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## The Chemical Tools for Imaging Dopamine Release

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### Summary

Dopamine is a modulatory neurotransmitter involved in learning, motor functions, and reward. Many neuropsychiatric disorders including Parkinson's disease, autism, and schizophrenia are associated with imbalances or dysfunction in the dopaminergic system. Yet, our understanding of these pervasive public health issues is limited by our ability to effectively image dopamine in humans, which has long been a goal for chemists and neuroscientists. The last two decades have witnessed the development of many molecules used to trace dopamine. We review the small molecules, nanoparticles, and protein sensors used with fluorescent microscopy / photometry, MRI, and PET that shape dopamine research today. None of these tools observe dopamine itself, but instead harness the biology of the dopamine system – its synthetic and metabolic pathways, synaptic vesicle cycle, and receptors – in elegant ways. Their advantages and weaknesses are covered here, along with recent examples and the chemistry and biology that allow them to function.

### eTOC Blurp

Post and Sulzer broadly review the molecules and approaches used to image dopamine release. The small molecules, nanoparticles, and protein sensors that have been developed for use with fluorescent microscopy / photometry, MRI, PET and MRI are covered. Each technique's advantages, disadvantages, and underlying chemistry and biology discussed.

### Graphical Abstract

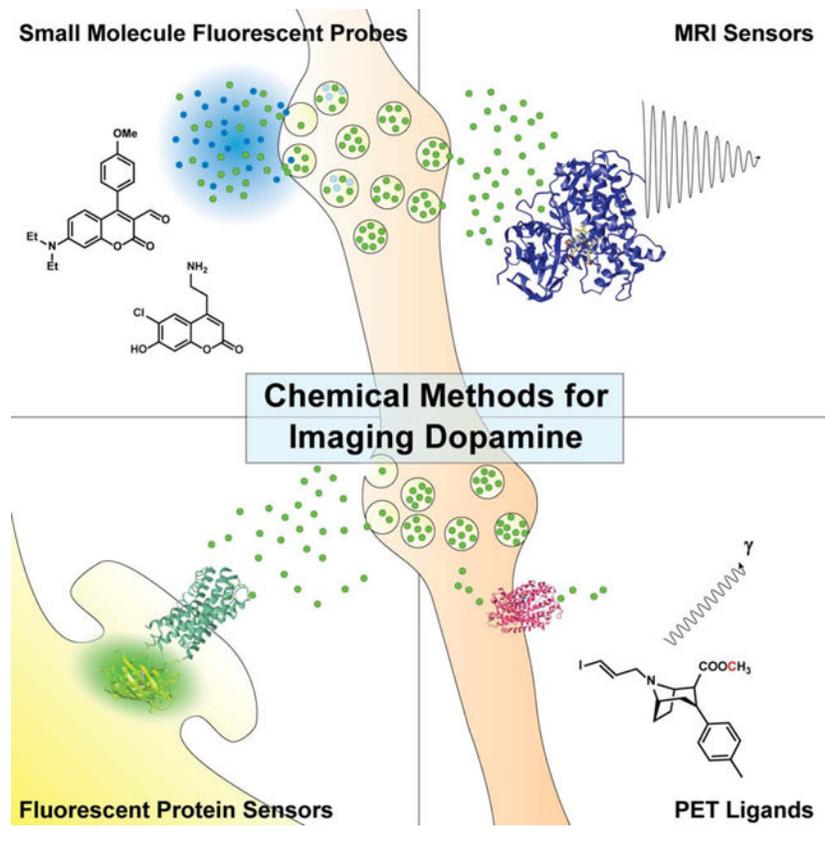
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Declaration of Interests

The authors declare no competing interests.

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## Introduction

Dopamine is a small molecule neurotransmitter that in contrast to glutamate and GABA, which interact with ionotropic receptors to inhibit or excite neurons, binds to G protein-coupled receptors to modulate neuronal responses (Berke, 2018; Surmeier et al., 2014). Dopamine's effects vary with respect to the dopamine receptor type: dopamine type-1 receptors (which include D1 and D5 dopamine receptors) were classically identified by enhancing cyclic AMP levels and are  $G_s$  protein-coupled, while dopamine type-2 receptors (encompassing D2, D3, and D4 receptors) were defined by their decrease of cyclic AMP, and are coupled to  $G_{i/o}$  (Beaulieu and Gainetdinov, 2011). Dopamine's activation of these receptors – which in the striatum are highly expressed on the primary spiny projection neurons (SPNs, also referred to as medium spiny neurons) – initiates chemical cascades that enhance or depress other signals, in some cases acting as a high-pass filter of other ionotropic synapses (Pignatelli and Bonci, 2015). Our understanding of dopamine continues to evolve, with new work suggesting it might act as a morphogen during development (Lieberman et al., 2018).

Classical studies used techniques in which a reaction of catecholamines with aldehydes, principally the Falck-Hillarp technique, produced fluorescent dopamine and norepinephrine (NE) derivatives, and were used to identify dopaminergic and norepinephrinergic neurons and projections in the brain (Falck et al., 1982). This approach indicated that the largest populations of dopaminergic neurons are those of the substantia nigra pars compacta (SNc)

and the neighboring ventral tegmental area (VTA) (Falck, 1962). SNc neurons mostly project into the dorsal striatum and are classically associated with the development and selection of motor function (Hodge and Butcher, 1980). Neurons in the VTA extend mostly into the ventral striatum, including the nucleus accumbens (NAc), as well as some cortical regions, and they are classically associated with acquisition reward-mediated behaviors (Morales and Margolis, 2017). Both populations of neurons contain pools of dopamine-filled synaptic vesicles throughout their axons, typically of 40–50 nm diameter, although larger vesicles are also present, particularly at apparent *en passant* presynaptic varicosities (Arluison et al., 1984). Upon depolarization, the vesicles fuse with the axonal membrane to release their contents at various points along the axon from sites that often appear to have limited presynaptic and postsynaptic specialization; this stands in contrast to the morphology of classical synaptic terminals at motor endplates or pyramidal projection neurons that innervate dendritic spines (Cragg et al., 2004; Rice et al., 2011). In the dorsal striatum and NAc, most of these dopamine synaptic vesicle clusters appear near the neck of dendritic spines, which would be consistent with a role in modulating the synapses of pyramidal neurons.

Imbalances in dopamine neurotransmission are implicated in the pathogenesis of a myriad of psychiatric and neurological disorders, including schizophrenia, Parkinson's disease (PD), autism, depression, drug dependence, and attention deficit disorders (Bohnen and Albin, 2011; Dunlop and Nemeroff, 2007; Howes and Kapur, 2009), and these diseases are treated by various drugs that regulate dopamine neurotransmission. However, except for the motor symptoms of PD, which are strongly linked to a loss of the neurons of the SNc, the roles of dopamine synapses in these disorders are unknown. All of these are pervasive public health issues; yet, understanding, diagnosing, and treating these disorders is limited by our ability to effectively analyze dopamine neurotransmission, particularly at the level of the synapse (Abi-Dargham and Horga, 2016). Such analysis requires the development of imaging approaches.

Over the past decade, many novel approaches to imaging dopamine release have been developed, some introducing entire new classes of molecules or elegant uses of chemical biology. This review is intended to provide a broad survey of the molecules and methods currently in use, including small molecules, nanoparticles, and biomolecules that are detected by fluorescence microscopy, positron emission tomography (PET) imaging, and magnetic resonance spectroscopy/imaging (MRS/MRI). While these techniques and modalities (summarized in Table 1) are quite diverse, they all take advantage of aspects of the basic biology of the dopaminergic system.

### Dopaminergic System and Non-imaging Approaches

Dopamine is synthesized in the cytosol from tyrosine, which is first hydroxylated by tyrosine hydroxylase (TH) to form L-DOPA and then decarboxylated by aromatic amino acid decarboxylase (AADC), as shown in Figure 1 (Molinoff and Axelrod, 1971). As mentioned, dopamine is stored in synaptic vesicles that cluster throughout the axon, often in apparent varicosities (Gaugler et al., 2012; Pereira et al., 2016). The synaptic vesicles are loaded by the vesicular monoamine transporter (VMAT2), which exchanges two protons for

one monoamine: this transporter is promiscuous in that it transports not only dopamine but also serotonin, NE, drugs and metabolites such as tyramine (an observation that introduced the term “false transmitters” by Irwin Kopin), amphetamine, and some neurotoxins (Fischer et al., 1965). The pH gradient that VMAT2 relies on is established by the vacuolar ATPase in the vesicular membrane (Edwards, 2007; Knoth et al., 1981; Lohr et al., 2017). After release, dopamine is either metabolized by monoamine oxidase (MAO) or accumulated back into the neuronal cytosol through the dopamine active transporter (DAT) where it can be repackaged into synaptic vesicles by VMAT2 (Hersch et al., 1997).

