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TITLE:

Combined Infusion and Stimulation with Fast-Scan Cyclic Voltammetry (CIS-FSCV) to Assess Ventral Tegmental Area Receptor Regulation of Phasic Dopamine

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KEYWORDS:

dopamine, ventral tegmental area, nucleus accumbens, rat, fast-scan cyclic voltammetry, nicotinic receptors, N-methyl-D-aspartate receptors, muscarinic receptors

SUMMARY:

The goal of this protocol is to directly manipulate ventral tegmental area receptors to study their contribution to subsecond dopamine release.

ABSTRACT:

Phasic dopamine (DA) release from the ventral tegmental area (VTA) to the nucleus accumbens plays a pivotal role in reward processing and reinforcement learning. Understanding how the diverse neuronal inputs into the VTA control phasic DA release can provide a better picture of the circuitry that controls reward processing and reinforcement learning. Here, we describe a method that combines intra-VTA cannula infusions of pharmacological agonists and antagonists with stimulation-evoked phasic DA release (combined infusion and stimulation, or CIS) as measured by in vivo fast-scan cyclic voltammetry (FSCV). Using CIS-FSCV in anesthetized rats, a phasic DA response can be evoked by electrically stimulating the VTA with a bipolar electrode fitted with a cannula while recording in the nucleus accumbens core. Pharmacological agonists or antagonists can be infused directly at the stimulation site to investigate specific VTA receptors' roles in driving phasic DA release. A major benefit of CIS-FSCV is that VTA receptor function can be studied in vivo, building on in vitro studies.

INTRODUCTION:

Phasic dopamine (DA) release from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) plays a vital role in reward-related behaviors. VTA DA neurons switch from a tonic-like firing (3–8 Hz) to a burst-like firing (>14 Hz)[1], which produces phasic DA release in the NAc. The VTA expresses a variety of somatodendritic receptors that are well-positioned to control the switch from tonic to burst-firing[2-5]. Identifying which of these receptors, and their respective inputs, control phasic DA release will deepen our understanding of how the reward-related circuitry is organized. The purpose of the methodology described here, combined infusion and stimulation with fast-scan cyclic voltammetry (CIS-FSCV), is to quickly and robustly assess the functionality of VTA receptors in driving phasic DA release.

The term combined infusion and stimulation (CIS) refers to pharmacologically manipulating receptors on a group of neurons (here the VTA) and stimulating those neurons to study the receptor's function. In the anesthetized rat, we electrically stimulate the VTA to evoke a large phasic DA signal (1–2 µM) in the NAc core, as measured by fast-scan cyclic voltammetry (FSCV). Infusions of pharmacological drugs (i.e., receptor agonists/antagonists) at the stimulation site can be used to measure the function of VTA receptors by observing the subsequent change in evoked phasic DA release. FSCV is an electrochemical approach that enjoys both high spatial (50–100 µm) and temporal (10 Hz) resolution, and is well-suited to measure reward-related, phasic DA events[6,7]. This resolution is finer than other in vivo neurochemical measurements, such as microdialysis. Thus, together, CIS-FSCV is well-suited to assess VTA receptor regulation of phasic dopamine release.

One common way to investigate VTA receptor function is by using a combination of electrophysiological approaches that address how those receptors alter the firing rate of neurons[1,8]. These studies are highly valuable in understanding what receptors are involved in driving DA firing upon activation. However, these studies can only suggest what might happen downstream at the axon terminal (i.e., release of a neurotransmitter). CIS-FSCV builds on these electrophysiological studies by answering how the output of VTA burst-firing, phasic DA release, is regulated by receptors located on VTA dendrites and cell bodies. Thus, CIS-FSCV is well-suited to build on these electrophysiology studies. As an example, nicotinic receptor activation can induce burst-firing in the VTA[9], and CIS-FSCV in the anesthetized rat was used to show that nicotinic acetylcholine receptor (nAChR) activation in the VTA also controls phasic DA release in the NAc[10,11].

Mechanistic examination of phasic DA regulation is also commonly studied using slice preparations alongside with bath application of drugs. These studies often focus on the presynaptic regulation of phasic DA release from dopamine terminals, as the cell bodies are often removed from the slice[12]. These preparations are valuable for studying presynaptic receptor effects on dopamine terminals, whereas CIS-FSCV is better suited to study somatodendritic receptor effects on dopamine neurons, as well as presynaptic inputs to the VTA. This distinction is important, because somatodendritic receptor activation in the VTA may have a different effect than NAc presynaptic receptor activation. Indeed, blocking dopaminergic presynaptic nAChRs in the NAc can elevate phasic dopamine release during burst-firing[13], whereas the opposite is true at VTA somatodendritc nAChRs[10,11].

CIS-FSCV is an ideal approach for studying the ability of VTA receptors to regulate phasic DA release. Importantly, this approach can be performed in an intact rat, either anesthetized or free moving. This approach is suitable for acute studies, to study receptor function in its baseline state[10,14] as well as long-term studies that can assess functional changes in a receptor after drug exposure or behavioral manipulation[11,15].

PROTOCOL:

All experiments were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by both Elizabethtown College and Yale University Institutional Animal Care and Use Committee (IACUC). This protocol is specific to the anesthetized rat preparation of utilizing CIS-FSCV.

1. Presurgical preparations

1.1. Electrode solution preparation

1.1.1. To make the electrode backfill solution, prepare a solution of 4 M potassium acetate with 140 mM potassium chloride[16].

1.2. Electrode preparation

1.2.1. Using vacuum suction, insert a T-650 carbon fiber (7 µm in diameter) into a borosilicate glass capillary (length = 100 mm, diameter = 1.0 mm, inside diameter = 0.5 mm).

1.2.2. Once the carbon fiber has been placed inside the glass capillary, place the glass capillary into a vertical electrode puller, with the heat element roughly in the middle of the capillary. Set the heater to 55 with the magnet turned off.

1.2.3. After the capillary is pulled, carefully raise the upper capillary holder so that the tip of the electrode is not surrounded by the heating element.

1.2.4. Using sharp scissors, cut the carbon fiber that is still connecting the two pieces of the capillary. This will result in two separate carbon fiber microelectrodes.

1.2.5. Under a light microscope, carefully cut the exposed carbon fiber with a sharp scalpel, so that the carbon fiber extends approximately 75–100 µm beyond the end of the glass.

1.2.6. Using a light microscope, ensure that the electrode is free of cracks along the capillary. Also ensure that the seal, where the carbon fiber exits the capillary, is difficult to notice and free from cracks.

NOTE: A good seal will help reduce noise during recordings. See published studies[17-19] for a more detailed protocol.

1.3. Reference electrode fabrication

1.3.1. Solder a gold pin to a 5 cm silver wire.

1.3.2. Attach the anode to a metal paper clip or other conductor, the cathode to a pin, and apply a voltage (~2 V) while the paper clip and silver wire is submerged in 0.1 M HCl.

1.3.3. Cease the voltage once a white coating (AgCl) appears on the silver wire.

1.4. Preparing electrode for implantation

1.4.1. Solder a gold pin to a thin insulated wire (~10 cm in length, <0.50 mm diameter).

1.4.2. Remove ~5 cm of insulation from the wire opposite to the gold pin.

1.4.3. Fill the electrode approximately halfway with electrode solution.

1.4.4. Insert insulated wire into the electrode.

NOTE: The wire should make contact with the carbon fiber inside the electrode.

2. Electrode implantations

2.1. Give adult, male, Sprague Dawley rats (250−450 g) an intraperitoneal injection (1.5 g/kg or 1 mL/kg volume) of 0.5 g/mL urethane dissolved in sterile saline. Start with an initial urethane dose of 1.0−1.2 g/kg. If the animal is still responsive to the noxious stimulus test (tail pinch) 20 min after urethane administration, administer an additional 0.3−0.5 g/kg urethane for a 1.5 g/kg total dose.

NOTE: For preparation of the 0.5 g/mL urethane solution, add 10 g of urethane to 10 g (~10 mL) of saline. Urethane is a carcinogen and must be handled with care. Urethane is an important anesthetic, as it does not alter levels of dopamine, as do other anesthetics such as ketamine/ xylazine and chloral hydrate[20,21].

2.2. Once the animal is deeply anesthetized and is not responsive to noxious stimuli (e.g., toe pinch), place it in the stereotaxic frame.

NOTE: This is a non-survival surgery, but good aseptic technique is encouraged.

2.3. Clean the rat's scalp using a two-stage scrub (i.e., an iodopovidone scrub followed by a 70% ethanol scrub; perform with a 3 cycle repetition).

2.4. Cut away the scalp tissue using sterilized needle nose tweezers and surgical scissors. Remove a significant amount of tissue to make room for the various implantations outlined below.

2.5. Gently clean the skull surface using sterilized cotton tip applicators. Then apply 2−3 drops of 3% hydrogen peroxide to help identify the lambda and bregma.

2.6. Using a stereotaxic or hand drill (1.0 mm, ~20,000 rpm), drill a 1.5 mm diameter hole 2.5 mm anterior to the bregma and 3.5 mm lateral to the bregma. Partially (about halfway, until it is firmly in place) implant a screw (1.59 mm O.D., 3.2 mm long) in this hole.

2.7. For the reference electrode, drill a 1.0 mm diameter hole 1.5 mm anterior and 3.5 mm lateral to the bregma, in the left hemisphere.

2.8. By hand, insert ~2 mm of reference wire into this hole, while wrapping the reference wire around and under the head of the implanted screw.

2.9. Fully implant the screw, pinning down the reference electrode in place.

2.10. In the right hemisphere, drill a 1.5 mm diameter hole 1.2 mm anterior and 1.4 mm lateral to the bregma.

2.11. Gently remove the dura using tweezers.

2.12. For the stimulating electrode, drill a square hole (2 mm anterior-posterior, 5 mm medial-lateral) centered at 5.2 mm posterior and 1.0 mm lateral to the bregma.

2.13. Using the stereotactic arm bars, lower the bipolar stimulating electrode/guide cannula 5 mm below the dura. In case of bleeding during the implantation of the electrode, use sterile cotton swabs and gauze to minimize bleeding.

NOTE: The bipolar stimulating electrode used in this method is prefitted with a guide cannula (**Table of Materials**). The internal cannula used with this item should be flushed with the prongs on the bipolar stimulating electrode when fully inserted into the guide cannula. This will allow the internal cannula to sit directly in between the two prongs of the stimulator, which sit about 1 mm apart. A similar protocol is described elsewhere[14].

2.14. Using the stereotactic arm bars, lower the carbon fiber microelectrode 4 mm below the dura. This location is at the most dorsal portion of the striatum.

2.15. Connect the reference wire and carbon fiber to a potentiostat.

2.16. Apply a triangular wave form (-0.4−1.3 V, 400 V/s) for 15 min at 60 Hz, and again for 10 min at 10 Hz.

NOTE: Typically, when applying waveforms to carbon fiber microelectrodes in the brain, oxide groups are added to the surface of the carbon fiber. Equilibrium of this reaction must be reached prior to recording; otherwise significant drift will occur[19]. Cycling the electrode at higher frequencies (60 Hz) allows the carbon fiber to achieve equilibrium faster.

3. Optimizing carbon fiber and stimulating electrode/guide cannula locations

3.1. Set the stimulator to produce a bipolar electrical waveform, with a frequency of 60 Hz, 24 pulses, 300 µA current, and pulse width of 2 ms/phase.

3.2. Gently lower the stimulator in increments of 0.2 mm from 5 mm to 7.8 mm below the dura. At each increment, stimulate the VTA.

