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Temporal dynamics of neuromodulator release in motor cortex revealed by cell-based optically-active probes

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Neurosciences

by

Victory Teresa Joseph

Committee in charge:

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2013
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2013
DEDICATION

For my father, who taught me to dream;
And for Steven, who makes my dreams reality.
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Sections within Chapters 1, 2 and 3, have been submitted for publication of the material. Muller, Arnaud*; Joseph, Victory*; Slesinger, Paul; Kleinfeld, David. The dissertation author was a primary investigator and author of this material.
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ABSTRACT OF THE DISSERTATION

Temporal dynamics of neuromodulator release in motor cortex revealed by cell-based optically-active probes.

by

Victory Teresa Joseph

Doctor of Philosophy in Neurosciences

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Professor Paul Slesinger, Chair
Professor David Kleinfeld, Co-Chair

Stimulus-to-action sequences are believed to involve the release of dopamine (DA) and noradrenaline (NA). However, the electrochemical similarity of these two monoamines has limited real-time measurements of their release. As such, the temporal dynamics of DA and NA release in the cortex during learning and behavior remain poorly understood. To meet the challenge of assessing DA and NA in cortex in real time, here I report the creation of two cell-based neurotransmitter fluorescent-
engineered reporters (CNiFERs) that discriminate nanomolar concentrations of DA and NA. These CNiFERs transform neurotransmitter receptor binding into a change in fluorescence and provides a direct and rapid optical read-out of local neurotransmitter activity. This generation of CNiFERs provides a new tool for examining the release of DA and NA in vivo and, by extension, the release of any molecule for which there is a G protein coupled receptor.

To probe the temporal patterns of neurotransmitter release, specifically DA, NA, and acetylcholine (ACh), during classical conditioning, CNiFERs were implanted into the motor cortex of mice and imaged using two-photon microscopy. The presentation of the unconditioned stimulus, a drop of sucrose solution, triggered the release of DA, NA, and ACh in the motor cortex. The release of ACh consistently tracked the time of licking. With conditioning, the onset of DA release and licking behavior monotonically shifted, in a correlated manner, from the time of the reward toward that of the cue, the conditioned stimulus. Concurrent release of NA, on the other hand, did not correlate with licking or the cue. Thus, by using newly realized CNiFERs to probe in vivo temporal dynamics of transmitter release, we were able to determine that DA, but not NA, release in motor cortex corresponds to the extent of learning, as defined by changes in the animals’ licking behavior and that ACh release in the motor cortex corresponds to the animal’s motor output.
Chapter 1: Introduction

Neuronal processing in the cortex is essential for the transformation of sensory perception to motor output and action. Slow-acting neuromodulators are involved in the execution of this transformation and in the plasticity and refinement of neuronal processing with increased experience (Adamantidis et al., 2011; Tsai et al., 2009). Neuromodulators such as dopamine (DA), noradrenaline (NA), and acetylcholine (ACh) are molecules that act through volume conduction in the extracellular space, allowing them to simultaneously affect multiple, diverse neurons within an area. This is in contrast to synaptic transmission in which a pre-synaptic neuron directly influences a post-synaptic neuron at dedicated synapses. Additionally, because neuromodulators signal through metabotropic G-protein coupled receptors (GPRCs), they typically act on longer time-scales such as seconds, as opposed to the millisecond scale of traditional synaptic transmission through ionotropic receptors. Importantly, however, they may also act over a much longer time scales, including hours and days, and may thereby play different functional roles across the range of time scales (Parikh et al., 2007). As such, neuromodulation undoubtedly plays a significantly different role in neural processing and computation than fast-acting neurotransmission.

1.1 Neuromodulators in sensory processing

Neuromodulators have primarily been implicated in a variety of sensory processes that are required for the brain to adapt to a changing environment including the formation of working memories, enhancement of decision-making
(Aston-Jones and Cohen, 2005; Bouret and Sara, 2005; Floresco, 2013), and perceptual learning (Bao et al., 2001; Bao et al., 2003; Edeline et al., 2011). Specifically, several lines of evidence suggest that the release of ACh, NA, and DA in the frontal cortex is critical for attention and task engagement to modulate these processes.

Evidence for the role of cortical ACh in attention comes primarily from direct measurements of ACh in the cortex and lesion studies in animals performing attention-demanding tasks such as cue-detection and serial choice. Several microdialysis studies have revealed increases in cortical ACh release associated specifically with attentional demand (Arnold et al., 2002; Passetti et al., 2000; Himmelheber et al., 2000). Parikh et al. (2007) measured this more precisely using electrochemical measurements of cholinergic activity to gain better temporal resolution and demonstrated that during a cued appetitive response task, ACh is released in the prefrontal cortex in response to cue-detection. However, ACh release was absent during missed cues, presumably when the animal was not attending to the task. ACh release was also absent during reward delivery and retrieval, suggesting its release was specifically tied to attention and not behavior. Additionally, lesions of the basal forebrain, which sends cholinergic inputs to the cortex, results in persistent impairment on attention-based performance (Muir et al., 1992; Muir et al., 1994; McGaughy et al., 2002).

Similarly, cortical NA is also thought to be a critical determinant in attention-based performance. Neurons in the locus coeruleus (LC), which sends noradrenergic projections to the cortex, transiently spike in response to task-relevant stimuli (Aston-Jones and Cohen, 2005; Bouret and Richmond, 2009). In these experiments, poorly
performing animals, which are inattentive or non-alert, exhibit low tonic firing in the LC, whereas high tonic firing is observed in poorly performing animals that are distractible. In contrast, phasic firing is observed in the LC during task engagement. Microdialysis measurements taken during a conditioning task, similar to those done in the recording experiments, have shown elevated levels of NA in the cortex during conditioning sessions relative to one hour prior to and one hour following the session (Feenstra et al., 2000). Additionally, pharmacological recruitment of NA by a receptor antagonist has also been shown to enhance attention-dependent performance on cue-switching tasks (Devauges and Sarah, 1990).

Dopamine has also been extensively studied and implicated in sensory processing and attention, specifically as it relates to reinforcement learning and the detection of salient stimuli. Dopamine neurons in the midbrain transiently spike during reinforcement learning (Schultz, 2013) and release DA in the striatum and prefrontal cortex (Feenstra, 2000; Tsai et al., 2009). Extensive studies in non-human primates (Romo and Schultz, 1990; Schultz et al., 1993) and rodents (Kosobud et al., 1994; Miller et al., 1981) demonstrated that classically conditioned cue-reward pairings increased neuronal firing in midbrain neurons with a temporal shift from the time of the reward, i.e., the unconditioned stimulus, to the time of the predictive cue, i.e., the conditioned stimulus. Similar to NA, the release of dopamine in the cortex, however, has not been measured with enough temporal precision to determine its behavioral triggers because of technical limitations in available methods. Nonetheless, it has been shown with microdialysis and voltammetry that DA is released in subcortical structures in response to occurrences of unexpected events, novel stimuli, and salient stimuli regardless of valence or motivational properties.
(Horvitz, 2000), again suggesting that it is triggered by attentional demand. Notably, the literature on DA is not limited to suggesting its role in attention, but also implicates DA in sensorimotor learning and the initiation and termination of sequences of movements (Jin and Costa, 2010). These disparate findings indicate that the function of dopamine neurons may be region and target-specific.

1.2 Neuromodulators in motor cortex

Interestingly, while ACh, NA, and DA have typically been associated with sensory processing in frontal cortices, they are released in motor cortex, an area typically involved in motor output and movement (Ferrier, 1874; Komiyama et al., 2010; Hill et al., 2011). This suggests that either sensory processing is also occurring in motor cortex or that the role of these neuromodulators cannot be fully described by their role in attention. As such, studying the release of these neuromodulators in the motor cortex provides a unique opportunity to challenge the canonical role of these neuromodulators in neural processing.

Motor cortex receives dopaminergic projections from the ventral tegmental area and substantia nigra (Hosp et al., 2011), noradrenergic projections from the locus coeruleus (Loughlin et al., 1986) and cholinergic projections from the basal forebrain (Connor et al., 2010) (Fig. 1). While it has been shown that disruption of ACh and DA transmission results in impaired plasticity and acquisition of motor skills (Connor et al. 2010; Molina-Luna et al. 2009) and that the neuronal activity in superficial layers of motor cortex changes with behavioral learning (Komiyama et al., 2010; Huber et al., 2012), very little is known about how this is regulated, and it may
in fact be regulated by attention or sensory processing. Specifically, what triggers the release of ACh, NA, or DA in the motor cortex is unknown. In this dissertation I ask if neuromodulator release in motor cortex is associated with attention.

![Figure 1: Schematic of neuromodulator projections to motor cortex.](image)

**Figure 1: Schematic of neuromodulator projections to motor cortex.** Motor cortex receives dopaminergic projections (black) from ventral tegmental area (VTA) and substantia nigra (SN), noradrenergic projection (green) from locus coeruleus (LC), and cholinergic projections (blue) from basal forebrain (BF).

