Persistent Electrophysiological Identity of Neurons in the Nucleus Accumbens Shell

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#### Abstract

Electrophysiology is an important technique using chronically-implanted electrode arrays, which allow for recording of neural activity *in vivo* during behavioral tasks that require cue and reward associations. Recordings are used to study how patterns of neural activity encode behaviorally-relevant information and how the disruption of this encoding can occur following cocaine experience. Behavioral testing and acute manipulations (such as chemogenetics) of associative behaviors can take place over several days. To precisely assess the progress of neural encoding over time, it is important to identify single unit neurons across multiple recording sessions. Here, we examined neurons in the nucleus accumbens (NAc) shell of Sprague-Dawley rats during a Pavlovian first-order conditioning task. To establish whether neurons are identifiable across multiple sessions, cells were identified using multiple metrics. These included principal component analysis, waveform voltage patterns, inter-spike interval patterns, and autocorrelation. We demonstrate that cells show specific, regular features across each metric and could therefore be identified across multiple sessions. Next, we analyzed the associative learning-related encoding of an individual neuron under two conditions: CNO-induced inhibition of hM4Di-expressing DREADDs in the shell on one day, and a control saline injection on the other. To demonstrate the efficacy of this approach, we us as an example a single unit that was determined to be the same across CNO and saline conditions by waveform and autocorrelogram analysis. We found that this cell exhibited differential firing patterns under each condition, indicating variable selectivity of cue encoding and motivated approaches to cues and foodcups.

## Introduction

Electrophysiology has been an irreplaceable practice that has influenced the study of any electrically excitable tissue system. It has been a pillar of neuroscience in particular ever since Otto Loewi electrically stimulated acetylcholine release in frog hearts, revealing that synaptic signaling was primarily bioelectrical. It has since become a staple of modern neuroscientific research and manages to evolve with the demands of ever more complex research questions. *In vivo* electrical recordings have provided unmatched temporal and spatial resolution in the study of the brain's bioelectrical encoding of complex behavior. Recording from a large population of neurons over time has yielded important clues into the aberrant nature of many brain pathologies and processes. The ability to record changes in electrophysiological properties of populations across multiple days is important in order to hone in on the potential neural mechanisms that underlie brain pathologies.

Unfortunately, this temporal specificity is not available to long term studies of discrete neurons. A caveat in electrophysiological research is the field's resistance to the claim that recording arrays may hold and record from the same neuron, or unit, across multiple sessions. The ability to do so would increase the field's understanding of complex factors that evoke changes in electrophysiological properties in response to manipulation or behavioral training. The specific roles of important brain regions could eventually be determined and their causal roles in mediating learning processes clarified. An example of how this limitation affects current research is the present parent experiment.

Here, we selectively inhibit nucleus accumbens (NAc) shell activity using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) while Sprague Dawley rats perform an associative conditioning task. Inhibitory (Gi) DREADDs are chemogenetically modified signaling proteins that respond to the presence of a specific binding molecule. As DREADDs are encoded in virally-transmitted DNA plasmids, which include particular sequences that ensure expression in a specific cell type, their action may be studied in brain regions of interest. Once bound, the ligand prompts the DREADD to acquire an active conformation, inducing signaling cascades that affect the generation of an action potential at the cell body, as is the case with the hM4Di DREADD (Roth, 2016). Such hM4Di-induced inhibitions are readily observed with electrophysiological recording arrays (Chang et al., 2015).

The hM4Di DREADD binds clozapine-n-oxide (CNO), a metabolite of the antipsychotic drug clozapine. The relative metabolites of CNO are functionally inert to all relevant nervous system targets (Whissel et al., 2016). CNO clears the system in a matter of hours, so its presence only activates the associated DREADD within a fixed time interval. As a result, it is possible to compare neural encoding under CNO and non-CNO (saline) treatments within the same animal. While the modulation of NAc activity induced by cocaine may be relatively persistent, hM4Di-induced inhibition of neurons is reversible, as cells can revert to normal function within a matter of hours.