NOTE: At more dorsal depths (5−6 mm), stimulation of the brain will typically (~80% of the time) cause the whiskers of the rat to twitch. At further depths, the whiskers will cease twitching, which occurs between 7.5−8.2 mm below the dura. When the whiskers cease twitching, the stimulating electrode will be near or at the VTA. This will not occur in every rat, and lack of whisker twitching should not be taken as a sign that the bipolar stimulating electrode/infusion cannula is misplaced. Whisker twitching may not occur for all anesthetics (e.g., isoflurane).

3.3. Continue to lower the bipolar stimulating electrode/guide cannula until a stimulation produces phasic DA release at the carbon fiber microelectrode (currently in the dorsal striatum).

NOTE: DA release in the dorsal striatum will not always occur if the bipolar electrode is implanted in the VTA, but observation of DA release in the dorsal striatum upon VTA stimulation is usually a good sign that a good signal will be observed in the NAc core.

3.4. Lower the carbon fiber microelectrode until it is at least 6.0 mm below the dura. This is the most dorsal part of the NAc core.

3.5. Stimulate the VTA and record the peak amplitude of the DA peak.

3.6. Lower or raise the carbon fiber microelectrode at the site that produces the greatest DA release.

3.7. Ensure that the peak of the DA response is a clear oxidation peak at 0.6 V and a reduction peak at -0.2 V. These peaks are indicative of DA.

4. Combination infusion and stimulation FSCV recording

NOTE: **Figure 1** shows the timeline for recording before and after VTA microinfusion.

4.1. Once the carbon fiber and stimulating electrode/guide cannula location has been optimized,

stimulate for ~20–30 min.

NOTE: Under the current stimulation parameters, do not stimulate any more than once every 3 min, to allow for vesicular reloading[22].

4.2. After achieving a stable baseline (<20% variation over five stimulations), gently lower the internal cannula by hand into the guide cannula that is prefitted into the bipolar stimulator.

4.3. Take an additional 2–3 baseline recordings to ensure that the cannula insertion itself did not cause a change in the evoked signal. In some cases, insertion and removal of the internal cannula can cause damage to the VTA. If the signal drastically changes over this baseline period (>20%), then take an additional 3–4 recordings until the baseline restabilizes.

4.4. Using a syringe pump and microsyringe, infuse 0.5 µL of solution (e.g., 0.9% saline, N-methyl-D-aspartate [NMDA], (2R)-amino-5-phosphonovaleric acid [AP5]) into the VTA over a 2 min period.

4.5. Postinfusion, leave the internal cannula for at least 1 min prior to removal.

NOTE: Some drugs may require leaving the internal cannula for a longer time based on the drug kinetics, and removal of the internal cannula may cause the drug to travel back up through the internal cannula. If there is concern, one could leave the internal cannula in the guide cannula during the entirety of the recording. Otherwise, recording can begin after this 1 min interval.

4.6. Continue recording every 3 min to measure postinfusion effects.

NOTE: If infusing a control solution, and no effect is observed, it is possible to infuse a second time[10]. If there is altered DA release caused by inserting the internal cannula or saline infusion, the signal typically recovers to baseline within 30 min.

5. Histological verification of electrode placement

5.1. At the end of the experiment, create a small lesion at the recording site using the carbon fiber microelectrode.

5.1.1. If the electrode must be preserved for postexperiment calibration, then use a tungsten wire placed in a glass capillary protruding ~100 µm beyond the capillary tip. In this case, raise the electrode from the brain, replace the recording electrode with the tungsten electrode, and lower it to the same dorsoventral coordinate.

NOTE: The carbon fiber may be used to lesion the brain as well and will provide a more accurate representation of the location of the recording site; however, the experimenter will lose the ability to calibrate these electrodes.

5.2. To lesion the recording site, apply voltage using a power supply. Start at 1 V and increase by 1 V every 10 s until 10 V is reached.

5.3. Using a lethal intraperitoneal injection of pentobarbital (150 mg/kg), euthanize the animal.

5.4. Perfuse the rat using a 4% formalin solution.

5.5. Remove the head from the rat using a sharpened guillotine.

5.6. Using rongeurs, remove the connective tissue and skull surrounding the brain, and gently dislodge the brain from any remaining tissue.

5.7. Store the brain in 4% formalin for 1 day and then transfer it to 30% sucrose.

NOTE: Perfusion with 4% formalin is not necessary to see the lesion site, although as a best practice it will improve the reconstruction of the lesion site.

5.8. Create 30 µm slices of the brain using a cryostat.

5.9. Mount the slices on slides and cover with a cover slip.

5.10. Denote the location of the carbon fiber microelectrode lesion and bipolar stimulator/infusion cannula location using a light microscope.

REPRESENTATIVE RESULTS:

CIS-FSCV was used to study the function of VTA N-methyl-D-aspartate receptors (NMDAR), nicotinic acetylcholine receptors (nAChRs), and muscarinic acetylcholine receptors (mAChRs) in driving phasic DA release in the NAc core. **Figure 2** shows representative data for a negative control, infusion of 0.9% saline, before (baseline) and 9 min postinfusion (saline). **Figure 2** shows a color plot with potential on the y-axis, time on the x-axis, and current (represented as false color) on the z-axis, current versus time traces (IvT), as well as a cyclic voltammogram taken at the peak evoked response to demonstrate that the analyte measured corresponds to DA. As expected, saline infusion did not alter the stimulated phasic DA release.

To demonstrate that CIS-FSCV can produce bidirectional effects when using agonists and antagonists, we compared the effects of infusion of the NMDAR agonist, NMDA (500 ng; **Figure 3A**) to the NMDAR antagonist, AP5 (1 µg; **Figure 3B**). Infusion of NMDA produced a robust increase in stimulated phasic DA release (**Figure 3A**, 9 min after infusion) while the NMDAR competitive antagonist, AP5 (1 µg), produced a robust decrease (**Figure 3B**, 9 min after infusion). To demonstrate the utility of CIS-FSCV using antagonists that target different classes of acetylcholine receptors, we compared the effects of infusion of the nonselective, noncompetitive nAChR antagonist mecamylamine (3 µg; **Figure 4A**) and the nonselective, competitive mAChR antagonist scopolamine (67 µg; **Figure 4B**). Both drugs produced robust decreases in stimulated phasic DA release (**Figure 4,** 9 min postinfusion). A summary of the results from **Figure 2**, **Figure**

3, and **Figure 4** are replotted in **Figure 5,** where the baseline period is averaged over five stimulations and the drug period is displayed as a percentage of the baseline average.

FIGURE LEGENDS:

Figure 1: Timeline for recording before and after VTA microinfusion.

Figure 2: Representative color and IvT plots of baseline (left) and saline (vehicle) infusion (right) on stimulated phasic DA release in the NAc core in a single male Sprague Dawley rat. Blue bar represents stimulation. The baseline recording occurs at t = 0, before the internal cannula was placed in the guide cannula in the bipolar stimulator. The saline recording was taken 9 min (t = 9) postinfusion. Cyclic voltammogram insets correspond to the peak of the IvT plots, showing a peak oxidation at 0.6 V and peak reduction at -0.2 V, indicative of DA. No change in stimulated evoked release should be observed after saline VTA infusion.

Figure 3: Effects of infusion of the NMDAR agonist (NMDA) and antagonist (AP5). (**A**) Representative color and IvT plots of baseline (left) and 500 ng of the NMDAR agonist infusion (right) on stimulated phasic DA release in the NAc core in a single male Sprague Dawley rat. NMDA infusion increased stimulated phasic DA release (recording taken 9 min postinfusion). (**B**) Representative color and IvT plots of baseline (left) and 1 μg of the NMDAR antagonist (2R)-amino-5-phosphonovaleric acid (AP5) infusion (right) on stimulated phasic DA release in the NAc core in a single male Sprague Dawley rat. AP5 infusion reduced stimulated phasic DA release (recording taken 9 min postinfusion). The baseline recordings occurred at t = 0, before the internal cannula was placed in the guide cannula in the bipolar stimulator.

Figure 4: Effects of infusion of mecamylamine and scopolamine. (**A**) Representative color and IvT plots of baseline (left) and 3 μg of the nonselective nicotinic acetylcholine receptor antagonist mecamylamine (MEC) infusion (right) on stimulated phasic DA release in the NAc core in a single male Sprague Dawley rat. (**B**) Representative color and IvT plots of baseline (left) and 67 μg of the nonselective muscarinic acetylcholine receptor antagonist scopolamine (SCOP) infusion (right) on stimulated phasic DA release in the NAc core in a single male Sprague Dawley rat. The baseline recording occurred at t = 0, before the internal cannula was placed in the guide cannula in the bipolar stimulator. MEC and SCOP recordings were taken 9 min postinfusion.

Figure 5: Data summary showing drug effects over time. Pre-infusion period (baseline) was averaged over five stimulations, and postinfusion period (starting at t = 3) is presented as a percentage of baseline. At 9 min postinfusion, we observed that the evoked DA signal was 103% of baseline after saline infusion, 196% after NMDA infusion, 18% after AP5 infusion, 49% after MEC infusion, and 43% after SCOP infusion. n = 1 per condition.

DISCUSSION:

CIS-FSCV provides a unique opportunity to investigate VTA receptor mechanisms underlying phasic DA release. There are two critical steps in order to ensure a proper recording. First, a stable baseline recording must be achieved, with little drift in the evoked DA signal. An important way

to increase the likelihood of establishing a stable recording is to ensure that the electrode has had plenty of time to cycle at both 60 Hz and 10 Hz (typically 15 min at 60 Hz, and 10 min at 10 Hz). As the carbon fiber is being cycled, the carbon fiber itself oxidizes, and becomes etched, decreasing the surface area but producing new surface for dopamine adsorption[23]. This can lead to an increase in sensitivity to DA. Thus, one might see a slight increase in stimulated dopamine release over time in the experiment due to this increased etching, rather than any pharmacological manipulation. Additionally, beginning a recording within 90 to 120 min of initial anesthesia will increase the likelihood of a stable recording over long periods of time. As such, as the rat nears death from anesthesia, it is typical for the evoked DA release to decrease slowly.

The second critical step in this procedure is to ensure that the infusion cannula is gently inserted into the bipolar stimulating electrode. The stereotaxic armbars can move if too much pressure is placed while inserting the internal cannula, and as a result, the dopamine signal might artificially increase or decrease, as the stimulation site may be different. If there is a significant change in evoked signal after cannula insertion, a new baseline period should be established. Moreover, if there is altered DA release caused by inserting the internal cannula or vehicle infusion, the signal typically recovers to baseline within 30 min. Should there be extensive alterations in DA release to vehicle infusion, the infusion rate or volume could be reduced. Investigators may also perform an additional recording after inserting the internal cannula before the infusion to assess whether insertion of the cannula itself can alter release. Relatedly, it is important to verify that the infusion occurred, and that there is no blockade of the internal cannula. One way to do this is to make a small bubble in the infusion tubing, and mark this with a pen or marker. The bubble should be further away from the marker after infusion. Another way to ensure that the infusion occurred properly is to turn on the infusion pump after the internal cannula has been removed from the brain, and if there is still solution forming at the tip, then a successful infusion likely has occurred.

CIS-FSCV can be adapted to study VTA receptors in both behaviorally naive and trained animals to study changes in receptor function over time[11]. CIS-FSCV can also be modified to measure 5-HT and norepinephrine (NE)[24,25]. CIS-FSCV is also highly suitable for awake, behavior experiments and can be integrated with optogenetic approaches[26,27]. It is important to note that electrically evoked release events are distinct from transient release events often observed in free-moving studies, and less often in the anesthetized preparation. Transient release events, for example, may not be necessarily be driven by direct depolarization of dopamine neurons unlike electrically evoked release events[28]. As such, phasic neural activity might be dissociable from the dopamine release events detected via FSCV. Moreover, optically evoked DA release has been shown to differ from electrically evoked DA release events. A recent comparison between optically and electrically evoked stimulation has revealed that electrically evoked stimulation produces multisynaptic regulation of phasic DA release, whereas optically evoked stimulation can limit stimulation to more specific circuits[29].

