The Property-Based Practical Applications and Solutions of Genetically Encoded Acetylcholine and Monoamine Sensors

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Neuromodulatory communication among various neurons and non-neuronal cells mediates myriad physiological and pathologic processes, yet defining regulatory and functional features of neuromodulatory transmission remains challenging because of limitations of available monitoring tools. Recently developed genetically encoded neuromodulatory transmitter sensors, when combined with superresolution and/or deconvolution microscopy, allow the first visualization of neuromodulatory transmission with nanoscale or microscale spatiotemporal resolution. *In vitro* and *in vivo* experiments have validated several high-performing sensors to have the qualities necessary for demarcating fundamental synaptic properties of neuromodulatory transmission, and initial analysis has unveiled unexpected fine control and precision of neuromodulation. These new findings underscore the importance of synaptic dynamics in synapse-, subcellular-, and circuit-specific neuromodulation, as well as the prospect of genetically encoded transmitter sensors in expanding our knowledge of various behaviors and diseases, including Alzheimer's disease, sleeping disorders, tumorigenesis, and many others.

Key words: acetylcholine; norepinephrine; serotonin; dopamine; neuromodulation; synaptic transmission; superresolution and deconvolution microscopy

Introduction

Acetylcholine (ACh), the first identified neurotransmitter, mediates communication among various neurons and non-neuronal cells. In the CNS, cholinergic neurons originate primarily from three major nuclei (i.e., the basal forebrain nuclei, brainstem pedunculo-pontine, and lateral dorsal tegmental nuclei), which project widely throughout cortical and subcortical regions to modulate complex brain functions, including attention, cognition, and sleep-wake cycles (Picciotto et al., 2012; Ballinger et al., 2016). In addition, a few smaller cholinergic neuron populations in the regions, such as medial habenula and striatum, contribute to behaviors related to movement, motivation, and stress (Picciotto et al., 2012; Ballinger et al., 2016). Dysregulation of central cholinergic transmission plays a role in major brain disorders, including addiction, Alzheimer's disease, depression, epilepsy, Parkinson's disease,

and schizophrenia (Picciotto et al., 2012; Kruse et al., 2014; Ballinger et al., 2016). Beyond CNS, neurons and non-neuronal cells may release ACh to relay neuromuscular synaptic transmission and regulate functions of a variety of other tissues and organs (e.g., the heart, liver, and pancreas) (Wessler and Kirkpatrick, 2008; Kruse et al., 2014). Dysregulation of peripheral and non-neuronal cholinergic signals may initiate a plethora of pathologic conditions, including cardiovascular diseases, diabetes, immunodeficiency, and cancer (Kruse et al., 2014; Herring et al., 2019).

Norepinephrine (NE) is a monoamine neurotransmitter synthesized by neurons in the locus coeruleus, which project to a large variety of areas in the CNS (Aston-Jones and Cohen, 2005; Sara and Bouret, 2012; Schwarz and Luo, 2015). Through these diverse projections, noradrenergic neurons regulate multiple high-level cognitive functions, including attention, cognition, sleep-wake cycles, movement, and stress, many of which are modulated by cholinergic neurons as well. Dysregulated adrenergic transmission profoundly disrupts arousal and cognition, leading to disorders similar to those caused by dysregulated cholinergic transmission, such as Alzheimer's disease, autism, sleep disorders, mood disorders, Parkinson's disease, and schizophrenia (Aston-Jones and Cohen, 2005; Berridge et al., 2012; Jacob and Nienborg, 2018). In peripheral organs, dysregulation of adrenergic signals is responsible for many health problems, including cardiovascular diseases, immunodeficiency, and cancer (Wood and Valentino, 2017; Dantzer, 2018).

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Figure 1. Profiling neuromodulatory transmission with genetically encoded sensors. **A**, Schematic of designs of GPCR- and bacterial PBP-based genetically encoded sensors for neuromodulatory transmitters. **B**, Schematic of expression, *in vivo* and *ex vivo* applications of genetically encoded sensors. **C**, Schematic of super-resolution and/or deconvolution microscopic analysis of image data obtained with genetically encoded sensors that allows nanoscale and microscale resolution of neuromodulatory transmission. **D**, Spatiotemporal profiling of neuromodulatory transmission with genetically encoded sensors that permits decoding of fundamental properties of neuromodulatory transmission, such as transmitter spatial diffusion extent, number of release sites, release pool size, release probability, quantal size, and refilling rate.

Serotonin (5-hydroxytryptamine [5HT]) in the brain is synthesized by neurons in the brainstem raphe nuclei that innervate nearly every area in the brain and spinal cord (Jacobs and Azmitia, 1992; Okaty et al., 2019). Serotonergic neurons influence diverse behaviors, including appetite, attention, emotion, cognition, motivation, perception, sleep, social interaction, and sexuality (Berger et al., 2009; Okaty et al., 2019). It has been hypothesized that 5HT may have a unified functional role involved primarily in aversive processing in the brain (Dayan and Huys, 2009; Hu, 2016). Indeed, dysregulation of central serotonergic transmission has been implicated in a large group of anxiety and mood disorders, including panic disorder, social anxiety disorder, generalized anxiety disorder, post-traumatic stress disorder, obsessive-compulsive disorder, major depressive disorder, bipolar disorder, and schizophrenia (Ravindran and Stein, 2010). Serotonergic signals also participate in functions of many other tissues and organs, and dysregulation of peripheral and non-neuronal serotonergic signals may cause a wide range of disorders, including cardiovascular diseases, respiratory defects, and metabolic and bowel disorders (Berger et al., 2009).