Animals in this experiment will perform the conditioning task under CNO-induced inhibition of the shell on one day and a non-CNO (saline) condition on the other. This design allows for electrophysiological recordings to be taken from the same animal under distinct treatment conditions, enabling animals to serve as their own controls. Recordings will both confirm CNO inhibition and assess changes in shell encoding over the course of task training. Recordings from the same affected neuron across sessions would provide a level of explanation unachievable with network data or data from different neurons each session. The goal of the present thesis is therefore to exploit the electrophysiological properties of units in order to establish a reliable metric for identifying and maintaining them across recording sessions. Using the parent study as a proxy for this electrophysiological analysis will enhance our understanding of nuanced alterations in NAc shell associative encoding during CNO-induced shell ablation. Here, we carry out electrophysiological recordings in order to identify and verify the continued identity of neural units in the shell during a conditioning task using both cocaine-experienced and naïve animals. Multiple metrics will assist in the identification of units across days. Electrophysiological properties of neurons, such as their waveform amplitude and shape (Chang et al., 2015), are sources for comparison and characterization. Autocorrelogram, principal component analysis (PCA), and inter-spike interval (ISI) histograms are further metrics to distinguish and establish the continued identity of recorded units.

Studies involving DREADDs have been particularly useful in characterizing functional heterogeneity of important brain nuclei implicated in reward encoding and reward seeking behaviors. The rostral and caudal ventral pallidum projections to the VTA seem necessary for cue and drug-induced reinstatement of cocaine-seeking, respectively. This was determined after CNO-induced inhibition of these projections actively blocked the reinstatement (Mahler et al., 2014). Furthermore, Chang et al. (2015) have shown that DREADD-induced inhibition of the ventral pallidum impairs the acquisition of sign-tracking during a conditioning task.

The present experiment seeks to analyze the effects of CNO-mediated inhibition on network encoding over time. A necessary first step toward this goal is establishing the discrete effects on individual neurons. Previous literature is scarce in its appreciation for this need. For example, Mahler et al. (2014) establish the effects of DREADD inhibition in single units in isolated *in vitro* slices, but not across multiple sessions or in the same unit. Furthermore, *in vivo* electrophysiological analysis was geared toward confirming inhibition prior to task training rather than studying unit dynamics over time in inhibited and control conditions. Chang et al. (2015) recorded across multiple sessions of the conditioning task but advanced electrodes in 40  $\mu$ m increments prior to each session to record from new units. This electrode advancement strategy was also used by Setlow et al. (2003).

The need for long term single unit analysis spawns from open questions that remain in the reward system literature. In particular, there is broad understanding that drugs of abuse, including cocaine, bring about deficits in associative learning that diminish an organism's ability to flexibly alter behavior (Calu et al., 2007; Saddoris & Carelli, 2014; Saddoris et al., 2016; Takahashi et al., 2007). Animal models of cocaine addiction have demonstrated that these neural and behavioral manipulations persist well into abstinence (Cornish & Kalivas, 2000; Saddoris et al., 2016). However, it is unclear how cocaine brings about these cognitive deficits.

Learning processes underlying motivated behaviors may evoke hedonic "liking" responses as well as motivational "wanting" responses. For example, pharmacological blockage of dopamine (DA) release into the nucleus accumbens (NAc) severely inhibits animals' approach to sucrose reward (wanting response), but not their consumption of sucrose (liking response) (Berridge & Robinson, 1998; Ikemoto & Panksepp, 1996). Activity within the NAc, or ventral striatum, thus seems to play a larger role in encoding the motivational aspect of reward (Ambroggi et al., 2008; Berridge & Robinson, 2003; Flagel et al., 2011). The NAc is further thought to prompt behavioral output geared at obtaining

reward following presentation of a reward-predictive cue (Setlow et al, 2003), which is consistent with the characterization of the NAc as a limbic-motor interface (Mogenson et al., 1980). It is therefore unsurprising that cocaine-induced changes in NAc encoding modulate the motivation for drug-seeking behaviors, even when the drug stops evoking a pleasurable response.

While the NAc has been consistently tied to the motivation of reward-directed behavior, dopaminergic projections from the ventral tegmental area (VTA) are likely required for making the behaviorally-meaningful association between cue and reward. Cocaine also manipulates VTA DA signals, facilitating rapid habitual responses to drug-associated cues (Saddoris et al., 2016). Cocaine abuse is implicated in dysfunctional reward-learning by blocking the reuptake of DA, thus increasing its extracellular concentration. DA is an effective modulator of medium spiny neuron (MSN) activity and fluctuations in basal levels of DA drive abnormal neural signaling and encoding (MacAskill et al., 2014; Smith et al., 2013). The NAc is composed of 95% MSNs (Smith et al., 2013) and is divided into core and shell sub regions, each of which works differently to update reward associations. Without proper NAc performance, animals lose the ability to effectively respond to motivating cues (Ambroggi et al., 2011; McDannald et al., 2013).