Some recent approaches have employed optogenetic and fluorescent methods to investigate the circuitry underlying rapid dopamine dynamics in vivo[30]. For example, recent work by Sun and colleagues showed that optogenetic stimulation of dopamine neurons in the substantia nigra produces rapid elevations of DA in the striatum, as measured via expression of G-protein coupled

receptor-activation-based DA ($GRAB_{DA}$) sensors[30]. Combined optogenetic and fluorescence approaches could be used to stimulate or inhibit specific afferent inputs to the VTA while measuring DA release in the NAc. CIS-FSCV cannot stimulate the afferents as specifically as optogenetic stimulation, but it has an advantage in that it can address questions about presynaptic and postsynaptic receptors within the VTA. While both fluorescent and FSCV approaches have sufficient temporal resolution (subsecond) and sensitivity to DA (1–10 nM) to comparably measure changes in phasic DA release[30,31], one advantage FSCV may have over fluorescent monitoring of phasic DA in vivo is that no genetic manipulations are required for recording. Indeed, a CIS-FSCV experiment can be completed within hours, whereas combined optogenetic and fluorescence approaches require sufficient time (weeks) for sufficient expression using viral constructs.

A key benefit of CIS-FSCV is that specific VTA receptor regulation of phasic DA release can be studied in the intact brain, building on other in vivo studies that either measure the electrophysiological properties of VTA neurons or in vitro studies that evaluate the presynaptic regulation of phasic DA release[3,12]. One caveat of CIS-FSCV is that these recordings must be done in a relatively DA-rich area. This is for two reasons: First, there are some limits to FSCV sensitivity, which can only detect DA concentrations in the nanomolar range and above[6,19]. Second, FSCV has trouble dissociating norepinephrine from DA, because their cyclic voltammograms are nearly identical. Thus, these studies may be limited to assessing areas with high DA, such as some parts of the medial prefrontal cortex, NAc, striatum, and the olfactory tubercle[32]. Future studies might be able to employ some of the advances FSCV approaches that allow for better discrimination between DA and NE, as well as other electroactive neurotransmitters such as adenosine[33] and serotonin[12].

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Grace, A. A., Bunney, B. S. The control of firing pattern in nigral dopamine neurons: burst firing. *Journal of Neuroscience*. **4** (11), 2877–2890 (1984).

2. Lester, D. B. et al. Midbrain acetylcholine and glutamate receptors modulate accumbal dopamine release. *Neuroreport*. **19** (9), 991–995 (2008).

3. Lodge, D. J., Grace, A. A. The laterodorsal tegmentum is essential for burst firing of ventral tegmental area dopamine neurons. *Proceedings of the National Academy of Sciences of the United States of America*. **103** (13), 5167–5172 (2006).

4. Li, C. et al. Mu Opioid Receptor Modulation of Dopamine Neurons in the Periaqueductal Gray/Dorsal Raphe: A Role in Regulation of Pain. *Neuropsychopharmacology*. **41** (8), 2122–2132 (2016).

5. Zhang, H. Y. et al. Expression of functional cannabinoid CB2 receptor in VTA dopamine neurons in rats. *Addiction Biology*. **22** (3), 752–765 (2017).
6. Wickham, R. J. et al. Advances in studying phasic dopamine signaling in brain reward mechanisms. *Frontiers in Bioscience*. **5**, 982–999 (2013).
7. Wightman, R. M. et al. Monitoring of transmitter metabolites by voltammetry in cerebrospinal fluid following neural pathway stimulation. *Nature*. **262** (5564), 145–146 (1976).
8. Grace, A. A., Bunney, B. S. The control of firing pattern in nigral dopamine neurons: single spike firing. *Journal of Neuroscience*. **4** (11), 2866–2876 (1984).
9. Mameli-Engvall, M. et al. Hierarchical control of dopamine neuron-firing patterns by nicotinic receptors. *Neuron*. **50** (6), 911–921 (2006).
10. Wickham, R. et al. Ventral tegmental area alpha6beta2 nicotinic acetylcholine receptors modulate phasic dopamine release in the nucleus accumbens core. *Psychopharmacology*. **229** (1), 73–82 (2013).
11. Solecki, W. et al. Differential role of ventral tegmental area acetylcholine and N-methyl-D-aspartate receptors in cocaine-seeking. *Neuropharmacology*. **75**, 9–18 (2013).
12. John, C. E., Jones, S. R. Fast Scan Cyclic Voltammetry of Dopamine and Serotonin in Mouse Brain Slices. In *Electrochemical Methods for Neuroscience*. Edited by Michael, A. C., Borland, L. M., CRC Press/Taylor & Francis. Boca Raton, FL (2007).
13. Rice, M. E., Cragg, S. J. Nicotine amplifies reward-related dopamine signals in striatum. *Nature Neuroscience*. **7** (6), 583–584 (2004).
14. Espana, R. A. et al. Hypocretin 1/orexin A in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration. *Psychopharmacology*. **214** (2), 415–426 (2011).
15. Addy, N. A. et al. The L-type calcium channel blocker, isradipine, attenuates cue-induced cocaine-seeking by enhancing dopaminergic activity in the ventral tegmental area to nucleus accumbens pathway. *Neuropsychopharmacology*. **43** (12), 2361–2372 (2018).
16. Hermans, A., Wightman, R. M. Conical tungsten tips as substrates for the preparation of ultramicroelectrodes. *Langmuir*. **22** (25), 10348–10353 (2006).
17. Borland, L. M., Michael, A. C. An Introduction to Electrochemical Methods in Neuroscience. In *Electrochemical Methods for Neuroscience*. Edited by Michael, A. C., Borland, L. M., CRC Press/Taylor & Francis. Boca Raton, FL (2007).
18. Mundroff, M. L., Wightman, R. M. Amperometry and cyclic voltammetry with carbon fiber microelectrodes at single cells. *Current Protocols in Neuroscience*. **6** (6), 14 (2002).
19. Rodeberg, N. T. et al. Hitchhiker's Guide to Voltammetry: Acute and Chronic Electrodes for in vivo Fast-Scan Cyclic Voltammetry. *ACS Chemical Neuroscience*. **8** (2), 221–234 (2017).
20. Sabeti, J., Gerhardt, G. A., Zahniser, N. R. Chloral hydrate and ethanol, but not urethane, alter the clearance of exogenous dopamine recorded by chronoamperometry in striatum of unrestrained rats. *Neuroscience Letters*. **343** (1), 9–12 (2003).
21. Masuzawa, M. et al. Pentobarbital inhibits ketamine-induced dopamine release in the rat nucleus accumbens: a microdialysis study. *Anesthesia & Analgesia*. **96** (1), 148–152 (2003).
22. Montague, P. R. et al. Dynamic gain control of dopamine delivery in freely moving animals. *Journal of Neuroscience*. **24** (7), 1754–1759 (2004).
23. Keithley, R. B. et al. Higher sensitivity dopamine measurements with faster-scan cyclic voltammetry. *Analytical Chemistry*. **83** (9), 3563–3571 (2011).

24. Jackson, B. P., Dietz, S. M., Wightman, R. M. Fast-scan cyclic voltammetry of 5-hydroxytryptamine. *Analytical Chemistry*. **67** (6), 1115–1120 (1995).
25. Park, J., Takmakov, P., Wightman, R. M. In vivo comparison of norepinephrine and dopamine release in rat brain by simultaneous measurements with fast-scan cyclic voltammetry. *Journal of Neurochemistry*. **119** (5), 932–944 (2011).
26. Wenzel, J. M. et al. Phasic Dopamine Signals in the Nucleus Accumbens that Cause Active Avoidance Require Endocannabinoid Mobilization in the Midbrain. *Current Biology*. **28** (9), 1392–1404 e5 (2018).
27. Spanos, M. et al. NMDA Receptor-Dependent Cholinergic Modulation of Mesolimbic Dopamine Cell Bodies: Neurochemical and Behavioral Studies. *ACS Chemical Neuroscience*. **10** (3), 1497–1505 (2019).
28. Cheer, J. F. et al. Cannabinoids enhance subsecond dopamine release in the nucleus accumbens of awake rats. *Journal of Neuroscience*. **24** (18), 4393–4400 (2004).
29. Melchior, J. R. et al. Optogenetic versus electrical stimulation of dopamine terminals in the nucleus accumbens reveals local modulation of presynaptic release. *Journal of Neurochemistry*. **134** (5), 833–844 (2015).
30. Sun, F. et al. A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice. *Cell*. **174** (2), 481–496 e19 (2018).
31. Robinson, D. L. et al. Monitoring rapid chemical communication in the brain. *Chemical Reviews*. **108** (7), 2554–2584 (2008).
32. Park, J. et al. Heterogeneous extracellular dopamine regulation in the subregions of the olfactory tubercle. *Journal of Neurochemistry*. **142** (3), 365–377 (2017).
33. Ganesana, M., Venton, B. J. Early changes in transient adenosine during cerebral ischemia and reperfusion injury. *PLoS One*. **13** (5), e0196932 (2018).

