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New optical methods for detecting monoamine neuromodulators

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Abstract

Monoamine neuromodulators such as dopamine, norepinephrine, serotonin (5-HT), octopamine, and tyramine are signaling molecules in the nervous system, where they play critical roles in both health and disease. Given the complex spatiotemporal dynamics, similar structural features, and multiple receptors, studying their dynamics has been limited using conventional methods such as microdialysis and electrochemistry. However, recent advances in optics have facilitated the development of imaging-based detection methods. In this review, we summarize current detecting approaches for specific monoamines, emphasizing their design strategies, detection properties, applications, and limitations. We highlight the genetically encoded GPCR–based sensors for DA and NE, which have high signal-to-noise ratio, selectivity and can be used *in vivo* in different living organisms. Finally, we discuss the potential for using this approach to generate new neuromodulator sensors with nonoverlapping spectra, which will ultimately pave the way for studying the interplay among various neuromodulators and neurotransmitters.

Keywords

Monoamine neuromodulators; Genetically encoded GPCR-based sensor

Introduction

The human brain performs a highly versatile array of functions via the concerted activity of its several billion neurons. The principal messengers for communication among these neurons is a wide variety of neurotransmitters and neuromodulators, which are released from the presynaptic terminal and are detected by receptors expressed by postsynaptic neurons. Monoamine neuromodulators contain an aromatic ring and an amino group

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connected by a two-carbon chain. They comprise an essential group of neuromodulators that include dopamine (DA), norepinephrine (NE), serotonin (5-HT), octopamine (OA), and tyramine (TA) (Figure 1A). In both vertebrates and invertebrates, these neuromodulators participate in a wide range of physiological processes, including learning, movement, sleep, and memory [1–4]. Impaired monoamine transmission has been correlated with a range of pathological conditions, including Parkinson's disease, attention deficit hyperactivity disorder, depression, and addiction [5–7].

Unlike glutamate (GLU) and γ-aminobutyric acid (GABA) that mediate fast synaptic transmission by targeting ionotropic receptors, monoamine neuromodulators primarily target metabotropic G protein–coupled receptors (GPCRs), thereby regulating distinct downstream signaling pathways. Although much is currently known regarding the properties of monoamine neuromodulators, their precise spatiotemporal dynamics during specific brain functions cannot be measured using traditional electrophysiological methods and are therefore poorly understood. To measure monoamines *in vivo*, several methods have been developed in recent decades, including analytical chemistry–based tools and electrochemical methods. Thanks to the recent advances in fluorescence imaging techniques, both genetically encoded sensors [8–10] and a nongenetically encoded nanosensor [11] have also been developed. In this review, we summarize the recent progress with respect to monitoring various monoamines (Table 1).

Dopamine

DA plays a key role in modulating motor control, reward signaling, decision making, and attention [1,2]. Impaired dopaminergic transmission has been correlated to several pathological conditions, including Parkinson's disease, addiction, and various psychiatric disorders [5]. Several approaches have been used to detect DA, including microdialysis [12,13], electrochemical methods [14,15], and cell-based methods [16]; however, these techniques have either low spatiotemporal resolution or insufficient molecular selectivity, as discussed in the following sections.

Fluorescent false neurotransmitters (FFNs) are a family of synthesized neurotransmitter analogs, which can be loaded into synaptic vesicles of mono-aminergic neurons or dopaminergic neurons through vesicular monoamine transporter 2 or dopamine transporter [17–19]. Loaded FFNs are irreversibly released along with synaptic vesicles and this vesicular destaining process is used as a readout of DA transmission. FFNs have been used to monitor electrically or pharmacologically evoked DA release from individual presynaptic terminals in cultured neurons and brain slices. However, the preloading and washing processes have limited cell-type specificity and are not amenable for *in vivo* studies. In addition, FFNs themselves do not report neurotransmitter identity, and the depletion of loaded FFNs makes it challenging for repetitive measurement over time.