As dopamine does not activate ionotropic receptors, its release cannot be straightforwardly detected by recording from a post-synaptic neuron using whole-cell electrophysiological recordings, the standard means by which glutamate or GABA release has been estimated. Instead, intensive research over several decades yielded two different non-imaging methods to study dopamine release.

Brain microdialysis is used to monitor dopamine release in awake behaving rats and was developed primarily by Urban Ungerstedt (Chefer et al., 2009; Justice, 1993). Here, a probe that contains a small stretch of microdialysis tubing is implanted and a cerebrospinal fluid-like solution is pumped through. Neurotransmitters and other molecules diffuse into the tubing, which is connected to an HPLC for monitoring the contents by electrochemical or mass spectrometry detection. This technique can be used to measure dopamine release on the timescale of minutes. Microdialysis is limited by low spatiotemporal resolution, which is due to the size of the probe, its surrounding dead-space, and a slow sample collection rate. For example, microdialysis has been used extensively to study reward-mediated behaviors such as drug addiction, and demonstrated an increase in extracellular dopamine but missed forms of rapid plasticity that were later detected by techniques with greater spatiotemporal resolution (Torregrossa and Kalivas, 2008),

The second technique takes advantage dopamine’s redox properties to measure release. Dopamine can be reversibly oxidized to dopamine-o-quinone (Figure 1) and is therefore amenable to amperometry and cyclic voltammetry. Fast-scan cyclic voltammetry (FSCV), introduced by Ralph “Buzz” Adams, Zygmunt Kruk, and Julian Millar, uses an electrode embedded amongst dopamine axons (Stamford et al., 1984). A major improvement in the technique was the introduction of the carbon fiber electrode by Francois Gonon (Gonon et al., 1980). Typically, the FSCV electrode applies a constant train of triangular voltage ramps from  $-0.4$  to  $1.0$  V and back. The current measured at  $0.6$  V, the peak oxidation potential of dopamine, is proportional to the concentration of dopamine present (Robinson et al., 2003). Due to its ability to measure extracellular concentrations as low as  $50$  nM at a sampling rate of  $10$  Hz, FSCV offers a vast improvement in temporal resolution compared to microdialysis, and has been a major workhorse for studying dopamine release in rodent primary dopamine neuron culture and acute brain slice. Work from Regina Carelli and Mark Wightman’s labs developed *in vivo* FSCV to couple the analysis of dopamine release with behavior. For example, rapid changes in dopamine concentration were observed in the NAc at different stages of cocaine-taking behavior in rats, and this behavior could be recapitulated by electrically stimulating the same area to induce dopamine release (Phillips et al., 2003).

Constant potential amperometry, a technique closely related to FSCV, is sensitive and rapid enough to detect the release of dopamine from the fusion of synaptic vesicles from an individual presynaptic site; it was used to observe the “quantal” release of 10,000 molecules of dopamine in less than 100  $\mu$ sec (Staal et al., 2004). Many additional discoveries and advances have been made with these electrochemical approaches, as recently reviewed by Roberts and Sombers (Roberts and Sombers, 2018). Furthermore, advances continue to be made in FSCV technology, from new fiber coatings to novel strategies to induce dopamine release (Rodeberg et al., 2017).

A downside of the electrochemical approaches is the limited spatial resolution in intact tissue, which is required to analyze how dopamine release is governed by release site properties or interactions on a synaptic scale. Additionally, electrochemical techniques are quite invasive when performed *in vivo*, making them difficult to translate into clinical research; although not impossible, as FSCV has been performed during open-brain surgery to demonstrate a relationship between dopamine and reward prediction error (Kishida et al., 2011, 2016).

In contrast, imaging techniques offer exquisite spatial resolution, in some cases without sacrificing temporal resolution. Ideally, dopamine could be mapped by directly observing it, as glutamate and GABA can be using MRS; however, dopamine’s brain concentration is orders of magnitude lower than glutamate and GABA, and this approach remains elusive (Rothman et al., 1993). Instead, the proteins involved in the life cycle of dopamine – TH, AADC, VMAT2, and DAT – as well as the dopamine receptors and dopamine itself can serve as targets for molecules that can then be imaged by a method appropriate for the experimental model at hand.

### Fluorescent Approaches (Optical Dopamine Sensors)

As established by the classical studies from Falck, Hillarp, and colleagues, fluorescent microscopy offers spatial resolutions that provide synapse- and circuit-level detail. Ideally, dopamine would be imaged directly, as it has a fluorescence excitation peak at 280 nm and an emission maximum of 315 nm; however, light at this ultraviolet wavelength is phototoxic (Wang, 2002). Two-photon (2P) microscopy – in which the fluorophore absorbs two lower-energy photons to reach an excited state – may provide a potential work-around, and there are publications that use 2P to observe dopamine release by exciting at 540 nm in mouse slice (Bera et al., 2018; Sarkar et al., 2014). However, even with the 2P approach, this excitation wavelength can cause photodamage and lacks sufficient biopenetration needed to image dopamine *in vivo*. Indeed, to observe a useful signal, the authors had to place their photomultiplier detector directly over the slice. Klioutchnikov et. al. recently developed a head-mounted three-photon microscopy system in rats, which they use to image a fluorescent calcium sensor (GCaMP, discussed below) (Klioutchnikov et al., 2020). Watt Webb and colleagues have shown serotonin can be imaged in cell culture natively using 3P excitation, so it may be possible to use such a system to image dopamine directly as well (Maiti, 1997).

While the direct observation of dopamine release would be ideal, multiple approaches have been developed to image dopamine indirectly. These novel techniques involve fluorescent

sensors that either co-transit with dopamine through the synaptic vesicle cycle or reversibly bind to dopamine resulting in a change in fluorescence; they are depicted in Figure 2 and summarized in Table 2 (Beyene et al., 2019a; Sames et al., 2013; Wang et al., 2018).

**Small Molecule Approaches**—Rather than image dopamine directly, efforts have been made to track its transit through the vesicle cycle using fluorescent small molecules. Taking advantage of the acidic pH of vesicles established by ATPase and used by VMAT to transport dopamine, the fluorescent weak base acridine orange (AO) was one of the first tools used to image and characterize large secretory vesicles in adrenal gland-derived chromaffin cells (Kuijpers et al., 1989; Steyer et al., 1997; Sulzer and Holtzman, 1989). AO gains an excitation peak at 460 nm at low pH: after diffusing across the membrane into an acidic compartment, it is protonated and cannot diffuse outward. Inducing cells to release the contents of their synaptic vesicles causes AO to enter a pH-neutral environment, resulting in a decrease in fluorescence. This technique was used in mast cells that secrete serotonin to prove that both full exocytosis and more transient ‘kiss-and-run’ occur, in this case at a one-to-two ratio (Williams and Webb, 2000). Another report using AO showed that applying a weak base drug such as chloroquine or amphetamine decreases fluorescence, indicating a collapse in the pH gradient. (Sulzer and Rayport, 1990)

The endocytic probe, FM1–43 (Figure 2, sensor 2), provided a major innovation for imaging neurotransmitter release, as recently reviewed by Wong et al (Wong et al., 2015). This hydrophilic dye binds to the outer surface of the cell membrane via its nonpolar dibutylamino group. When applied to axons that are then stimulated, the probe is taken up into the lumen of synaptic vesicles following endocytosis, and in the acute brain slice, an ADVASEP-7 wash is used to remove non-specific labeling, effectively labeling the recycled vesicle pool (Betz and Bewick, 1992). After a subsequent stimulus, the recycling synaptic vesicles fuse again to the cell membrane, allowing the dye to diffuse into the extracellular membrane, thereby decreasing total fluorescence intensity. Importantly, FM1–43 does not specifically label particular neurotransmitter systems; rather, it concentrates amongst any recycling synaptic vesicle pool after the first stimulation. Therefore, in order to make conclusions about any dopamine-related activity, the dye must be co-imaged with some sort of dopamine marker. For example, Jomphe et al. use FM4–64, a red-shifted FM1–43 analogue, in a mouse expressing GFP driven by the TH promoter (TH-GFP), which specifically labels dopaminergic axons, to study exocytosis in primary dopaminergic culture (Jomphe et al., 2005). Another non-specific approach uses the dye NeuO, which selectively labels live neurons *in vivo* – studies in mice and zebrafish have been published – but does not discriminate between neurotransmitter types, and therefore requires post-hoc analysis or colocalization with a dopaminergic marker (Er et al., 2015; Chai Lean Teoh et al., 2015).

