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Current and emerging methods for probing neuropeptide transmission

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Abstract

Neuropeptides comprise the most diverse category of neurochemicals in the brain, playing critical roles in a wide range of physiological and pathophysiological processes. Monitoring neuropeptides with high spatial and temporal resolution is essential for understanding how peptidergic transmission is regulated throughout the central nervous system. In this review, we provide an overview of current non-optical and optical approaches used to detect neuropeptides, including their design principles, intrinsic properties, and potential limitations. We also highlight the advantages of using G protein–coupled receptor (GPCR) activation–based (GRAB) sensors to monitor neuropeptides *in vivo* with high sensitivity, good specificity, and high spatiotemporal resolution. Finally, we present a promising outlook regarding the development and optimization of new GRAB neuropeptide sensors, as well as their potential applications.

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Introduction

Neuropeptides are evolutionarily ancient molecules typically produced and secreted by neurons, serving as neuromodulators and hormones in a wide range of species from nematodes to mammals [1]. Neuropeptides are the most diverse family of neuronal signaling molecules in the brain, ranging in size from only three amino acid residues such as thyrotropin-releasing hormone (TRH) to considerably longer polypeptides such as the 112-residue agouti-related peptide (AGRP). In neurons, neuropeptides are stored in large dense-core vesicles (LDCVs) and can be released from the axon, soma, and dendrites upon high neuronal activity and/or mobilization of intracellular calcium (Ca^{2+}) [2,3]. Unlike classic neurotransmitters such as glutamate, neuropeptides are believed to diffuse relatively slowly in the extrasynaptic space due to the lack of an effective mechanism for their recycling at the synaptic cleft, and they modulate neighboring cells primarily by binding to G protein–coupled receptors (GPCRs) [3].

Neuropeptides play an important role in regulating a wide range of physiological processes in the central nervous system. To name just a few examples, oxytocin and vasopressin are involved in social behaviors [4]; orexigenic AGRP and anorexigenic α-melanocyte-stimulating hormone (α-MSH), released by two distinct subtypes of neurons in the hypothalamus, have been shown to antagonistically regulate energy homeostasis and food intake in rodents [5]; gastrin-releasing peptide (GRP) and substance P, produced by sensory neurons in the spinal cord, are thought to code for itch and pain, respectively [6,7]; and endogenous opioid peptides have been implicated in analgesia and substance abuse [8]. Conversely, altered peptidergic signaling has been implicated in a variety of pathological conditions such as obesity, addiction, cataplexy, autism spectrum disorder, and schizophrenia [9].

Although numerous seminal studies have contributed important insights into the functional role of peptidergic transmission, many fundamental questions remain largely unexplored, including when and where neuropeptides are released in the neuronal network, the spatiotemporal dynamics of their release patterns in health and disease, and how neuropeptide release is regulated with respect to the underlying molecular mechanisms. These questions have been difficult to answer, however, as detecting peptidergic transmission is challenging due to the low extracellular concentration

(in the picomolar to nanomolar range) of neuropeptides and their relatively short half-life (on the order of several minutes to tens of minutes) [2]. In addition, neuropeptides likely act extrasynaptically via volume transmission [3]. Thus, answering these key questions requires the ability to directly monitor neuropeptides within the brain, ideally using minimally invasive techniques that have good sensitivity and high spatiotemporal resolution. Thanks to pioneering work by countless researchers, a number of molecular detection technologies have been developed, providing complementary approaches for measuring neuropeptides in various platforms. Here, we summarize the design principles, properties, advantages, and potential limitations of these methods (Figure 1).

