NEURAL CORRELATES OF CUE-INDUCED COCAINE SEEKING IN THE NUCLEUS ACCUMBENS OF LONG-EVANS RATS.

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ABSTRACT OF THE DISSERTATION

Neural correlates of cue-induced cocaine seeking in the nucleus accumbens of long-evans rats.

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Male and female Long-Evans rats were trained to self-administer (SA) cocaine in a two-week SA experiment. Availability of cocaine was signaled by a discrete tone cue and drug administration was contingent upon a nose-poke response during the period when the tone was presented. Firing rates on trials preceding the first infusion (missed opportunities), were compared to the trial immediately leading to first self-administered infusion (first hit).

When movement-free, tone-evoked, firing rates of core and shell neurons were analyzed, it was observed that both core and shell showed robust task-related changes in firing. NAc neurons of both males and females showed differences in tone-evoked firing between all of the missed trials prior to the first infusion, vs the first hit of the day. Consistent with learning by core and shell

neurons of association between tone and cocaine, only late sessions showed the difference between missed trials and the tone immediately leading to the first self-administered infusion of the day. For males the effect of neuron subregion did not modify the neural response to “Hits” vs “Misses”, whereas for females there was a major increase for shell neurons, however only to “Missed” trials. Together these results suggest that the core is not the first and only stage of processing of limbic inputs necessary for cue-induced drug seeking in nucleus accumbens, but rather processing of those cues might involve NAc shell, which is anatomically upstream of the core.

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1. INTRODUCTION

According to the National Institute on Drug Abuse (NIDA) each year between 26 and 35 million people in the United States struggle with substance abuse. The annual cost associated with drug addiction exceeds 740 billion dollars. Those costs include, but are not limited to, costs of drug-associated crime and unemployment, as well as health care-related costs.

In 1991 33.5% of all crimes were shown to be committed under the influence of a substance (alcohol or illicit drugs), and approximately 5% under the influence of stimulants. In particular, cocaine has been tied to the abrupt increase in inner city violent crime of the 1980s and 1990s (Trainor, 2017). This has led to increased criminalization of drug use – in 2016 arrests for violent crimes constituted 25% of arrests related to drug possession. Although overall rates of cocaine overdose have decreased, there is evidence that among non-hispanic black Americans cocaine is the most common cause of drug-overdose (Shiels et al., 2017).

Over the past two decades our understanding of drug addiction has steadily increased. Drug addiction is no longer considered a moral failure, but rather a brain disorder with both: biological and environmental causes. NIDA defines drug addiction as a relapsing brain disorder, characterized by compulsive drug seeking, despite negative, harmful consequences to an individual.

Despite the fact that the focus in recent years has shifted from criminalizing drug use, to addiction treatment, treatment of drug addiction still poses major challenges. Statistics show that on average, a person entering treatment has about a 40-60% chance of relapsing (NIDA statistics), once out of treatment, back into regular drug use. Thus, in order to treat addiction effectively, it is necessary to understand neural and environmental mechanisms that are involved in relapse.

1.1 Characteristics of Addiction to Cocaine

Cocaine is an alkaloid produced from Coca leaves which are grown at high altitude in Columbian, Argentinian and the Bolivian Andes. It is primarily consumed in the US and Western Europe and can be ingested intranasally, intravenously, or smoked in its free-base form. Psychological effects of cocaine include alertness, excitement, euphoria and increased confidence. At first, the positive effects on mood are the primary incentive for drug use. A feeling of “high”, or a positive affective state, is an immediate result of taking cocaine. However, this feeling is short-lived and subsequent withdrawal is associated with a prolonged feeling of depression and anxiety (Koob & Volkow, 2010).

Drug addiction is often defined as an affective disorder (Khantzian, 1987; Cheetham, 2010). It appears that changes in affective state are a salient factor underlying a pattern of drug use. Thus, drug addiction theories often relate motivational aspects of drug use to its impact on individuals’ affective states. According to one theory, it is the positive affective states that trigger and sustain drug use. However, the positive affective states are closely followed by negative

affective states (withdrawal/depression), which often outweigh the subjective effects of the initial high (Wise, 1988; Koob 1996). Despite that, drug use is continued. Another theory proposes an opponent process through which positive affective states are followed by “compensatory” states of opposite valence (Koob, 1996). This theory states that drug use is associated with an intertwined series of ups and downs. After prolonged use, the initial positive affective states eventually fade, whereas negative states may increase in strength (Koob 1996). Thus, although initial drug use is driven by positive affect, with continued use the urge to escape from negative affect becomes the more salient motivator (Solomon, 1980).

Often initial use is intranasal, but with chronic intake, drug use escalates. (Ahmed and Koob, 2008). As tolerance builds, higher doses are required to obtain the same reinforcing effect or ‘high’ (Ambre et al., 1988). Thus, users often transition into intravenous use, or smoke free-base cocaine, which are both associated with a faster action of the drug and stronger subjective effect of the same dose. Importantly at some point a shift occurs from controlled to compulsive drug use (Ahmed & Koob, 1998). At this latter stage, the user often persists taking drugs despite severe negative consequence triggered by drug use. Although most drug users voluntarily or involuntarily experience abstinence for short periods of time, 60-90% of those users eventually relapse into drug use (Mendelson & Mello, 1996).

Reinforcing mechanisms via which cocaine affects the brain are relatively well known, but the mechanism that drives the compulsion to resume drug taking

remains less well known. Chronic relapsing is thought to be triggered by drug-associated cues, and is at the core of the disease. Thus, understanding neural correlates of persistent relapse triggered by drug-cues is a major public health challenge concerning drug addiction research (O'Brien, 1998).

1.2 Drug Abuse in Females

It is important to point out that abuse of drugs by females and males differs in a number of respects. Although females initiate drug use later than males, studies show that females transition faster into drug addiction, -marked by a faster progression from the first use to developing clinical symptoms of addiction (Hernandez-Avila et al., 2004; Lewis, et al., 2014). Despite a shorter span of drug use, females admitted into drug abuse programs typically display more severe symptoms of addiction (Longshore et al., 1993; McCance-Katz et al., 1999), and have higher rates of drug associated emergencies, such as cocaine or heroin induced cardiac arrest (Dudish et al., 1996) or alcohol induced liver failure (Ashley et al., 1977). There are also some underlying neurobiological differences between sexes. Males show greater release of dopamine in ventral striatum correlated with increased positive affect after amphetamine consumption. (Munro et al., 2006). Quantitative analysis of pre- and post-synaptic densities in male and female rodents following cocaine treatment indicate morphological sex differences in NAc structure, such that females show greater Medium Spiny Neuron (MSN) spine densities in both core and shell subregions in comparison to males (Forlano and Woolley, 2010). There is are also evidence for an effect of estrogen on D2/D3

receptor-induced G protein activation in NAc and VTA, which could underlie increased behavioral sensitization to cocaine seen in female rodents (Febo et. al, 2003).

1.3 Propensity to Relapse in Females

As shown by Griffin et al. (1989), female cocaine abusers experience more negative affect and have shorter periods of abstinence from cocaine. Also, McKay et al. (1996) found that females who relapse to cocaine report increases in negative affective states immediately prior to relapse than male drug addicts. This heightened negative affective state associated with drug withdrawal could potentially contribute to poorer treatment outcomes in abstinence-based therapies for females (Becker et al., 2017). A number of epidemiological studies showed an increased risk for female patients to drop out of treatment, which suggests a poor prognosis for maintenance of abstinence (Sayre et al., 2002; King and Canada, 2004; McCaul et al., 2001). These data suggest that females are potentially more at risk of drug craving due to depression or a stressful event (McKay et al., 1996; Miczek et al., 1996; Elman et al., 2001). In a study by Robbins et al. (1999), females but not males, reported experiencing increases in craving in response to drug associated cues. A subsequent PET study by Kilts et al. (2004) showed differences between male and female processing of drug associated cues. Cocaine craving in women was shown to be associated with less activity in amygdala, insula, orbitofrontal cortex and ventral cingulate cortex as well as greater activity in the central sulcus and widely distributed activation in frontal

cortical areas. Interestingly, Calipari and colleagues describe an estradiol-dependent mechanism in the female NAc via which, during the estrous phase, cocaine binds to dopamine transporter with greater affinity and more potently inhibits dopamine re-uptake (Calipari et al., 2017).

Together these results indicate that females are potentially at greater risk for more severe consequences of drug use, and possibly at higher risk of relapse.

1.4 Animal Models of Drug Abuse

Animal models have been crucial for understanding the mechanisms by which reinforcing effects of cocaine are achieved in the mesolimbic dopamine system. Experimental animals self-administer (SA) the same substances that humans typically self-administer. Koob and Ahmed (1998) showed that animals who undergo prolonged SA during 6-hour daily sessions markedly escalate their drug intake over days, in comparison to those who have only 1-hour daily cocaine access. Also, the pattern of SA in animals resembles that of humans, in that inter-infusion-intervals are inversely related to the administered dose (Panlilio et., al, 2003; Zimmer et al., 2011; Zimmer et al., 2013). Long daily access, compared with short access, to a drug over an extended period of time leads animals to develop symptoms resembling those of drug addiction: 1) Animals display problems with ceasing drug use – they continue to seek drugs even when drugs are not available, 2) They display very high motivation to take drugs – are willing to work harder to obtain an infusion, as shown by higher break points on progressive ratio tasks, 3) They persist in self-administering drugs despite negative consequences, such as

foot shock (Deroche-Gamonet et al., 2004). Most importantly, experimental animals show changes in their affective state in response to drugs of abuse that are similar to humans.

1.5 Effects of Cocaine on Central Nervous System

As mentioned earlier, the acute effects of cocaine on the central nervous system have been extensively studied. Cocaine is known to increase levels of the extracellular monoamine neurotransmitters: dopamine, serotonin and noradrenaline. This is achieved by blocking the dopamine, serotonin and noradrenaline transporters (DAT, SERT and NET). Although cocaine binds to all three neurotransmitter-transporters with equal affinity, its reinforcing effects have been primarily linked to increased dopamine neurotransmitter levels in the mesolimbic reward pathway (Schmidt & Pierce, 2006). Dopamine activity in the nucleus accumbens (NAc) appears to be crucial, as administration of dopamine antagonists directly into NAc blocks the reinforcing effects of stimulant drugs, and animals self-administer cocaine directly into the NAc shell (Marie et al., 2012). Lesions of the dopaminergic pathway by injecting 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle leads to cessation of cocaine SA by experimental animals (Roberts et al., 1980). It has also been demonstrated that experimental animals such as rhesus monkeys would SA a D1 dopamine receptor agonist (Kamien & Woolverton, 1989). A D1 agonist can reinstate cocaine seeking, but neither serotonin or noradrenaline reuptake blockers or receptor agonists have this ability (Schmidt & Pierce, 2006)

1.6 Crucial CNS Circuits for Cocaine Addiction

The mesolimbic dopamine pathway includes the ventral tegmental area (VTA), NAc), and medial pre-frontal cortex (mPFC). VTA projects dopamine to NAc (which is a mesolimbic component of the reward pathway) as well as GABA. MSNs - the principal neurons of the NAc - are GABA-ergic and in response to dopamine, inhibit GABA-releasing neurons of ventral pallidum. This in turn disinhibits neurons in medio-dorsal nucleus of the thalamus, which projects to the limbic prefrontal cortex (Pierce & Kumaresan, 2006). VTA also sends dopamine projections to mPFC (which is a mesocortical component of the pathway) and amygdala.(Figure a) VTA also projects GABA to hippocampus. Both amygdala and hippocampus send excitatory glutamate projections to NAc and mPFC. Some proportion of GABA-ergic MSNs in NAc project back to VTA. (Steffensen et al., 1998, Joel et al., 2000)

Overall, NAc dopamine activity is necessary for the reinforcing properties of cocaine. Considering the NAc's dense dopaminergic inputs from VTA, which converge with glutamatergic inputs from the limbic system, and its ventral pallidal outputs, the NAc fulfills necessary conditions to convey information regarding reward from limbic to motor areas (Morales and Margolis, 2017). Thus, NAc might potentially be connecting motivational signals with desired goal-directed motor output (Haber et al., 2000).

1.7 NAc Core and Shell

NAc is not a homogenous structure, and can be further subdivided into two main compartments: NAc core and NAc shell. Tract tracing studies suggest that information flowing through the compartments of the NAc is processed sequentially. Further evidence from a number of tract tracing experiments suggests an ascending projection “spiral”: via thalamocortical and mesencephalic pathways, shell projects to core, and core projects to central striatum, which then projects to dorsolateral (sensorimotor) striatum – it is theorized that these projections provide a necessary basis for a limbic-motor interface (Haber et al., 2000).

NAc sub-components, core and shell, differ in terms of their connectivity, as well as neurochemical and morphological properties (Dreyer et al., 2016). Firstly, the two components can be differentiated by distribution of calcium binding protein – calbindin. Accumbens core is more strongly immunoreactive to calbindin than shell (Groenwegen et al., 1999; Zahm, 1999), and a calbindin stain results in a darker staining core subregion of NAc (Meredith et al., 1996). Further neurochemical differences between the two regions, suggest that NAc shell is more chemically diverse than NAc core. It has been demonstrated that NAc shell possess 5-HT_4, and $dopamine_3$ receptors which are immunoreactive to calretinin, cocaine and amphetamine. Core, on the other hand, is strongly reactive to $GABA_A$. (Groenwegen et al., 1999). There are also morphological differences between the

two regions. Core MSNs (in rat, but not in mice) appear to be bigger with richer arborizations and axon collaterals than those of shell MSNs (Heimer et al., 1997).

In terms of connectivity, accumbens shell is considered to be a part of the extended amygdala. Both core and shell receive projections from basolateral amygdala (BLA), but the topology of those projections differs. Caudal BLA projects mostly to medial shell, whereas rostral BLA projects to lateral shell. In particular the parvicellular-caudal part of BLA projects to dorsomedial part of the shell, whereas the magnocellular part of caudal BLA to ventral shell (Wright, Beijer, & Groenewegen, 1996). Thus projections from BLA to NAc shell rese10mble those received by the Bed Nucleus of the Stria Terminalis or central nucleus of amygdala.(Figure b) Other inputs to NAc include ventral subiculum of the hippocampus, which preferentially innervates the medial shell (Bossert, et al., 2016).

