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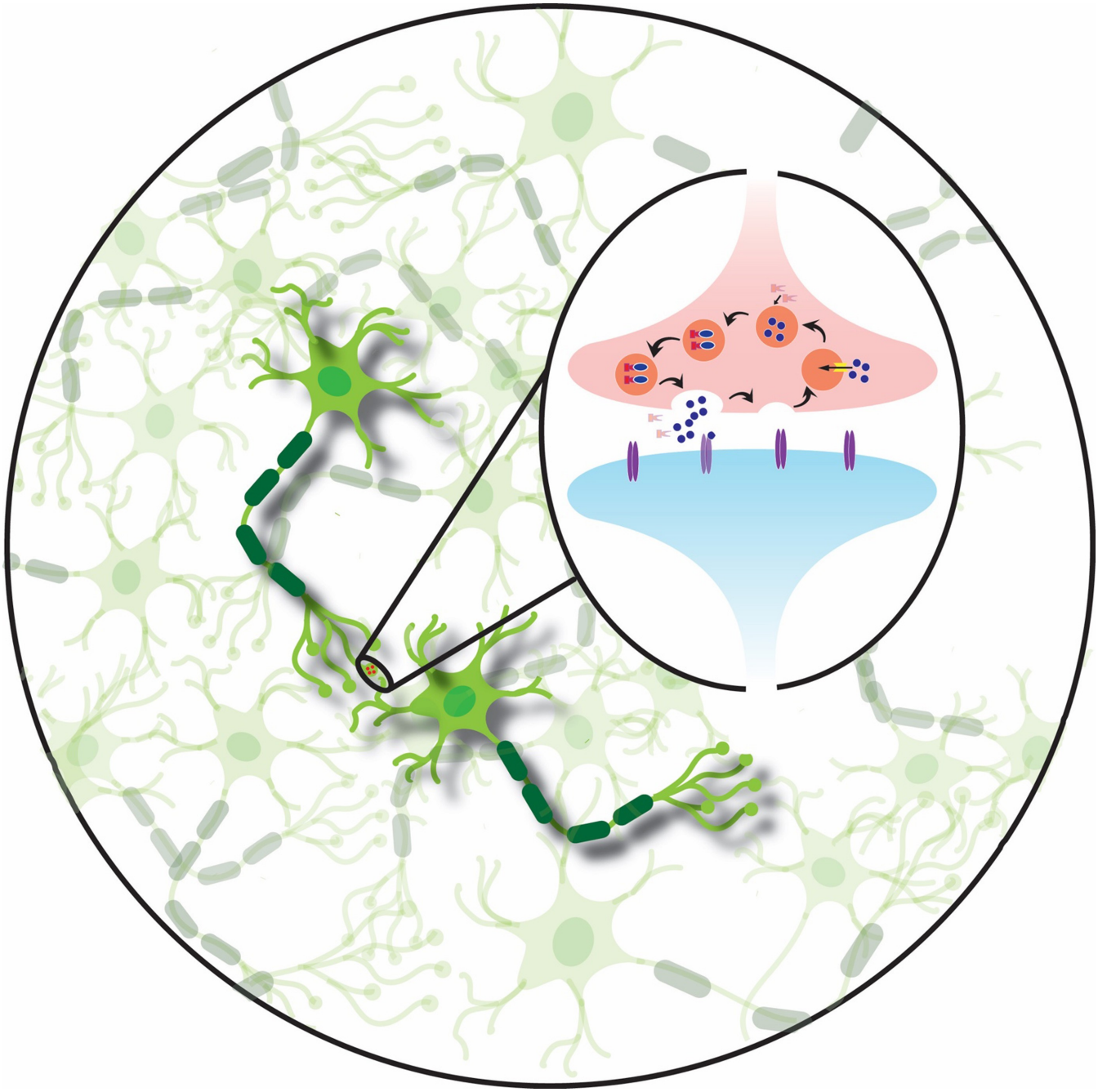
Neurotransmitter Sensors

Imaging Neurotransmitters with Small-Molecule Fluorescent Probes

*Anusha Bade, Peeyush Yadav, Le Zhang, Ramesh Naidu Bypaneni, Ming Xu, and Timothy E. Glass**



Abstract: Neurotransmitters play a crucial role in regulating communication between neurons within the brain and central nervous system. Thus, imaging neurotransmitters has become a high priority in neuroscience. This minireview focuses on recent advancements in the development of fluorescent small-molecule fluorescent probes for neurotransmitter imaging and applications of these probes in neuroscience. Innovative approaches for probe design are highlighted as well as attributes which are necessary for practical utility, with a view to inspiring new probe development capable of visualizing neurotransmitters.

1. Introduction

Neurotransmitters are endogenous small-molecule chemical messengers synthesized within neurons and stored in synaptic vesicles at high concentration (0.2–1.0 M).[1] Synaptic vesicles containing neurotransmitters undergo a series of processes, including docking and priming, as they move towards the axon terminal (Figure 1).[2] When an action potential occurs, these vesicles fuse with the membrane proteins, leading to the release of neurotransmitters into the synaptic cleft through exocytosis.[3] Released neurotransmitters traverse the synaptic cleft to bind to postsynaptic receptors on another neuron, triggering a new impulse.[4] The vesicles are recycled by endocytosis and eventual refilling. Neurotransmitters are categorized based on their difference in chemical structures,[5] including catecholamines like dopamine (DA), norepinephrine (NE) and epinephrine (EP); amino acids such as glutamate (Glu) and γ-aminobutyric acid (GABA); aromatic monoamines which include serotonin and histamine; and acetylcholine (Ach) (Figure 2). In addition, there are other types of neurotransmitters like peptide neurotransmitters, purine-based neurotransmitters and gasotransmitters which are outside the scope of this review.