Non-optical methods for measuring peptidergic transmission

Microdialysis

The classic microdialysis method is considered the gold standard for measuring the absolute concentration of neurochemicals in cerebrospinal fluid and other fluids in animal models [10]. With microdialysis, a semipermeable membrane is used to selectively collect signaling molecules that fall within a specific molecular weight range. After the dialysis probe is implanted in the target brain region, the neuropeptides present in the extracellular fluid diffuse into the dialysate along their concentration gradient. The dialysate can then be analyzed using quantitative methods such as radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), or high-performance liquid chromatography coupled with mass spectrometry (HPLC-MS). Microdialysis has improved considerably over the past several decades and has been widely used for the *in vivo* detection of numerous neuropeptides, including—but by no means limited to—oxytocin [11], hypocretin/orexin [12], and corticotropin-releasing factor (CRF) [13]. Microdialysis can be used to repeatedly measure dozens of neurochemicals simultaneously in multiple brain regions in freely moving animals. Nevertheless, due to the detection limit of biochemical analyses, this method requires enrichment to achieve sufficient amounts of dialysate in order to identify specific neuropeptides; consequently, microdialysis has a relatively slow sampling rate, typically taking ∼5−10 min depending on the collection system used and the animal's brain size [14]. In addition, the embedded probe is relatively large, with an outer diameter on the order of hundreds of microns, too large to achieve single-cell resolution and rendering it unsuitable for use in small animal models such as *Drosophila* and *C. elegans*. Furthermore, implanting the probe can damage the brain tissue and can affect the blood-brain barrier's permeability [15]. Therefore, this method cannot be used to measure dynamic changes in neuropeptides under normal physiological conditions with sufficient spatiotemporal precision.