The fluorescent false neurotransmitter (FFN) approach (Figure 2, sensor 1), developed by the Sames and Sulzer labs, has been used to image specific neurotransmitter accumulation and release. The dopaminergic FFNs consist of a coumarin scaffold substituted with an amino-ethyl group thought to confer VMAT2- and in some cases DAT-recognition. The first of these probes developed, FFN511, effectively accumulated in synaptic vesicles in acute mouse brain slice, appearing as puncta that colocalized with TH-GFP. Puncta formation could be blocked by both VMAT2 and DAT inhibitors, and the puncta disappeared

following electric stimulation or application of amphetamine in slice (Gubernator et al., 2009; Zhang et al., 2009). More recently, FFN511 was imaged in chromaffin granules using stimulated emission depletion (STED) microscopy to monitor vesicle shrink-fusion. (Shin et al., 2020) FFN511, however, is relatively non-selective compared to later generations of FFN and labels additional non-dopaminergic synapses. A more selective probe, FFN102, was designed to be pH-sensitive by utilizing chlorine and hydroxy ring substituents. With a pKa of 6.2, the probe is protonated when sequestered in acidic synaptic vesicles and deprotonated when in the cytoplasm or extracellular milieu (Lee et al., 2010; Rodriguez et al., 2013). The deprotonation introduces a new resonance form with increased fluorescence. When synaptic vesicles fuse to release FFN102, such as in response to electric stimulation or application of a secretagogue, this is observed as an optical “flash”.

FFN200, a substrate for VMAT2 but not DAT, offered a DAT-independent way of loading dopaminergic synaptic vesicles, making it useful in both the brain slice and dopaminergic primary neuronal culture (Pereira et al., 2016). The lack of pH-dependence is also useful for tracking the probe during both dopamine storage and release, as it maintains constant brightness through the entire cycle. FFN200 provided the observation of presynaptically silent synapses – axonal varicosities that accumulate FFN but do not form active release sites – in the lateral dorsal striatum and subsequently the globus pallidus (Meszaros et al., 2018; Pereira et al., 2016). Dopamine release ‘hot spots’ had previously been theorized based on FSCV experiments exploring ethanol- and sucrose-directed behaviors (Cacciapaglia et al., 2011; Robinson et al., 2009), and these FFN experiments offer an example of the insights gained when imaging at the scale of individual synapses using fluorescence microscopy.

FFN270 is more selective for the norepinephrine transporter (NET) than DAT, but it is transported by both as well as by VMAT2 (Dunn et al., 2018), and it is pH-sensitive like FFN102 (and ES517, see below). It has been used to image cortical NE neurotransmission *in vivo* using 2P microscopy at a cranial window, demonstrating release in response to both electrical stimulation and amphetamine (Dunn et al., 2018). Imaging striatal dopamine release *in vivo* using a cranial window is complicated by the need to deliver FFN to the striatum of a living mouse while simultaneously imaging such a deep structure, but is feasible.

Fluorescent probes of the dopamine system can also be useful for high throughput bioassays. FFN206 and APP+, a fluorescent analog of the toxin MPTP (available from Molecular Devices as their Neurotransmitter Transporter Uptake Assay Kit), have been developed as VMAT- and DAT-specific fluorescent probes (Bernstein et al., 2012; Hu et al., 2013; Karpowicz et al., 2013). While originally reported to be only DAT-specific, APP+ has been used in medium- and high-throughput assays that identified novel VMAT1 and VMAT2 inhibitors along with FFN206 (Bernstein et al., 2012). Additionally, FFN206 has been useful for *Drosophila* imaging as well as VMAT1 structure-function studies (Freyberg et al., 2016; Sato et al., 2019).

The Neurosensor dyes (Figure 2, sensor 4), developed by Timothy Glass’ lab, offer a somewhat different method from FFNs, in that these probes selectively bind to monoamine neurotransmitters within the synaptic vesicle, which results in a large increase

in fluorescence. The first iteration, NS521, was used to image NE, dopamine, and glutamate release in acute mouse brain slice (Hettie et al., 2013). The next generation, Exosensor or ES517, features pH-sensitivity, so that it is only fluorescent when bound to neurotransmitter and in a pH-neutral environment and is therefore useful for imaging exocytosis (Klockow et al., 2013).

Notably, these dyes have faced hurdles for use *in vivo*, as they mostly operate in the blue-green range of the spectrum, which often causes photodamage and lacks biopenetration. These challenges could be addressed in part by red shifting these small molecule dyes toward more biopenetrant near-infrared (IR) excitation wavelengths (800–122 nm). A near-IR Neurosensor selective for serotonin, NS715, was reported in 2016, but no small molecule DA sensor has been reported at these wavelengths (Hettie and Glass, 2016). Instead, nanoparticle sensors have filled this niche.

**The Nanoparticle Approach**—Nanoparticles offer a broader palette of functionality and photophysical properties than small molecules. There is a plethora of examples of probes where a graphene oxide or quantum dot core is functionalized to react specifically with dopamine, resulting in a decrease in fluorescence. Kamal Eddin and Wing Fen recently reviewed many of these probes with a focus on the engineering principles involved (Kamal Eddin and Wing Fen, 2020) Most are intended to serve as a lab-on-a-chip sensor component, including one where tyrosinase was immobilized on a quantum dot to achieve selective dopamine-induced fluorescent quenching (Arkan et al., 2019). Only a few nanoparticle probes have been tested in cells, such as in a recent report where graphene oxide was functionalized with tyrosine and iron and could detect dopamine in cell culture via fluorescence quenching (Jeon et al., 2019). Other nanoparticle probes have used surface plasmon resonance or Raman scattering to detect the presence of dopamine and other neurotransmitters (Choi et al., 2014; Pathak and Gupta, 2019; Yu et al., 2018).

Carbon nanotubes are promising for detecting specific neurotransmitter release; work originating in the Strano lab on near-IR catecholamine sensors (nIRCats, Figure 2, sensor 3) provides an elegant example (Beyene et al., 2019b). The nIRCats consist of a single-walled carbon nanotube functionalized by single stranded DNA that experiences more than a ten-fold increase in fluorescence in the presence of dopamine. This change is specific for dopamine – GABA, glutamate and acetylcholine do not generate a change in fluorescence, while NE does but with a maximal response and affinity that is much lower than DA and therefore not problematic – in a sequence-dependent manner, with (GT)<sub>6</sub> showing the greatest selectivity for dopamine (Kruss et al., 2014). The sequence can be changed to select for other neurotransmitters, such as serotonin (Jeong et al., 2019). The mechanism remains unsolved, but experiments have ruled out dopamine forming a covalent bond to the probe or reactive oxygen species playing a role. A redox mechanism or  $\pi$ - $\pi$  interactions between the DNA bases and the catechol ring of dopamine remain plausible (Beyene et al., 2018). nIRCats have been used to image dopamine in brain slice from wild-caught mice, demonstrating its use as an alternative to genetically introduced dopamine sensors and applicability across species (Beyene et al., 2019b).

**Genetically Targeted Protein Sensor Approaches**—Genetically targeted fluorescent protein sensors in neurons have been in wide use since the first report of GCaMP by Nakai, Ohkura and Imoto in 2001 (Nakai et al., 2001). The GCaMP construct is a circularly permuted green fluorescent protein (cpGFP) fused to the calcium-binding motif of calmodulin on the C-terminal end, and its binding partner, myosin light chain RS20 peptide, on the N-terminal end (Sun et al., 2013). When calcium is present, the resulting structural change in the calmodulin portion forces the GFP into a fluorescent conformation (Wang et al., 2008). The GCaMP family (there are now at least 10 generations in multiple colors) has become a workhorse for studying neuronal activity in culture, in slice, and *in vivo*, using either head-fixed microscopy or free-moving fiber photometry, wherein an optical fiber is implanted in the brain that can measure fluorescence (Girven and Sparta, 2017). Dopamine release can be inferred by targeting GCaMP expression to SPNs, which upon exposure to dopamine, experience a change in intracellular calcium (Calipari et al., 2016; Muir et al., 2018).

