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# Measuring brain chemistry with genetically encoded fluorescent sensors

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## Abstract

To understand the precise roles of chemical messengers in the brain for communication, we need tools to measure the concentration and release events of neurotransmitters and neuromodulators with great precision. Fluorescent protein-based biosensors with high molecular specificity have been recently developed for direct, optical recording of brain chemistry. Here, we discuss the engineering and applications of the most recently developed genetically encoded neuromodulator sensors for *in vivo* studies.

## Highlights

- Development of genetically encoded calcium indicators (GECIs) paved the way for design and characterization of other single-fluorescent protein (FP)-based indicators
- Single-FP based indicators for small molecules using either bacterial periplasmic binding proteins (PBPs) or G-protein coupled receptors (GPCRs) as scaffolds are discussed
- An increasing number of neurotransmitters/neuromodulators are targetable by emerging genetically encoded and chemi-genetic sensors

## Keywords

molecular imaging, neurotransmitter, neuromodulators, fluorescent protein, genetically encoded fluorescent indicators, GPCRs

## Introduction

To understand brain's structure and function in health and disease, we must have the ability to measure the communication between neurons on both large and small scales of distance and time. Molecular imaging of neuronal transmissions is a powerful, non-invasive method promising higher spatial and/or temporal resolution compared to other methods such as microdialysis, electrophysiology recordings or electrochemical detection. A large number of molecular probes, such as small molecule dyes and genetically encoded indicators, have been developed to image synaptic transmission. Calcium and pH sensitive dyes, quantum dots, and fluorescent false neurotransmitters (FFN's) have been employed to monitor exocytosis of synaptic vesicles providing an indirect (and unspecific) way to observe neurotransmitter release. Recently, to achieve cell-type specificity, high-quality fluorescence protein-based sensors that report calcium, voltage or transients of neurotransmitter/modulators have recently been developed and are widely used in cells, tissues and whole organs. A more detailed discussion of various design strategy of genetically encoded sensors based on either cell reporter systems, Förster resonant energy transfer (FRET) between fluorescent proteins, or between fluorescent proteins and dyes can be found in refs [1,2]. Here, we mainly focus on the development of single-fluorescent protein (single-FP) based sensors for glutamate and neuromodulators, which permits specific, direct, long-term imaging of samples *in vivo* with high signal-to-noise ratio (SNR), fast kinetics, and subcellular resolution.

## Calcium sensors: where it all started

Genetically encoded calcium indicators (GECIs) based on recombinant fluorescent proteins have been engineered to report calcium transients in living cells and organisms. Through observation of calcium, these indicators also report neural activity; action potential (AP) firing triggers large influxes of  $\text{Ca}^{2+}$  through voltage-gated calcium channels located throughout the cell, and neurotransmitter receptors cause local  $\text{Ca}^{2+}$  influx in the dendritic spines and shaft, thus coupling the spatiotemporal pattern of intracellular  $[\text{Ca}^{2+}]$  to neural activity [3]. A GECI is a single-FP sensor comprising a calcium-binding recognition element allosterically coupled to a circularly permuted (cp), or split (sp), FP. The green fluorescent protein (GFP) is robust to circular permutations, and it became the most widespread FP used in calcium sensors since the first GECIs inception [4]. Typical recognition elements employed are calmodulin (CaM), or troponin-C (TnC), together with the M13, or RS20, peptide from myosin light chain kinase, or with the rat CaM-dependent kinase kinase peptide (ckkap). In a GECI, the recognition elements are fused at the N- and C-termini of the cpFP (or spFP), and upon binding of calcium a large conformational change in the binding element is mechanically transmitted to the FP, thereby modulating its fluorescent properties (emission wavelength or intensity) by changing the chromophore protonation state or strain [5–7]. The structural details of this process were first described in ref. [8–10]. In the past decade, the intrinsic properties of GECIs, including brightness, color-spectrum, dynamic range, calcium sensitivity, response linearity and kinetics, have been iteratively optimized and improved via various protein engineering efforts, that significantly expanded the toolbox of calcium indicators (Table 1). For example, optimizing the linkers at the interface between the cpFP and the sensing domain can significantly improve the dynamic range, while directed mutagenesis in the ligand binding domain tunes affinity and response kinetics [3]. Additionally, directed evolution of the FP domain often improves features such as brightness and dynamic response [3,11,12] and replacing cpGFP with circularly permuted FPs in other colors significantly expanded the color-spectrum of GECIs. Broad dissemination of high-quality GECIs combined with advanced microscopy now permit large-scale imaging of genetically defined neuronal populations with >10Hz temporal and cellular/subcellular resolution (for comprehensive reviews of calcium imaging applications, please see [3,13]). Here we list representative protein engineering efforts in GECIs optimization:

- Continuous improvement of GCaMP for increased linearity, high sensitivity and ultrafast detection of calcium transients [14–17]
- Red-shifted circularly permuted protein scaffolds for deeper tissue imaging (mApple [18,19], mRuby [19,20], mCherry [21], FusionRed [12])
- A near-infrared (NIR), non-GFP derivative, fluorescent protein for mesoscale widefield imaging of AP in mouse sensorimotor cortex [22]
- Signal integration of  $\text{Ca}^{2+}$  release using the photoconvertible protein mEos (CaMPARI) [11,23]
- Exploration of new topologies using a split GFP and a smaller sensing domain derived from Troponin C (NTnC sensor series) [24–26]
- Specific targeting of sensors to the axons of neurons to reduce background from the soma during *in vivo* imaging [27]
- Prevention of interferences of calcium sensors with transcription signaling and  $\text{Ca}^{2+}$  dysregulation by using a “protected” CaM module (GCaMP-X) [28]
- Introduction of a bioluminescent protein in the sensor construct, for the detection of activity deeper in tissues without an external illumination source [29]

The development of GECIs has pushed our knowledge forward in terms of sensor design, optimization, characterization and validation from concept to purified proteins, and to application in imaging neural activity in behaving animals, which paved ways for the development of genetically encoded indicators for other ligands.

### **Sensors for neurotransmitters/neuromodulators**

Direct measurement of neuronal communication goes well beyond imaging spikes. A comprehensive understanding of brain mechanisms requires precise measurement of neurotransmitter release at the synaptic level. Attempts at engineering FRET-based sensors for small molecules, and in particular neurotransmitters, were indeed successful in some cases [1]. However, their usefulness has been limited to cultured cells due to suboptimal signal-to-noise ratio for *in vivo applications*. High-resolution, direct, and specific measurement of neurotransmitter has been recently made possible by the development of several high-quality, intensity-based,

genetically encoded indicators. These sensors are generally categorized by two major ligand-binding scaffolds: bacterial periplasmic binding proteins (PBP) and G-protein coupled receptors (GPCRs).

### **PBP-based genetically encoded indicators**

Microbial periplasmic binding proteins (PBPs) is a protein superfamily with a large number of members, with binding specificity ranging across numerous classes of small molecules and peptides [30], thus offering appealing scaffolds for engineering genetically encoded sensors. PBPs are typically stable, soluble, and well-tolerated when expressed in other cell types, and the availability of genomes from hyperthermophiles allows the facile discovery of incredibly stable homologues for most given PBPs. The general structural fold of PBPs consists of two domains connected through a hinge region that undergo a Venus flytrap-like conformational change upon ligand binding (Figure 1A). This feature is highly conserved and facilitates homology modeling for the sensor design and engineering using PBPs whose structure are yet unknown. The paradigm for engineering single-wavelength sensor is based on the insertion of a circularly permuted green fluorescent protein (cpGFP) into a region of the PBP in which large local conformational rearrangements upon ligand binding occur. Using this framework, high signal-to-noise ratio (SNR) sensors for maltose [31], organophosphorus [32], glutamate [33], acetylcholine [34], glucose [35,36], gamma-aminobutyric acid (GABA) [37], and nicotine [38], among others, have been developed. iGluSnFR [33], in particular, is the first single-FP based sensor capable of detecting the excitatory neurotransmitter glutamate *in vitro* and *in vivo* with a large response and highly specific signal. iGluSnFR consists of a cpEGFP inserted in a loop of the interdomain hinge region of the glutamate transporter protein GltI from *E. coli*. Membrane expression for extracellular glutamate sensing was achieved by fusion of iGluSnFR to the transmembrane-anchoring domain of platelet-derived growth factor receptor (PDGFR). iGluSnFR has been used to monitor the primary motor cortex of mice *in vivo* during active tasks, with glutamatergic activity resolution at the level of single spines. Further rational engineering provided faster sensors capable of recording multiple glutamate releases at single boutons in mouse brain slices (iGlu<sub>f</sub>, iGlu<sub>u</sub>) [39], as well as in individual dendritic spines in the ferret visual cortex [40]. A constant effort is ongoing to ameliorate the kinetic features of iGluSnFR for better temporal response to record fast synaptic release and uptake of glutamate: the iGlu<sub>f</sub> and iGlu<sub>m</sub> variants by Coates *et al.*