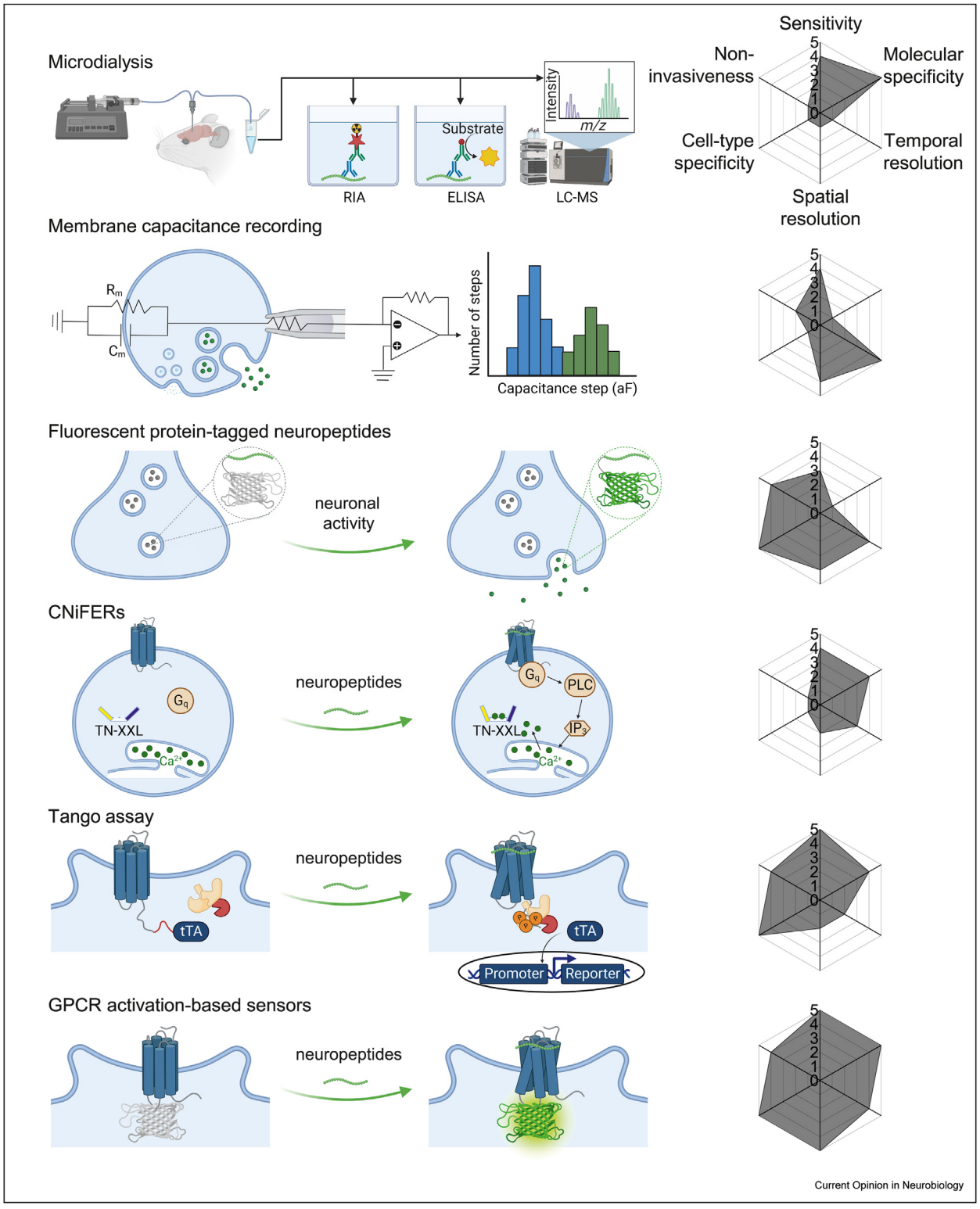
Electrophysiology

The release of classic neurotransmitters such as glutamate and γ-aminobutyric acid (GABA) can be detected with relatively high precision by recording currents generated by their corresponding ionotropic receptors. However, because most neuropeptide receptors are metabolic GPCRs, their release cannot be observed by measuring electrical signals. Nevertheless, some neuropeptides bind to $G_{i/o}$-coupled GPCRs, thereby activating G protein−gated inwardly rectifying potassium (GIRK) channels via their coupled $G_{\beta\gamma}$ G protein subunit; the resulting K^+ efflux causes hyperpolarization of the postsynaptic membrane. Using patch-clamp recording in *in vitro* preparations, the release of inhibitory neuropeptides such as neuropeptide Y [16] and somatostatin [17] can be measured indirectly by measuring changes in GIRK currents mediated by activation of the peptides' corresponding GPCRs. In addition, it is believed that activation of the oxytocin receptor can induce a sustained inward current via the opening of nonspecific cationic channels or the closure of K^+ channels through a mechanism that remains partially characterized [18,19], potentially serving as a means to detect oxytocin. Although this method can provide a snapshot of when peptides bind to their receptors—due to the high temporal resolution of electrophysiology—this low-throughput method cannot provide information regarding the diffusion properties of peptidergic transmission. Moreover, currents through GIRK channels are not linearly correlated with the number of released neuropeptides, due to the activity of various intracellular signaling cascades. Finally, this method lacks molecular specificity, has limited scalability, and is highly invasive, thereby making it poorly suited for use under physiological conditions and in *in vivo* preparations.

Most classic neurotransmitters are packaged in recyclable synaptic vesicles with a diameter of approximately 40 nm, while neuropeptides are stored in non-recycling LDCVs with a diameter of approximately 100−500 nm. Both synaptic vesicles and LDCVs release their contents by fusing with the presynaptic membrane, with each vesicle type causing a specific change in the area of the presynaptic membrane. Thus, membrane capacitance (which is correlated with membrane area) can be recorded in order to specifically measure LDCV fusion events with high sensitivity and high temporal resolution [20,21]; however, this approach is invasive, lacks neuropeptide specificity and high throughput, and is therefore suitable primarily for use in *in vitro* and *ex vivo* applications.

In addition to microdialysis and electrophysiology, fast-scan cyclic voltammetry (FSCV) has also been used successfully in non-optically detecting neuropeptides, such as methionine-enkephalin [22,23] and dynorphin

Figure 1



Schematic overview of the currently available methods for detecting peptidergic transmission. For each method, the design principle is shown on the left, and radar graphs summarizing the method's properties, including sensitivity, molecular specificity, temporal resolution, spatial resolution, cell-type specificity, and non-invasiveness, are summarized on the right, with each property ranging from 0 to 5. See the text for further details. The cartoons were created with BioRender.com.

[24]. So far, the detection of the majority of peptides in the brain remains unfeasible by means of FSCV. Moreover, the application of FSCV necessitates the implantation of a relatively large probe into the brain tissue, which inherently constrains the attainment of highly accurate and spatially precise measurements of neuropeptides *in vivo*.