Dopamine (3,4-dihydroxyphenethylamine [DA]) is a monoamine transmitter that seems to functionally counteract 5HT. Dopaminergic neurons primarily cluster in nine major cell groups in the brain, with those situated in the midbrain SNc and VTA best characterized (Bjorklund and Dunnett, 2007; Morales and Margolis, 2017). In contrast to other monoaminergic transmitters, dopaminergic neurons project to limited, specific brain regions, mainly the striatum, PFC and orbitofrontal cortex, amygdala, and associated structures (Bjorklund and Dunnett, 2007; Morales and Margolis, 2017). Dopaminergic transmission is critical for motor control and appetitive processing, involving particularly the reward-associated liking, wanting, and learning behaviors (Hu, 2016; Schultz, 2016). Disturbance of dopaminergic transmission contributes to a number of psychiatric conditions, such as addiction, Parkinson's disease, Tourette's

syndrome, attention-deficit/hyperactivity disorder, and schizophrenia (Maia and Frank, 2011; Grace, 2016; Volkow et al., 2019).

Years of research have made an impressive progress in our understanding of neuromodulation, and the findings highlight the pivotal role of neuromodulation in myriad behaviors and diseases. However, many key features of cholinergic and monoaminergic transmission remain unresolved (Barbour and Hausser, 1997; Eban-Rothschild et al., 2018; Mohebi et al., 2019; Okaty et al., 2019), which has hampered efforts to define the physiological and pathologic mechanisms for the behaviors and diseases. For example, whether cholinergic and adrenergic signals differentially control attention and movement, and how they individually affect behaviors are still in debate. Likewise, whether serotonergic and dopaminergic signals act individually or complementarily to regulate aversion and appetite, and how they balance their efforts in different contexts remain elusive. Moreover, what synaptic properties of neuromodulatory transmission are altered under disease conditions and how the alterations may lead to the pathogenesis of neuromodulation-linked diseases are largely unknown.

The slow advance in understanding of synaptic properties of neuromodulatory transmission is due primarily to limitations of available tools that may quantitatively analyze cholinergic and monoaminergic transmission. Although the detection sensitivity of microdialysis, a frequently used method, has been improved in recent years, its poor spatial and temporal resolution remains an obstacle that precludes accurate assessment of the signal dynamics of cholinergic and monoaminergic transmission (Olive et al., 2000; Darvesh et al., 2011). With remarkable sensitivity and temporal resolution, patch-clamp recordings have served as the gold standard to delineate synaptic properties of glutamatergic and GABAergic transmission (Neher, 2015; Jackman and Regehr, 2017). However, this electrophysiology application is severely compromised when dealing with cells with minimal or no net neuromodulator-induced electrophysiological responses (Dani and Bertrand, 2007; Nadim and Bucher, 2014). Fast scan cyclic voltammetry provides excellent nanomolar sensitivity and millisecond temporal resolution, but this detection approach is hindered by its poor spatial resolution and inability to distinguish NE and DA (Robinson et al., 2008). Recent efforts have led to the development of fluorescence resonance energy transfer- and cell-based fluorescent ACh and monoamine sensors (Villardaga et al., 2003; Muller et al., 2014). However, their low sensitivity and/or low resolution permit detection of only volume-size transmission, and are unable to resolve synaptic properties of cholinergic and monoaminergic transmission. These limitations inspire the desire to engineer user-friendly and broadly applicable genetically encoded neurotransmitter sensors that permit tissue-specific high-resolution measurements of neuromodulatory transmission (Lin and Schnitzer, 2016).

Sensor properties

In the last few years, close collaborations among tool engineers and biologists have created and validated several intensity-based genetically encoded sensors for ACh and monoamines. These sensors consist of a conformationally sensitive circularly permuted GFP (cpGFP) and a ligand-binding protein that alters cpGFP fluorescence by inducing conformational changes upon

Table 1. Key properties of genetically encoded sensors for ACh and monoamines^a

Sensors	Sensitivity (μM)	$\Delta F/F$ (%)	SNR (puff)	Kinetics	Selectivity
ACh sensors					
GRAB _{ACh2.0}	$EC_{50} \sim 1\text{--}2 \mu\text{M}$	~ 90	~ 14	$\tau_{on} \sim 250 \text{ ms}; \tau_{off} \sim 700 \text{ ms}$	High
GRAB _{ACh3.0}	$EC_{50} \sim 2 \mu\text{M}$	~ 280	—	$\tau_{on} \sim 100 \text{ ms}; \tau_{off} \sim 900 \text{ ms}$	High
iAChSnFR	$K_d \sim 1 \mu\text{M}$	~ 1200	~ 50	$k_{on} = 0.62 \mu\text{M}^{-1}\text{s}^{-1}; k_{off} = 0.73 \text{ s}^{-1}$	$K_d \sim 45 \mu\text{M}$ (choline)
NE sensors					
GRAB _{NE1m}	$EC_{50} \sim 2 \mu\text{M}$	~ 230	~ 75	$\tau_{on} \sim 70 \text{ ms}; \tau_{off} \sim 700 \text{ ms}$	$EC_{50} \sim 1400 \mu\text{M}$ (DA)
GRAB _{NE1h}	$EC_{50} \sim 0.1 \mu\text{M}$	~ 130	~ 10	$\tau_{on} \sim 35 \text{ ms}; \tau_{off} \sim 2000 \text{ ms}$	$EC_{50} \sim 0.6 \mu\text{M}$ (DA)
5HT sensors					
GRAB _{5HT0.5}	$EC_{50} \sim 0.25 \mu\text{M}$	~ 280	~ 50	$\tau_{on} \sim 60 \text{ ms}; \tau_{off} \sim 3300 \text{ ms}$	High
GRAB _{5HT1.0}	$EC_{50} \sim 0.02 \mu\text{M}$	~ 250	—	$\tau_{on} \sim 200 \text{ ms}; \tau_{off} \sim 3000 \text{ ms}$	High
iSeroSnFR	$EC_{50} \sim 1.5 \mu\text{M}$ $K_d \sim 350 \mu\text{M}$	~ 1000	—	$\tau_{fast} \sim 4 \text{ ms}; \tau_{slow} \sim 100 \text{ ms}$	High
DA sensors					
GRAB _{DA1m}	$EC_{50} \sim 0.13 \mu\text{M}$	~ 90	—	$\tau_{on} \sim 60 \text{ ms}; \tau_{off} \sim 700 \text{ ms}$	$EC_{50} \sim 1.5 \mu\text{M}$ (NE)
GRAB _{DA1h}	$EC_{50} \sim 0.01 \mu\text{M}$	~ 90	—	$\tau_{on} \sim 140 \text{ ms}; \tau_{off} \sim 2500 \text{ ms}$	$EC_{50} \sim 0.1 \mu\text{M}$ (NE)
GRAB _{DA2m}	$EC_{50} \sim 0.1 \mu\text{M}$	~ 340	—	$\tau_{on} \sim 40 \text{ ms}; \tau_{off} \sim 1300 \text{ ms}$	$EC_{50} \sim 1.2 \mu\text{M}$ (NE)
GRAB _{DA2h}	$EC_{50} \sim 0.03 \mu\text{M}$	~ 280	—	$\tau_{on} \sim 50 \text{ ms}; \tau_{off} \sim 7300 \text{ ms}$	$EC_{50} \sim 0.07 \mu\text{M}$ (NE)
rGRAB _{DA1m}	$EC_{50} \sim 0.1 \mu\text{M}$	~ 150	—	$\tau_{on} \sim 80 \text{ ms}; \tau_{off} \sim 770 \text{ ms}$	$EC_{50} \sim 2.2 \mu\text{M}$ (NE)
rGRAB _{DA1h}	$EC_{50} \sim 0.02 \mu\text{M}$	~ 100	—	$\tau_{on} \sim 60 \text{ ms}; \tau_{off} \sim 2150 \text{ ms}$	$EC_{50} \sim 0.06 \mu\text{M}$ (NE)
dLight _{1.1/1.2}	$K_d \sim 0.3\text{--}0.7 \mu\text{M}$	~ 300	—	$\tau_{on} \sim 10 \text{ ms}; \tau_{off} \sim 100 \text{ ms}$	$K_d \sim 20 \mu\text{M}$ (NE)
dLight _{1.3}	$K_d \sim 2.0 \mu\text{M}$	~ 900	—	—	—
RdLight ₁	$K_d \sim 2.0 \mu\text{M}$	~ 250	—	$\tau_{on} \sim 15 \text{ ms}; \tau_{off} \sim 400 \text{ ms}$	$K_d \sim 20\text{--}100 \mu\text{M}$ (NE)

