Protein-based tools for studying neuromodulation

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ABSTRACT: Neuromodulators play crucial roles in regulating neuronal activity and affecting various aspects of brain functions, including learning, memory, cognitive functions, emotional states and pain modulation. In this account, we describe our group's efforts in designing sensors and tools for studying neuromodulation. Our lab focuses on developing new classes of integrators that can detect neuromodulators across the whole brain while leaving a mark for further imaging analysis at high spatial resolution. Our lab also designed chemical- and light-dependent protein switches for controlling peptide activity to potentially modulate the endogenous receptors of the neuromodulatory system in order to study the causal effects of selective neuronal pathways.

1. INTRODUCTION

Neurotransmitters control brain activity and functions by relaying signals across neurons through both fast and slow transmissions[1]. Common fast-acting neurotransmitters, such as glutamate and Gamma-aminobutyric acid (GABA), act on ligand-gated ion channels at the synapse within a millisecond[1]. Slow-acting neurotransmitters, also known as neuromodulators, can cause effects on the order of milliseconds to minutes to modulate the firing ability of the neurons[1]. More than one-hundred neuromodulators have been identified, including amino acids, monoamines, neuropeptides, and small signaling molecules[9]. Most of the neuromodulators target G protein-coupled receptors (GPCRs), which can cause both immediate and long-term changes of the synaptic activity and plasticity[1, 9].

Neuromodulators play crucial roles in regulating neuronal activity. Hence, many neurological disorders are associated with the mis-regulation of neuromodulators, including Parkinson's Disease, Schizophrenia, addiction and Alzheimer's Disease[10-12]. Many drugs have been designed to target the endogenous neuromodulatory system, demonstrating the importance of understanding this complex system. However, many questions remain regarding the regulation and transmission of the neuromodulators and their effects on behaviors.

When neurons undergo depolarization, the majority of neurotransmitters are released into the synaptic cleft from synaptic vesicles through a calcium-dependent mechanism. In contrast, neuropeptides are released from dense core vesicles near the synapse in a depolarization-induced, calcium-dependent manner. Following their release, fast-acting neurotransmitters are cleared from the synaptic cleft immediately via reuptake or enzymatic degradation mechanisms, while slow-acting neuromodulators can diffuse away and affect a large volume, causing volume transmission.

Bioanalytical methods including microdialysis[13] and fast-scan cyclic voltammetry[14] have enabled the detection of neuromodulators, but only in bulk brain volume, lacking cellular resolution. Recent development of genetically encoded fluorescent sensors for real-time detection of neuromodulators[14-18], along with microscopic imaging and analysis technology advancement, have significantly facilitated the investigation of neuromodulator release and transmission in live animals with cellular resolution. Further, genetically encoded integration sensors (abbreviated as integrators hereon) have also been designed to detect neuromodulators and leave a permanent mark on the neurons, detecting neuromodulators with cellular resolution across large brain volumes. While a large panel of real-time sensors are available and have been transformative, there are a limited number of integrators available for detecting neuromodulators. Our lab focuses on designing genetically encoded sensors to record the cellular location of the neuromodulators

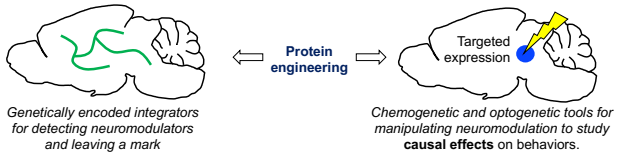

Figure 1: Our group focuses on designing genetically encoded integrators as well as chemogenetic and optogenetic tools for studying neuromodulation.

across a large brain volume to study their release and transmission.

While neuromodulator detection provides correlation of neuromodulator signaling and transmission to behaviors, there is also a need for tools that can manipulate the endogenous receptors in selective neuronal populations to study their causal effects in various physiological processes and animal behaviors. Our lab also designs genetically encoded

chemogenetic and optogenetic protein switches to enable the modulation of endogenous GPCR activity in a defined neuronal population and the study of their functional roles.

2. SENSORS FOR NEUROMODULATORS

Studying when and where neuromodulators are released and transmitted is crucial for a better understanding of how they exert their effects on targeted neuronal populations[19, 20].

