of a specific type of neurotransmitter using optical imaging, a protein-based optical reporter must include two key features. First, it must be able to recognize or bind directly and specifically to its cognate neurotransmitter and change its conformation. Second, the reporter must produce an optical signal that is sensitive to the change in the protein's conformation. To achieve the first goal, various scaffolds have been generated, including mammalian neurotransmitter receptors (either ionotropic or metabotropic), neurotransmitter-binding proteins isolated from bacterial periplasm, and enzymes that utilize the neurotransmitter as the substrate (*e.g.* acetylcholinesterase, which catalyzes the breakdown of acetylcholine). The corresponding optical output (*i.e.* the signal) can be generated using various approaches that include a change in first, the fluorescence of a synthetic dye, or a single fluorescent protein, second, a FRET/ BRET (fluorescence/bioluminescence resonance energy transfer) signal produced by a pair of donor and acceptor proteins, or third, expression of an optical reporter gene (*i.e.* a luciferase). Based on their genetical encodability, we can classify each of these methods as either a hybrid sensor composed of a chemical synthetic dye with a protein binding partner, or a fully genetically encoded sensor.

Hybrid sensors for imaging neurotransmitters and neuromodulators

The hybrid sensor E(glutamate) optical sensor (EOS) was developed to report glutamate, the predominant excitatory neurotransmitter in the mammalian brain; this sensor contains a chemically linked fluorescent dye (Oregon Green) located near the glutamate-binding pocket of the GluR2 metabotropic glutamate receptor [10]. The dynamic range of the first-generation EOS was approximately 20%, and it could only report glutamate signals in simple cultured preparations. To overcome these practical limitations, a series of optimized EOS variants were developed with increases in both the dynamic range of the fluorescent signal and the affinity for glutamate, enabling researchers to monitor the dynamics of extrasynaptic glutamate in the brain during physiological stimuli [11].