Optical methods for measuring peptidergic transmission

Unlike the non-optical methods discussed above, optical methods allow the researcher to measure neuropeptides with relatively low invasiveness and higher throughput. These methods use fluorescent signals as a readout, identifying changes in neuropeptide concentration or in their GPCR. Using this principle, several optical tools have been developed to visualize peptidergic transmission, including fluorescent protein-tagged neuropeptides, cell-based neurotransmitter fluorescent engineered reporters (CNiFERs), the Tango assay, and GPCR activation–based (GRAB) sensors (Table 1).

Fluorescent protein-tagged neuropeptides

Most neuropeptides are comprised of 3−∼100 amino acids and can be directly labeled by recombinant fusion with a fluorescent reporter protein in order to track their transport or image LDCV fusion events. Fluorescent reporters such as green fluorescent protein (GFP), the GFP-based pH-sensitive indicator pHluorin [25], fluorogen-activating protein (FAP) [26], and fluorescent Ca^{2+} indicators have been used for this purpose. For example, GFP-tagged neuropeptides have been used to detect the activity-dependent release of neuropeptide Y (NPY) [27] and atrial natriuretic factor (ANF) [28] in cultured PC12 cells, in which the release of LDCVs causes a decrease in fluorescence intensity. pHluorin has been used to image the exocytosis and recycling of synaptic vesicles due to its change in fluorescence in response to the difference in pH between the vesicle lumen and the extracellular space [25]. In addition, NPY-pHluorin has been combined with total internal reflection fluorescence (TIRF) microscopy to visualize the exocytosis of single LDCVs [29]. In addition to its acidic pH, the LDCV lumen also has a considerably lower Ca^{2+} concentration compared to the extracellular fluid [30]; capitalizing on this property, neuropeptide release receptors (NPRRs)—consisting of the neuropeptide of interest fused to the fluorescent Ca^{2+} indicator GCaMP6s—were developed for detecting the release of ANF at the neuromuscular junction in *Drosophila* larvae [31]. In addition, the fluorescent reporter protein can be fused to the luminal side of LDCV-specific membrane proteins such as cytochrome *b*561, providing a versatile tool for monitoring LDCV release in brain slices and freely moving mice, albeit at the expense of molecular specificity [32]. Overall, these probes can provide optical imaging of neuropeptide transmission at sub-second resolution, have cell-type specificity, and have the potential for use in *in vivo* applications. However, a remaining challenge is ensuring that the peptide's transport and diffusion properties are not affected by fusing the fluorescent reporter, which can have a molecular weight 10−100 times that of the neuropeptide itself.

CNiFERs

The majority of neuropeptides identified to date selectively bind to their corresponding GPCRs and modulate specific downstream signaling pathways, providing an amplification process that can be used to detect neuropeptide release. For example, HEK293 cells co-expressing G_q-coupled GPCRs and genetically encoded Ca^{2+} indicators can be implanted in specific brain regions in order to detect extracellular increases in neuropeptide concentration [33]. Although only a subset of GPCRs couple to the Gq-mediated Ca^{2+} pathway, chimeric G proteins can be developed in order to expand the platform to include Gq-independent GPCRs [34]. Thus, several highly sensitive CNiFERs have been developed for detecting various neuropeptides such as oxytocin [35], vasoactive intestinal peptide (VIP) [36], vasopressin [37], and somatostatin [38] by reporting an increase in cytosolic Ca^{2+} levels. However, the need to implant cells and potential interference by non-specific intracellular Ca^{2+} signals make this method highly invasive and lacking in both molecular and cell-type specificity.

Tango assay

The Tango assay is commonly used to detect GPCR ligands by measuring GPCR activation–mediated β-arrestin recruitment [39,40]. This assay involves fusion of the C-terminal tail of the GPCR to the transcription factor tTA via a 7-amino acid cleavage site that can be cleaved by the tobacco etch virus (TEV) protease fused to β-arrestin. Upon GPCR activation, the protease is recruited, causing the release of tTA, which then drives the expression of a tTA-dependent reporter gene such as luciferase or GFP. A number of neuropeptides, including vasopressin, substance P, and cholecystokinin (CCK), have been detected in HEK293 cells using this strategy [39]. The next-generation iTango2 system contains a light-controlled element that exposes the TEV recognition site in response to blue light and has been used to detect oxytocin release during social interactions [41] and maternal behaviors [42] in mice. Although this assay provides better control using light delivery, it still takes at least several hours for sufficient expression of the reporter gene and therefore has relatively low temporal resolution.