Research focused on neural systems that encode information about motivation and associative learning frequently employs chronically-implanted electrode arrays to record from neurons during motivated behavioral training in awake and behaving animals. Such studies have implicated important brain regions like the NAc in specific phases of reward processing. For example, electrophysiological studies of rats that learn the correlation between an odor and water reward show progressively greater NAc neural encoding of the odor (Setlow et al., 2003).

Simultaneously, chronic cocaine self-administration impairs an animal's ability to encode first-order and second-order cues associated with non-drug related rewards (Saddoris & Carelli, 2014). The NAc therefore encodes cues, rewards, and cue-reward associations. Consequently, its functionality is necessary in learning a Pavlovian first-order conditioning (FOC) task. During FOC, a stimulus such as a light or tone (CS+) temporally precedes the delivery of food reward. Control animals learn this association aptly and approach the site of reward delivery, or foodcup, upon CS+ presentation. Second-order conditioning (SOC) occurs in the absence of reward. A second stimulus (SOC+) temporally precedes the CS+. So, an animal must perform a higher-order cognitive task and use the established value of a CS+ to respond to an SOC+. Control animals will approach the foodcup upon effective SOC-FOC association (Saddoris & Carelli, 2014).

Cocaine experienced rats, while able to learn FOC, fail to learn SOC (Saddoris & Carelli, 2014). It is puzzling that electrophysiological recordings of phasic neurons, which fire or are inhibited in response to a behaviorally meaningful event, show normal encoding of reward in NAc neurons of cocaine-experienced rats. This suggests that cocaine-experienced rats maintain a certain level of associative learning and reward encoding, but that higher order cognitive associations between cues is diminished due to some encoding deficit during FOC.

NAC shell neurons of cocaine-experienced rats fail to increase encoding patterns in response to cues compared to controls (Saddoris & Carelli, 2014; Saddoris et al., 2016). Control groups express progressively greater numbers of cue-encoding neurons while cocaine-experienced rats do not. In control animals, shell cue-encoding is positively correlated with behavior. This relationship disappears in cocaine-experienced groups. Furthermore, cocaine-experienced animals become biased towards physical interactions with first-order cues, a behavior known as "sign-tracking" (Flagel et al., 2011), rather than attending reward receptacles (Saddoris et al., 2016). This is somewhat paradoxical, as cocaine-experienced rats seem motivated towards reward seeking but lack the neural means to maintain complex reward associations.

While cocaine-associated loss of NAc shell task encoding strongly parallels these learning deficits, a causal relationship has not yet been shown. Cocaine alters shell DA levels as well as shell cue encoding. It remains unclear whether a loss of shell activity, brought about by changes in DA, underlies these associative learning impairments. The literature would benefit from the electrophysiological study of controllable NAc shell inhibition during the acquisition of FOC learning. This would reveal the connection between cocaine, DA, and encoding, revealing whether disruptions in encoding are sufficient to induce poor FOC task performance. If so, then this result would serve as a proxy to implicate cocaine-induced modulation of DA in the encoding deficits responsible for poor task performance.

The above literature suggests that DREADD activity can block acquisition, as well as maintenance, of behavior-related neural activity without damaging regions of interest. However, they lack the power of depicting the mechanism by which CNO interferes with encoding because they do not record from single units more than once. Techniques that identify and verify CNO-affected units, like waveform analysis, can be extended to study the same units over time and across treatment conditions. Given the success of the above studies, and the complex nature of cocaine-DA interactions in the shell, it is likely that assessing shell encoding during FOC training under CNO and saline conditions will specify the role of unit

encoding in associative learning. A reasonable path of investigation would seek to study inhibited shell activity under CNO and saline conditions across FOC sessions and determine if the associative learning deficits resemble those that occur following cocaine experience. Such information would clarify whether cocaine induces poor behavioral performance by way of ablating shell neural encoding. It is necessary to study the effects of treatment on a single unit over time so that nuanced changes in encoding and firing patterns may be observed. This would specify the role CNO plays in altering a region's encoding with more precision and clarity than recording from different units each session. Specific changes in encoding and firing can be identified and used to explain both loss of associative encoding and, ultimately, behavior.