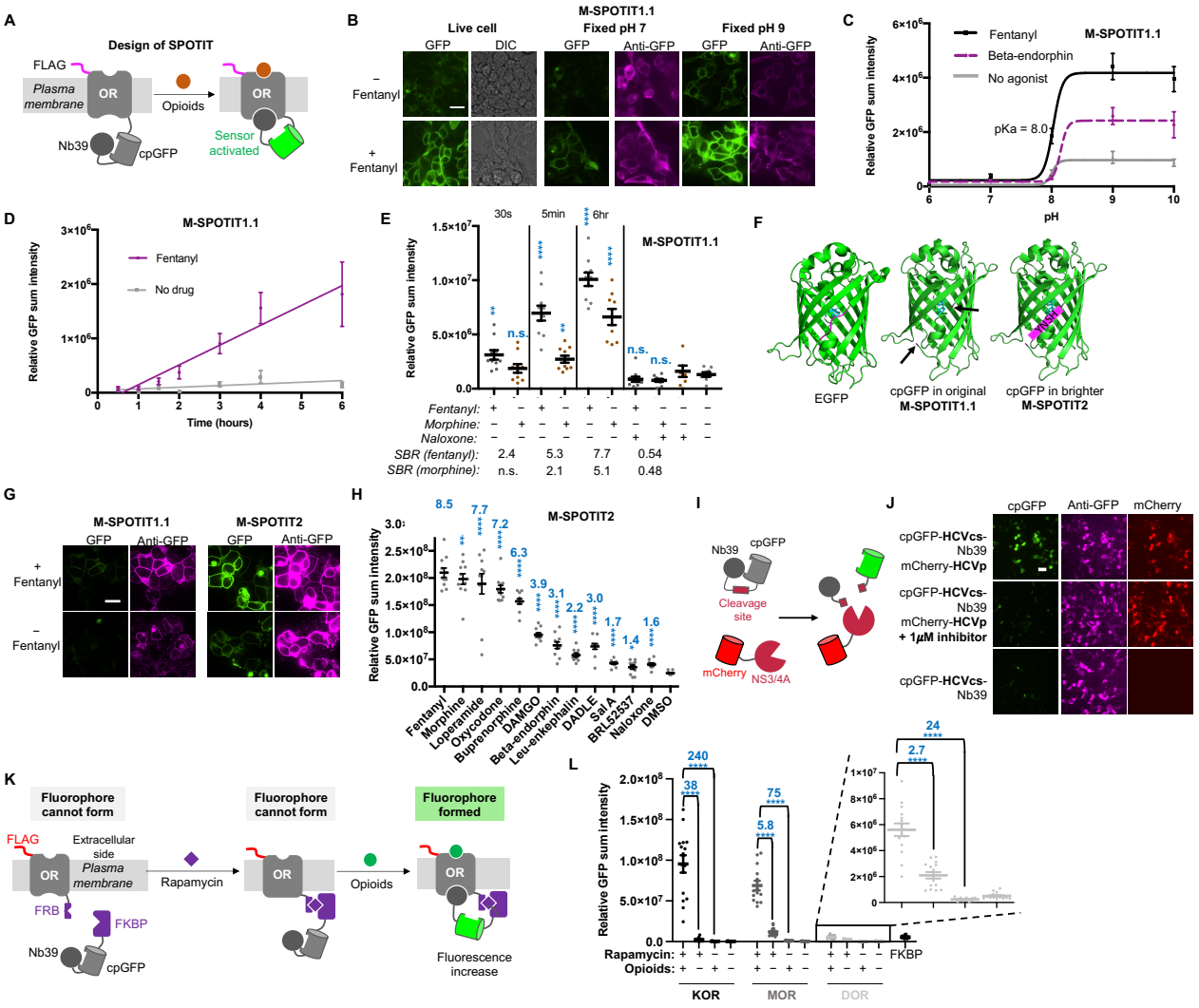

Figure 2: (A) SPOTIT design. **(B)** M-SPOTIT1.1 under different imaging conditions. Two days after infection with lentiviruses expressing SPOTIT1.1, cells were stimulated with 10 μM fentanyl for 24 hours. Cells were imaged either live or fixed at pH 7 or 9, respectively. Scale bar, 20 μm. **(C)** pH titration analysis of M-SPOTIT1.1 stimulated with 10 μM fentanyl or beta-endorphin in fixed HEK293T cells. **(D)** Characterization of M-SPOTIT1.1 maturation time dependence. **(E)** Characterization of M-SPOTIT1.1 agonist exposure time dependence. M-SPOTIT1.1 was exposed to 10 μ fentanyl and DAMGO for different times before the agonists were washed away. Cells were imaged while fixed at pH 9, 24 hours post-stimulation. The MOR antagonist naloxone, was added after 30 s-stimulation to test the inhibition of sensor maturation. SBR was calculated by dividing the sum GFP intensity against that in the no agonist condition. **(F)** Comparison of cpGFP in M-SPOTIT1.1 and 2. **(G)** M-SPOTIT2 is much brighter than M-SPOTIT1.1 in HEK293T cells. **(H)** Characterization of M-SPOTIT2 against a variety of ligands. **(I)** Schematic of SPOTon for protease activity detection. **(J)** HEK293T cell imaging characterization of the SPOTon reporter for detecting HCVp protease activity. 1 μM of the HCVp inhibitor BILN-2061 was used. **(K)** Schematic and **(L)** Analysis of SPOTon for opioid detection with chemical-gated temporal control based on KOR, MOR and DOR. Scale bars, 20 μm. Panel A-E were reproduced with permission from ref. 3. Copyright 2021 Elsevier. Panel F-H G were reproduced with permission from ref. 7. Copyright 2021 Royal Society of Chemistry. Panel I-L were reproduced with permission from ref. 8. Copyright 2022 American Chemical Society.

In this account, we will discuss our group's work in designing genetically encoded integrators and both chemogenetic and optogenetic tools for studying neuromodulation (**Fig. 1**). We have designed new classes of integrators and tools that offer enhanced capabilities in dissecting the neuromodulatory system. The integrators and tools presented here can be combined with other sensors and tools to further explore and investigate the complexities of the neuromodulatory system.

Bioanalytical methods such as microdialysis and fast-scan cyclic voltammetry (FSCV) are frequently used to detect neuromodulators in live animals. Microdialysis is used to extract interstitial tissue fluid from live animals, which can be further analyzed using mass spectrometry[13, 21]. Microdialysis can detect many neuromodulators at one time

and offers temporal resolution on the order of minutes. In FSCV, a microelectrode is implanted in the brain tissue to monitor the neuromodulator release and transmission. FSCV allows for real-time monitoring of neuromodulators, including dopamine, serotonin and dynorphin with sub-second kinetics[22, 23]. Unfortunately, neither FSCV nor microdialysis methods can provide cellular resolution across a brain region.

Genetically encoded fluorescent sensors can be expressed in the brain and provide high spatial resolution when analyzed by fluorescence imaging. Genetically encoded fluorescent sensors can be largely grouped into real-time sensors and integrators. Real-time sensors have been successfully designed to detect various neuromodulators, including dopamine[15, 16], serotonin[17], oxytocin[24, 25], norepinephrine[26], and neuropeptides[27]. These real-time sensors have significantly facilitated the study of neuromodulation by enabling monitoring of neuromodulation dynamics at high temporal and spatial resolution. However, detection by real-time sensors is often limited to a small brain region at a time in large vertebrate animals. Since many neuromodulators can transmit a long distance from their release sites, large brain volume detection is desired. Additionally, real-time sensors do not allow further analysis or manipulation of the identified group of neurons.

In contrast, integrators can detect neuromodulators across a large brain volume in live animals, leaving a fluorescent mark to enable detection for imaging analysis at high spatial resolution post-mortem. This permanent mark allows for further molecular and functional analysis to enable comprehensive understanding of neuromodulator's effects brain-wide. However, integrators cannot monitor the kinetics of the neuromodulator release and transmission. Therefore, real-time sensors and integrators complement each other and can be applied together to study different aspects of the neuromodulatory system.