Each neurotransmitter serves a different role.[6] Dopamine is associated with motivation, reward, and pleasure,[7] while serotonin plays a key role in regulating mood and digestion.[8] Norepinephrine affects attention and the response to stress.[9] Acetylcholine is involved in muscle movement and cognitive abilities.[10] Glutamic acid, the main excitatory neurotransmitter, is crucial for cognitive functions such as learning and memory.[11] In contrast, GABA, the primary inhibitory neurotransmitter in the brain, helps manage fear and anxiety.[12] Abnormal levels of neurotransmitters are associated with various neurological diseases. Therefore, measurements of the distribution, uptake, storage, release and utilization of neurotransmitters are of substantial significance to uncover novel biological pathways for signal transmission and investigate the mechanism behind neurological diseases. Thus, a great number of methods have been developed for the detection of neurotransmitters. Among these techniques, capillary electrophoresis is notably useful with high resolution and efficiency at low concentrations of neurotransmitters.[13] However, this method is rather invasive. The emergence of fluorescent imaging has significantly addressed this challenge, and offers clear advantages compared to other methods for non-invasive detection of neurotransmitters with spatial resolution.[14]

We note that there are well-developed genetically-encoded fluorescent probes for monitoring

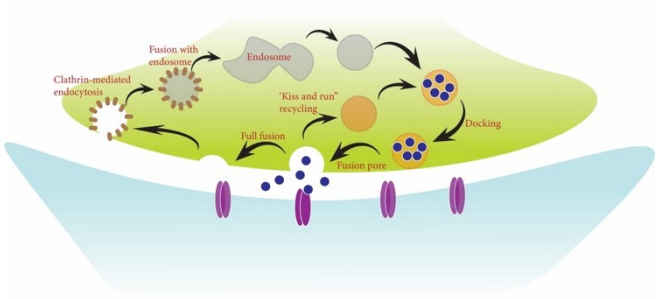


Figure 1. The life cycle of synaptic vesicles.

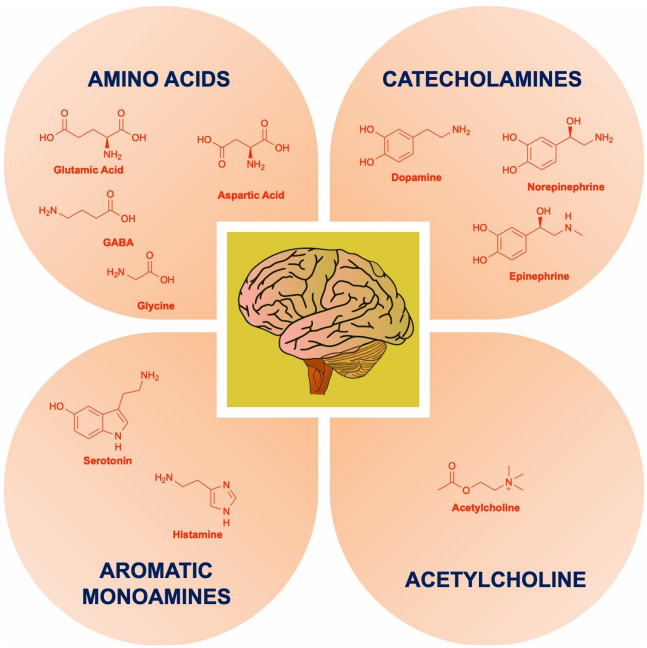


Figure 2. Structures of representative neurotransmitters.

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neurotransmitters.[15] Genetically-encoded fluorescent probes can achieve remarkable selectivity for targeted neurotransmitters by incorporating specific neurotransmitter receptors.[16] These probes can be localized within particular cellular compartments or selectively expressed in specific cell types to monitor neurotransmitter release or uptake at precise locations,[17] which could minimize cross-reactivity with other neurotransmitters and biogenic or cellular molecules. Furthermore, some genetically-encoded fluorescent probes demonstrate robust resistance to photobleaching, allowing for prolonged imaging processes under microscope.[18] Yet they are limited by the requirement for genetic modification of organisms. Small-molecule fluorescent probes offer complementary advantages. Cell permeable probes are simple to use and have the capability to monitor neurotransmitters across diverse cellular compartments and environments by freely diffusing across cell membranes, therefore enabling them to interact with neurotransmitters wherever they are present rather than being limited to specific locations.[19] This versatility allows them to provide a broad view of neurotransmitter dynamics.[20]

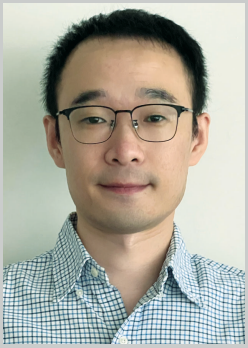
In the past few years, increasing numbers of fluorescent probes have been developed and used to image neurotransmitters in live cells, tissue and animals. This minireview aims to examine recent advancements in the development of small-molecule fluorescent probes for imaging neurotransmitters. We explore the methodologies involved in designing fluorescent probes that target various neurotransmitters. Consideration is given to properties that are required for practical utility. Lastly, we delve into innovative strategies for constructing new scaffolds, providing insights to inspire the creation of new more sophisticated probes.