## Methods

#### **Contribution and Study Site**

Research was conducted in the Saddoris Lab at the University of Colorado, Boulder. Note that surgery and behavior were run in cooperation with graduate students and that some data referenced in this thesis was analyzed and supplied by the Principal Investigator (P.I.). My role was to assist in behavioral training sessions, surgeries, and to collect and analyze electrophysiological data.

## Animals

Sprague-Dawley rats were used for this experiment. Rats were individually housed in standard home cages on a 12 h light/dark cycle with ad libitum access to food and water. The animals habituated to this environment for seven days prior to behavioral testing. Rats were

water deprived (20-25 ml/day) for the duration of the self-administration period and food deprived (15 g chow/day) for the duration of Pavlovian First-Order Conditioning (FOC). Testing was carried out according to guidelines set by the University of Colorado Institutional Animal Care and Use Committee.

#### Surgery

Rats were anesthetized (2% isoflurane) for all surgical procedures, which were performed aseptically. To prepare for the Pavlovian conditioning sessions, the scalp was opened, retracted, and the head leveled. Infusion holes were drilled 1.8 mm anterior and .8 mm bilateral to bregma (Paxinos and Watson, 2014). To induce hM4Di expression in the nucleus accumbens (NAc) shell, a Hamilton Microliter Syringe delivered 0.6  $\mu$ l infusions of AAV8hSyn-hM4Di(Gi)-mcherry (UNC Vector Core) virus into to the shell at a depth of 6.2 mm. The syringe was left at this depth for 10 minutes post-infusion to facilitate virus diffusion.

In a later surgery, animals were fitted with electrode recording arrays. Surgery procedures were the same as those described above. Two eight-wire arrays, consisting of parallel rows of four Teflon-coated stainless steel wires (50 µm diameter; 500 µm tip separation; 1x1 mm span; NM Labs, Denison, TX) were implanted bilaterally into the NAc shell of either hemisphere. The coordinates from the previous surgery were used for this implantation. A ground wire was also placed in each hemisphere distal to the shell. Dental acrylic was fixed to screws attached to the skull to secure the array. Animals were given the antibiotic Baytril (2 mg/kg) and the analgesic Meloxicam (1 mg/kg) immediately following all surgeries and were allowed to recover for seven days before beginning either self-administration or Pavlovian conditioning.

## **Behavior**

**Pavlovian conditioning.** Following a seven day post-operative rest period, animals were trained on a modified first-order Pavlovian conditioning task (FOC). The Pavlovian training chambers were discernable from the self-administration chambers. A food cup was situated in the center of the rightmost wall, with lights and retracted lever ports flanking the food cup on either side. A center LED was situated above the food cup.

During normal FOC, animals are exposed to two visually discernible light stimuli, a CS+ and CS-. The CS+ occurs to the left of the food cup and the CS- occurs on the right. On 12 out of 14 days, the CS+ is reinforced by the delivery of 3 sucrose pellets (45 mg; Purina TestDiet) whereas the CS- is never reinforced. After 10 days, animals reliably approach the food cup after presentation of the CS+ but not the CS-.

To take advantage of the power a within subject experiment brings, we aimed to test every animal in both of two conditions. In one condition, we injected animals with clozapinen-oxide (CNO; 3 mg/kg) and in the other, animals received saline injections (.9%). All animals received these injections in the same order of pseudo randomly assigned days throughout the FOC paradigm.

For this experiment, we counterbalanced the types of stimuli animals experienced during two FOC cases and animals received distinct pairs of stimuli (CS+ and CS-) between CNO days and saline days. An animal's injection for a given day (CNO or saline) would determine the case to be run for that day. For half the animals, CNO days were Case 1 days and saline days were Case 2 days (Table 1). For example, rats in the Case 1 group received a steady-illuminated panel light on the right side of the chamber for the food-associated CS+ and a flashing multicolor three-LED panel centrally located over the foodcup for the CS- on days in which rats received CNO injections. In contrast, the same rats received a flashing panel light on the left side as the CS+ and a rotating three-LED over the foodcup as the CS- on saline injection days. Thus, each rat learned about one pair of stimuli exclusively under CNO conditions (e.g. Case 1) and another pair of stimuli conditions under control saline conditions (e.g. Case 2). Animals underwent twenty days of FOC training in total (n=10 each, Case 1 and Case 2).

