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Synaptic optical imaging platforms: Examining pharmacological modulation of neurotransmitter release at discrete synapses

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Abstract

Chemical synapses are not only fundamental functional units of the brain but also anatomical and functional biomarkers of numerous brain disorders. Therefore, new experimental readouts of synaptic function are needed – with the spatial resolution of single synapses and the scale to image large ensembles of synapses in specific circuits – for the study of both acute and chronic effects of pharmacological agents on synaptic plasticity in living mammals. In this article we discuss the design and use of fluorescent false neurotransmitters (FFNs) as an important step in the development of versatile synaptic imaging platforms.

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Keywords

Fluorescent probes; Synapses; *In vivo* imaging; Brain receptors; Synaptic transmission

Synaptic connections are the essential components of neural circuits in the brain. Numerous studies have demonstrated that synaptic plasticity is required for information coding by the brain, (Sadegh et al., 2014) synapse loss is a pathological hallmark of neurodegenerative diseases (Terry et al., 1991; Overk and Masliah, 2014; Selkoe, 2002), and synaptic dysfunction underlies neuropsychiatric disorders, including autism, schizophrenia, and mood disorders (Tang et al., 2014; Kang et al., 2012; Karayannis et al., 2014; Lu et al., 2013). Consequently, development of new experimental methods for monitoring synaptic function across a wide variety of experimental systems is of central importance in neuroscience and neuropharmacology. The ability to image the effects of activation (or inhibition) of central

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nervous system (CNS) receptors or signaling pathways on the dynamics of discrete synapses, and their ensembles, in specific circuits represents an important future goal in the field of neuropharmacology. We further propose that studying the effects of pharmacological agents on synaptic function ought to become a common experimental readout in the process of development and evaluation of new drug candidates.

In this article we focus on the synaptic release of monoamine neurotransmitters ([NTs] dopamine, norepinephrine, and serotonin) which play important roles in modulating the strength of both excitatory and inhibitory neurotransmission via activation of their corresponding receptors (Bamford et al., 2004; Ellender et al., 2011; Cai et al., 2013). Monoamine neurons, located in small nuclei of the midbrain and brain stem, project their axons throughout the brain and regulate diverse brain functions including arousal, stress, emotion, reward, learning, and cognition. Aberrations in monoamine neurotransmission have been implicated in numerous neurological and neuropsychiatric disorders including Parkinson's disease, schizophrenia, attention deficit hyperactivity disorder (ADHD), drug addiction, depression, and anxiety (Sulzer, 2011; Rothman and Baumann, 2003; Tye et al., 2013; Vazey and Aston-Jones, 2012). Furthermore, monoamines also modulate responsivity of their target cells and synapses (Aston-Jones and Cohen, 2005; Wise, 2004). As a specific example, dopamine (DA) release in the dorsal striatum and the basal ganglia in general is essential for motor coordination and habit formation (Graybiel, 2005). One of us (D. Sulzer) proposed a synaptic mechanism underlying motor and movement coordination where dopaminergic synapses and dopamine release “filter” active glutamatergic excitatory inputs originating in the motor cortex. Cortical glutamatergic and nigral dopaminergic synapses converge on GABAergic medium spiny neurons in the dorsal striatum (Fig. 1) where the dopamine inputs inhibit the activity of the majority of cortico-striatal glutamatergic synapses, enabling the emergence of a few strong cortico-striatal connections (Bamford et al., 2004). However, in a living mammalian brain, monoamine neurotransmission has only been studied on the bulk level of large ensembles of monoaminergic synapses (via microdialysis or voltammetry) (Kita and Wightman, 2008), as ***there have been no experimental tools to examine the NT release characteristics of individual presynaptic boutons, a fundamental physiological parameter*** (Sames et al., 2013).

Fluorescence microscopy offers sufficient spatial and temporal resolution for examination of single presynaptic boutons and their activity (approx. 1 μm in size, milliseconds to seconds). Introduction of fluorescent calcium ion indicators and membrane voltage-sensitive sensors based on organic dyes greatly enabled research in neuronal and synaptic activity (Tsien, 1989; Knöpfel et al., 2006). Despite the importance of these probes, their use in living tissue is limited by their lack of selectivity for specific cellular types. The selective targeting was addressed by developing genetically encoded calcium indicators (GECIs), such as GCaMPs, which have rapidly become the standard tool for measuring neuronal activity in cell bodies, dendrites, and axonal extensions (Mank and Griesbeck, 2008; Chen et al., 2012). Similarly, development of protein based voltage sensors is advancing rapidly, which will enable optical measurement of electrophysiological parameters of neural circuits, individual neurons and synapses (St-Pierre et al., 2014; Kralj et al., 2012; Hochbaum et al., 2014; Peterka et al., 2011). Despite the importance and broad utility of GECIs and fluorescent voltage sensors,