NAc core receives less dense projections from the rostral and caudal divisions of BLA. “Patches” of NAc core receive inputs from caudal parvicellular and magnocellular BLA, in a very similar manner as dorsal striatum, i.e., caudate and putamen (Brog et al., 1993; Zahm and Heimer., 1993). Accumbens core also receives inputs from prehippocampal regions (Groenewegen et al., 1999; French and Totterdell, 2003; Voorn et al., 2004; Ito and Hayn, 2011) and shell (Van Dongen et al., 2005). Thus, whereas the shell is included in the extended amygdala, the core is considered more “striatal like”.

Core and shell project to the VTA, ventral pallidum, as well as lateral hypothalamus; their spiraling projections, however, show vastly different topology. The majority of shell projections reach subcomissural ventral pallidum, VTA, lateral hypothalamus and extended amygdala, whereas core projections target dorsolateral ventral pallidum, substantia nigra and entopeduncular nucleus (Heimer, 1991).

1.8 Functional Differences Between NAc Core and NAc Shell

The structural and neurochemical differences between the two regions are followed by differences in their respective function. The functional differences in relationship to SA have been demonstrated in a number of experimental paradigms. It has been shown that NAc shell neurons are more sensitive to stimulants than NAc core neurons (McKinzie et al., 1999,;Ikemoto et al., 1997; Di Chiara et al., 2004). Evidence to support this include the finding that increased CREB activity in shell facilitates cocaine SA (Larson et al., 2011). Intracranial infusion of D1 agonists into shell can support SA, and intravenous SA can be diminished by intracranial administration of D1 and D2 receptor antagonists into shell (Ikemoto et al., 1997). Additionally, lesions of medial shell have been linked to a decrease in amphetamine induced conditioned place preference (Sellings and Clarke, 2003). On the other hand, excitotoxic lesions of NAc core have been shown to dramatically impact drug seeking driven by conditioned reinforcers, such as drug-associated cues (Fritz et al., 2011). However, the question remains to be answered: when responding for conditioned cues is diminished by interfering with

core, it appears that core is both necessary and sufficient for cocaine cues to induce relapse; but is core the key starting point of cue processing, or is it downstream in the spiral circuit in which striatal processing of presumably limbic inputs may actually start in NAc shell?

1.9 Reinstatement Model

As mentioned previously, the immediate effects of cocaine on the CNS are well understood. However, the neural mechanisms underlying a “relapsing” nature of the disorder are unknown. A reinstatement model can be used to investigate the neural mechanisms of relapse to substance of abuse in controlled laboratory conditions (Crombag, et al. 2002). In reinstatement models, animals are taught to self-administer a drug signaled by a visual or auditory cue. Following training sessions, responding is diminished by repeated sessions of non-reinforcement, in the absence of both cue and drug. During the reinstatement test, return of drug seeking is measured as responding is reinforced by presentation of the drug associated cue. In 1980 de Wit and Stewart reported that drug priming or exposure to the drug-associated cue leads to reinstatement of previously extinguished responding for cocaine. Subsequent studies have demonstrated that SA drugs, drug-cues, or stressors can reinstate drug seeking even after prolonged period of abstinence (Crombag, et al. 2002). Thus, the same factors that trigger relapse can be used to trigger reinstatement in laboratory conditions; animal models of reinstatement are considered a valid model when predicting relapse. This lends credibility to further neural and behavioral findings implementing this model. For

example, evidence from the reinstatement model suggests that drug craving can increase throughout a period of abstinence. This has been evidenced by increases in drug seeking triggered by drug-associated cues with increasing times of abstinence – such an effect is labeled “incubation” (Grimm et al., 2001). Those findings demonstrate that incubation develops over 60-90 days and can last for several months after initial withdrawal. Thus, data suggest that, during the initial months of abstinence, an individual is increasingly susceptible to drug-associated cues, and exposure to those can easily trigger relapse. The neural basis of this incubation of susceptibility has been studied. One region possibly involved in cue induced relapse is Basolateral Amygdala (BLA). Selective inactivation of BLA and dorsomedial PFC has been shown to entirely abolish cue induced drug seeking (McLaughlin & See, 2003). Fuchs et al. 2004 have shown that inactivation of both: NAc core and NAc shell decreases context cue-induced responding for cocaine. Multiple studies have implicated NAc core as a structure specifically responsible for cue induced relapse (Fuchs et al., 2004). However, Ghitza and colleagues (2003) have shown that after several weeks of abstinence, under extinction conditions, only shell, but not core, neurons selectively responded to drug associated cues (tones). In contrast, firing patterns of core, but not shell, neurons were correlated with executing the drug-seeking response itself (Ghitza et al, 2004). This suggests that drug-association encoded within the cue persists after weeks of abstinence and could potentially drive relapse to cocaine. But it also leads to theory that both core and shell neurons might be necessary for responding

to discrete drug-associated cues. Because cocaine-associated cues were presented under extinction conditions, it remains to be studied whether NAc shell responses to the cue were driven by new learning that the cue predicts non-availability of drug, or whether their activity was driven by a maintained cocaine representation. A second issue needing study is whether NAc core neurons are the starting point of cue processing in the mesolimbic pathway that triggers responding to drug-associated cues. Clarifying these issues is important for developing therapies aimed at reducing cue-induced craving/relapse.

1.11 Summary

As suggested above, there are some issues with the particular way reinstatement designs strive to model relapse. For example, the majority of electrophysiological studies using reinstatement models have been conducted exclusively using male rats. This is a major deficit, as we do not know whether the observed effects generalize to the female population. Other issues pertain to face validity of the model. For example, stress-triggered relapse in laboratory conditions is usually operationalized via means of electric shock or administration of yohimbine (pharmacological stressor), which might not adequately model the type of persistent, low level stress that recovering addicts might typically be subject to (Epstein et al., 2006). In reinstatement models, drug associated cues have been shown to lead to drug seeking and have been linked to increases in dopamine activity in NAc (Volkow et al., 2008). This is consistent with self-reports of drug users who claim that drug-related cues such as drug paraphernalia (pipes,

needles, powder), or places (bars, alleys, intersections, etc.) can induce drug craving, which are related to increases in NAc dopamine activity (Volkow, et al. 2011). In a reinstatement model, however, drug is absent and thus subjects undergo extinction training during a test in which the drug stimulus is displayed hundreds of times without the expected reinforcement. In animal models, extinction sessions prior to the test of cued reinstatement can require up to three weeks to achieve low criterion response rates. It is important to emphasize that there are few real-world circumstances where a human drug-addict would be exposed to drug-associated cues in such a manner without drug availability. This particular aspect causes the reinstatement model, although having many advantages, to differ in important respects from true relapse.

1.12 Goals of The Current Project

The following study included two main goals. The first goal was to record neural correlates of cue-evoked drug-seeking. No study to date has looked at cue-evoked activity at the moment of cue-induced drug-seeking in a drug free state, and assess cue-evoked firing in NAc core and shell neurons with drug available. It was expected that some percent of both core and shell neurons will show modified activity in response to the drug-associated tone cue in rats trained to SA cocaine. The specific hypothesis was that, in agreement with Ghitza et al. (2003), shell neurons will show a greater change in firing, in comparison to core neurons, in response to the drug cue prior to the first infusion of the session. In addition, it was hypothesized that NAc neurons will show these changes also during the load-up

phase (10th infusion of cocaine), when positive affect is most prevalent (Barker et al., 2014).

Our second goal was to conduct the first electrophysiological study of female NAc core and shell neuron firing patterns during chronic access to cocaine SA. In particular, we expect increased changes in cue-evoked firing in female shell neurons.

Thus, we conducted a study of NAc core and shell correlates of true cue-evoked daily relapse to, or resumption of cocaine seeking/taking, which includes the first electrophysiological study of NAc core and shell in females during cocaine SA.

2. METHODS

2.1 Subjects

Male and female Long-Evans rats (Charles River, Raleigh, NC) at least six months old at surgery (14 males and 12 females) were used in this study. Prior to surgery rats underwent testing for sign tracking/goal tracking for one week; those results are undergoing analysis, and are not focus of the present study. Subjects were housed, throughout the duration of the experiment, individually in the SA chambers, and fed standard lab chow diet daily, after conclusion of all experimental procedures. Mean weight for females in this experiment was 0.266 kg(+/-0.016), whereas average male weight was 0.368 kg(+/-0.05) (Figure 1).

2.2 Surgery

Animals were initially anesthetized with ketamine xylazine (1.4 ml/kg). Anesthesia was maintained by administering ketamine hydrochloride (60 mg/kg, i.p.) whenever necessary. Glycoparylyte (10 mg/kg, i.p.) and penicillin G (75,000 U/0.25 ml, i.m.) were administered before surgery to prevent respiratory arrest and infection.

Animals were implanted with a jugular vein catheter. A rectangular array of 16 stainless steel microwires was implanted on the same day as the jugular catheter, targeting the right nucleus accumbens core and shell (0.0-2.8mm AP; 1.6-2.2mm ML; -6.6 mm DV from Bregma) according to the atlas of Paxinos and

Watson (1997). Following surgery an eight day recovery period was allowed before any experimental procedures were initiated. After surgery animals were given restricted food to maintain them at 330-350 g males, 220-250 g females, and ad libitum access to water.

2.3 Electrophysiological Data Recording

Single-unit activity was recorded approximately every other day for a period of 15 days. Neural signals from all 16 microwires were amplified at the level of the head stage using a harness with four quad-channel operational amplifiers (MB Turnkey Design, Hillsborough, NJ, USA). Each harness was connected to a fluid and electrical swivel (Plastics One Inc., Roanoke, VA, USA) through which signals were fed to a preamplifier and filter (MB Turnkey Design). The preamplifier differentially amplified the signal on each recording electrode against a ground wire implanted array that did not display a neuron. The filter then amplified and band-passed signals between 450 Hz and 10 kHz with a roll off of 1.5 dB per octave below 1 kHz and 6 dB per octave above 11 kHz. Finally, signals were digitized at a 50-kHz sampling frequency and were recorded using DATAWAVE Technologies hardware and software (Longmont, CO, USA). All signals were then stored for offline sorting and analysis. During each session, electrophysiological recordings began at the same time as the start of the self-administration session and terminated at the end of the session.

2.4 Pre-processing of Neural Data

In order to separate neural signals on each wire from background noise, neural spikes belonging to NAc core or shell neurons were played back and sorted based on their waveform shape. Waveforms were considered to belong to a single neuron if: 1) signal-to-noise ratio was less than 2 to 1; 2) no inter-spike-intervals were shorter than 1.6 ms; 3) recorded waveforms were similar in shape. All spike-sorting analyses were performed using a DataWave software. The timestamps from sorted neural signals were saved for further quantitative analysis.

2.5 Cocaine Self-Administration Sessions.

Six hour long self-administration (SA) sessions took place 7 days/week for a period of two weeks (long-access model of Ahmed & Koob, 1998). Neural data were recorded every other day (on odd sessions). Animals were plugged into a recording harness daily, so that procedures leading to the beginning of SA were kept identical every day of the experiment, regardless of neural recording. Each self-administration session began at 10:30 am, at the onset of 12/12 light/dark cycle. During the sessions, a 70 dB tone discriminative stimulus was presented for 30 seconds. A photo-cell device was mounted outside the corner of the experimental chamber. If the animal performed a nose poke response during the tone, breaking the photo-cell beam, a 7.5 second infusion of 0.24 mg of cocaine solution was administered through a jugular catheter (average 0.69 mg/kg males; 0.96 mg/kg females). After the nose poke response, registered by photocell device, the tone was immediately terminated, and a fixed interval of 40 seconds time-out

was initiated. Nose-pokes during the inter-tone intervals (ITI), although registered, had no programmed consequence. After the first 10 self-administered infusions, the fixed ITI was replaced by variable intervals of 3-6 minutes. The dose and timing were designed to produce a “hit” rate of approximately 50% of trials, based on well-established patterns in which rats “titrate” their cocaine level during SA (Zimmer et al., 2011). The session ended after 360 minutes or 80 self-administered infusions, which ever occurred first.

2.6 Abstinence.

After 14 consecutive days of SA, a period of ‘abstinence’ began. All procedures at the onset of each daily 12/12 light cycle were maintained the same: boxes were cleaned, water bottles were removed and animals were plugged into the recording harness. After the onset of the light cycle no tones were played and any nose pokes, although registered, did not result in cocaine delivery. Animals were fed daily after conclusion of experimental procedures at 4:30 pm. Abstinence lasted for 3 consecutive days after the last day of SA-training. Each day of abstinence was identical.

2.7 Drug-Seeking after 3-Day Abstinence

On the 4th day after the last SA session, a typical SA session was repeated: in the morning, animals were plugged into a recording harness and after the onset of the light cycle a normal SA session began and breaking the photocell during the tone resulted in a cocaine infusion. Sessions following 3-day abstinence were

collapsed together and analyzed with all remaining sessions, as a 15th Session of SA.

2.8 Immunohistochemistry

After the last SA session, animals were perfused with a 4% solution of para-formaldehyde. Brains were extracted and placed overnight in 4% para-formaldehyde. Subsequently, for cryoprotection, brains were placed into 30% sucrose for a period of at least 24 hours. After the 24 hour period brains were sectioned into 50 µm thick sections. Immunohistochemistry was carried out under gentle agitation using a horizontal rotator (Laboratory-Line, Fisher, Pittsburgh, PA). Free-floating sections were washed for 10 minutes three times in Phosphate Buffer (PB), and then were rinsed for 15 minutes in a 0.3% H_2O_2 solution to quench endogenous peroxidase. Sections were then washed in PB for 3 × 10 min before treatment with blocking solution (4% bovine albumin + 0.3% triton X-100 in PB) for 1h. Sections were subsequently transferred into the primary antibody solution containing anti-calbindin d28k diluted in blocking solution (Immunostar, Inc., Hudson WI) at a 1:5000 ratio for a minimum of 16h at 4°C (gently agitated and refrigerated overnight). Following incubation in the primary antibody, sections were rinsed for 3 × 10min in PB and transferred to a solution containing biotinylated secondary antibody against rabbit immunoglobulin (1:200 dilution; Vector Laboratories inc., Burlingame, CA, USA) in blocking solution for 1h at room temperature. Again, sections were rinsed in PB (3 × 10 min) prior to incubation with a solution containing avidin biotinylated horseradish peroxidase complex

(ABC kit; Vector Laboratories Inc.) for 1h. Finally, sections were rinsed in PB (3 × 10 min) before developing the peroxidase reaction with 0.05% 3,3-diaminobenzide-4 HCl (DAB; Vector laboratories Inc.) and 0.003% H_2O_2 for 15 minutes.