## Electrophysiology

**Recording.** Rats were connected to a harness through two eight-channel Omnetics connectors which were attached to a unity-gain headstage (Plexon Inc). A recording cable connected to this interface on one end and to a commutator at the other (Crist) allowing the rat free range of motion. Recordings took place during Pavlovian FOC training. Plexon software was used to record and store data (Plexon Inc) while spikes were sorted according to principal component analysis (PCA) on waveforms. The Offline Sorter software (Plexon Inc) was used to perform data analysis. Electrical events were recorded into Plexon software, thereafter sorted and displayed according to PCA in Offline Sorter.

Unit identification followed a three step process. In steps one and two, units were distinguished from noise and from each other. Unit clusters were selected according to their distance from the origin and distinguishability from unsorted clusters generated by noise (Figure 2A, B). Once a candidate unit was selected, its waveform was examined for properties that typically indicate voltage changes resulting from neuron action potentials which would serve to verify the unit's identity. These properties include the amplitude of the waveform in the y-axis region < 0 (peak), the refractory period in the y-axis region > 0 (trough), and the

overall width of the waveform. These properties were further compared against those waveforms generated by unsorted noise (Figure 3C, D top). Inter-spike interval (ISI) patterns were also examined (Figure 3C, D bottom). Units with distinct histogram shapes were classified apart, and distinguished from noise by the absence of event counts near the y-axis origin.

Correlograms (Figure 4A, B) were examined to further distinguish putative units from surrounding noise and to more specifically characterize units. Autocorrelograms were examined for features that typify neuronal activity, such as a marked trough period that approached 0 on the y-axis, indicating a refractory period. These were compared against other units and noise via cross correlogram analysis (Figure 4A, B top and middle). Cross correlograms that lacked substantial refractory periods indicated the distinctiveness of the compared units. Units were also required to show a sufficiently large difference between max and min y-axis values.

In step three of this process, a single unit was analyzed in order to test a metric for establishing its persistent identity across multiple recording sessions. To do this, a similar analysis was performed as outlined in steps one and two. First, the waveforms of the unit in question (13a) were compared against each other for both treatment days (Figure 5A). Second, the waveform from this unit was compared to the waveforms from all other units recorded during the saline treatment (Figure 5B). Third, 13a's normalized autocorrelograms from each treatment were compared to each other (Figure 5C). Finally, the normalized autocorrelograms from all units collected on the saline day were compared with 13a (Figure 5D). Autocorrelograms were normalized by dividing the difference in maximum and minimum y-axis value by the original value for a certain time bin.

**Neural analysis.** Recorded units were analyzed in relation to behavioral events on NeuroExplorer software (Nex Technologies). Perievent raster plots and histograms were first generated in order to assess the firing frequency of 13a during behavioral events of interest (cue and reward presentation, Figure 6A). Perievent raster plots were also examined for neural properties distinct from noise. 13a was examined for excitatory or inhibitory firing by comparing spike events 10 seconds pre and post behavioral event (baseline for an event) to spike events during event presentation. 13a firing frequencies during these events were then compared to one another across treatment conditions to assess the effects on CNO inhibition on event encoding (Figure 6B-G). Then, event encoding was analyzed in comparison to baseline firing rates over the entirety of the session (the cumulative time for which no behavioral events occurred). This comparison was used to determine the effects of CNO inhibition on encoding during an animal's entry into event zones (the foodcup or location of the cue) relative to baseline (Figure 7A-F). Finally, firing rates during session baseline and per event were compared across treatment conditions (Figure 8A-C).

#### Histology

Following termination of Pavlovian conditioning, animals were sacrificed, perfused, and their brains collected for confirmation of electrode placement and DREADD expression. Brains were stored in a sucrose solution for at least 24 h prior to freezing in -80C in isopentane, after which they were sliced in 30 µm increments in a cryostat (-20C) and mounted to microscope slides. A brightfield microscope was used to confirm electrode placement (Figure 1).