1.1. Significant Challenges and Opportunities

One of the unusual opportunities of neurotransmitters is that they are stored in vesicles at high concentration (0.2–1 M).[1] Even when expelled into the synapse, their concentration can reach 1 mM.[21] When designing a useful sensor, it is common to try to match the dissociation constant of the sensor to the average concertation of the analyte. In this case, an ideal sensor would not need to have high affinity.

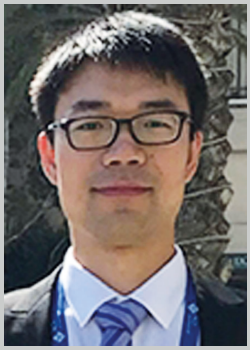
Anusha Bade received her Ph.D degree under supervision of Professor Timothy E. Glass at University of Missouri – Columbia in the United States. She subsequently joined Eurofins Biopharma Product Testing – Columbia as a Senior Scientist II. Her research interests include development of fluorescent probes targeting biological molecules, supramolecular chemistry and ^{14}C radiolabelling.

Peeyush Yadav received his Master's in Chemistry from University of Delhi, India. He moved to the US and joined Prof. Timothy Glass' research group at University of Missouri-Columbia as a doctoral student. His main research interest is the design and synthesis of novel fluorescent probes for neurotransmitters.



Le Zhang obtained Ph.D. degree under guidance of Professor Timothy E. Glass at University of Missouri-Columbia in United States. He subsequently joined Professor Eric V. Anslyn research group at the University of Texas at Austin as a postdoctoral associate and he is currently a postdoctoral associate at the Rockefeller University. His research interests include fluorescent probes development for biological applications, chemoproteomics and protein degradation.

Ramesh Naidu Bypaneni joined the Glass group in 2019 at University of Missouri-Columbia to pursue his Ph.D. in Organic chemistry. His research interests include developing fluorescent probes for neurotransmitters, cell labelling and imaging.



Ming Xu completed his PhD in 2020 at the University of Missouri-Columbia under the supervision of Professor Glass. Currently, University of California, San Diego. His research focuses on chemical biology, synthetic organic chemistry, and drug discovery.

Timothy Glass is the Associate Dean for Research in the College of Arts & Science and Professor of Chemistry at the University of Missouri, Columbia. His work focusses on the design and synthesis of receptors and sensors for biologically important analytes.

Indeed, imaging stored neurotransmitters requires rather low affinity. Moreover, presynaptic vesicles are acidic (pH of 5.5) which provides opportunity for probes to be localized there using weakly basic groups.[22] Finally, there has been some work taking advantage of the fact that the vesicle environment is acidic whereas the synapse itself is at neutral pH. Thus, a probe which has low fluorescence at low pH, but high fluorescence at neutral pH can be used to image exocytosis of neurotransmitters.[23]

The major challenge of neurotransmitters is that they are all amines. Thus selectivity poses significant challenges. For example, catecholamine neurotransmitters share most of the same structural features with subtle variations (Figure 2).[24] Hence, sophisticated considerations are required for probe design to achieve selectivity.

2. Amino Acids

2.1. Glutamate and Aspartate

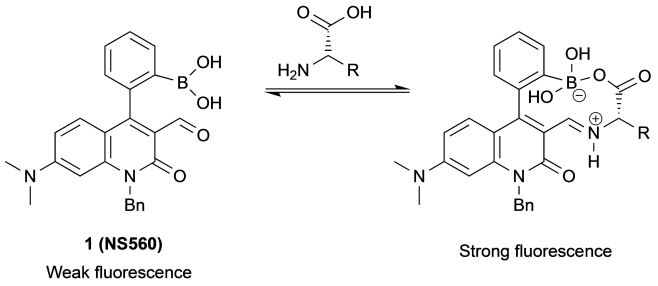
Glutamate and aspartate are structurally quite similar, thus most small molecule sensors do not distinguish them well. The Glass group has pioneered the development of fluorescent sensors for amine-containing compounds based on a coumarin-aldehyde scaffold.[25] Recently, a quinolone-based sensor **1** (NS560) was developed for amino acid detection (Scheme 1).[26] The aldehyde associates with the analyte primary amine group by reversible iminium ion formation and the boronic acid reversibly forms a boronate with the free alpha-carboxylate. The quinolone fluoresces from an internal charge transfer (ICT) state. Formation of the iminium ion stabilizes the ICT state and shifts the wavelength of absorbance from 424 to 462 nm, allowing the bound and unbound forms of the sensor to be independently monitored by appropriate selection of the excitation wavelength. Upon interaction of this sensor with glutamate, a roughly 800-fold increase in fluorescence at 560 nm was observed. NS560 showed good binding affinity towards all amino acids except for proline and tryptophan, hence, it is described as a pan-amino acid sensor. Since glutamate is the most abundant amino acid in the brain, NS560 would elicit strong fluorescence with glutamate compared to other amino acids.

