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DOUBLE DISSOCIATION OF THE ANTERIOR AND POSTERIOR DORSOMEDIAL CAUDATE-PUTAMEN IN THE ACQUISITION AND EXPRESSION OF ASSOCIATIVE LEARNING WITH THE NICOTINE STIMULUS

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by

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Tobacco use is the leading cause of preventable deaths worldwide. This habit is not only debilitating to individual users but also to those around them (second-hand smoking). Nicotine is the main addictive component of tobacco products and is a moderate stimulant and a mild reinforcer. Importantly, besides its unconditional effects, nicotine also has conditioned stimulus effects that may contribute to the tenacity of the smoking habit. Because the neurobiological substrates underlying these processes are virtually unexplored, the present study investigated functional involvement of dorsomedial caudate putamen (dmCPu) in the conditioning processes with nicotine as a conditioned stimulus. Rats were trained using the discriminated goal-tracking task where nicotine injections (0.4 mg/kg; SC) were paired 100% of a time with intermittent (36 per session) sucrose deliveries; sucrose was not available on alternative saline days. Pre-training excitotoxic or post-training transient lesions of anterior or posterior dmCPu were

used to elucidate the role of these areas in acquisition or expression of associative learning with nicotine stimulus. Pre-training lesion of p-dmCPu inhibited acquisition while post-training lesions of p-dmCPu attenuated the expression of associative learning with the nicotine stimulus. On the other hand, post-training lesions of a-dmCPu evoked nicotine like responding following saline treatment indicating the role of this area in disinhibition of learned motor behaviors. These results for the first time show the role of a- and p-dmCPu in various stages of associative learning using nicotine stimulus and provide an initial account of neural plasticity underlying these learning processes

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I would like to thank my mentor and advisor Dr. Rick Bevins for his tremendous help throughout my graduate studies at the University of Nebraska-Lincoln. His insight and guidance helped to shape my identity as a researcher and educator in general. I also would like to thank all the members of my dissertation committee, Dr. Daniel Leger, Dr. Ming Li, and Dr. You Zhou for their thoughtful comments and guidance throughout the process of conceptualizing and executing the work presented in this dissertation. And finally, I would like to thank my wife, Augusta Jamin, for her love, help, and moral support all throughout these tumultuous times of being a graduate student.

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CHAPTER 1

INTRODUCTION

Preface

Tobacco use is the leading cause of preventable deaths worldwide (WHO, 2011). This habit is not only debilitating to individual users but also to those around them (e.g., second-hand smoking). Nicotine is the main addictive component of tobacco products and is a moderate stimulant and a mild reinforcer. Importantly, besides its unconditioned effects, nicotine also has conditioned stimulus effects that may contribute to the tenacity of the smoking habit. Investigation of learning processes involving nicotine as a conditioned stimulus (CS) is an understudied area relevant to nicotine dependence. Understanding these associative processes with the interoceptive effects of nicotine is of importance in order to develop a comprehensive theory of addiction and, hence, develop better prevention and treatment strategies. Excitatory conditioning with nicotine stimulus, including its neurobiological etiology, has been one of the less studied areas of nicotine dependence. This chapter will provide rationale for this dissertation project by detailing behavioral and neurobiological mechanisms which are known to contribute, or theorized to be involved, in the excitatory learning with the nicotine stimulus. Furthermore, this section will also propose a functional approach to elucidating neurobiological substrates involved in the critical phases of associative learning with the nicotine stimulus.

The ensuing chapters will detail two experiments of this dissertation. In general, the focus of this dissertation work is to better understand the conditioned stimulus effect of nicotine and specifically its neurobiological substrates. The neural substrates of the nicotine-evoked conditioned response (CR) are essentially unexplored (only one study from our laboratory). The experiments in this dissertation will begin to identify areas mediating acquisition and expression of the nicotine-evoked CR, setting the foundation for future studies detailing neural processes governing learning with nicotine as a CS.

Our laboratory has extensively studied associative learning with nicotine stimulus (for reviews please see Bevins *et al*, 2012; Bevins and Murray, 2011; Bevins and Palmatier, 2004) and recently, we have started investigating involvement of neural mechanisms in this excitatory learning with nicotine. Specifically, the CR evoked by nicotine CS preferentially induced c-Fos expression in the dorsomedial regions of rat's caudate-putamen (Charntikov *et al*, 2012). Importantly, expression of c-Fos protein among rats challenged with nicotine on the test day was dependent on learning history with nicotine (nicotine as a CS vs. non-CS nicotine control). One of the limitations of that study is that c-Fos expression does not provide functional evidence for the role of the dorsomedial caudate-putamen (dmCPu) in the control of the nicotine-evoked responding. Rather, c-Fos expression provides a correlational account of heightened neuronal activity in the area. Therefore, experiments presented in this dissertation will provide a comprehensive and systematic assessment of this area

as a possible mediator of the nicotine-evoked CR. This assessment includes programmatic investigation of the role of this area in the acquisition and expression of conditioned responding controlled by the CS effect of nicotine. Findings from these experiments will fill an important gap in the scientific literature related to the neurobiological processes potentially contributing to the tenacity of tobacco dependence. Such an understanding will aid in formation of comprehensive theories of addiction that encompass conditioning processes involving interoceptive conditioning with drugs.

Associative Learning with Nicotine as a Conditioned Stimulus

Associative learning is the area of psychology investigating processes involved in the formation of association between stimuli or a behavior when they are presented together. Most of the knowledge about associative learning is derived from animal studies. Animal research provides a vital foundation for understanding basic biology, biology of diseases, cognition, and mental disorders, to name a few. The value of animal research in elucidating underpinnings of learning has been noted more than a century ago by Thorndike (1898), who wrote: *"The main purpose of the study of the animal mind is to learn the development of mental life down through the phylum, to trace in particular the origin of human faculty"*. Thorndike, who studied associative learning using animal models, played a vital role in the development of a theory of associative learning based on his studies of instrumental conditioning (Thorndike, 1898). In his experiments, hungry animals (cats, dogs, or chicks) were placed in the enclosures

equipped with a manipulandum (lever, hanging cord, or a platform) which, if activated, released the door and provided access to food. Thorndike (1898) argued that improvement in escape time over the number of trials was the evidence of strengthening an association between the act leading to escape (e.g., pressing a lever) and food outside the enclosure. Thus learning, according to Thorndike and later adopted by Hull (1943), is the strengthening of association between stimulus and response, where response is followed by a reward. Though this view of learning, where learning is thought to represent strengthening of stimulus-response connections, is still prominent to this day, the focus gradually has been expanded to stimulus-stimulus associations. Furthermore, new models began to emerge that attempted to explain learning behavior. For example, Skinner's (Skinner, 1969) view differed from S-R or S-S formulations as he theorized that learning, in a form of operant conditioning, establishes relationship between behavior (B), context (A), and consequences (C). Thus the AB relationship is reliant upon C and represents the effect of antecedent conditions on behaviors. This antecedent-behavior-consequence relationship has been termed "three term contingency".

Understanding stimulus-stimulus connections is the primary focus of Pavlovian conditioning researchers. Pavlovian conditioning allows greater control over the training parameters in contrast to instrumental learning where animals are in control of the delivery of reward through their actions (e.g., a lever press). In Pavlovian conditioning, where stimulus (e.g., sound of metronome) is paired

with reward (e.g., food), experimenter is in control of each stimuli presentation. The evidence of learning in the Pavlovian conditioning is the acquired conditioned response. In this case, a salivation to the auditory stimulus. Thus Pavlovian learning is naturally viewed as a strengthening of stimulus-stimulus connections which serves as a basis of a prominent Rescorla and Wagner (1972) associative learning model. In this elegant model, the magnitude of conditioned response depends on the strength of stimulus-stimulus (CS-US) connection and is further explained by a mathematical model predicting the change in the strength of this connection based on the parameters derived from a conditioning trial.