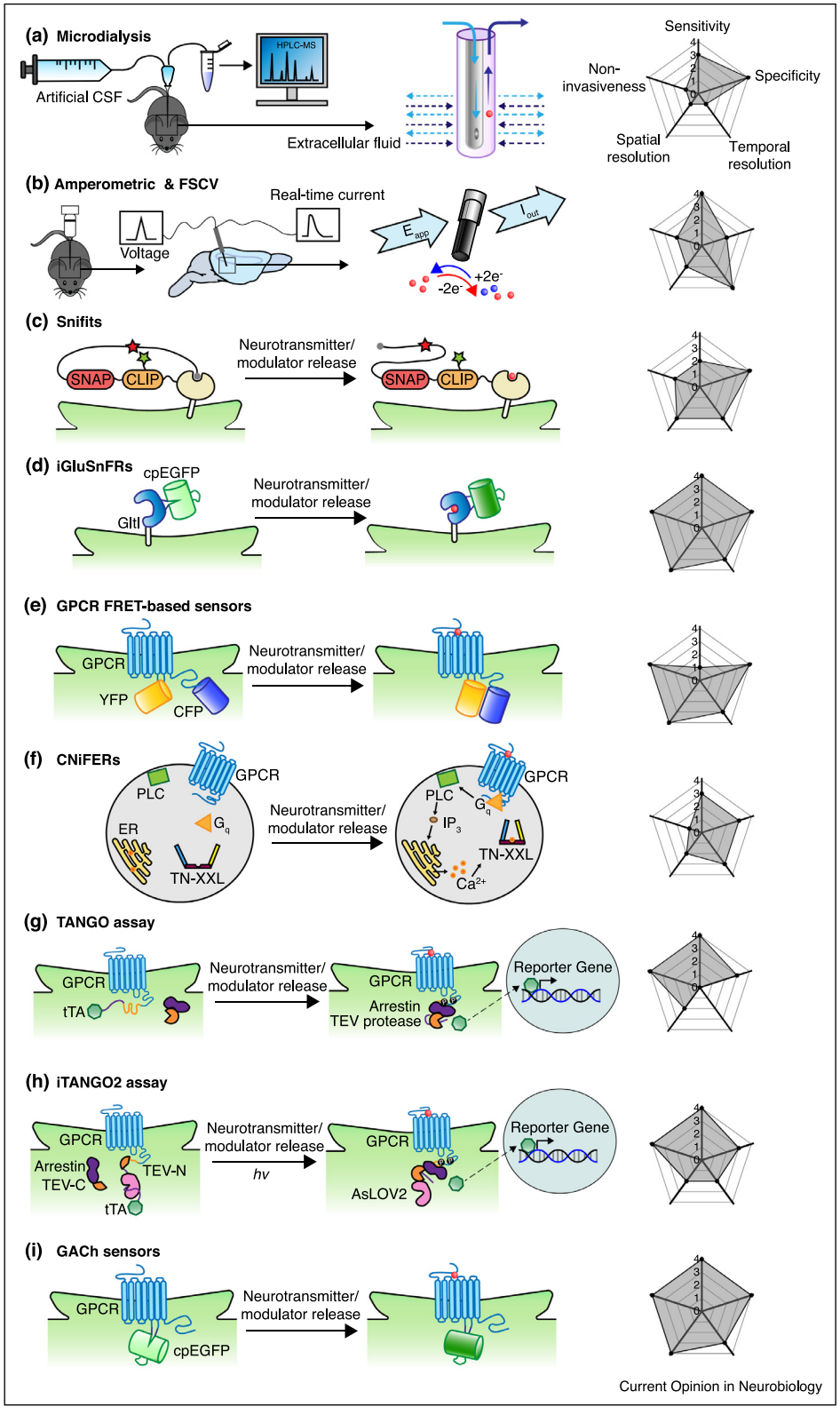
Another class of hybrid neurotransmitter sensors, Snifits (SNAP-tag based indicator proteins with a fluorescent intramolecular tether), was constructed by labeling the neurotransmitter-binding protein with a SNAP-tag-linked fluorescent ligand and a CLIP-tag-linked fluorophore (see Table 1). The fluorescent ligand and the fluorophore form a FRET pair, and the energy transfer efficiency shifts when the endogenous ligand competes for binding (Figure 1c). Snifits have been used in cultured cells to image the dynamics of several transmitters, including GABA [12], glutamate [13], and acetylcholine (ACh) [14•], yielding significant changes in the FRET ratio. However, Snifits have not been tested *in vivo*; therefore, whether they have enough sensitivity to detect transmitter dynamics in the intact brain is currently unknown. Another group of hybrid sensors using the FlAsH-CFP FRET pair inserted in GPCRs will be elaborated below in GPCR-based sensors.

Overall, hybrid sensors provide relatively sensitive and selective detection of specific neurotransmitters. However, because they require a synthetic dye to label the genetically encoded tag, their usage in behaving animals is limited due to low tissue penetrance and relatively high background signals.

Genetically encoded sensors for imaging neurotransmitters and neuromodulators

To avoid the need of exogenously applying a synthetic fluorophore for labeling, genetically encoded optical reporters provide cell-specific expression and transmitter detection, which is essential for monitoring specific neurotransmitters and neuromodulators in the context of the highly complex central nervous system. Based on the type of neurotransmitter-binding protein, these sensors are generally classified as either bacterial periplasmic-binding protein (PBP)-based sensors or GPCR-based sensors.

Figure 1



Schematic overview of the current methods and tools for detecting neurotransmitters and/or neuromodulators. The design principle is shown on the left, and the performance features (*e.g.* sensitivity, specificity, resolution) are summarized at the right. The scale (ranging from 0 to 4) in the radar graphs reflects performance in each specific feature. Microdialysis **(a)**, amperometry and FSCV **(b)**, Snifits **(c)**, iGluSnFR **(d)**, GPCR FRET-