The BODIPY scaffold is very robust and can be manipulated to modulate its fluorescence properties to serve many applications. Using this scaffold, Adhikari et al. developed a fluorescent probe, **2**, for the rapid detection of aspartic acid or glutamic acid in water (Scheme 2).[27] Sensor **2** was constructed by appending two basic groups to the BODIPY core. Sensor **2** is non-fluorescent due to PET quenching from the N,N-diethylamino group. However, addition of aspartic acid or glutamic acid to Sensor **2** results in photoinduced electron transfer (PET) inhibition in water and thus results in fluorescence enhancement of the dye (Scheme 2). Various analytes and amino acids were tested of which only Glu, and Asp resulted in a ~6-fold increase in fluorescence intensity at 524 nm.

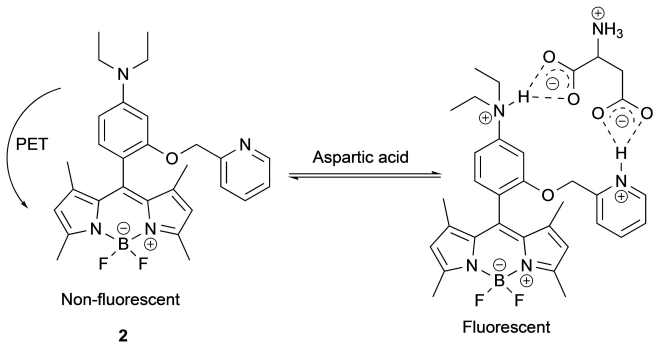
2.2. Glutamate and Zinc Ion

Certain synaptic vesicles of glutamatergic neurons in the mammalian brain co-package zinc ions and glutamate. Evidence shows that the zinc in these neurons may be involved in the control of important synaptic targets like the glutamate receptor and transporter.[28] So it is important to image those synaptic vesicles with both glutamate and zinc. Two representative fluorescent sensors for the detection of glutamate and zinc ion are shown in Scheme 3.[29,30] Compound **3** is a two-input logical gate.[30] This sensor uses an aldehyde for the binding of the amine group from glutamate, and also contains a benzimidazole group which, combined with the two carboxylate groups of the glutamate makes an excellent ligand for zinc ion. Addition of either glutamate or zinc to the sensor produces a fluorescence decrease. However, a 4.5-fold fluorescence increase at 478 nm was observed only when both glutamate and zinc are introduced into the sensor system.

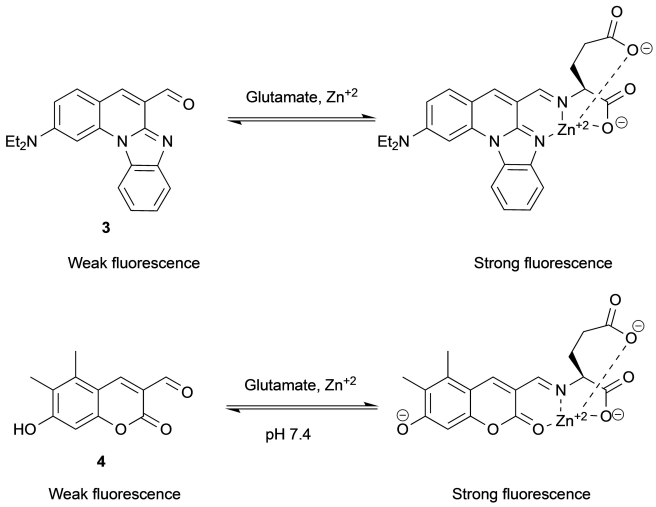
Compound **4** is a fluorescent sensor with a three-input logical gate for the detection of both glutamate and zinc ion at pH 7.4.[31] The aldehyde group in this sensor is used to bind with the amine group from glutamate. When the aldehyde reacts with the amine group, it can provide appropriate binding sites for the coordination of zinc ion. Even with both zinc and glutamate bound, the system has a relatively low fluorescence until the pH is changed from 5.5 to 7.4 as happens upon exocytosis. So, there are three logical inputs for this sensor: 1) addition of glutamate; 2) addition of zinc ion; 3) changing of pH from 5.5 to 7.4. The sensor



Scheme 1. Mechanism of interaction of **1** (NS560) with amino acids.



Scheme 2. Interaction of aspartic acid with sensor **2**.



Scheme 3. The detection mechanisms of sensors **3** and **4** for glutamate and zinc.

system shows an 11-fold fluorescence increase at 470 nm only if all the three logical inputs were achieved. However, when only one or two of these inputs is present only low fluorescence can be detected.

The Pu group has recently synthesized compound **5**, which is both chemoselective and enantioselective towards detection of both L-Glu/L-Asp (Scheme 4).[32] Sensor **5** showed almost 82-fold fluorescence enhancement at 365 nm upon interaction with L-glutamate and 40-fold fluorescence enhancement with L-aspartic acid, but no change with any other amino acid. The proposed binding is rather unusual and utilizes addition of the glutamate carboxylates to each of the aldehyde groups. The sensor was designed to provide discrimination between enantiomers of glutamate and was not intended for cell imaging. Yet, this strategy may be relevant to other types of fluorescent sensors.