2.9 Estrous Cycle

Each day when data were collected, following a recording session, a sample of vaginal cells was collected using a pipette probe and placed on gelatin coated slides. Slides were stained using craysole blue and estrous cycle stage was determined on the basis of shapes and types of cells present.

3.10 Behavioral Data

Behavioral variables (latency to first response, amount of drug consumed) were analyzed separately for males and females as a function of training. For females, the effect of estrous stage on SA behavior was determined.

2.11 Video Analysis

In order to assess pure tone-evoked activity without any movement-related changes, video analysis was conducted on all Pre and Load-Up trials. Video analysis involved viewing a video of the animal's movement activity prior to the onset of each tone, as well as immediately following the onset of the tone. The onset of any tone-evoked change in behavior, as well as any distinct shift in behavior prior to, or following the onset of the tone, was noted. This movement-free period was the basis for calculating the tone-evoked and baseline FRs on

each trial. For a small proportion of trials video was not available; for those trials a fixed duration of 200 ms was used for calculation of both baseline and tone-evoked FRs, thus avoiding changes in firing related to tone-evoked movement.

2.12 Neural Data Analysis

Peri-event time histograms (PETHs) were constructed to visualize tone-evoked activity for identified core and shell neurons. PETHs were centered at the onset of the tone and created separately for tones preceding the first infusion ("Pre"), and separately for the tones from the first infusion on, until the tenth infusion ("Load-up"). Baseline firing patterns (prior to the onset of the tone for 1.0 second or as long the trial was free of tone-evoked movement) and firing following the tone onset were used in the analyses and quantified as Pre, vs Load-up.

Statistical analysis consisted of four separate (Pre trials males, Pre trials females, Load-up trials males, Load-up trials females;) generalized linear mixed models (GLMM) via penalized quasi likelihood with absolute (baseline - tone-evoked firing rates) as the dependent measure. The reason for using absolute value of a difference of baseline and tone-evoked firing was that preliminary analysis indicated that distribution of FR changes from baseline (increases vs decreases) was symmetrical (Figure 2). In addition there was a steady, linear relationship between baseline and tone-evoked FRs (Figure 3). Thus, increasing baseline FRs did not lead to increasing changes from baseline (Figures 4).

Because the distribution of NAc core and shell firing rates is positive (any neuron's firing rate cannot < 0), continuous, with a point mass at zero, a Tweedie

distribution for zero-inflated, positive, continuous data was used to model the absolute Firing Rate changes. A power index parameter for the Tweedie distribution was found for each of the four distinct models via maximum likelihood estimation. Each of the constructed models included the following three fixed factors: Subregion (core vs. shell), Session [‘Early’: sessions 1-5 vs ‘Late: sessions 7-15)] and Response (hit or miss). Individual neuron was included as a random effect. Post-hoc analyses consisted of follow-up simple effects for any statistically significant interactions and F-tests for main effects in the omnibus models. To limit the number of comparisons for all significant interactions with Subregion, effects of Response and Sessions were tested within each Subregion. The Sidak-Holm correction for multiple comparisons was used to protect against inflated Type-I error rate. Because in the presence of higher order interactions, interpretation of lower order effects can be misleading (Faraway, 2016), interpretation of the results focused mainly on highest order significant effects.

2.13 Tracking The Same Neuron over Days

Due to the fact that well trained animals may respond to the very first tone, leaving only one trial to assess Pre tone-evoked FR, trials were combined over days to estimate, and to average out, trial-to-trial variance. Previous studies conducted by our group show a between-session stability of neurons recorded on the same wire. Based on the available literature using chronic microwire implants under similar circumstances as presented here, it is reasonable to assume that the same neuron can be recorded over days (Thompson & Best, 1990; Greenberg &

Wilson, 2004; Schmitzer-Torbert et al., 2005; Jackson & Fetz, 2007; Lutcke et al., 2013). Medium spiny neurons in ventral striatum are homogenously distributed (spaced apart rather than packed into layers), with radially symmetric dendrites. They produce small amplitude spikes (~170 µV) that decay rapidly over distance. Based on Rall's (1962) calculations and our own unpublished observations with moveable electrodes, it can be demonstrated that their waveform declines by 170 µV over 34 µm distance. Thus one microwire is extremely unlikely to pick up signals generated by multiple neurons, but rather one single neuron. This is consistent with our observations that 1) a microwire typically records uniform waveforms indicative of one neuron (ISI shows no discharges in the first 2 ms, consistent with a neuron's natural refractory period); 2) the waveform remains stable during a single 6-hour long session; the same stability is observed over multiple days. In order to be able to assume that recorded data over multiple sessions belong to a single neuron, the following requirements had to be met:

1) Waveforms recorded over multiple days maintain their similarity, and thus have a high correlation coefficient > 0.95 between two consecutive days; 2) Average peak time does not differ by more than 0.04 ms between waveforms recorded on two consecutive days. 3) Average difference in spike height between two consecutive days does not exceed 20%. Waveforms recorded on the same wire that fulfill all three criteria, were considered representing the same neuron recorded over days (Coffey et al, 2015).

3. RESULTS

3.1 Behavioral Analysis

Both males and females exhibited escalation of their drug intake, as amount of consumed drug increased over subsequent sessions. (Figure 5 & 6)

Animals learned to discriminate the tone, as they increased, over sessions, responding during tone-on periods. There was a steady decrease in the rate of responding during tone-free periods. The one-way repeated measures ANOVA confirmed a significant main effect of tone [$F(1,550)=8.31$, $p<0.005$) “Tone-on” vs ”Tone-off”], but no interaction Session by Tone [$F<1$] and no main effect of Session (1:15) [$F<1$].(Figure 7).

Behavioral data of males and females were analyzed separately using Ordinary Least Squares (OLS) linear regression. Consumed Drug per kilogram of weight was defined as an outcome variable and ‘Session’ and ‘Latency to the first response’ (measured from the onset of the first tone presented during the session) were used as continuous predictors. In models for both males and females, session (1-15 day) and latency to first response were significant predictors of the amount of drug consumed (Table 1 & 2 for detailed results). Analysis revealed that as latency was decreasing, the amount of consumed drug per session increased (Figure 8). In addition, the amount of consumed drug was increasing as a function of session (Figure 9, Figure 10).

A separate ANOVA with animal as a random effect was conducted on amount of Consumed Drug per kg with Estrous Cycle phase a grouping variable. Estrous Cycle stage did not have any effect on amount of consumed drug (Figure 11). For this reason neural data were collapsed with respect to Estrous Cycle stage ($F<1$) (Table 3).

3.2 Neural Findings

3.2.1 Histology

Out of 512 microwires implanted, 294 were localized to nucleus accumbens. Two hundred and two of those wires detected a unit and were used for all subsequent data analysis. Ninety seven neurons included into analysis were identified as core neurons, whereas 105 were identified as shell neurons. Three neurons of females, identified as shell and one neuron of male, identified as core, have been excluded from analysis. Those neurons exhibited very high, tonic baseline firing rates (greater than 20 spikes per second) and thus, most likely represented striatal interneurons, rather than MSNs (Figure 12).

3.2.2 Video Analysis

Video analysis was performed on 8778 trials. The analysis resulted in less than 3.2% of trials with video-determined latencies shorter than 200 ms. About 13% of video-analyzed trials, were movement free for less than 0.5 second, whereas 76% of trials were determined to be movement free for a duration of up to 1 second.

Trials with video-determined latencies shorter than 100 ms (0.36% of all trials) were excluded from analysis of neural firing rates.

Firing rates computed using a fixed 200 ms interval were compared to firing rates computed based on video-scored, movement free, intervals. There was a clear linear relationship between tone-evoked firing rates computed using the two different methods (Figure 13). Based on duration from Video-analysis, data were broken down into five distinct categories: below 300 ms, from 300 ms to 500ms, from 500 ms to 700 ms, from 700 ms to 900 ms, above 900ms. In each of the five distinct duration categories, there was a strong linear relationship ($r>0.8$) between firing rates computed based on 200 ms latency, and firing rates computed based on video-scored duration (Table 4).

As expected, the strongest linear relationship ($r=0.97$) was in the shortest duration category. This linear relationship decreased as video-scored durations increased ($r=0.84$ in above 900 ms category).

In the next step, percent of non-zero firing for each duration category was computed. In the shortest duration category (below 300 ms) only 0.5% of trials with zero firing rates, were non-zero when video-analyzed duration was used to calculate FRs. This proportion increased to 5% for video durations from 300 ms to 500 ms and increased further to 17% for trials for which video-analyzed FRs were computed based on duration of one second.

To sum-up, video-analysis increased power to detect differences, by decreasing zero-inflation. However, the fixed 200 ms provided a very good approximation whenever video-analysis was not available. Thus for the days for which video-analyzed data were not available, the fixed 200 ms duration was used (about 16% of trials).

3.3 Analysis of Firing Rates

3.3.1 Zero-Inflation

The average firing rates of core and shell neurons were very low. On day 1 of SA training, mean baseline firing rate for core neurons was 0.32 (+/-0.015) spikes per second, whereas shell neurons showed an average firing rate of 0.24 (+/-0.012) spikes per second Interestingly, baseline activity of shell neurons increased during later sessions of SA, relative to baseline activity of core (Figure 14). This was particularly true for drug-free trials of males from session 3 onwards. Although about 71% of trials had no firing during baseline or during “tone-on” period (‘0-0’ trials), Pre-drug trials of males showed a marked reduction in these ‘0-0’ trials (56% of trials) (Figure 15). The proportion of ‘0-0’ trials was relatively similar between core (30% of all trials) and shell (26% of all trials). In comparison, Load-up trials for either sex, or ‘Pre-drug’ trials for females, contained about 70% of ‘0-0’ firing rates (females:73%, males:70%, Pre-drug trials of females: 75%). There was a small trend toward a decrease in ‘0-0’ trials during ‘Late’ sessions in comparison to ‘Early’ sessions, specifically in core during ‘Load-up’ trials of females and ‘Pre-drug’ trials of males (Figure 16).

Interestingly, an increase in tone-evoked firing (difference from baseline) was observed for shell neurons around session ‘7’ for females. For males a much smaller increase was observed during sessions ‘1-5’ only in Pre-drug trials (Figure 17). The increase in tone-evoked change in firing appeared to be greater in shell, particularly during Pre-drug trials of females. For all four data sets, Pre-drug and Load-up trials of males and females, there was an observed trend for increased tone-evoked change in firing on ‘Hits’ in comparison to ‘Misses’ across sessions (Figure 18). For the following analysis both zero inflation and magnitude of change were studied.

3.3.2 Linear Mixed Models

3.3.2.1 Female Pre-drug data: NAc neurons show tone-evoked changes in firing during tones leading to the first self-administered infusion of the day

Analysis of tone-evoked firing patterns of females revealed an overall increase in tone-evoked change in FR from early to late sessions in shell neurons [‘Early’ sessions: 0.37+/-0.18, whereas ‘Late’ sessions: 0.97+/-0.17 spikes per second, $t(5290)=41.20$, $p<0.0001$]. Core neurons exhibited an opposite pattern: a small overall decrease in tone-evoked change in FR from “Early” to “Late” sessions [‘Early’ sessions: 0.45(+/- 0.11) spikes per second, whereas ‘Late’ sessions: 0.22 +/-0.12 spikes per second,$t(5290)=3.43$, $p=0.014$]. This effect was confirmed by a two-way interaction effect of Type by Session [$F(1,529)=145.32$, $p<0.0001$]. Further analysis revealed that this interaction effect was further modified by

Response factor: increase in tone-evoked change in FRs in shell, during “Late” sessions was driven by large tone-evoked changes in FR for “Missed” tones (“Misses”: 1.7 +/-0.16 spikes per second, ‘Hits’: 0.62+/-0.17 spikes per second). The opposite pattern of results was observed in core neurons: greater tone-evoked change in FR for “Hits” (0.5+/-.2 per second) was observed, in comparison to “Missed” trials (0.1+/-0.2 spikes per second). This effect was confirmed by a significant three-way interaction of Response by Subregion by Session [$F(1,5290)=23.07$, $p<0.0001$]. Post-hoc tests confirmed that there was a significant difference between ‘Hit’ and ‘Miss’ trials during ‘Late’ sessions, in both: core $t(5290)=4.744$, $p=0.0001$] and shell neurons [$t(5290)=29.49$,$p<0.0001$]. However, the direction of those differences was opposite between the subregions. Shell neurons have shown greater tone-evoked change in FR during ‘Missed’ trials, whereas core neurons during ‘Hit’ trials. Interestingly, increase in tone-evoked change in firing during ‘Missed’ trials was much larger than in any other experimental conditions (Figure 19.a) To sum up, shell neurons show more ‘robust’ tone-evoked changes in FR, than core neurons. As tone-evoked changes in FR increased in shell from ‘Early’ to ‘Late’ sessions, tone-evoked changes in FR decreased in core.

3.3.2.2 Male Pre-drug data: Male core and shell neurons show tone-evoked increases in FR change on trials leading to the first self-administered infusion of the day.

Both shell and core neurons exhibited greater tone-evoked change in FR on 'Hits' (Hits:0.77 +/-0.14 spikes per second), in comparison to 'Missed' trials (0.54 +/-0.11 spikes per second) during 'Late' sessions. This effect was confirmed by significant Response by Session interaction [$F(4281)=7.05, p=0.008$]. Post-hoc analysis of this interaction confirmed that increases for 'Hits' relative to 'Missed' trials, were significant only during late sessions ($t(4281)=2.72$, $p=0.03$), but not during early sessions ($t<2$). Thus, during 'Late' sessions NAc neurons of males exhibited greater tone-evoked change in in FR during 'Hit' trials in comparison to 'Missed' trials.