## Results

Histological verification of the recording site from a single animal confirmed successful electrode implantation in the NAc shell (Figure 1). PCA identified four recognizable clusters during both saline and CNO conditions on FOC day 9 (Figure 2A, B). Three units from the channel were selected during the saline condition (Figure 2A) and two units during the CNO condition (Figure 2B). The remainder of clusters were classified as unsorted noise. Unit cluster waveforms were examined and compared against unsorted. Each of the three units' waveforms from the saline day were distinct enough from unsorted waveforms to constitute neurons (Figure 3A, C). Two units from the CNO day were identified apart from noise waveforms (Figure 3B, D).

Analysis of ISI histograms further discriminated units from noise (Figure 3C, D bottom). All ISI histograms displayed features consistent with an action potential refractory period, which was unshared by noise ISI histograms. Autocorrelogram analysis identified three distinct units on the saline day (Figure 4A) and two distinct units on the CNO day (Figure 4B). Refractory periods were clear and distinct from the maximum autocorrelogram y-axis value. Cross-correlograms further confirmed the uniqueness of identified units (Figure 4A, B). The lack of refractory periods in unit-noise cross correlograms confirmed their neural identity (Figure 4A, B top row). Furthermore, unit-unit cross correlograms were sufficiently different to distinguish units from one another (Figure 4A, B middle rows).

A single unit, 13a, was isolated for analysis to determine its persistent identity across FOC day 9. First, the unit was compared with itself across CNO and saline conditions. Waveform analysis indicated that 13a expressed similar waveform amplitude, width, and peak coordinates such that it was indeed the same unit across conditions (Figure 5A). No significant differences were discovered between the autocorrelograms on CNO and saline conditions, further confirming this finding (Figure 5C). To further distinguish 13a, its waveform was contrasted against all other units from separate channels that were recorded from this animal during FOC day 9 saline. Waveform amplitude, width, and peak values were dissimilar from all other units recorded on this day (Figure 5B). Similarly, autocorrelogram comparisons on this day revealed marked differences between 13a and all other units (Figure 5D). Unit 13a is easily discernible on both waveform and autocorrelogram metrics.

In saline conditions, 13a revealed excitatory firing patterns during CS+ and reward events (Figure 6A right). CNO effectively inhibited this excitatory firing for both CS+ and reward (Figure 6A left). Furthermore, 13a showed differential firing rates relative to the CS+ and CS- (Figure 6B). 13a was selective to the CS+ and reward delivery relative to baseline firing (Figure 6C, D), but not for the CS- (Figure 6C). These differences in firing frequency were not observed on the CNO day (Figure 6E) and all selective firing for cues and reward was attenuated by CNO (Figure 6F, G).

Saline conditions further revealed that 13a showed specific firing patterns during conditioned approach to the CS+ and reward zones relative to baseline approaches. Specifically, pre and post foodcup zone entry times showed distinct ramping activity during the CS+ and sustained inhibition during reward-related entry (Figure 7A). This firing during CS+ presentation at the foodcup was paralleled in CS+ zone approaches (Figure 7C). Firing during CS- zone approach showed a significant inhibition in the pre entry period but not post entry (Figure 7E). Such specific pre and post firing for the CS+ during foodcup zone approaches was abolished with CNO treatment (Figure 7B). Modest inhibition during reward-triggered foodcup approach was observed under CNO, but no differences occurred during the CS+ period compared to baseline (Figure 7B). Again, pre and post ramping for the CS+ was attenuated during CS+ zone approaches (Figure 7D). No encoding was observed for CS- approaches (Figure 7F).

Across most metrics, CNO induced dampening of overall unit firing (Figure 8). Significant differences in firing rate were observed during baseline for cues (Figure 8A), both cue presentations (Figure 8B), and for reward delivery (Figure 8C) but not for the post CSinterval (Figure 8C).

## Discussion

The present findings provide justification for the assumption that neurons identified by electrophysiological analysis are the same across recording days. Specifically, the electrophysiological properties that software such as Plexon's Offline Sorter use to distinguish units from unsorted noise may further be used to distinguish units from one another. Sorting by principal components adequately identifies multiple distinct unit clusters (Figure 2). Electrical properties such as the sum of waveform amplitudes, waveform peak position, and width of waveform may serve as x, y, and z coordinates in PCA and thus is a reliable demarcation criteria. However, a unit is lost on the following CNO day. Though the disappearance of this unit is difficult to account for, multiple factors may influence the appearance of a given unit. For instance, a recording session with a particularly high amount of noise may drown out the unit signal. Or, as is the case on the saline day, a unit cluster may contain a small amount of spike events (Figure 3C, unit 13c). In some cases, this may be insufficient to mark the activity as neuronal.