3. Catecholamines

One of the challenges of catecholamine sensing is that catechol is electron-rich aromatic system and acts as a strong PET quencher of fluorescence. It has, therefore, been challenging to develop 'turn-on' fluorescent probes for catecholamines. To avoid quenching issues, the Sames group designed fluorescent false neurotransmitters. FFNs are fluorescent analogs of neurotransmitters and are taken up by VMAT2 from the cytoplasm into synaptic vesicles (Figure 3a). These probes mimic the catecholamine rather than bind to it, thus avoiding quenching affects. FFN102 (**6**), initially developed to visualize dopamine release from individual presynaptic terminals, could not be used to monitor destaining kinetics in cultured neurons, but could be observed in striatal brain slices.[33], [34] Subsequently, FFN200 (**7**), was developed to label dopaminergic neurons in both culture and acute brain slices to investigate the spatial dynamics and synaptic heterogeneity of individual dopamine boutons (Figure 3b).[35]

The library of FFN molecules was further expanded by the development of FFN270 (**8**), a probe that mimics NE.[36] The probe was designed by combining norepinephrine and coumarin moieties, and was validated and shown to be taken up into hNET-transfected cells and VMAT2-transfected cells, indicating NET and VMAT2 specificity. The use of FFN270 may contribute to a better understanding of the neurobiological mechanisms underlying NE neurotransmission.

Falck and Hillarp[37], [38] discovered that when dry brain tissue was exposed to formaldehyde vapor, a bluish-green fluorescence was observed in monoaminergic cells. Based on this work, Maiti et al. used *ortho*-phthalaldehyde (OPA), **9**, to image chatecholamine neurotransmitters.[39] *ortho*-Phthalaldehyde forms an isoindole with the primary amine group of the neurotransmitters, creating a fluorophore out of the catecholamine (Scheme 5). Fluorescence studies showed that the DA-OPA complex showed fluorescence

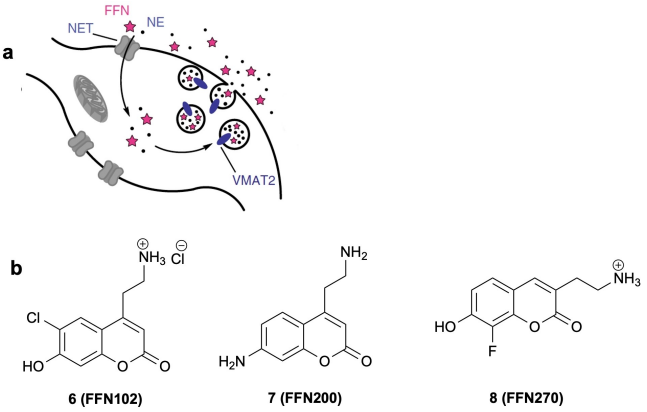
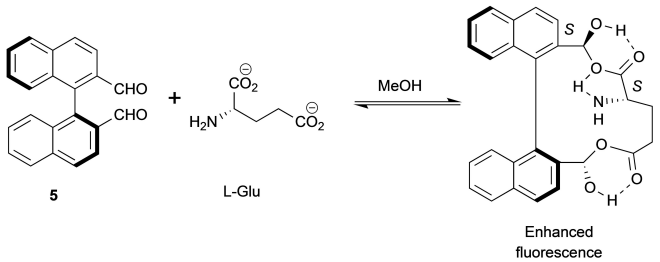
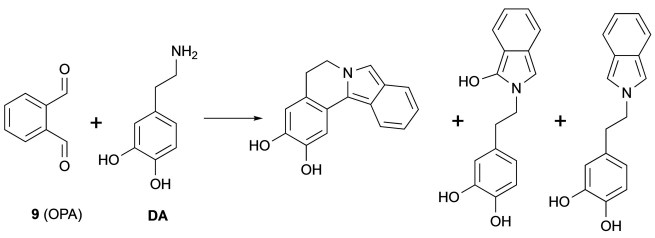


Figure 3. Structures of FFNs.



Scheme 4. Proposed mechanism for the reaction of **5** with L-Glu.



Scheme 5. Plausible adducts from the reaction of dopamine and sensor **9**.

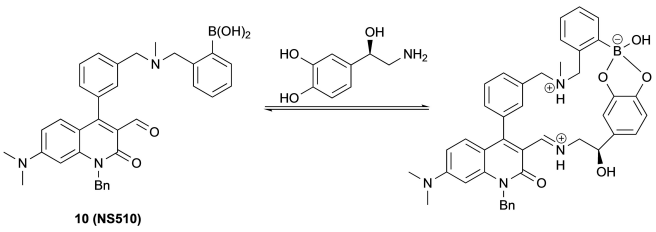
enhancement of up to 20 times compared to that of OPA alone. OPA was used to image catecholamines in live cells. These experiments validated the utility of the probe, although little selectivity is expected, particularly between catecholamines.

The Glass group developed **10** (NS510) as a turn-on fluorescent sensor for catecholamines (Scheme 6).[40] In this case, the substituents on the fluorophore were tuned to avoid quenching by the electron rich catechol group. This sensor has a boronic acid recognition element at a distant position compared to sensor **1**, giving it larger binding cavity resulting in a high affinity for catecholamines. **10** gave only a 2-fold fluorescence increase upon exposure to norepinephrine. Bovine adrenal chromaffin cells were stained with **10** and showed punctate staining of the secretory vesicles. In this case, because the sensor is neutral and membrane permeable and the bound complex is charged and cannot cross membranes, the sensor is trapped inside the vesicle. Thus, although the sensor has relatively small fluorescence changes upon binding norepinephrine, localization aids in neurotransmitter staining.