these tools do not indicate synaptic NT release. Exocytotic neurotransmitter release at individual axonal boutons is a probabilistic event, and thus release does not occur with each action potential or subsequent calcium transient [described by release probability at each synapse (Branco and Staras, 2009)]. Furthermore, the calcium and voltage sensors provide no information about presynaptic processes such as NT redistribution and release via reverse transport induced by psychostimulant drugs (Rothman et al., 2001, 2005). Therefore, ***calcium and voltage indicators alone are not well suited for studying release properties from individual presynaptic boutons and their ensembles.***

As a result, ***many questions of fundamental interest with regard to synaptic control of NT release in the brain remain unanswered.*** For example, how does NT release change during the two major physiological modes of firing, tonic (continuous action potential pulses at low frequency of 1–4 Hz) versus phasic [1–5 action potential bursts at 15–20 Hz (Grace and Bunney, 1984)]?; how does release correlate with voltage and calcium transients?; are there silent terminals, i.e., those that uptake NT, receive action potentials, but do not release? (Crawford and Mennerick, 2012); do releasing boutons change position along the axon?; do release properties change at different cellular targets?; how are all of these parameters modulated by specific auto- and heteroreceptors (such as mGluRs, nAChRs) or dysregulated in brain disease models?

Prompted by these questions and the limitations of existing methods, we developed a new class of imaging probes, termed ***fluorescent false neurotransmitters (FFNs)***, which act as optical tracers of dopamine and enable labeling of dopaminergic presynaptic boutons. FFNs have provided the first means for optical imaging of synaptic vesicular content release at individual DA presynaptic boutons and provide a novel approach for studying synaptic plasticity in the brain (Gubernator et al., 2009; Rodriguez et al., 2013). We approached the task of optical imaging of NT release by fluorescent “labeling” of synaptic vesicle content with fluorescent NT tracers. This was achieved by designing fluorescent substrates of the vesicular transporter (VMAT2) (Hu et al., 2013), and more recently dual substrates of VMAT2 and the plasma membrane dopamine transporter (DAT) (Rodriguez et al., 2013), as exemplified by the probe FFN102 (Fig. 2). In addition to its properties as a dual transporter substrate, we designed FFN102 as a pH sensor (Lee et al., 2010), affording a fluorescence increase during exocytosis due to the change of pH between the acidic lumen of synaptic vesicles and the neutral extrasynaptic milieu. Thus, FFN102 contains a triple design feature – it functions as a VMAT2 substrate, a plasma membrane DAT substrate, and an optical pH sensor - rendering a new class of FFNs we termed “flashing FFNs”. This was accomplished by systematic molecular design and chemical synthesis via merging the key structural features of monoamine NTs and coumarin chromophore cores (Rodriguez et al., 2013). Many other fluorophores have been examined but failed for various reasons (e.g., large size or high background labeling of brain tissue) (Karpowicz et al., 2013).

FFNs Provide Two New Functional Synaptic Parameters. After subtraction of the extrasynaptic signal (released probe), FFNs permit the measurement of the rate of vesicular content depletion, expressed as $t_{1/2}$, for each bouton from the exponential destaining curves (Gubernator et al., 2009; Rodriguez et al., 2013). The measured fluorescence signal indicates the remaining FFN in the presynaptic boutons. Flashing FFNs, such as FFN102, provide a

new parameter based on imaging the released tracer (Rodriguez et al., 2013). Current efforts suggest that it will be possible to measure the single action potential-induced release (measured as F/F) both at the bouton and in its vicinity and thus characterize discrete release sites under both tonic and phasic firing regimes. Characterization and use of FFN102 (as well as other FFNs) in acute slice have revealed that individual dopaminergic presynaptic boutons in the dorsal striatum differ widely in terms of their release kinetics. We also found unexpectedly that the majority of FFN-positive boutons (80–90%) do not destain FFN102 under electrically induced stimulation (Rodriguez et al., 2013). The future efforts in our laboratories will be addressing exciting questions including whether these non-destaining terminals are indeed silent terminals, the mechanisms underlying silencing and “unsilencing” of presynaptic terminals, and the potential physiological relevance of this synaptic on/off switch.