Figure1

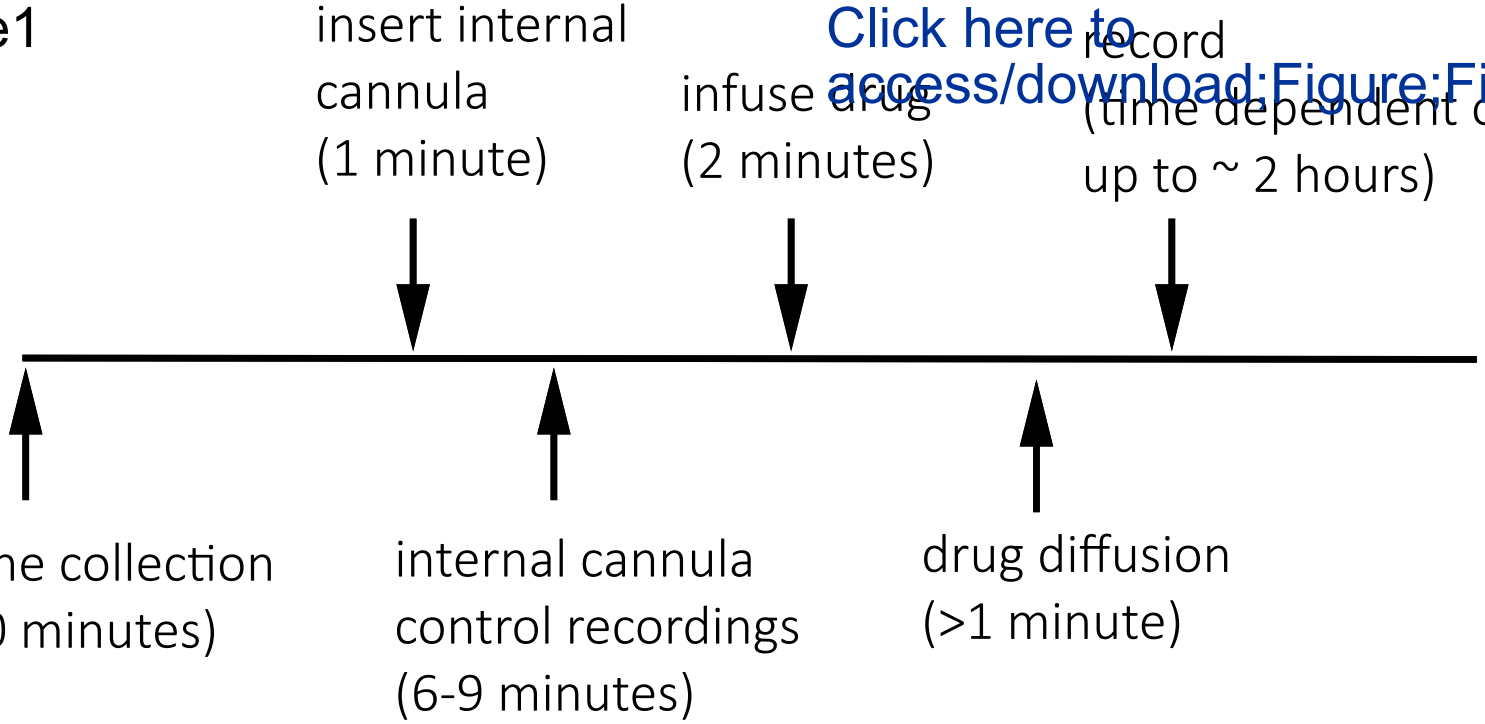


Figure2

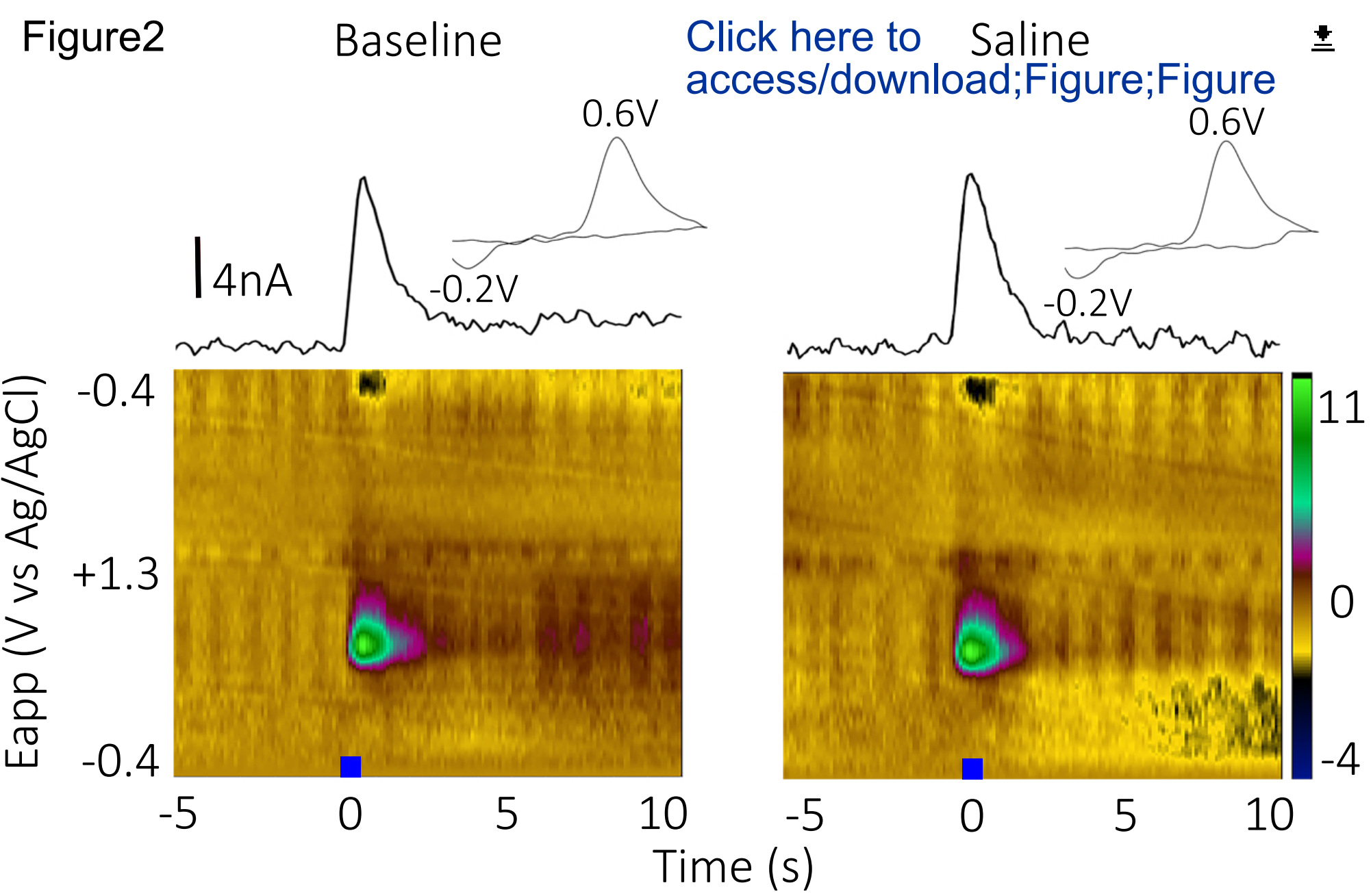
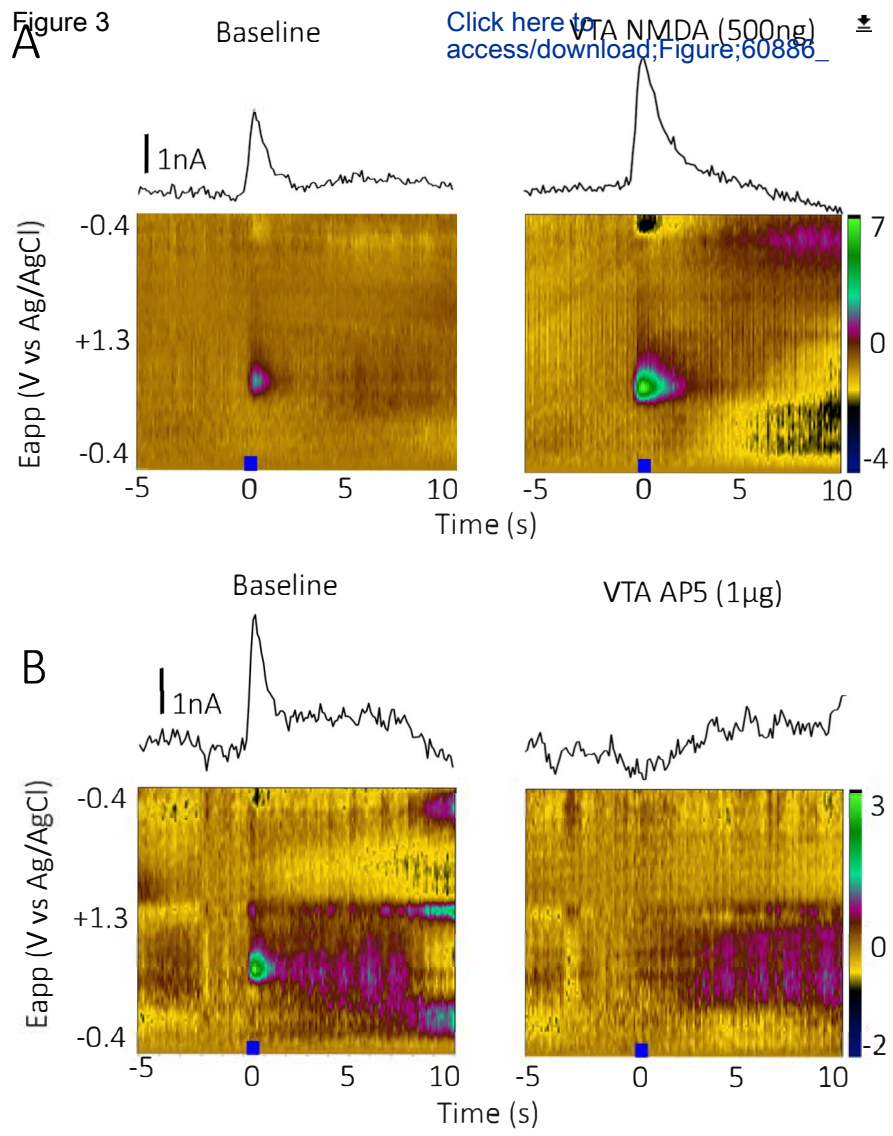

Baseline
Click here to access/download;Figure;Figure
Saline
0.6V
4nA
-0.2V
0.6V
-0.2V
-0.4
+1.3
-0.4
Eapp (V vs Ag/AgCl)
11
0
-4
-5
0
5
10
-5
0
5
10
Time (s)