promise extremely fast binding and dissociation *in vitro*, which would be useful to observe transients at the synaptic level [41]. Color-variants of iGluSnFR were produced by mutating cpEGFP into cpAzurite, cpTurquoise2, and cpVenus [40], thus demonstrating the robustness and modifiability of the design. A red-shifted version of iGluSnFR is available, although its performance is not yet comparable with the blue/green versions. The R-iGluSnFR uses cpmApple as a fluorescent reporter, however it shows an “inverted behavior” : the brightness decreases with glutamate binding [42], which leads to high levels of background noise in *in vivo* imaging. Additionally, “inverted sensors” tend to suffer from faster photobleaching compared with positive ones.

New indicators derived from PBPs have been recently added to the toolbox for the detection of small molecules. The GABA sensor iGABASnFR is derived from the protein Pf622, identified in *Pseudomonas fluorescens*. This sensor tightly and selectively binds GABA ( $K_d$  in the  $\mu\text{M}$  range, *in vitro*), while providing sufficient contrast for recording GABA spiking in mouse brains (in hippocampal slices and in the visual cortex) during epileptic seizures induced with pilocarpine, and in zebrafish cerebellum during motorial activity [37]. A very interesting application of a PBP-based sensor is provided by Shivange and coauthors [38]: they coupled the acetylcholine-binding OpuBC protein from *Thermoanaerobacter spX513* with cpGFP, and through optimization of the binding site and the linkers between OpuBC and cpGFP produced a sensor highly specific for nicotine. The resulting iNicSnFR shows a 10-fold increase in fluorescence (*in vitro*) upon ligand binding, and it was used to observe for the first time the dynamics of nicotine entry into the endoplasmic reticulum, a phenomenon connected to the “inside-out” pathway of upregulation of acetylcholine receptors (AChRs) that leads to drug dependency [38]. In the same work [38], there is an intriguing mention of a sensor for acetylcholine (iAChSnFR). A preprint that uses iAChSnFR for high speed imaging is available [34], although no details are yet provided regarding its design and engineering.

From the same family, iATPSnFR is a sensor for ATP based on the epsilon subunit of the  $F_0F_1$ -ATP synthase from *Bacillus subtilis* and coupled with cpGFP [43]. This sensor successfully detects changing levels of ATP in the cytosol of HEK cells, neurons and astrocytes. Although not exclusively aimed at neuroscience research,

the sensor paves the way for further development of tools useful in studies of cellular metabolism, as well as of the purinergic signaling system in the brain.

The large conformational change upon ligand binding offered by PBPs makes them great scaffolds for engineering small molecule sensors. Additionally, PBPs are versatile and can further be mutated to bind neurotransmitters for which there are no naturally occurring PBPs. In addition to the PBP sensor discussed above, we and other labs are currently expanding the toolbox of PBP based sensors for other neurotransmitters and neuromodulators.