### 1.3 Techniques for measuring neuromodulator release

While deciphering how neuromodulators act is critical, it has been a challenge to develop methods to detect the release of the relevant molecular transmitters *in vivo* with sufficient chemical specificity, spatial resolution, and temporal resolution (**Fig. 2**). The most common technique to measure the extracellular concentration of neuromodulators is microdialysis. Here, a dialysis probe is stereotaxically implanted into the brain, and samples are collected for identification by high-performance liquid chromatography and/or mass spectrometry. This technique can accurately identify and differentiate between monoamines (Greco et al., 2013; Ji et al., 2008); yet it requires the collection of relatively large samples and has poor temporal resolution, exceeding ten minutes per sample in the case of monoamines (Mingote et al., 2004). Dialysis studies have shown efflux of both DA and NA in the cortex during conditioning (Feenstra, 2000; Mingote et al., 2004), but these measures lacked
sufficient temporal resolution to determine the relationship of their release to specific stimuli and cognitive processes within conditioning trials. Furthermore, the insertion of microdialysis probes can disrupt monoaminergic activity near the probe track (Wang and Michael, 2012). An alternative method, fast-scan cyclic voltammetry, provides excellent temporal resolution but lacks chemical selectivity as DA and NA differ chemically by a single hydroxyl side-group and have indistinguishable cyclic voltammetry signatures (Robinson et al., 2003). Thus, the use of fast-scan cyclic voltammetry has been limited to areas of the brain in which only one of DA or NA is thought to be present. For example, cyclic voltammetry is appropriate for the striatum, which receives a strong DA projection (Park et al., 2011). In contrast, measurements of DA and NA in neocortex, which a priori have roughly equal [DA] and [NA], remains a challenge for cyclic voltammetry.

To meet the challenge of assessing [DA] and [NA] in cortex in real time, here we expand on a previously developed cell-based neurotransmitter fluorescent engineered reporter (CNiFER) that was created to measure acetylcholine release in vivo (Nguyen et al., 2010). These biophotonic sensors are clonal cell-lines engineered to express a specific G_{q}-type G protein coupled receptor (GPCR) that triggers an increase in intracellular [Ca^{2+}] which is rapidly detected by a genetically encoded FRET-based Ca^{2+} sensor. This system transforms neurotransmitter receptor binding into a change in fluorescence and provides a direct and rapid optical read-out of local neurotransmitter activity. Furthermore, by utilizing the natural receptor for a given transmitter, CNiFERs gain the chemical specificity and temporal dynamics present in vivo. Based on these principles, and chimeric forms of G-proteins, we created the next generation of CNiFERs to detect DA and NA in vivo.
1.4 Hypothesis

Neuromodulators have primarily been implicated in a variety of sensory processes that are required for the brain to adapt. However, they are released in motor cortex, an area typically involved in motor output and movement. In this dissertation I ask if neuromodulator release in motor cortex is associated with attention. I hypothesized that the release of DA, NA, and ACh in motor cortex would reflect changes in attention due to associative learning. To test this, we trained mice on a classical conditioning task in which an auditory cue was paired with the presentation of reward after a fixed interval. The retrieval of reward required the mice to perform a motor behavior, licking. I demonstrate real-time, concurrent measurements of DA, NA, and ACh release in murine motor cortex during conditioning using newly designed and realized CNiFERs. I then determined whether the release of transmitter tracks the motor behavior or instead is triggered by the reward-predictive cue. I predicted that the release of DA, NA, and ACh would be triggered in response to the reward-predictive cue, reflecting changes in attention due to associative learning.
In Chapter 2, I detail the development and application of CNiFER technology. In Chapter 3, I test the hypothesis that DA, NA, and ACh release in motor cortex is triggered by attention and challenge the canonical role of these neuromodulators in the brain.

Chapter 1, in part, is a reprint of the material that has been submitted for publication. Muller, Arnaud*; Joseph, Victory*; Slesinger, Paul; Kleinfeld, David. The dissertation author was a primary investigator and author of this material.
Chapter 2: CNiFERs

To meet the challenge of assessing [DA] and [NA] in cortex in real time, here we expand on a previously developed cell-based neurotransmitter fluorescent engineered reporter (CNiFER) that was created to measure acetylcholine release in vivo (Nguyen et al., 2010). CNiFERs are clonal HEK293 cell-lines engineered to express two proteins: (i) a specific G-protein coupled receptor (GPCR) and (ii) TN-XXL, a genetically encoded FRET-based [Ca^{2+}] sensor utilizing a component of troponin to bind Ca^{2+} (Nguyen et al., 2010; Yamauchi et al., 2011). Activation of a metabotropic G_{q}-coupled receptor triggers an increase in cytosolic [Ca^{2+}] through the inositol trisphosphate pathway. Calcium binds to TN-XXL and induces a conformational change that permits fluorescence resonance energy transfer (FRET) between enhanced cyan fluorescent protein (eCFP) and Citrine cp174 fluorescent protein (YFP) (Fig. 3A). The change in fluorescence provides a direct and rapid optical read-out of the change in neurotransmitter levels (Fig. 3A,B). CNiFERs can be chronically implanted in neocortex and imaged through a cranial window with in vivo two-photon laser scanning microscopy (Nguyen et al., 2010) at depths up to several hundred micrometers below the pial surface.

2.1 Creation of CNiFERs selective to DA versus NA

To develop new CNiFERs to detect DA and NA, we selected two GPCRs with high affinity and selectivity; the D2 dopaminergic receptor and the α1a adrenergic receptor (Fig. 3A,B). The D2 dopaminergic receptor is G_{i/o}- and not G_{q}-coupled. To link the D2 receptor with the inositol trisphosphate pathway, we first created a clonal
HEK293 cells that expressed a chimeric G protein, Gqi5, in which the last five amino acids of the carboxyl terminal of the Gi/o alpha subunit were replaced with that of Gq subunit (Conklin et al., 1993). The Gqi5 chimera couples to the D2 receptor, but redirects signaling to the inositol trisphosphate pathway (Fig. 3A,B), to form a D2-CNiFER. The α1a adrenergic receptor is Gq-coupled and was used in HEK293 cells that expressed TNXXL, but were otherwise unmodified, to form a α1a-CNiFER.

As a qualitative, in vitro demonstration of the response of these new CNiFERs, presentation of a pulse of dopamine to a cluster of D2-CNiFERs led to opposing changes in eCFP versus YFP fluorescent emission, as expected for the FRET pair (Fig. 3C, left panel). The calculated fractional change in fluorescence, ΔF/F, for each signal (Fig. 3C, upper panel) was used to obtain a FRET ratio, denoted ΔR/R (Fig. 3C, lower panel), that is largely insensitive to motion artifacts (Nguyen et al., 2010) (see section 2.5: Methods). Similar results were obtained for the α1a-CNiFERs (Fig. 3C, right panel).
Figure 3: Design of D2- and α1a-CNIFERs. (A) Schematic of CNiFER signaling pathway. Dopamine (DA) stimulation of D2 GPCR, leads to activation of Gq/5 chimeric proteins. Noradrenaline (NA) stimulation of α1a GPCR, leads to activation of Gq G-proteins. Activated Gq stimulates phospholipase C (PLC), producing signaling intermediate inositol triphosphate (IP3) which stimulates release of Ca^{2+}, detected by the FRET based TN-XXL calcium sensor. (B) Depiction of DA activating D2 receptor (left, black) and NA activating α1a receptor (right, green) to induce IP3-mediated Ca^{2+} cytoplasmic influx detected by TN-XXL. Fluorescence from laser-excited enhanced cyan fluorescent proteins and citrine fluorescent proteins flanking TN-XXL is collected for the FRET signal. (C) FRET response of D2-CNIFER to a 2.5 s pulse of 100nM DA (left) and α1a-CNIFER to a 2.5 s pulse of 100nM NA (right). Example of transmitter-induced concurrent, opposing responses in citrine (530 nm) and cyan (475 nm) fluorescence (top) represented as a FRET ratio (bottom).
2.2 *In vitro* characterization of D2- and α1a-CNIFERs

We tested the sensitivity and specificity of the D2- and α1a-CNIFERs with a high-throughput fluorometric plate reader. The D2-CNIFERs displayed nanomolar sensitivity to DA, with $EC_{50} = 2.5 \pm 0.2$ nM (mean ± SE), and were ~30 times less sensitive to NA ($EC_{50} = 81 \pm 10$ nM) ([Fig. 4A, left panel](#)). Similarly, the α1a-CNIFERs exhibited nanomolar sensitivity to NA, with $EC_{50} = 19 \pm 2$ nM, and only responded to DA at non-physiological concentrations ($EC_{50} = 1.4 \pm 0.1$ µM ([Fig. 4A, right panel](#))). Furthermore, the dynamic range of the respective sensitivities of the CNIFERs were comparable to basal levels of monoamines measured in rodent brain, as found with microdialysis (Engelman et al., 2006; Ihalainen et al., 1999; Mingote et al., 2004). To test for non-specific receptor activation, we screened both CNIFERs against a panel of common neurotransmitters ([Fig. 4B](#)). The D2-CNIFERs responded weakly to somatostatin ($\Delta R/R = 0.2$), acetylcholine ($\Delta R/R = 0.1$) and VIP ($\Delta R/R = 0.1$) at high, non-physiological concentrations (1 µM). The α1a-CNIFERs did not show appreciable responses to other agonists.