Figure 3



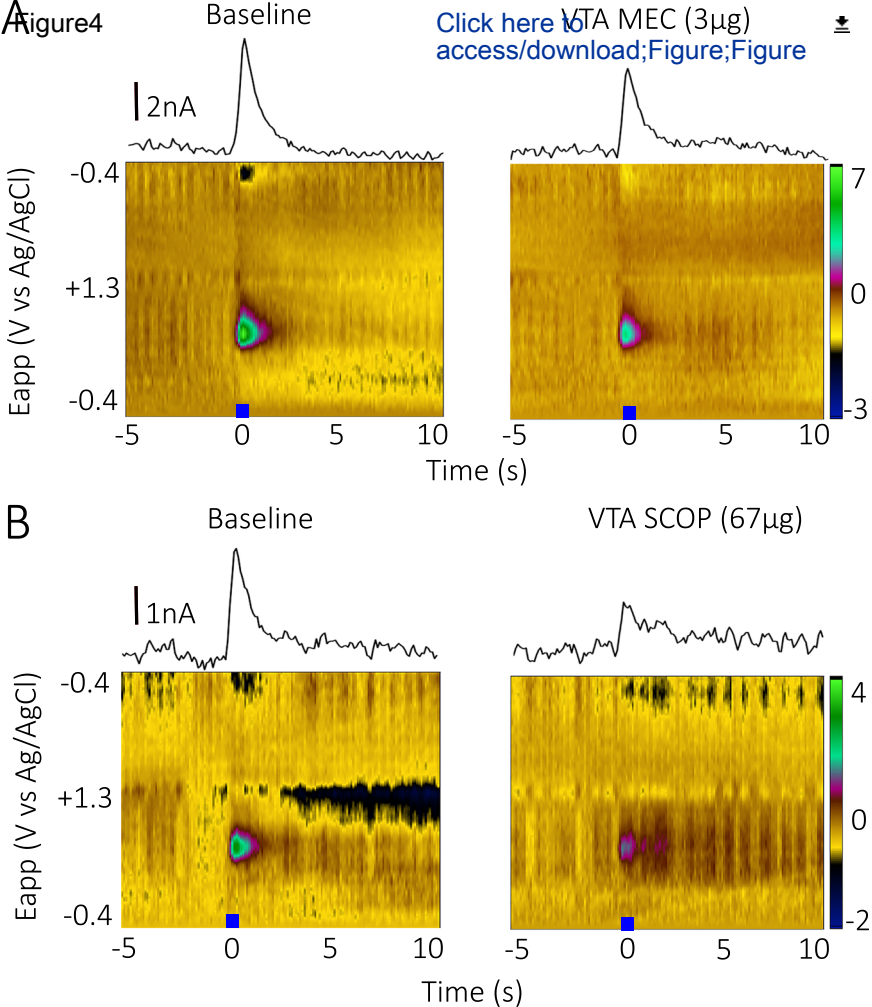
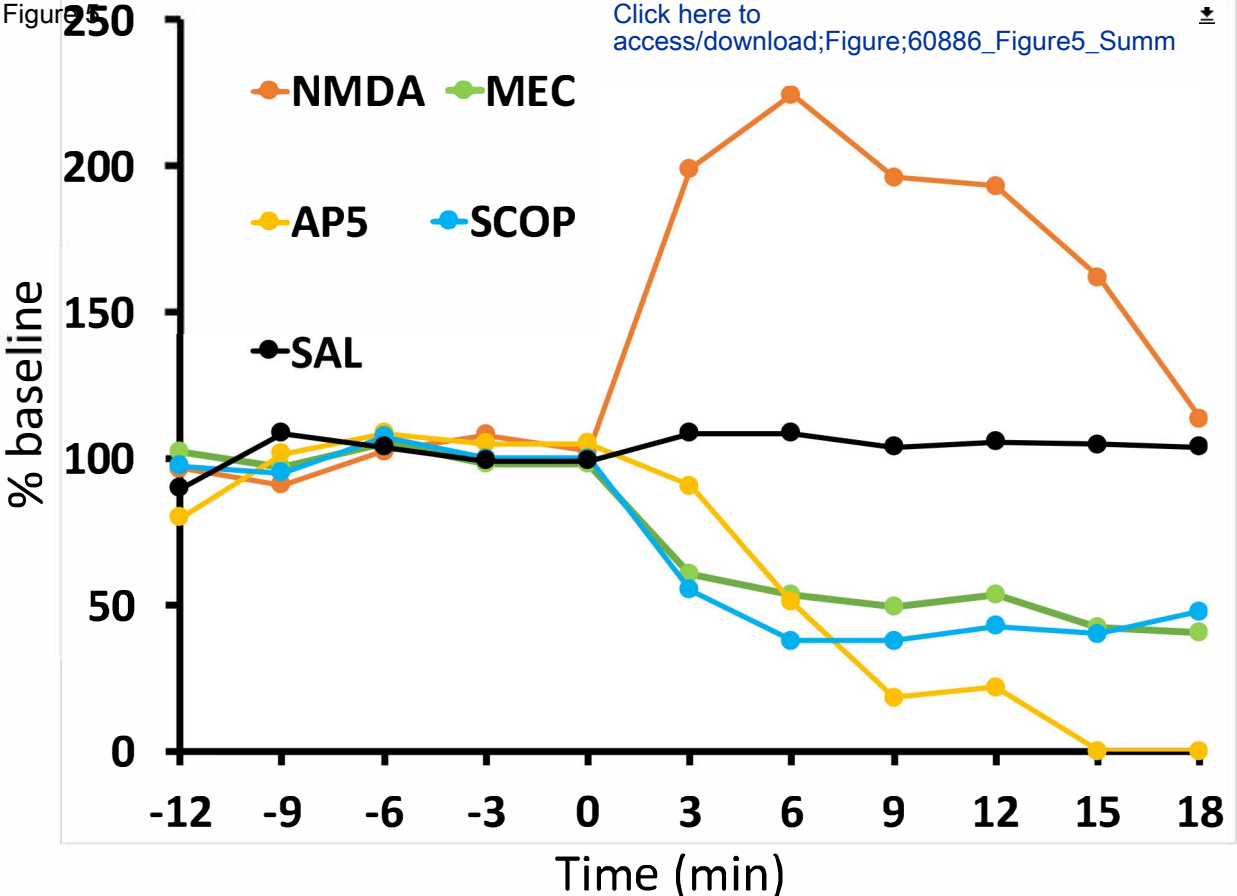


Figure4
Click here to access/download;Figure;Figure
A
Baseline
VTA MEC (3µg)
2nA
-0.4
+1.3
-0.4
Eapp (V vs Ag/AgCl)
7
0
-3
-5
0
5
10
Time (s)
B
Baseline
VTA SCOP (67µg)
1nA
-0.4
+1.3
-0.4
Eapp (V vs Ag/AgCl)
4
0
-2
-5
0
5
10
Time (s)

Figure 5



Name
Electrode Filling Solution/Supplies
Micropipette
Potassium Acetate
Potassium Chloride
Electrode Supplies
Carbon fiber
Electrode puller
Glass capillary
Insulated wires for electrodes
Light Microscope (for viewing and cutting electrode)
Pin
Putty
Scalpal Blade
Silver Wire
FSCV Hardware/Software
Faraday Cage
Potentiostat
Stimulating electrode
TarHeel HDCV Software
UEI breakout box
UEI power supply
Stimulator Hardware
Neurolog stimulus isolator
Infusion/Stimulation Supplies
Infusion Pump
Internal Cannula
Microliter Syringe
Tubing
Surgical Supplies
Cannula Holder
Cotton Tip Applicators
Electrode Holder
Heating Pad
Povidone Iodine

Screws
Silver wire reference with AgCl
Square Gauze
Stereotax
Histological Supplies
Formulin
Power supply
Sucrose
Tungsten microelectrode
Drugs for infusions
((2*R*)-amino-5-phosphonovaleric acid
N-methyl-D-aspartate
Mecamylamine hydrochloride (M9020-5mg)
Scopolamine hydrobromide (S0929-1g)

Company	Catalog Number
World Precision Instruments	MF286-5 (28 gauge)
Sigma	236497-100G
Sigma	P3911-25G
Thornel	T650
Narishige International	PE-22
A-M systems	626000
Weico Wire and Cable Incorporated	UL 1423
Fischer Scientific	M3700
Phoenix Enterprises	HWS1646
Alcolin	23922-1003
World Precision Instruments	500239
Sigma	327026-4G
U-Line	H-3618 (36" x 24" x 42")
Univ. of N. Carolina, Electronics Facility	
PlasticsOne	MS303/2-A/SPC
University of North Carolina-Chapel Hill	-
Univ. of N. Carolina, Electronics Facility	
Univ. of N. Carolina, Electronics Facility	
Digitimer Ltd.	DS4
New Era Syringe Pump	NE-300
PlasticsOne	C315I/SPC INTERNAL 33GA
Hamilton	80308
PlasticsOne	C313CT/ PKG TUBING 023 X 050 PE50
Kopf Instruments	1776 P-1
Vitality Medical	806
Kopf Instruments	1770
Kent Scientific	RT-0501
Vitality Medical	29906-004

Stoelting	Bone Anchor Screws/Pkg.of 100
InVivo Metric	E255A
Vitality Medical	441408
Kopf Instruments	Model 902 (Dual Arm Bar)
Sigma	1004960700
BK Precision	9110
Sigma	80497
MicroProbes	WE30030.5A3
Sigma Aldrich	A5282
Sigma Aldrich	M3262
Sigma Aldrich	M9020
Sigma Aldrich	S0929

Comments		
Note: horizontal pullers can be used as well		
Length; 10 cm; diameter,0.4mm; must get custom made; insulated material should cover 5 cm of the wire		
To be soldered onto the insuled electrode wire and reference electrode; connects to heads		
Used to place electrode on while cutting the carbon fiber		
For cutting carbon fiber to the apprpriate length		
when ordering, request a 22 mm cut below pedestal		
https://chem.unc.edu/critcl-main/criticl-electronics/criticl-		
https://chem.unc.edu/critcl-main/criticl-electronics/criticl-electronics-hardware/ for ordering information		
https://chem.unc.edu/critcl-main/criticl-electronics/criticl-		
Neurolog 800A		

1.59 mm O.D., 3.2 mm long		

stage

Dear Journal of Visualized Experiments Editorial Team and Reviewers,

We thank you for your thoughtful feedback for improving the clarity of the methodology instructions and providing a richer context for how this method fits within the field. Below, you will find a line-by-line response to your feedback. We have made every attempt to fully address and incorporate and your suggestions, and we are excited about the prospect of publishing this improved version of our manuscript in the Journal of Visualized Experiments.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We have thoroughly reviewed the text to identify spelling and grammatical errors.

• **Textual Overlap:** Significant portions show significant overlap with previously published work. Please re-write lines 110-121, 125-129, 265-280 to avoid this overlap.

This text has been rewritten to reduce overlap between previous submissions.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 2.1: Mention animal age, sex, weight, strain.
The following has been added to this line:
"Using adult, male, Sprague Dawley Rats (250-450g)…

2) 2.6: Mention drill bit size and speed.

The following has been added to this line:
"Use a stereotaxic or hand drill, drill (1.00mm, 30,000 r.p.m)"

3) 2.7: What kind of screw? Mention specifications.

The following has been added to this line:
"Partially (about halfway, until it is firmly in place) implant a screw (1.59mm O.D., 3.2mm long) in this hole"

4) 2.8.,2.11: How? By drilling? See previous comment on drilling.

These suggestions have been accommodated by replacing the word "drill" instead of "make"

5) 5.4: Briefly describe brain extraction or cite a reference.

The following sections have been added and rewritten to expand on brain extraction:

5.5 Remove the head from the anesthetized rat by using a sharpened guillotine.
5.6 Using rongeurs, remove the connective tissue and skull surrounding the brain, and gently dislodge the brain from any remaining tissue
5.7 Store the brain in 4% formalin for 1 day. The brain should be transferred to 30% sucrose afterwards.

Note: in our hands, perfusion with 4% formulin is not necessary to see the lesion site, although as a best practice will improve the reconstruction of the lesion site.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have significantly reworked and reorganized the discussion to better incorporate these five sections.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

N/A: this work has not been published in any other publication.

Comments from Peer-Reviewers:

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The Addy lab provides a brief description of the methodology by which a scientist could perform combined microinfusions + electrochemical recording via fast-scan cyclic voltammetry (FSCV). Dr. Addy trained under the founder of this electrochemical technique and is a good candidate to make a video detailing how it should be performed. Moving forward with a video would make a useful contribution to the field. Of note, there are several nuances regarding urethane anesthesia, where to order specific parts, etc., that are not otherwise available in current publications on the technique. However there are several places the written text could be expanded, in addition to numerous grammatical errors and typos that require a round of judicious editing by the authors.

Major Concerns:
The authors should note in the discussion that transient release events are distinct from electrically-evoked release events (PMID: 15128853 fig. 3; this may not directly depolarize dopamine neurons) and these, in turn, are dissociable from optically-evoked release events (PMID: 26011081). It might also be worth noting that phasic neural activity detected using extracellular electrophysiological recordings might be dissociable from the dopamine release events detected using FSCV.

This is an important distinction to integrate into the discussion. The following has been incorporated into the discussion to highlight the distinction between transient, electrical, and optically evoked dopamine release:

"It is important to note that electrically evoked release events are distinct from transient release events often observed in freely-moving studies, and less often in the anesthetized preparation. Transient release events, for example, may not be necessarily be driven by direct depolarization of dopamine neurons unlike electrically evoked release events[30]. As such, phasic neural activity might be dissociable from the dopamine release events detected via FSCV. Moreover, optically evoked DA release has been shown to differ from electrically evoked DA release events A recent comparison between optically and electrically evoked stimulation has revealed that electrically evoked stimulation produces multi-synaptic regulation of phasic DA release whereas optically evoked stimulation can limit stimulation to more specific circuits" [31].

Urethane dosing is complicated and could be clarified here. Some detailed description in determining final solution and injection dosage for urethane would be helpful. Substantial solute is required in the case of urethane. What is the concentration of urethane solution the authors use? Is the 1g/kg dose based on a concentration after the solute is added or before? It might also be helpful to note that urethane is a carcinogen/mutagen/teratogen and why it is used over less dangerous anesthetics.

Your suggestion is very useful in clarifying the complexities of urethane dosing. We have amended the protocol to incorporate a clearer, step-by-step explanation on urethane dosing and achieving an anesthetized plane.