^aSensors with the highest SNR and fastest kinetics provide the best spatial and temporal resolutions critical for resolving synaptic properties of neuromodulatory transmission. For the sensors without SNR measurements, those with the sensitivity comparable to the concentration of endogenous neuromodulators (which offers the optimal baseline fluorescence and SNR) and highest $\Delta F/F$ might perform best.

transmitter binding (Fig. 1A). Two major groups of genetically encoded transmitter sensors have been created: G protein-coupled receptor (GPCR)- and bacterial periplasmic binding protein (PBP)-based sensors.

GPCR-based sensors are typically engineered by replacing the third intracellular loop of G-protein-coupled ACh and monoamine receptors with a cpGFP. Evolution has tuned GPCRs to have appropriate affinity for detecting cognate molecules. GPCR-based sensors, which typically preserve the affinity, usually yield the balanced baseline and signal-evoked fluorescence best for imaging experiments. Moreover, GPCR-based sensors frequently inherit excellent membrane surface trafficking and pharmacological properties from primogenitor GPCRs (Wang et al., 2018; Andreoni et al., 2019); and researchers have taken advantage of the pharmacology to verify the specificity of recorded fluorescence responses (Jing et al., 2018; Piatkevich et al., 2018; Sun et al., 2018; Feng et al., 2019; Wan et al., 2020). Engineering GPCR-based sensors with large dynamic ranges and fast kinetics is crucial for effectively monitoring endogenous signals, but this can sometimes be challenging because of the primogenitors' slow kinetics and limited conformational changes associated with ligand binding (Wang et al., 2018; Andreoni et al., 2019).

PBPs belong to a diverse superfamily of proteins that recognize a plethora of small molecules, including ACh and monoamines (Dwyer and Hellinga, 2004). Since PBPs have evolved to help microbes navigate nutrient gradients and frequently have lower affinities, creating the PBP-based sensors with sensitivity matching the endogenous transmitter levels can be a daunting task (Marvin et al., 2013; Helassa et al., 2018). Because PBPs are soluble proteins, they are amenable to targeted expression in other subcellular compartments, albeit their membrane surface trafficking may require improvement. Fast kinetics and large dynamic ranges are the main advantages of PBP-based sensors, which could be vital for detecting fast dynamics of transmission. Finally, because PBP-based sensors are orthogonal to (or unaffected by) most cholinergic and monoaminergic drugs, they are

compatible with certain pharmacological studies, such as drug screening (Shivange et al., 2019; Unger et al., 2020).

Although creating top-performing GPCR- and PBP-based sensors typically requires generations of evolution, this task has become less arduous with improved molecular evolution approaches, including machine-learning-guided protein redesign and directed evolution, and automated screening and analysis (Piatkevich et al., 2018; Vilette et al., 2019). The improved molecular evolution approaches certainly accelerate the expansion of sensor toolbox (Table 1).

ACh sensors

Jing et al. (2018) reported the first family of user-friendly and broadly applicable genetically encoded GPCR activation-based ACh (GRAB_{ACh}) sensors by incorporating cpGFP into the third intracellular loop of human muscarinic receptors, which minimizes downstream G protein- and β -arrestin-coupled signaling (Wang et al., 2018). The same strategy has worked well in engineering GPCR-based sensors for DA, NE, and 5HT (Sun et al., 2018; Feng et al., 2019; Wan et al., 2020), indicating a generalizable approach. A more recently reported GRAB_{ACh3.0} sensor had a fluorescence response $\Delta F/F \sim 180\%$ larger than that of previous versions, which enhanced its ability to detect miniscule cholinergic signals (Jing et al., 2020).