Table 1

Overview of optical tools for detecting neuropeptides.

Sensor	Ligand	Binding protein	Optical reporter	Maximum $\Delta F/F_0$	Affinity (EC_{50} (nM))	*In vivo* application	Refs.
NPY-GFP	NPY	-	GFP	–74.5 ± 4.5%[a]	-	ND	[27]
ANF-GFP	ANF	-	GFP	–24.3 ± 4.1%[a]	-	ND	[28]
NPY-pHluorin	NPY	-	pHluorin	~2300%[a]	-	ND	[29]
NPRRs	ANF	-	GCaMP6s	~60%[b]	-	Fly	[31]
CybSEP	-	-	SEP	~15%[a]	-	Mouse	[32]
CNiFER	OT	hOTR	R-GECO	13.3 ± 0.04%[c]	1.5[d]	ND	[35]
CNiFER	VIP	VPAC2R	jRCaMP1b	~40%[d]	ND	ND	[36]
CNiFER	AVP	AVPR1a	R-GECO	163.0 ± 7.5 (F/F_0*s)[d]	7.2[d]	ND	[37]
CNiFER	SST	SSTR2	Twitch 2 B	~200% ($\Delta R/R$)[d]	4.4[d]	Mouse	[38]
AVPR2-Tango	AVP	AVPR2	SEAP	ND	4.3[d]	ND	[40]
OXTR-iTango2	OT	hOTR	EGFP	~18.4 ($\Delta R/R$)[d] ~15.9 ($\Delta R/R$)[e]	ND	Mouse	[41]
kLight	κ-opioid	KOR	cpEGFP	60 ± 4%[d] (U50488)	ND	Mouse	[50,63]
mLight	μ-opioid	MOR	cpEGFP	37 ± 2%[d] (DAMGO)	ND	ND	[50]
OxLight1	orexin	OX2R	cpEGFP	906 ± 28%[d] (orexin-A) 859 ± 27%[d] (orexin-B)	75±6[d] (orexin-A) 47±5[d] (orexin-B) 127 ± 13[e] (orexin-A) 110 ± 10[e] (orexin-B)	Mouse	[56]
$MTRIA_{OT3.0}$	OT	meOTR	cpEGFP	~735%[d]	20.5[d] 20.2[e]	Mouse	[57]
$GRAB_{OT1.0}$	OT	bOTR	cpEGFP	~392%[d] ~454%[e]	1.6[d] 3[e]	Mouse, rat	[58]
$GRAB_{CRF1.0}$	CRF	CRF1R	cpEGFP	~1205%[d] ~1334%[e]	33[d] 18[e]	Mouse	[59]
$GRAB_{SST1.0}$	SST	SSTR5	cpEGFP	~431%[d] ~447%[e]	70[d] 130[e]	Mouse	[59]
$GRAB_{CCK1.0}$	CCK	CCKBR	cpEGFP	~844%[d] ~907%[e]	4[d] 5.5[e]	ND	[59]
$GRAB_{NPY1.0}$	NPY	NPY1R	cpEGFP	~490%[d] ~424%[e]	40[d] 0.7[e]	ND	[59]
$GRAB_{NTS1.0}$	NTS	NTSR1	cpEGFP	~380%[d]	6[d]	Mouse	[59,60]
$GRAB_{VIP1.0}$	VIP	VIPR2	cpEGFP	~357%[d] ~324%[e]	120[d] 19[e]	ND	[59]
grpLight1.2	GRP	GRPR	cpEGFP	~350%[d]	103[d]	Mouse	[61]
GLPLight1	GLP-1	GLP1R	cpEGFP	~528%[d] ~455%[e]	27.6[d] 9.3[e]	ND	[62]
M-SPOTIT1.1	μ-opioid	MOR	cpEGFP	~570% (S/N)[d] (fentanyl) ~460%(S/N)[e] (fentanyl)	15[e] (fentanyl)	ND	[64]
M-SPOTIT2	μ-opioid	MOR	cpEGFP	~850% (S/N)[d] (fentanyl) ~680%(S/N)[e] (fentanyl)	30 ± 10[d] (fentanyl)	ND	[65]
NOPLight	N/OFQ	NOPR	cpEGFP	~388%[d] ~378%[e]	28.65 ± 5.1[d] 42.81 ± 5.4[e]	Mouse	[66]