Table 1

Overview of genetically encoded neurotransmitter sensors

Genetically encoded neurotransmitter sensor	Ligand	Reporter	Maximum ($\Delta F/F_0$) *in vitro*/on neurons	Affinity (Kd) *in vitro*/on neurons	Time constant (τ_{ON}/τ_{OFF})	Ref.
GABA-Snifit	GABA	DY-547/Cy5	0.5	100 μM	1.5 s/2.8 s	[12]
Snifit-iGluR5	Glutamate	DY-547/Cy5	0.93	15 μM	3–4 s (perfusion time)	[13]
ACh-Snifit	ACh	Cy3/Cy5	0.52	20 mM	2.4 s/4 s	[14•]
FRET-based M_1, M_2, M_3, and M_5 muscarinic receptor sensors	ACh	CFP/FlAsH	−0.05 to 0.09	0.2–1.5 μM	60 ms/0.8 s	[23•,24]
α_{2A}AR-cam	NE	CFP/YFP	−0.05	17 nM	40 ms	[21]
PTHR-cam	PTH	CFP/YFP	−0.2	16 nM	1 s	[21]
M_1-cam5	ACh	CFP/YFP	−0.1	ND	0.5 s	[38]
M_1-CNiFER	ACh	TN-XXL	0.18 (tonic) 1.1 (phasic)/0.3	11 nM	∼2 s	[25]
D2-CNiFER	DA	TN-XXL	0.57/0.24	2.5 nM/30 nM	<7 s	[26•]
α_{1A}-CNiFER	NE	TN-XXL	0.90/0.25	20 nM/100 nM	<5 s	[26•]
TANGO	GPCR ligands	Reporter genes	>10	∼1 nM	∼Hours	[27]
iTANGO2	GPCR ligands	Reporter genes	16.5/8.9	ND	∼Minutes	[29••]
FLIPE	Glutamate	CFP/YFP (Venus)	ND	0.6 μM/ND	$k_{on} = 10.0 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{off} = 60\ s^{-1}$	[15]
SuperGluSnFR	Glutamate	CFP/YFP (Citrine)	0.44	2.5 μM/2.5 μM	$k_{on} = 3.0 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{off} = 75\ s^{-1}$/13 ms	[18]
iGluSnFR	Glutamate	cpEGFP	4.5/1.03	110 μM/4.9 μM	∼5 ms/∼92 ms	[19••]
GACh	ACh	cpEGFP	0.9/0.90	0.78 μM/1.99 μM	∼280 ms/∼760 ms	[34••]

ACh, acetylcholine; PTH, parathyroid hormone; PTHR, PTH receptor; NE, norepinephrine; DA, dopamine; ND, not determined.

PBP-based sensors

Bacterial periplasmic-binding proteins (PBPs) comprise a large, diverse superfamily of proteins that bind various chemicals, including neurotransmitters; thus, these proteins provide a suitable scaffold for biosensor engineering. The *Escherichia coli*-derived glutamate-binding PBP GltI (also known as ybeJ) was used to generate a series of glutamate-sensing fluorescent indicators (FLIPE and GluSnFRs) by fusing the CFP-YFP FRET pair to the protein [15,16]. Further engineering refinements, including changing the insertion site of the FRET pairs and random mutagenesis, have increased the signal's dynamic range to nearly 50% [17,18]. Although these optimized GltI/FRET-based glutamate sensors have high affinity and rapid kinetics (around the 10-ms level), their application *in vivo* has been hampered by their relatively low signal-to-noise ratio.

To increase the signal of PBP-based sensors, a new intensity-based fluorescent glutamate sensor (iGluSnFR) was engineered by combining circular permutated EGFP (cpEGFP) with the PBP GltI [19••] (Figure 1d). This next-generation sensor produces a bright, rapid, and specific fluorescence increase upon glutamate binding, with a large dynamic range (peak $\Delta F/F_0$ values of 4.5 and 1.0 in cultured HEK-293 cells and neurons, respectively). The ability of iGluSnFR to detect glutamate release *in vivo* was demonstrated using several model organisms, including *Caenorhabditis elegans*, zebrafish, and mice [19••]. Recent developed red version R-iGluSnFR1 further expanded the color palette of glutamate sensors [20]. Overall, the iGluSnFR sensor provides high sensitivity and specificity for detecting glutamate in a cell-specific manner, thereby providing important information regarding the *in vivo* dynamics of glutamatergic transmission.