Additional post hoc tests are useful in specifying units. Waveform properties such as peak and trough values are not only contrasted with noise, but are used as a benchmark when determining whether a separate cluster is a unique neuron or simply an outlying feature of a greater trend in a single unit's spike firing. The unit in question, 13a, was consistently labeled as such due to the consistency of waveforms it supplied in Offline Sorter. Furthermore, these waveforms were readily distinguished from other candidate units and thus justified our examination of its changing firing patterns across treatments.

Autocorrelogram analysis is also instructive. The depth of the center refractory period, as well as the relative height of either flank, are generally thought to be unique features of a single neuron. Clear refractory periods in cross correlograms of separate unit clusters are indications that the units are the same (Figure 4A), though this relationship does not hold on the CNO day (Figure 4B). Furthermore, the shape of 13a's autocorrelogram does not deviate noticeably across CNO and saline conditions. The shape of this autocorrelogram to all other units is noticeably deviant, however (Figure 5D), and is distinct from that of 13b. In light of this consideration, 13a was consistently labeled across recording days.

The justification provided by this rough comparison of properties outlines the beginnings of a method by which the assumption of identity may evolve into a confident and well supported claim. Upon further refinement of this method, a metric for detailed comparisons of electrophysiological properties outlined in this thesis may inform future research. For instance, statistical analyses that compare differences in waveform peaks, troughs, and width could serve as a component of a program which assesses a unit's similarity to others and to "itself" across recording days. The program's output would be a statistically informed determination that would, with a certain degree of confidence, classify

units across days as same or different. Waveform recognition methods have the advantage of being able to distinguish between different types of neurons, which often generate contrasting waveforms (Nadásdy et al., 1998).

Given the nature of the within subjects design addressed in this thesis, the ability to make determinations on the identity of neurons across recording days is crucial. The informative power DREADDs supply is vested in their effects on single units. If researchers are unable to examine data from a single neuron across CNO and saline conditions, they are incapable of making specific claims about the effects of DREADD manipulations on a region's encoding. In other words, it seems as if they must analyze data from a different neuron during each recording session.

Without the analysis outlined in this thesis, researchers are left with limited ability to claim that CNO affects the same units on more than one occasion. Thus, claims about the effectiveness of DREADD manipulation on neural encoding turn out somewhat empty. On any given day, recordings may simply be from neurons that are phasic or silent by chance. More crucially, analyzing single units across days provides enormous insights into the intricacies of CNO's effects on encoding. On saline days, 13a shows robust and complex responses to cues, reward, and cue-reward associations. We can now say, confidently, that the same unit lost such nuanced encoding in the presence of CNO. Such claims are not possible when analyzing different units each session.

In order to benefit from the FOC design, we must have confidence that neurons affected by CNO can be sampled continuously. The findings of this thesis justify that claim. However, further investigation is required to strengthen the justification. Histological verification of DREADD expression localized to the shell would provide more powerful and relevant evidence for identity across recording days. However, the strategy applied here should apply to neurons regardless of their treatment condition and is nevertheless informative. For instance, initial analyses of 13a show that CNO disrupts its firing frequency during cue and reward encoding (Figure 6). Similarly, saline conditions facilitate significant differences in firing during approaches to cue and foodcup zones during relevant events as opposed to baseline approaches (Figure 7, 8). Such selective encoding for cue and reward is consistent with previous literature (Ambroggi et al., 2011; Saddoris & Carreli, 2014) and seems to disappear upon CNO treatment (Figure 6E, F).

Overall, the method for identifying and holding cells across days has enormous explanatory potential. 13a showed complex and interesting changes in firing patterns following CNO-induced inhibition. This observation on its own shows the importance of long term single cell analysis. If a hypothetical unit, 13x, had been examined on a saline day and compared to 13a on the CNO day, entirely different firing patterns may have been compared and a level of detail in understanding unit inhibition would have been lost.