We next confirmed the pharmacological receptor specificity of each CNIFER since HEK293 cells can endogenously express GPCRs ([Fig. 4C](#)). We observed that the response of the D2-CNIFER to 20 nM DA was not significantly altered following pre-incubation with the D1 receptor antagonist SCH23390 (100 nM; normalized, $\Delta R/R = 0.75$), but was fully blocked by pre-incubation with the D2 receptor antagonist eticlopride (50 nM; normalized $\Delta R/R = 0.04$). This confirms that the DA response of the D2-CNIFER is specific to the D2 receptor. For the α1a-CNIFER, we observed that the response to 50 nM NA was not significantly altered by pre-incubation with the β-
adrenergic receptor antagonist sotatol (5 \( \mu \text{M} \); normalized \( \Delta R/R = 0.82 \)) but was strongly suppressed by pre-incubation with the \( \alpha_1 \)-antagonist WB4101 (50 nM; normalized \( \Delta R/R = 0.09 \)). This confirms that the NA response of the \( \alpha_1 \)-CNiFER is specific to the \( \alpha_1 \) receptor.

We next determined the temporal resolution of the new CNiFERs. We used a fast perfusion system to apply agonists at saturating concentrations (Fig. 4D). The agonists were co-loaded with Alexa-594 to determine the precise perfusion time. Both D2- and \( \alpha_1 \)-CNiFERs responded robustly within 2 s of agonist application and, critically, could be distinguished by pulses of agonist with an inter-stimulus interval as short as 5 s. Lastly, under the condition of saturating agonist concentration, both CNiFERs required \( \sim \) 20 s for their FRET signal to return to baseline. Previous studies using FRET probes (Hoffmann et al., 2005) and bioluminescence resonance energy transfer signals (Falkenburger, Jensen et al. 2010) have shown that this slow recovery after agonist wash-off results from intrinsic properties of the GPCR signaling pathway.

A final analysis determined if the response of the CNiFERs desensitizes. To test this, we delivered long, repeated pulses of agonist, \textit{i.e.}, a pulses of 60 s duration every four minutes for 40 minutes (Fig. 4E). We observed a small adaptation in the D2-CNIFE response between the first and the second pulse of agonist, but a stable signal thereafter (\( n = 3 \)) (Fig. 4E, left panel). We observed no adaptation for the \( \alpha_1 \)-CNiFER, with a reproducible response across pulses (\( n = 3 \)) (Fig. 4E, right panel). This demonstrates that neither new CNiFER appreciably desensitizes to repeated neurotransmitter exposure.
Figure 4: *In vitro* characterization of CNiFERs. (A) Dose response of D2-CNiFER (left) and α1a-CNiFER (right) to DA (black) and NA (green). (B) Summary of D2-CNiFER (left) and α1a-CNiFER (right) FRET responses to panel of endogenous neurotransmitters. NA, noradrenaline; DA, dopamine; VIP, vasoactive intestinal peptide; GABA, gamma-aminobutyric acid. Error bars (A-B) represent standard deviation. (C) Left, D2-CNiFER FRET response to 20 nM DA in the presence or absence of D1-receptor antagonist SCH 23390 (100nM, blue), or D2-receptor antagonist eticlopride (50nM, red). Right, α1a-CNiFER response to 50nM NA in presence or absence of β-adrenergic receptor antagonist, sotatol (5µM, blue), or α1a-receptor antagonist, WB4101 (50 nM, red). CNiFER response to agonist alone normalized to one. (D) Left, discrimination of D2-CNiFER FRET-based responses to delivery of a two 2.5 s pulse of 100nM DA with variable interstimulus intervals. Right, discrimination of α1a-CNiFER responses to two pulses of 100 nM NA. (E) D2-CNiFER (left) and α1a-CNiFER (right) FRET responses to repeated 60 s pulses of 50 nM DA (black dashes) or 500 nM NA (green dashes) followed by 180 s of ACSF. Error bars and shaded grey areas (C-E) represent standard error.
2.3 *In vivo* characterization of D2- and a1a-CNiFERs

We re-tested the sensitivity of the new CNiFERs *in vivo* by implanting them into mouse primary motor cortex. To confirm the presence of direct projections from both dopaminergic and noradrenergic nuclei to the motor cortex, we first injected a neuronal retrograde tracer, Fluorogold™, into the motor cortex and searched for neurons co-labeled with Fluorogold™ and tyrosine hydroxylase, a biosynthetic enzyme for both DA and NA (Fig. 5A,B).

Three-dimensional reconstructions of the histological data (n = 2 brains) revealed that the majority of dopaminergic projections originated from substantia nigra (SN) (Fig. 5C, left panel), confirming previous studies that have shown projections from the SN to the prefrontal and motor cortex in rat (Loughlin and Fallon, 1984) and monkey (Hoover and Strick, 1999; Middleton and Strick, 2002). We observed relatively few neurons co-labeled for Fluorogold™ and tyrosine hydroxylase in the mouse ventral tegmental area, although such projections to primary motor cortex have been reported in rats (Hosp et al., 2011). Notably, Hosp et al. reported cortical projections primarily originating from the lateral VTA whereas we observe projections primarily from the medial SN. There is likely large overlap between these areas given the lack of clearly defined boundaries, both anatomically and physiologically, in this area of the midbrain, and given the difference in species.

Three-dimensional reconstruction also revealed that the majority of noradrenergic inputs originated from the locus coeruleus (LC) (Fig. 5C, right panel), corroborating previous studies with monkey (Gatter and Powell, 1977) and rat (Loughlin et al., 1986).
Figure 5: Identification of dopaminergic and noradrenergic projections to cortex. (A) Immunostaining for tyrosine hydroxylase (green) and fluorogold tracer (magenta) injected ~200µm deep into frontal cortex (+1.5mm A/P, +1.5mm M/L). Coronal sections including substantia nigra (SN) (left, A/P -3.5 mm) or locus coeruleus (LC) (right, A/P -5.6 mm). Threshold adjusted to display weak auto-fluorescence in order to show brain structure. (B) Co-labeling (white) of tyrosine hydroxylase and Fluorogold in SN (left) or LC (right), magnified from blue boxes in (A). (C) Position of co-labeled cell bodies in SN (left) or LC (right) indicated by red dots imposed on three-dimensional reconstructions as outlined by grey in (A).
Having confirmed the appropriate projections sites, we then surgically implanted CNiFERs into mouse motor cortex at discrete sites 200 to 300 µm below the cortical surface, *i.e.*, in layers 2/3 (Fig. 6B). The typical two-photon imaging plane contained five to ten CNiFERs per site. In response to a single burst of electrical stimulation in the SN, the FRET ratio for the D2-CNIFERs increased within 2 s of the stimulation. The amplitude of the FRET response varied with the amplitude of the stimulation, with a $\Delta R/R = 0.04 \pm 0.02$ (mean ± SE) for near-threshold 50 µA stimulation and $\Delta R/R = 0.21 \pm 0.06$ for 300 µA stimulation (Fig. 6A).

Similarly, the $\alpha_{1a}$-CNiFER response increased following electrical stimulation of the LC (Fig. 6B), with a $\Delta R/R = 0.06 \pm 0.02$ for 50 µA stimulation and $\Delta R/R = 0.24 \pm 0.05$ for 150 µA stimulation. Thus, both D2- and $\alpha_{1a}$-CNIFERs appear to exhibit a dynamic range *in vivo* suitable to measure release of DA and NA in behaving mice. The duration of the response varied between 20 s for the weakest stimulation to more than a minute for the strongest stimulation (Fig. 6A,B; *left panels*). This is longer than the recovery time observed *in vitro* after a short pulse of agonist at saturating concentration, suggesting an extended presence of the neurotransmitter in the narrow extracellular space. Lastly, we confirmed receptor-specificity *in vivo* with systemic injection of receptor-specific antagonists that blocked CNIFER responses to electrical stimulation (Fig. 6A,B; *orange traces*).
Figure 6: In vivo characterization of D2- and α1a-CNIFERs. (A) D2-CNIFER FRET responses evoked in motor cortex by increasing amplitude of SN electrical stimulation before (black traces) and after i.p. injection of D2-receptor antagonist eticlopride (1mg/kg, orange trace). Left, example of raw traces used to calculate average peak responses (right) for each stimulation intensity. (B) α1a-CNIFER FRET responses evoked by LC stimulation before (green traces) and after i.p. injection of α1a-receptor antagonist, WB4101 (2mg/kg, orange trace). Example traces (left) and average peak responses (right, n=4). Error bars represent standard deviation.

2.4 Applications

Here we report the expansion of the family of cell-based reporters of neurotransmitter release to potentially include any molecule for which there is a GPCR. This advances the technology from the previous state, where only GPCRs that couple to $G_q$ and ultimately lead to a calcium rise in the cell, could be used. We constructed a D2-CNIFER to detect dopamine, for which the GPCR normally couples to $G_i/o$, as well as a $\alpha 1a$-CNIFER, for which the GPCR couples to $G_q$ (Fig. 3). These
new biosensors exhibit nanomolar sensitivity to their respective agonists, a temporal resolution of seconds, and a dynamic range that make them suitable to discriminate between DA and NA in vivo (Fig. 4). As CNiFERs are derived from human cells, it may be necessary to suppress the immune response as was done here. Yet the concept may be extended to species specific cell types.