Borden et al. (2020) created the other family of genetically encoded ACh sensors, iAChSnFRs, based on a bacterial choline-binding protein. cpGFP was inserted into the choline-binding protein at positions determined by quantitative structural analysis of local conformational changes induced by ligand binding, the same strategy developed for engineering iGluSnFRs (Marvin et al., 2013, 2018). Both green and yellow fluorescent versions of these sensors are available, and the latter is ideal for two-photon imaging.

Parameters of GPCR- and PBP-based sensors, often obtained under different experimental conditions in different laboratories, may not be comparable (Table 1). Experiments examining endogenous cholinergic transmission in the same medial entorhinal

cortical preparation with both GRAB_{ACh} and iAChSnFR sensors provided a direct comparison and revealed key differences between these two families of sensors (Borden et al., 2020; Zhu et al., 2020). GRAB_{ACh} sensors produced large fluorescence responses, yet their inherited slow kinetics prevented them from faithfully following high-frequency cholinergic signals (Jing et al., 2018, 2020). On the other hand, the slow kinetics of GRAB_{ACh} sensors might integrate ACh signals over time. This property, together with their large fluorescence responses, could make them excellent somewhat all-or-none-like binary signal detectors. iAChSnFRs rivaled GRAB_{ACh2.0} in detecting low-frequency cholinergic signals, but surpassed GRAB_{ACh2.0} in following high-frequency cholinergic signals (e.g., see Borden et al., 2020, their Fig. S25). Moreover, iAChSnFRs had a large response dynamic range, allowing further improvement of fluorescence response (Borden et al., 2020). Notably, iAChSnFRs exhibited sufficient spatial and temporal resolution to allow characterization of fundamental synaptic properties of cholinergic transmission, including transmitter diffusion extent, number of release sites, release pool size, release probability, quantal size, and refilling rate, in various preparations (Borden et al., 2020; Lin et al., 2021; Zhu et al., 2020).

NE sensors

Using the same strategy that created GRAB_{ACh} sensors, Feng et al. (2019) engineered a family of GRAB_{NE} sensors for NE, GRAB_{NE1m}, and GRAB_{NE1h}. Overall, GRAB_{NE1m} performed better in monitoring endogenous adrenergic signals, yielding larger responses and following signals more faithfully because of its larger fluorescence responses, faster kinetics, and better specificity (Table 1). A GRAB_{NE1m} minor variant, sometimes dubbed “GRAB_{NE2m}” by researchers, has almost identical properties and performance compared with GRAB_{NE1m} (Kwak et al., 2020). A few experiments compared performance of iAChSnFR and GRAB_{NE1m} in monitoring endogenous cholinergic and adrenergic transmission in the same amygdalar preparation (Zhu et al., 2020). While GRAB_{NE1m} performed worse than iAChSnFR in speed and fluorescence response, the sensor was able to follow the slower adrenergic signals (Zhu et al., 2020), raising the hope that GRAB_{NE1m} may at least resolve some synaptic features of adrenergic transmission, although the experiments could be challenging because of the small fluorescence response.

5HT sensors

In addition to GRAB_{ACh} and GRAB_{NE} sensors, Wan et al. (2020) recently introduced a family of GRAB_{5HT} sensors that are highly selective for 5HT. Both GRAB_{5HT0.5} and GRAB_{5HT1.0} effectively detected endogenous serotonergic signals in populations of cells, yet GRAB_{5HT0.5} appeared to perform better in detecting serotonergic signals at the single-cell and subcellular levels (Wan et al., 2020; Zhu et al., 2020), presumably because its kinetics and affinity match more closely to the dynamics and concentration of endogenously released 5HT (Table 1). The results also showed that GRAB_{5HT0.5} served as an excellent tool to dissect fundamental synaptic properties of serotonergic transmission (Zhu et al., 2020).

Guided by a machine-learning-based binding-pocket redesign strategy, Unger et al. (2020) recently converted an early version of iAChSnFR into an iSeroSnFR sensor for 5HT (Table 1). iSeroSnFR reported robust endogenous serotonergic signals in populations of cells (Unger et al., 2020); yet given its low sensitivity, it will be important to see whether the sensor can detect single-cell and subcellular serotonergic transmission.

DA sensors

The Sun and Patriarchi teams independently developed two families of GPCR-based DA sensors: (1) GRAB_{DA}, based on human D₂ dopamine receptors (Sun et al., 2018); and (2) dLight, based on human D₁ dopamine receptors (Patriarchi et al., 2018, 2019) (Table 1). More recently, Sun et al. (2020) reported the second generation of GRAB_{DA} sensors (Table 1). Compared with GRAB_{DA1m} and GRAB_{DA1h}, the new green versions of GRAB_{DA2m} and GRAB_{DA2h} have significantly improved $\Delta F/F$, whereas the first red versions of rGRAB_{DA1m} and rGRAB_{DA1h} have slightly improved $\Delta F/F$. Likewise, the Tian team (Patriarchi et al., 2020) reported a red version DA sensor, RdLight₁, which had similar $\Delta F/F$ compared with green dLight sensors (Table 1). All other properties of these new DA sensors remain largely unchanged (Patriarchi et al., 2020; Sun et al., 2020).

Direct experimental comparison showed that GRAB_{DA2m} produced slightly larger single-cell dopaminergic responses with a better SNR compared with dLight₁ in the striatum (Sun et al., 2020; Zhu et al., 2020). Given the extremely low baseline dLight₁ fluorescence that frequently precluded identification of individual expressing cells, GRAB_{DA2m} was handier in analyzing single-cell dopaminergic signals. On the other hand, dLight₁ appears to have slightly higher selectivity for DA over NE than GRAB_{DA2m} (Table 1). GRAB_{DA} and dLight both worked well in observing dopaminergic signals from populations of cells in the striatum, which receives the heaviest dopaminergic innervations and probably largest dopaminergic signals (Bjorklund and Dunnett, 2007; Morales and Margolis, 2017). It waits additional experimentation to determine whether GRAB_{DA} and dLight are capable of detecting modest single-cell dopaminergic signals in other brain areas, such as the PFC and hippocampus.