Our lab focuses on the design of integrators for detecting neuromodulators. When we started in 2018, there were limited integrators available for detecting neuromodulators, primarily the sensors TANGO[28-30], iTANGO[31] and SPARK[32-36]. All of these integrators rely on protein-protein interaction (PPI)-dependent protease cleavage to activate a transcription factor, providing versatile transcriptional reporter readouts. While TANGO has been widely applied in high-throughput GPCR ligand screening[30] and successfully demonstrated trans-synaptic tracing in *Drosophila*[28], it does not have a temporal control for detecting neuromodulators. iTANGO and SPARK both have light-dependent temporal control to record neuromodulator activity within a specific time window. Among these integrators, only iTANGO has been applied in vertebrate animal brain. Therefore, there is a need for further development of integrators for detecting neuromodulators. My lab has so far designed two new classes of molecular integrators, which have the potential to be applicable for large brain volume detection in live animals, followed by post-mortem analysis[3, 5, 7, 8, 35-37].

Single-chain fluorescence integration sensors for detecting opioids. We started by engineering fluorescence integrators for detecting opioids due to the prevalence of opioid signaling in the animal nervous system and its important roles in various physiological processes, including pain, addiction, respiration and sleep[12]. We designed SPOTIT: Single-chain Protein-based Opioid Transmission Indicator Tool[3, 7]. SPOTIT contains a new sensor motif that is composed of the circularly permuted green fluorescent protein (cpGFP) and the nanobody Nb39 (**Fig. 2A**)[3, 7]. Nb39 was originally designed to stabilize the agonist-bound conformational state of the opioid receptor (OR)[38], but was found to inhibit the fluorophore maturation of cpGFP intramolecularly. In the presence of opioids, the agonist-activated OR interacts with Nb39, which removes Nb39 from cpGFP, allowing the cpGFP fluorophore to mature and become fluorescent.

We designed SPOTIT based on mu, kappa and delta OR (MOR, KOR and DOR), abbreviated as M-, K- and D-SPOTIT. M- and K-SPOTIT show a signal-to-background ratio (SBR) of ~2-10-fold. D-SPOTIT shows high background signal even in the absence of opioids, possibly due to the high basal activity of DOR for Nb39. Importantly, we truncated the C-terminal portion of the OR tail that is critical for activity-dependent phosphorylation, preventing sensor internalization and degradation.

Since MOR is the dominant target for opioid-based pain medications, we focused our characterizations on M-SPOTIT. The first version of M-SPOTIT is called M-SPOTIT1.1. As shown in **Fig. 2B**[3], the fluorophore formed in M-SPOTIT1.1 is bright when imaged in live cells. However, the fluorescence becomes much dimmer when fixed in formaldehyde. pH titration reveals the pKa of the fluorophore in fixed tissues to be 8.0 (**Fig. 2C**)[3]. Since the fluorophore is much more fluorescent in the deprotonated state, we usually image M-SPOTIT1.1 in fixed tissues in buffers of pH > 9 to ensure the majority of cpGFP fluorophore is deprotonated.

Since fluorophore maturation involves irreversible chemical transformations, SPOTIT activation is therefore irreversible and acts as an integrator for opioid transmission. Due to the fluorophore maturation mechanism, M-SPOTIT1.1 activation takes hours (**Fig. 2D**)[3]. However, short pulses of opioid incubation (30 seconds to 5 minutes) are sufficient for detecting potent agonists since stable agonist-bound MOR-Nb39 complex is formed (**Fig. 2E**)[3].

To increase the brightness of M-SPOTIT1.1, we designed SPOTIT2 by introducing four amino acids, YNSH, which are missing in cpGFP but play an important role in enclosing the fluorophore in the beta barrel (**Fig. 2F**)[7]. M-SPOTIT2 is much brighter than M-SPOTIT1.1 (**Fig. 2G**) and is selective for MOR agonists, showing low activation with antagonist naloxone or KOR agonists (**Fig. 2H**)[3]. M-SPOTIT2 also shows significant activity for DOR agonists, unsurprisingly, as it was known that these DOR agonists can also activate MOR.

The new sensor motif, cpGFP-Nb39, is highly versatile and can be used to detect protease activity and PPIs[8]. A protease cleavage site was introduced in between cpGFP and Nb39 (**Fig. 2I**)[8] so that protease cleavage will remove Nb39 from proximity, allowing cpGFP fluorophore maturation and fluorescence increase (**Fig. 2J**)[8]. This suggests the interaction between Nb39 and cpGFP is weak and requires Nb39 to be in proximity.

To harness the sensor's modularity, we split SPOTIT into two parts: the GPCR part and the cpGFP-Nb39 part. The reconstitution of SPOTIT requires a high-affinity PPI to bring the cpGFP-Nb39 to the proximity of the GPCR.

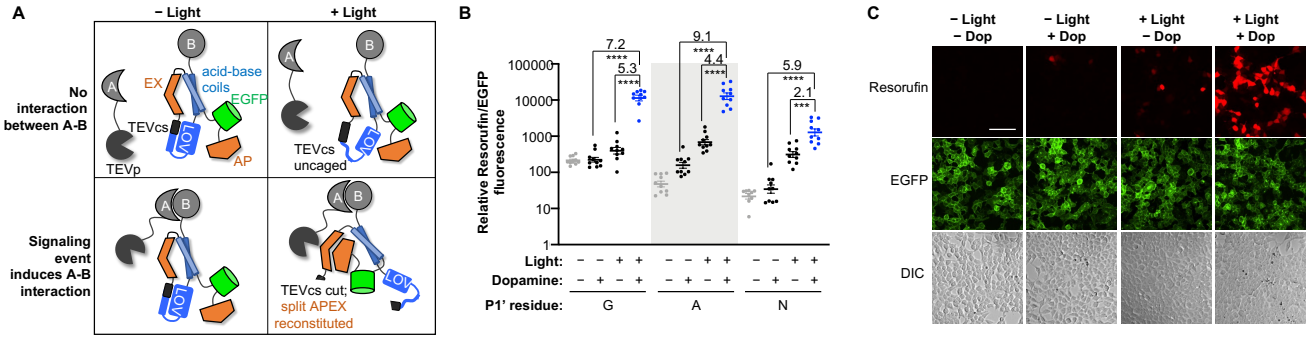

Figure 3: (A) Design of LiPPI-CLAPon1.0 for detecting PPIs with light gating. LOV, light, oxygen and voltage sensing domain. **(B)** Dot plot analysis of LiPPI-CLAPon with different TEVcs (ENLYFQ | X, X = G, A and N). **(C)** Representative fluorescent images of LiPPI-CLAPon1.0 for detecting DRD1-arrestin interaction with Amplex UltraRed labeling. TEVcs ENLYFQA and fast TEVp were used. 10 min stimulation. Dop, dopamine. Scale bar, 100 μm. Reproduced with permission from ref. 5. Copyright 2022 American Chemical Society.