2.1 Using adult, male, Sprague Dawley Rats (250-450g) give 1.5 g/kg (1 ml/kg volume) intraperitoneal injection of urethane dissolved in sterile saline

2.2 The concentration of the urethane solution is 0.5g/ml w/v (weight per volume). For preparation, add 10g of Urethane to 10g (~10ml) saline. The initial urethane dose administered to the rat is 1.0 to 1.2g/kg. If the animal is still responsive to the noxious stimulus test (tail pinch) 20 minutes after urethane administration, an additional 0.3 to 0.5g/kg urethane can be administered for a 1.5g/kg total dose. It is important to note that urethane is a carcinogen, but also note the importance of its use as an anesthetic as it does not alter levels of dopamine, as do other anesthetics such as ketamine/ xylazine and chloral hydrate[22, 23].

Squires electronics was purchased by Kauffman engineering I believe; this point should be checked and updated in the authors' part description spreadsheet if necessary. Also, a unit should be provided for length. But, a reference of parts, etc., is sorely lacking and makes it difficult for investigators unfamiliar with the field to set-up this technique. This reference alone appeals to me.

Thank you for identifying that Squire Electronics is no longer in business. We have also updated the materials sheet that will be more useful to those who are unfamiliar with this approach.

Might perfusion of the animal improve histological reconstruction of lesioned tissue in this 'best-practices' tutorial?

We agree that perfusion could certainly improve histological reconstruction. In our hands, we do not need it to see the reconstruction site, but we included a note that perfusion will aid in reconstructing the lesion site:

5.3 Using a lethal injection of pentobarbital (150mg/kg, i.p,), euthanize the animal.
5.4 Perfuse the rat using a 4% formalin solution
5.5 Remove the head from the rat by using a sharpened guillotine.
5.6 Using rongeurs, remove the connective tissue and skull surrounding the brain, and gently dislodge the brain from any remaining tissue.
5.7 Store the brain in 4% formalin for 1 day. The brain should be transferred to 30% sucrose afterwards.

Note: in our hands, perfusion with 4% formulin is not necessary to see the lesion site, although as a best practice will improve the reconstruction of the lesion site.

Minor Concerns:
It is unclear why the electrical stimulation section is still highlighted in yellow; is this incomplete?

This section is highlighted yellow as part of JOVE's instructions for highlighting which sections of the protocol are most important to be video-taped.

There is a spacing error in 1.4; 1.4.2
This has been addressed

At times the bullets end with a sentence; in others they do not.
All bullets now end with a period.

There are other minor grammatical errors that should be checked by the authors.
We have reviewed the manuscript more thoroughly to avoid these grammatical errors.

Reviewer #2:
Manuscript Summary:
The manuscript "Combined infusion and stimulation with fast-scan cyclic voltammetry (CIS-FSCV) to assess ventral tegmental area receptor regulation of phasic dopamine" by Wickham et al. described the method of CIS-FSCV and its application in anesthetized rats. This method provides specific VTA receptors' role in modulating phasic dopamine release in its terminal regions.

Minor Concerns:
1. Line 43 in Abstract and Line 66: Suggest to verify that the stimulation is "electrical" stimulation.
This suggestion has been incorporated, and now reads as:

Abstract: "a phasic DA response can be evoked by electrically stimulating the VTA with a bipolar electrode"

Introduction: "Here, in the anesthetized rat, we electrically stimulate the VTA to evoke a large phasic DA signal (1-2µM) in the NAc core, as measured by fast-scan cyclic voltammetry (FSCV)"

2. Line 89-91: Suggest to clarify that presynaptic receptors are in the dopamine terminal or cell body regions in the VTA.

Thank you for this suggestion—this section has been re-written to incorporate your feedback:

"Mechanistic examination of phasic DA regulation is also commonly studied using slice preparations alongside with bath application of drugs. These studies often focus on the presynaptic regulation of phasic DA release from dopamine terminals, as the cell bodies are often removed from the slice[12]. These preparations are valuable for studying presynaptic receptor effects on dopamine terminals, whereas CIS-FSCV is better suited to study somatodendritic receptor effects on dopamine neurons as well as presynaptic inputs to the VTA. This distinction is important, as somatodendritic receptor activation in the VTA may have a different effect than NAc presynaptic receptor activation. Indeed, blockade of

dopaminergic presynaptic nAChRs in the NAc can elevate phasic dopamine release during burst-firing[13], whereas the opposite is true at VTA somatodendritc nAChRs[10, 11].”

3. In the Protocol: need to clarify that the protocol is for only anesthetized animal experiments. For examples, 1.0 mm outer diameter of glass capillary and filling the electrode with electrolyte solution is for the anesthetized animal experiments.

This is an important clarification point, since freely-moving and anesthetized preparations require different capillaries. We have added the following line to clarify that this protocol is for use in the anesthetized preparation:

“This protocol is specific to the anesthetized rat preparation of utilizing CIS-FSCV”

4. Line 110: The protocol describes making "electrode solution" as a Tris buffer. This physiological solution is used in post calibration rather than backfill solution. Backfill "electrode" solution consists of 4 M potassium acetate and 150 mM potassium chloride (A. Hermans, R.M. Wightman, Langmuir 2006).

This error has been fixed and reference updated. This section now reads as:

1.1.1. ***“To make the electrode backfill solution, prepare a solution of 4M potassium acetate with 150mM potassium chloride[3].”***

5. Line 132: Suggest to change "electrode fabrication" to "electrode preparation"
This suggestion has been implemented.

6. Line 174 and 187: suggest to add "for reference electrode" and "for carbon-fiber microelectrode". In addition 1.5 mm for reference and 3.0 mm holes are too big for the electrodes. Especially, 3.0 mm hole for carbon fiber microelectrodes will cause misplacement of the carbon microelectrode in the brain. Recommend to change to 1.5 mm hole. Additionally, it may be advisable to not mention "anywhere in the left hemisphere" but rather give an approximate anterior-posterior axis coordinate as to not place items too close to the CIS or working electrode.

Thank you for these suggestions—we have incorporated your feedback below. Drilling the holes for the stimulating electrode now comes prior to lowering the working electrode into the brain, per your suggestion:

2.6 Use a stereotaxic or hand drill, (0.1.00mm, ~20,000 r.p.m), drill a 1.5mm diameter hole 2.5mm anterior to bregma and 3.5mm lateral to bregma. Partially (about halfway, until it is firmly in place) implant a screw (1.59mm O.D., 3.2mm long) in this hole.

2.7 For the reference electrode, drill a 1.0mm diameter hole 1.5mm anterior to bregma and 3.5mm lateral from bregma (in the left hemisphere), anterior to bregma and in the left hemisphere.

2.8 By hand, insert ~2mm of reference wire into this hole, while wrapping the reference wire around and under the head of the screw previously implanted.

2.9 Fully implant the screw, pinning down the reference electrode in place.

2.10 In the right hemisphere, drill a 1.5mm diameter hole at 1.2mm anterior and 1.4mm lateral to Bregma.

2.11 Gently remove the dura, using tweezers.

2.12 For the stimulating electrode, drill a square hole (2 mm anterior-posterior, 5 mm medial-lateral) centered at 5.2 mm posterior and 1.0 mm lateral to Bregma.

2.13 Using the stereotactic arm bars, lower the bipolar stimulating electrode/guide cannula 5 mm below dura. There may be bleeding during the implantation of the electrode. Utilize sterile Q-tips and gauze to minimize bleeding.

Note: The bipolar stimulating electrode used in this method is pre-fitted with a guide cannula (see materials sheet). The internal cannula used with this item should be flush with the prongs on the bipolar stimulating electrode when fully inserted into the guide cannula. This will allow the internal cannula to sit directly in between the two prongs of the stimulator, which sit about 1 mm apart. A similar protocol is described in [16].

2.14 Using the stereotactic arm bars, lower the carbon fiber microelectrode 4mm below dura. This location is at the most dorsal portion of the striatum.

7. Line 200: It is unclear why the author's suggest drilling holes while the electrode is implanted in the brain.

The order of drilling has been re-arranged (see previous comment) so that this no longer occurs in the protocol.

8. Line 213: 300 uA stimulating current is typically used for anesthetized animal experiments. 150 uA is for freely moving animal experiments.

Thank you for the correction—we have modified it accordingly.

9. Line 218: Suggest to clarify that "electrical stimulation" induces twitching in the rat's

whiskers.

We have clarified this point below:

3.2 Gently lower the stimulator in increments of 0.2mm from 5mm below dura to 7.8mm. At each increment, stimulate the brain.

Note: <u>At more dorsal depths (5-6mm), stimulation of the brain will typically (~80% of the time) cause the whiskers of the rat to twitch</u>. At further depths, the whiskers will cease twitching, which occurs between 7.5-8.2 mm below dura. When the whiskers cease twitching, the stimulating electrode will be near or at the VTA. This will not occur in every rat, and lack of whisker twitching should not be taken as a sign that the bipolar stimulating electrode/ infusion cannula is misplaced. Whisker twitching may not occur for all anesthetics (e.g., isoflurane).

10. Line 297: Suggest to verify that the drugs were in fact infused into the VTA.

We have incorporated this suggestion by adding in addition to verifying carbon fiber placement, also to verify the location of the stimulator histologically:

5.10 Denote the location of the carbon fiber microelectrode lesion and bipolar stimulator/infusion cannula location using a light microscope.

11. Line 355: etching the carbon surface decreases the surface area but produces new surface.

We have added this correction accordingly:

Discussion, First Paragraph

As the carbon fiber is being cycled, the carbon fiber itself oxidized, and becomes etched, decreasing the surface area but producing new surface for dopamine adsorption

12. In Discussion: Suggest to provide the following additional information.
"If the altered DA released caused by inserting *the* internal cannula or saline will be gradually recovered to the baseline within 30 min.

We have made this incorporation accordingly. This is very important.

Discussion, Second Paragraph
"Moreover, if there is altered DA released caused by inserting the internal cannula or vehicle infusion, the signal typically recovers to baseline within 30 minutes."

Reviewer #3:
Manuscript Summary:

This work is about how to perform a drug infusion at the place where the stimulation electrodes are located during a fast-scan cyclic voltammetry procedure. The proposed protocol is for the ventral tegmental area of the rat brain while quantifying the release of dopamine in the nucleus accumbens. The possibility of infusing drugs, agonist or antagonist ligands, allows studying the influence of homologous and heterologous neurochemical systems in controlling the phasic release of dopamine (or another catecholamine) using FSCV. Although the methodology has been previously described and used, the value of generating a visual protocol plus an exhaustive description of the technique can be very significant for people who want to implement it in their laboratories.
This work adequately addresses the challenge and shows a convincing protocol, although there are some aspects that should be improved.

Major Concerns:
1.- The most important part of the protocol is how to reach the VTA with a cannula through which it is infused a solution containing drugs. This is not explained. How do they do it? How do they introduce the cannula? Important details are missing. The authors should explain in a detailed way how they lowered the cannula, how they ensure that the infusion cannula is close to the tip of the stimulation electrode in the VTA. Did they use a needle to infuse the solution?

Thank you for this feedback. We have incorporated additional details to clarify this point. In short—the description of the bipolar stimulating electrode, which is fitted with a guide cannula, is expanded upon. We have added the following to the method sections:

> ***2.14 Using the stereotactic arm bars, lower the bipolar stimulating electrode/guide cannula 5 mm below dura. There may be bleeding during the implantation of the electrode. Utilize sterile Q-tips and gauze to minimize bleeding.***

Note: The bipolar stimulating electrode used in this method is pre-fitted with a guide cannula (see materials sheet). The internal cannula used with this item should be flush with the prongs on the bipolar stimulating electrode when fully inserted into the guide cannula. This will allow the internal cannula to sit directly in between the two prongs of the stimulator, which sit about 1 mm apart. A similar protocol is described in [16].