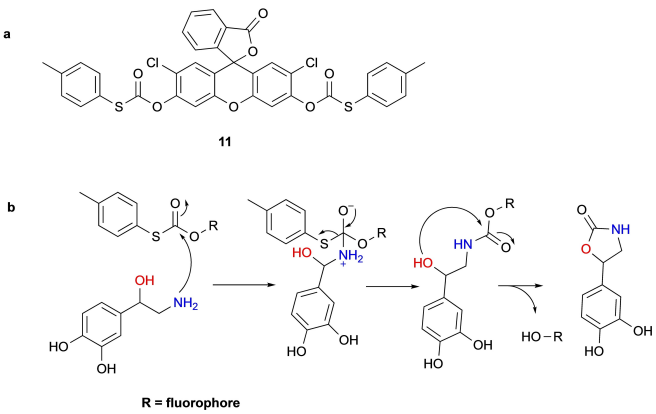
The Yin group developed **11** as a fluorescent probe for specific detection and visualization of norepinephrine using a "protect-deprotect" strategy[41] (Scheme 7). The probe can undergo an irreversible cascade of nucleophilic substitution reactions on exposure to norepinephrine, and in the process releasing the fluorophore which fluoresces at 520 nm. The probe was able to successfully label noradrenergic neurons in brain tissue. As this mechanism requires the hydroxyl group as well as a primary amine, the probe is selective for NE. In addition, since the probe releases the fluorophore from the NE, quenching by the catechol is avoided. However, it should be noted that this probe does not fluorescently tag NE.

The strategy of "protect-deprotect" was further utilized to develop a NIR-NE probe and an endoplasmic reticulum targeting NE probe.[42], [43] To improve the probe response rate and specificity to NE the Yin group utilized a "hunting and shooting" strategy to develop the probe **12**, SXU-NEQ, (Scheme 8).[44] The probe employs an aldehyde to react quickly with NE releasing the free fluorophore giving strong fluorescence at 635 nm. Through in situ imaging of mouse brain, findings indicated an increase in fluorescence intensity (indicating increased NE levels) during epileptic seizure compared to the pre-seizure state.

To monitor the transient NE dynamics in living systems Mao et al. developed a novel small molecule fluorescent probe, **13**, that can anchor on neuronal cytomembranes and exhibits high temporal resolution by specifically responding to norepinephrine on a 100-ms timescale (Scheme 9).[45] The probe works on a dual acceleration mechanism, involving molecular conformational folding and water bridging, which significantly reduced the activation energy of the probe's NE detection reaction, making it ultrafast and highly sensitive, though responding with a fluorescence decrease at 488 nm. The probe was used to monitor NE dynamics in single-neuron levels and in acute mouse brain tissue slices by monitoring fluorescence decreases.



Scheme 6. Structure of **10** (NS510) and its binding with norepinephrine.

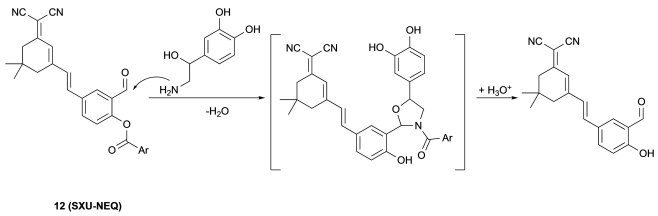


Scheme 7. (a) Structure of probe **11** (b) Mechanism of reaction of FCS with norepinephrine.

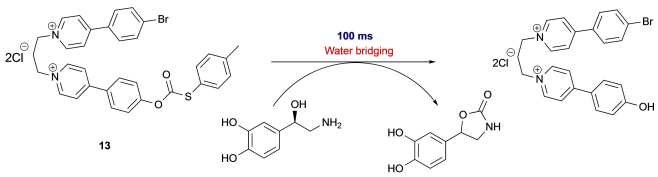
4. Aromatic Monoamines

4.1. Serotonin

Serotonin contains a 5-hydroxyindole group which is even more prone to PET quenching of standard fluorophores than catechol. Thus, probes for serotonin must overcome



Scheme 8. Structure of probe **12**, SXU-NEQ, and its mechanism of action with norepinephrine.



Scheme 9. Structure of the probe **13** and the active fluorophore.

significant quenching effects. The Glass group developed **14** (NS667) with the quinolone-3-aldehyde fluorophore with THQ framework and a boronic acid appended for enhanced binding.[46] Sensor **14** is only moderately quenched by electron rich guests and has a high affinity towards serotonin (K_a 2619 M^{-1}). The formation of a boronate ester between the boronic acid and the hydroxyl of serotonin is the major contributor to the high affinity observed with serotonin in addition to iminium ion formation (Scheme 10).

Similar to FFNs for dopamine and norepinephrine, the Sames group developed a serotonergic FFN (Figure 4). They first introduced a prototype, **15** (FFN246), which served as a dual substrate for both SERT and VMAT2.[47] However, the probe's lack of specificity for SERT over DAT and NET and high lipophilicity resulted in substantial background interference, thereby preventing identification of serotonergic dendrites and axons. Their next FFN, **16** (SERTlight), selectively labels 5HT neurons via SERT.[48] **16** is highly selective for SERT, with low affinity for other transporters like DAT, NET, VMAT2.