Without this PPI, the cpGFP-Nb39 will not be recruited to the GPCR, because the interaction between Nb39 and agonist-activated MOR is too weak, with a K_D of 0.1-1 μM[39]. We demonstrated that a rapamycin-dependent FKBP-FRB interaction could be used to reconstitute SPOTIT (**Fig. 2K**), and both rapamycin and opioids are therefore required for SPOTIT activation[8]. The rapamycin-dependent reconstitution of SPOTIT (**Fig. 2K, L**) could potentially provide a chemical-dependent temporal control for SPOTIT detection of opioids, which still needs to be tested in animal models.

In summary, the newly discovered cpGFP-Nb39 sensor is highly modular and enabled the design of SPOTIT for detecting opioids. The sensor platform could potentially be extended to detect other neuromodulators.[40] SPOTIT are the first single-chain fluorescent integrators and have the potential to allow robust applications in animal models.

Light- and PPI-dependent integrators for detecting neuromodulators. Fast temporal gating in integrators is desired to label neuromodulation events within a short time window during specific behavioral context. Light- and PPI-gated integrators such as SPARK[32-34] and iTANGO[31] provide fast time gating and versatile transcriptional readout for detecting various signaling events. The transcriptional readout even allows further manipulation of the labeled cells. However, the >12 hour delay between the signaling event detection and reporter protein expression makes transcriptional reporters unsuitable for terminal surgery behavioral experiments.

To address the limitations of transcriptional reporters, we designed a new class of integrator, a light- and PPI-dependent enzymatic reporter, called LiPPI-CLAPon (**Li**ght- and **PPI**-dependent **C**leavage of **L**eucine zipper-caged **AP**EX for Protease detecti**ON**)[5]. The key in LiPPI-CLAPon is a new protease cleavage-dependent enzymatic sensor motif we designed called CLAPon. In CLAPon, the reconstitution of the split APEX2 (enhanced ascorbate peroxidase) is sterically blocked by acid-base coils (**Fig. 3A**). Protease cleavage releases the steric hindrance so that within minutes split APEX reconstitutes, allowing for further fluorescent labeling and analysis in live or fixed tissues[5]. LiPPI-CLAPon achieves light- and PPI-dependence similar to the SPARK design[32]. As shown in **Fig. 3A**, the tobacco etch virus protease (TEVp) cleavage site (TEVcs) is sterically blocked by the light, oxygen, and voltage sensing domain (LOV) in the dark. Upon light irradiation, LOV undergoes a conformational change and TEVcs becomes accessible. The light-uncaged TEVcs is most efficiently cleaved when the TEVp is brought into proximity of the TEVcs via a pair of interacting proteins.

We demonstrated LiPPI-CLAPon's application in detecting dopamine using the Dopamine Receptor D1 (DRD1)-arrestin interaction pair in HEK293T cells (**Fig. 3B, C**)[5]. Screening of different TEVcs shows that the ENLYFQA peptide sequence gives the best SBR for LiPPI-CLAPon (**Fig. 3B**), while CLAPon for detecting PPI without a light control (PPI-CLAPon) gives the best SBR with the TEVcs ENLYFQN[5]. Therefore, it is always good to re-evaluate the TEVcs and TEVp pair used in the TEVp-based proximity cleavage reporters.

LiPPI-CLAPon is the first genetically encoded time-gated PPI integrator that generates a reporter readout within minutes of PPI detection. CLAPon requires only two components, eliminating the need of co-infection with a reporter gene construct as in SPARK and iTANGO, which will simplify the viral delivery and co-infection in animal brain. It also enables sub-cellular spatial resolution detection of PPI and detection of extracellular PPIs. However, CLAPon currently has low sensitivity and has only been demonstrated in cell cultures with prolonged stimulation (5-10 minutes) at high expression levels. Further optimization of the sensor sensitivity and SBR is required for demonstrations in animal models.

In vitro assay for detecting GPCR activation. Besides detecting neuromodulators in vivo, we have also developed an in vitro assay for detecting GPCR activation in cell lysates[4]. Live cell-based assays have significantly advanced the screening and characterization of GPCR ligands[41-48]. However, there is a lack of in vitro GPCR assays that are convenient to use and have readily accessible reagents that can be prepared in large batches, frozen and stored. In vitro assays can also be used in applications where live cell-based assays cannot, such as characterization of extracted GPCRs or in portable assays for detecting GPCR ligands outside the lab.

The in vitro assay we designed for characterizing a GPCR's activity is called IGNiTR (In vitro GPCR split NanoLuc lIgand Triggered Reporter)[49]. IGNiTR is based on the use of conformation-specific binders for the activated GPCR conformational state and the proximity-dependent split NanoLuciferase (NanoLuc).[50] Split NanoLuc fragments SmBiT and LgBiT are each fused to the conformation-specific binder and the GPCR of interest (**Fig. 4A**)[49].