### **GPCRs-based genetically encoded indicators**

G-protein coupled receptors belong to a large superfamily of membrane proteins divided in 6 classes (A, B, C, D, E, F). While each class has different functions, they have highly conserved common structural features [44]: seven bundled transmembrane helices (TM1-7), three extracellular loops (ECL1-3) and the N-terminus composing the ligand binding site (the most structurally variable part of the protein), and three intracellular loops (ICL1-3) and the C-terminus comprising the cytosolic side. When a ligand docks in the binding pocket, a cascade of conformational rearrangements occurs within the transmembrane helices: TM6 usually undergoes the largest motion, which translates to structural changes of ICL3 and relative movements of this loop and the C-terminus[44]. ICL3 transitions from a disordered to an ordered state, a crucial step in recruiting G-proteins. The mechanism that induces intracellular conformational changes is conserved among several members of the GPCR family [45]. The use of these conformational changes to drive movements of fluorescent proteins was pioneered by pharmacologists and structural biologists to study mechanistic details of GPCR protein activation, kinetics, and drug response [46]. In those cases, a FRET-based approach was used: a fluorescent protein acting as a FRET donor (CFP) was inserted in ICL3, and a second fluorescent protein acting as a FRET acceptor (YFP) was linked to the C-terminus. Relative motions of the loop and the C-terminus upon ligand binding induce FRET between CFP and YFP. However, a broad application of FRET-based GPCR sensors is limited for *in vivo* imaging, majorly due to their relatively low SNR.

A new class of indicators for small molecules based on GPCRs was very recently developed to enable high-resolution imaging of neuromodulators *in vivo*, one created by inserting cpGFP into the ICL3 of GPCRs (Figure 1B). Two groups independently engineered sensors for dopamine (DA): Patriarchi *et al.* developed the dLight series based on three human Dopamine Receptors (DRD1, DRD2 and DRD4) [47], while Sun *et al.* developed the GPCR-activation-based-DA sensor series (GRAB<sub>DA</sub>) based on DRD2 [48]. The designs of dLight1 and GRAB<sub>DA</sub> use different linker compositions and ICL3 insertion sites, which lead to different sensor properties. dLight1 suite offers various combinations of dynamic range and DA affinity to enable versatile applications in various brain regions (for details please see Table 2).

The versatility of the GPCR-based approach is further demonstrated by applying the design strategy of dLight1 to develop a class of intensity-based sensors for various neurotransmitters, neuromodulators and neuropeptides. G<sub>s</sub>-coupled  $\beta_1$  and  $\beta_2$  adrenergic receptors (B1AR and B2AR), G<sub>i</sub>-coupled  $\kappa$ - and  $\mu$ -type opioid receptors (KOR, MOR) and  $\alpha_2$  adrenergic receptor (A2AR), and G<sub>q</sub>-coupled 5- hydroxytryptamine (serotonin) receptor-2A (5HT2A) and melatonin type-2 receptor (MT2). Systematic optimization and characterization are still needed before broad applications for *in vivo* imaging. In addition, due to the highly conserved structural features, beneficial mutations may be portable across sensors [47].

The GRAB family was recently enlarged by probes for norepinephrine [49] and acetylcholine [50]: GRAB<sub>NE</sub> and GRAB<sub>ACh</sub>, respectively. The former uses the  $\alpha$ -adrenergic receptor ( $\alpha$ 2AR), whereas the latter employs the human muscarinic receptor 3 (M<sub>3</sub>R) as a sensing moiety. Both sensors, as with their GRAB<sub>DA</sub> analog, were suitable for *in vivo* expression and activity recording, although with some limitation for GRAB<sub>ACh</sub>, which showed a reduced dynamic range and sparse response when compared to other GRAB sensors.

Though the abundance of GPCRs is advantageous for sensor design, GPCRs, as endogenous receptors, can trigger signaling cascades. Therefore, it is important to engineer them as inert sensors and maintain membrane expression. In the case of dLight1, the whole IC3 was replaced with cpGFP, which completely abolished endogenous signaling through G-proteins and engagement of  $\beta$ -arrestins for internalization. In the GRAB family, cpGFP was inserted into IC3, which may not completely suppress downstream signaling as

evidenced by a weak activation of the  $G_q$ -dependent calcium signaling pathway observed in GRAB<sub>ACh</sub> [50]. Future characterization may focus on examining physiological properties of cells with and without sensor expression such as resting potentials, membrane resistance and capacitance, synaptic transmission and circuit properties.