GPCR-based sensors

G protein-coupled receptors (GPCRs) constitute the majority of receptors for neurotransmitters and neuromodulators, with a conserved structural topology and high specificity for endogenous neurotransmitters. In 2003, Vilardaga and colleagues generated a series of ratiometric FRET-based sensors called GPCR-cam by inserting a pair of FRET proteins in the receptor's third intracellular loop and C-terminal domain (Figure 1e). Upon binding the ligand, the resulting conformational change of the receptor shifts the distance and orientation between the FRET pair, thereby changing FRET efficiency. Using this strategy, PTHR-cam and α_{2A}AR-cam were generated based on the parathyroid hormone receptor and α_{2A}-adrenergic receptor, respectively [21]. However, reminiscent of the FRET sensors created with PBPs, the change

(Figure 1 Legend Continued) based sensors **(e)**, CNiFERs **(f)**, the TANGO assay **(g)**, the iTANGO2 assay **(h)**, and GACh sensors **(i)** are shown. Further details are provided in the text. CSF, cerebrospinal fluid; FSCV, fast-scan cyclic voltammetry; GPCR, G protein-coupled receptor; FRET, fluorescence resonance energy transfer.

in GPCR-cam FRET efficiency is relatively small (<10%) [21], and trafficking of the GPCR-cam protein is affected by the presence of two relatively large fluorescent proteins. Therefore, to improve the expression and delivery of these sensors to the plasma membrane, and to increase the resulting FRET signals, a different FRET pair — CFP and the fluorophore FlAsH — was used. Using this FRET pair, sensors for detecting epinephrine and acetylcholine were engineered with the β_2-adrenergic receptor [22] and the muscarinic acetylcholine receptor [23•,24], respectively, as the scaffold. These FRET sensors have high binding affinity for their respective ligands and millisecond temporal resolution; however, the relatively small change in FRET signal and the need to load the FlAsH-CFP pair with an exogenous fluorescent dye limits their feasibility in *in vivo* systems.

To overcome the relative small conformational change induced by neurotransmitter binding to the GPCR, the signal can be amplified by detecting the downstream signaling molecules activated by the GPCR, or by inducing the expression of a reporter gene via activating a transcription factor. Thus, a series of cell-based neurotransmitter fluorescent engineered reporters (CNiFERs) were developed in order to report the change in intracellular Ca^{2+} following GPCR activation (Figure 1f). Cultured HEK cells expressing specific GPCRs and the ratiometric fluorescent Ca^{2+} sensors were then implanted into specific regions in the brain, serving as a reporter unit to transmit extracellular neurotransmitter signals into a change in fluorescence. Using this approach, CNiFERs have been used successfully to sense acetylcholine [25], dopamine, and norepinephrine [26•] *in vivo*. Despite their ability to report specific neurotransmitters with high sensitivity, the invasive nature of cell implantation required in CNiFERs hinders their broad application.

Based on the downstream signal transduction cascades activated by GPCRs, the TANGO assay was designed to capture and transmit GPCR activation into a stable intracellular signal [27] (Figure 1g). In this system, the transcription factor tTA is fused to the C-terminal domain of the GPCR by a peptide sequence containing a TEV (tobacco etch virus) protease cleavage site, which is cleaved when TEV protease-fused β-arrestin is recruited upon GPCR activation. This cleavage event releases tTA from the receptor, allowing it to translocate to the nucleus and initiate expression of a reporter gene (*e.g.* luciferase or a fluorescent protein) for optical detection. By signal amplification, the TANGO assay provides single-cell resolution, nanomolar sensitivity for specific neurotransmitters. Importantly, this system also circumvents the confounding effects associated with implanting exogenous cells in CNiFERs, as the signaling molecules are genetically encoded and could be target-expressed in desirable cells by various genetic techniques. This method has been used successfully to detect *in vivo* dopaminergic signaling in both *Drosophila* and mice [28,29••], and very recently a derived *trans*-Tango method has been used to map anterograde synaptic circuits in olfactory and gustatory systems of *Drosophila* [30].