Practical considerations

The majority of genetically encoded neuromodulatory transmitter sensors seem to work well in various tissue preparations. A few top-performing sensors (e.g., iAChSnFR, GRAB_{NE1m}, and GRAB_{5HT0.5}) detected robust but variable single-cell responses in every *ex vivo* and *in vivo* neuronal and non-neuronal tissues examined (Feng et al., 2019; Borden et al., 2020; Wan et al., 2020; Zhu et al., 2020), supporting their applicability in general tissue preparations. Genetically encoded DA sensors could detect single-cell dopaminergic responses in the striatum *ex vivo* and *in vivo* (compare Patriarchi et al., 2018; Sun et al., 2018, 2020; Zhu et al., 2020), but their efficacy in other brain areas remains unknown.

Genetically encoded sensors are powerful tools for resolving synaptic properties of neuromodulatory transmission if properly used. Because genetically encoded sensors perform better than the previously available monitoring tools in terms of fluorescence response and targeted expression, many laboratories have already combined them with imaging approaches (e.g., photometry) to monitor cholinergic and monoaminergic signals, as advocated by recent reviews (Wang et al., 2018; Andreoni et al., 2019; Jing et al., 2019; Sabatini and Tian, 2020). However, the top-performing genetically encoded sensors possess properties that empower them to do more. In addition to being able to follow rapid transmitter signals, fortuitously and welcomingly, these sensors emit a large amount of photons upon binding of neuromodulators, and their fluorescence responses are largely independent of expression levels (Feng et al., 2019; Borden et al., 2020; Wan et al., 2020; Zhu et al., 2020). These properties, when combined with superresolution and/or deconvolution

microscopic analysis algorithms, permit dissection of the fundamental synaptic properties of neuromodulatory transmission, such as the transmitter diffusion extent, number of release sites, release pool size, release probability, quantal size, and refilling rate (Neher, 2015; Pulido and Marty, 2017). These synaptic parameters, which are essential for determining the mechanisms underlying behaviors (Henley and Wilkinson, 2016; Sudhof, 2018), could not be extracted effectively by electrophysiological recordings because neuromodulators typically produce only small and rapidly desensitizing neuromodulatory current responses (Dani and Bertrand, 2007; Nadim and Bucher, 2014). By determining these synaptic parameters, it should be possible to investigate the regulation, adaption (i.e., short- and long-term plasticity), and function of neuromodulatory transmission. Moreover, after defining the normal baseline, analyzing synaptic alterations of synapse-, subcellular-, and circuit-specific signals under disease conditions should shed new light on the neuromodulatory pathogenesis of a variety of diseases (Barbour and Hausser, 1997; Eban-Rothschild et al., 2018; Mohebi et al., 2019; Okaty et al., 2019).

Good experimental practice is essential for high spatiotemporal resolution imaging that is required to resolve synaptic properties of neuromodulatory transmission. Our experience with genetically encoded sensors highlights the importance of (1) mitigation and correction for image drift and fluctuations; (2) minimization of photobleaching, autofluorescence, and noise; and (3) optimization of light diffraction correction. Adapting stable recording systems, such as electrophysiological setups designed for *ex vivo* and *in vivo* multiple patch-clamp recordings (Zhu, 2009; Jiang et al., 2013; Zhang et al., 2018), can mitigate experimental drifts and fluctuation, and thus allow acquisition of stable living-cell images easily correctable with an intensity-based registration function (Wang et al., 2015a; Zhu et al., 2020). Running optimized correction algorithms can minimize photobleaching, autofluorescence, and noise (Zhu et al., 2020). Getting the accurate point spread function and using optimized analysis algorithms are vital for reversing optical diffraction to “reassign” the light to its original place (Sibarita, 2005; Guo et al., 2020).

Our experiments have shown that iAChSnFR, GRAB_{NE1m}, and GRAB_{5HT0.5} have the essential qualities required for high spatiotemporal resolution image analysis of endogenously released neuromodulators, and they can resolve synaptic properties of neuromodulatory transmission (Feng et al., 2019; Borden et al., 2020; Wan et al., 2020; Zhu et al., 2020); but the performance of several other sensors requires further evaluation. Fortunately, all genetically encoded neuromodulatory transmitter sensors come from a few rigorous research groups. Thus, one may roughly deduce the relative performance of sensors generated from the same groups based on their reported sensor specificities (e.g., sensitivity, $\Delta F/F$, SNR, and kinetics) (Table 1).

Various expression methods have helped in the engineering, validation, and application of genetically encoded transmitter sensors (Fig. 1B). Transfection provides a quick and convenient method for expression as genetically encoded sensors are being developed. Transfected cultured non-neuronal cells and neurons are perfect for quickly screening and rating properties of genetically encoded sensor variants. However, the best-performing sensor variants in cultured cells may not be the top performers in tissue slice and intact brain preparations. Therefore, viral expression in *ex vivo* and *in vivo* preparations is indispensable for screening and ranking the best-performing sensor variants, and subsequently validating their application. Three viral