### **Engineering new sensors: PBPs or GPCRs, which one to choose?**

PBP-based sensors are amenable to high-throughput screening in bacteria, and easily allow detailed characterization on purified protein as well as structural determination through crystallography. They can be expressed cytoplasmically to study the activity of transporters, or as membrane exposed proteins through integration with a transmembrane domain (e.g. PDGFR) to visualize extracellular secretion. Their orthogonality to host cells makes them compatible with pharmacological studies, since the chances of drugs aimed at physiological receptors interacting with PBP-based sensors are negligible.

GPCRs, being membrane proteins, are somewhat more difficult to characterize and screen *in vitro*, and present more challenges in the determination of structural information during sensor-development. However, the readily available receptors for human neurotransmitters and neuromodulators eliminates the need to re-engineer their binding pocket to increase their selectivity, specificity and optimizing affinity, and the high conservation of structure adds to the ease of developing new tools. GPCR-based sensor can also serve as a drug screening platform for the evaluation, *in vitro*, of the binding and action of new potential allosteric ligands.

### **Future perspectives**

Looking at the current toolbox of fluorescent sensors for neurobiology, the picture that emerges is that we are just at the beginning of an era where new and exciting molecular tools for specific neurotransmitters and neuromodulators will become available. Whether these are PBP- or GPCR-based, we think that both approaches have their strengths, adaptability, and room for improvement. Bacterial proteins are limited by the type of ligands that they are often suitable for, but the development of iNicSnFR [38], using powerful protein engineering efforts, showed it possible to drive their evolution toward binding with non-native substrates. GPCRs certainly offer scaffolds already compatible with signaling molecules in the brain, but are more

cumbersome to screen; although, new approaches for direct screening in mammalian cells may speed up this task [51].

Independently from the scaffold of choice, other tools are also being sought after, the most compelling possibly being a new generation of red-shifted sensors, the creation of which requires both application of available red fluorescent proteins to the newly developed sensors (SnFR, dLight, or GRAB family), as well as an effort to improve red proteins to make them comparably as bright and reliable as GFP. Deep brain imaging beyond the cortex is the main, but not the only goal: a bathochromic shift of the sensors means improvement in their compatibility with optogenetics tools that often require blue/green excitation.

Going beyond the use of fluorescent proteins as reporters, chemigenetic approaches, using split variants of self-labeling proteins including Halo-, SNAP- and CLIP-tag [52,53], remain a rich ground for engineering new molecular probes. The use of a chemigenetic strategy would introduce complementary and orthogonal features in the existing toolbox. These would, for example, be suitable for tagging active subpopulations of neurons on an intermediate time scale between the fast release of neurotransmitters (milliseconds), and the slow rearrangement of gene expression (hours to days). Modulation of the activity of SNAP-, CLIP- or Halo-tag through insertion in a ligand binding domain would be necessary to achieve this goal: the evidence that the protein can be split certainly makes a case for designing circularly permuted versions to be used to this aim. An aspect where we see that the field might be lagging behind compared to other applications such as imaging, genomic profiling, or drug discovery is the use of machine learning techniques. Screening of hundreds of thousands of variants at a time does the job, but important structural and mechanistic relationships between mutations might be missed in the vast sea of numbers. Very recent work in the general field of protein evolution has moved steps toward algorithmic-based directed evolution [54], and we believe that sensor design should be the next in line.

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**Conflict of interest statement**

L.T. is a co-founder of Seven Biosciences.