Pavlovian conditioning is an important aspect of associative learning because it provides a mechanism by which humans and non-human animals can adapt to biologically significant events (Hollis *et al*, 1989). Unfortunately, this learning can be maladaptive as it serves as a basis for abnormal behaviors like anxiety disorders (Bouton, 2000) and drug abuse (Siegel, 1989) to name a few. Like the exteroceptive stimuli discussed above, interoceptive stimuli can also come into association with other stimuli in the environment, subsequently changing the behavior of the organism (Bevins *et al*, 2012; Bevins *et al*, 2011; Bevins *et al*, 2004; Charntikov *et al*, 2012; Wooters *et al*, 2009). This section of the Introduction will further detail the associative learning with pharmacologically induced interoceptive stimuli and will focus in particular on the associative learning with nicotine as an interoceptive stimulus.

Nicotine is the principle addictive component of tobacco. When a smoker inhales vapors of combusted tobacco or vaporized nicotine, nicotine's physiological effects (the unconditioned stimulus - US) can come into association with a variety of stimuli that co-occur (CSs; e.g., throat irritation, smell or taste of tobacco, situational cues, etc.). After a number of pairings, these conditioned stimuli are able to induce cravings and induce relapse in those trying to stay abstinent (Niaura *et al*, 1992; Payne *et al*, 1991; Tiffany and Drobes, 1990). However, as mentioned above, nicotine (or nicotine-induced interoceptive effect) is also able to function as a CS. In our laboratory, we have established a protocol where the interoceptive stimulus effects of nicotine come to guide rat's anticipatory approach to a location where reward (the US) has occurred in the past (i.e., goal-tracking; Boakes, 1977; Farwell and Ayres, 1979). In this discriminated goal-tracking task (DGT), rats receive nicotine (the CS) paired with intermittent access to sucrose (the US); on intermixed saline days sucrose is not available. Across sessions, nicotine comes to evoke a goal-tracking CR (Besheer *et al*, 2004; Murray and Bevins, 2007b; Palmatier *et al*, 2005). Behaviorally, this learning follows many of the postulates of Pavlovian conditioning (Murray and Bevins, 2011a; Murray *et al*, 2009) and likely simulates conditioning processes in human smokers (Glautier *et al*, 1996). For example, following consumption of a tobacco product, nicotine's interoceptive effects (CS) can come into association with commonly co-occurring appetitive USs (e.g., post-meal satiety, alcohol, coffee, work breaks, social interaction, stress relief, etc.). In this model, after

repeated nicotine CS-US pairings, nicotine alone would be able to evoke an appetitive conditioned response (CR) that likely contributes to the tenacity of the nicotine addiction.

Our laboratory has made considerable efforts in elucidating behavioral and neuropharmacological processes mediating the CS effects of nicotine (see Bevins *et al*, 2012; Bevins *et al*, 2011). For example, a number of nicotine doses (0.05 - 0.4 mg/kg) can function as a reliable nicotine cue in the discriminated goal-tracking task (Besheer *et al*, 2004; Murray and Bevins, 2007a; Murray *et al*, 2007b; Polewan *et al*, 2013; Wilkinson *et al*, 2006). Nicotine can serve as a stimulus indicating presence (CS+) or absence (CS-) of the reward (Besheer *et al*, 2004; Murray *et al*, 2011b). The magnitude of the conditioning effect with nicotine stimulus in the discriminated goal-tracking task depends on the salience of the nicotine stimulus (nicotine dose), salience of the US (sucrose concentration), and the number of CS-US (nicotine-sucrose) pairings during the training session (Murray *et al*, 2007a; Murray *et al*, 2007b; Murray *et al*, 2009; Wilkinson *et al*, 2006). Withholding reward after a period of training (extinction) results in gradually diminished conditioned responding over repeated daily extinction sessions (Besheer *et al*, 2004). Extinction rates of nicotine-evoked conditioned responding also depend on the salience of nicotine stimulus (nicotine dose) during the training phase (Murray *et al*, 2007b) and the number of reward presentations during training (Wilkinson *et al*, 2006). In these extinction tests, rats trained with higher nicotine dose (0.4 mg/kg) show more

persistence during extinction (higher rates of goal-tracking) than rats trained with lower nicotine doses (0.1 and 0.2 mg/kg; Murray *et al*, 2007b). Goal-tracking is also more resistant to extinction with more nicotine-sucrose pairings during the training (Wilkinson *et al*, 2006). Importantly, learning with nicotine as an appetitive conditioned stimulus is not state-dependent (see Bevins *et al*, 2007). The state-dependent theory proponents may argue that performance in the discriminated goal-tracking task (DGT) is specific to the physiological state and the responding is dependent on the association between contextual cues in the testing environment and the unconditioned reward. Under this premise, increased goal-tracking on the nicotine days is a result of nicotine stimulus facilitating recall of reward availability in this particular setting (i.e., chamber). In the context of DGT task, state-dependent theory predicts that shift from one training state to different test state would disrupt elevated goal-tracking . However, rats receiving sucrose in either nicotine or saline state do not show disruption in goal-tracking when tested in the alternate state [e.g., trained in nicotine - tested in saline and vice versa (Bevins *et al*, 2007)]. This effect renders state-dependent assumption very unlikely and supports the notion that nicotine functions as an interoceptive conditioned stimulus in the discriminated goal-tracking task.

Receptor Substrates Involved in Learning with Nicotine Stimulus

In order to better understand mechanisms contributing to chronic tobacco use and nicotine dependence, there is a need for a better understanding of

neurobiological processes modulating learning with nicotine as an interoceptive stimulus. At the moment, there is a limited understanding of neural substrates involved in learning with the nicotine stimulus. What is currently known about neurobiology of learning with nicotine stimulus comes from our general understanding of nicotine pharmacodynamics, instrumental learning, and Pavlovian conditioning studies with the nicotine stimulus. Nicotine exerts its pharmacological actions by binding to nicotinic acetylcholine receptors (nAChRs). Nicotinic acetylcholine receptors comprise of various homomeric or heteromeric combinations of twelve distinct α and β subunits (α_2–α_{10} and β_2–β_4). Most of research assessing receptor specificity of the nicotine stimulus has used the two-lever operant drug discrimination paradigm (Smith and Stolerman, 2009; Wooters *et al*, 2009). In this task, rats learn to discriminate which lever will be reinforced based on the availability of the drug induced interoceptive stimulus. For example, when rats are pretreated with nicotine prior to the training session, one of the levers (let's say right) will be reinforced with food on some schedule of reinforcement. On the other non-drug days, when rats are pretreated with saline, response on the other lever (left) will be reinforced on a comparable schedule. The behavior is said to be under the control of interoceptive stimulus of the drug when the internal drug cue evokes appropriate lever responses at least 80% of the time. Food can be eliminated as a control stimulus by testing the response in extinction where food is not available and thus cannot be used as a stimulus to guide the behavior.

A number of studies, using two-lever drug discrimination procedure, established that the interoceptive stimulus effects of nicotine are primarily mediated by the centrally located nAChRs. In the operant discrimination studies, nornicotine, a primarily centrally active nicotinic agonist and dopamine transporter inhibitor (Middleton *et al*, 2007), dose-dependently substitutes for the interoceptive stimulus effect of nicotine whereas the peripherally active nicotinic agonist - methylcarbamylcholine does not (Desai *et al*, 1999). That is, nornicotine evokes at least 80% of nicotine-appropriate lever responding during the substitution test. Moreover, centrally and peripherally acting nAChR antagonists like mecamylamine and dihydro-β-erythroidine (DHβE) fully block nicotine-appropriate responding. On the other hand, antagonists (chlorisondamine or pentolinium) that do not readily cross the blood brain barrier do not block nicotine-appropriate responding unless administered intracerebroventricularly (Kumar *et al*, 1987).