The Tango assay is designed to report GPCR activation through the expression of reporter genes [20]. This assay includes two critical components: (i) a GPCR tandemly attached to a transcription factor via a tobacco etch virus cleavage site and (ii) a β-arrestin fused to tobacco etch virus protease. Activation of the GPCR recruits β-arrestin and triggers

the release of the transcription factor, which then drives the expression of a reporter gene. The Tango assay has been used to identify circuit-level dopaminergic modulation in the fly brain [21]. The next-generation iTango2 [22] and SPARK [23] reporters provide temporally precise labeling by similarly introducing a light-gated motif into the system. The interchangeable reporter gene in this system significantly expands its applications with respect to both the reporter protein and the ability to incorporate chemogenetics/optogenetics; thus, this system has been used to monitor and manipulate behaviorally relevant DA-responsive neuronal populations in mice *in vivo*. On the other hand, the relatively long gene expression time (in the order of hours) precludes its use in detecting rapid dynamic changes and in longitudinal experiments.

Recently, two series of genetically-encoded single-fluorophore DA sensors are developed by introducing an environment-sensitive circularly permutated enhanced green fluorescent protein (cpEGFP) into the third intracellular loop of the DA receptors (Figure 1C) called $GRAB_{DA}$ [10] and dLight [9]. A pair of D2 receptor–based sensors with either medium ($GRAB_{DA1m}$, EC_{50}: 130 nM) or high ($GRAB_{DA1h}$, EC_{50}: 10 nM) apparent DA affinity were used to rapidly and specifically detect DA *in vivo* in several living organisms, including flies, fish, mice, and songbirds [24]. In addition, the family of D1, D2, and D4 receptor–based sensors (dLight1.1 to dLight1.5) has an apparent DA affinity in the nanomolar to micromolar range, and both dLight1.1 and dLight1.2 have been used *in vivo* in mice. Both the $GRAB_{DA}$ and dLight sensors inherit the intrinsic selectivity for DA over NE from the corresponding scaffold DA receptors, with the D2-based $GRAB_{DA}$ sensor and the D1-based dLight1.1 sensor (Figure 1D) having 10-fold and 60-fold higher affinity for DA compared with NE. In addition, they have minimal effect on cellular physiology due to the negligible coupling with the downstream signaling pathways of GPCRs.

Recently, a near-infrared catecholamine (nIRCat) carbon nanoparticle–based probe was developed (Figure 1B) [11]. This sensor has an emission spectrum of 1000–1300 nm, which has minimal light scattering, and is therefore suitable for imaging deep tissues. nIRCat has been used to detect striatal DA release in brain slices with temporal resolution comparable with that of electrochemical methods. Comparing with GPCR-based fluorescent sensors, nIRCat has limitations with respect to selectivity for DA over NE and lacks cell-type specificity, but it has unique advantages to be useable in the presence of DA receptor ligands. Given the nongenetically encoded nature, it also provides an additional tool for studying organisms that are currently not amenable to genetic manipulation; moreover, it serves as an orthogonal approach that can be combined with protein-based sensors in several model organisms.

Norepinephrine

NE, a key biogenic monoamine neuromodulator, is generated from dopamine by the enzyme dopamine β-hydroxylase. Consistent with the extensive projections of noradrenergic neurons from the locus coeruleus to the entire brain, NE plays an important role in a wide range of physiological processes throughout the central nervous system, including modulating sensory information, regulating attention, and controlling both sleep and arousal [3]. It is therefore not surprising that impaired noradrenergic signaling has been linked to a wide

range of psychiatric disorders and neurodegenerative diseases, including stress and anxiety [6].

In the past few decades, scientists have studied the dynamics of NE *in vivo* using low-throughput microdialysis coupled with biochemical analysis [25]. Taking advantage of the oxidative nature of monoamine neuromodulators such as NE, electrochemical amperometry can be used to detect these molecules by applying a steady voltage potential and quantifying the oxidative currents. Although amperometry is sensitive and offers high temporal resolution, the relatively high voltage applied can also oxidize many other molecules, thus limiting the molecular selectivity of this approach. With the improved electrochemical method fast-scan cyclic voltammetry (FSCV) monoamines are scanned using a specific voltage wave, and the chemical can be identified by analyzing the specific shape of the voltammogram; nevertheless, FSCV is unable to distinguish between structurally similar monoamines such as NE and DA [15,26] or OA and TA [27] (Figure 1A).