## Tables and Figures

**Table 1:** overview of GECIs currently available for fast, high SNR imaging of calcium *in vivo*.

GECI	Exc/Em (nm)	$\Delta F/F$ in vitro (%)	$K_d$ in vitro (nM)	n (Hill coefficient)	$k_{off}$ (s <sup>-1</sup> )	$t_{peak\ rise}$ (ms) <sup>a</sup>	$t_{1/2\ decay}$ (ms) <sup>a</sup>	Applications <i>in vivo</i>	Ref.
GCaMP6	497/515	3600	158	NA	NA	62	457	<i>C. elegans</i>	[55]
GCaMP6f	497/515	5200	380	2.3	3.93	45 <sup>b</sup>	140 <sup>b</sup>	<i>Drosophila</i> , mouse	[56,57]
GCaMP6s	497/515	6300	140	2.9	1.12	179 <sup>b</sup>	550 <sup>b</sup>		
GCaMP6m	497/515	3810	167	3.0	2.06	80 <sup>b</sup>	270 <sup>b</sup>		
Fast-GCaMP6f-RS06	488/512	1500	320	3.0	10.2	~60	131	Mouse	[15]
Fast-GCaMP6f-RS09	488/512	2500	520	3.2	13.8	~60	104		
GCaMP6f <sub>u</sub>		510	890	3.0	89	<50 <sup>d</sup>	40 <sup>d</sup>	NA	[16]
jGCaMP7s	497/515	3900	68	NA	2.86	NA	1260	<i>Drosophila</i> , mouse	[14,58]
G-GECO1.2	498/513	2300	1150	3.0	0.700	NA	NA	<i>C. elegans</i>	[59]
CAMPARI2 (Green)	502/516	780	199	2.8	1.43	NA	NA	Zebrafish, mouse	[11,58]
CaMPARI2 (Red)	562/577	780	199	2.8	1.43	NA	NA		
R-GECO1	561/589	1600	480	1.6	0.752	110	800	<i>C. elegans</i>	[55,59]
R-CaMP2	565/583	480	69	1.2	1.00	40	170	Mouse, <i>C. elegans</i>	[18,56]
jRGECO1a	562/590	1100	150	1.8	3.4	NA	390	<i>C. elegans</i> , <i>Drosophila</i> , Zebrafish, mouse	[56,60]
jRCaMP1a	570/595	320	141	1.5	2.2/0.32	50	580	<i>Drosophila</i> , zebrafish, mouse	[19,56]
K-GECO1	565/590	1100	165	1.1	NA	Similar to jRGECO1a <sup>c</sup>		Zebrafish, mouse	[12,58]
XCaMP-Gf <sub>o</sub>	494/514	1980	128	1.3	NA	40	<100	Mouse	[17]
XCaMP-Gf	492/514	1170	115	1.4	NA	50	148		
XCaMP-R	561/593	560	97	1.1	NA	25	200		
XCaMP-Y	503/527	620	81	1.5	NA	68	220		

Notes: <sup>a</sup>1 AP in mouse or rat slices, unless stated otherwise; <sup>b</sup>1AP, *in vivo*, mouse; <sup>c</sup>cultured dissociated neurons; <sup>d</sup>5 AP, mouse brain slice.