To further understand the pharmacological specificity of the nicotine stimulus, a number of ligands selective for various nicotinic receptors subtypes have been tested using this two-lever discrimination task. One of the nAChR combinations that seems to be critical for the detection of the nicotine stimulus is the α4β2-containing receptor subtype. nAChR agonists like TC-2559, ABT-594, and A-85380 have relatively high specificity for the α4β2-containing receptors and fully substitute for nicotine's interoceptive effects (Smith *et al*, 2007). Partial agonists for α4β2-containing receptors like cytisine and varenicline partially

generalize for the nicotine stimulus; they dose-dependently evoke higher rates of nicotine-appropriate responding but only to a maximum of about 60%. Although cytisine is also active at the α3β4 and varenicline at the α7 receptor subtypes (Smith *et al*, 2007). On the contrary, agonists with specificity to the α3β4 (WO 03/062224) or α7 (WO 01/60821A1, GTS-21) receptor subtypes do not evoke nicotine-appropriate responding suggesting their limited role in neuropharmacology of the nicotine stimulus (Smith *et al*, 2007). In sum, two lever discrimination studies effectively demonstrated that nicotine's interoceptive stimulus effects are mediated by the centrally located nicotinic receptors amongst which α4β2-containing receptor subtype seems to play a critical role in the perception of this stimulus.

Another approach to studying the neuropharmacology of the nicotine stimulus is to use the previously described DGT task. A variety of receptor types and subtypes have been assessed for substitution for the nicotine stimulus using this task. These substitution studies yielded mostly comparable results to the findings from the two-lever drug discrimination studies (Murray *et al*, 2007a; Murray *et al*, 2009; Reichel *et al*, 2010; Struthers *et al*, 2009; Wooters *et al*, 2009). Following a period of training with nicotine stimulus using DGT task ligands can be tested for their generalization to the nicotine stimulus. In these brief substitution tests (4 min), a ligand is administered prior to test session and the goal-tracking response is assessed in the absence of sucrose reward. Full substitution is declared when a ligand evokes goal-tracking response comparable

to nicotine. Using this substitution protocol, ABT-418, varenicline, and nornicotine fully generalize to nicotine as they evoked goal-tracking comparable to nicotine (Reichel *et al*, 2010). Although these ligands do not bind exclusively to a single receptor type, the neurophamacology of their effect can be inferred from their binding profile. For example, ABT 418 receptor subunit specificity for nAChRs is $\alpha4\beta2 > \alpha3\beta4 > \alpha3\beta2 > \alpha7$ (Hahn *et al*, 2003), while varenicline binds to $\alpha4\beta2 > \alpha3\beta4 > \alpha7$ (Smith *et al*, 2007). In the same way, nornicotine binds to $\alpha6/3\beta2\beta3 > \alpha7 > \alpha4\beta2 > \alpha3\beta4 > \alpha3\beta2h\alpha5 > \alpha3\beta2\beta3 > \alpha3\beta2$ nAChRs (Papke *et al*, 2007). Inferring from the receptor binding profile of these ligands, the $\alpha4\beta2$ and $\alpha3\beta4$ subtypes seem to be critically involved in the expression of the goal-tracking response evoked by the nicotine stimulus. On the other hand, the $\alpha7$ receptor subtype does not seem to contribute to the nicotine's stimulus effect because the $\alpha7$ antagonist MLA does not block nicotine-evoked goal-tracking response (Struthers *et al*, 2009). In concordance with the two-lever discrimination studies, these effects appear to be centrally mediated because nicotine-evoked conditioned response in the DGT task can be antagonized by the centrally and peripherally nAChR antagonist mecamylamine and not by the hexamethonium - a mostly peripheral nAChR antagonist (Besheer *et al*, 2004; Struthers *et al*, 2009).

Neurobiology of the Nicotine Stimulus

Current understanding of the neurobiological loci involved in mechanisms mediating nicotine effects is largely derived from studies investigating acute,

chronic, or primary reinforcing effects of nicotine on the central nervous system (Balfour, 2009; Placzek and Dani, 2009). The reinforcing effects of nicotine, and subsequent dependence, have been linked to nicotine's ability to induce mesolimibic dopaminergic tone. Like many other drugs of abuse (Di Chiara *et al*, 1992; Koob, 1992; Robinson and Berridge, 1993; Wise, 1996), nicotine stimulates ventral tegmental area (VTA; Calabresi *et al*, 1989; Clarke *et al*, 1985; Grenhoff and Johnson, 1996; Mansvelder and McGehee, 2002; Pidoplichko *et al*, 1997; Wooltorton *et al*, 2003) which gives rise to mesocortical and mesolimbic pathways releasing dopamine at the end terminals (Figure 1). Mesocortical projections innervate prefrontal cortex by the way of nucleus accumbens, while efferent fibers of mesolimbic pathway connect to nucleus accumbens, hippocampus, and amygdala. The ventral tegmental area and nucleus accumbens are predominant sites of nicotine actions when its direct rewarding effects are investigated (Corrigall and Coen, 1989; Corrigall *et al*, 1994; Corrigall *et al*, 1992; Di Chiara, 2000). For example, interruption of dopaminergic input from ventral tegmental area to nucleus accumbens, or antagonism of intraccumbal dopaminergic receptors, blunt nicotine self-administration (Corrigall *et al*, 1989; Corrigall *et al*, 1994; Corrigall *et al*, 1992; Di Chiara, 2000). Albeit a number of other limbic areas are activated by either acute or chronic nicotine treatment (Pagliusi *et al*, 1996), their involvement in the nicotine evoked stimulus effect is unclear.
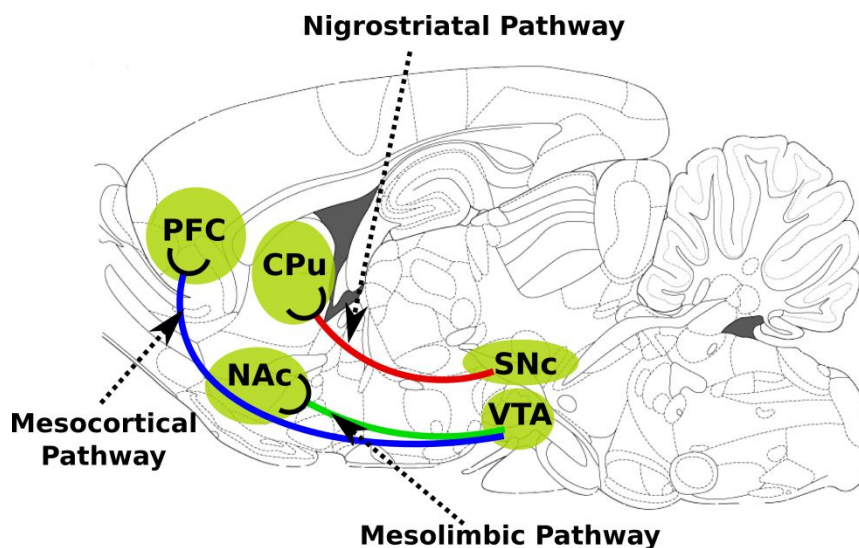
Figure 1. Graphical representation of three major dopaminergic pathways projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc; Mesolimbic Pathway), from VTA to the prefrontal cortex (PFC; Mesocortical Pathway), and from substantia nigra pars compacta (SNc) to the caudate putamen (CPu; Nigrostriatal Pathway).