Optical imaging methods have emerged as an ideal alternative, providing high spatial resolution and minimal invasiveness. For example, HEK293 cells stably expressing a cell-based neurotransmitter fluorescent engineered reporter (CNiFER) have been implanted in mice and used to detect NE and DA in real time [16]. The NE reporter α_{1A}-CNiFER is an engineered cell line that expresses the α_{1A} adrenergic receptor (α_{1A}R) as the NE-sensing module, a chimeric Gq protein that couples α_{1A}R activation to stimulation of Phospholipase C and increase of cytosolic Ca^{2+} concentration, and the Förster resonance energy transfer (FRET)–based Ca^{2+} sensor TN-XXL as the reporter. The NE reporter a1A -CNiFER responds to NE with an EC_{50} of approximately 19 nM which is 70-fold more sensitive to NE compared with DA. After the cells are implanted in the mouse's target brain region, they can be used to detect NE dynamics *in vivo*. On the other hand, the use of a tumor-derived cell line limits subcellular spatial specificity to approximately 100 μm and has the potential for inducing an undesired immune response.

These limitations can be overcomed using a genetically encoded probe, which provides cell-type specificity for monitoring neuromodulator dynamics [28]. One such example is sensors based on FRET; however, these sensors have a relatively low signal-to-noise ratio, which limits their applicability, particularly *in vivo* [29,30]. Recently, a single-fluorophore GPCR-based NE sensor called $GRAB_{NE}$ was generated by inserting a single cpEGFP into the third intracellular loop of the α_2 adrenergic receptor (Figure 1C), yielding a 230% fluorescence increase in response to NE [8], with a 700-fold higher sensitivity to NE compared with DA (Figure 1E). Importantly, $GRAB_{NE}$ has been used to rapidly and specifically detect NE with high spatiotemporal resolution in a variety of systems, including cultured cells, awake zebrafish larvae, and freely behaving mice.

Serotonin

Serotonin is highly conserved from invertebrates to humans and plays a key role in a wide range of physiological processes, including mood control, appetite, the sleep–wake cycle, learning, and memory [4]. Malfunction of serotonergic system has been linked to several psychiatric disorders, including depression [7], and many clinically prescribed

antidepressants target the serotonergic system (e.g., the selective serotonin reup-take inhibitors).

Combining microdialysis with high-performance liquid chromatography and mass spectrometry is a widely used technique for the continuous *in vivo* detection of various biomolecules in mammals, including 5-HT [31] and other monoamine neuromodulators. Using this approach, a microdialysis probe of ~200-μm diameter is implanted into the brain region of interest, and biomolecules are collected via passive diffusion through the probe's semipermeable membrane. Although this technique can achieve nanomolar-level sensitivity, the relatively slow sampling rate of approximately 10 min makes it unsuitable for monitoring the rapid dynamic changes of 5-HT, which occur on the order of seconds or even subseconds.

Another powerful tool for detecting 5-HT is FSCV, which can be used to distinguish between 5-HT and other monoamine neuromodulators with high temporal resolution in mouse brain slices and in freely moving mice during social behaviors [32,33]. However, because the spatial resolution depends on both the size (~70–300 μm in diameter) and position of the implanted probe, it can only report volume-averaged signals without providing cell specificity.

Yet, another tool is the genetically encoded FRET-based 5-HT sensor 5HT-CC, which was engineered by inserting two fluorescent proteins, one in the third intracellular loop and one in the C-terminus of the 5-HT1b receptor [34]. This sensor has good selectivity for 5-HT and produces an approximately 4% change in the FRET ratio in response to a saturated concentration of 5-HT, with an EC_{50} of approximately 428 nM. To date, the 5HT-CC sensor has not been used in *in vivo* applications, possibly because of the relatively low signal-to-noise ratio associated with FRET-based sensors.