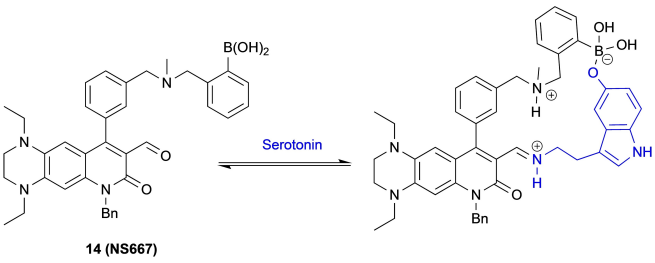
4.2. Histamine

Minami et al. demonstrated that fluorescent cucurbituril-based hosts can be used to distinguish various basic amino acids and their derivatives.[49] This system was one of the earliest turn-on sensing arrays developed in water employing a macrocycle. Here, probe **17** is constructed using a rigid cucurbit[6]uril whereas probe **18** is an acyclic flexible cucurbituril (Figure 5). Both probes have a naphthalene moiety as a fluorophore incorporated in its skeleton. Eu^{3+} is used initially to complex the macrocyle which results in quenching off the fluorescence of naphthalene due to an "antenna effect". However, when the guest molecules are added, they displace Eu^{3+} leading to a turn-on response. The key feature of these probes is that the restoration of fluorescence is dependent on the structure of the guest, which makes them unique. Irrespective of which probe is used, high affinity towards histamine over histidinol or histidine was observed.

Carboxylato-pillar[6]arenes WP6 (**19**), are another class of macrocycles used to detect histamines via a fluorescence indicator displacement (FID) strategy.[50] This water-soluble anionic host was combined with a fluorescent indicator and titrated with various monoamine neurotransmitters such as ACh, choline, histamine, dopamine, norepinephrine, epinephrine and serotonin (Figure 6). ^{1}H NMR titrations and fluorescence assays indicated that this host-sensor system has higher selectivity towards histamine over other neurotransmitters.

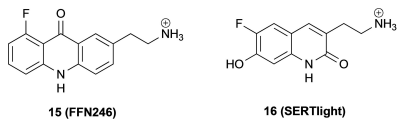
Other sensors for histamine rely on the reactivity of the imidazole group. One example is mesoionic acid fluoride known as "Histamine Blue" (compound **20**), synthesized by Chang et al. This probe produces a "turn-on" response upon interaction with histamine.[51] A condensation reaction occurs between the primary amine of histamine and mesoionic acid fluoride through presumably catalytic action of the imidazole group to generate an amide substituted product (Scheme 11). PET quenching gets blocked in the resultant amide substituted product leads to a turn-on fluorescence response. A bathochromic shift from 406 nm to 432 nm along with 14-fold increase in the fluorescence emission was observed when histamine reacted with the probe.

The Bhattacharya group developed a versatile dual-mode optical sensor based on fluorescein for the detection of histamine (compound **21**).[52] The fluorescein sensor contains a reactive aldehyde and is naturally fluorescent



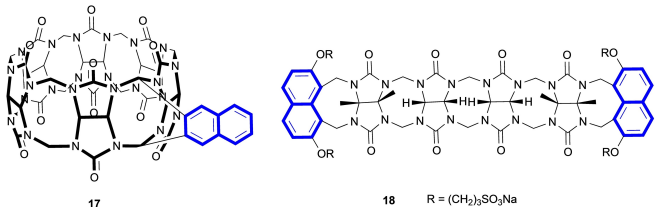
14 (NS667)

Scheme 10. Interaction between **14** (NS667) and serotonin.



15 (FFN246) 16 (SERTlight)

Figure 4. Structures of serotonin FFNs.



17 18 R = $(CH_2)_3SO_3Na$

Figure 5. Structures of water-soluble cucurbituril based sensors.

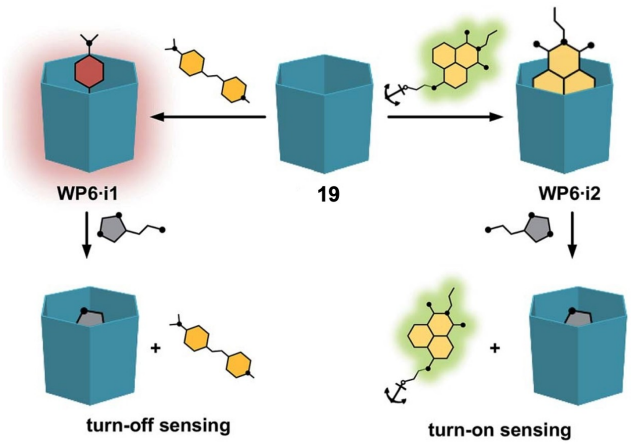
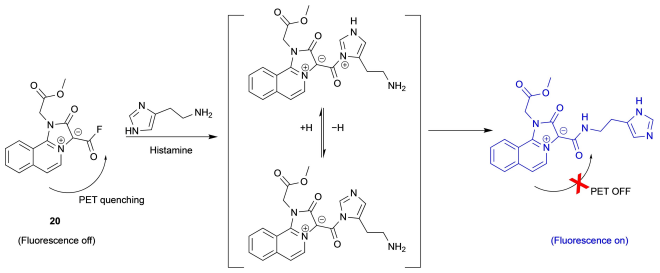
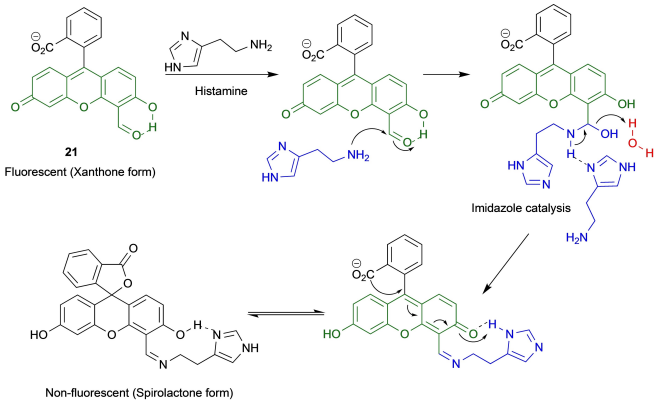