With respect to neuropharmacology, FFNs also enable visualization of synaptic content release by psychostimulants such as amphetamine (a widely prescribed and abused compound). Amphetamine induces dopamine and norepineprine release via reverse transport through DAT/NET (Rothman et al., 2001, 2003, 2005). FFNs enable visualization of this pharmacologically induced redistribution of NT pools (Gubernator et al., 2009; Rodriguez et al., 2013; Hu et al., 2013). Further, using FFN102 together with amperometric measurements in striatal slices, it was also found that methylphenidate, another frequently prescribed psychostimulant to treat ADHD, as well as cocaine inhibit synaptic dopamine release, an unexpected effect that occurs at low micromolar and higher concentrations of the drugs (Federici et al., 2014). The NT release inhibition is masked in the sub-micromolar and low micromolar range by the well-established dopamine neurotransmission enhancing effects of methylphenidate and cocaine via blockage of DAT, but the inhibition effect becomes dominant at concentrations of 10 μ M and higher (Fig. 3). It was suggested by this study that the inhibition effect is related to decreasing synapsin I phosphorylation and thus inhibiting the synaptic exocytotic machinery. The psychostimulants methylphenidate and cocaine exert two distinct effects, at least in brain tissue *ex vivo*, which reshape the strength and temporal domain of dopaminergic neurotransmission depending on the drug concentrations. Recent measurements and estimates suggest that 10–20 μ M range is reached in the brain under conditions that produce subjective effects (Zheng and Zhan, 2012) and maintain self-administration (Zimmer, Dobrin, & Roberts, 2011) due to accumulation of these compounds in the brain. Therefore these findings may have physiological relevance. This recent study illustrates the potential of FFNs and related synaptic probes in neuropharmacology, revealing the effects of drugs on synaptic NT transmitter release (Fig. 3).

Hitherto the majority of our studies with FFN probes have been conducted in acute mouse brain slices (an acute *ex vivo* experimental system) in one brain area (the dorsal striatum). However, our current efforts indicate that FFNs will be applicable *in vivo* in living rodents, in many different brain areas including neocortical regions that are readily accessible via multi-photon microscopy. Further, we focus on design of FFN probes that target other monoamine synapses, most notably the cortical noradrenergic release sites to expand the FFN concept to other NT systems. We are thus approaching the reality of the envisioned *in vivo* synaptic imaging platforms. Although it is desirable for FFNs and other probes and

sensors to be applicable in a number of widely used experimental systems such as *in vitro* neuronal culture and acute brain slices, *in vivo* microscopic methods in living rodents are rapidly assuming a prominent role in neuroscience. This trend is likely to continue, as many properties of intact circuitry in the entire mammalian brain cannot be recapitulated *in vitro*. The chronic cranial window (Holtmaat et al., 2009) will enable repetitive multi-photon microscopy imaging of the same presynaptic boutons, during various stimulation protocols in anesthetized animals. The ability of selective stimulation of specific neurons and their axonal projections is now readily approachable via optogenetic techniques and thus the intersection of specific stimulation sequences and pharmacological modulation of discrete synapses will become accessible. The chronic cranial window will also enable long-term studies of synaptic plasticity in conjunction with specific behavioral and treatment paradigms in awake animals. Therefore, observing the activity and regulation of specific synaptic boutons -which represent long standing questions in neuroscience and neuropharmacology (see above) - will be accessible under both acute and chronic pharmacological treatments.

In the broader outlook, we see FFNs as the first step in the process of gaining experimental access to multiple functional synaptic parameters in the brain. *FFNs are “chemical” probes* that enable imaging of both the microanatomy and the uptake/release parameters of individual presynaptic terminals by simply incubating brain tissue with the probe (or by microinjection of the probe into the brain) *with no need for prior chemical or genetic tissue manipulation*. In contrast to voltage and calcium dyes, FFNs are selective for dopaminergic neurons, dendrites and synapses (Sames et al., 2013). Further, the photophysical properties are favorable (2-photon excitation with ~760 nm light and emission in the blue region ~ 450 nm), which renders ***FFNs compatible with common fluorescent protein-based markers and sensors*** (including green/GFP, yellow/YFP, and red/mCherry fluorescent proteins and related sensors). In addition to these strengths, FFNs, like any probes, suffer from some shortcomings. Most notably, FFNs as synaptic vesicle content markers do not actually measure the concentration of NTs in the synaptic cleft or in the extrasynaptic areas. This issue will be addressed by NT sensors, which will complement FFNs in this regard very well (Muller et al., 2014). Currently, there are protein-based sensors available only for glutamate (Marvin et al., 2013), however this state of affairs is likely to change in the near future. We expect that a variety of genetically encoded fluorescent sensors will be developed for many NTs including the monoamines (Sames et al., 2013).