Cell-based neurotransmitter fluorescent engineered reporters, or CNiFERs (Figure 2, sensor 5), utilized a different calcium protein-based sensor to detect dopamine release. Originally developed to detect acetylcholine, CNiFERs use HEK cells that express a  $G_q$  protein-coupled receptor and the FRET-based  $Ca^{2+}$  sensor TN-XXL (Nguyen et al., 2010). In the case of DA-CNiFERs, D2R is transfected in HEK cells that express a modified  $G_{q/i5}$  protein chimera, which is activated by  $G_{i/o}$ -coupled receptors but recruits the  $G_q$  signaling pathway. When dopamine is present, it binds to D2 receptors, activating  $G_{q/i5}$  and therefore a PLC/IP<sub>3</sub> cascade that results in the release of stored  $Ca^{2+}$  (Lacin et al., 2016). The increased cytoplasm calcium concentration induces a conformational shift in TN-XXL that results in an increase in the FRET ratio,  $R/R_0$ . To use CNiFERs *in vivo*, the HEK cells must be implanted into the brain area of interest, and  $R/R_0$  can be monitored using fiber photometry.

DopR-Tango (Figure 2, sensor 7), used in *Drosophila*, is a novel dopamine imaging approach which relies on  $\beta$ -arrestin recruitment that occurs after dopamine binds to D1Rs. Here, flies are genetically modified to express three constructs: a TEV-protease fused to  $\beta$ -arrestin, D1R linked to the LexA transcription factor via a TEV-protease cleavage site, and a Lex-A promotor-driven membrane-bound GFP reporter gene. When dopamine is present, it binds to the modified D1R, causing  $\beta$ -arrestin-TEV recruitment, which in turn breaks the TEV cleavage site linkage, releasing LexA and driving GFP expression (Inagaki et al., 2012). Forty-eight hours after inducing expression of these genes, GFP expression was observed in the  $\beta$  and  $\gamma$  lobes of the mushroom body, a fly brain region innervated by dopaminergic neurons. In flies that were administered SCH23390, a D1R antagonist, GFP expression was significantly reduced.

Over the last decade, the cpGFP-calmodulin strategy was adapted for the direct detection of neurotransmitter release. The first example of this strategy was iGluSnFR, which replaced the calmodulin domain of GCaMP with a glutamate-binding domain, and this approach has since been optimized for higher sensitivity and greater temporal resolution in addition to blue, cyan, green, yellow, and red variants (Marvin et al., 2013, 2018; Wu et al., 2018).

More recently, the approach was adapted for dopamine. Two similar constructs have been introduced: the dLight sensors from the Tian lab and the GRAB<sub>DA</sub> sensor from the Li lab (Figure 2, sensor 6). dLight consists of a dopamine receptor with cpGFP taken from a GCaMP6 sensor inserted into the third intracellular loop, so that the GFP fluorescence increases when dopamine is bound (Patriarchi et al., 2018). The first generation, dLight1.1, used D1R, while dLight1.2 used D4R. dLight1.1 has an EC<sub>50</sub> value of 1.3  $\mu$ M and experiences a change in fluorescence intensity upon activation ( $F/F$ ) of 182% in response to a single pulse stimulation in mouse slice. Fluorescence is blocked by the D1R antagonist SCH-23390. dLight1.1 and 1.2 have been used as fiber photometry sensors to study heroin addiction and sleep cycles (Corre et al., 2018; Dong et al., 2019). Another variant, dLight1.3, which contains an F129A mutation resulting in an improved  $F/F$  of 660% but a higher EC<sub>50</sub>, was recently used to study effects of thirst on dopamine release (Augustine et al., 2019). It is unclear whether the different dLight1 variants have practical differences; one report combined results from 1.1 and 1.2 after finding no statistical difference (Mohebi et al., 2019).

GRAB<sub>DA</sub> has a similar design but uses cpEGFP inserted into the third intracellular loop of the D2R (Sun et al., 2018). There are two GRAB<sub>DA</sub> variants with EC<sub>50</sub> values of 10 nM and 130 nM respectively. Both versions have a  $F/F$  of 90% and can be blocked by the D2R antagonists haloperidol and eticlopride but not SCH-23390. The high affinity GRAB<sub>DA</sub> is also responsive to NE, which may complicate attempts to use it in areas of the brain outside the striatum. GRAB<sub>DA</sub> has been used to measure dopamine release in mice using cultured neurons, acute slice, and fiber photometry, and in fruit flies and zebrafish.

Both dLight and GRAB<sub>DA</sub> are exciting developments that continue to evolve; recent studies have introduced a red fluorescent protein variant for each, as well as versions that can sense other neurotransmitters, allowing complex neurotransmitter interactions to be studied simultaneously (Jing et al., 2020; Nakamoto et al., 2020; Patriarchi et al., 2020; Sun et al., 2020). Most importantly, using these probes in behaving mice is at this point straightforward, as fiber photometry is already in wide use for GCaMP, thus providing a means for dopamine detection adaptable in labs already measuring calcium (Labouesse et al., 2020).

**Voltage Sensing Probes**—In contrast to measuring dopamine release by sensors within synaptic vesicles or the extracellular space, alternative approaches have inferred activity by measuring the voltage across the membrane, using voltage-sensing probes. The use of these small molecule dyes began in the early 1970's in turtle retina (Baylor and Fuortes, 1970). This was followed by work in the 80's, when Grinvald et al. used a merocyanine-rhodamine dye, WW401, which has a  $\pi$ -wire region of the molecule that spans the cell membrane and a more polar fluorescent headgroup (Grinvald et al., 1981). They initially developed these approaches in squid axons and tested them in cell culture and mouse hippocampal slice (Grinvald et al., 1982). Over the last few years, new rhodamine dyes have been developed, such as one from the Miller lab which can achieve  $-44\%$   $F/F$  per 100 mV (Boggess et al., 2019; Kulkarni et al., 2018). A downside of this class of dyes is that they are not neurotransmitter-specific, and therefore to image dopamine activity, they require genetically expressing a fluorescent marker or co-labeling with a small molecule dye such as an FFN.

The Voltage Dye Molecular Recognition (VoLDeMo, Figure 2, sensor 9) probe aimed to surmount this challenge by linking a voltage-sensitive dye to a cocaine-like DAT anchor. This dye construct was found to label dopamine axon terminals in the striatum specifically, and could sense changes in voltage in DAT-expressing HEK cells that were manipulated with patch-clamp electrophysiology, although change in signal was far lower than the dye alone, at  $-4.7\%$  F/F per 100 mV (Fiala et al., 2020).

Similar to dLight and GRAB<sub>DA</sub>, an entire class of genetically encoded voltage indicators (referred to as GEVIs in the literature) have been developed to monitor changes in membrane voltage in a targeted way, including the highly optimized ASAP3, somArchon, and Voltron, reviewed recently by Xu et al. and Bando et al. (Bando et al., 2019; Xu et al., 2017). The latter is especially interesting because it utilizes a hybrid genetic/small molecule approach (Abdelfattah et al., 2019). The Voltron sensors (Figure 2, sensor 8) fuse the rhodopsin Ace2N with an intracellular halo-tag. The halo-tag can form covalent bonds with small molecule dyes that contain a bio-orthogonal alkyl-halide group – the authors used JaneliaFluor rhodamine-based dyes. When Ace2N experiences a depolarizing change in membrane potential, its native retinal fluorophore undergoes a reversible increase in absorbance, becoming a FRET acceptor for the nearby halo-tag bonded dye. Thus, a decrease in FRET signal is observed of about  $-23\%$  F/F per 100 mV in cultured neurons. In the initial report, Voltron was imaged during *in vivo* experiments in mice, zebrafish, and the dopaminergic neurons of *Drosophila*. In a follow-up report, the authors were able to invert the signal to yield an increase in FRET intensity (Abdelfattah et al., 2020). Because they rely on FRET, these probes are impractical for two-photon microscopy (the non-linear optics makes FRET measurements quite challenging)(Tao et al., 2015), an obstacle to their use for *in vivo* imaging.

### MRI and PET Approaches

The fluorescent imaging approaches discussed above provide powerful tools to study model organisms but are not practical for use in humans due to the depth of dopamine neurons within the brain and the limited biopentrance of UV and visible light. In contrast, MRI, MRS, and PET offer modalities to image the deeper brain structures necessary to assess dopaminergic function in humans.

The direct detection of dopamine by MRS would be an ideal way to quantify dopamine storage and study its release. MRS is similar to MRI in that a subject is placed in a strong magnetic field and nuclei (typically protons) are excited with a radio frequency pulse (Passe et al., 1995). With MRS, additional information is generated in the form of a spectrum, where individual peaks termed chemical shifts and measured in radio frequency or parts per million (ppm) can be assigned to each nucleus (Öngür, 2013; Prost, 2008). The identity and relative concentrations of molecules can be deduced by the heights and ppm values of each peak in the spectrum. MRS is analogous to an organic chemist's use of NMR to solve the structure of a molecule and has been used successfully for the measurement of several neurotransmitters including glutamate, glutamine, and GABA as well as other important small molecule metabolites such as creatine and phosphocreatine, N-acetylaspartic acid, lactate, choline, and myo-inositol (Bogner et al., 2017). Importantly, MRS signal is directly

proportional to the number of nuclei present in the voxel, and therefore metabolites found at lower concentrations or those that lack several nuclei in the same chemical environment (such as the trimethyl group of choline) are impossible to detect with current state-of-the-art instruments. Unfortunately, dopamine falls into both categories; thus, as with the fluorescent approaches, elegant solutions have been developed to detect dopamine and its associated networks using indirect methods.