Although nicotine's rewarding effects in the central nervous system have been extensively studied, the neural loci involved in mechanisms mediating interoceptive stimulus effects of nicotine remain an understudied areas of research. To this date, there are scant published reports from two-lever operant drug discrimination field investigating neural substrates involving the nicotine stimulus. Initial reports confirmed that centrally located nAChRs mediate nicotine's interoceptive effects (Chance *et al*, 1978; Miyata *et al*, 2002; Schechter, 1973). Infusions of nicotine directly into the lateral ventricle of the brain, causing infusate to disperse indiscriminately throughout the central nervous tissue, substitute for the nicotine stimulus in the two-lever task (Chance *et al*, 1978;

Miyata *et al*, 2002; Schechter, 1973). When nicotine is infused into the dorsal hippocampus, it evoked partial substitution in some studies (Meltzer and Rosecrans, 1981; Shoaib and Stolerman, 1996), but failed to do so in other studies (Miyata *et al*, 2002). Partial substitution for the nicotine stimulus was also observed when nicotine was infused into the ventral tegmental area, whereas full substitution was seen after nicotine infusion into the medial prefrontal cortex (Miyata *et al*, 2002). On the other hand, there were mixed results showing the role of the nucleus accumbens, an area critically involved in reward and motivation (Everitt *et al*, 2001; Ito *et al*, 2004; Schultz, 1998; Wise, 2002), in mediation of the nicotine stimulus. Shoaib and Stolerman (1996) reported that infusion of 1-8 µg of nicotine into the nucleus accumbens did not prompt nicotine appropriate responding. In contrast, Miyata *at al.*, (2002) had full substitution though at much higher infusion doses (i.e., 20-40 µg). Although limited, these published reports indicate that nicotine's interoceptive effects: a) are centrally located, b) engage the mesolimbic system, and c) are largely mediated by the medial prefrontal cortex - an area involved in decision making and executive functions.

Very little is known about neurobiology of the conditioned stimulus effects of nicotine. One of our recent projects began to elucidate the neurobiological loci involved in appetitive conditioning with the nicotine stimulus (Charntikov *et al*, 2012). In that experiment, magnitude of rapidly developing c-Fos protein was used as a measure of neuronal activity and a marker of area activation. Rats in

the main condition of interest (see group nicotine-CS in Figure 2) reliably

acquired a differential goal-tracking CR controlled by the nicotine CS. That is,

throughout training (32 total daily sessions), nicotine administration (0.4 mg

base/kg; SC) for this group was paired 100% of a time with intermittent access to

sucrose (36 per session); sucrose was not available on intermixed saline days.

Two additional carefully designed conditions served as controls. One control

condition (chamber-CS) had equal exposure to nicotine and sucrose, but nicotine

was not reliably paired with the sucrose US (only half of nicotine sessions paired

with the sucrose US; 25% of all reinforced sessions). The second control

condition had exposure to nicotine in a manner identical to the nicotine-CS and

chamber-CS conditions; however, sucrose was never available for this CS-alone

control.
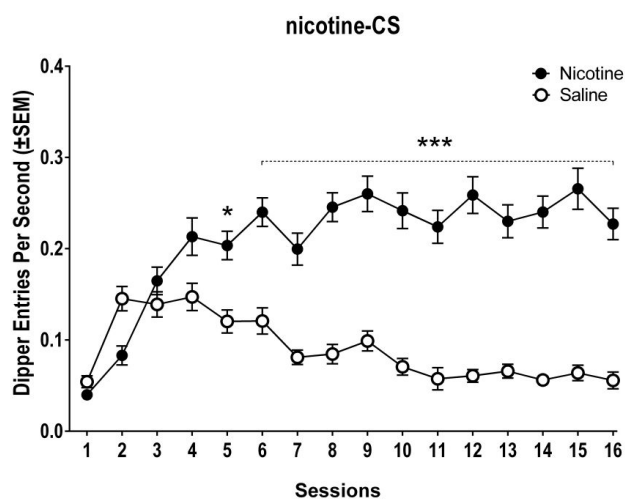
Figure 2. Mean (±SEM) number of dipper entries (goal-tracking evoked by a nicotine CS) during 2 min prior to initial sucrose delivery on nicotine sessions or equivalent time on saline sessions. Nicotine and saline sessions were administered on separate days and were pseudorandomly intermixed. *Significant from saline session(s) [*p<0.05, ***p<0.001]. Partially adapted from Charntikov *et al*, (2012).

Following training, rats in all conditions were challenged with either nicotine or saline (3 × 2; condition × test drug factorial design) and assessed in the absence of sucrose reward for their goal-tracking behavior during a brief 4-min test. Following the test, brains were removed and selected areas were processed for c-Fos immunohistochemsitry - a marker of neuronal activation. Nuclei selected for the c-Fos assessment represented brain regions implicated in the rewarding and/or incentive motivational effects of drugs of abuse [e.g., caudate-putamen, nucleus accumbens, ventral pallidum, ventral tegmental area, substantia nigra], learning and memory [e.g., hippocampus, amygdala], and executive and cognitive functions [e.g., prelimbic cortex, orbitofrontal cortex, anterior cingulate cortex] (Everitt and Wolf, 2002; Robbins, 2005; Robinson and Berridge, 2003). With these controls in mind, among rats challenged with nicotine, rats in the nicotine-CS condition (i.e., those expressing a nicotine-evoked CR) had significantly higher c-Fos expression in the medial CPu when compared to the chamber-CS and the CS-alone conditions (Figure 3).
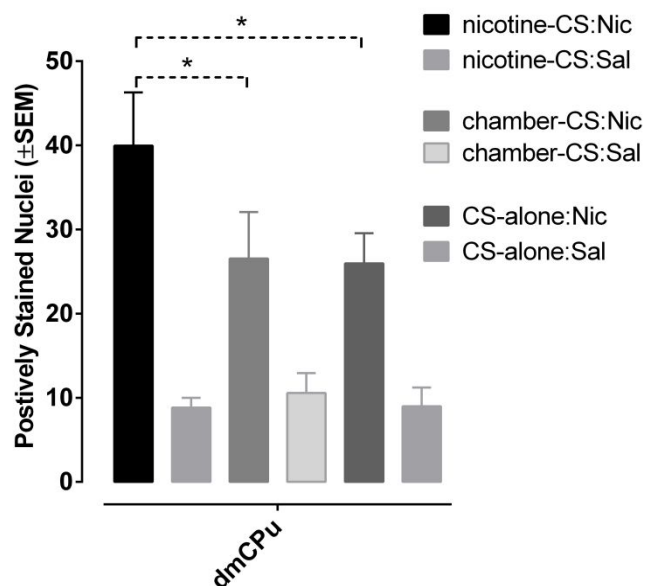
Figure 3. Means (±SEM) of positively labeled c-Fos nuclei in the dmCPu following a nicotine or saline challenge test. *Significant difference between groups indicated by dashed brackets (p <0.05). Partially adapted from Charntikov *et al*, (2012).

Results of this preliminary study provide a first account of possible neurobiological loci involved in conditioning processes with interoceptive stimulus effects of nicotine. Indeed, this area of the brain has been previously associated with stimulus-response (S-R) instrumental processes and has been argued to be critically involved in acquisition of automatic or habitual responding (Everitt and Robbins, 2005; Everitt *et al*, 2002; Ito *et al*, 2000; Ito *et al*, 2002). The elegant work by Everitt and others, combined with our initial c-Fos findings, lead us to believe that it is very likely that the dmCPu is functionally involved in conditioning mechanisms (learning and expression of the CR), when nicotine serves as a CS, guiding appetitively-motivated behaviors. These preliminary

findings placed us in a strong position to test the hypothesis that dmCPu is the locus for acquisition and expression of nicotine-evoked CR. Testing of this hypothesis is the chief goal of this dissertation.