3.3.2.3 Load-up trials, females: Load-Up trials in females show an increase in tone-evoked change in FR in NAc during 'Hit' trials, in comparison to 'Missed' trials.

There was an overall greater tone-evoked change in FR for shell neurons (shell: 0.58+/-0.1 spikes per second) in comparison to core neurons (core: 0.26+/-0.1 spikes per second). This was confirmed by a main effect of Subregion [$F(1,24294)=119$, $p<0.0001$]. A greater tone-evoked change in FR was observed during 'Hit' trials ('Hit': 0.78 +/-0.11, spikes per second) in comparison to 'Missed' trials ('Missed':0.58 +/- 0.09, spikes per second). This observation was confirmed by a significant main effect of Response [$F(1,24294)=37.59$, $p<0.0001$]. When

effect of response was separated by Session, there was a greater tone-evoked change in FR for ‘Hits’ relative to ‘Misses’ during ‘Late’ sessions (‘Hits’ 0.89+/-0.11, ‘Misses’:0.64 +/- 0.09 spikes per second), but not during ‘Early’ sessions (‘Hits’: 0.49 +/ -0.11, ‘Misses;: 0.45+/-0.10 spikes per second). This was confirmed by a significant two-way interaction effect of Response by Session [$F(1,24294)=14.56$, $p<0.0001$]. Post-hoc analysis confirmed that the effect of Response was significant only for ‘Late’ sessions ($t(24294)=6.49, p<0.0001$), but not during ‘Early’ sessions ($t<1$). Thus, female Load-up trials exhibited greater tone-evoked change in FR on ‘Hits’ in comparison ‘Misses’. The three-way interaction of Response by Session by Subregion was however not significant.

3.3.2.4 Load-up trials, males: Core neurons exhibited decreased ‘tone-evoked’ activity during late sessions. Shell neurons show increases in tone-evoked change in FR for ‘Hits’ relative to ‘Misses’ on ‘Late’ sessions.

Analysis of Load-up trials for males revealed a slight decrease in tone-evoked change in FR for core neurons during ‘Late’ sessions (core, ‘Late’ sessions: 0.45 +/-0.1 spikes per second), in comparison to ‘Early’ sessions (core, ‘Early’ session: 0.66 +/- 0.12 spikes per second). This was confirmed by a significant two-way interaction of Subregion by Session [$F(1,24895)=79.89$, $p<0.0001$]. Post-hoc testing of this effect confirmed a decrease in tone-evoked activity during ‘Late’ sessions, in comparison to early sessions for core neurons ($t(24895)=4.01, p=0.0004$), but not for shell neurons [$t<1.5$].

Importantly, there was greater tone-evoked change in FR for ‘Hits’ during ‘Late’ sessions [Hits:0.64 +/- 0.09 per second, Misses: 0.38 +/- 0.7 spikes per second]. This was confirmed by significant two-way interaction of Response by Session [$F(1,24895)=7.31$, $p=0.007$]. Post-hoc tests confirmed that the effect of Response was significant during ‘Late’ sessions [$t(24895)=6.448$, $p<0.0001$], but not during ‘Early’ sessions [$t<1.5$]. In addition, this effect differed across the subregion, such that effect of Response on ‘Late’ trials was only significant for shell neurons [Hits: 0.63+/-0.13, Misses: 0.32+/-0.13 spikes per second, $t(24895) = 8.65$, $p<0.0001$], but not in core neurons ($t<1$). This was confirmed by a three-way interaction effect of Session by Subregion by Response [$F(1,24895)=5.45$, $p=0.02$]. Just like during Pre-drug trials there was an increase in tone-evoked change in FR on ‘Hits’ during ‘Late’ sessions, but this increase was only significant in shell neurons. To sum up, results show decreasing tone-evoked changes in FR in core neurons, in contrast to increasing tone-evoked changes in FR in shell neurons across sessions, specifically for ‘Hit’ trials. Thus, shell neurons show more ‘robust’ tone-evoked changes in FR, than core neurons.

4. DISCUSSION

A number of studies have pointed toward NAc core as possibly crucial for cocaine cue-induced relapse. Inactivation of NAc shell does not alter cue induced relapse, whereas inactivation of NAc core impairs cue-induced reinstatement of drug seeking. Neurons that project to NAc core, but not NAc shell, express Fos during cue-induced reinstatement of cocaine seeking (Fuchs et al., 2007, Ito et. al. 2004, McFarland and Kalivas, 2001, McGlinchey et al. 2016). Thus NAc core has been implicated in processing drug-associated cues. However, cue-induced drug seeking includes both the initial cue processing, followed by initiation of the behavioral response. Therefore, a question remains whether NAc core is the starting point of striatal cue processing, or rather a downstream node in a circuit in which striatal processing of, most likely, limbic inputs, starts in NAc shell? NAc core is more “striatal-like”, whereas shell is part of the extended amygdala. The core may be more involved in (pre)motor processing, and thus temporal isolation of cue vs motor processing is necessary for adequate investigation of shell and core involvement in cue-induced drug seeking. The present study accomplished this by combining the high temporal resolution of single unit recording with video analysis. Video analysis of all tone-trials ensured that any change in FR was due to tone processing and was not triggered by tone-evoked changes in the animal’s behavior, such as onset of approach to the manipulandum. Thus, this design allowed discerning effects of the cue from any possible motor confounds.

The design of the current study also enabled investigation of NAc core and shell firing rates in a drug-free state, in order to approximate relapse in an abstinent cocaine abuser when encountering a cocaine-associated cue. Further, the tone discriminative stimulus, S^D, only signals cocaine *availability*, contingent upon a drug-seeking response, in order to assess cue processing that actually triggers resumption of drug seeking. In contrast, in other studies the tone acts as a conditioned stimulus, CS, automatically predicting cocaine delivery, following the *already-emitted* drug seeking operant response.

4.1 Zero Inflation of Firing Rates in Nucleus Accumbens

The primary goal of the study was to investigate differences in how core and shell neurons process the tone that leads to the first response of the day. The first important observation was that NAc firing rates, when calculated based on the movement free period, are very low. In fact, in about 79% of all trials there was no firing during the baseline period. When looking at tone-evoked activity, this number decreased to 77%. In about 71% of all trials there was no firing on either baseline, nor during the tone-on period (termed “0-0”). Further analysis showed that there was a decrease in ‘0-0’ trials during the drug-free Pre period for males (56% vs about 70% for all other segments). In addition, for females during drug-free period trials there was a decrease in ‘0-0’ trials during late sessions, relative to early sessions in core. The decrease in ‘0-0’ trials in Pre-drug trials, combined with increase in baseline firing in shell, could reflect changes in plasticity in NAc neurons that take place with repeated exposure to cocaine. Some studies suggest

that repeated exposure causes changes in K^+ channels in MSNs (Kourrich et al., 2015), and those changes could influence membrane excitability of MSN neurons, which could in turn, lead to changes in baseline firing of those neurons with repeated exposure to cocaine. Those changes could be an important contributor to evolution of addiction.

4.2 Core and Shell Tone Evoked Changes in FRs

Relative to core neurons, Pre-drug shell neurons of females showed an abrupt increase in absolute FR-change around session 7 of SA (Figure 14.a & 14.b). Male shell neurons showed a similar, but more protracted, increase in shell activity relative to core.

Similar changes were present in Load-up data of both males and females, although the magnitude of increase was greater for shell relative to core neurons (Figure 24.c & 24.d). Interestingly the effects seemed greater in magnitude in females, compared to males. The reason for this could be that some males, included in the study, learned to self-administer already on day 1 of SA. For those males, the differences in core and shell firing could have occurred earlier, or could have been mediated via a different mechanism. Most females began to consistently self-administer after session 5, and thus the increase in change in firing in shell correlated with escalation of drug intake.

A crucial comparison revealed that both core and shell neurons showed differences between ‘Missed’ trials preceding first infusion of the day, vs the one

trial, or first ‘Hit’, immediately leading to the first infusion of the day. Interactions with subregion were driven by shell neurons which, overall, showed greater changes in firing than core neurons. NAc shell neurons responded to the tone, before the onset of drug seeking, more strongly than core neurons. This finding reaffirms that of Ghitza et al (2003), and extends it to rule out any influence of extinction, which was absent from the present study. It appears initial striatal processing of cues leading to resumption of cocaine taking after abstinence, occurs in the shell whereas core activity decreases with increasing exposure to cocaine.

Analysis of Load-up trials indicated that tone-evoked firing changes persist even when the animal begins to self-administer cocaine. However, effects of tone cue seemed blunted, especially in males. Previous studies have shown that trained animals are sensitive to interoceptive cues (Wise et al., 2008). In the maintenance phase of self-administration, they are able to maintain their drug level within a narrow range. (Zimmer et al., 2011, Zimmer et al., 2013). This could possibly be mediated via phasic and tonic changes in dopamine levels in NAc core and shell during SA. Tonic levels of dopamine can go up by 800% of baseline levels, whereas phasic changes can increase by 50% of the tonic elevation (Wise et al. 1995). Thus it seems likely that while under the influence of drugs, environmental cues become a less salient driver of drug seeking, while interoceptive cues primarily drive responding.

Our second goal was to conduct the first electrophysiological study of female NAc core and shell neuron firing during chronic access to cocaine SA. We expected activity in female core and shell to parallel that observed in males.

Females, just like males, developed reliable self-administration behavior. This was evidenced by individual drug curves, as well as by the linear increase in drug consumed, with decreasing latencies to the first response. Female shell neurons, during the drug free state, showed changes between the tone leading to the first infusion of the day, vs all 'missed' tones during late sessions. This effect was also significant in core neurons, but with opposite direction – core neurons exhibited an increase in activity for "Hits" in comparison to "Misses". During drug-free trials of males, both core and shell neurons showed parallel changes on late sessions.

The observed difference is a possible sex difference, however the current study avoids making any direct comparison of males and females, because a multitude of factors could have influenced self-administration behavior of the two groups, making any differences difficult to interpret.

In conclusion, we show that female NAc neurons in parallel with escalation of drug intake, show changes in tone-evoked firing. In fact shell neurons show a large decrease from baseline, immediately prior to the first infusion of the day, whereas core neurons show an increase immediately prior to the first Hit. Because we studied firing patterns during the drug free state, and the effect of the cue is

carefully isolated from the any motor effects on firing, our interpretation is that these effects underlie drug-seeking in females. The current study is not only the first study to investigate electrophysiological activity of NAc core and shell during SA in females, but also the first study to carefully isolate correlates of cue-induced drug-taking in females. The observed differences could potentially underlie increased susceptibility to drug-associated cues in females.

4.3 Concluding Remarks

The present results provide evidence of learning of a tone cue predicting cocaine availability by NAc core and shell neurons. Specifically both shell, and to a lesser extent, core neurons showed changes in activity prior to the first drug infusion of the day in both males and females.

Past studies have shown that successful reinstatement of drug seeking requires activation in multiple distinct structures: nucleus accumbens, basolateral amygdala, pre-frontal cortex and ventral tegmental area (LaLumiere et al, 2012). Multiple studies have suggested a dissociation between NAc core and shell involvement in drug-seeking. NAc shell is thought to be necessary for cocaine reward and drug seeking driven by contextual cues, whereas NAc core is thought to be necessary for drug-seeking driven by discrete cues. The current experiment suggests that in circumstances where a cue that is signaling cocaine availability is presented during drug-free state, both NAc core and shell neurons show changes in firing, preceding drug-seeking. Interestingly in female Pre-drug trials, a decrease in activity was observed for shell neurons during first “Hit” and large increase during

“Misses”. Thus it appears that shell neurons are active during “Missed” tones and decrease their tone-evoked activity during the first “Hit” tone of the day. These patterns are projected to the ventral pallidum, but are not predictive of simple inversion by GABAergic connections (Root et al, 2013), and require further study.

Further exploration of data shows a trend for an increase in baseline activity for shell during late, relative to early sessions. There is also an observed trend for an increase in baseline firing immediately prior to the first “Hit” of the day, relative to preceding ’Missed’ trials (Figure 20). Given these data it is possible that the decision to self-administer the first infusion of the day occurs earlier, perhaps during preceding “Missed” tones.

The observed results lead us to propose a theory according to which *relapse* is a gradual and continuous-time evolving, stochastic process in which neural responses to subsequent presentations of drug-associated tone cue, are a function of previous neuronal responses to that same cue and its baseline firing rate (that follows Brownian Motion). Tone-evoked changes in firing in BLA, VTA, PFC subiculum and NAc core and shell, would be directly observable, interconnected sequential nodes of a network. At each trial, tone-evoked changes in activity in those regions, would lead to an update of probability of relapsing [$T_{ij} = P(z_i = x | z_{i-1,i-2,i-n} = y_{1n})$], with baseline firing as correlated noise in this model.

Thus, there is a continuous evolving likelihood of relapse based on tone-evoked activity in shell. As this probability exceeds a critical point, the subject is

ready and prepared to make the first response of the day. Thus even though drug-seeking behavior has not yet occurred, it becomes more likely in the near future. Core neurons increase their firing rate at the onset of the first “Hit” tone of the day. Given the known anatomical connections of the NAc, it is possible that initially limbic inputs to the shell underlie processing of drug-associated cues, whereas later, when an association with the appropriate motor response is formed, core becomes involved in cocaine-cue discrimination. Whether such learning can occur without involvement of NAc shell is an important question, which cannot be answered solely on the basis of results of the current study. Nonetheless, the present results clearly demonstrate that, after chronic long-access cocaine SA, core is not the only, or even the strongest, location within the NAc of processing the cocaine cue.

4.4 Future Directions

Numerous questions follow from the results of this study. Which particular inputs into NAc shell are correlated with observed cocaine-cue associated firing changes in NAc shell? Three major glutamatergic inputs into NAc are: basolateral amygdala, medial prefrontal cortex and ventral hippocampus. Questions arise whether disabling any of those inputs during cocaine SA training could potentially impair learning of cocaine-associated drug cues. Importantly, another question is whether changes observed in NAc core are dependent on those observed in shell. Will NAc core, under the same circumstances when the cue signals cocaine availability contingent upon an operant response, show the same differences if

shell neuron activity were prevented?