The development of this technology has already proven useful for testing GPCR activation in vitro, ex vivo, and in vivo, positioning it as a powerful tool for a variety of applications. Here we demonstrated that CNiFERs could be utilized for high-throughput screening of receptor binding in vitro by using specific agonists, antagonists, and a panel of common neurotransmitters against the D2- and α1a-CNiFERs (Figs. 4B and 4C). While our experiments used known compounds, CNiFERs could also be used to screen novel compounds for a given receptor. This would be particularly useful in the development of receptor-targeted therapeutics. Similarly, CNiFERs could be made from orphaned GPCRs and then screened against ligand libraries as a novel approach to de-orphanization of the receptors. High-throughput screening using CNiFERs in vitro thereby provides numerous pharmaceutical applications.

CNiFERs can also be used with ex vivo preparations in order to measure GPCR activation by released molecules from tissue. As a demonstration of this, we plated D2-CNiFER cells onto glass coverslips and measured FRET responses with an inverted fluorescent microscope. A 250 µm thick, horizontal section containing the mouse VTA was placed directly on top of the cells and held in place with a weighted harp. Robust FRET responses were measured during depolarization of the section by fast perfusion of 25mM KCl suggesting the release of DA from the sample (Fig. 7).
Similarly, Ford et al. (2012) have demonstrated the use of CNiFERs in *ex vivo* retinal preparations to measure spontaneous waves of acetylcholine release by placing CNiFERs on top of the tissue sample. Notably, *ex vivo* preparations are often used to study specific brain structures and/or circuits with electrophysiology, however, it is difficult to simultaneously measure the release of transmitter while recording, as this requires the collection of the perfusion solution. Using CNiFERs in parallel with electrophysiological recordings would facilitate our understanding of circuit dynamics such that we can begin to understand both how endogenously released neuromodulators effect the surrounding neural activity and how neural activity effects the release of neuromodulators within specific circuits.

![Figure 7](image)

**Figure 7:** Demonstration of D2-CNiFER response elicited from *ex vivo* tissue. Normalized D2-CNiFER FRET responses evoked by depolarization of a 250 µm horizontal section of mouse brain, including the VTA. Depolarization was elicited by three applications of 25mM KCl by fast perfusion for a fixed duration of one minute each (black bars).
Finally, CNiFERs provide a novel approach to studying \textit{in vivo} neuropharmacology as well as the dynamics of neuromodulator release in the brain during behavior. Currently, we utilize two photon microscopy to image CNiFERs \textit{in vivo} because in this type of fluorescent imaging excitation is highly localized, resulting in several advantages including excellent spatial and temporal resolution, increased imaging depth, and confined photo-damage. As such, it has become an important tool for imaging biological samples, particularly \textit{in vivo}, allowing for experimentation in awake animals. The ability of the D2-CNiFER and $\alpha_{1a}$-CNiFER to chemically discriminate NA from DA, while retaining fast temporal resolution, provides a unique opportunity to analyze the dynamics of DA and NA release on a trial-by-trial basis during learning (\textbf{Fig. 6}). This is discussed in detail in the next chapter. Currently, voltammetry techniques are often used to measure DA and NA with fast temporal resolution, but this method has poor discrimination between DA and NA as a result of their similar redox profiles (Robinson, Venton et al. 2003). Further, CNiFER technology could also be extended to detect \textit{in vivo} neurotransmitter release and G-protein receptor activation in deeper brain areas by using optical fibers or microendoscopic objectives. This could also be paired with simultaneous recordings of neural activity with either electrophysiology or calcium imaging. CNiFERs can also be used to determine pharmacokinetics and metabolism of a drugs in the brain, as well as its effects on a specific GPCR. Delivering therapeutic agents to the brain presents a major challenge to treatment of most brain disorders; CNiFERs will be able to test their pharmacological efficacy \textit{in vivo}.

Here we have described a significant advancement of CNiFER technology to include detection of a larger family of GPCRs, however, there are still several point
that can be improved upon. First, while CNiFERs provide faster read-out of neurotransmitter release than microdialysis in vivo, they still require several seconds to detect changes in local transmitter concentrations. Furthermore, the decay of the response is too long to discriminate discrete pulses of transmitter that occur within several seconds of each other. While this is in part due to the natural dynamics of the GPCR used, the downstream kinetics of the CNiFER response could be improved, utilizing other calcium detectors for example, to allow for better on and off signal detection and therefore better temporal resolution overall.

Additionally, it would useful to create CNiFERs with different FRET pairs. This would allow for the simultaneous detection of different CNiFERs from the same location. Currently, different CNiFERs must be injected into discrete neighboring sites in order to dissociate their signals. However, CNiFERs of different colors could be dissociable even if injected as a mixed population into the same site.

Lastly, in order to apply CNiFERs to a wide range of in vivo questions, it needs to be detectable in deeper brain structures. This can likely be achieved with an implantable optical fiber. While this has yet to be demonstrated with CNiFERs, optical fibers have successfully been used to measure signal from other genetically encoded calcium detectors, such as GCamp3 (Cui et al., 2013), or indicators such as Oregon green BAPTA-1 (Stroh et al., 2013). Given that FRET indicators are typically brighter than single wavelength sensors (Whitaker 2010), it is likely they will be detectable by an optical fiber as well. Currently, the detection of CNiFERs in the brain is significantly limited. They can only be detected in animals that are head-fixed, under a two-photon microscope, and at depths within 300 µm of the cortical surface if imaged through a thin skull window and up to 500 µm from the cortical surface if imaged through an
open skull window. Detecting the CNiFER FRET signal with an optical fiber removes the necessity for a two photon microscope, allows the animal to be freely moving, and gives access to deep brain structures thereby making CNiFERs more applicable to both a wider range of users and scientific questions.

2.5 Methods

**Stably expressing cell lines.** ‘TN-XXL only CNiFERs’ were created using HEK293 cells transduced with replication deficient lentivirus to express a TN-XXL calcium indicator (Mank et al., 2008). Lentiviral particles were produced by the UCSD Vector Development Laboratory (Atsushi Miyano, UCSD) using HIV-based backbone cloning plasmids (System Biosciences). Clonal separation and selection were based on fluorescence intensity using flow cytometry (FACSaria, BD Biosciences), and response to internal calcium concentration increase in the presence of 3 µM ionomycin (Sigma). After selection of a single clone, ‘TN-XXL only CNiFERs’ were transduced with either the α1α adrenergic receptor or a combination of the D2 dopaminergic receptor and the chimeric Gq₅₅ protein (Conklin et al., 1993). Clonal separation and selection was based on fluorescence intensity using flow cytometry. Single α1α- and D2-CNIFER clones were selected based on their dose response curves to both dopamine and noradrenaline (Sigma). All CNiFER cells were maintained at 37°C with 5 % (v/v) CO₂. Upon confluence, cells were trypsinized, tritutrated, and seeded into new flasks using Dulbecco’s Modification of Eagle’s Medium (Cellgro®; Mediatech) with 10 % (v/v) of Fetalplex™ serum (Gemini Bio-Products), 100 U/ml of penicillin and 100 µg/ml of streptavidin (Gibco).
**In vitro high-throughput testing.** D2- and α1a-CNIFER FRET responses to different neurotransmitters were measured *in vitro* using a high-throughput fluorometric plate reader (FlexStation3, Molecular Devices). The day before experiments, CNIFERs were plated on fibronectin-coated 96-wells plates. Thirty minutes before experiments, media in each well was replaced with 100 µl artificial cerebral spinal fluid (ACSF; 125 mM NaCl, 5 mM KCl, 10 mM D-glucose, 10 mM HEPES, 3.1 mM CaCl$_2$, 1.3 mM MgCl$_2$, pH 7.4) and plates were loaded into the FlexStation3. Experiments were conducted at 37°C using 435 nm excitation light. Light was collected at 485 nm and 527 nm every 3.8 s. After 30 s of baseline, 50 µl of drug diluted in ACSF was delivered to each well. Background measurements taken from wells without cells were subtracted, fluorescence intensities were normalized to pre-stimulus baselines, and peak responses were measured from the ratio of the 527 nm and 485 nm channels.

**In vitro characterization.** D2- and α1a-CNIFERs were trypsinized and plated on fibronectin-coated coverslips. The following day, CNIFERs were placed in a cell culture chamber (RC26; Warner Instruments) and perfused with gravity-fed ACSF. Chamber fluid temperature was kept at 35°C by a temperature controller (TC-324B; Warner Instruments). To test the receptor specificity of the D2-CNIFER, FRET responses were measured during two 60 s presentations of 20nM dopamine. The second presentation was preceded by 60 s of either 100 nM D1-receptor antagonist, SCH23390 (Tocris) or 50nM D2-receptor antagonist, eticlopride (Tocris). Percent response remaining was calculated by subtracting the peak FRET response during the second DA presentation (in the presence of antagonist) from the first (in the absence of antagonist). α1a-CNIFER receptor specificity was similarly tested using
two 60 s presentations of 50nM noradrenaline with the second presentation being preceded by 180 s of either 5 µM β-adrenergic receptor antagonist, sotatol (Tocris) or 50 M α1a-receptor antagonist, WB4101 (Tocris). For repeat pulse experiments, D2- and α1a-CNIFERs were given 10 presentations of either 60 s 50 nM dopamine or 500 nM noradrenaline, respectively, followed by 180 s of ACSF alone. For temporal discrimination experiments, cells were imaged with a 2-photon microscope (see next section), and rapid drug presentation was achieved with a fast perfusion stepper (SF-77B; Warner Instruments). The drug pipette was co-loaded with Alexa-594 to determine precise perfusion time.