Role of Caudate Putamen in Reward Processes

The basal ganglia is a group of nuclei located in the base of the forebrain spreading from telencephalon, to diencephalon and midbrain. These nuclei are interconnected through a set of networks receiving major excitatory input from the cerebral cortex and further relaying the predominantly inhibitory output from the striatum, via direct or indirect pathways, projecting to the complex of nuclei comprised of substantia nigra pars reticulata (SNr) and internal segment of globus pallidus (GPi; Figure 4). The basal ganglia is thought to be involved in a variety of processes including locomotion, cognition, reward, motivation, and learning (Albin *et al*, 1989; Graybiel *et al*, 1994; Kimura, 1995; Knowlton *et al*, 1996; Schultz, 1998). Rodent striatum, consisting of both caudate and putamen, is one of the most prominent structures within basal ganglia. Because there is no clear distinction between caudate and putamen in rodents, unlike what is seen in primates (Hassani *et al*, 2001; Hauber, 1998), the structure is commonly referred to as caudate-putamen (CPu). Caudate-putamen is a large subcortical structure, often divided to anterior and posterior compartments, involved in modulation of a major excitatory inputs from the cerebral cortex, amygdala, substantia nigra, and thalamus (cf. Figure 4; Hauber, 1998; Kelley *et al*, 1982). On the other hand, efferent neurons of the CPu, projecting to the output structures, release a primary

inhibitory γ-amino-butyric acid (GABA) neurotransmitter at their terminals with a combination of neuropeptides including substance P, dynorphine, and enkephalin. The activity of inhibitory efferent projections is mediated by the D1 (D1, D5) and D2 (D2, D3, D4) families of dopaminergic receptors. These two families of dopamine receptors give rise to two distinct pathways: direct – D1, and indirect – D2 activated. Therefore, neurons of the direct pathway project directly from CPu to the SNr and are activated by stimulation of a D1 family of dopaminergic receptors while neurons of the indirect pathway project from CPu to the external segment of the globus pallidus (GPe) and are activated by stimulation of the D2 family of dopaminergic receptors. Thus, the location and the functional connections of caudate-putamen indicate its integral role in the integration, mediation, and modulation of prominent afferent cortical and efferent sub-cortical signals.
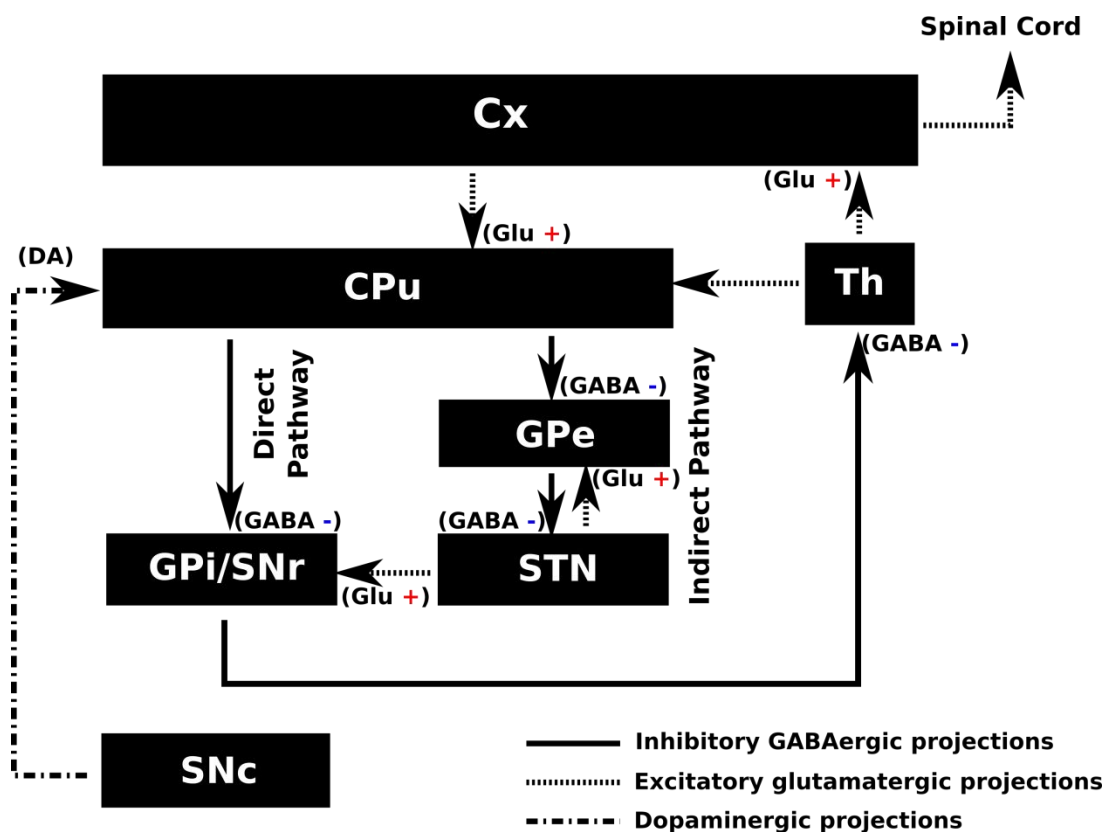
Figure 4. Graphical representation of circuitry in the basal ganglia. Cx, cerebral cortex; CPu, caudate putamen; GPe, external segment of globus pallidus; GPi, internal segment of globus pallidus; SNc, substantia nigra pars compacta, SNr, substantia nigra pars reticulata, Th, thalamus (partially adapted from Nambu, 2008).

Caudate-putamen plays a major role in mediating behaviors associated with motivation and reward. One of the canonical tests in evaluating a role of a particular brain region in the behavior of interest is a transient or permanent inactivation of that region, followed by a test of the behavior of interest. Though this procedure is not as precise as current cutting-edge optogenetic or designer receptor mediated inactivation techniques, it provides excellent gross assessment of the area involvement in the mechanism of interest. One of the most prominent behavioral tests currently available to assess the reinforcing effects of drugs is the

self-administration procedure. In drug self-administration, an operant response on the operandum (typically a lever in the operant chamber), under some schedule of reinforcement, results in intravenous infusion of the drug (Charntikov *et al*, 2013; Donny *et al*, 2000; Neisewander *et al*, 1996; Wise, 2002). After a period of training, animals will preferentially respond on the designated active lever, delivering a drug infusion after meeting a schedule requirement. In this protocol, rats are typically considered sufficiently trained when responding on the active lever is significantly higher than on the inactive lever and a number of infusions per session reaches a predetermined criterion. Selective inactivation or blockade of the ventral caudate-putamen, also referred to as nucleus accumbens, disrupts established self-administration of major drugs of abuse like cocaine and heroin (Ito *et al*, 2004; Pettit *et al*, 1984; Zito *et al*, 1985). For example, destruction of the dopaminergic terminals in the nucleus accumbens with 6-hydroxydopamine (6-OHDA) attenuated cocaine and, to lesser degree, heroin self-administration (Pettit *et al*, 1984). Self-administration of cocaine in these 6-OHDA lesioned rats was reduced to 30% of pre-lesion responding while self-administration of heroin gradually recovered to 76% of pre-lesion baseline. Furthermore, mesolimbic dopamine depletion disrupts cocaine self-administration, but does not disrupt food-reinforced behavior (Caine and Koob, 1994). This attenuation of cocaine self-administration through mesolimbic dopamine blockade seems to be specific to rewarding effects of cocaine and cannot be attributed to the general disruption of the operant behavior or reward

perception by this regional dopamine depletion manipulation.