Octopamine and tyramine

Both OA and TA are key monoamine neuromodulators in invertebrates, serving as counterparts to the adrenergic neuromodulators present in vertebrates [35]. Both OA and TA are derived from tyrosine, and TA is the precursor of OA. The physiological roles of OA in the invertebrate nervous system have been well documented, showing that OA controls a wide range of processes, including the sleep–wake cycle, oviposition, aggression, and associative learning. In contrast, the role of TA is less known; however, studies of mutant *Drosophila* and *Caenorhabditis elegans* have revealed that TA plays a role in several processes, including olfaction, courtship, movement, and cocaine sensitization. Both OA and TA are classified as trace amines in the nervous system of vertebrates, as they are approximately 100-fold less abundant than other monoamine neuromodulators [36]. Although the function of trace amines in vertebrates is currently unclear, the identification of their respective GPCRs in the central nervous system supports the notion that they function as neuromodulators [37].

Previously, OA and TA were measured in homogenates prepared from the *Drosophila* brain using a combination of separation and detection techniques. Initially, gas chromatography

was coupled with mass spectrometry and used to detect several monoamines in the *Drosophila* brain; interestingly, TA was detected, whereas OA was not detectable [38]. Subsequently, high-performance liquid chromatography [39] and micellar electrokinetic capillary chromatography [40] have been used to isolate monoamines from samples, with highly sensitive electrochemical amperometry used for detection. Given the low molecular specificity associated with amperometry, additional genetic and/or pharmacological approaches are needed to confirm the chemical's identity. Overall, methods used to separate and detect chemicals from homogenates provide a snapshot of the amounts of OA and TA present in the brain; however, the preparation is highly destructive and precludes taking repeated measurement in individual animals.

Recently, researchers used electrochemical techniques to monitor the endogenous release of OA from intact tissues such as the *Drosophila* larval body wall and ventral nerve cord [41,42]. Importantly, FSCV can be used to distinguish OA and TA from other monoamines, but it cannot distinguish between OA and TA, as — similar to DA and NE — they differ by only one hydroxyl group (Figure 1A) [27,43].

Conclusions and future perspectives

Detecting monoamine neuromodulators in freely behaving animals is essential for understanding the role that these chemicals play in both health and disease. Given their release mode and chemical nature, probing the dynamics of monoamine neuromodulators in the intact nervous system has been challenging. First, monoamine neuromodulators — particularly DA — have two release modes: (i) constitutive tonic release, which is induced by the spontaneous activity of dopaminergic neurons, thereby setting the basal level; and (ii) phasic release, which is triggered by action potentials and typically lasts several seconds [44]. Thus, a rapid and robust detection method is needed to fully capture both release modes. A second challenge is that once released from the nerve terminal, monoamine neuromodulators are not restricted to the synaptic cleft, but can diffuse and reach neighboring cells and/or synapses [45]. Because the dynamics of this volume transmission has not been characterized, a suitable probe should have high spatial resolution, ideally in the subcellular range. Finally, neurons that contain different neuromodulators are intermingled in the same brain area; for example, both DA and NE are released in the prefrontal cortex [46]. To tease apart the interplay between structurally similar monoamine neuromodulators the sensors used should have sufficient molecular selectivity.

Except for aforementioned DA, NE, 5-HT, OA and TA, there are some additional monoamine neuromodulators in the nervous system, including histamine as well as trace amines (e.g. phenethylamine and tryptamine). The dynamics of these monoamines can also be studied with existing detection methods. For example, both electrochemistry [47] and Tango assays [48] have been adopted to monitor histamine dynamics in fly and in mouse. However, classic methods used to detect monoamines, including microdialysis, amperometry/FSCV, CNiFER, FFN, and Tango assay, all lack sufficient spatiotemporal resolution and/or molecular selectivity. Moreover, FRET-based sensors have limited dynamic range and are less suitable for use in tissues and *in vivo* imaging. On the other

hand, the recently developed nongenetically encoded nanosensor nIRCat uses low-scattering infrared fluorescence, but cannot distinguish DA and NE.