The results would be fortified by analyzing greater numbers of units. Some units are more problematic to identify than others, and are difficult to disentangle from noise. Though there are drawbacks to any recording method, PCA has the challenge of teasing apart conflicting electrical information. However, for clearly defined units the method seems promising. Further post hoc statistical analysis would also support the creation of a neuronal "fingerprint". T-tests might be used to study the relatedness between unit's waveform peaks, troughs, widths, and amplitudes. This would further validate the metrics used in this thesis.

Establishing a unit's constant identity across condition days is a necessary preliminary step in making causal claims about the role of a brain region in reward processing and behavioral output. The implications of the method described here have bearing on research outside of the addiction field. I propose that this method is a potential way for electrophysiology to adapt to the demands of modern research by facilitating intricate study of network changes over time.

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# **Tables and Figures**

## Tables

| Group 1 | CNO    | Case 1 | CS1+ | Right solid     |
|---------|--------|--------|------|-----------------|
|         |        |        | CS1- | Center solid    |
|         | Saline | Case 2 | CS2+ | Left flashing   |
|         |        |        | CS2- | Center rotating |
| Group 2 | Saline | Case 1 | CS1+ | Left solid      |
|         |        |        | CS1- | Center solid    |
|         | CNO    | Case 2 | CS2+ | Right flashing  |
|         |        |        | CS2- | Center rotating |

Table 1. Behavioral design for first-order conditioning (FOC) training. Cues according to FOC Case for a given injection day. Half of animals were assigned to Group 1 and the other half to Group 2.

# Figures



Figure 1. Verification of electrode recording site in the nucleus accumbens (NAc) shell. Orange arrow indicates electrode tip. ac = anterior commissure.



Figure 2. Principal component analysis of electrical events during FOC day 9. (A) Saline condition shows four recognizable clusters, three of which are putative units. (B) CNO condition shows four recognizable clusters, two of which are putative units. Note unit cluster distances from origin and noise cluster. Red = unit 13a, blue = unit 13b, purple = unit 13c.



Figure 3. Waveform features of units identified on FOC day 9 saline/CNO. (A) Example of three units sorted on one channel during FOC day 9 saline. (B) Example of two units sorted on one channel during FOC day 9 CNO. Waveforms for FOC day 9 saline (C) and CNO (D) reported with ISI histograms (C, D bottom) and number of spikes per unit. Red = unit 13a, blue = unit 13b, purple = unit 13c.





Figure 4. Autocorrelograms are consistent across condition days. Cross (center and rightmost images on each row) and auto (leftmost images on each row) correlograms for units identified on FOC day 9 saline (A) and CNO (B).



Figure 5. Distinct unit shares waveforms and autocorrelograms across conditions but is differentiated from other units. (A) Waveform comparison of FOC day 9 CNO/saline for 13a. (B) Waveform comparison of 13a against all other identified units on FOC day 9 saline (C) Autocorrelograms for CNO/saline days (D) Autocorrelogram of 13a compared with other units on FOC day 9 saline.



Figure 6. Unit 13a changes in firing rate during CNO and saline conditions. (A) Perievent histogram (bottom) and raster plot (top) of unit 13a, FOC day 9 saline (left) and CNO (right) during cue (CS+) and reward (Rew) presentation. Selective firing for cue (CS+/-) (B, C) and Rew (D) during saline condition. (E, F, G) Lack of selective firing for cue or reward during CNO condition. \*\*p<0.01



Figure 7. CNO disrupts selective encoding of cues (CS+/-) and reward during approach to food cup and cue zones. Firing changes during saline (A) and CNO (B) conditions during approach to food cup. (C) Firing during entry of CS+ zone during saline conditions. (D) Unit firing during approach to CS+ and CS- zones during CNO conditions. (E) Unit firing during approach to CS- zone in saline condition. (F) Unit firing during approach to CS- zone in CNO conditions. Red bar = CS+ significantly different from baseline, p<0.05. Orange bar = Reward significantly different from baseline, p<0.05.



Figure 8. CNO dampens firing rate during baseline (BL), cue (CS), and reward (Rew) events. (A) CNO diminishes firing during the ten second period (BL) prior to cue onset. (B) CNO diminishes firing during presentation of both CS types. (C) CNO diminishes firing during Rew presentation. \*\*p<0.01 \*p<0.05.