Studies outlined above provide ample evidence that the mesolimbic dopamine pathway plays a critical role in mediating rewarding effects of various drugs of abuse. Though there is a limited number of studies investigating similar effects with nicotine, there is evidence that mesolimbic dopamine pathway may also mediate nicotine's reinforcing effects. For example, acute intravenous or subcutaneous injections of nicotine increases dopaminergic tone in the nucleus accumbens shell but not core (Cadoni and Di Chiara, 2000; Iyaniwura *et al*, 2001; Pontieri *et al*, 1996). Nicotine repeatedly administered (subcutaneously) non-contingently by the experimenter, overtime, sensitizes dopamine release in the core of the nucleus accumbens (Cadoni *et al*, 2000; Iyaniwura *et al*, 2001). On the contrary, when nicotine is self-administered by the animal it induces dopaminergic release in the shell portion of the nucleus accumbens (Lecca *et al*, 2006).

Role of Caudate Putamen in Learning Processes

The dorsal caudate putamen receives inputs from nucleus accumbens, prefrontal cortex, and substanta nigra pars compacta (SNc). Dorsal striatum has been identified as a critical area involved in encoding of reward prediction error in associative learning tasks. Schultz and colleagues (1998) elegantly demonstrated this effect in monkeys using common Pavlovian conditioning task. These experiments demonstrated that some subsets of neurons in anterior striatum of *macaca fascicularis* (macaque) monkeys initially activated (increased

responding) by the presentation of the unconditioned stimulus - juice or water delivery into the mouth. During subsequent training, a visual stimulus (CS) was paired with the delivery of reward. After repeated parings, neurons previously activated by the presentation of reward, gradually shifts their responding to the stimuli reliably paired with the reward (CS) and stop responding to the reward itself. After establishing reliable response of these neurons to the CS, omission of the reward or presentation of a conditioned inhibitor (stimulus reliably paired with the absence of reward) reduced the activity of these neurons.

In addition to the neurons that respond to conditioned stimuli, there are other neuronal ensembles that respond based on the reward expectation. For example, some neurons respond when occurrence of the reward is unpredicted; that is, there is no previous stimulus-reward association. Yet, other neurons stop responding when the reward is fully predicted by the CS. Finally, a third distinct subset of neurons depress their responding when previously predicted reward fails to occur following the CS. This neural plasticity associated with learning processes using natural rewards and reward predictors form a basis of prediction error theory. Rich data from these experiments played a critical role in understanding the neural plasticity underlying associative learning processes and provided a fertile ground for computational modeles investigating neural mechanisms of uncertainty (Schultz, 2004; Schultz, 2006; Schultz *et al*, 2008).

Dorsal striatum is also involved in mediation of goal-directed actions controlled by instrumental contingencies. Unlike Pavlovian stimulus-reward

learning, where from a procedural perspective a stimulus-reward association is established independently of the subject's behavior, in the instrumental learning paradigm, reward is contingent upon an experimentally prescribed response. Instrumental behavior can be further categorized into two classes: instrumental behavior controlled by the response-reward contingency, which is sensitive to the reward degradation, and habitual behavior, which is unaffected by the reward or outcome devaluation (Yin *et al*, 2008). Two regions of dorsal CPu are differentially involved in the goal-directed and habitual instrumental behaviors. Whereas dorsolateral region of the CPu (dlCPu) is involved in the habitual behaviors, the dmCPu controls goal-directed actions (Yin *et al*, 2004).

Previous research established that dorsal CPu is critically involved in the acquisition and expression phases of instrumental learning. For example, Yin *et al.* (2004) trained rats with excitotoxic or sham lesions to dlCPu to lever press for sucrose reward on the random interval schedule of reinforcement. In that experiment, rats in both groups were able to acquire appropriate lever-press responding, which on that schedule of reinforcement typically progresses to habitual behavior. In the next phase of experiment, sucrose was devalued using a conditioned taste aversion procedure. Both dlCPu and sham lesioned rats were then returned for extinction session to assess the effect of sucrose devaluation on lever responding. In these tests, rats with lesions to dlCPu responded less than sham controls; responding of sham controls was unaffected by the devaluation procedure. The unaffected level of responding by the sucrose devaluation is taken

to indicate habibual responding. Yin e*t al.* (2004) concluded that because lesioned rats modulated their lever responding based on the reward evaluation, dlCPu must be involved in the habit formation facilitated by the instrumental learning phase. Notably, this effect was specific to the dlCPu as lesions to the dmCPu, in a companion experiment, did not disrupt habitual performance following reward devaluation.

On the other hand, lesions to dmCPu impair acquisition of action-outcome association and subsequent sensitivity to reward devaluation (Yin *et al*, 2005b). Specifically, lesions to posterior part of dmCPu (p-dmCPu), and not anterior dmCPu (a-dmCPu), slowed initial acquisition of instrumental lever responding for the sucrose reward. Furthermore, rats with lesions to p- but not a-dmCPu were insensitive to subsequent reward devaluation and contingency degradation. In the contingency degradation tests, reward is delivered non-contingently of the instrumental response (free reward delivery) resulting in reduction of lever pressing in control rats but not in those with p-dmCPu lesions. In addition to mediating acquisition of instrumental behaviors, p-dmCPu is also involved in the expression of instrumental learning. For example, post-training lesions of p-dmCPu produced a drastic deficits in both tests of action-outcome contingency (reward devaluation and contingency degradation). In summary, the aforementioned series of experiments that were designed to elucidate the role of dorsal striatum in instrumental learning, concluded that dlCPu was involved in mediating habitual behaviors whereas dmCPu is involved in acquisition and

expression of goal-directed actions (for review see Yin *et al*, 2008).

Functional Approach to Understanding Neurobiological Substrates

of Nicotine CS

Tobacco users have many opportunities to experience nicotine's interoceptive effects paired with various appetitive unconditioned stimuli throughout the duration of their habit. As previously discussed, the interoceptive stimulus effects of nicotine can come into association with peer interactions, food, alcohol, work breaks, and other rewarding unconditioned stimuli. Later, nicotine alone may evoke an appetitive CR – an effect that could be a contributing factor to chronic smoking and nicotine dependence. As described earlier, in a rat model, nicotine stimulus is readily available to serve as a CS for appetitive rewards like liquid sucrose (Besheer *et al*, 2004; Bevins *et al*, 2011; Bevins *et al*, 2004). In these studies, after repeated pairings of nicotine (CS) with intermittent access to sucrose (US), nicotine acquires the ability to evoke a CR (anticipatory food-seeking response or goal-tracking). Furthermore, we found that the dorsomedial caudate putamen (dmCPu) was involved (as evident by elevated levels of c-Fos activation) in processing of this nicotine-evoked CR (Charntikov *et al*, 2012). The research in this dissertation work programmatically builds on these previous finding by examining more closely the role of dmCPu in the excitatory conditioning with the nicotine stimulus. Specifically, two Aims were designed to accomplish this goal:

Aim 1: Examine the involvement of the anterior or posterior dmCPu in the <u>acquisition</u> of the nicotine CS evoked CR

Aim2: Examine the involvement of the anterior or posterior dmCPu in the <u>expression</u> of the nicotine CS evoked CR

To accomplish Aim 1, we used excitatory NMDA lesions, along with sham controls, to permanently inactivate either a- or p-dmCPu prior to any experimental manipulations. Our preliminary studies indicate that lesion integrity stays intact for at least 30 days after the procedure - the duration of standard nicotine discriminated DGT training. That is, when lesion sites are stained for neuronal bodies (Anti-NeuN; EMD Millipore Chemicals, MA, USA) 30 days after the lesion is made, there is no reduction in size in comparison to 7 day old lesions. Following recovery after the inactivation surgeries, rats were trained using our standard DGT protocol with nicotine as the stimulus. All rats were able to acquire the discrimination, although, acquisition of rats with lesions to the posterior but not anterior dmCPu were more blunted relative to sham controls. That is, goal-tracking (primary dependent measure) of rats with lesions to the p-dmCPu were, throughout the training phase, generally lower than sham controls. This outcome indicates that the posterior and not the anterior dmCPu seems to be involved in the acquisition of the appetitive excitatory conditioning with nicotine as a conditioned stimulus.