expression systems, including adeno-associated virus (AAV), lenti, and Sindbis viruses, are routinely used in screening and validating genetically encoded sensors. Several serotypes of AAV viruses have been extensively used because of their minimal toxicity, persistent expression (≥ 6 months), and possibility for non-invasive delivery (Samulski and Muzyczka, 2014; Wang et al., 2019); these features are particularly suitable for long-term physiological and behavioral tests. However, the low viral production efficiency and slow expression time (≥ 3 weeks) of AAV preclude its use in screening sensor variants, and the small payload for a transgene (up to ~ 4.5 –5 kb) limits its applications in coexpression with other transgenes (Samulski and Muzyczka, 2014; Wang et al., 2019). Although the rapid serotypes of AAV viruses could drive expression within a week, they come with the price of increased cell toxicity (Jing et al., 2018). Lentiviruses have relatively faster production, shorter expression time (~ 1 –2 weeks), and long-lived expression (multiple months) (Matrai et al., 2010; Alfranca et al., 2018), making them excellent choices in sensor screening and validation experiments. Lentiviruses allow expression of a transgene of up to ~ 8 –10 kb (Matrai et al., 2010; Lim et al., 2017; Alfranca et al., 2018), permitting coexpression with other transgenes and/or knockdown siRNAs. As with AAVs, modifications of envelope components, regulatory elements, and administration routes allow lentiviruses to achieve targeting expression in various types of cells and tissues (Borden et al., 2020; Zhu et al., 2020). Sindbis virus has the most efficient production time (~ 1.5 d), shortest expression time (~ 8 –16 h), and highest expression levels (particularly for membrane proteins), but it has a relatively short viable expression time (~ 3 –5 d) (Malinow et al., 2010; Uyaniker et al., 2019). These characteristics make Sindbis virus perfect for rapid screening of potentially workable sensors and validation of their properties. We note that the Sindbis-driven high sensor expression can be particularly advantageous for less-than-optimal sensors (e.g., GRAB_{NE1m} and dLight₁) to achieve high-resolution image analysis (Feng et al., 2019; Zhu et al., 2020). In addition, the large Sindbis payload of up to 15 kb enables combination with additional genetic and imaging applications (Wang et al., 2015b; Zhang et al., 2018). Although Sindbis has a strong neurotropism toward excitatory neurons, local administration permits expression in GABAergic interneurons and non-neuronal cells (Jing et al., 2018; Zhu et al., 2020). Importantly, viral expression can be used with cre mice to achieve more controllable expression of genetically encoded sensors (Borden et al., 2020; Unger et al., 2020). In addition, transgenic mice expressing genetically encoded neuromodulatory transmitter sensors are being created, which will add additional convenience to the sensors' applications.

Biological applications

A substantial number of recently published “proof-of-principle” tools or methods failed to work for biology because they were not rigorously validated (e.g., see Meister, 2016; Wang et al., 2020). Several genetically encoded neurotransmitter sensors have gone through rigorous validation that demonstrates their applicability in resolving a few fundamental biological questions (Jing et al., 2018; Borden et al., 2020; Zhu et al., 2020). For example, the new sensors delivered a direct answer to the long-standing neurobiological question of whether the primary neuromodulatory transmission mode is restricted or volume transmission (Barbour and Hausser, 1997; Sarter et al., 2009). The dominant theory for neuromodulatory transmission proposed three decades ago postulated that

the primary mode of intercellular neuromodulatory communication is volume transmission among groups of cells within a region, rather than restricted transmission between specific cells that form direct circuits or contacts (Agnati et al., 1992; Zoli et al., 1999). Specifically, the volume transmission model purports that ACh and monoamines diffuse into local areas, affecting many different types of nearby cells, and that neuropeptides travel even farther, influencing both local cells and cells millimeters away (Agnati et al., 1992; Borroto-Escuela et al., 2018). However, this theory is based primarily on the postulation that endogenously released neuromodulatory transmitters behave similarly to exogenously applied ones (that diffuse more freely in the extrasynaptic space), an assumption that has not yet been corroborated by direct experimental evidence (Barbour and Hausser, 1997; Sarter et al., 2009). Because previous imaging techniques did not allow direct visualization of transmitter release and diffusion at individual release sites (Sykova and Nicholson, 2008; Zheng et al., 2017), some researchers had ingeniously used mathematical models to simulate the evoked releases. Although this approach could not distinguish single and multiple closely packed release sites, it gave decent estimations of neuromodulatory transmitter spread areas with radius of ~ 5.0 – $10.0\ \mu\text{m}$ (Beckstead et al., 2004; Courtney and Ford, 2014, 2016). Unfortunately, these studies were underappreciated, due presumably to their indirect calculation approaches and/or their dependence on simulation assumptions. Genetically encoded neuromodulatory transmitter sensors, in combination with superresolution (Sauer, 2013; Li and Vaughan, 2018) and/or deconvolution (Arigovindan et al., 2013; Weigert et al., 2018) microscopic analysis strategies (Fig. 1C), permitted the first visualization of spatial diffusion of neuromodulatory transmitters at individual release sites (Fig. 1D). The analysis yielded precise diffusion spread length constants of $\sim 0.75\ \mu\text{m}$ for both ACh and monoamines (Zhu et al., 2020), with the cholinergic spread length constant independently verified in the intact brain (Borden et al., 2020). These results indicate that highly restricted, nonvolume neuromodulatory transmission is a key mode for intercellular communication.

The finding of restricted neuromodulatory transmission has multiple implications. Like several other transmission parameters, such as the amount of released transmitter, width of synaptic clefts, and location of postsynaptic transmitter receptors across various synapses (Savtchenko and Rusakov, 2007; Savtchenko et al., 2013; Haas et al., 2018), spatial spread length constants of neurotransmitters (i.e., glutamate, ACh, monoamines, and neuropeptides) are almost identical across various cell types (Lin et al., 2021; Zhu et al., 2020). These results support the idea that synapses optimize their nanoscale presynaptic and postsynaptic organizational elements to maximize efficacy and precision.

The restricted diffusion of released neuromodulators also indicates that neuromodulatory transmission resembles fast-acting glutamatergic and GABAergic transmission in fine spatial regulation. Interestingly, as with the fast-acting transmitters glutamate (e.g., via NMDA receptors) (Rusakov et al., 1999) and GABA (e.g., via δ subunit-containing GABA_A receptors) (Brickley and Mody, 2012), neuromodulatory transmitters may likewise engage high-affinity receptors (Kellar et al., 1985; Dani and Bertrand, 2007) and/or large clusters of release sites to achieve certain volume transmission effects under physiological and pathologic conditions. Thus, the previous results, together with new imaging findings (Barbour and Hausser, 1997; Jensen et al., 2019; Zhu et

al., 2020), support an emerging concept that a key mode of intercellular communication for both fast-acting (i.e., glutamate and GABA) and slow-acting neurotransmitters is highly restricted transmission, and this mode is complemented by volume transmission under certain conditions.