NPY, neuropeptide Y; ANF, atrial natriuretic factor; OT, oxytocin; VIP, vasoactive intestinal peptide; AVP, arginine vasopressin; SST, somatostatin; N/OFQ, nociceptin/orphanin-FQ peptide; GRP, gastrin-releasing peptide; GLP-1, glucagon-like peptide-1; CRF, corticotropin-releasing factor; CCK, cholecystokinin; NTS, neurotensin; hOTR, human oxytocin receptor; meOTR, medaka oxytocin receptor; bOTR, bovine oxytocin receptor.

[a] Measured in PC12 cells with the treatment of high K^+.

[b] Measured in the *Drosophila* larval neuromuscular junction with the treatment of electrical stimulation.

[c] Measured in rat slices with optogenetic stimulation.

[d] Measured in cell lines with the application of ligands.

[e] Measured in cultured neurons with the application of ligands. ND, not determined.

GPCR activation–based (GRAB) sensors

In addition to activating downstream signaling pathways, GPCRs also undergo a rapid outward movement on transmembrane 6 (TM6) upon ligand binding [43,44]. Building on the context-based sensitivity of circularly permutated green and red fluorescent proteins (cpEGFP and cpRFP, respectively), researchers have developed sensors in which this ligand-induced change in GPCR conformation causes a detectable change in fluorescence. This so-called GPCR activation–based

(GRAB) approach has been used successfully to develop genetically encoded sensors for detecting a wide range of neurochemicals, including acetylcholine [45,46], monoamines [47–52], purines [53,54], and cannabinoids [55]. In addition to small molecules, several sensors have also been generated to detect neuropeptides such as orexin [56], oxytocin [57,58], CRF [59], somatostatin (SST) [59], CCK [59], neurotensin (NTS) [59,60], NPY [59], VIP [59], GRP [61], glucagon-like peptide-1 (GLP-1) [62], and opioids [50,63–66] in cultured cell lines, cultured neurons, isolated tissues, and/or freely moving rodents with high specificity and spatiotemporal resolution.

Here, we use our recently reported $GRAB_{OT1.0}$ oxytocin sensor as an example to illustrate how GRAB neuropeptide sensors are designed, optimized, and then applied both *in vitro* and *in vivo* [58]. $GRAB_{OT1.0}$ consists of the oxytocin receptor as the ligand-recognition module connected via linker sequences to cpEGFP as the fluorescent module. To generate this sensor, a wide range of oxytocin receptors derived from various vertebrate species were systematically screened by inserting the fluorescent module into the receptor's third intracellular loop (ICL_3). The performance of each chimeric protein was then measured with respect to its membrane trafficking, brightness, and maximum change in fluorescence (i.e., peak $\Delta F/F_0$) in response to oxytocin. Based on the results of this screen, an intermediate version (referred to as $GRAB_{OT0.5}$) based on the bovine oxytocin receptor was first identified, and then optimized by truncating the ICL_3 to modify the cpEGFP insertion site, resulting in the $GRAB_{OT1.0}$ sensor, which produces a peak $\Delta F/F_0$ of ~400% and has a high affinity for oxytocin, with a median EC_{50} of ~3 nM. Using pharmacology combined with two-photon imaging in acute brain slices, we then used $GRAB_{OT1.0}$ to show that the molecular mechanisms underlying oxytocin release differ between axonal and somatodendritic compartments. We also showed that $GRAB_{OT1.0}$ had a specific dosage-dependent response to increasing concentrations of oxytocin injected intraventricularly and had a robust $\Delta F/F_0$ in response to the optogenetically induced release of endogenous oxytocin in freely moving mice and rats. Importantly, we also used $GRAB_{OT1.0}$ to identify differences in the dynamics of oxytocin release in discrete brain regions in male mice during specific mating behaviors [58]. So far, other GPCR-activation based neuropeptide sensors have been developed using corresponding backbone receptors (Table 1) but sharing similar construction strategies, including the optimization on insertion sites, linker length, and the cpEGFP module.