**Early attempts and fluorodopamine**—Attempts to image dopamine indirectly by MRI were first published by Kenneth Kirk's lab at the NIDDK and others at the NIMH in the 1980s. Rather than observing protons, as is typical with MRI, they used  $^{19}\text{F}$ -MRS to detect fluorinated dopamine analogs in a false neurotransmitter-like approach. Imaging  $^{19}\text{F}$  offers several advantages: it has a spin  $\frac{1}{2}$  and a gyromagnetic ratio similar to a proton, it is 100% abundant in nature but completely absent from the brain, it experiences chemical shifts over the span of 100 ppm, and standard industry instruments can be used with limited additional equipment (Ruiz-Cabello et al., 2011; Waiczies et al., 2019). The Kirk lab synthesized and characterized 2-, 5-, and 6-fluorodopamine and tested the latter in intact guinea pig neural sacs. They observed chemical shifts at 36.5 ppm (relative to hexafluorobenzene), 37.2 ppm, 36.2 ppm, and 44 ppm, corresponding to 6F-dopamine, 6F-dihydroxyphenylacetic acid (DOPAC, a dopamine metabolite), 6F-NE (NE is metabolized from DA by dopamine-beta-hydroxylase,  $\text{D}\beta\text{H}$ ), and 6F-dihydroxymandelic acid, a NE metabolite (Eisenhofer et al., 1989; Goldberg et al., 1980). Notably, the 6F-dopamine peak had a linewidth twice that of 6F-DOPAC, suggesting it was observed in a much less mobile environment such as within a synaptic vesicle. When the neural sacs were pre-incubated in reserpine and pargyline, VMAT2 and monoamine oxidase inhibitors respectively, the 6F-dopamine peak had a linewidth and height similar to 6F-DOPAC peak observed in the untreated sacs.

Despite initial promise, because NE and DOPAC were the predominant peaks in these experiments, and due to dopamine's inability to cross the blood brain barrier (BBB) as well as the lack of sensitivity of the MRIs at the time, this work was abandoned in favor of PET imaging of [ $^{18}\text{F}$ ]-fluorodopamine (Chiueh et al., 1983).  $^{18}\text{F}$  is a positron-emitting isotope of fluorine that must be synthetically derived and incorporated into probes immediately prior to imaging. [ $^{18}\text{F}$ ]-fluorodopamine presented an especially difficult synthesis requiring  $^{18}\text{F}$ - $^{18}\text{F}$  gas, until very recently when Vavere et al. published a new route (Vavere et al., 2018).

[ $^{18}\text{F}$ ]-Dopamine has similar challenges due to a poor penetration across the BBB, but has proven useful in imaging neuroblastoma tumors in which the BBB is damaged, as well as for pheochromocytomas in the adrenal gland (Ilias et al., 2003; Vavere et al., 2018). Yanagisawa et al. revived the  $^{19}\text{F}$ -MRI effort in 2017, publishing a study that imaged eight different fluorinated compounds, and found some success with  $^{19}\text{F}$ -L-DOPA using  $^{19}\text{F}$ -MRS on PC-12 cell culture (an immortalized adrenal gland cell line that expresses TH and VMAT1) and *ex vivo* whole brain imaging, but no *in vivo* studies have been reported (Yanagisawa et al., 2017).

Stemming from this history, the field has been mostly dedicated to developing PET sensors that indirectly measure dopamine capacity by specifically binding to the enzymes, transporters, and receptors for which dopamine is a ligand. Kanthan et al. have written an outstanding perspective and history of the chemistry of these molecules (Kanthan et al., 2017). Here, we focus on the functionality of these PET ligands (Figure 3), organized by target, and discuss recent examples of innovative uses.

**Dopamine Receptor Ligands**—The most widely used PET ligand for imaging dopamine release is [ $^{11}\text{C}$ ]-raclopride, a D2R antagonist and antipsychotic drug (Ehrin et al., 1985; Farde et al., 1986). Raclopride quantifies dopamine release by acquiring images of the striatum before and immediately after amphetamine administration (Ross and Jackson, 1989). The first image provides a baseline level of D2R binding. The amphetamine causes high levels of dopamine release and inhibits reuptake (Sulzer et al., 2005), and the extracellular dopamine competes with raclopride at the D2R ligand-binding site. The second scan quantifies how much raclopride remains bound to the D2Rs and therefore indirectly measures how much dopamine was released. Subjects with greater dopamine release capacity exhibit a larger decrease in [ $^{11}\text{C}$ ]-raclopride signal following the amphetamine challenge. This technique has been used for studying multiple disorders and behaviors, most prominently schizophrenia, wherein elevated dopamine release has been observed compared to control subjects (Abi-Dargham, 2020; Laruelle et al., 1996). In addition to amphetamine, other stimuli have been explored. Lippert et al. report using milkshakes in place of amphetamine and imaged dopamine release on a sub-second timescale (Lippert et al., 2019). Cox et al. measured dopamine release by merely exposing habitual cocaine users to cues they associate with taking cocaine (Cox et al., 2017).

A different technique can be used where the second scan is acquired much later after the amphetamine challenge, as a way of measuring D2R internalization following a stimulus (Skinbjerg et al., 2010). Here, a second bolus of raclopride is administered before the post-amphetamine image is taken. With this approach, a decrease in signal is not related to the amount of dopamine that was released, but to the level of receptor internalization that occurred.

For regions where dopamine release is sparse, higher-affinity D2R PET ligands have been developed. These include [ $^{18}\text{F}$ ]-fallypride, [ $^{11}\text{C}$ ]-FLB-457, and [ $^{11}\text{C}$ ]-MNPA, the latter of which is actually selective for the high-affinity D2R isoform (Farde et al., 1997; Hernaus and Mehta, 2016; Seneca et al., 2008). The first two have been used to image D2Rs in the cortex: Aalto et al. used [ $^{11}\text{C}$ ]-FLB457 to measure extrastriatal dopamine release in response to amphetamine, while Ceccarini et al. measured task-induced striatal and cortical dopamine release with [ $^{18}\text{F}$ ]-fallypride (Aalto et al., 2009; Ceccarini et al., 2012).

Similar approaches have been attempted using D1R ligands. An early attempt used an isotope of a common D1R antagonist, [ $^{11}\text{C}$ ]-SCH23390, but the ligand was not replaced by dopamine as is raclopride (Chou et al., 1999). [ $^{11}\text{C}$ ]-N-methyl-NNC01-0259 is better at labeling D1Rs, but also fails to measure dopamine release (Finnema et al., 2013). D3R ligands, such as [ $^{11}\text{C}$ ]-PHNO, are useful for imaging the NAc where that receptor is more prevalent. Examples include a study of impulse control disorder in PD patients – those with

symptoms had 20% lower [ $^{11}\text{C}$ ]-PHNO signal than those without – as well as the effect of the atypical antipsychotic cariprazine in schizophrenia patients (Girgis et al., 2016; Payer et al., 2015).

**AADC, VMAT2, and DAT ligands**—Whereas the PET ligands discussed above have focused on postsynaptic targets, a great deal of work has also gone into imaging presynaptic processes by targeting AADC, VMAT2, and DAT. Indeed, a recent review by Kaasinen and Vahlberg contains a meta-analysis of 142 such studies in PD (Kaasinen and Vahlberg, 2017).

[ $^{18}\text{F}$ ]-L-DOPA is converted to [ $^{18}\text{F}$ ]-dopamine by AADC as the rate-limiting step, prior to transport into synaptic vesicles. Therefore, lower PET signal with [ $^{18}\text{F}$ ]-DOPA is due to lower AADC concentrations and indicates a decreased capacity for dopamine synthesis. For example, [ $^{18}\text{F}$ ]-L-DOPA was administered to chronic cannabis users and AADC activity was inversely correlated to a measure of apathy (Bloomfield et al., 2014). [ $^{18}\text{F}$ ]-L-DOPA was also used to track the efficacy of stem cell grafts in PD patients over the course of a year, and indicated an increase in dopamine synthesis capacity over time (Ma et al., 2010).