However, the high signal amplification provided by this assay comes at a cost: temporal resolution is poor (around hours or more), making this approach unsuitable for real-time neurotransmitter/neuromodulator measurements. An improved iTANGO and its simplified version iTANGO2 were recently developed [29••], where a clever light-controlled system was additionally implemented to gate the protease cleavage and the subsequent transcription factor activation (Figure 1h). Similar in design principle, SPARK method developed by Ting's group was shown to yield up to 37-fold signal increase upon activation by specific neuromodulators, with ~5-minute temporal resolution [31]. In comparison to the previous TANGO approach, iTANGO2 and SPARK reduce background signals and yield better temporal resolution (~ minutes) to detect neuromodulators, that is, dopamine. Judicious usage of dCas9 [32] or split dCas9 [33] based transcription amplification systems in a TANGO style design could achieve the flexibility to activate endogenous genes at will and could further increase relative signal strength over background. These improved strategies have not been validated *in vivo* yet, however, the sub-second or second kinetics of neurotransmitters/neuromodulators is still beyond the temporal resolution of above transcription assays and the irreversible nature of protease cleavage precludes this method to continuously monitor the dynamics of neurotransmitters/neuromodulators in real time.

In summary, genetically encoded neurotransmitter sensors provide improved ligand selectivity, spatial sensitivity, and cell specificity especially for *in vivo* applications, although majority existing sensors have their own constrains in either signal-to-noise ratio or temporal resolution (Figure 1).

Future directions

The famous South African molecular biologist and Nobel laureate Sydney Brenner stated that 'Progress in science depends on new techniques, new discoveries, and new ideas, probably in that order.' Indeed, understanding the complex nature of the mammalian brain calls for new research tools that can measure the dynamics of key neurotransmitters and neuromodulators with high specificity, single-cell spatial resolution, and physiologically relevant temporal resolution, ideally in an *in vivo* setting. Genetically encoded fluorescent sensors fulfill these criteria and can provide important information regarding the functional properties and dynamics of neurotransmitters in the brain. In general, the scaffold used to engineer genetically encoded sensors need to provide high ligand specificity and can — at least in principle — be adapted

for sensing all neurotransmitters and neuromodulators. With respect to PBP-based sensors, the iGluSnFR represents an important step forward in terms of providing a highly specific and sensitive *in vivo* sensor for glutamate; however, whether the same strategy can be scaled up and applied to measure other neurotransmitters or neuromodulators, and whether these transmitters/modulators can be reliably detected under physiological conditions, remain open questions, especially for peptide neuromodulators that do not have a corresponding cognate PBP in bacteria. On the other hand, GPCRs are evolutionarily conserved and have retained both specificity and affinity for nearly all neurotransmitters; thus, GPCRs may provide a better scaffold for developing the next generation of sensors.

Indeed, by tapping into the GPCR scaffold, our group recently developed an ACh sensor with high signal-to-noise ratio [34••]. Taking advantage of the environmental sensitive cpEGFP to report the conformational change during GPCR activation, we incorporated cpEGFP into a human muscarinic GPCR (Figure 1i). By optimizing the membrane trafficking as well as the conformational coupling between the GPCR and cpEGFP through iterative site-directed mutagenesis, we generated an GACh sensor (short for the G-protein-coupled receptor activation-based AChsensors) that could readily achieve 90% $\Delta F/F_0$ fluorescence increase upon ACh application in both cultured HEK293T and cortical neurons. GACh sensors also have micro-molar affinity and high specificity to ACh as well as sub-second response kinetics (see Table 1). GACh sensors were further validated in multiple *in vivo* systems including *Drosophila* and mice, capable of detecting the endogenous ACh dynamics in physiological relevant settings [34••]. Importantly, the rich structural information for various GPCRs in both active and inactive states currently available reveals that diverse GPCRs share similar activation mechanisms [35–37]. Thus, the strategy to generate GACh sensors [34••], in principle, could be extended and applied to develop other neurotransmitter/neuromodulator sensors with high sensitivity and specificity, despite the fact that considerable challenges still remain for engineering seven-transmembrane proteins to harness their subtle ligand-induced conformational changes. As a note, attention needs to be paid to ensure the GPCR based sensors do not perturb the intrinsic physiology in the cell of interest, given a plethora of intracellular signaling pathways GPCRs involved. Looking ahead, in addition to PBPs and GPCRs, neurotransmitter transporter proteins may also serve as a viable scaffold for new sensors, particularly due to their high specificity and affinity for their respective ligands, as well as their conserved structures. Colorwise, a single-wavelength sensor cannot be used to map the entire spectrum of neurotransmission, as several neurotransmitters can interact simultaneously at a single site or at different subcellular compartments, including axons, dendrites, cilia, and axon initial segments, thereby providing the network with highly precise functional control. Therefore, using multicolor imaging of sensors with non-overlapping spectrums — or using bioluminescence, which provides better tissue penetration — will likely yield valuable information regarding the processes through which neurotransmitters are spatially and dynamically controlled in order to coordinate their complex functions within the brain. Moreover, the simultaneous application of optical actuators such as channel rhodopsin-based optogenetic tools may help to bridge the cause-and-effect relationship between specific neurotransmitters and behavioral output. Finally, probing the dynamics of specific neurotransmitters in various disease models may provide researchers with important information regarding the underlying pathogenic mechanisms, thereby yielding new targets for rational drug design and new therapeutic approaches.