In summary, the GPCR activation–based strategy for designing genetically encoded sensors provides a highly sensitive set of tools for detecting specific neuropeptides with relatively high spatiotemporal resolution and minimal invasiveness, making these sensors well suited for *in vivo* applications.

Future directions

Despite the important physiological roles that neuropeptides play in both health and disease, many fundamental questions regarding their regulation and release remain poorly understood due to a lack of sufficiently precise sensors. In this review, we summarized the pros and cons of currently available techniques for detecting neuropeptides, suggesting that each method has its own specific strengths and can be used judiciously in various platforms to meet the needs of specific applications.

Although the repertoire of optical tools has been expanded to include sensors for a wide range of neuropeptides with a large dynamic range *in vitro*, several key neuropeptides still remain undetectable. However, given that the conformational change upon ligand binding is common among GPCRs, GRAB strategies can be exploited to develop new sensors for detecting additional neuropeptides that bind to GPCRs. Interestingly, transplanting the cpEGFP module and linker sequences from previously optimized GRAB sensors into the ICL_3 of other peptide GPCRs has been used to expedite the development of new neuropeptide sensors, as we discussed in our recent work of genetically encoded neuropeptide sensors [59]. With respect to peptides that bind to receptor tyrosine kinases (RTKs) such as insulin and neurotrophic factors, the activation-induced conformational change between RTK subunits can be exploited to develop sensors based on fluorescence resonance energy transfer (FRET) and/or bioluminescence resonance energy transfer (BRET) [67,68].

Despite their many advantages, the recently developed GRAB sensors can lack sufficient sensitivity to detect endogenous peptides at low concentrations. Thus, improvements in both their dynamic range and their apparent affinity should be introduced into the next generation sensors. Several optimizing approaches are possible, for example, screening GPCRs derived from other subtypes and/or species, performing random mutagenesis in the linker sequences and cpEGFP module, and structure-guided optimization of the ligand-binding site. Furthermore, some naturally occurring GPCRs may lack the specificity to distinguish analogous neuropeptides derived from a common precursor; thus, using rational design to modify critical residues in the ligand-binding pocket based on resolved and/or predicted structures may help increase the selectivity of these sensors. In addition, developing ratiometric fluorescent neuropeptide sensors can help reduce non-specific artifacts and can be used to quantify

extracellular concentrations. Finally, expanding the spectra of GRAB sensors can allow the excitation and emission wavelengths to penetrate deeper into tissues, increase the ability to image several neurochemicals simultaneously, and provide compatibility with opsin-based optogenetic tools. Large-scale screening of variants is required for these directions, and the development of a high-throughput screening platform in mammalian systems is expected to accelerate the process [69].

Combining these cutting-edge indicators with high-resolution imaging tools such as one-photon and multi-photon microscopy will provide researchers with the tools needed to solve many important questions regarding peptidergic transmission. For instance, these tools can be used to examine whether inhibitory interneurons release neuropeptides in both cortical and hippocampal microcircuits, and—if so—what roles they play in processing sensory information, learning, and memory. Moreover, the presence of both neuropeptides and classic neurotransmitters in the same neuron is ubiquitous throughout the central nervous system [70], yet neuropeptides produced by specific cell types may provide pharmacologically specific and controllable modulation of surrounding neurons, unlike widespread small-molecule transmitters. Thus, understanding the molecular mechanisms that underlie neuropeptide release may provide valuable new insights into the precise control of the functional properties of neural circuits, ultimately revealing new, highly specific drug targets for treating neurological diseases.

In summary, current and future methods for detecting neuropeptides offer a clear path to increasing our understanding of peptidergic signaling at the molecular, circuit, and whole-brain levels under both physiological and pathophysiological conditions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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- • of special interest
- •• of outstanding interest

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