As previously mentioned, direct comparison of males and females was not possible in this study. All previous electrophysiological investigations of NAc core and shell during cocaine SA have been conducted in male rats. Cocaine addiction, however, does not spare females. In fact there is strong evidence suggesting that females who abuse cocaine are at higher risk of disastrous health consequence of addiction. Thus we included females in the present study and show robust changes in NAc shell and core triggered by cocaine associated cues when the animal is in a drug-free state. The next question would be whether those differences are sex specific.

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6. FIGURES

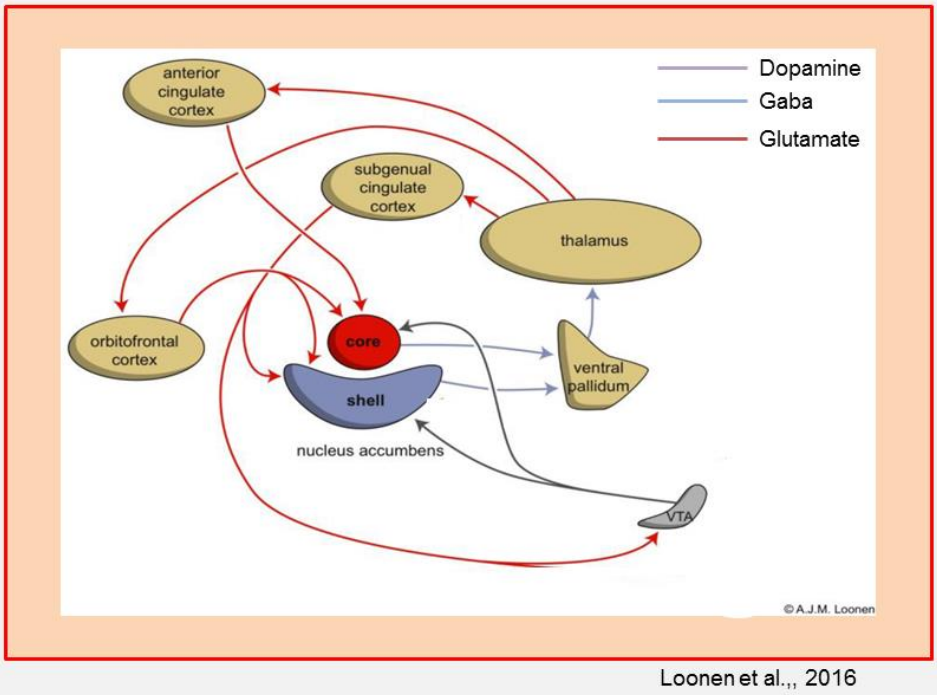

Figure a.Diagram of mesolimbic dopamine pathway. Flow of information through the mesolimbic dopamine pathway is indicated with arrows. Dopamine, glutamate and GABAergic projections are separated.

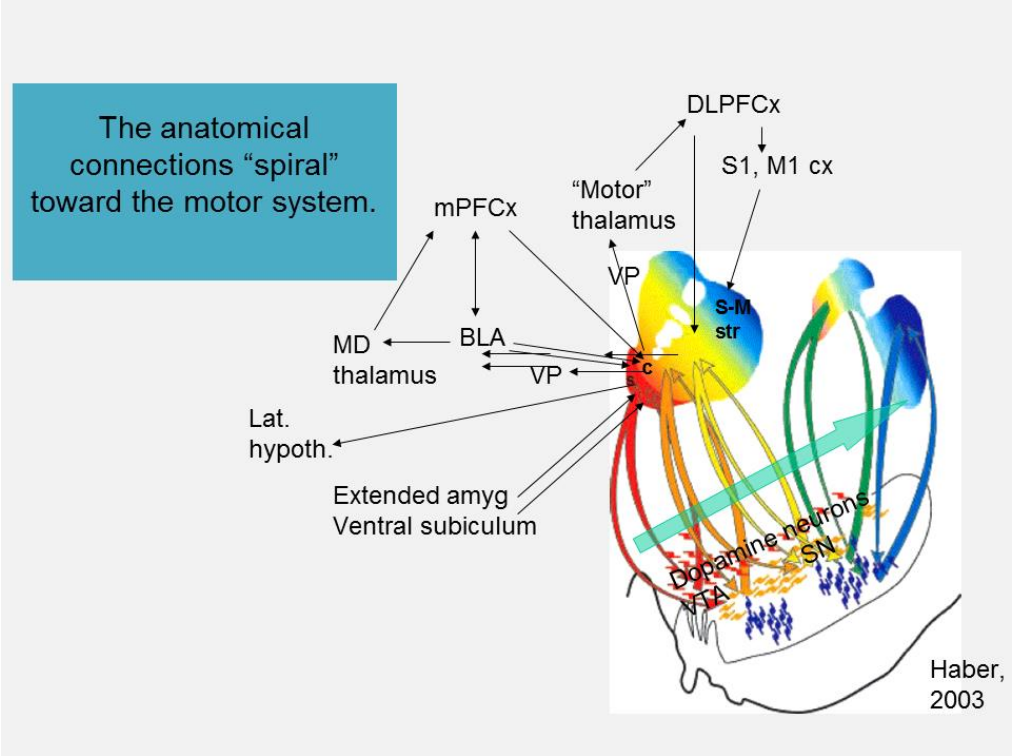

Figure b. Flow of information through nucleus accumbens core and shell. Diagram presents ascending projection “spiral”: via thalamocortical and mesencephalic pathways, shell projects to core, and core projects to central striatum, which then projects to dorsolateral (sensorimotor) striatum. It is theorized that these projections provide a necessary basis for a limbic-motor interface.

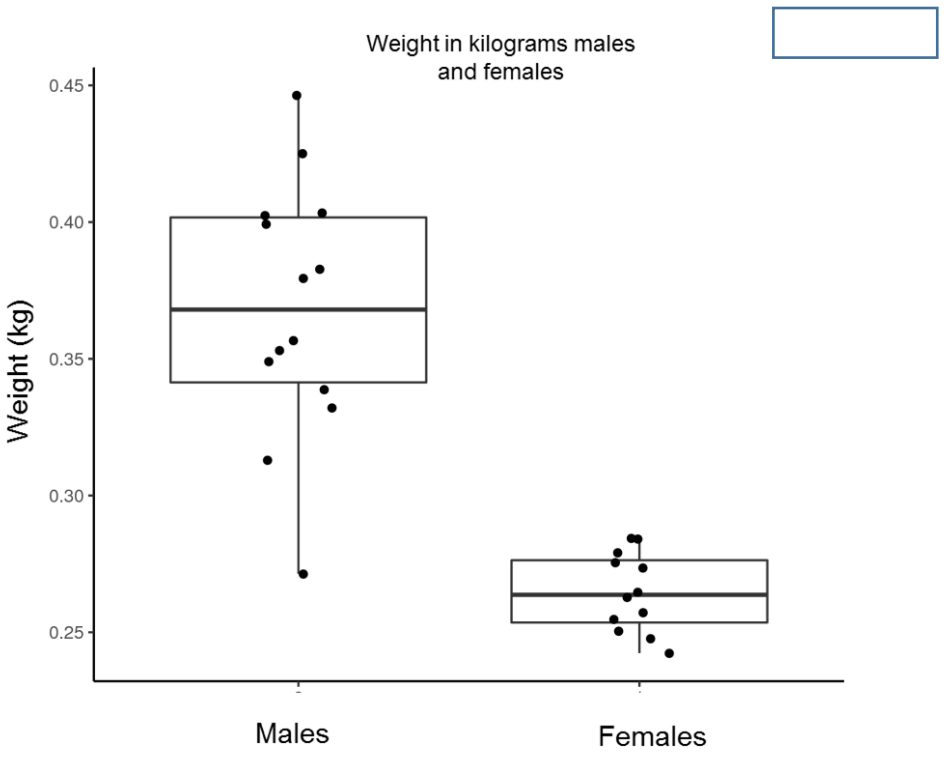

Figure 1. Distribution of weights of males and females included in this study. Females on average weight less than males included in the study

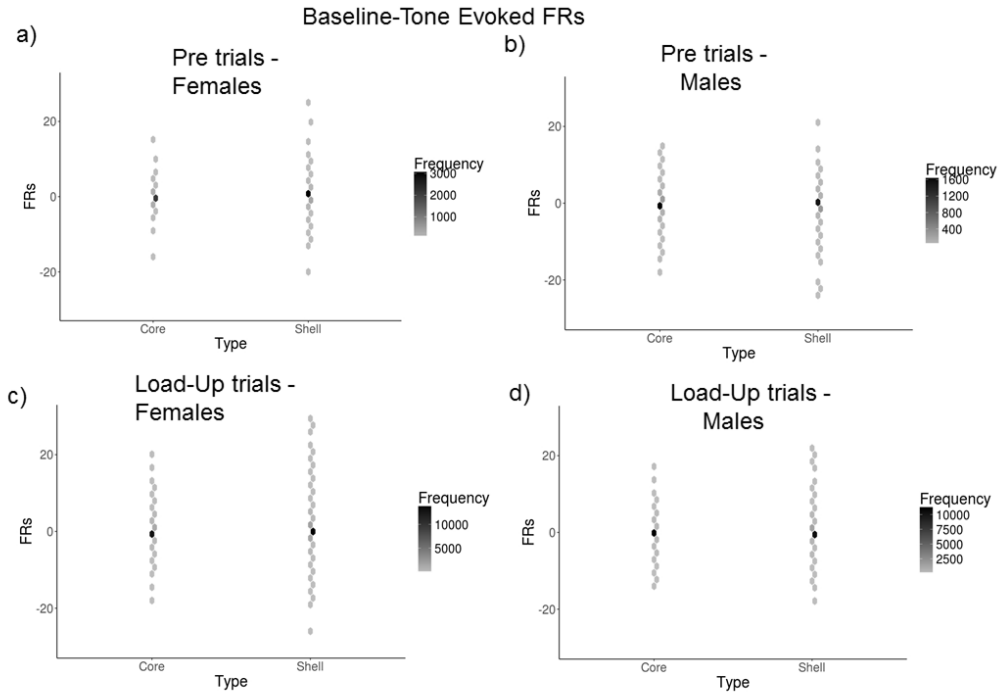

Figure 2. Raw FR difference per experimental condition: Subregion (core, shell). The responses are symmetrical both increases and decreases are observed per experimental condition.

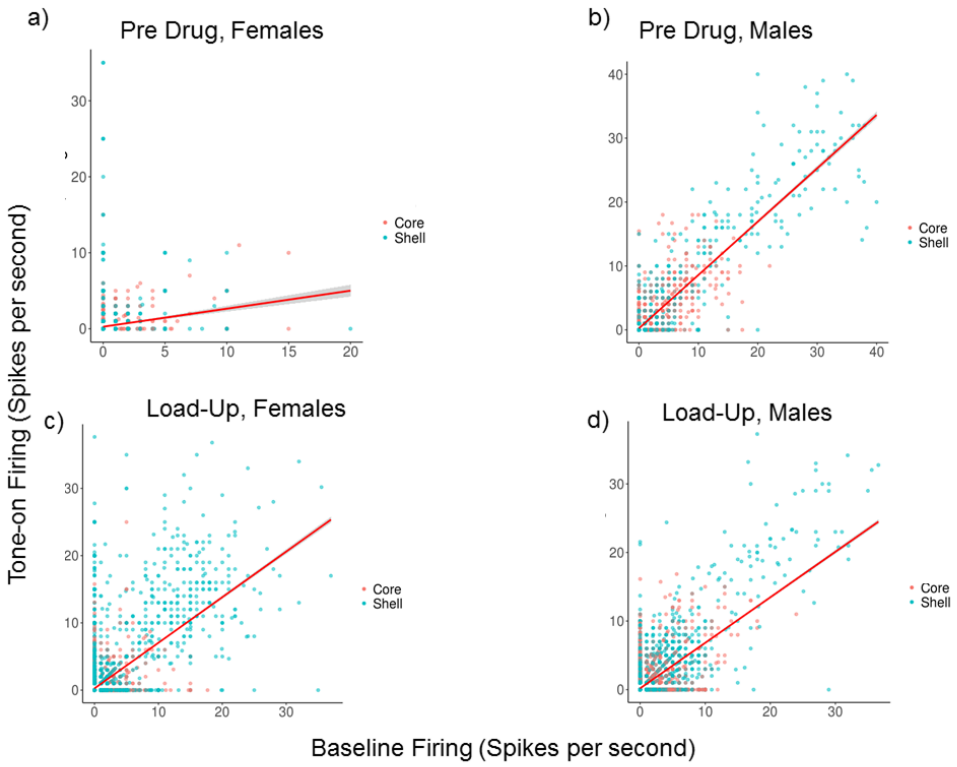

Figure 3. Baseline firing rate vs tone evoked firing. There is a linear relationship between baseline FRs and tone-evoked FRs; with increasing baseline FR, tone-evoked-FRs are increasing. Pre-drug trials of females are an exception and it looks like the relationship between firing during baseline and during tone-on is not as strongly linear.