To accomplish Aim 2, we first trained all rats using the DGT protocol with the nicotine as a CS and sucrose as a US. Following training, cannulae extending

to the a- or p-dmCPu were implanted and after a period of recovery all rats were once again retrained on the DGT task. Having cannulae permanently targeting the areas of interest, we were able to utilize mixed $2 \times 2 \times 2$ factorial design with region (a- or p-dmCPu) as between-subjects factors, and transient lesion (lidocaine or distilled water) and test drug (nicotine or saline) as the within-subjects factors. In this factorial design, all rats receiving anterior or posterior infusions (lidocaine or distilled water) would experience all possible lesion/test drug combinations (4 total infusions). Thus, on test days, lidocaine or saline was infused into a- or p-dmCPu prior to testing. Subsequently, goal-tracking was assessed following nicotine or saline injections in the brief 4-min test sessions during which sucrose was withheld. Temporary inactivation of a-dmCPu produced an increase in goal-tracking rates on saline sessions, yet it did not affect nicotine-evoked responding. This finding suggests that the a-dmCPu mediates acquired discriminated responding to the nicotine stimulus by inhibiting responding on non-reinforced saline sessions or removing inhibition in the nicotine state. Therefore, inactivation of a-dmCPu disinhibited (increased) conditioned responding on saline test days.

On the other hand, temporary inactivation of the p-dmCPu inhibited nicotine-evoked responding; the responding on saline test days was not affected. These results suggests that a- and p-dmCPu are differentially involved in the expression of responding maintained by the nicotine stimulus. That is, following extensive training with nicotine as conditioned stimulus and being exposed to

this training context on the test day, a-dmCPu inhibits conditioned responding

when nicotine cue is not detected. On the contrary, intact function of the p-

dmCPu is needed to facilitate expression of the nicotine-evoked responding.

CHAPTER 2

GENERAL METHODS

Subjects

Subjects were experimentally naive male Sprague-Dawley rats (total
n=105) purchased from Harlan Industries (275-290 g; Indianapolis, IN, USA).
Rats were housed individually in a temperature- and humidity-controlled colony
(12hr:12hr light:dark cycle; lights on at 6 am). Water access was freely available
in the home cages; access to chow (Harlan Teklad Rodent Diet; Harlan,
Indianapolis, IN, USA) was restricted to maintain rats at 85% of their free-
feeding body weight. This 85% target weight was increased by 2 g every four
weeks from beginning of the study. The night before and for two days following
surgery, food was freely available. Rats in all experiments were handled for a
minimum of 2 min per each of three consecutive days before all experimental
procedures. Experimental protocols were approved by the University of
Nebraska-Lincoln Institutional Animal Care and Use Committee.

Apparatus

Behavioral testing was conducted in commercially available chambers
(ENV-008CT; Med Associates, Inc., St. Albans, VT, USA) enclosed in sound- and
light-attenuating cubicles equipped with an exhaust fan. Each conditioning
chamber had aluminum sidewalls, metal rod floors with polycarbonate front,
back, and ceiling. A recessed receptacle (5.2 × 5.2 × 3.8 cm; l × w × d) was

centered on one of the sidewalls. A dipper arm, when raised, provided access to 0.1 ml of 26% (w/v) sucrose solution in the receptacle. Access to the dipper was monitored by an infrared beam mounted 1.2 cm into the receptacle and 3 cm above the chamber floor. Beam breaks for dipper entries were monitored using Med Associates interface and software (Med-PC for Windows, version IV).

Drugs

(−)-Nicotine hydrogen tartrate, buprenorphine hydrochloride, and sodium pentobarbital (Sigma; St. Louis, MO, USA) were dissolved in 0.9% saline. NMDA and lidocaine hydrochloride (Sigma) were dissolved in sterile distilled water. Nicotine pH was adjusted to 7.0 ± 0.2 with a dilute NaOH solution. Nicotine dose (reported as base) and the 5 min injection-to-placement interval was selected based on previous research (Charntikov *et al*, 2012; Murray *et al*, 2007a; Murray *et al*, 2007b).

Discriminated Goal-Tracking Task

Rats were injected with 0.4 mg/kg nicotine subcutaneous (SC) for three consecutive days before training to attenuate initial locomotor suppressant effects of nicotine (Besheer *et al*, 2004; Charntikov *et al*, 2012). For each daily training session, all rats were injected SC with either nicotine (0.4 mg/kg) or saline 5 min before placement in the conditioning chamber for a 20-min session. During training, each rat received equal number of nicotine and saline sessions. Sessions were assigned using a unique pseudorandom order of nicotine and

saline sessions for each rat with the condition that no more than two of the same session type occur in a row. On nicotine sessions, the interoceptive stimulus effects of nicotine were paired with intermittent access to sucrose. Access to sucrose was initiated between 124 to 152 s from the start of the session with 4 possible onset times randomized throughout the training phase. There were 36 separate 4-sec deliveries of sucrose per nicotine session. Time between sucrose deliveries ranged from 4 to 80 s (mean = 25 s) and was intermixed for each session. For intermixed saline sessions, sucrose was withheld.

Testing

To test the effects of lesions in Aim 2 on the nicotine-evoked goal-tracking, rats were injected with either nicotine or saline 5 min before the start of the test session and placed in the conditioning chambers for 4 min. Dipper entries and locomotor beam crosses were recorded, but sucrose was withheld.

Surgical Procedures

Permanent dmCPu Inactivation. Rats were anesthetized with 1 ml/kg ketamine (100 mg/ml)/xylazine (20 mg/ml) mixture (2:1 ratio; IM) and placed in the stereotaxic apparatus (David Kopf Instruments, CA, USA). Two bilateral craniotomies were performed and NMDA (0.5 μl/side; 0.12 M [~17.65 mg/ml] concentration) or vehicle (distilled water) was injected into either anterior (A/P +1.2, M/L ±1.6, D/V +4.2) or posterior dmCPu (A/P -0.36, M/L ±2.4, D/V +4.2) (coordinates from Paxinos and Watson, 2007; NMDA dose and coordinates

adapted from Yin *et al*, 2005). Injections were made using a 28 gauge cannula (Plastics One, Roanoke, VA, USA) attached via tubing to a Hamilton microsyringe (10 µl; Reno, NV, USA) mounted on a single infusion pump (Fisher Scientific; Pittsburg, PA, USA). Infusions were made at a constant rate of 0.1 µl/min and cannula was left in place for an additional 5 min. Anesthesia was terminated using IM injection of 0.5 mg/kg atipamezole diluted in saline (Charntikov *et al*, 2013; Wee *et al*, 2006). Buprenorphine hydrochloride (0.1 mg/kg) was injected SC immediately following surgery and the next day (am and pm) for pain management.