Some genetically encoded neurotransmitter sensors exhibit both fast kinetics and photostability that permit stable measurements of synaptic responses with high temporal resolution, providing an opportunity to improve quantitative understanding of neuromodulatory synaptic properties (Neher, 2015; Thanawala and Regehr, 2016). Traditionally, patch-clamp recordings, which make reliable measurements of excitatory and inhibitory currents after high-frequency activation, serve as the prime method to define synaptic properties of glutamatergic and GABAergic transmission (Elmqvist and Quastel, 1965; Schneggenburger et al., 1999; Ruiz et al., 2011; Thanawala and Regehr, 2013). However, small and rapidly desensitizing neuromodulatory currents make electrophysiology an ineffective approach to determine synaptic parameters of neuromodulatory transmission. Some genetically encoded transmitter sensors possess kinetics fast enough to follow slow neuromodulatory synaptic releases evoked by moderate (actually, more physiological) rates of stimulation over a prolonged period (Borden et al., 2020; Lin et al., 2021; Zhu et al., 2020). This should permit reasonably accurate estimation of neuromodulatory synaptic properties (e.g., number of release sites, release pool size, release probability, quantal size, and refilling rate) (Neher, 2015; Pulido and Marty, 2017). Our preliminary analysis indicated that neuromodulatory transmission at times acted very differently from classic fast glutamatergic and GABAergic transmission (Borden et al., 2020; Zhu et al., 2020), inspiring new ideas about neuromodulatory engagements in various behaviors and diseases (see Discussion below).

Clinical applications in Alzheimer's disease, sleeping disorders, and tumorigenesis

New insights into neuromodulatory transmission explain some perplexing clinical observations and suggest novel potential therapeutic interventions for various neurologic disorders, including Alzheimer's disease (Fig. 2). For example, the only available therapy for Alzheimer's disease is based on the finding of diminished ACh release and deteriorating cholinergic neurons in Alzheimer's brains: the cholinergic hypothesis (Mash et al., 1985). Currently, all FDA-approved Alzheimer's drugs directly or indirectly inhibit acetylcholinesterase (AChE) to boost cholinergic signals. These medicines have limited efficacy in cognitive improvement, and on medication termination, induce irreversible, accelerated deterioration (Zemek et al., 2014; Ashford, 2015). The unexpected fine regulation of cholinergic transmission illustrated by genetically encoded sensors sheds light on these two clinical observations (Borden et al., 2020; Zhu et al., 2020). First, AChE inhibitors might reduce the physiological precision of cholinergic transmission (Barbour and Hausser, 1997; Sarter et al., 2009), explaining the limited cognitive improvement. Second, long-term application of AChE inhibitors might homeostatically upregulate AChE levels in Alzheimer's patients and/or downregulate presynaptic ACh release (Ashford, 2015), explaining the accelerated deterioration upon medication termination.

Like disrupted cholinergic signaling, dysregulated adrenergic transmission also appears as an early pathologic correlate of cognitive decline in Alzheimer's disease (Mather and Harley, 2016; Simic et al., 2017). Genetically encoded sensors

revealed unexpected adrenergic synaptic properties that seem to run counter to the natural tendency of synapses to achieve fine-tuned linear input-output computational processes (Zhu et al., 2020). These properties distinguish adrenergic transmission from all other neuronal transmission, including the fast glutamatergic and GABAergic transmission (Jackman and Regehr, 2017) as well as cholinergic and other monoaminergic transmissions (Zhu et al., 2020). The findings explain the unique involvement of adrenergic transmission in fine-tuning of attention (Carter et al., 2010), optimization of behavior in complex social and physical environments (Gompf et al., 2010), and deficits in complex mental processes (e.g., reasoning and abstract thinking) in Alzheimer's patients (McKhann et al., 2011). The new understanding of cholinergic and adrenergic transmission would immediately suggest multiple synaptic mechanisms as potentially effective targets for intervention and set the physiological transmission reference for future medication testing and development.

Genetically encoded sensors may help to solve the mystery of sleep, and such understanding should suggest effective treatments for sleep disorders. Sleep is one of the most mysterious yet ubiquitous animal behaviors, and sleep disorders are among the most common clinical problems, causing a number of health issues that range from cognitive and immune deficiencies to cardiovascular diseases and obesity (Weber and Dan, 2016; Scammell et al., 2017; Eban-Rothschild et al., 2018). Many factors modulate sleep-wake behaviors, but ACh and monoamines play complex and central roles (Fig. 2). While experimental data consistently show that cholinergic nuclei have important roles in initiating and maintaining wakefulness (Buzsaki et al., 1988; Fuller et al., 2011), existing evidence is inconclusive as to whether cholinergic neurons are necessary for wakefulness (Scammell et al., 2017; Eban-Rothschild et al., 2018). Genetically encoded ACh sensors illustrated that neuronal activity could induce large initial and small sustained ACh releases (Jing et al., 2018; Borden et al., 2020; Zhu et al., 2020), providing a biophysical base for cholinergic involvement in initiation and maintenance of wakefulness. It was proposed that combining optogenetic and chemogenetic techniques with new monitoring tools, which allow direct assessment of cholinergic releases at natural sleep-wake cycles (e.g., genetically encoded ACh sensors), might delineate the exact cholinergic contributions in sleep-wake behaviors (Scammell et al., 2017). Unlike the cholinergic nuclei, loss of locus coeruleus cells did not stop wakefulness but instead impaired wakefulness under socially and physically complex environments (Gompf et al., 2010). Visualization of adrenergic transmission with genetically encoded NE sensors illustrated that adrenergic synapses operated in a linear computational mode (Borden et al., 2020; Zhu et al., 2020), perfect for the possible adrenergic role of finely tuning wakefulness and attention (Carter et al., 2010). Making linear signal amplification, which runs against the natural tendency of synapses that weakens during activation (Jackman and Regehr, 2017), renders adrenergic transmission to be vulnerable to system runaway. To avoid such risk, adrenergic synapses seemed to set a small release pool and a tiny refill rate to ensure transmitter

Figure 2. Genetically encoded sensors hold the key to unlock secrets of behaviors and diseases. Genetically encoded sensors, which permit high spatiotemporal resolution imaging of neuromodulatory transmission in synapse-, subcellular-, and/or circuit-specific manners under various healthy and diseased conditions, hold the key to unlock the regulatory or pathogenic mechanisms of a variety of behaviors and diseases.

depletion after a certain amount of neuronal activity (Borden et al., 2020; Zhu et al., 2020), creating an emergency break point presumably responsible for the observed behavioral arrests (Carter et al., 2010). A comprehensive analysis of adrenergic synaptic properties with genetically encoded NE sensors should define adrenergic roles in sleep-wake cycles, and unveil the pathogenesis of behavioral arrests and sleep disorders.