TPLSM imaging. CNIFER cells were imaged with a custom-built two-photon laser-scanning microscope. Control of scanning and data acquisition was achieved through the MPScope software suite (Nguyen et al., 2009). 820 nm excitation light was used to excite the eCFP portion of TN-XXL. Fluorescence was collected by either a 25X water objective (HCX-IRAPO, Leica) for in vivo experiments or a 10X air objective (PLAN-NEOFLUAR, Zeiss) for in vitro experiments. The fluorescent signal was split into two channels: 450 to 490 nm for measurement of emission by eCFP and 515 to 555 nm for emission by Citrine.

Animal preparation. Adult C57BL/6 mice, age P60 to P90, were maintained in standard cages on a natural light-dark cycle. The Institutional Animal Care and Use Committee at the University of California San Diego approved all protocols. For surgery, mice were anesthetized with isoflurane (Butler Schein). Body temperature was monitored and maintained at 37°C. Subcutaneous injections of 5 % (w/v) glucose in saline were given every 2 h for rehydration. Buprenorphine (0.02 mg/kg, Butler Schein) was administered i.p. for post-operative analgesia.
**Retrograde labeling.** After anesthesia, mice were placed in a stereotaxic frame. A small craniotomy was performed where CNiFERS were typically injected (+1.5 mm A/P, +1.5 mm M/L). Using a 10 µm inner-diameter glass pipette connected to a Nanoinjector II (Drummond), 200 nl of Fluorogold™ (Fluorochrome), prepared as 1 % (w/v) in 0.1 M cacodylate buffer, was injected (20 nl every minute) in the cortex 200 µm from the surface. After 7 days, the mice were transcardially perfused. Histological sections were scanned at 1 µm spatial resolution using a Nanozoomer (Hamamatsu) digital slide scanner. Using the Neurolucida software (Microbrightfield™), outlines of midbrain, brainstem, and cerebellum were drawn and sections were aligned based on anatomical borders to yield three-dimensional reconstruction. Outlines of substantia nigra, ventral tegmental area and locus coeruleus were defined by tyrosine hydroxylase labeled neurons. Cells double-labeled for tyrosine hydroxylase and Fluorogold were marked and co-labelling was confirmed by confocal microscopy.

**Histology.** Mice were perfused with phosphate buffered saline (PBS), immediately followed by 4 % (w/v) paraformaldehyde (PFA) in PBS. Brains were post-fixed overnight at 4°C followed by immersion in 30 % (w/v) sucrose. Brain sections, 30 or 50–µm thick, were cut using a sliding microtome. Primary antibodies (mouse anti-tyrosine hydroxylase, 1:1000, Millipore and rabbit anti-fluorogold, 1:5000, Millipore) were diluted in a buffer that consisted of 10 % (v/v) goat serum (Vector Labs) and 0.1 % (v/v) Triton X-100. Free-floating sections were then incubated overnight under slow rotation at 20°C in antibody solution, washed 3-times with PBS and incubated with secondary antibody (Alexa 488 anti-mouse and Alexa 598 anti-
rabbit, 1:2000, Molecular Probes) for 2h. Sections were then washed and mounted with Fluoromount™-G (Southern Biotechnology Associates).

**CNiFER implantation.** CNiFERs were harvested without trypsin from 80% confluent culture flasks, centrifuged, and re-suspended in ACSF for injection. After a ‘thinned skull’ craniotomy (Drew et al., 2010), CNiFER cells were loaded into a 40 µm inner-diameter glass pipette connected to a Nanoinjector II (Drummond) and injected into neocortex through the thinned skull ~200 µm from the cortical surface. CNiFERs were injected into adjacent sites within the following stereotaxic coordinates: +1 to +2 mm A/P; +1 to +2 mm M/L. After implantation in several adjacent sites (typically two injection sites per CNiFER variant), the craniotomy was sealed with a glass coverslip. A custom-built head-bar was attached to the skull with C&B-METABOND (Parkell Inc.), and the preparation surrounding the imaging window was covered with dental cement (Dentsply). Mice were immunosuppressed by daily cyclosporine injection (20 µl/100 g, i.p., Belford Laboratories;).

**Electrical stimulation and in vivo pharmacology.** Mice were prepared and injected with CNiFERs as described. Additionally, a 0.1 MΩ tungsten bipolar stimulating electrodes with a tip separation of 500 µm (Microprobes Inc.) was implanted into either substantia nigra (-3.2 mm A/P, -1.3 mm M/L, -4.4 mm D/V) or locus coeruleus (-5.3 mm A/P, -0.9 mm M/L, -3.4 mm D/V). After a day of recovery, imaging was performed under isoflurane anesthesia. Experimental runs consisted of 30s baseline followed by electrical stimulation (200 µs pulses of 50-300 µA at 50 Hz for 500 ms). To test the specificity of the response, eticlopride (1mg/kg, Sigma) or WB4101 (2 mg/kg, Tocris) were injected i.p. 10 minutes before the electrical stimulation.
Data analysis. All the TPLSM data analysis was done using Matlab (MathWorks). TN-XXL fluorescence intensities were background-subtracted and normalized to pre-stimulus baselines. Regions of interest were drawn around either the D2- or the α1a-CNiFER implants. Responses were quantified as the fractional change in the FRET ratio \( \Delta R/R \), where \( \Delta R \) is the change in the ratio of fluorescence intensities of the two channels, denoted \( F_{530\text{ nm}} \) and \( F_{475\text{ nm}} \) respectively, and \( R \) is the normalized baseline ratio, so that:

\[
\Delta R(t)/R = \left[ F_{530\text{ nm}}(t)/F_{530\text{ nm}}(\text{baseline}) \right]/\left[ F_{475\text{ nm}}(t)/F_{475\text{ nm}}(\text{baseline}) \right] - 1
\]

Responses were measured at the peak of \( \Delta R/R \) after low-pass filtering. For in vitro high-throughput testing, the peak responses were determined using Matlab, and the \( EC_{50} \) and Hill coefficient were calculated using Prism software (GraphPad). Statistical analyses were calculated using Prism software (GraphPad).

Chapter 2, in part, is a reprint of the material that has been submitted for publication. Muller, Arnaud*; Joseph, Victory*; Slesinger, Paul; Kleinfeld, David. The dissertation author was a primary investigator and author of this material.
Chapter 3: Neuromodulator Release During Learning

We hypothesized that the release of DA, NA, and ACh in motor cortex would reflect changes in attention due to associate learning. To test this, we trained mice on a classical conditioning task in which an auditory cue was paired with the presentation of reward. The retrieval of reward required mice to perform a motor behavior, licking. We asked whether the release of transmitter would track this motor behavior or instead be triggered by the reward-predictive cue. We predicted that release of DA, NA, and ACh would be triggered in response to the cue, reflecting changes in attention due to associative learning. Having established the feasibility of using D2- and α1a-CNIFERs to measure DA and NA in vivo (Chapter 2), we used these CNIFERs and the M1-CNIFER (Nguyen et al. 2010), to detect and discriminate DA, NA, and ACh release during classical conditioning. Here we demonstrate real-time, concurrent measurements of DA, NA, and ACh release in murine motor cortex during conditioning.

3.1 Simultaneous measurement of neuromodulators during conditioning

CNIFERs were injected into layers 2/3 of motor cortex (Fig. 8A,b) and mice were allowed to recover for 2 days (13 mice). The CNIFERs were then imaged through a thinned-skull window and licks recorded with a lickometer while mice learned to associate a 5 s tone, the conditioned stimulus (CS), with delivery of a drop of 10 % sucrose solution, the unconditioned stimulus (US), after a fixed delay of 3 s from the end of the tone. We implemented a long, i.e., 8 s, training interval between cue onset and reward to facilitate dissociation between release of transmitter in
response to the CS and release of transmitter in response to the animal’s behavior given the 2 s temporal resolution of the CNiFERs (Fig. 8A). The delay between cue offset and reward, \(i.e.,\) 3 s, was kept short to facilitate associative learning (Pavlov, 1927). Although the delivery of the reward was not dependent on the animal’s behavior, the dropper was positioned just beyond the animal’s mouth such that the animal was required to make a motor act, tongue protrusion and licking, in order to retrieve the drop of sucrose.