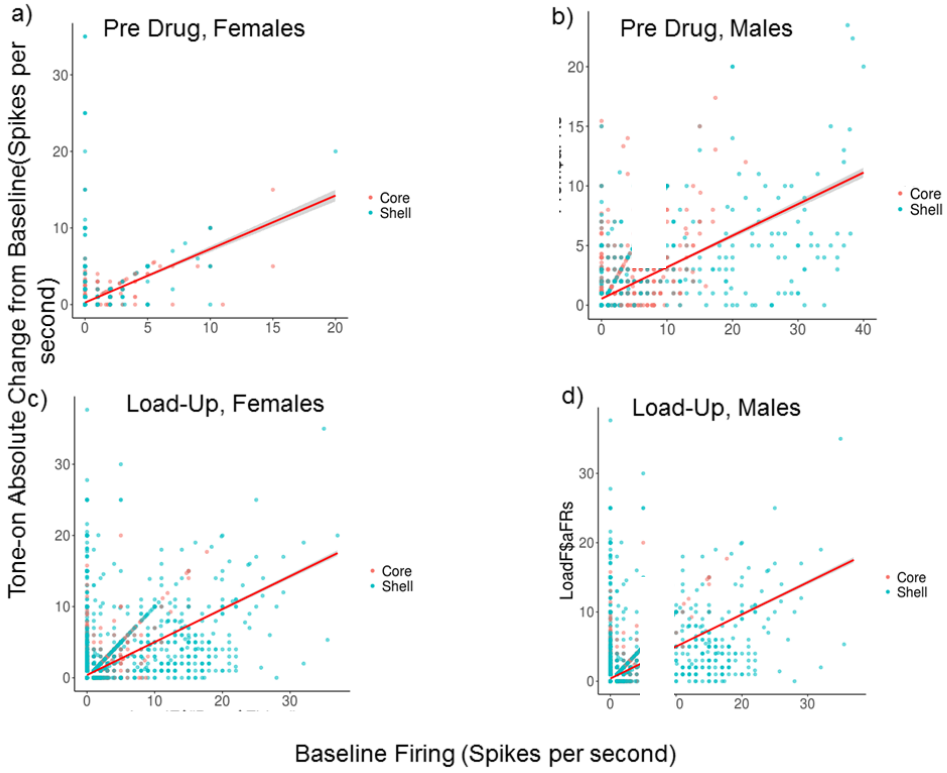

Figure 4. Baseline FR vs absolute differences between baseline and tone-evoked FR. Linear relationship is decreasing when baseline FR is compared to absolute difference (tone-evoked FR).

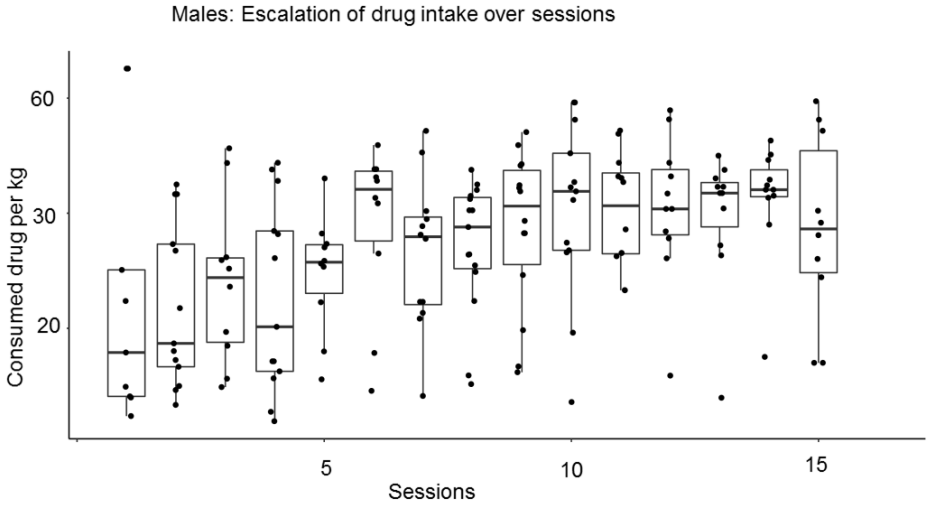

Figure 5. Amount of drug per kg of weight consumed by males over the subsequent sessions of SA. Males have escalated their drug intake.

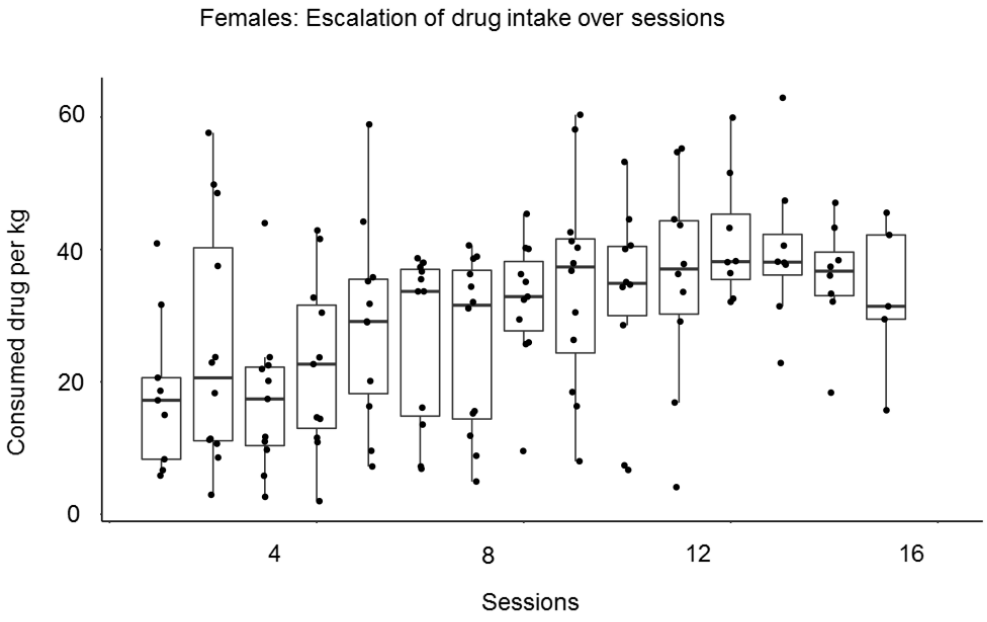

Figure 6. Amount of drug per kg of weight consumed by females over the subsequent sessions of SA. Females, just like males, show and escalation of drug intake. There is a large increase in drug consumed from session 5 to session 7. From session 7 onward, drug level remains constant.

Figure 2

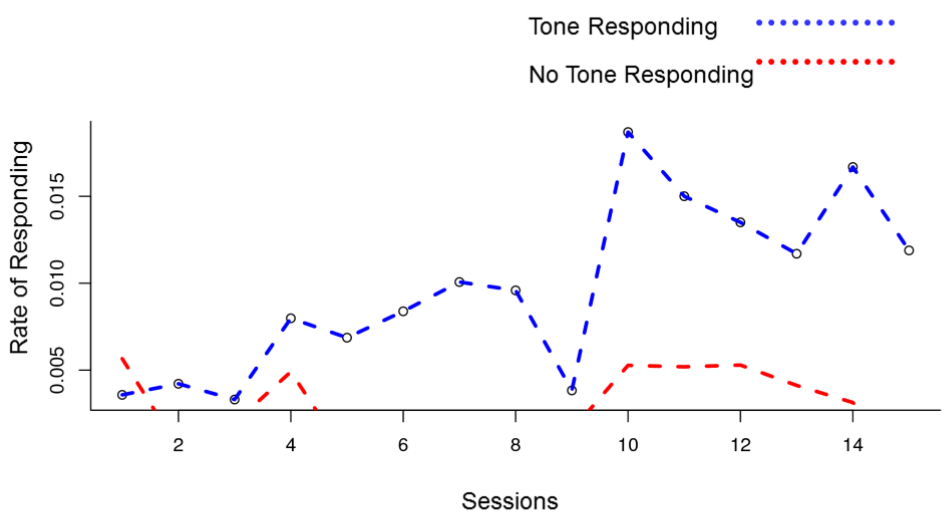

Figure 7. Evidence of Tone-learning. Animals decreased their rate of responding during tone-off period (drug-free) and over-time increased their responding during tone on period. The main effect of tone (Tone on responding vs Tone-off responding) is significant, confirming that animals are more likely to respond during tone-on period.

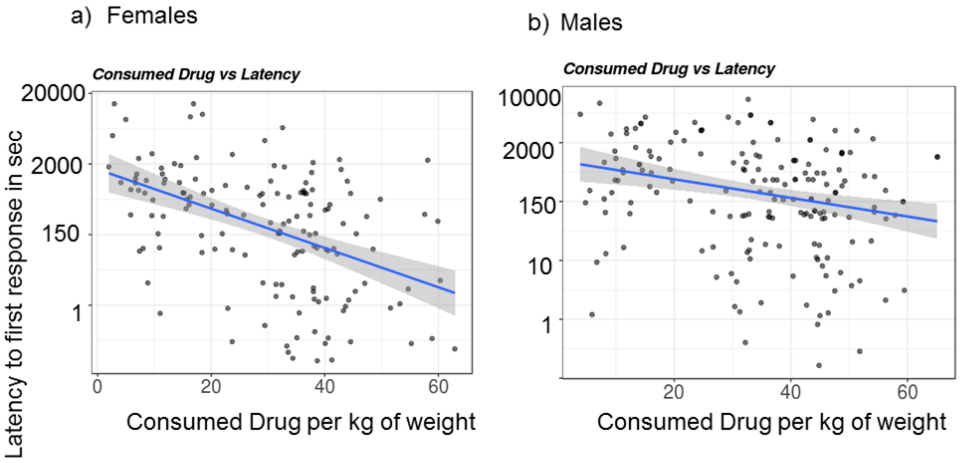

Figure 8. Amount of consumed drug by males and females vs latency to the first response on logarithmic scale. There is a negative linear relationship, such that with increasing amounts of consumed drug, latencies are decreasing.

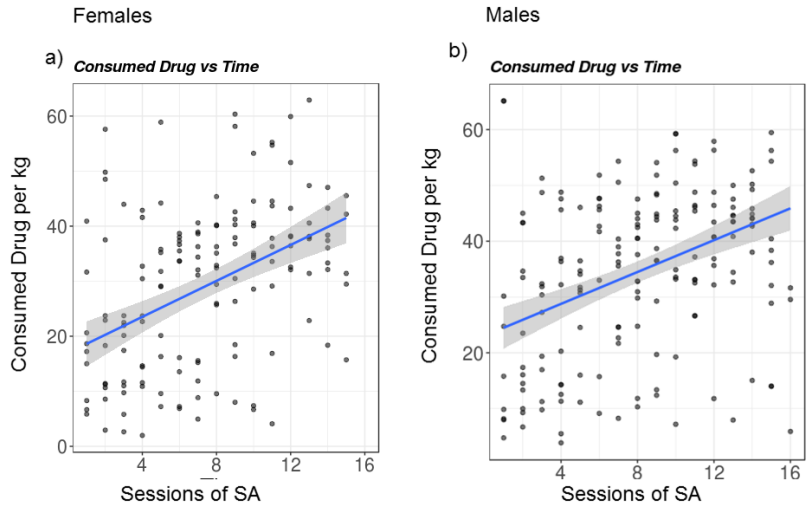

Figure 9. Amount of drug consumed vs sessions. With increasing time there was a linear increase in the amount of drug consumed for both: a) females and b) males. For females this relationship appears to be stronger: the least squares line between Sessions and Consumed Drug per kg has a steeper slope.

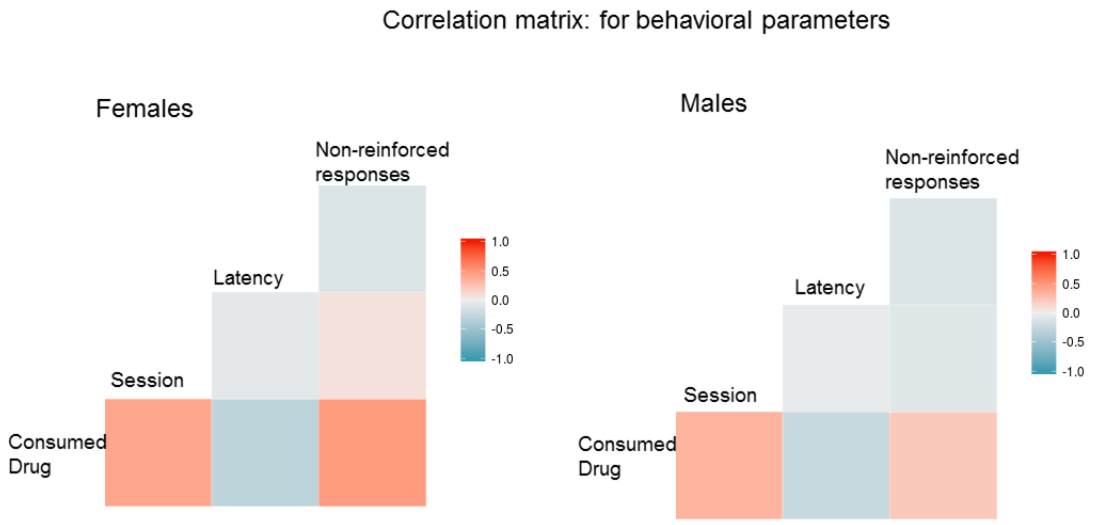

Figure 10. Heat-map of correlation matrix for both males and females included in the study. Colors vary from blue (dark blue representing the strongest negative relationship), to dark red (strongest positive relationship). Females, included in this study, show stronger linear relationship between each pair of parameters: “Latency” (Latency to first response), “Session”, “Non-reinforced responses” (number of non reinforced responses per session) and “Consumed Drug” (Drug consumed per kg of weight”.

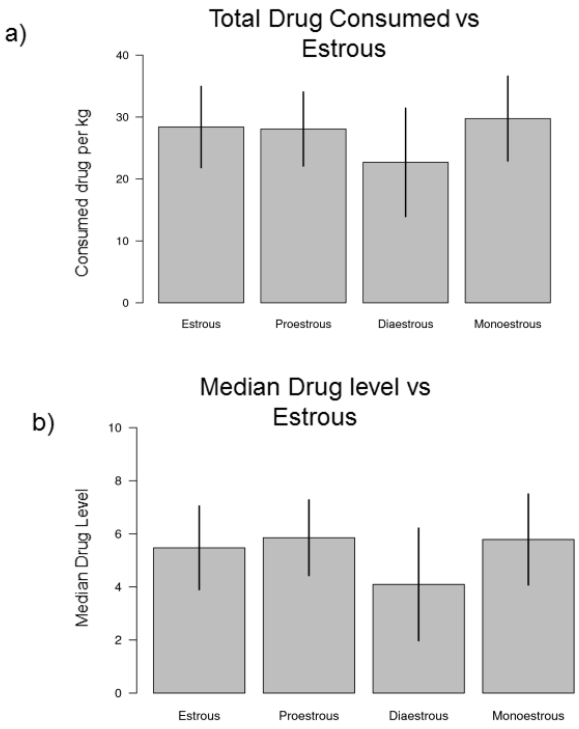

Figure 11. Drug consumed (a) and median drug level (b) did not differ between different phases of estrous cycle.