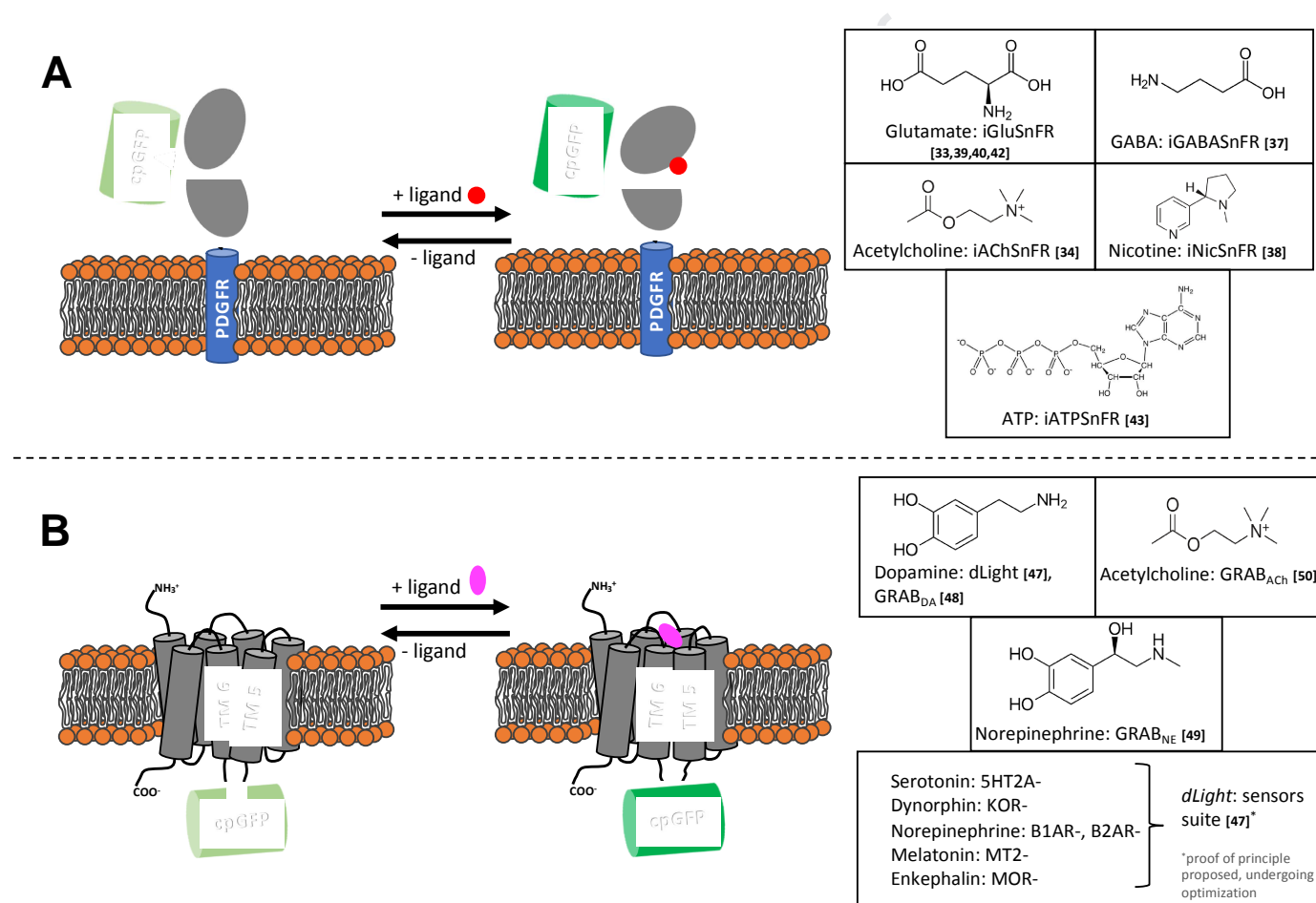
**Table 2:** overview of genetically encoded indicator for neurotransmitters and small molecules with neuromodulating activity based on periplasmic binding proteins (PBPs) or G-protein coupled receptors (GPCRs).