Figure 6. Sensing mechanism of **19**.



Scheme 11. Imidazole catalyzed reaction between Histamine Blue and histamine.

(λ_{em} = 514 nm). Upon interaction with histamine, imidaloze catalyzed imine formation occurs along with subsequent secondary hydrogen bonding interaction which leads to the formation of the spirolactone form, which is non-fluorescent (Scheme 12). Hence, this is a "turn-off" sensor. The sensor was used for detection of histamine in water at pH 7.0 as well as in different biological media such as blood serum and human urine.



Scheme 12. Imidazole-catalyzed imine formation reaction of **21** with histamine.

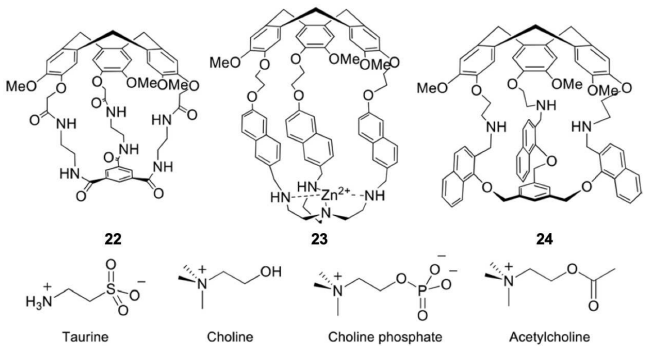


Figure 7. Structure of hemicryptophanes.

5. Acetylcholine

Due to the lack of functional group which allow for direct binding, all acetylcholine sensors and receptors are based around macrocycles which have a cavity that matches the choline group. Thus selectivity between ACh and choline is the primary challenge.

The earliest chemosensors for the detection of acetylcholine (ACh) were based on fluorescent-indicator displacement (FID) strategy, which include tetracyanoresorcin-[4]arene[53] and *p*-sulfocalix[n]arenes[54] as the macrocylic hosts. More recently, fluorescent cyclotriveratrylene (CVT) hosts have been used to detect ACh.[55] A fluorescent hemicryptophane cage was synthesized by Long et al., for selective detection of ACh over choline with a turn-on signal.[56] The cage is built by combining cyclotriveratrylene moiety with naphthalene unit as shown in Figure 7. The hemicryptophane binds Ach giving a 3-fold enhancement in the fluorescence emission at 362 nm. Downfield shifts of ACh peaks in the ^{1}H NMR experiments showed that ACh binds inside the host cavity.

The Jarosz group reported fluorescent molecular cages constructed with sucrose and cyclotriveratrylene (CTV) units in their backbone which are connected to each other via naphthalene bridges for the recognition of acetylcholine.[57] Studies with compound **25** (Figure 8) demonstrated high selectivity towards acetylcholine over choline with binding affinity of K_a 5.6 $\times 10^3$ M^{-1} and $K_{ACh}/K_{Ch} = 3.1$. Fluorescence titrations revealed that the host **25** showed a turn-on response at 330 nm when bound to the acetylcholine, clearly indicating the encapsulation of ACh inside the CTV cavity. The same group made fluorescent molecular cages wherein the sucrose and cyclotriveratrylene units were linked via phenylene units which did not show any selectivity between acetylcholine and choline.[58] This is indeed a great improvement over previous fluorescent molecular cages reported by the same group.

6. Outlook

In summary, the progress in developing organic small-molecule fluorescent probes, as discussed in this review, has been remarkable. Some probes have demonstrated high

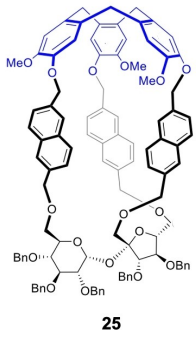


Figure 8. Structure of compound **25**.

affinity and selectivity or promising applications in neuroscience. To better tailor these probes for real-world application, it is imperative to pursue the development of more sophisticated probes for cell, tissue or even in vivo imaging to monitor neurotransmitter dynamics relying on current progress of probe development, for instance, seeking more photostable fluorophores such as fluorescein, rhodamine or silicon rhodamine that are favored by biologists, extending the emission wavelength to the near infrared region, developing novel chemical strategies to enhance selectivity or some combination. While the ongoing progress of organic small-molecule fluorescent probes is promising, we believe optimization of these probes could significantly improve neurotransmitter imaging for disease mechanisms and novel signaling pathways investigation.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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