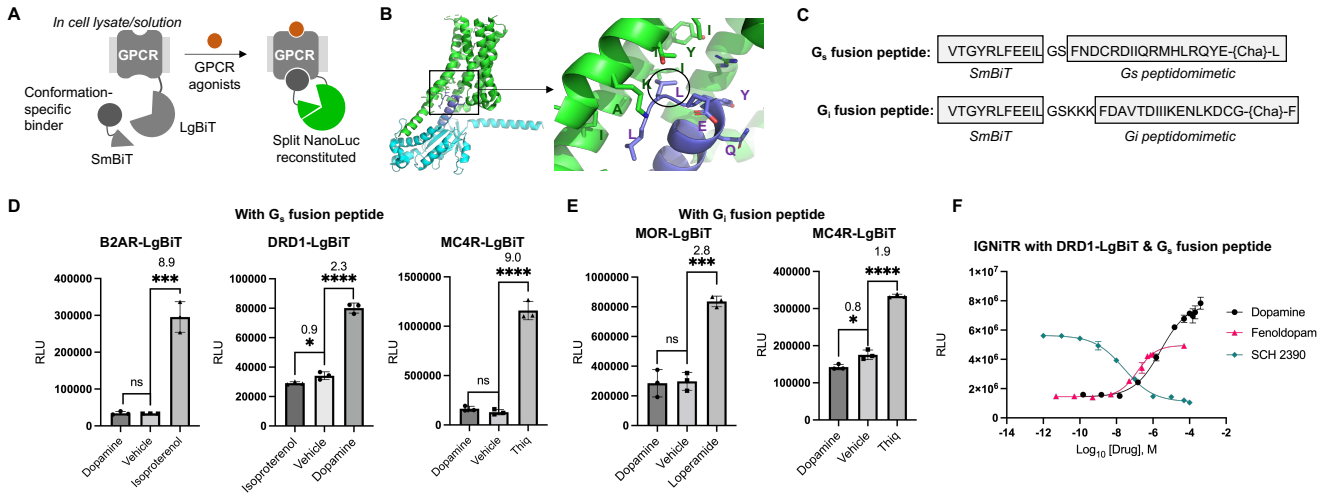

Figure 4: (**A**) Schematics of IGNiTR. (**B**) Crystal structure of LY3154207-bound dopamine receptor D1 (DRD1) (PDB: 7X2F). DRD1, green. Gs protein's α-5-helix is highlighted in purple. The penultimate position is enclosed inside the circle. (**C**) Amino acid sequences for the Gs and Gi fusion peptides composed of Gs and Gi peptidomimetics fused with SmBiT. (**D**) Characterization of IGNiTR with the Gs fusion peptide and (**E**) the Gi fusion peptide for the GPCRs indicated. Gs and Gi fusion peptide, 2 μM. Drug, 10 μM. (**F**) Dose-response curve of DRD1 IGNiTR with dopamine, fenoldopam and SCH 23390. Reproduced with permission from ref.4. Copyright 2023 American Chemical Society.

Upon addition of GPCR agonists, the GPCR undergoes a conformational change and recruits the conformation-specific binder, reconstituting the split NanoLuc. We tested three classes of conformational specific binders: conformation specific nanobodies, miniG proteins and G protein peptidomimetics[49]. G protein peptidomimetics were designed based on the α-5 helix of the G protein, with an unnatural amino acid cyclohexylalanine (Cha) introduced in the penultimate position to enhance the peptide's interaction with the activated GPCR (**Fig. 4B, C**). While nanobodies and miniG proteins can be co-expressed along with the GPCR of interest and provide robust readout of the conformational change of GPCR activation, the use of G protein peptidomimetics enable the incorporation of unnatural amino acids for designing high affinity peptide binders.

We demonstrated IGNiTR assay with three Gs-coupled and two Gi-coupled GPCRs (**Fig. 4D-F**)[49]. We also showed its versatile applications, including high-throughput GPCR ligand screening, easy detection of opioids using a simple camera and characterization of the functionality of extracted GPCRs[49]. IGNiTR is the first GPCR activity in vitro assay that can be easily scaled up and performed with only a one-step protocol. IGNiTR will find broad applications in high-throughput GPCR ligand screening and in vitro GPCR activity characterization.

Summary and Future Outlook. In summary, we have designed two major classes of integrators for detecting neuromodulators. One is the cpFP-Nb39-based single protein chain fluorescent integrators, including SPOTIT for detecting opioids. The other class is the light- and PPI-dependent enzymatic reporter, called CLAPon.

SPOTIT sensors are the first single-chain fluorescent integrators for detecting neuromodulators. They can be packaged into one AAV viral vector and therefore will be easier to use than multi-component systems that require co-infection of multiple AAV viruses, such as CLAPon, SPARK and iTANGO. In the future, further optimization of M-SPOTIT2 can be performed to enhance the SBR, brightness, fluorophore maturation rate and agonist EC_{50}.

Upon optimization of SPOTIT, our goal is to apply the SPOTIT sensors to provide brain-wide mapping of opioid peptide release events to study where endogenous opioid peptides are released in response to pain and reward, and where the synthetic opioids target in the brain at high spatial resolution. We will perform RNA profiling of the labeled neurons to help elucidate the molecular differences in the opioid-based pain and reward circuits.

Other SPOTIT-based designs can also be explored to extend SPOTIT to detect a wide range of neuromodulators with high activation efficiency and brightness. They can also be designed for detecting orphan GPCR ligands for whole body mapping of the orphan GPCR activation in mouse models to search for their endogenous ligands and functions.

CLAPon and SPARK provide high temporal resolution on the order of seconds to minutes. CLAPon provides an immediate readout of APEX activation after detecting the neuromodulators, therefore providing a shorter experimental timeline suitable for terminal behavioral assays. Further, CLAPon requires co-delivery of only two AAV viruses while SPARK requires three, which would potentially make CLAPon in vivo application optimization easier than SPARK systems. On the other hand, SPARK provides versatile transcriptional readout, including fluorescent proteins, opsins[51, 52] and DREADDs[53, 54] to enable further manipulation of the labeled neurons. Therefore, CLAPon and SPARK can be used for a broader range of applications.

SPOTIT and SPOTIT-based sensors can provide large volume and whole-body mapping of neuromodulators at cellular resolution. CLAPon and SPARK can then be used in specific brain regions to study the neuromodulator release during specific time window. Ultimately, we will apply SPARK and CLAPon to study neuromodulator release during specific behaviors with temporal resolution of seconds to minutes, after brain-wide mapping with SPOTIT-based sensors. These integrators can be used together with real-

time sensors and bioanalytical methods to study neuromodulator release and transmission dynamics and its interaction with the other neuromodulatory systems.

Lastly, the in vitro IGNiTR assay provides a new platform for screening GPCR ligands. IGNiTR reagents can be stored frozen in large batches to ensure consistency across experiments.