Concurrent measurements of transmitter release and licking in head-fixed mice were performed on a pair-wise basis to reveal the potential engagement of the DA, NA, and ACh systems in cortex during classical conditioning (Fig. 8). We simultaneously measured the FRET responses of D2- and \(\alpha_{1a}\)-CNiFERs, along with licking, and demonstrated that the full time-course transient changes in [DA] and [NA] can be detected during single trials of conditioning with a high signal-to-noise ratio (Fig. 8C). The D2- and \(\alpha_{1a}\)-CNiFERs responded on the majority of trials. Simultaneous measurements of the D2- and M1-CNIFER responses, and concomitant licking, were also observed on a single trial basis with a high signal-to-noise ratio during conditioning trials (Fig. 8D). The transient increases in [DA] and [NA] were similar in duration, with a full-width at half maximum amplitude of 25 ± 11 s (mean ± SD; 13 mice) and 28 ± 13 s (8 mice) for [DA] and [NA] respectively (Fig. 8C,D; Fig. 9A). In contrast, [ACh] transients to a burst of licks persisted for a much shorter interval, \(i.e.,\) 15 ± 9 s (4 mice) (Fig. 8D; Fig. 9A).
Figure 8: Simultaneous measurement of neurotransmitter release. (A) Procedure to measure licking behavior and CNIFER fluorescence in head-restrained mice during classical conditioning. Conditioning trials consisted of a 5 s tone (conditioning stimulus, CS) followed by a 3 s delay and delivery of a drop of 10% sucrose water reward (unconditioned stimulus, US). (B) D2-CNIFERs (green) implanted into frontal cortex. Coronal section stained with NeuroTrace 435/455 (magenta). (C) Simultaneous measurement of D2- (left, black) and α1a-CNIFER (right, green) FRET responses and licking (pink) during a single conditioning trial. CS (grey bar) and US (dashed red line). (D) Simultaneous measurement of D2- (left, black) and M1-CNIFER (right, blue) responses and licking.
Figure 9: Duration of CNiFER responses during conditioning trials. (A) Mean CNiFER response duration, quantified as the full-width at half maximum amplitude, across all animals and conditioning trials over five days. (B) Distribution of CNiFER response durations across all animals and trials. (C) Mean D2-CNIFER response duration across all animals for each day of conditioning. (D) Mean $\alpha_{1a}$-CNIFER response duration across all animals for each day of conditioning. (E) Mean M1-CNIFER response duration across all animals for each day of conditioning. (A-E) Error bars represent standard deviation. Black bars, response of D2-CNIFERs. Green bars, response of $\alpha_{1a}$-CNIFERs. Blue bars, response of M1-CNIFERs.

3.2 Changes in transmitter release and behavior during conditioning

I hypothesized that the release of DA in the frontal cortex shifts from the time the reward is presented toward the earlier time when the predictive cue occurs (Figs. 8A and 10A). This hypothesis follows from extensive studies have demonstrated that classically conditioned cue-reward pairings increased neuronal firing from its projection nuclei with a temporal shift from the time of the reward. First, we noted that the onset time of licking across multiple days of training shows a monotonic shift from the time of the reward to that of the cue, with slope $=-0.4 \pm 0.14$ s per day (mean $\pm$ SE, $p = 0.05$; 13 mice)
(Fig. 10B). These data confirmed that animals learned to associate the CS with the US. Does the release of DA also show such a monotonic shift? Consistent with my hypothesis, we observed a strong, statistically significant decrease in the mean onset time of the FRET response across multiple days of training, with slope = -1.1 ± 0.14 s per day (p = 0.02; 13 mice) (Fig. 10C). The observed shift in DA onset time was consistent across individual animals but with varying slope indicating varying rates of change in onset time (Fig. 11). Thus, the release of DA monotonically shifts from the time of the reward to the time of the cue, similar to licking behavior (Fig. 10B, Fig. 11).

As an additional control to confirm that animals had learned the pairing between the conditioned and unconditioned stimulus, we looked for licking during probe trials in which the CS was presented alone, without reward following. We observed anticipatory licking and licking at the expected time of reward during probe trials (data not shown).

In contrast with the D2-CNIFER responses, the onset time of the α1a-CNIFER FRET responses did not show an appreciable change across conditioning days (p = 0.6; 7 mice) (Fig. 10D). Notably, the onset times were highly variable both within a set of trials for a given animal as well as across animals over conditioning days (Fig. 12).

The release of ACh consistently tracked the time of licking during reward presentation, as measured by the onset time of M1-CNIFER FRET responses across conditioning trials, with slope = -0.2 ± 0.05 s per day across
animals (p = 0.04; 4 mice) (Fig. 10E). This trend was consistent across individual animals (Fig. 13). This release occurred regardless of whether there was anticipatory licking with conditioning (Fig. 10B). Of interest, we observed ACh transients when the animal engaged in bouts of high frequency licking similar to reward retrieval but absent of any stimuli (data not shown).
Figure 10: Changes in transmitter release and behavior with conditioning. (A) Schematic of CNiFER FRET response and onset time measurement during a single conditioning trial. For (B-E), upper panels depict individual mice in different colors and lower panels depict population averages of response onset times per day of conditioning. Error bars represent standard error. (B) Licking onset times during conditioning trials (CS, grey bar; US, dashed red line) across five days of conditioning. (C) D2-CNIFER FRET response onset times during conditioning. FRET onset times are measured relative to CS onset. (D) α1a-CNIFER onset times during conditioning. (E) M1-CNIFER onset times during conditioning.
Figure 11: Changes in DA release with conditioning for individual animals. Responses for an individual animal are represented in each graph for every trial (CS, grey bar; US, dashed red line), across five days of conditioning. Licking (orange) and D2-CNIFER (black) onset times are plotted relative to CS onset. Black dots, CNIFER onset times. Black line, linear regression through CNIFER onset times. Orange dots, licking onset times. Orange line, linear regression through licking onset times. Slopes are denoted in the top right corner of each graph with asterisks marking significances at $p<.05$. 

$^{\ast}$DA = -2
Lick = -0.08

$^{\ast}$DA = -0.1
Lick = -0.05

$^{\ast}$DA = -0.05
Lick = -0.08

$^{\ast}$DA = -0.2
Lick = -0.07

$^{\ast}$DA = -0.09
Lick = -0.02

$^{\ast}$DA = -0.07
Lick = -0.02

$^{\ast}$DA = -0.1
Lick = -0.04

$^{\ast}$DA = -0.2
Lick = -0.05

$^{\ast}$DA = -0.2
Lick = -0.08
Figure 12: Changes in NA release with conditioning for individual animals. Responses for an individual animal are represented in each graph for every trial (CS, grey bar; US, dashed red line), across five days of conditioning. Licking (orange) and α1a-CNIFER (green) onset times are plotted relative to CS onset. Green dots, CNIFER onset times. Green line, linear regression through CNIFER onset times. Orange dots, licking onset times. Orange line, linear regression through licking onset times. Slopes are denoted in the top right corner of each graph with asterisks marking significances at p<.05.

Figure 13: Changes in ACh release with conditioning for individual animals. Responses for an individual animal are represented in each graph for every trial (CS, grey bar; US, dashed red line), across five days of conditioning. Licking (orange) and M1-CNIFER (blue) onset times are plotted relative to CS onset. Blue dots, CNIFER onset times. Blue line, linear regression through CNIFER onset times. Orange dots, licking onset times. Orange line, linear regression through licking onset times. Slopes are denoted in the top right corner of each graph with asterisks marking significances at p<.05.
3.3 Correlation of DA and NA release with behavior

The timing of cortical DA release shifts from the onset of the US to that of the CS as mice learn to associate the tone with reward (Fig. 10C). On a trial-by-trial basis, the onset of licking and that of DA release are correlated (Fig. 14A); six of 13 animals showed significant trial-by-trial correlations with a group correlation coefficient of \( r = 0.41 \) \((p < 0.0001; 435 \text{ trials across 13 mice})\). In contrast, on a trial-by-trial basis the onset of licking and that of NA release are weakly, albeit significantly, correlated (Fig. 14B); only one of eight animals showed significant trial-by-trial correlations with a group correlation coefficient of \( r = 0.29 \) \((p < 0.001; 227 \text{ trials across 8 mice})\). Thus DA release is a much more reliable indicator of learning than NA release.

![Figure 14: Trial-by-trial correlation of DA and NA release with behavior.](image)

The correlation at the level of the average CNiFER responses and behavior between animals was robust. Mice that only exhibited a small albeit significant change in the timing of DA release did not show significant anticipatory licking (Fig. 13A, left). By contrast, mice that demonstrated a stronger significant shift in DA
release towards the time of the CS also demonstrated significant anticipatory licking (Fig. 15A, right). We quantified this trend and found that the rate of change in the release of DA is strongly and significantly correlated with the rate of change in anticipatory licking as an average across all animals and trials ($r = 0.8$, $p = 0.003$; 13 mice) (Fig. 15B). In contrast, there was no significant correlation between the release of NA and the animal’s licking behavior ($p = 0.10$). All told, the new CNiFERs allowed us to determine that DA release, but not NA release, corresponds to the extent of learning, as defined by changes in the licking behavior.