> ***4.2 After achieving a stable baseline (<20% variation over 5 stimulations), gently lower the internal cannula by hand into the guide cannula that is pre-fitted into the bipolar stimulator.***

2.- Is the drug infusion cannula attached to the stimulant electrode? If it's not how the cannula gets in? I miss a third arm in stereotaxic to do this. The first work to my knowledge describing the infusion of drugs in the VTA during FSCV experiments is the paper of España et al. 2011 (Hypocretin 1 / orexin A in the ventral tegmental area improves dopamine responses to cocaine and promotes cocaine self-administration. Psychopharmacology (Berl). March 2011; 214 (2): 415-26. doi: 10.1007 / s00213-010-2048-8). In this paper the cannula is fused to the stimulant electrode. Authors should cite this paper and compared with their own protocol.

This protocol is similar to the one outlined in Espana et al.,. This reference has been added accordingly, in addition to clarifying this component of the methodology (see previous response)

3.- Important details regarding the protocol description are missing. How long it is necessary to wait after the infusion to perform the first registration?

Thank you for this helpful suggestion. Additional details regarding how long one should wait post-infusion has been added below.

4.4 Post-infusion, leave the internal cannula for at least 1 minute prior to removal. Some drugs may require leaving the internal cannula for longer based on the drug kinetics, and removal of the internal may cause the drug to travel back up through the internal. If there is concern, one could leave the internal cannula in the guide cannula during the entirety of the recording. Otherwise, recording can begin after this 1-minute interval.

Minor Concerns:
1.- The last sentence of the summary (Additionally, CIS-FSCV produces very robust effects since the levels of phasic DA produced through VTA stimulation higher than spontaneous or stimulus-evoked events observed in freely-moving studies), should be modified, since it is a conjecture and does not correspond to a conclusion that emerges from the data provided by work.

This sentence has been removed from both the abstract and discussion—we agree that this statement is too strong and there is not sufficient evidence to support this.

2.- Line 55 should say VTA DA neurons switch…..

This has been corrected accordingly.

3.- lines 87-88, and 92-93, sentences are confusing. What the authors try to say with somatodendritic versus presynaptic control should be better explained. It cannot be ruled out that drugs infused directly into the VTA may have an effect on presynaptic terminals and not on the somatodendritic tree of the dopaminergic neurons.

We agree that this section could be further clarified, and have made edits to this section accordingly, outlined below.

Introduction Paragraph Four
"Mechanistic examination of phasic DA regulation is also commonly studied using slice preparations alongside with bath application of drugs. These studies often focus on the presynaptic regulation of phasic DA release from dopamine terminals, as the cell bodies are

often removed from the slice[12]. These preparations are valuable for studying presynaptic receptor effects on dopamine terminals, whereas CIS-FSCV is better suited to study somatodendritic receptor effects on dopamine neurons as well as presynaptic inputs to the VTA. This distinction is important, as somatodendritic receptor activation in the VTA may have a different effect than NAc presynaptic receptor activation. Indeed, blockade of dopaminergic presynaptic nAChRs in the NAc can elevate phasic dopamine release during burst-firing[13], whereas the opposite is true at VTA somatodendritc nAChRs[10, 11]."

4.- Indicate the model of potentiostat (line 195) and stimulator (line 212)

These models have been added to the text accordingly

2.16 Connect the reference wire and carbon fiber to a potentiostat (University Electrochemistry Instrument, University of North Carolina Chapel Hill Electronics Facility).

3.1 ***Set the stimulator (Neurolog 800A, Digitimer) to produce a bipolar electrical waveform, with a frequency of 60Hz, 24 pulses, 300 µA current, and pulse width of 2ms/phase****.*

5.- Give an explanation for step 2.15 (Why is it important?)

We have added additional details to this step to provide some additional background as to why this step is taken.

2.17 Apply a triangular wave form (-0.4 to 1.3V, 400V/s) for 15 minutes at 60 Hz, and again for 10 minutes for 10 Hz. Typically, when applying waveforms to carbon fiber microelectrodes in the brain, oxide groups are added to the surface of the carbon fiber. Equilibrium of this reaction must be reached prior to recording, otherwise significant drift will occur[21]. Cycling the electrode at higher frequencies (60Hz) allows the carbon fiber to achieve equilibrium faster.

6.- Figure 4 define MEC

These have been done so in the figure legend, as well as indication of the dose used. The figures have also been re configured; this figure is now Figure 4A.

7.- Figure 5 define SCOP

These have been done so in the figure legend, as well as indication of the dose used. The figures have also been re configured; this figure is now Figure 4B.

Reviewer #4:
Manuscript Summary:
The manuscript by Wickman et al. describes the use of fast-scan cyclic voltammetry at a carbon-fiber microelectrode coupled with a combined stimulating electrode and injection cannula to

assess afferent control of mesolimbic dopamine neurons at the receptor level. The manuscript is well written and organized. The technique is clearly and comprehensively described. Presented data support the utility of the approach. Overall, the manuscript provides a valuable description of a useful technique for studying afferent control of mesolimbic dopamine neurons.

Major Concerns:
1. While the Introduction provides a good description comparing technical approaches for studying mesolimbic dopamine neurons, it needs to be cast broader by inclusion of more recent optogenetic and fluorescent approaches. In particular, how does the voltammetric approach with infusion of receptor targeting drugs compare with optogenetically activating or inhibiting afferent input and monitoring dopamine in striatal regions fluorescently with, e.g., GCaMP3, GRABDA or dLight?

Thank you for this very helpful suggestion in broadening the scope of this work. We have added a discussion in the introduction section comparing the pros and cons of CIS-FSCV with combined optogenetic and fluorescent approaches, as described below. We recognize your suggestion is for the introduction section, and we feel that it is more appropriate for the discussion section given the editorial comments and guidelines placing comparisons of approaches in the discussion section.

Discussion Paragraph 4

Some recent approaches have employed optogenetic and fluorescent methods to investigate the circuitry underlying rapid dopamine dynamics in vivo[14]. For example, recent work by Sun and colleagues showed that optogenetic stimulation of dopamine neurons in the substantia nigra produces rapid elevations of DA in the striatum, as measured via expression G-protein coupled receptor-activation-based DA ($GRAB_{DA}$) sensors[14], Combined optogenetic and fluorescence approaches could be used to stimulate or inhibit specific afferent inputs to the VTA, while measuring DA release in the NAc. CIS-FSCV, on the other hand, cannot stimulate the afferents as specifically as optogenetic stimulation, but has the advantage in that it can address questions about presynaptic and postsynaptic receptors within the VTA. While flourescent and FSCV approaches both have sufficient temporal resolution (subsecond) and sensitivity to DA (1-10nM) to comparable measure changes in phasic DA release[14, 15], one advantage FSCV may have over fluorescent monitoring of phasic DA in vivo is that no genetic manipulations are required for recording. Indeed, a CIS-FSCV experiment can be completed within hours, whereas combined optogenetic and fluorescence approaches require sufficient time (weeks) for sufficient expression using viral constructs.

2. The discussion of altering the levels of electrically evoked dopamine by changing stimulating parameters is confusing. Yes, increasing stimulus intensity will yield larger-amplitude dopamine

signals that are easier to measure, but these same signals may not be physiologically relevant. Thus, receptor drug effects on this dopamine signals may be challenging to interpret faithfully.

We have omitted this section for clarity.

Minor Concerns:
1. Whisker twitching elicited by electrical stimulation may not occur with all anesthetic (e.g., isoflurane).

We have added a line accordingly incorporating this suggestion:

"Whisker twitching may not occur for all anesthetics (e.g., isoflurane".

Reviewer #5:
Manuscript Summary:
In this paper, the authors are proposing a methodology that combines infusion and stimulation with fast-scan cyclic voltammetry (CIS-FSCV), to assess the functionality of VTA receptors in driving phasic DA release. For that, they included:

1) A general description, step-by-step, about how the experiment should be carried out.
2) Data and figures for voltammetric recordings using the proposed CIS-FSCV system after infusion of different drugs

The authors conclude that "Using CIS-FSCV, pharmacological agonists or antagonists can be infused directly at the stimulation site to investigate specific VTA receptors' role in driving phasic DA release in the NAc". They also claim that using CIS-FSCV, the" VTA receptor the function can be studied in vivo, building on in vitro studies".

In general terms, the protocol is including most of the critical details in an organized fashion. However, several aspects need to be completed, added and/or explained to give the reader a more complete and accurate idea about the procedure.

Comments:
1. Electrode preparation (1.2.) steps are in general complete. However, the reference provided for more detailed info is not informative at all since it is not even including any detail about electrode preparation. Considering that this is a very important step for the outcome (good quality recordings), it requires better sources regarding electrode preparation. I suggest checking on the references below that, together, can offer a better perspective about this critical step.

https://www.ncbi.nlm.nih.gov/books/NBK1847/
https://www.ncbi.nlm.nih.gov/pubmed/18428562
https://www.ncbi.nlm.nih.gov/pubmed/28127962

Thank you for these references—we have incorporated them into the manuscript to aid the would-be experimenter to craft electrodes. Specifically, at the end of the electrode fabrication, we conclude by saying

1.2.5 Using a light microscope, ensure that the electrode is free of cracks along the capillary. Additionally, the seal, where the carbon fiber exits the capillary should be difficult to notice and should also be free from cracks. A good seal will help reduce noise during recordings. For a more detailed protocol, see. [19-21].

2. There are some critical concerns about electrode implantation (2) related to the order of those steps.

*Since there is not too much space between electrodes and the carbon fiber electrode is very delicate, implanting this electrode (step 2.13.) before making all the holes (step 2.16.) increases the risk very much for broking it with all the complications it implies. Maybe, this is not a problem for more experimented researchers but, it could be for beginners. I will not recommend to implanting the carbon fiber electrode until all the other electrodes (reference and stimulation) were already implanted.

We have amended the method section accordingly. Now, the implantation of the electrode occurs at the very end. These steps have been re configured as follows:

2.15 Use a stereotaxic or hand drill, (1.00 mm, ~20,000 r.p.m), drill a 1.5 mm diameter hole 2.5 mm anterior to bregma and 3.5mm lateral to bregma. Partially (about halfway, until it is firmly in place) implant a screw (1.59 mm O.D., 3.2 mm long) in this hole.

2.16 For the reference electrode, drill a 1.0 mm diameter hole 1.5 mm anterior to bregma and 3.5 mm lateral from bregma (in the left hemisphere), anterior to bregma and in the left hemisphere.

2.17 By hand, insert ~2mm of reference wire into this hole, while wrapping the reference wire around and under the head of the screw previously implanted.

2.18 Fully implant the screw, pinning down the reference electrode in place.

2.19 In the right hemisphere, drill a 1.5 mm diameter hole at 1.2 mm anterior and 1.4 mm lateral to Bregma.

2.20 Gently remove the dura, using tweezers.

2.21 For the stimulating electrode, drill a square hole (2 mm anterior-posterior, 5 mm medial-lateral) centered at 5.2 mm posterior and 1.0 mm lateral to Bregma.

2.22 Using the stereotactic arm bars, lower the bipolar stimulating electrode/guide cannula 5 mm below dura. There may be bleeding during the implantation of the electrode. Utilize sterile Q-tips and gauze to minimize bleeding.

*Please check on the cycling parameters you are using/proposing in the protocol. I suggest take a look at (Rodeberg et al. 2017 https://www.ncbi.nlm.nih.gov/pubmed/28127962). It is well known that "the required time to reach equilibrium differs across electrodes and implantations" However, most of the protocols about cycling suggest a minimum of ~15 min at 60 Hz followed for ~10 min at 10 Hz before starting recording.