A major disadvantage to [ $^{18}\text{F}$ ]-L-DOPA, and PET, is that the [ $^{18}\text{F}$ ]-DOPA signal is indistinguishable from its metabolic products, including [ $^{18}\text{F}$ ]-dopamine, [ $^{18}\text{F}$ ]-DOPAC, and [ $^{18}\text{F}$ ]-norepinephrine. Therefore, PET signal in the cortex and cerebellum is prevalent due to these metabolites, and signal from the striatum can be difficult to interpret. For this reason, [ $^{11}\text{C}$ ]-Me-mTyr, an AADC ligand which is not metabolized into NE, was recently developed. In monkeys, this probe shows lower cortical and cerebellar signal than [ $^{18}\text{F}$ ]-L-DOPA (Kanazawa et al., 2015; Tsukada et al., 2016).

PET ligands that target DAT have been used to assess the health of dopaminergic axons in the striatum, often to assess the progression of PD. A lower signal is inferred to be the result of less DAT on the axonal membrane, which is a sign of neurodegeneration. Tropanes serve as a common scaffold for the PET ligands such as [ $^{11}\text{C}$ ]-cocaine or [ $^{11}\text{C}$ ]-PE2I. The latter was used to study DAT levels in  $\alpha$ -synuclein knockout mice, showing an increased signal in the caudate putamen of these mice compared to wild-type (Levigoureux et al., 2019).

DAT is also a common target for single-photon emission computed tomography (SPECT, a less technically demanding technique similar to PET but with lower resolution) ligands, including [ $^{123}\text{I}$ ]- $\beta$ -CIT, [ $^{18}\text{F}$ ]- and [ $^{123}\text{I}$ ]-FP-CIT, as well as [ $^{11}\text{C}$ ]-CFT. The N-fluoropropyl-tropanes are the most common DAT ligands: [ $^{18}\text{F}$ ]-FP-CIT, for example, was used to compare PD patients with and without hyposmia, a common early symptom of PD (Oh et al., 2018), and [ $^{123}\text{I}$ ]-FP-CIT (marketed as DaTSCAN) was approved by the FDA for clinical use in 2011 (Park, 2012). DaTSCAN was recently utilized in a case study assessing the impact of SARS-CoV-2 on a PD patient (Méndez-Guerrero et al., 2020).

VMAT2-targeting PET ligands are mostly tetrabenazine derivatives, including [ $^{11}\text{C}$ ]-dihydro-tetrabenazine and [ $^{18}\text{F}$ ]-AV-133, which is a fluoro-tripropyl analog. Both tracers have been used to study  $\beta$ -cell masses in pancreatic cancer, as well as tracking the effects of 6-hydroxydopamine in rats over time (Veluthakal and Harris, 2010; Walker et al., 2013;

Wang et al., 2010). The latter has also been used with the MPTP mouse model (Toomey et al., 2012) and in PD patients (Okamura et al., 2010).

**MRI Contrast Agents**—Historically, the imaging field moved from MRI to PET ligands because the technology surrounding PET advanced at a faster rate. With major advances in computing and the arrival of stronger magnets – 14T magnets are now used for small animal experiments, while 9T magnets are beginning to be used with humans – MRI and MRS present major opportunities for creative solutions for imaging dopamine. Two recent approaches suggest what is possible.

One utilizes the paramagnetic iron atom found in the heme group of BM3h, a member of the bacterial cytochrome P450-BM3 family. Shapiro et al. used directed evolution on the enzyme BM3h, which normally metabolizes arachidonic acid, to create analogues that selectively bind to dopamine with  $K_d$  values in the micromolar range (Shapiro et al., 2010). In the absence of ligand, water molecules exchange in the empty coordination site, promoting T1 relaxation. When dopamine is present, it binds to this site, blocking water, therefore decreasing T1 relaxation. The authors tested two BM3h variants, BM3h-8C8 and BM3h-9D7, to quantify dopamine release from PC-12 cells. They then directly injected 8C8 into anesthetized rat striatum via cannula, along with either high or low  $K^+$  buffer. The rats underwent continuous T1-weighted MRI scans during this time, and a significant change in signal intensity with high  $K^+$  was found in voxels surrounding the cannula. The other variant, 9D7, was tested in the NAc in response to electrical stimulation of the medial forebrain bundle (Lee et al., 2014). The 9D7 variant was then imaged simultaneously with blood-oxygen-level-dependent (BOLD) fMRI to compare dopamine release to system-wide brain activity following electric stimulation of the lateral hypothalamus, which projects to the VTA (Li and Jasanoff, 2020). While the BM3h dopamine sensors have only been tested by direct infusion into the brain, because they are proteins, one can imagine system-wide dopamine imaging via genetic engineering.

The second technique uses neuromelanin (NM), the dark pigment for which the SNc and locus coeruleus receive their names, as an MRI contrast agent. NM is the product of the iron-dependent oxidation of dopamine that builds up within autophagic lysosomes over the course of a neuron's lifespan, and it is only cleared by microglia after cell death occurs (Fasano et al., 2006; Monzani et al., 2019). Notably, NM contains high concentrations of iron chelated by oxidized catechol derivatives and is therefore paramagnetic. The effect is a shortened T1 relaxation time (Tosk et al., 1992; Trujillo et al., 2017). In NM-sensitive MRI protocols, high concentrations of NM appear as higher signal intensity. In healthy patients, the NM signal in the SNc grows more intense with age, but in PD patients, whose dopamine neurons are cleared by microglia as neurodegeneration progresses, the signal decreases over the course of the disease (Sulzer et al., 2018; Zecca et al., 2002). This effect has been shown to occur the locus coeruleus (Sasaki et al., 2006), and the signal intensity has been correlated with DatSCAN PET imaging, although there is some controversy, as Saari et al. reported no correlation between DatSCAN levels and post-mortem NM levels (Saari et al., 2017).

Cassidy and colleagues advanced the technique, showing that NM-MRI signal is indeed correlated to dopamine release capacity (Cassidy et al., 2019). They first demonstrated a

relationship between the NM-MRI contrast-to-noise ratio in the SNc and the change in [<sup>11</sup>C]-raclopride PET intensity in the striatum following a dose of amphetamine. They then compared cerebral activity using fMRI to NM-MRI signal and found that individuals with higher NM-MRI signals had a higher rate of cerebral brain flow. Finally, they compared individuals with a history of psychosis, and found that they were more likely to have higher NM-MRI signals, suggesting that the technique could be a useful biomarker for both PD and psychotic disorders such as schizophrenia. A follow-up to that study applied the technique to participants with cocaine use disorder and found an increased signal compared to participants with no cocaine use (Cassidy et al., 2020). That the technique does not rely on any exogenous small molecules or genetic manipulation also makes it promising for wide use in patients, especially over long timespans, and possibly in children, although their signals are far lower.

## Conclusion

The last twenty years have witnessed a boom in the methods available to assess dopaminergic function and health. These methods each have advantages as well as limitations. Fluorescent techniques provide synaptic-level observations and are applicable to a wide range of model cell lines and organisms but are not adaptable to humans. PET techniques offer high sensitivity but lack temporal-spatial resolution and require ligands with short half-lives. MRI techniques can distinguish ligands from their metabolites but are relatively new, less sensitive than PET or fluorescence, and not yet fully optimized for clinical use. Many techniques are undergoing a broad array of innovations and show great promise for the future. As new approaches continue to emerge, direct comparison studies will be important, so that results from these different techniques can be understood in context, and so that the field can eventually coalesce around some common protocols. It is also likely that soon, the limiting factor for imaging dopamine will no longer be the chemical biology required to detect it, but rather the technology necessary to detect and analyze signals. We expect that as computing and detector technology continue to advance, our ability to image dopamine storage and release will increase dramatically.

While the imaging modalities are vastly different, these approaches effectively target the same protein infrastructure involved in dopamine synthesis, storage, release, and reception. As technology continues to improve, direct detection of dopamine may eventually be possible. Until then, creative uses of chemistry and biology for indirect detection provide a broad range of opportunities.