Type	Sensor	Ligand	Exc/Em (nm)	$\Delta F/F$ (%)	K <sub>d</sub> ( $\mu$ M)	k <sub>on</sub> ( $\mu$ M <sup>-1</sup> s <sup>-1</sup> ) <sup>a</sup>	k <sub>off</sub> (s <sup>-1</sup> ) <sup>a</sup>	$\tau_{on}$ (ms) <sup>b</sup>	$\tau_{off}$ (ms) <sup>b</sup>	Applications <i>in vivo</i>	Ref.
PBP	iGluSnFR	Glu <sup>c</sup>	490/510	100	4.9	799 <sub>(lim)</sub> (s <sup>-1</sup> )	233	15	92	<i>C. elegans</i> , zebrafish, mouse	[33,39]
	SF-iGluSnFR-A184S	Glu <sup>c</sup>	490/510	69	0.6	6.0	25	85	450	Mouse, ferret	[40]
	SF-iGluSnFR-S72A	Glu <sup>c</sup>	490/510	250	34	0.58	108	5	11	NA	[40]
	SF-Venus-iGluSnFR	Glu <sup>c</sup>	515/528	66	2.0	NA	NA	NA	NA	Mouse	[40]
	iGlu <sub>u</sub>	Glu <sup>c</sup>	490/510	170 <sup>d</sup>	53 <sup>d</sup>	1493 <sub>(lim)</sub> (s <sup>-1</sup> )	1481	0.7	2.6	NA	[39]
	R-iGluSnFR1	Glu <sup>c</sup>	562/588	-490 <sup>b</sup>	1 <sup>b</sup>	NA	NA	NA	NA	NA	[42]
	iGABASnFR	GABA	485/510	75	30	<i>Bi-exponential</i>	NA	~25 <sup>e</sup>	~60 <sup>e</sup>	Zebrafish, mouse	[37]
	iNicSnFR	Nicotine	485/535	~300	10	0.07	2.0	~1000	NA	NA	[38]
	iATPSnFR1.0	ATP	490/512	240 <sup>f</sup> 150	120 <sup>f</sup> 630	0.47	0.58	NA	NA	Mouse	[43]
GPCR	dLight1.1	DA	490/516	230 <sup>d</sup>	0.33 <sup>d</sup>	NA	NA	10	100	Mouse	[47]
	dLight1.2	DA	490/516	340 <sup>d</sup>	0.77 <sup>d</sup>	NA	NA	9.5	90	Mouse	[47]
	dLight1.3b	DA	490/516	~900 <sup>d</sup>	1.6 <sup>d</sup>	NA	NA	NA	NA	Mouse, rat	[47,61,62]
	nLight	NE	490/516	~150	1.2	NA	NA	NA	NA	Mouse	[47, personal communication]
	sLight	5-HT	490/516	80	0.65	NA	NA	NA	NA	Mouse	[47, personal communication]
	GRAB <sub>DA1m</sub>	DA	490/510	90 <sup>d</sup>	0.13 <sup>d</sup>	NA	NA	80	3100	Mouse	[48]
	GRAB <sub>DA1h</sub>	DA	490/510	90 <sup>d</sup>	0.01 <sup>d</sup>	NA	NA	110	17150	Mouse	[48]
	GAC <sub>h</sub> 2.0	ACh	490/510	90	2	NA	NA	280 <sup>d</sup>	760 <sup>d</sup>	Mouse	[50]
	GRAB <sub>NE1m</sub>	NE	490/510	230	1.9	NA	NA	72 <sup>d</sup>	680 <sup>d</sup>	Mouse, zebrafish	[49]
	GRAB <sub>NE1h</sub>	NE	490/510	150	0.093	NA	NA	36 <sup>d</sup>	1890 <sup>d</sup>	NA	[49]

Exc: excitation wavelength; Em: emission wavelength;  $\Delta F/F$ : maximum change in fluorescence from pre-ligand to post-ligand bound states, measured in dissociated neurons unless otherwise stated; K<sub>d</sub>: ligand dissociation constant measured in dissociated neurons, unless otherwise stated; ND: data not available

Notes: <sup>a</sup>measured *in vitro*, purified protein; <sup>b</sup>measured in acute brain slices, unless stated otherwise; <sup>c</sup>also shows comparable affinity to Asp; <sup>d</sup>measured in HEK cells; <sup>e</sup>estimated from published 1 AP trace in cultured neurons (see ref. [58]); <sup>f</sup>measured for sensor expressed in the cytosol of U373MG astroglia.

**Figure 1** A) Left: illustration of the design and working mechanism of the SnFR sensors based on a circularly permuted GFP inserted in a hinge region of a periplasmic binding protein. Extracellular sensing is achieved by expressing the sensors linked to PDGFR for membrane incorporation. Right: panel of neurotransmitters and brain-relevant molecules for which a SnFR sensor is available. B) Left: schematics of the design and working mechanism of GPCR-based sensors obtained through incorporation of cpGFP in the intracellular loop 3, between transmembrane domains 5 and 6, of a receptor. Right: panel of molecules for which GPCR-based sensors are available.



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

\* of special interest

\*\* of outstanding interest

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**Conflict of interest statement**

L.T. is a co-founder of Seven Biosciences.