We have amended the method section accordingly, and included the reference you suggested:

2.17 Apply a triangular wave form (-0.4 to 1.3V, 400V/s) for 15 minutes at 60 Hz, and again for 10 minutes for 10 Hz. Typically, when applying waveforms to carbon fiber microelectrodes in the brain, oxide groups are added to the surface of the carbon fiber. Equilibrium of this reaction must be reached prior to recording, otherwise significant drift will occur[21]. Cycling the electrode at higher frequencies (60Hz) allows the carbon fiber to achieve equilibrium faster.

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*I appreciate the note in 2.17. about bleeding since that is something very frequent.

3. The note in 3.2. must be clarified. Is it something always expected? If so, you must add a reference. If not but, that is something you observed very frequently, please make it clear. During surgery, there are some "signs" that are not common for every animal. Because that note is part of the protocol, beginners can expect that "sign" as an indicator of a "good placement" and decide based on that which is not necessarily right.

Thank you for this suggestion—we have clarified this section according to your recommendation.

3.2 Gently lower the stimulator in increments of 0.2mm from 5mm below dura to 7.8mm. At each increment, stimulate the brain.

Note: At more dorsal depths (5-6mm), stimulation of the brain will typically (~80% of the time) cause the whiskers of the rat to twitch. At further depths, the whiskers will cease twitching, which occurs between 7.5-8.2 mm below dura. When the whiskers cease twitching, the stimulating electrode will be near or at the VTA. This will not occur in every rat, and lack of whisker twitching should not be taken as a sign that the bipolar stimulating electrode/ infusion cannula is misplaced. Whisker twitching may not occur for all anesthetics (e.g., isoflurane).

4. Infusion after stable baseline (4.2.) requires some additional steps. When lowering infusion cannula, even gently, some damage can be produced in local VTA neurons that have been

stimulated, leading to some variation in the evoked DA release signal. As mentioned before, those changes can be observed in some animals and not in others. However, it implies to make, at least, two or more additional stimulations to check if the signal was affected for the cannula. If no changes are observed, infusion (4.3.) can be started. However, when DA signal changes, a new baseline (same parameters: no more than 20% variation in at least the 3 last stimulations) should be established. Otherwise, any apparent change after infusion will not be reliable.

*Also, before starting an experiment, it is necessary to infuse one animal with an innocuous solution. This is because, similarly that cannula entrance, infusion itself can produce changes in DA signal, changes that are not expected after the innocuous solution. If changes are observed, maybe the rate of infusion (speed/time) needs to be modified accordingly.

*Post-infusion (4.4) step suggests leaving the internal cannula for 1 minute. Depending on the drug kinetics, adsorption must require more than just 1 minute, and removal can produce the drug to traveling up through the needle track. If not additional infusions are planned, I suggest just leave at place the cannula for the rest of the experiment to avoid any change in the local environment around the *infusion* spot that could lead to changes in DA signal.

We thank you for this technical suggestion, and have incorporated your feedback into the manuscript accordingly. As a side note, we do not typically record after cannula insertion, mainly because we see little change in evoked release after infusion of vehicle. However, you are raising the point that if it is a problem for some, so it should be part of this manuscript as best practices. Please find the amended sections below:

4.2 After achieving a stable baseline (<20% variation over 5 stimulations), gently lower the internal cannula by hand into the guide cannula that is pre-fitted into the bipolar stimulator.

4.3 Take an additional 2-3 baseline recordings to ensure that the cannula insertion itself did not cause a change in the evoked signal. In some cases, insertion and removal of the internal cannula can cause damage to the VTA. If the signal drastically changes over this baseline period (>20%), then take an additional 3-4 recordings until the baseline re-stabilizes

4.5 Post-infusion, leave the internal cannula for at least 1 minute prior to removal. Some drugs may require leaving the internal cannula for longer based on the drug kinetics, and removal of the internal may cause the drug to travel back up through the internal. If there is concern, one could leave the internal cannula in the guide cannula during the entirety of the recording. Otherwise, recording can begin after this 1-minute interval.

5. For historical verification (5), please also include the VTA.

This suggestion has been incorporated accordingly.

Other comments

RESULTS

1. The section would benefit from an experimental outline explaining the order and time for each step after carbon fiber/stimulation electrode optimization.

Thank you for this suggestion. We have now added a new figure to explain this timeline. This will be the new Figure 1.

2. Please think about the option to combine figures according to its pharmacological targets (saline, NMDA and ACh drugs). Also, when possible use the same Y-axis scale for IvT and color plots, at least for each pharmacological target.

Thank you for this suggestion. We have combined figures per your suggestion. Figure 3 combined NMDA and AP-5 infusion while Figure 4 combines MEC and SCOP infusion.

3. Figure legends require more work. Please include the number of animals (N) for each drug, the nature of the drug (agonist/antagonist) and the time (in minutes) when the baseline was recorded before the drug infusion. Also, because your results are showing changes in DA signal after drug infusion, including the percentage of change, compared with baseline.

Thank you for these suggestions to clarify the figure legends. We are only reporting 1 animal per condition, and this information has been clarified in each figure legend. We also included in the figure legends the nature of the drug, as well as identifying at which time points for baseline and post-infusion illustrated by the figure images.

4. Please include the rationale for using agonist/antagonist for NMDA receptors and just antagonist for Ach receptors

We have included this rationale in the following section:

Representative Results Paragraph 2
To demonstrate that CIS-FSCV can produce bidirectional effects when using agonists and antagonists, we compared the effects of infusion of the NMDAR agonist, NMDA (500ng; Figure 3A) to the NMDAR antagonist, AP5 (1µg; Figure 3B). Infusion of NMDAproduced a robust increase in stimulated phasic DA release (Figure 3A, 9 minutes after infusion) while the NMDAR competitive antagonist, AP5 (1 µg), produced a robust decrease (Figure 3B, 9 minutes after infusion). To demonstrate the utility of CIS-FSCV using antagonists that target different classes of acetylcholine receptors, we compared the effects of infusion of the

nonselective, noncompetitive nAChR antagonist mecamylamine (3 µg; Figure 4A) and the nonselective, competitive mAChR antagonist scopolamine (67 µg; Figure 4B). Both drugs produced robust decreases in stimulated phasic DA release (Figure 4, 9 minutes post-infusion).

5. If you did do recordings after introducing the canula and before the infusion, please include a color plot and IvT as an additional figure showing any change (or no changes) in the DA signal and, what must be done in both cases. It will complement the suggestions mentioned in the protocol (4).

Unfortunately, we did not do this recording. We do not typically see changes as a result of internal implantation, but we have incorporated your suggestions regarding ensuring that the internal does not cause changes to evoked signals as well as the steps needed to address this issue should it occur (see discussion responses below)

DISCUSSION

1. In line 366 is mentioned that, as a result of pressure while inserting the internal cannula (protocol 4 and comment 4) the dopamine signal might artificially increase or decrease, as the stimulation site may be different. While this critical step is mentioned in the discussion, no suggestions are included to fix or handle this situation. Please add the potential options mentioned before in the comments.

Thank you for this suggestion—we have incorporated these troubleshooting tips accordingly:

Discussion Paragraph 2
Should there be a significant change in evoked signal after cannula insertion, a new baseline period should be established. Moreover, if there is altered DA released caused by inserting the internal cannula or vehicle infusion, the signal typically recovers to baseline within 30 minutes. Should there be extensive alterations in DA release to vehicle infusion, the infusion rate or volume could be reduced. Investigators may also perform an additional recording after inserting the internal cannula, but before the infusion to assess whether insertion of the cannula itself can alter release

2. Because FSCV is also used for measuring different analytes like 5-HT or Norepinephrine, CIS-FSCV could potentially be used for the same purposes as presented in this paper/protocol. Please add these additional possibilities to the discussion.

This suggestion has been incorporated below.

Discussion Paragraph 3
CIS-FSCV can also be modified to measure 5-HT and norepinephrine[8, 9].

1. Cheer, J F, K M Wassum, M L Heien, et al., *Cannabinoids enhance subsecond dopamine release in the nucleus accumbens of awake rats.* J Neurosci, 2004. 24(18): p. 4393-400.
2. John, C E and S R Jones, *Fast Scan Cyclic Voltammetry of Dopamine and Serotonin in Mouse Brain Slices*, in *Electrochemical Methods for Neuroscience*, A.C. Michael and L.M. Borland, Editors. 2007: Boca Raton (FL).
3. Hermans, A and R M Wightman, *Conical tungsten tips as substrates for the preparation of ultramicroelectrodes.* Langmuir, 2006. 22(25): p. 10348-53.
4. Espana, R A, J R Melchior, D C Roberts, et al., *Hypocretin 1/orexin A in the ventral tegmental area enhances dopamine responses to cocaine and promotes cocaine self-administration.* Psychopharmacology (Berl), 2011. 214(2): p. 415-26.
5. Rodeberg, N T, S G Sandberg, J A Johnson, et al., *Hitchhiker's Guide to Voltammetry: Acute and Chronic Electrodes for in Vivo Fast-Scan Cyclic Voltammetry.* ACS Chem Neurosci, 2017. 8(2): p. 221-234.
6. Sun, F, J Zeng, M Jing, et al., *A Genetically Encoded Fluorescent Sensor Enables Rapid and Specific Detection of Dopamine in Flies, Fish, and Mice.* Cell, 2018. 174(2): p. 481-496 e19.
7. Robinson, D L, A Hermans, A T Seipel, et al., *Monitoring rapid chemical communication in the brain.* Chem Rev, 2008. 108(7): p. 2554-84.
8. Jackson, B P, S M Dietz, and R M Wightman, *Fast-scan cyclic voltammetry of 5-hydroxytryptamine.* Anal Chem, 1995. 67(6): p. 1115-20.
9. Park, J, P Takmakov, and R M Wightman, *In vivo comparison of norepinephrine and dopamine release in rat brain by simultaneous measurements with fast-scan cyclic voltammetry.* J Neurochem, 2011. 119(5): p. 932-44.

Wickham Bio

Dr. Wickham is a behavioral neuroscientist with research interests in the brain mechanisms underlying drug relapse and the role of menthol in nicotine addiction. He received his Ph.D. from Yale University, where he worked with Dr. Nii Addy. There, Dr. Wickham investigated the role of midbrain nicotine receptors in driving drug relapse behaviors as well as how adding flavorants (e.g., candy or menthol) to tobacco products play a role in nicotine consumption. Dr. Wickham is currently and Assistant Professor of Psychology and Neuroscience at Elizabethtown College.

Mitchell Bio

Ms. Mitchell is a senior psychology major with a broad interest in many fields such as neuroscience, neuropharmacology and preclincal/clinical trials of pharmaceuticals, where she is aspiring to have an exciting career.

Lehr Bio

Ms. Lehr is a senior psychology major with a broad interest in clinical psychology and neuroscience. Ms. Lehr is hoping to acquire a PhD in Clinical Psychology and serve as a clinican-scholar.

Addy Bio

Dr. Addy is a behavioral neuroscientist with research interests in the brain mechanisms underlying drug relapse and reward mechanisms broadly. He received his Ph.D. from Yale University and trained in Dr. Mark Wightman's lab at the University of North Carolina- Chapel Hill. Dr. Addy is an expert in developing novel methods to investigating dopaminergic functions in behavior, as well as broadly understanding the role of dopamine in reward-related behavior. He is currently an Associate Professor of Psychiatry and Cellular and Molecular Physiology at Yale School of Medicine.