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Howes OD, Kapur S: **The dopamine hypothesis of schizophrenia: version III — the final common pathway**. *Schizophr Bull* 2009, **35**:549-562.

2. Bohnen NI, Albin RL: **The cholinergic system and Parkinson disease**. *Behav Brain Res* 2011, **221**:564-573.

3. Dunlop BW, Nemeroff CB: **The role of dopamine in the pathophysiology of depression**. *Arch Gen Psychiatry* 2007, **64**:327-337.

4. Gaffield MA, Betz WJ: **Imaging synaptic vesicle exocytosis and endocytosis with FM dyes**. *Nat Protoc* 2006, **1**:2916-2921.

5. Zhang Q, Cao YQ, Tsien RW: **Quantum dots provide an optical signal specific to full collapse fusion of synaptic vesicles**. *Proc Natl Acad Sci U S A* 2007, **104**:17843-17848.

6. Gubernator NG, Zhang H, Staal RG, Mosharov EV, Pereira DB, Yue M, Balsanek V, Vadola PA, Mukherjee B, Edwards RH *et al.*: **Fluorescent false neurotransmitters visualize dopamine release from individual presynaptic terminals**. *Science* 2009, **324**:1441-1444.

7. Li Z, Burrone J, Tyler WJ, Hartman KN, Albeanu DF, Murthy VN: **Synaptic vesicle recycling studied in transgenic mice expressing synaptopHluorin**. *Proc Natl Acad Sci U S A* 2005, **102**:6131-6136.

8. Li Y, Tsien RW: **pHTomato, a red, genetically encoded indicator that enables multiplex interrogation of synaptic activity**. *Nat Neurosci* 2012, **15**:1047-1053.

9. Miesenbock G, De Angelis DA, Rothman JE: **Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins**. *Nature* 1998, **394**:192-195.

10. Namiki S, Sakamoto H, Iinuma S, Iino M, Hirose K: **Optical glutamate sensor for spatiotemporal analysis of synaptic transmission**. *Eur J Neurosci* 2007, **25**:2249-2259.

11. Okubo Y, Sekiya H, Namiki S, Sakamoto H, Iinuma S, Yamasaki M, Watanabe M, Hirose K, Iino M: **Imaging extrasynaptic glutamate dynamics in the brain**. *Proc Natl Acad Sci U S A* 2010, **107**:6526-6531.

12. Masharina A, Reymond L, Maurel D, Umezawa K, Johnsson K: **A fluorescent sensor for GABA and synthetic GABA(B) receptor ligands**. *J Am Chem Soc* 2012, **134**:19026-19034.

13. Brun MA, Tan KT, Griss R, Kielkowska A, Reymond L, Johnsson K: **A semisynthetic fluorescent sensor protein for glutamate**. *J Am Chem Soc* 2012, **134**:7676-7678.

14. • Schena A, Johnsson K: **Sensing acetylcholine and anticholinesterase compounds**. *Angew Chem Int Ed Engl* 2014, **53**:1302-1305.

ACh-Snifits (2013). In this study, the authors used selective chemical-labeling reactions combined with a genetically encoded acetylcholinesterase scaffold to generate semi-synthetic ACh analog that could be targeted to the cell surface and used to measure ACh. The underlying principle behind Snifits can be generalized and used to develop probes to detect other molecules, for example, the generation of fluorescence signals can be generically produced by an endogenous true ligand that displaces a pre-bound fluorescent 'false' ligand.