Compatibility of FFNs with protein-based and other sensors will render many exciting possibilities and imaging capabilities. For example, the simultaneous use of FFNs and calcium and voltage sensors deployed in the same presynaptic terminals will permit detailed examination of the interplay between electrical activity, subsequent calcium transient spiking, and NT release in specific synaptic ensembles of chosen circuits. Selective expression of calcium or voltage sensors in the postsynaptic cells or adjacent synapses will allow for the study of synaptic plasticity in the context of synaptic microcircuits such as the striatal system shown in Fig. 1.

Historically, pharmacological modulation of CNS receptors and its consequences on brain function has been the defining subject of neuropharmacology. In this context, many exciting

possibilities emerge when considering the judicious application of FFNs (and or other current and future synaptic probes) with fluorogenic receptor activation readout mechanisms and fluorescent ligands discussed in this special issue of Neuropharmacology. Namely, it will be possible to study the effects of specific receptor activation events on synaptic NT release as well as other synaptic functional parameters in circuits of interest, with the spatial resolution of discrete synapses and temporal resolution relevant for the synaptic processes at hand.

With the growing interest in mapping and understanding behavior in terms of circuits and their dynamics, there is an equal need to advance our understanding of the effects of specific molecular perturbation (via pharmacological agents) on the circuits. In brain research, one essential link between these different complexity levels (behavior, circuits, cells) is the chemical synapse. We therefore foresee that versatile synaptic imaging tools and platforms will be an essential component of modern neuropharmacology and brain sciences in general.

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Fig. 1. Excitatory and dopaminergic inputs into the dorsal striatum (dSTR)

(A) Sagittal slice of the mouse brain showing glutamatergic inputs (yellow) into the dSTR (blue region) from layer 5 of the motor cortex (MCTX5, yellow region) and dopaminergic inputs (purple) into the dorsal striatum from the substantia nigra (SN, pink region). Glutamatergic axons from the cortex (shown) and thalamus (not shown) as well as dopaminergic axons from the SN (shown) spread widely and form complex arborizations in the dSTR (estimated 10^5 synaptic boutons per SN neuron). Image of the background sagittal slice is adapted from the Allen Developing Mouse Brain Atlas. **(B)** Graphic showing the complex regulation of individual synapses on medium spiny neurons (MSNs) in the dSTR. Glutamate (GLU, yellow circles) modulates MSNs by binding to the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, the N-methyl-D-aspartic acid (NMDA) receptor and the metabotropic glutamate receptors (not shown). Dopamine (DA, purple circles) modulates MSNs by binding to D1 and D2 like receptors. The MSNs are further modulated by cholinergic synapses (via the muscarinic acetylcholine receptor M1) and GABAergic synapses (not shown) as well as a variety of neuropeptides. Both dopaminergic and glutamatergic neurons are regulated presynaptically by D2 receptors and nicotinic and muscarinic acetylcholine receptors. The glutamatergic inputs are further regulated by cannabinoid release from MSNs.

Fig. 2. Fluorescent False Neurotransmitters (FFNs) are optical tools that enable the visualization of synaptic vesicle content release at individual presynaptic terminals

FFN102 is a fluorescent dopamine transporter (DAT) substrate, which selectively labels presynaptic dopamine terminals in the dorsal striatum of the mouse brain. It is accumulated into synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2). Its fluorescence is attenuated (blue circles) inside the acidic synaptic vesicles and unquenched (blue stars) upon stimulation when it is released to the extracellular physiological pH. FFN102 therefore allows for optical measurement of released vesicle content.

Fig. 3. Unexpected effect of methylphenidate on the release of dopamine and FFN102 in mouse brain

(A) Structure of methylphenidate. (B) Acute striatal slices loaded with FFN102 were electrically stimulated at a frequency of 10 Hz starting at time point $t = 0$. Stimulation continues until the final time point. Error bars represent S.E. The change in fluorescence signal obtained by 2-photon microscopy (field of view of $37.5 \times 37.5 \mu\text{m}$ including ~ 150 presynaptic boutons) upon stimulation was $19.6 \pm 1.4\%$ in control slices (mean $\pm$ S.E., $n = 5$) and $8.8 \pm 1.2\%$ in slices with $30 \mu\text{M}$ methylphenidate (MPH, mean $\pm$ S.E., $n = 5$). Methylphenidate inhibited the evoked increase in fluorescence ($p < 0.0001$, two-way ANOVA; $p < 0.0001$ for methylphenidate versus control). Dopamine release (measured by constant potential amperometry) was inhibited by the same extent (not shown).