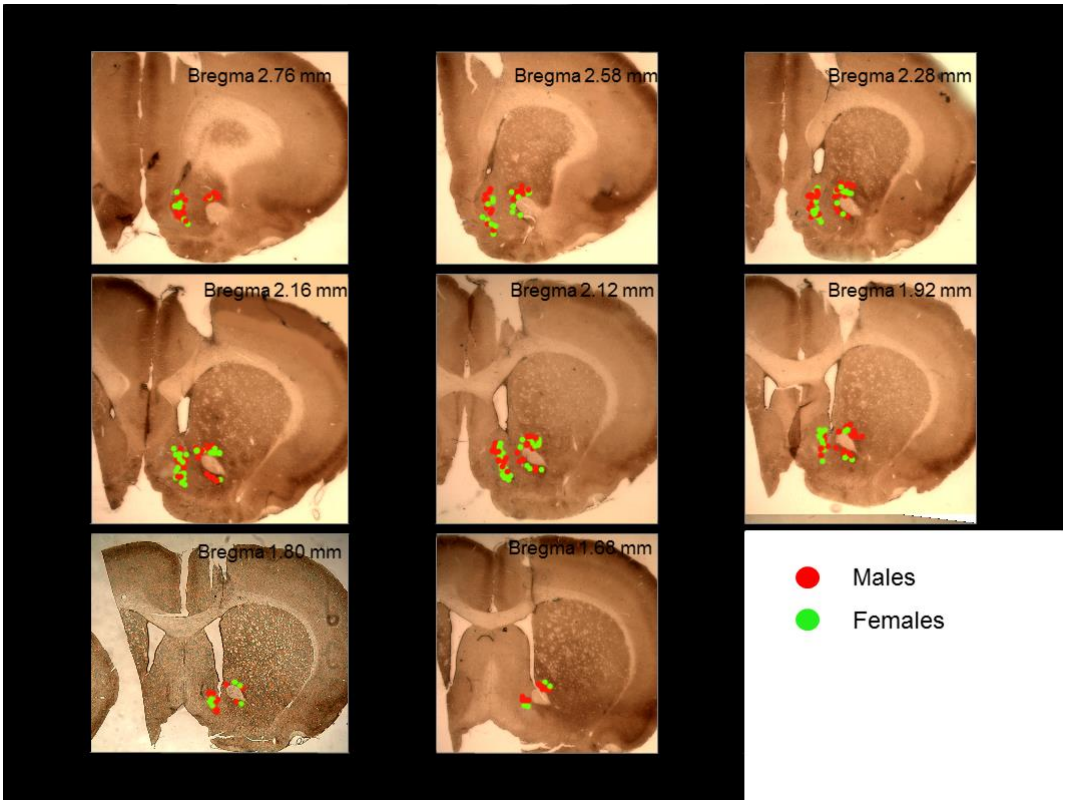

Figure 12. Schematic representation of microwire placement for male and female, core and shell neurons included in the analysis.

6.14. Linear relationship between video calculated FRs and 200 m calculate FRs

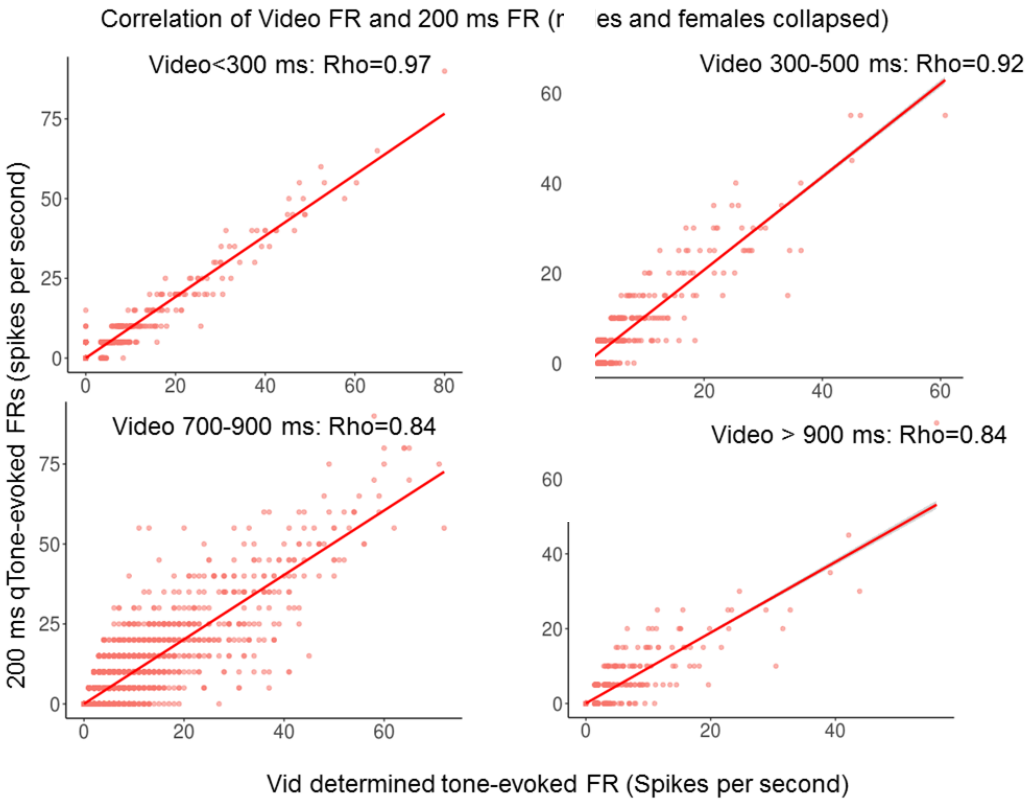

Figure 13. There is a strong linear relationship between tone-evoked FRs calculated based on video-scored latencies and fixed 200 ms interval. The relationship is almost ideally linear in the lowest range of video-scored latencies and is decreasing, as video-scored latencies are increasing.

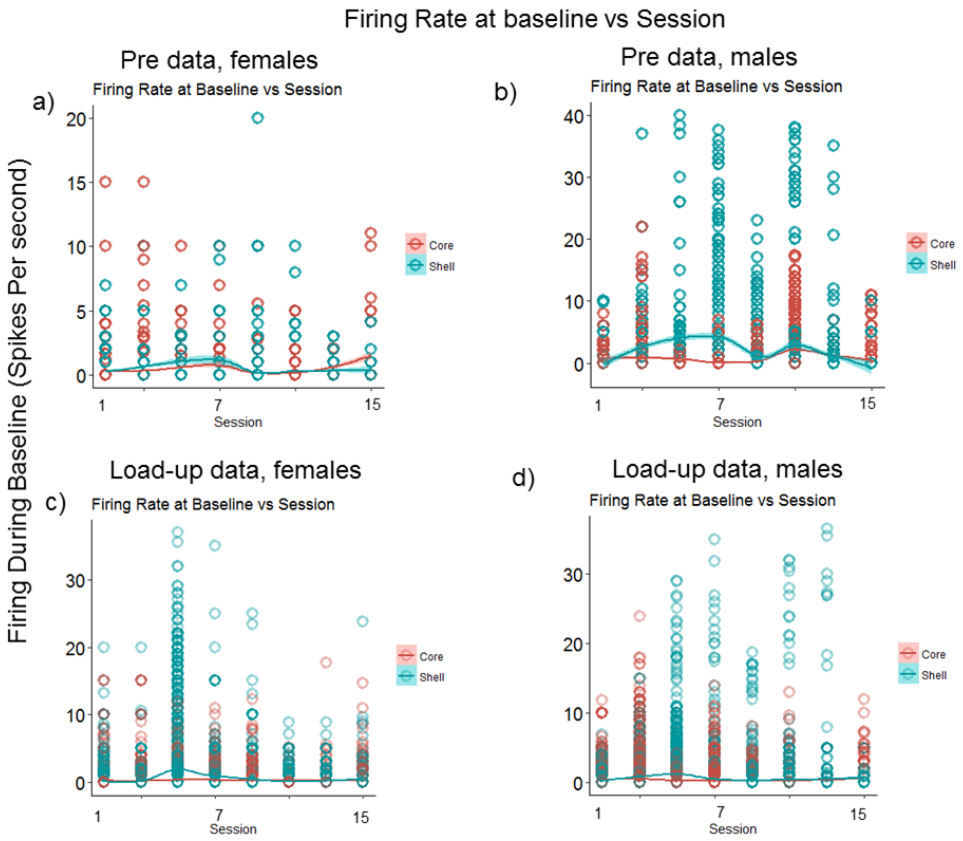

Figure 14. Baseline firing vs sessions. Shell neurons in Pre-drug trials of males show a large increase in FRs in comparison to Load-up trials of males or females or Pre-drug trials of females. Red and blue line represent a line of non-linear fit to the data, separately for core and shell neurons, with confidence interval around it.

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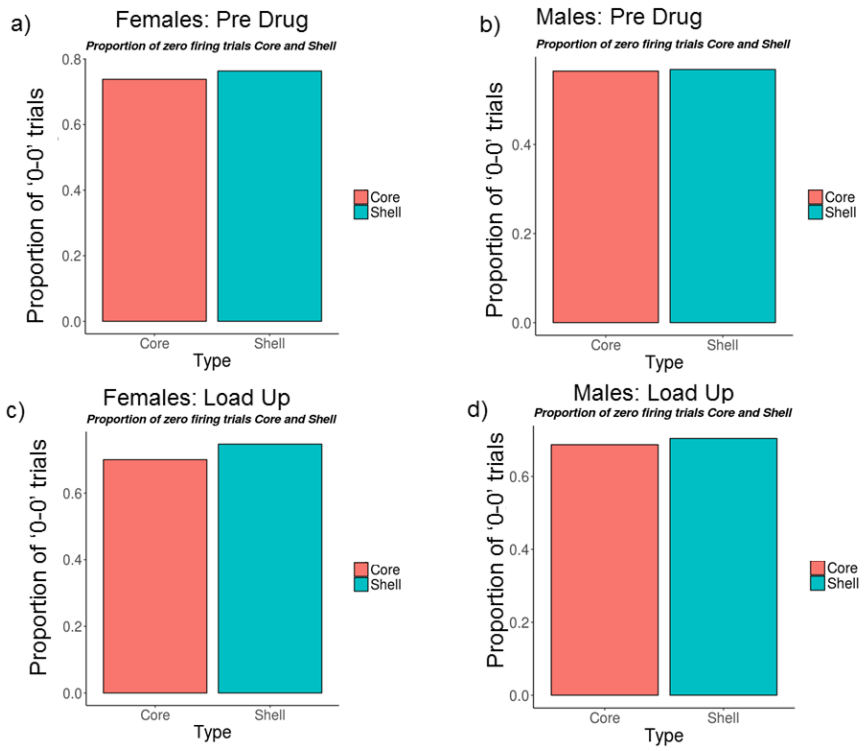

Figure 15. Distribution of '0-0' FRs between different experimental conditions. There is a similar pattern of '0-0' responses for all experimental conditions. There is marked decrease in '0-0' trials for males in Pre-drug trials.

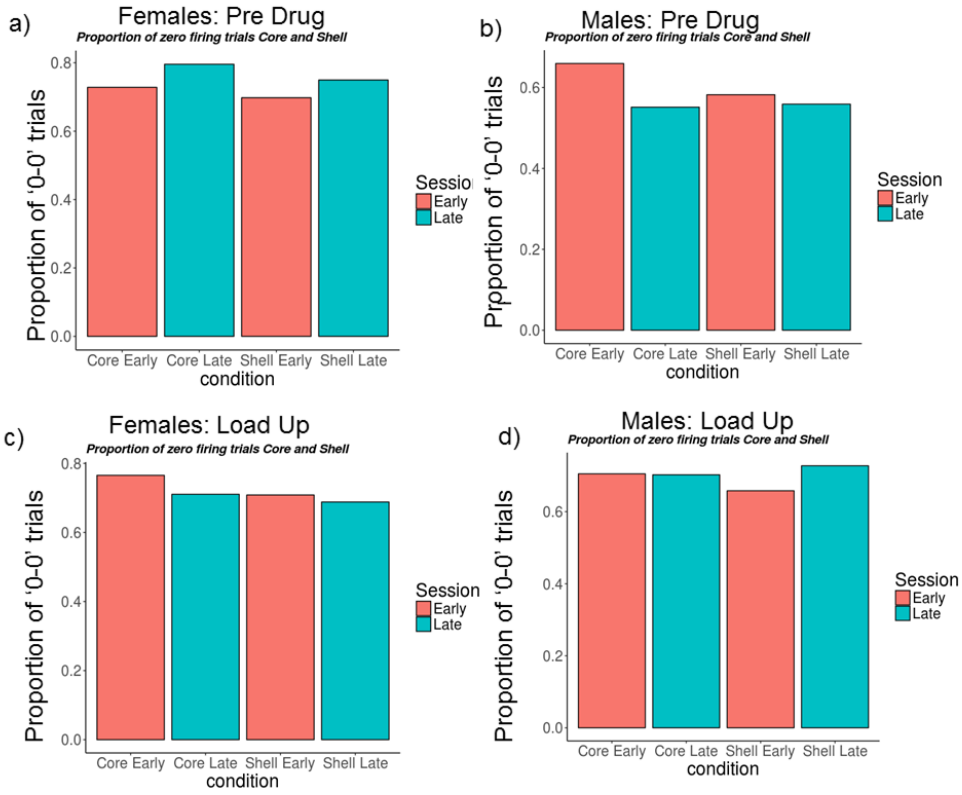

Figure 16. Distribution of '0-0' trials for males and females. There is a visible decrease in '0-0' firing rates for males on late trials for both core and shell neurons in addition, there is decrease in '0-0' early for shell. There is a large decrease in '0-0' trials for Pre-drug trials of males for core neurons.