15. Okumoto S, Looger LL, Micheva KD, Reimer RJ, Smith SJ, Frommer WB: **Detection of glutamate release from neurons by genetically encoded surface-displayed FRET nanosensors**. *Proc Natl Acad Sci U S A* 2005, **102**:8740-8745.

16. Tsien RY: **Building and breeding molecules to spy on cells and tumors**. *FEBS Lett* 2005, **579**:927-932.

17. Deuschle K, Okumoto S, Fehr M, Looger LL, Kozhukh L, Frommer WB: **Construction and optimization of a family of genetically encoded metabolite sensors by semirational protein engineering**. *Protein Sci* 2005, **14**:2304-2314.

18. Hires SA, Zhu Y, Tsien RY: **Optical measurement of synaptic glutamate spillover and reuptake by linker optimized glutamate-sensitive fluorescent reporters**. *Proc Natl Acad Sci U S A* 2008, **105**:4411-4416.

19. •• Marvin JS, Borghuis BG, Tian L, Cichon J, Harnett MT, Akerboom J, Gordus A, Renninger SL, Chen TW, Bargmann CI *et al.*: **An optimized fluorescent probe for visualizing glutamate neurotransmission**. *Nat Methods* 2013, **10**:162-170.

iGluSnFR (2013). This study reports the development of the intensity-based glutamate fluorescent sensor iGluSnFR by combining the glutamate-binding protein GltI with cpEGFP. This sensor was then used to visualize glutamate dynamics in several model animals. This was the first time that a fluorescent neurotransmitter sensor was developed with significantly improved signal-to-noise ratios that is capable of reporting endogenous glutamate dynamics *in vivo*.

20. Wu J, Abdelfattah AS, Zhou H, Ruangkittisakul A, Qian Y, Ballanyi K, Campbell RE: **Genetically encoded glutamate indicators with altered color and topology**. *ACS Chem Biol* 2018.

21. Vilardaga JP, Bunemann M, Krasel C, Castro M, Lohse MJ: **Measurement of the millisecond activation switch of G protein-coupled receptors in living cells**. *Nat Biotechnol* 2003, **21**:807-812.

22. Nakanishi J, Takarada T, Yunoki S, Kikuchi Y, Maeda M: **FRET-based monitoring of conformational change of the beta(2) adrenergic receptor in living cells**. *Biochem Biophys Res Commun* 2006, **343**:1191-1196.

23. • Maier-Peuschel M, Frolich N, Dees C, Hommers LG, Hoffmann C, Nikolaev VO, Lohse MJ: **A fluorescence resonance energy transfer-based M2 muscarinic receptor sensor reveals rapid kinetics of allosteric modulation**. *J Biol Chem* 2010, **285**:8793-8800.

ACh-FRET (2010). The authors developed FRET-based ACh sensors that retain the functional-coupling capability of original GPCR. Ligand binding to the muscarinic ACh receptor causes a conformational change in the receptor, which changes the FRET signal between the donor (CFP) and the acceptor (FlAsH, Fluorescein Arsenical Hairpin binder). Although the resulting signal is relatively small, this method has generally high temporal resolution.

24. Ziegler N, Batz J, Zabel U, Lohse MJ, Hoffmann C: **FRET-based sensors for the human M1-, M3-, and M5-acetylcholine receptors**. *Bioorg Med Chem* 2011, **19**:1048-1054.

25. Nguyen QT, Schroeder LF, Mank M, Muller A, Taylor P, Griesbeck O, Kleinfeld D: **An in vivo biosensor for neurotransmitter release and in situ receptor activity**. *Nat Neurosci* 2010, **13**:127-132.

26. • Muller A, Joseph V, Slesinger PA, Kleinfeld D: **Cell-based reporters reveal in vivo dynamics of dopamine and norepinephrine release in murine cortex**. *Nat Methods* 2014, **11**:1245-1252.

CNiFER (2014). Using cell-based reporters, the authors showed that dopamine and norepinephrine release can be detected and discriminated in mice. Their cell-based reporters utilize GPCRs that are activated by specific ligands and trigger intracellular Ca^{2+} signaling, which is then detected by an intracellular fluorescent Ca^{2+} sensors. This innovative design can be adapted to develop sensors for other ligands that activate GPCRs, although complex cell transplantation is required for *in vivo* detection.