Serotonergic and dopaminergic mechanisms in regulating sleep-wake cycles remain elusive. Early research suggested that 5HT might initiate and maintain sleep, whereas later work found that serotonergic neurons promoted wakefulness (Scammell et al., 2017; Eban-Rothschild et al., 2018). Because serotonergic signals regulate a variety of physiological processes, whether the observed serotonergic effects on sleep-wake cycles are original or secondary is still unclear. Genetically encoded 5HT sensors may connect synaptic dynamics of serotonergic transmission with specific sleep-wake events, and thus define the precise serotonergic roles in sleep-wake behaviors (Wan et al., 2020). Dopaminergic neurons act as key modulators of sleep and wakefulness. Sleep, like food, water, and sex, appears to serve as a primary reinforcer, and existing experimental data indicate that dopaminergic neuronal activity and extracellular DA levels correlate with circadian oscillations and sleep-orienting behaviors (Korshunov et al., 2017; Rial et al., 2018). However, whether and how DA regulates, and/or is regulated by, the circadian clock and other sleep-wake regulators remain mysterious because of modest and varied DA release changes in the midbrain, hypothalamus, and other related brain areas over the sleep-wake cycle. This underlines the significance of monitoring cell type- and projection-specific dopaminergic contributions to sleep-wake regulation (Scammell et al., 2017; Eban-Rothschild et al., 2018). Genetically encoded DA sensors have fast kinetics to track synaptic dopaminergic signals (Patriarchi et al., 2018; Sun et al., 2018, 2020) and permit

visualization of dopaminergic transmission with the high spatial resolution (Zhu et al., 2020). Given the modest DA release associated with sleep-wake cycles (Eban-Rothschild et al., 2018), the next generation of DA sensors with improved fluorescence responses may aid synapse-, subcellular-, and circuit-specific dissection of dopaminergic contributions to sleep-wake behaviors.

Genetically encoded sensors can advance understanding and treatment of cancer because recent studies recognize a major neuromodulatory contribution to cancer development and metastasis (Servick, 2019; Zahalka and Frenette, 2020), and revive an ancient idea that perineuronal invasion processes are central for tumor cells to gain access to migratory routes and pro-survival components (Ernst, 1905). In particular, there is now good evidence indicating that adrenergic fibers promote cancer cell survival during the initial stage of cancer development via adrenergic receptors, while cholinergic fibers contribute to later phases of cancer invasion (i.e., migration and metastases) (Magnon et al., 2013; Mauffrey et al., 2019). Similarly, dopaminergic and serotonergic inputs play a role in tumor proliferation and dissemination (Jiang et al., 2020). These findings rendered surgical or chemical denervation and pharmacological inhibition of neuromodulation promising antitumor therapeutic options. However, preclinical and clinical trials reported conflicting results of these treatments in controlling tumor development (Jiang et al., 2020; March et al., 2020), underscoring the need for decoding neuromodulatory roles in multiple biological behaviors of cancers, such as cancer cell survival, proliferation, resistance to apoptosis, invasion, metastasis, angiogenesis, and stromal cell transition in tumor initiation and progression. Recently developed genetically encoded neuromodulatory transmitter sensors provide viable tools for direct visualization of neuromodulatory communications among nerve, cancer, immune, and/or endothelial cells during tumor proliferation and dissemination. This line of research is likely to lead to better understanding of tumorigenesis and effective neuromodulation-based antitumor therapies.

In conclusion, the impressive amount of prior work on neuromodulation has underscored the importance of neuromodulatory transmission in various behaviors and diseases, yet our understanding of the regulation and properties of neuromodulatory transmission remains primitive because of limitations of available tools that can quantitatively analyze the transmission. Recently engineered genetically encoded neuromodulatory transmitter sensors power up our toolbox with a set of tools that allow quantification of synaptic parameters of neuromodulatory transmission. Because qualifying synaptic properties of neurotransmission is essential for understanding the physiology and pathology of behaviors and diseases (Bayes et al., 2011; Volk et al., 2015; Henley and Wilkinson, 2016; Sudhof, 2018), we expect these new sensors to rapidly change the landscape of neuromodulation research.

Indeed, initial experimental work validates that genetically encoded sensors, when combined with superresolution and deconvolution microscopic analysis, can decode the fundamental synaptic properties of neuromodulatory transmission (Borden et al., 2020; Lin et al., 2021; Zhu et al., 2020). It is important to note that both high-performing sensors and good practical solutions are critical for the success of biological applications (Lin et al., 2021; Zhu et al., 2020). We discussed how genetically encoded neuromodulatory transmitter sensors might advance our understanding of Alzheimer's disease, sleep-wake cycle and related sleep disorders, and tumorigenesis. The

applications of genetically encoded sensors certainly do not stop here since ACh and monoamines play critical roles in a wide range of behaviors and diseases, including addiction, autism, cardiovascular regulation and diseases, mood disorders, schizophrenia, immune regulation and deficiency, metabolic regulation, and eating disorders (Fig. 2). Therefore, we expect that combining newly developed genetically encoded sensors with superresolution and deconvolution microscopic analysis algorithms will shed new light on the physiology or pathology of a large variety of behaviors and diseases.

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