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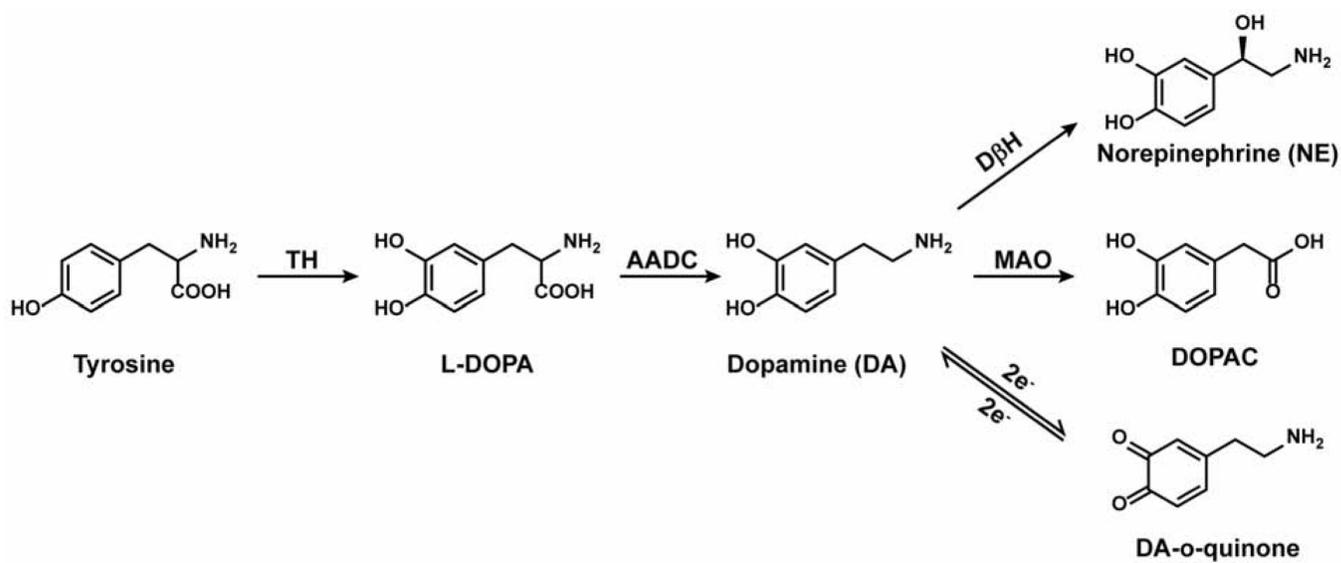
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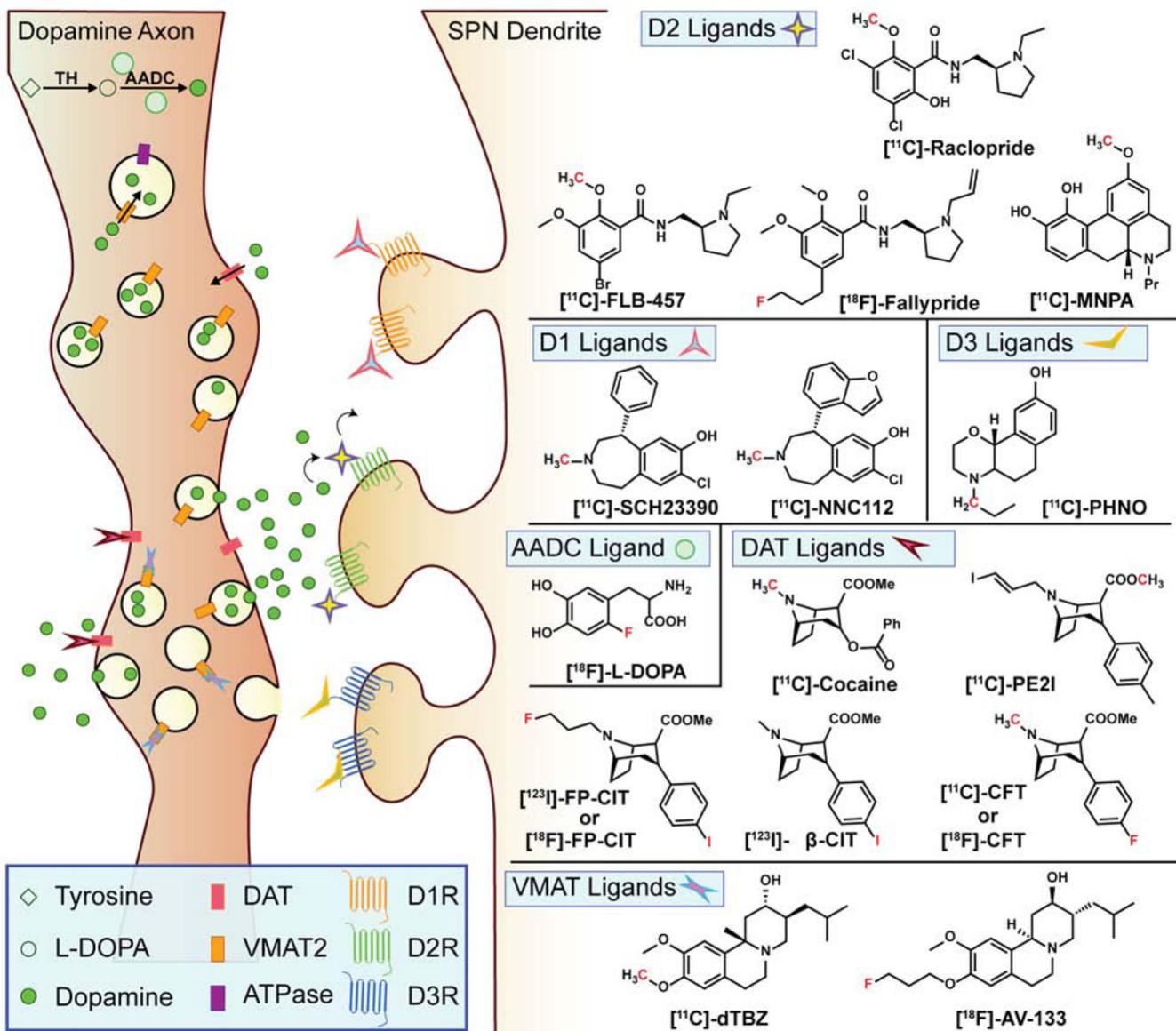
**Highlights**

- New imaging techniques critical to understanding dopamine disorders are reviewed
- The last 20 years have seen many new molecules developed to image dopamine
- Fluorescence, MRI, and PET approaches all show promise