**Figure 15: Correlation of DA release with behavior.** (A) Onset time of D2-CNIFER FRET response (black) and licking (orange) across conditioning trials for two mice. Solid lines, best fit linear regressions of CNIFER responses and licking. Grey area, time of CS presentation. (B) Correlation between rate of change in DA onset and rate of change in licking onset across conditioning trials. Each point represents the rate relationship for one animal with standard deviation (grey error bars). Black line, linear regression with 95% confidence intervals (grey shaded area).
3.4 Discussion

We found that the presentation of the unconditioned stimulus triggered release of DA, NA, and ACh in the motor cortex (Fig. 8). The release of NA, did not show an appreciable change across conditioning days (Fig. 10D) and was notably highly variable both within a set of trials for a given animal as well as across animals over conditioning days (Fig. 12). As predicted, the release of DA monotonically shifted from the time of the reward to the time of the cue (Fig. 10B), consistent with a role in attention and reinforcement learning. In contrast, the release of ACh consistently tracked the time of licking during reward presentation (Fig. 10E) and when the animal engaged in bouts of high frequency licking similar to reward retrieval but absent of any stimuli, suggesting a role in enhancing motor behavior.

Noradrenaline

NA release occurred but was highly variable between and within mice (Fig. 10D). It is possible that the release of NA is triggered by stimuli that could not be discriminated by our behavior paradigm. Just within the realm of sensory processing, noradrenaline has been implicated in a range of cognitive operations, including arousal, attention, and uncertainty (Devauges and Sara, 1990; Aston-Jones and Cohen, 2005; Bouret and Sara, 2005; Yu and Dayan, 2005), that may have been engaged and account for much of the variability. For example, there was likely variability in arousal levels between and within animals from session to session. Additionally, animals may have attended to different stimuli that varied across trials and between animals. Inconsistent stimuli during the training paradigm may have triggered this type of engagement, including noise outside the imaging chamber, which was not sound attenuated and likely highly variable. However, if noise outside
the chamber were the main source of variability, we would have expected to see the release of NA during the inter-trial intervals more frequently and in equal proportion to within trial intervals, which is not what we observed.

Aston-Jones and Cohen (2005) have proposed that the firing rate of noradrenergic neurons in the LC is variable as a function of the animal's attention and fluctuates between tonic firing and phasic firing. According to their research, when the animal is inattentive or distractible there is low or high tonic firing, respectively. However, when the animal is engaged in a task, there is specific phasic firing time-locked to stimuli presentation. Their experiments were performed in well- and over-trained animals (receiving hundreds of trials). Given their results, it is possible that during the initial phase of conditioning the NA response is highly variable as the animal’s attention is pulled in multiple directions trying to learn the task, but with significant training the response may become more precisely associated with only particular stimuli. Therefore, we might have seen a change in the release of NA in the motor cortex if we had continued to train our animals beyond 5 days of conditioning (approximately 50-75 trials).

Alternatively, Yu and Dayan (2005) proposed that the release of NA plays a role in the computation of unexpected uncertainty. Under this model, the release of NA would become less apparent to the cue as the animals became better trained. They suggested that the release of NA is instead triggered by unexpected events such as the absence of an expected reward, for example during probe trials or during extinction, a hypothesis also proposed by Mason and Iverson (1977) based on slower rates of extinction following lesions to the dorsal noradrenergic bundle. However, our observations did not support this role for NA in the motor cortex. During probe trials,
the release of NA was not significantly different than its release during normal trials in onset time, amplitude, or duration. Similarly, we trained one group of animals on our conditioning task until they displayed significant anticipatory licking on the majority of trials. We then presented these animals with repeated trials of the cue absent reward (extinction). Again we did not observe a significant difference in the release of NA versus normal trials, and responses continued to be highly variable both across and within animals.

We were not able to determine a precise trigger for the release of NA in motor cortex with our behavioral task. However, it is clear that NA is released during attention-demanding and stimuli-rich periods of activity, i.e. within trials. Therefore, our results provide some evidence that NA is involved in attention and sensory processing in the motor cortex, but further experiments are required to disambiguate this role more precisely.

**Dopamine**

As predicted, the release of DA in motor cortex was triggered in response to the cue after conditioning (Fig. 10B), reflecting changes in attention due to associate learning. The gradual shift in the release of DA from the time of the US to that of the CS is consistent with the temporal difference model proposed by Schultz and colleagues (Montague et al., 1996; Schultz et al., 1997), in which DA neurons respond to reward-predicting cues. Notably, on a trial-by-trial basis, the onset of dopamine was only weakly dependent on the onset of the licking. Furthermore, the release of DA was not observed when animals engaged in bouts of high frequency licking absent conditioned stimuli or reward. Thus, DA release in the motor cortex is
consistent with a role in attention and reinforcement learning but inconsistent with a major role in movement.

Although our results were largely consistent with reinforcement learning models, there were some important differences between our results and those from studies of dopamine neuron activity. First, while previous studies of midbrain DA neurons showed a shift in the time of firing (from cue to reward), the increase in firing rate occurred either just after the reward or just after the cue, implying a discrete instead of monotonic shift (Pan et al., 2005). This difference could be explained by the use a longer CS in our experiments that may have facilitated the observation of a more gradual shift of the response to the start of the cue. Alternatively, there may be large differences between the firing of DA neurons in the midbrain and the actual release of DA in the cortex, or the recorded neurons in previous studies may not project to the motor cortex.

Second, we did not observe a change in cortical [DA] in the absence of reward. We randomly interleaved probe trials, in which animals received the CS without subsequent delivery of reward, during the last two days of conditioning. While we observed release of DA in response to the cue on these trials, we did not observe an appreciable decrease in [DA] at the expected time of reward. Midbrain DA neurons, however, exhibit decreased firing rates, relative to baseline, at the expected time of reward under these conditions (Shultz et al., 1997). However, the absolute decrease in firing rate is small, therefore it is possible that this does not significantly change [DA] in the motor cortex or rather that the change is too small to be detected by our sensor.
**Acetylcholine**

The release of ACh was triggered when animals engaged in bouts of high frequency licking either at the time of reward presentation (Fig. 10E) or absent any stimuli, suggesting a role in enhancing motor behavior. This was not predicted given its canonical role in sensory processing as described in other cortical areas, particularly prefrontal cortex (Arnold et al., 2002; Himmelheber et al., 2000; Parikh et al., 2007). Instead it suggests that the role of ACh may be consistent, but better described more broadly, as modulating the signal to noise ratio within a given cortical area to enhance the specific type of information processing done there.

Interestingly, we only observed ACh release during high frequency (≥8 Hz) licking bouts and not during single licks or licking at lower frequencies that we typically observed during anticipatory licking. Our results suggest a model in which acetylcholine release enhances signal processing in the motor cortex, leading to vigorous movements.

### 3.5 Methods

**TPLSM imaging.** CNiFER cells were imaged with a custom-built two-photon laser-scanning microscope. Control of scanning and data acquisition was achieved through the MPScope software suite (Nguyen et al., 2009). 820 nm excitation light was used to excite the eCFP portion of TN-XXL. Fluorescence was collected by a 25X water objective (HCX-IRAPO, Leica). The fluorescent signal was split into two channels: 450 to 490 nm for measurement of emission by eCFP and 515 to 555 nm for emission by Citrine.
**Animal preparation.** Adult C57BL/6 mice, age P60 to P90, were maintained in standard cages on a natural light-dark cycle. The Institutional Animal Care and Use Committee at the University of California, San Diego approved all protocols. For surgery, mice were anesthetized with isoflurane (Butler Schein). Body temperature was monitored and maintained at 37°C. Subcutaneous injections of 5 % (w/v) glucose in saline were given every 2 h for rehydration. Buprenorphine (0.02 mg/kg, Butler Schein) was administered i.p. for post-operative analgesia.

**CNiFER implantation.** CNiFERs were harvested without trypsin from 80 % confluent culture flasks, centrifuged, and re-suspended in ACSF for injection. After a ‘thinned skull’ craniotomy (Drew et al., 2010), CNiFER cells were loaded into a 40 µm inner-diameter glass pipette connected to a Nanoinjector II (Drummond) and injected into neocortex through the thinned skull ~200 µm from the cortical surface. CNiFERs were injected into adjacent sites within the following stereotaxic coordinates: +1 to +2 mm A/P; +1 to +2 mm M/L. After implantation in several adjacent sites (typically two injection sites per CNiFER variant), the craniotomy was sealed with a glass coverslip. A custom-built head-bar was attached to the skull with C&B-METABOND (Parkell inc.), and the preparation surrounding the imaging window was covered with dental cement (Dentsply). Mice were immunosuppressed by daily cyclosporine injection (20 µl/100 g, i.p., Belford Laboratories;).

**In vivo awake imaging and behavior.** After one day of recovery from surgery, mice were water deprived (23 h/day). Conditioning started the following day. Animals were placed in a stationary head-frame and imaged while being presented with a 5 s tone (CS), followed by a 3 s delay and a drop of 10 % (w/v) sucrose-water (US), with an average inter-trial interval of three minutes. Two CNIFER variants (α1a-
and D2-CNIFERs or M1- and D2-CNIFERs) were imaged simultaneously. Licking behavior was recorded using a custom-built, conductance-based sensor. Mice were imaged for 10 to 15 trials and then returned to their home cage. Animals were imaged 5 consecutive days.