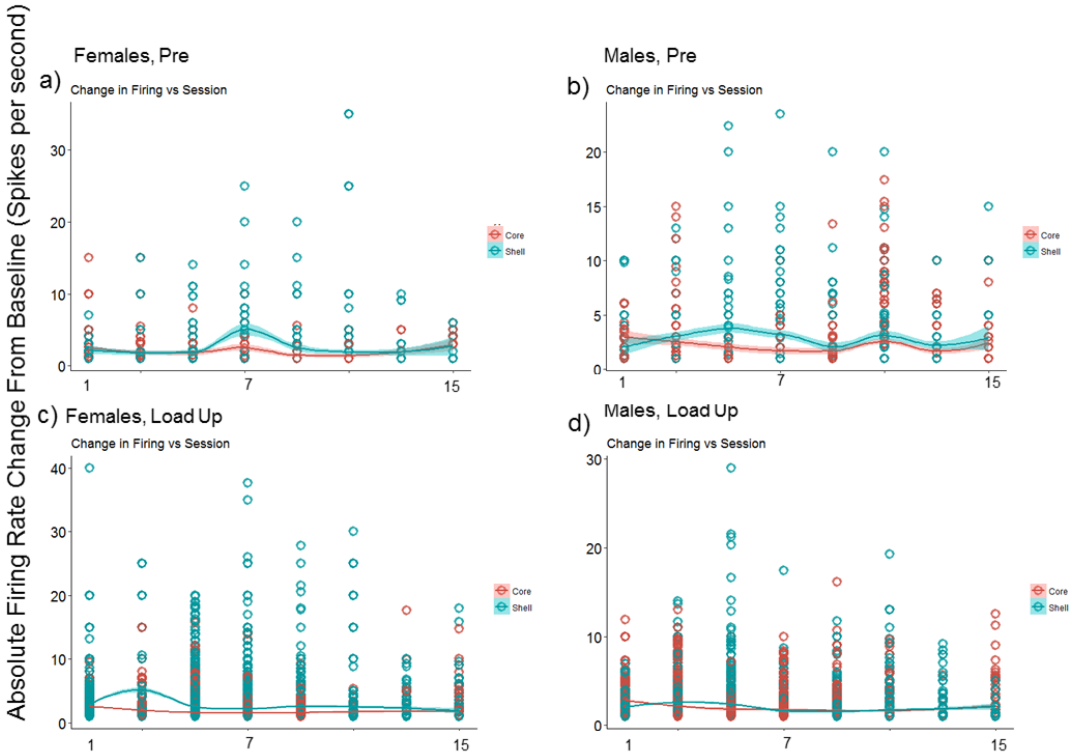

Figure 17. Change in Firing Rate for Load-up and Pre data for males and females per session. Core and shell neurons are marked with different colors. During Session 1, Pre-drug Trials core and shell neurons show the same change from baseline. There is an increase in change for shell relative to core during late sessions. Red and blue line represent line of non-linear fit to the data, separately for core and shell neurons, with corresponding confidence interval around it. Trials with zero changes were deleted for the purpose of this analysis, as they would drag all the possible trends toward zero.

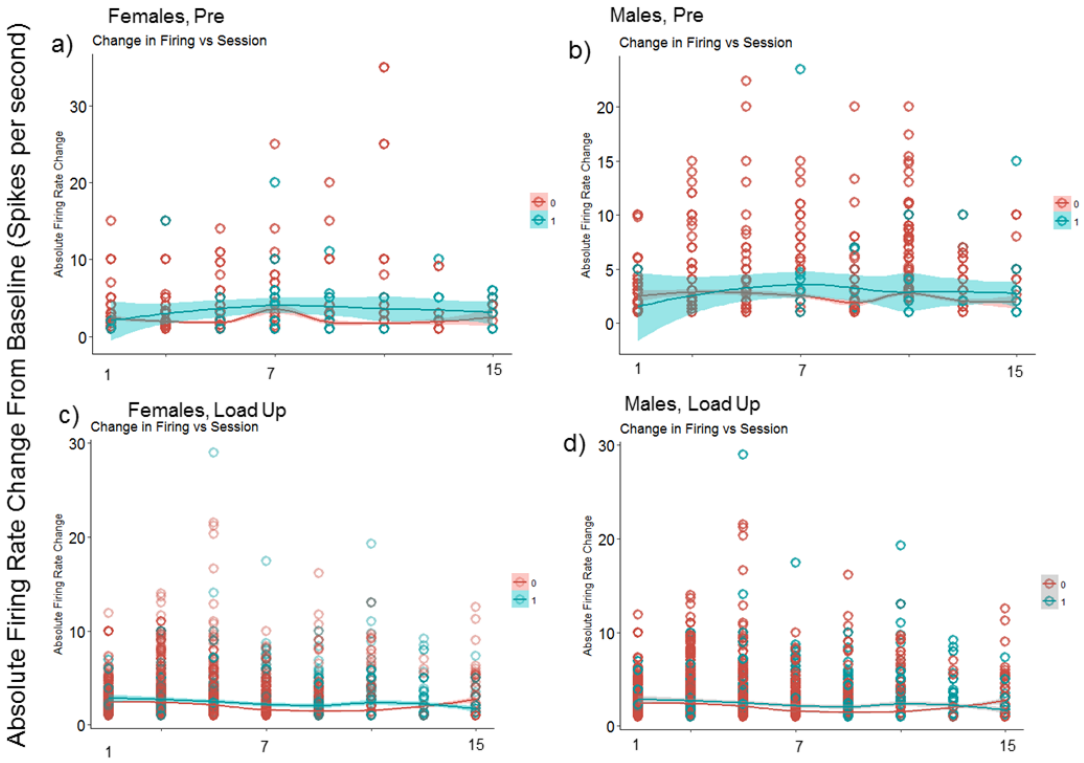

Figure 18. Firing per Session by Response factor("Hits": 1, "Misses": 0). Curve-fit indicates that there is a cross-over effect for both males and females: 'Hits' are more positive than 'Misses' during middle sessions of SA (Sessions 5-9), whereas on later days 'misses' become more positive (Session 15). Red and blue line represent line of non-linear fit to the data, separately for 'Hits' and 'Misses', with corresponding confidence interval around it. Trials with zero changes were deleted for the purpose of this analysis, as they would drag all the possible trends toward zero.

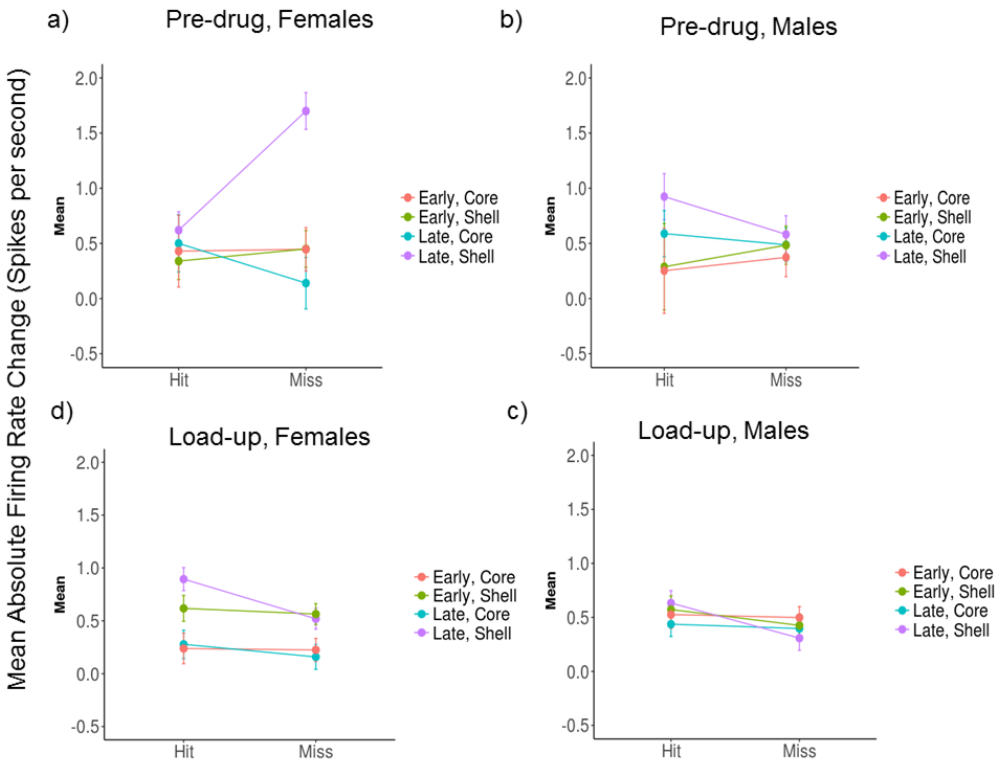

Figure 19. Interaction of Response by Subregion by Session for males and females for: Pre-drug and load-up trials. Pre drug-trials are associated with a greater variability in FR-changes.

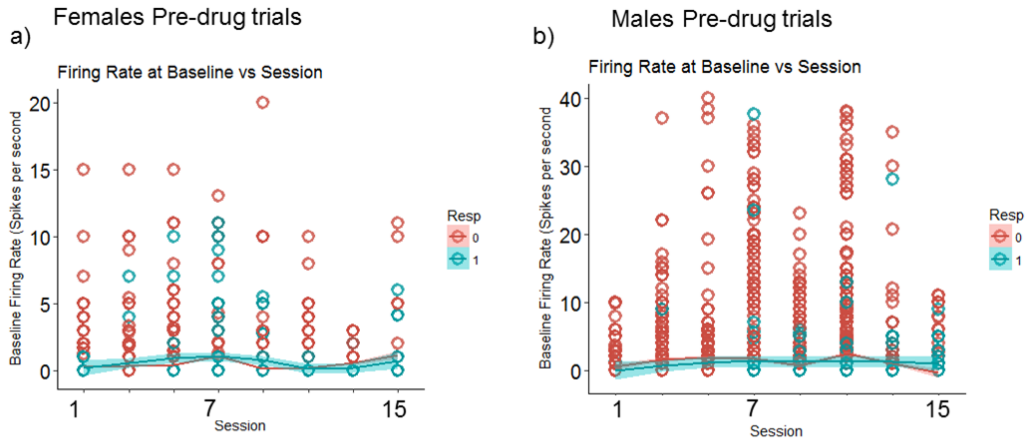

Figure 20. Baseline firing rates during "Hits"('Hit': 1) and "Misses"('Miss': 0) by Session during Pre-drug trials of males and females. There is a slight increase in activity for "Hits" for females on and prior to session 7

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7. TABLES

7. 1. Regression analysis on Latency to Response and Session of males.

	Estimate	Standard Error of the estimate	p-value
Intercept	27.0608	2.3103	<0.0001
Latency to Response	-0.0021	0.0006	<0.001
Session	1.1276	0.0241	<0.0001

Table 1. Results of regression of Latency to Response and Session for Males included in this study

7.2. Regression analysis on Latency to Response and Session of females.

	Estimate	Standard Error of the estimate	p-value
Intercept	20.5489	2.3103	<0.0001
Latency to Response	-0.0018	0.0004	<0.001
Session	1.4234	0.2586	<0.0001

Table 2. Results of regression of Latency to Response and Session for Females included in this study

7.3. Results of Anova on drug consumed per session vs estrous stage

	DF	Mean Sq	F value	p-value
Estrous Stage	4	353	1.51	0.20
Residuals	90	232		

Table 3. Results of ANOVA on drug consumed per session, vs estrous stage.

7.4. Congruence between video-scored FRs and 200ms FRs

Video Duration Category	Mean Video Scored FR	Proportion of 0 Firing Rates	Correlation with 200 ms FRS (Pearson's R)
<300 ms	0.856	0.907	0.97
300-500 ms	0.903	0.838	0.92
500-700 ms	0.796	0.805	0.86
700-900 ms	0.868	0.733	0.83
900-1000 ms	0.611	0.754	0.83

Table 4. Congruence between FRs computed based on video-analysis and fixed "200ms" interval.

7.5. Estimated effects per experimental cell

Response	Subregion	Sessions	Mean	SE	Dataset
Miss	Core	Early, Core	0.374	0.175	Pre-Drug, Males
Miss	Core	Late, Core	0.488	0.153	Pre-Drug, Males
Hit	Core	Early, Core	0.253	0.386	Pre-Drug, Males
Hit	Core	Late, Core	0.589	0.209	Pre-Drug, Males
Miss	Shell	Early, Shell	0.484	0.173	Pre-Drug, Males
Miss	Shell	Late, Shell	0.582	0.168	Pre-Drug, Males
Hit	Shell	Early, Shell	0.288	0.391	Pre-Drug, Males
Hit	Shell	Late, Shell	0.924	0.208	Pre-Drug, Males
Miss	Core	Early, Core	0.447	0.196	Pre-Drug, Females
Miss	Core	Late, Core	0.140	0.233	Pre-Drug, Females
Hit	Core	Early, Core	0.430	0.325	Pre-Drug, Females
Hit	Core	Late, Core	0.500	0.259	Pre-Drug, Females
Miss	Shell	Early, Shell	0.450	0.167	Pre-Drug, Females
Miss	Shell	Late, Shell	1.700	0.167	Pre-Drug, Females
Hit	Shell	Early, Shell	0.340	0.167	Pre-Drug, Females
Hit	Shell	Late, Shell	0.620	0.167	Pre-Drug, Females
Miss	Core	Early, Core	0.225	0.109	Load-Up Females
Miss	Core	Late, Core	0.159	0.117	Load-Up Females
Hit	Core	Early, Core	0.238	0.144	Load-Up Females

Hit	Core	Late, Core	0.278	0.134	Load-Up Females
Miss	Shell	Early, Shell	0.564	0.099	Load-Up Females
Miss	Shell	Late, Shell	0.519	0.099	Load-Up Females
Hit	Shell	Early, Shell	0.618	0.122	Load-Up Females
Hit	Shell	Late, Shell	0.894	0.109	Load-Up Females
Miss	Core	Early, Core	0.499	0.102	Load-Up Males
Miss	Core	Late, Core	0.397	0.104	Load-Up Males
Hit	Core	Early, Core	0.526	0.128	Load-Up Males
Hit	Core	Late, Core	0.437	0.113	Load-Up Males
Miss	Shell	Early, Shell	0.426	0.107	Load-Up Males
Miss	Shell	Late, Shell	0.308	0.113	Load-Up Males
Hit	Shell	Early, Shell	0.572	0.127	Load-Up Males
Hit	Shell	Late, Shell	0.632	0.115	Load-Up Males

Table 5. Estimate of means per each of the combinations of levels in experimental design and each phase of SA: Pre-drug trials and Load-up trials.