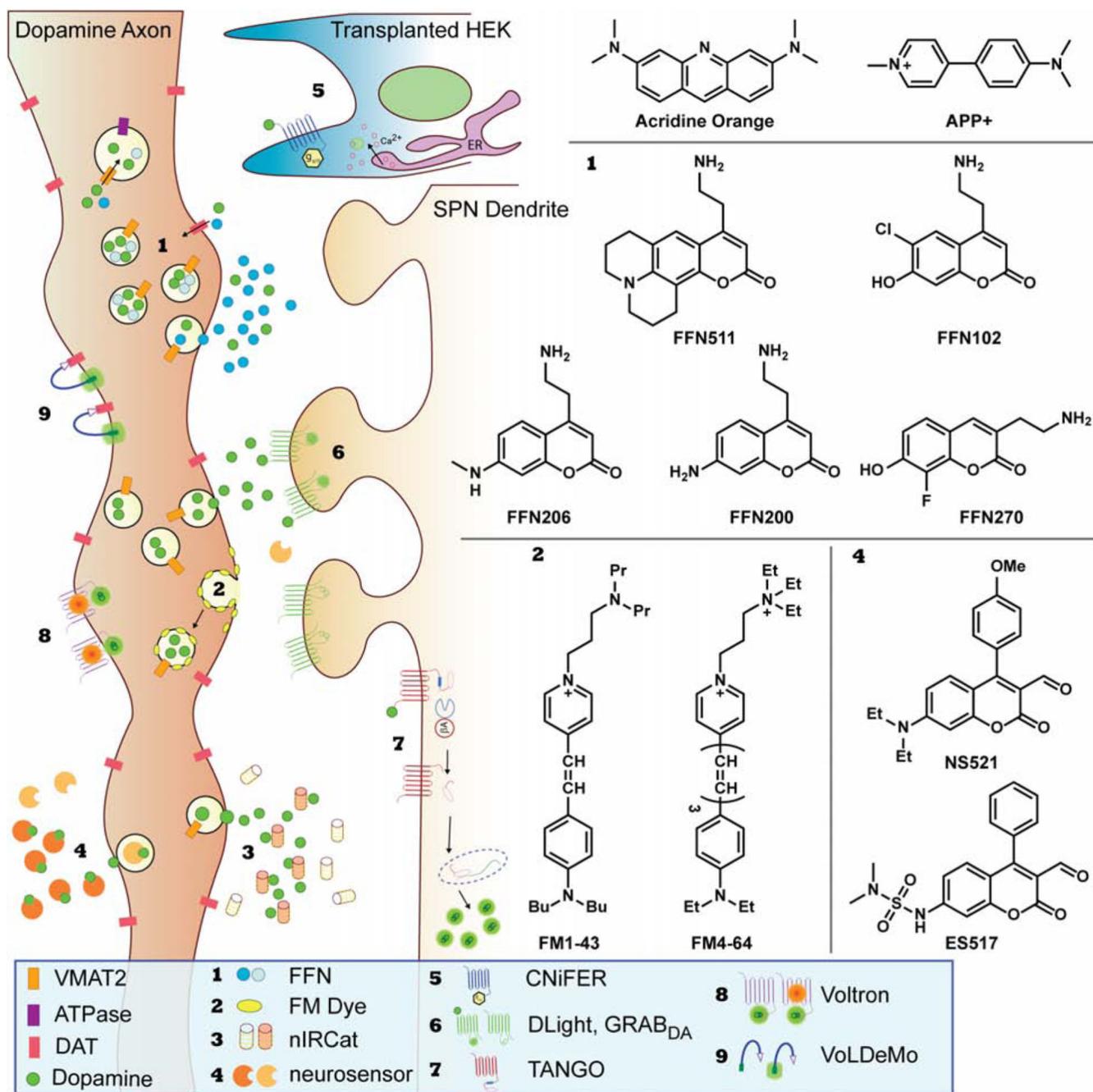
**Figure 1. Dopamine Pathways.**

The biosynthetic, metabolic, and redox pathways of dopamine



**Figure 2. Fluorescent Approaches**

A schematic of the different fluorescent techniques used to image dopamine in the brain and the corresponding chemical structures of the small molecule dyes referenced throughout this review



**Figure 3. PET Approaches.**

A schematic of the PET ligands used to image dopamine systems in the brain with their corresponding chemical structures; note that red-colored atoms represent their positron-emitting isotopes, i.e.  $^{11}\text{C}$ ,  $^{18}\text{F}$ , and  $^{123}\text{I}$  respectively.

Table 1:

## Summary of Dopamine Sensing Modalities

Modality	Description	Spatial Resolution / Level of Analysis	Temporal Resolution	Advantages	Limitations
Microdialysis	A small dialysis probe is surgically implanted into the brain and analytes are collected over time	mm-cm / brain regions (e.g. dorsal lateral striatum)	seconds - minutes	HPLC separation allows complete analysis of small molecules within dialysate; can be used in an awake, behaving, free moving animal	Poor spatiotemporal resolution
FSCV	A redox electrode is inserted into slice or surgically implanted and the concentration of dopamine is electrochemically measured	mm / brain region; specific circuits if coupled with electro- or optogenetic stimulation	milliseconds	High temporal resolution; can be used in acute brain slice or behaving animals	Poor spatial resolution; difficult to implement <i>in vivo</i>
Fluorescent Microscopy	Small molecule- or protein-based sensors are used to indirectly monitor or measure dopamine release in slice or culture	$\mu\text{m}$ - mm / synapses (e.g. release sites, cell bodies and projections)	milliseconds - seconds	High spatiotemporal resolution; diverse set of fluorescent dyes and protein sensors	Low biopentrance and small wavelengths can be phototoxic; limited use <i>in vivo</i>
2P Microscopy	Fluorescent sensors are imaged using two-photon excitation, typically at twice the wavelength	$\mu\text{m}$ - mm / synapses	milliseconds - seconds	Longer wavelengths used in 2P allow higher biopentrance and less phototoxicity; <i>in vivo</i> imaging is possible	Expensive and complicated to implement; biopentrance is still limited to 10s – 100s of $\mu\text{m}$ ; incompatible with FRET sensors
Fiber Photometry	A fiberglass fluorescence probe is surgically implanted into the brain; changes in fluorescence intensity of genetic probes are measured over time	mm / brain region; specific circuits	milliseconds - seconds	Protein sensors can be used <i>in vivo</i> in behaving animals; widely adaptable in labs already measuring GCaMP	Limited spatial resolution
PET	Positron-emitting ligands can target specific receptors in order to monitor dopaminergic activity	mm / brain region	minutes	High sensitivity (i.e. small amount of ligand needed) with a diverse set of ligands; can be used in humans and coupled with behavior	Ligands are expensive, difficult to synthesize, and can have short half-lives; poor spatiotemporal resolution, impossible to resolve ligand signal from its metabolite signal
MRI	Magnetic resonance is used to image endogenous dopamine metabolites or exogenous MR-active ligands	sub-mm - mm	milliseconds - seconds	Potentially non-invasive, can be used in humans and coupled with behavior; ligands can be used to increase spatiotemporal resolution or highlight specific structures	Information is largely structural and not functional
MRS	Magnetic resonance is used to generate spectra that can identify specific metabolites and their relative concentrations	mm-cm	seconds - minutes	Can identify and resolve multiple molecules and their relative concentrations within the same voxel, including ligands from their respective metabolites	Highly specialized technique, lower spatiotemporal resolution than MRI and PET; certain nuclides ( $^{13}\text{C}$ , $^{15}\text{N}$ , and $^{31}\text{P}$ ) can have very long scan times

Table 2.

## Summary of fluorescent dopamine sensors

Name	Category	Functionality / excitation wavelength	Target	Advantages	Limitations
Dopamine (self)		2P microscopy/540 nm		direct detection of dopamine	low biopentrance, sensitivity
Acridine orange	small molecule	fluorescence microscopy/460 nm	acidic compartments (synaptic vesicles)	pH sensitive; can detect release	only reported in cell culture; not specific to dopamine
FM1-43	small molecule	fluorescence microscopy/472 nm	cell membrane (synaptic vesicles, post-stimulation)	labels recycling vesicle pool	not neurotransmitter specific
FM4-64	small molecule	fluorescence microscopy/515 nm	cell membrane (synaptic vesicles, post-stimulation)	red, can be used in conjunction with blue and green sensors	not neurotransmitter specific
NeuO	small molecule	fluorescence microscopy/468 nm	live neurons	can be used <i>in vivo</i> ; reported in mice and zebrafish	not neurotransmitter specific
FFN511	small molecule	fluorescence microscopy/406 nm; 2P/760 nm	DAT and VMAT2	selective for dopamine axons	less specific than later FFNs; most FFNs are limited to practical use <i>ex vivo</i>
FFN102	small molecule	fluorescence microscopy/406 nm; 2P/760 nm	DAT and VMAT2	pH sensitive; can detect release	not as bright when stored in vesicles
FFN200	small molecule	fluorescence microscopy/406 nm; 2P/760 nm	VMAT2	DAT independent, can detect silent synapses	longer incubation period and wash than other FFNs
FFN270	small molecule	fluorescence microscopy/406 nm; 2P/760 nm	NET, DAT, and VMAT2	pH sensitive; can detect release; has been used <i>in vivo</i>	more potent at NET than DAT
FFN206	small molecule	fluorescence microscopy/406 nm; 2P/760 nm	DAT, VMAT1, and VMAT2	useful for high- throughput studies, and fly imaging	less effective in mice
APP+	small molecule	fluorescence microscopy/416 nm; 2P/800 nm	DAT, NET, SERT	good monoamine sensor/ high-throughput studies	not DA specific; labels mitochondria
NS521	small molecule	fluorescence microscopy/488 nm	monoamines	survives fixation	not DA specific
ES517	small molecule	fluorescence microscopy/488 nm	monoamines	adds pH sensitivity to NS521, can image release	not DA specific
nIRCat	nanoparticle	fluorescence microscopy/785 nm; 2P/1600 nm	dopamine, norepinephrine	near-IR sensor allows high biopentrance; DNA sequence is tunable	not widely available; requires intracranial injection
CNiFER	HEK cell/ protein sensor	fluorescence microscopy and fiber photometry/436 nm (FRET)	dopamine	does not require genetic manipulation	requires exogenous cell implant
DopR-Tango	genetic protein sensor	Postmortem analysis	DIR activation	good spatial resolution for post-synaptic analysis	requires 48 h postexcitation analysis
Name	Category	Functionality/excitation wavelength	Target	Advantages	Limitations
dIight/GRAB <sub>DA</sub>	genetic protein sensor	fluorescence microscopy and fiber photometry/488 nm	Dopamine	Easy to introduce into mice and analyze dopamine release during behavior; newer generations come in red	newer approach, less characterized

<b>Name</b>	<b>Category</b>	<b>Functionality / excitation wavelength</b>	<b>Target</b>	<b>Advantages</b>	<b>Limitations</b>
VoLDeMo	targeted voltage-sensing dye	2P microscopy/920 nm	DAT; changes in voltage	can sense changes in voltage in DAT-expressing membranes (axons)	targeted probes have less dynamic range than untargeted voltage-sensing dyes
Voltron	genetic voltage-sensing protein + small molecule	fluorescence microscopy (FRET)/variable	changes in voltage	allows for fine experimental control based on which halo-tag dye is used	FRET approach is not amenable to 2P microscopy

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