3. PROTEIN SWITCHES FOR DESIGNING GENETICALLY ENCODED TOOLS TO CONTROL CELL SIGNALING

Genetically encoded chemical- and light-dependent systems (chemogenetic and optogenetic tools) are the most commonly used genetically encoded tools to modulate cellular signaling with temporal control. Genetic encoding with existing genetic tools (e.g. transgenic mice with Cre recombinase, promoter- and enhancer based expression) allows cell-targeted expression. Optogenetic and chemogenetic tools enable activation or inhibition of selective neuronal populations with temporal control to dissect their functional roles in learning, memory, cognitive functions and specific behavioral paradigms.[51, 53, 55, 56]

Chemogenetic and optogenetic tools have complementary strengths. Chemogenetic tools enable manipulation throughout the brain and in deep tissues and do not require complicated setups (such as optical fiber implant). However, chemogenetic tools are limited by their poor temporal control, on the order of hours. Optogenetic tools offer fast temporal control on the order of milliseconds to minutes, enabling studies in acute behaviors, but light has poor tissue penetration. Therefore, optogenetic and chemogenetic tools complement each other and are suitable for different applications.

Temporally controlled manipulation of endogenous GPCRs in selected neuronal populations is desired to study the causal effect of endogenous neuromodulation on various physiological processes and behaviors. There are only a few tools available for manipulating endogenous GPCR activity with temporal control to investigate the effect of neuromodulation. Pharmacological approaches can manipulate endogenous receptors' activity but lack cell population or cell type selectivity. Most of the existing chemogenetic and optogenetic tools for modulating neuronal activity utilize the activity of exogenously introduced ion channels[51, 52] or receptors[53, 54, 57, 58], and therefore do not exactly recapitulate the effect of a specific endogenous receptor's activation and downstream signaling pathways.

Membrane-tethered synthetic ligands via self-labeling proteins such as HaloTag[59] and SNAP-tag[60] can be applied to modulate endogenous GPCR's activity[61]. DART (drugs acutely restricted by tethering)[62, 63] is composed of a synthetic drug with reduced potency attached to a HaloTag ligand via a PEG linker. The drug-HaloTag ligand conjugate is then anchored to the cell surface, where HaloTag is expressed. DART has been designed for modulating endogenous α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor and Gamma-Aminobutyric Acid Type A Receptor activity, but not yet for modulating endogenous GPCR activity. Furthermore, DART becomes active only when the tethered drug reaches high enough concentration via HaloTag reaction and, once labeled, remains active until the HaloTag protein is degraded. Consequently, DART has slow on and off kinetics.

Another membrane-tethered ligand addresses this limitation: maPORTL, or membrane-anchored photoswitchable orthogonal remotely tethered ligand. maPORTL tethers a photoswichable ligand to the cell membrane and thus has fast on and off kinetics and. This system has been demonstrated in the modulation of endogenous DRD1 in vivo[64].

While versatile and powerful, DART and maPORTL require usage of complicated drug conjugates that are difficult to synthesize and do not cross the blood brain barrier easily. Therefore, to avoid usage of complicated drug conjugates, new approaches to design chemogenetic and optogenetic tools for modulating endogenous receptors' activity with simpler chemicals are needed.

Existing chemical-dependent protein switches have been applied in causing exogenous receptor activation, protein dimerization, dissociation, and protein degradation.[56] However, when we started our research group, there was a lack of generalizable chemical switches to cage the N- or C-terminal of different peptides. These switches can be used to control the activity of peptides to modulate endogenous receptors.

Neuropeptides comprise a large family of the neuromodulators that regulate many GPCRs. Controlling the peptide agonist's accessibility via chemical- and light-dependent protein switches will provide new chemogenetic and optogenetic tools for controlling endogenous receptor activity to study their causal effects on behaviors and physiological processes. The LOV domain has been widely applied to control peptide activity by fusion of the C-terminus of the LOV domain to the N-terminus of a peptide, regulating the accessibility of the N-terminal portion of the peptide[65]. However, many neuropeptides (e.g., peptide agonists for MOR, KOR and DOR) require the N-terminal portion to be free of fusion to enter the GPCR binding pocket, so the LOV domain cannot be used. Therefore, new light-dependent protein switches were needed that can fuse to the C-terminus of a peptide.

To address the above needs, our lab has designed both chemical-activatable[2] and photo-switchable[6] protein domains for controlling short peptide activity. These protein switches can be used to cage peptides' agonist activity so that they cannot activate the receptor. Upon addition of chemical addition or light irradiation, the peptide agonist will be uncaged and can activate the endogenous GPCR of interest within proximity.

New chemical-dependent protein switches for controlling the terminal accessibility of a peptide. To address the need for chemical-dependent protein switches, we designed a pair of switches called CAPs (Chemically Activated Protein domains) to control the accessibility of the N- or C-terminal region of a peptide embedded within the protein domain (**Fig. 5A**)[2]. CAPs are based on FK506 binding protein mutant, FKBP12(F36V), and its low nanomolar affinity chemical ligand Shield-1[66]. Shield-1 should have minimal effect on endogenous proteins since it has micromolar binding affinity for FKBP12. Additionally, shield-1 was reported to be able to cross the blood brain barrier[67].

Via yeast cell surface display, we selected peptide binding sequences that can bind to the ligand binding pocket of