A promising new strategy is the development of single-fluorophore GPCR-based sensors, which has been used successfully to generate sensors for detecting both DA and NE, thereby providing a wide dynamic range, rapid kinetics, single-cell resolution, and — most importantly — exquisite selectivity. Given the large variety of neuromodulator/neurotransmitter-sensing GPCRs in the nervous system, this strategy can be used to develop sensors for a wide range of molecules, including 5-HT, OA, and TA. Moreover, by attaching different fluorescent proteins, sensors with nonoverlapping spectra can be developed and then used to study the complex interplay between different neuromodulators. Thus, using simultaneous multicolor imaging to measure the release of different monoamines in real time will provide important insights into how these neuromodulators function in the brain.

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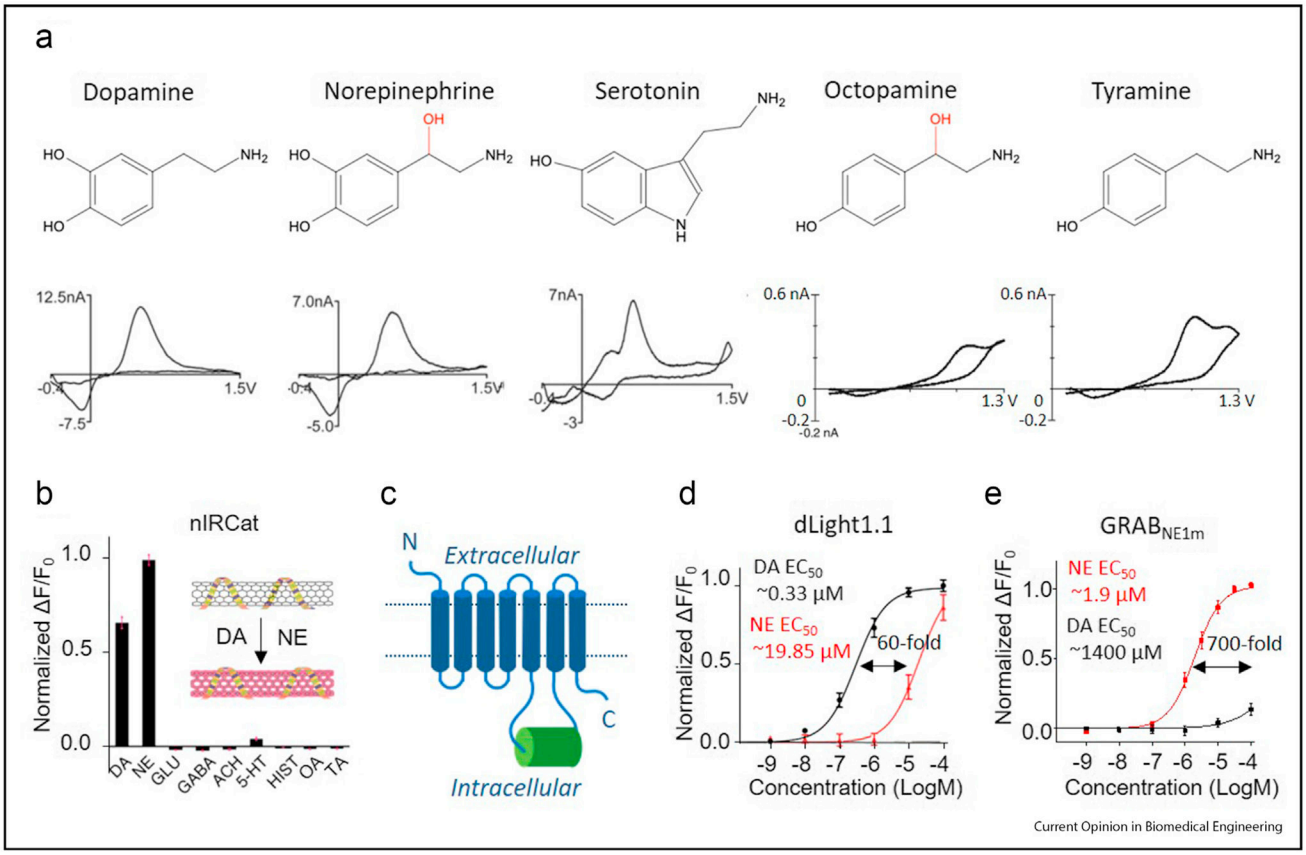

Figure 1.