27. Barnea G, Strapps W, Herrada G, Berman Y, Ong J, Kloss B, Axel R, Lee KJ: **The genetic design of signaling cascades to record receptor activation**. *Proc Natl Acad Sci U S A* 2008, **105**:64-69.

28. Inagaki HK, Ben-Tabou de-Leon S, Wong AM, Jagadish S, Ishimoto H, Barnea G, Kitamoto T, Axel R, Anderson DJ: **Visualizing neuromodulation in vivo: TANGO-mapping of dopamine signaling reveals appetite control of sugar sensing**. *Cell* 2012, **148**:583-595.

29. •• Lee D, Creed M, Jung K, Stefanelli T, Wendler DJ, Oh WC, Mignocchi NL, Luscher C, Kwon HB: **Temporally precise labeling and control of neuromodulatory circuits in the mammalian brain**. *Nat Methods* 2017, **14**:495-503.

iTANGO (2017). In this study, the authors cleverly added a light-sensitive component to gate the ligand-gated TANGO assay, thereby improving the assay's temporal precision. iTANGO was used to mark neurons that received dopamine release in the mouse brain *in vivo* and one added feature is its ability to control/manipulate the marked neuron by engaging distinct downstream effectors.

30. Talay M, Richman EB, Snell NJ, Hartmann GG, Fisher JD, Sorkac A, Santoyo JF, Chou-Freed C, Nair N, Johnson M *et al.*: **Transsynaptic mapping of second-order taste neurons in flies by trans-tango**. *Neuron* 2017, **96** 783-795.e784.

31. Kim MW, Wang W, Sanchez MI, Coukos R, von Zastrow M, Ting AY: **Time-gated detection of protein–protein interactions with transcriptional readout**. *Elife* 2017:6.

32. Kipniss NH, Dingal P, Abbott TR, Gao Y, Wang H, Dominguez AA, Labanieh L, Qi LS: **Engineering cell sensing and responses using a GPCR-coupled CRISPR-Cas system**. *Nat Commun* 2017, **8**:2212.

33. Baeumler TA, Ahmed AA, Fulga TA: **Engineering synthetic signaling pathways with programmable dCas9-based chimeric receptors**. *Cell Rep* 2017, **20**:2639-2653.

34. •• Jing M, Zhang P, Wang G, Jiang H, Mesik L, Feng J, Zeng J, Wang S, Looby J, Guagliardo NA *et al.*: **A genetically-encoded fluorescent acetylcholine indicator**. *Nat Biotechnol* 2018.

GACh (2018). The authors developed a family of fluorescent reporters for sensing ACh with high signal-to-noise ratio, by incorporating cpEGFP to the intracellular loop of human muscarinic ACh GPCRs; they then used these sensors to probe ACh dynamics both *in vitro* and *in vivo* of mice and transgenic flies. This study shows, for the first time, the design principle behind GPCR-based sensors with a single-fluorescence-wavelength cpEGFP, an approach that can be expanded for developing of sensors to image other neurotransmitters and/or neuromodulators.

35. Huang W, Manglik A, Venkatakrishnan AJ, Laeremans T, Feinberg EN, Sanborn AL, Kato HE, Livingston KE, Thorsen TS, Kling RC *et al.*: **Structural insights into micro-opioid receptor activation**. *Nature* 2015, **524**:315-321.

36. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D *et al.*: **Crystal structure of the beta2 adrenergic receptor-Gs protein complex**. *Nature* 2011, **477**:549-555.

37. Kruse AC, Ring AM, Manglik A, Hu J, Hu K, Eitel K, Hubner H, Pardon E, Valant C, Sexton PM *et al.*: **Activation and allosteric modulation of a muscarinic acetylcholine receptor**. *Nature* 2013, **504**:101-106.

38. Markovic D, Holdich J, Al-Sabah S, Mistry R, Krasel C, Mahaut-Smith MP, Challiss RA: **FRET-based detection of M1 muscarinic acetylcholine receptor activation by orthosteric and allosteric agonists**. *PLoS ONE* 2012, **7**:e29946.