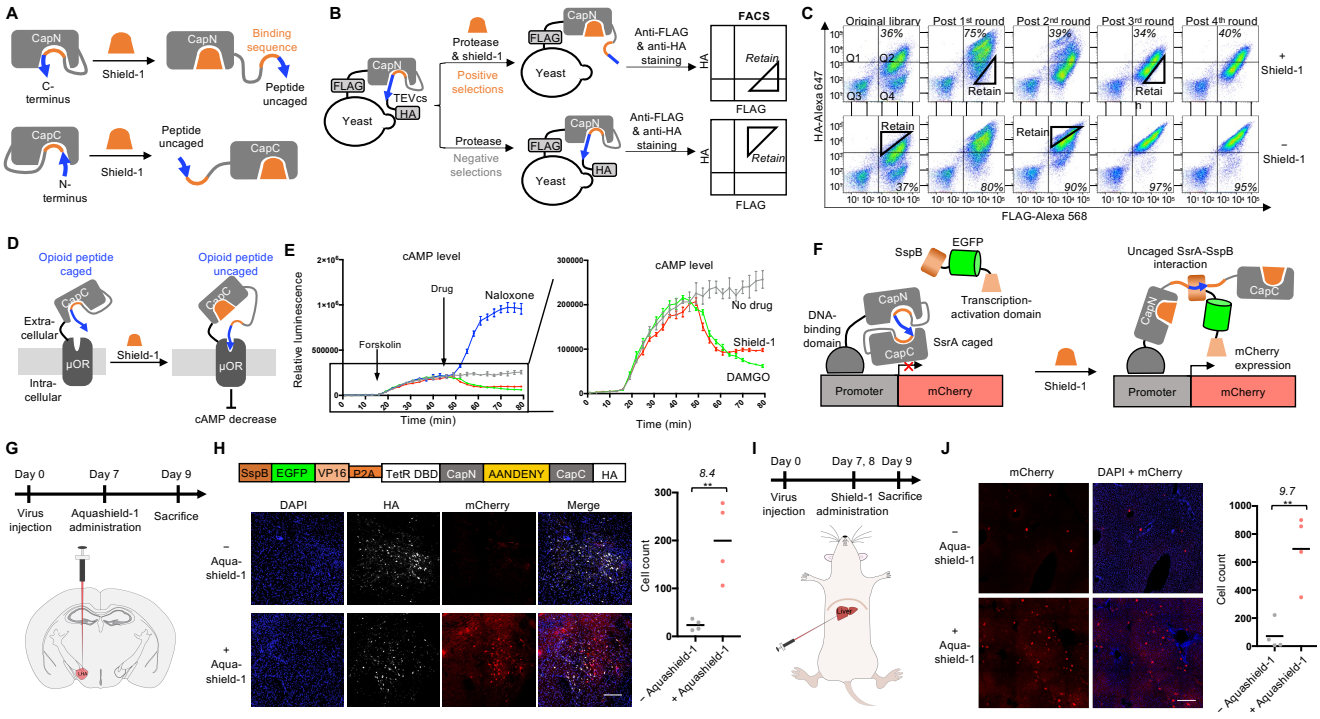

Figure 5: **(A)** Design of CAPs. **(B)** Labeling schematics and **(C)** FACS characterization of CapN library-caged TEV protease cleavage site selection. Populations within the triangles on the FACS plots are retained. TEVcs, TEV protease cleavage site ENLYFQG. FLAG and HA are epitope tags. Values are percentage of cells in Q2 over (Q2 + Q4). **(D)** Scheme of CapC-controlled opioid peptide and **(E)** cAMP level characterization in response to different drugs in HEK 293T cells. A cAMP biosensor, GloSensor™, was co-transfected to indicate cAMP level. **(F)** Scheme of shield-1-induced gene transcription. **(G)** Timeline for the aquashield-1-induced transgene expression in mouse brains. AAV is locally injected to lateral hypothalamic area (LHA). Water soluble shield-1, aquashield-1, is locally administered to mice (1 μL, 1 mM). **(H)** Representative fluorescence images of brain sections of the LHA and quantification based on the number of mCherry expressing cells. Scale bar, 200 μm. **(I)** Timeline for the aquashield-1-induced transgene expression in mouse liver. AAV is locally injected to liver. Aquashield-1 is administered to mice via two intraperitoneal (IP) injections (40 mg/kg) with 24 hours apart. **(J)** Representative fluorescence microscopy images of liver sections from injection site and its quantification based on the number of mCherry expressing cells. Scale bar, 200 μm. Reproduced with permission from ref. 2. Copyright 2022 Springer Nature.

FKBP12(F36V) such that a peptide that is directly fused to the binding sequence will be sterically blocked until shield-1 addition (**Fig. 5B, C**)[2]. Since CapN and CapC can block the accessibility of the N- and C-terminal portion of a peptide, CapN and CapC can be used together to cage both terminal portions of a peptide to achieve high chemical-dependence. We demonstrated the generality of the CAPs system in controlling the activity of four peptides in HEK293T cells[2].

We demonstrated that CAPs can cage the protease cleavage site TEVcs and achieve shield-1-dependent protease cleavage[2]. We also demonstrated CAPs' ability to cage the nucleus localization signal peptide for shield-1-dependent translocation of a protein to the nucleus[2] and the seven amino acid-SsrA peptide that dimerizes with SspB protein to achieve shield-1-dependent protein dimerization[2]. Further, we showed that CapC cages the C-terminal portion of enkephalin, an opioid receptor peptide agonist, to achieve shield-1-dependent activation of MOR that leads to a decrease of cAMP level (**Fig. 5D, E**)[2]. Lastly, we showed that CAPs-caged SsrA can be used to control transcription factor reconstitution and transcription of a reporter gene (**Fig. 5F**) with a shield-1-dependence of up to 150-fold in cell cultures[2]. The shield-1-dependent transcription was also demonstrated in the mouse brain and liver (**Fig. 5G-J**)[2]. CAPs are the first generally applicable chemical-dependent protein switches capable of temporally controlling peptide function, which will significantly expand the chemogenetic toolbox.

Design of new optogenetic tools by engineering circularly permuted LOV (cpLOV). The LOV domain has been widely applied to control peptide function by sterically blocking the fused peptide in the dark and uncaging it in blue light.[65] The LOV domain can be activated by blue light within seconds and reset to the dark state within minutes.[68] However, the LOV domain's application was limited to caging the N-terminal region of a peptide. To increase the versatility of light-sensing proteins, we have designed cpLOV to cage the C-terminal region of a peptide as well (**Fig. 6A, B**)[6].

cpLOV has been successfully applied to cage the TEVcs in the DRD1-SPARK system (**Fig. 6C-E**)[8]. We demonstrated the tandem use of both LOV and cpLOV domain to further improve the light-dependent dynamic range (**Fig. 6F-H**)[6]. cpLOV will significantly expand the toolbox of optogenetic tools by controlling either or both terminal regions of a peptide in a light-dependent manner. A similar work on cpLOV design was reported at a similar time[69], demonstrating the feasibility and utility of cpLOV domain.