Distinguishing monoamine neuromodulators using fast-scan cyclic voltammetry (FSCV) and GPCR-based single-fluorophore sensors **(a)** The chemical structures (top) and corresponding FSCV voltammogram traces (bottom) of the indicated monoamines [27,57]. The hydroxyl group that differs between DA and NE and between OA and TA is shown in red **(b)** Schematic diagram of carbon nanoparticle–based catecholamine sensor nIRCat and its normalized response to 100 mM DA, NE, GLU, GABA, acetylcholine (ACH), 5-HT, histamine (HIST), OA and TA [11] **(c)** Schematic diagram of a generic GPCR-based single-fluorophore sensor; the fluorophore is inserted in the third intracellular loop and is shown in green (**d** and **e**) Dose–response curves for the dLight1.1 expressed in HEK293T cells [9] and $GRAB_{NE1m}$ expressed in cultured cortical neurons [8] in response to DA and NE; the difference in EC_{50} between DA and NE is also shown for each sensor. (Figures are modified from original research paper with permission from the publisher).

Table 1

Overview of the currently available tools and techniques for detecting monoamine neuromodulators.

	DA	NE	5-HT	OA &TA
GC/HPLC/MEKC-electrochemistry/MS	Musshoff et al., 2000 [49] Carrera et al., 2007 [50]	Kuhlenbeck et al., 2000 [51] Carrera et al., 2007 [50]	Carrera et al., 2007 [50]	Watson et al., 1993 [38] Powell et al., 2005 [40] Hardie et al., 2006 [39]
Electrochemistry	Robinson et al., 2008 [15] Dreyer et al., 2016 [14]	Robinson et al., 2008 [15] Bucher et al., 2015 [26]	Marcinkiewcz et al., 2016 [32] Saylor et al., 2019 [33]	Cooper et al., 2009 [27] Fang et al., 2011 [43] Majdi et al., 2015 [42] Pyakurel et al., 2016 [41]
Microdialysis-biochemistry	Gu et al., 2015 [12] Nesbitt et al., 2015 [13]	McReynolds et al., 2010 [25]	Gardier et al., 2013 [31]	N.A.
FFNs	Gubernator et al., 2009 [17] Rodriguez et al., 2013 [19] Pereira et al., 2016 [52]	N.A.	Henke et al., 2018 [52]	N.A.
Tango assay	Barnea et al., 2008 [20] Inagaki et al., 2012 [21] Lee et al., 2017 [22] Kim et al., 2017 [23]	Hanson et al., 2009 [53] Kroeze et al., 2015 [54]	Hanson et al., 2009 [53] Kroeze et al., 2015 [54]	N.A.
CNiFER	Muller et al., 2014 [16]	Muller et al., 2014 [16]	Yamauchi et al., 2011 [55]	N.A.
FRET-based sensors	N.A.	Hoffmann et al., 2005 [29] Vilardaga et al., 2003 [30]	Candelario et al., 2012 [34]	N.A
GRAB/dLight	Sun et al., 2018 [10] Patriarchi et al., 2018 [9]	Feng et al., 2019 [8]	N.A.	N.A.
Nanosensor	Kruss et al., 2017 [56] Beyene et al., 2019 [11]	N.A.	N.A.	N.A.

DA, dopamine; NE, norepinephrine; 5-HT, 5-hydroxytryptamine (serotonin); OA, octopamine; TA, tyramine; N.A., not available; GC, gas chromatography; HPLC, high-performance liquid chromatography; MEKC, micellar electrokinetic capillary chromatography; MS, mass spectrometry; FFNs, fluorescent false neurotransmitters; CNiFER, cell-based neurotransmitter fluorescent engineered reporter; FRET, Förster resonance energy transfer; GRAB, GPCR activation-based sensor.