**Data analysis.** All the TPLSM data analysis was done using Matlab (MathWorks). TN-XXL fluorescence intensities were background-subtracted and normalized to pre-stimulus baselines. Regions of interest were drawn around either the D2- or the α1a-CNIFER implants. Responses were quantified as the fractional change in the FRET ratio ∆R/R, where ∆R is the change in the ratio of fluorescence intensities of the two channels, denoted F_{530 nm} and F_{475 nm} respectively, and R is the normalized baseline ratio. Responses were measured at the peak of ∆R/R after low-pass filtering. For behavioral experiments, the onset of the FRET signal was scored manually using Matlab on traces with the experimenter blind to the time of the CS and the US. Only trials in which animals responded after CS presentation and within 30 s of US presentation were used for analysis. Statistical analyses were calculated using Prism software (GraphPad). Best-fit slopes were determined by linear regression through the data with p-values indicating significant difference from a slope equal to zero (i.e. no relationship between variables). Pearson correlation coefficients were calculated and evaluated with a two-tailed t-test for significance.

Chapter 3, in part, is a reprint of the material that has been submitted for publication. Muller, Arnaud*; Joseph, Victory*; Slesinger, Paul; Kleinfeld, David. The dissertation author was a primary investigator and author of this material.
Chapter 4: Discussion

The precise cognitive operations supported by cortical release of DA, NA, and ACh are still debated. Extensive research has suggested that they play a role in attention and more broadly sensory processing (reviewed in Chapter 1). Research has been limited, in part, because of the low temporal resolution of measures of DA and NA release using microdialysis, which has prohibited the attribution of these changes to specific behavioral and/or cognitive operations. Notably, as Parikh et al (2007) describe, “such measures of release supported the traditional notion that neuromodulator systems act at a timescale of minutes to influence cortical ‘arousal’ states”.

However, it is known that cholinergic, dopaminergic, and noradrenergic systems can signal phasically (Parikh et al. 2007; Tsai et al., 2009; Aston-Jones and Cohen, 2005). And further, it is known that these neuromodulators systems are present in the motor cortex (Hosp et al., 2011; Loughlin et al., 1986; Connor et al., 2010), an area of the brain typically associated with motor output and not sensory processing. This suggests that the function of these systems is not sufficiently described by traditional notions of their role in attention.

Here I asked what determines the release of DA, NA, and ACh, in the motor cortex. I tested whether these neuromodulators participated in sensory processing in the motor cortex or if instead their role is specific to the brain region they are released in, i.e. motor output when released in the motor cortex. By testing these opposing ideas I sought to better understand the precise function of neuromodulators in the cortex.
I used the new D2- and a1a-CNiFERs (Chapter 2) to simultaneously detect release of DA, NA and, using the original M1-CNiFER, ACh in awake, behaving mice during classical conditioning (Chapter 3); and I predicted that the release of DA, NA, and ACh in motor cortex would be triggered in response to the cue after conditioning, reflecting changes in attention due to associate learning.

As predicted, the release of DA was triggered by cue presentation after conditioning (Fig. 10B), consistent with a role in attention and reinforcement learning. In contrast, the release of ACh tracked the time of licking (Fig. 10E), suggesting a role in enhancing motor behavior. The release of NA was highly variable and did not show appreciable change with conditioning (Fig. 10D). These results challenge existing hypotheses about neuromodulator release in the cortex.

4.1 CNiFERs

To meet the challenge of assessing [DA] and [NA] in cortex in real time, we expand on a previously developed cell-based neurotransmitter fluorescent engineered reporter (CNiFER) that was created to measure acetylcholine release in vivo (Nguyen et al., 2010). Using newly develop CNiFERs were are able to dissociate the release of DA and NA, previously not possible with conventional technology. The use of FSCV in these experiments would have confounded the DA and NA signals and the significance of the DA signal would have been lost within the observed variability of the NA signal. Alternatively, the use of microdialysis could not have captured the fast temporal dynamics of the DA and NA signal. By simultaneously measuring these signals, we demonstrated that DA, NA, and ACh can be released
phasically, they have distinct response profiles, and that they likely underlie different processes in motor cortex.

The creation of these new CNiFERs also advances the technology from the previous state, where only GPCRs that couple to $G_q$ and ultimately lead to a calcium rise in the cell, could be used. The development of these CNiFERs is proof of principle that the family of cell-based reporters of neurotransmitter release can include any molecule for which there is a GPCR. Of particular interest, CNiFERs can be developed to detect various neuropeptide for which there are very limited means of detection currently available.

**Future Directions**

The development of this technology has already proven useful for testing GPCR activation *in vitro, ex vivo, and in vivo*, positioning it as a powerful tool for a variety of applications (section 2.4). However, the technology can be improved in several ways to expand its versatility. The CNiFER response could be improved by utilizing other calcium detectors to allow for better on and off signal detection and therefore better temporal resolution overall. The development of CNiFERs with different FRET pairs would allow for the simultaneous detection of different CNiFERs from the same location. And lastly, in order to apply CNiFERs to a wide range of *in vivo* questions, it needs to be detectable in deeper brain structures which can likely be achieved with an implantable optical fiber. This would also remove the necessity for a two photon microscope and allow experimental animals to be freely moving thereby making CNiFERs more applicable to both a wider range of users and scientific questions.
4.2 Cortical release of neuromodulators

We challenged existing hypotheses about NA, DA, and ACh function by measuring their release in the motor cortex of behaving mice. We were not able to determine a precise trigger for the release of NA in motor cortex with our behavioral task. However, it is clear that NA is released during attention-demanding and stimuli-rich periods of activity, i.e. within trials. Therefore, our results provide some evidence that NA is involved in attention and sensory processing in the motor cortex, but further experiments are required to disambiguate this role more precisely. We observed that the release of DA and ACh in the motor cortex have dissociable triggers. Surprisingly, despite lesions of the cholinergic system inducing impairments in attention, we found the release of ACh to be specifically correlated with motor output. Also notable was our finding that DA release is specifically correlated with the presentation of rewarding sensory stimuli in the motor cortex, despite lesions of the dopaminergic system inducing impairments in motor control.

While our DA results are expected based on the prediction that DA plays a role in attention, it is perhaps still surprising given the literature on DA in motor control. Several studies have shown that the loss of DA neurons, by experimental lesion or disease, can cause motor impairments (Kirik et al., 2011; Lee et al., 1996; Plowman et al., 2011). Furthermore, dopamine plays a critical role in motor learning and plasticity in the motor cortex (Bao et al., 2001; Molina-Luna et al., 2009). However, the activity of dopamine neurons generally does not track movement per se (Romo and Schultz, 1990; Schultz et al., 1993), and our results suggest that even in the motor cortex dopamine release is not associated with movement. Thus, dopamine’s role in movement is likely indirect, through its role in attention (Horvitz,
2000), reinforcement learning (Schultz, 2013) and/or the initiation/termination of movement sequences (Jin and Costa, 2010) – depending perhaps on the region in which it is released. Our results are consistent with a model in which dopamine acts as a reinforcement signal in the motor cortex, strengthening synapses that were active before presentation of reward-predictive stimuli or reward itself.

While superficially it appears that ACh and DA have opposing roles in the motor cortex, perhaps the function of these neuromodulators is better described by how they affect neural circuits, independent of the particular type of information that is processed locally during their release (e.g., motor vs. sensory information). Across cortical regions, ACh may enhance information processing while DA may be necessary for plasticity that underlies reinforcement learning.

**Future Directions**

Additional experiments are necessary to test these ideas more directly. To determine whether ACh is necessary for motor processing, it would informative to control cholinergic inputs (via lesion, stimulation, pharmacologically, or optogenetically) to motor cortex and observe changes in neural activity and behavior. For example, we were only able to detect release of ACh during high frequency licking. It is possible that the amount of ACh released is dependent on or proportional to the intensity of the motor behavior. It would be interesting to test whether driving ACh could directly drive behavioral output once it is initiated. Our conclusions also predict that there would be an observable change in the neural activity following release of ACh, as has been shown in sensory cortices (Kilgard and Merzenich, 1998; Kilgard et al., 2001); and this could be tested in motor cortex. This would also
test the possibility that cholinergic dependent information processing underlies plasticity in motor cortex.

Similarly, our conclusion suggests that DA is necessary for plasticity in the motor cortex that underlies reinforcement learning. Huber and colleagues (2012) monitored ensembles of motor cortex neurons while animals learned to associate sensory information with a motor behavior. They found that neuronal population stabilized with learning and suggested that the motor cortex, specifically neuronal populations in layer 2/3, is important for learning sensorimotor associations. As it has been shown that DA in motor cortex is necessary for long term potentiation and synaptic plasticity (Molina-Luna et al., 2009) and that DA can induce cortical remodeling in sensory cortices (Bao et al., 2001), it would be interesting to test whether DA in the motor cortex is necessary for the changes in neural activity observed with learning (Huber et al., 2012; Komiyama et al., 2010). This could be achieved via lesion, stimulation, pharmacologic or optogenetic control of DA terminals in the motor cortex. It is possible that this would also have behavioral consequences. The removal of DA, for example, may impair the development of anticipatory licking.

These experiments would help clarify the role of neuromodulators in the motor cortex and our understanding of their role throughout the brain. Importantly, the roles of these neuromodulators are not only a function of their release, but also the resident neuronal population, their receptors, and their metabolizing enzymes. The unique combination of these variables may allow the role of the neuromodulators to be highly dynamic, versatile, and at times, seemingly contradictory.
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