Summary and Future Outlook. We have designed new chemical- and light-dependent protein switches. CapN and CapC, are the first chemical-dependent protein switches that can be generalizable for caging the N- and C-

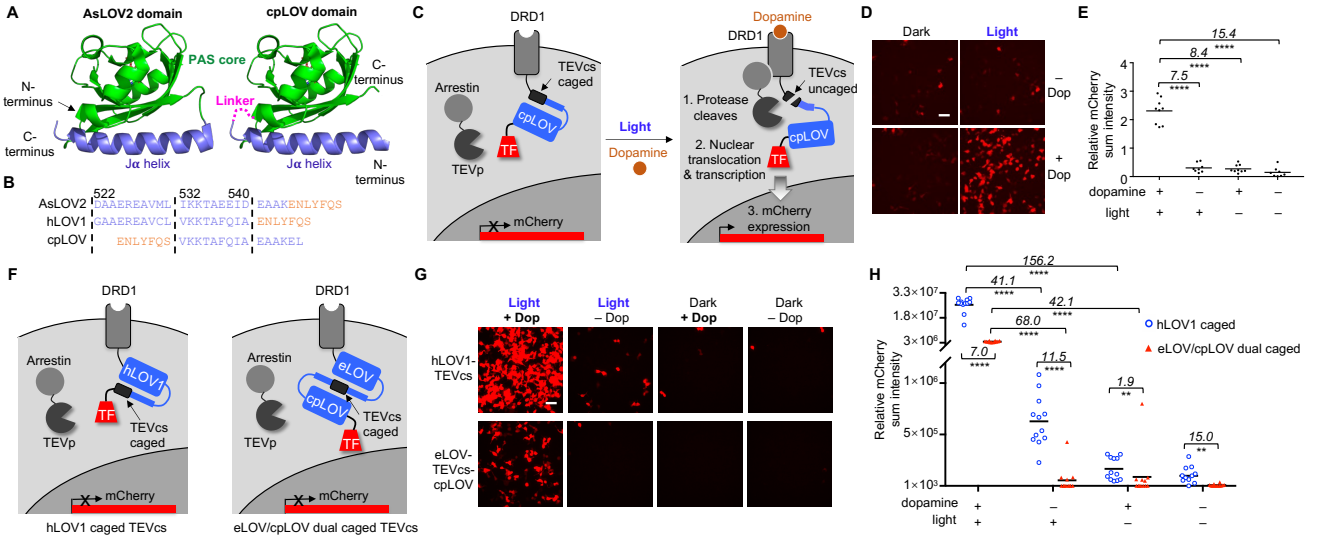

Figure 6: (A) Design of the circularly permuted AsLOV2 (cpLOV) based on the AsLOV2 domain (PDB: 2V1A). **(B)** The Jα helix sequences and truncation sites for AsLOV2, hLOV, and cpLOV when fused to TEVcs sequence, ENLYFQS. **(C)** Scheme of cpLOV-based light- and dopamine-dependent transcriptional assay in HEK293T cells, which is similar to the SPARK reporter, except that cpLOV replaces LOV in caging the TEVcs ENLYFQM. (**D**) Confocal fluorescence imaging characterization and (**E**) quantification of the transcriptional assay in (C) under the four conditions. The cells were stimulated with light and/or 100 μM dopamine for 10 minutes. (**F**) Scheme of single hLOV1- and dual-caged transcriptional assays in HEK293T cells. TEVcs, ENLYFQM. (**G**) Confocal fluorescence imaging characterization and (**H**) quantification of the single- and dual-caged transcriptional assay in HEK293T cells. The cells were stimulated with light and/or 100 μM dopamine for 10 minutes. Scale bar, 50 μm. Reproduced with permission from ref.6. Copyright 2021 Royal Society of Chemistry.

terminal portion of different peptides. We have demonstrated CAPs' applications through multiple proof-of-principle applications. The CapC-caged enkephalin is the first demonstration of genetically encoded, chemical-activatable short peptide agonist for controlling GPCR activity. While further optimization and demonstration in vivo is needed, the CAPs system provides a new class of chemical-dependent protein switches and new approaches for modulating endogenous receptor activity. The improved CapC-caged opioid peptide will provide the first chemogenetic tool to regulate endogenous MOR in selective cell populations and to read out its effects in pain modulation, addiction and respiration.

Overall, the caging efficiency of CAPs could be further optimized in the future, along with fine-tuning the caging for each peptide. For example, gradual truncation of the peptide could be performed so that the critical residues are directly fused to the CAPs for most efficient steric blocking. Further optimization via directed evolution could also be performed to customize the CAPs system for the peptide of interest. The same approach for designing CAPs could also be applied to develop new pairs of chemical-switchable protein domains by other small molecules, providing multiplexed control by different chemicals.

In future work, we will apply cpLOV to cage GPCR peptide agonists and achieve fast temporal control of endogenous GPCR activity in a reversible manner. The cpLOV-caged peptide agonists will enable fast and reversible control of GPCRs to study their functional output in acute behaviors. These genetically encoded and temporally gated GPCR peptide agonists will greatly facilitate the manipulation and investigation of endogenous neuromodulation.

4. SUMMARY AND FUTURE PERSPECTIVE

In this account, we describe our lab's effort in designing genetically encoded integrators and chemogenetic and optogenetic tools for studying the neuromodulatory system. Neuromodulators regulate the activity of various neuronal pathways and play important roles in all aspects of our brain functions, including learning, memory, cognitive functions, emotional states, sleep and pain modulation. Many recently designed sensors have greatly facilitated studies on neuromodulation at high spatiotemporal resolution, uncovering new biology. Since neuromodulation is highly complicated, different tools are still needed for addressing different aspects of the biological question.

Multiplexed imaging and detection will be important to study the interplay of the different neuromodulatory system. While many real-time sensors perform well with green fluorescence readout, designing robust and bright sensors with red and far-red fluorescence readout will be important for multiplexed imaging. Coupling neuromodulatory tools with sensors will allow targeted manipulation and monitoring to systematically study the effect of neuromodulation on different neuronal populations. However, most light-controlled tools for modulating the endogenous neuromodulatory system are violet or blue light-dependent, which have reduced tissue penetration. Designing red or far-red light-dependent molecular tools for modulating the neuromodulatory system will improve tissue penetration and achieve large volume and deep tissue modulation. Lastly, neuromodulators have varied basal levels in different brain regions. Therefore, sensors with different sensitivity and EC_{50} will be needed to detect neuromodulators spanning a wide range of concentrations.

The genetically encoded tools can also be coupled with bioanalytical methods and pharmacological approaches. Overall, this is a very exciting time for studying brain

signaling and neuromodulation. The swift expansion and advancement of our arsenal of tools and technologies will rapidly evolve our understanding of brain circuitry and functions.

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Notes

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