Cannulae Implantation. Anesthesia and scull preparation for craniotomies were performed as described above. Stainless steel single guide cannulae (22 gauge; Plastics One, Roanoke, VA, USA) were implanted 2 mm above the anterior or posterior dmCPu (see coordinates above). Guide cannulae were fixed in place using stainless steel anchor screws, cyanoacrylate gel, and followed by dental cement. Stainless steel stylets (Plastics One) were used to seal guide cannulae until the time of infusion. Post-surgical care was administered as described above.

Transient dmCPu Inactivation

Lidocane dose and its infusion volume for this experiment were selected from previously published studies to functionally block an area in size comparable to dmCPu (area of interest for this study) with high inactivation rate (>90% of neurons) within that area (Hiranita *et al*, 2006; Kantak and Nic

Dhonnchadha, 2011; Sandkuhler and Gebhart, 1984; Sandkuhler *et al*, 1987;

Tehovnik and Sommer, 1997). Lidocaine (100 µg/0.5 µl/side) or vehicle (distilled

water/0.5 µl/side) were infused in a room distinct from the testing environment

and especially equipped for this procedure. Stainless steel stylets were replaced

by 28 gauge infusion cannulae (Plastics One) which extend 2 mm below the guide

cannulae. Hamilton microsyringes (10 µl), attached to two single infusion pumps

(Fisher Scientific, Pittsburg, PA, USA), bilaterally infused assigned solution over

3 min and were left in place for additional 2 min after infusion.

Histology

All rats were overdosed with sodium pentobarbital (150 mg/kg) at the end

of the experimental procedures and then transcardially perfused with ice cold

0.9% saline immediately followed by 4% paraformaldehyde. Brains then were

rapidly removed, post-fixed (4% paraformaldehyde) for an additional 24 hrs, and

cryoprotected in 30% sucrose for another 72 hrs. Immediately after, brains were

flash-frozen on dry-ice and stored at -80° C until sectioning. Coronal sections (40

µM) were taken using cryostat microtome (Leica CM-1900, Nussloch, Germany)

and stored for no more than 48 hrs in 0.02 M phosphate-buffered saline (PBS)

containing 0.1% sodium azide. Coronal sections of lesioned site from Experiment

1 were further processed for Neu-N immunolabeling while coronal sections of

lesions from Experiment 2 were stained with thionin. Images of stained lesioned

areas were taken with a light microscope (Olympus CX41RF microscope, Japan)

and assessed for cell loss (Experiment 1) or for tissue damage (Experiment 2)

from surgically implanted cannulae in order to verify their location. Detailed staining procedures and tissue assessment techniques are further described in their respective chapters.

Prior to Experiment 2, we conducted a small dye dispersion study (anterior n=3; posterior n=2) to estimate the appropriate infusion volume based on the extent of vehicle dispersion 5 min after the infusion. Rats implanted with guide cannulae were infused with food dye diluted 1:10 in vehicle (distilled water) following exact procedures described above (see Testing subsection). Five minutes after the withdrawal of injectors and resealing guide cannulae with the stillets, rats were decapitated, their tissue was rapidly removed (< 1 min) and rapidly frozen on dry ice. Subsequently, frozen tissue was sectioned using cryostat microtome and the images of the exposed tissue revealing the dispersion sites were taken (see Figure 11 left panel).

Statistical Analysis

An omnibus analysis of variance (ANOVA) preceded all planned comparisons. Higher-order interactions were further analyzed by one- or multi-way ANOVAs and followed, if necessary, by multiple group post-hoc comparisons. Violations of Mauchly's tests of Sphericity were followed by Spericity corrections tests. Statistical significance for all tests was set to $p<0.05$. Specific analysis for each experiment is further described in their respective chapters.

CHAPTER 3

EXPERIMENT 1

THE INVOLVEMENT OF THE ANTERIOR OR POSTERIOR dmCPu IN THE

<u>ACQUISITION</u> OF LEARNING WITH THE NICOTINE STIMULUS

Based on our preliminary findings, dmCPu appears to be involved in the

expression of the CR evoked by the nicotine CS (Charntikov *et al*, 2012). This

finding is based on the measurements of expression of the immediate early gene

c-Fos – a marker of neuronal activity. Although increased c-Fos activity is enough

to ascertain elevated neuronal activity in the area, the assumption that this area is

functionally involved in the behavioral process is based merely on correlational

data. A more functional approach is needed to further elucidate its specific

involvement in the conditioning processes involving nicotine as a CS. Importantly,

the anatomical connections within anterior-posterior axis of rat dorsal striatum

are not homogeneous (Kelley *et al*, 1982) and can differ in their control of

learning and conditioning processes. For example, lesions to the posterior and

not anterior dmCPu disrupt acquisition and expression of goal-directed actions

(Yin *et al*, 2005a). On the other hand, anterior but not posterior dmCPu is

involved in latent inhibition (Jeanblanc *et al*, 2003), early learning stages

(Hikosaka *et al*, 1999), and reward encoding in primates (Samejima *et al*, 2005).

Because of this differential involvement of anterior and posterior regions of

dmCPu in various aspects of learning, Experiment 1 was designed to investigate

the role of anterior dmCPu and posterior dmCPu in the <u>acquisition</u> of the CR to

an appetitive nicotine CS.

Procedures

 Permanent dmCPu Inactivation. Following acclimation to the colony, rats received permanent excitotoxic (NMDA) or sham lesions of the a- or p-dmCPu (see general methods for details). Discrimination training commenced following 7 days of recovery from surgery.

 Discriminated Goal-Tracking Task. A 2 × 2 factorial design was used for this experiment with lesion (NMDA or vehicle) and region (a- or p-dmCPu) as between-subjects factors. All rats in this experiment received similar training with the nicotine stimulus where nicotine was reliably paired with access to sucrose and saline signaled non-reinforced sessions (see General Methods for details). Rats received 10 nicotine and 10 saline training sessions over 20 consecutive days.

 Histology. The day after the last training session, all rats were overdosed with sodium pentobarbital (150 mg/kg) and transcardially perfused with 0.9% saline following by 4% paraformaldehyde. Brains were rapidly removed and processed for the NeuN immunoreactivity as previously described (Charntikov *et al*, 2012; Zhao and Li, 2010). Briefly, following perfusion, tissue was post-fixed (4% paraformaldehyde) for an additional 24 hrs and then cryoprotected in 30% sucrose for another 72 hrs. Immediately after, brains were frozen on dry-ice and stored at -80° C until sectioning. Coronal sections (40 µM) were taken using

cryostat microtome and stored for no more than 48 hrs in 0.02 M phosphate-buffered saline (PBS) containing 0.1% sodium azide. For NeuN immunohistochemistry, brain sections were blocked for 1 hr with 10% normal horse serum (NHS; Vector Laboratories, CA, USA), 1% bovine serum albumin (BSA), and 0.3% Triton X-100 in 0.02 M PBS before 30-min incubation in 1.5% hydrogen peroxide and 50% methanol. Sections were then washed three times for 10 min in a wash buffer (0.02 M PBS containing 0.05% NHS and 0.3% Triton X-100). Sections were then incubated for 48 hrs at +4 $^{\circ}$C with anti-NeuN monoclonal primary antibody (clone A60; 1:5000 dilution; EMD Millipore Chemicals, MA, USA) diluted in PBS containing 0.3% Triton X-100, 1% NHS, and 1% blocking reagent (Roche Diagnostics, Mannheim, Germany). Following primary immunoreaction, sections were rinsed in a wash buffer three times for 10 min and incubated for 2 hrs on ice with a biotinylated horse anti-mouse secondary antibody (1:200 dilution; Vector Laboratories, CA, USA) diluted in PBS containing 1% NHS. Sections were then rinsed with 0.02 M PBS and incubated for 1 hr on ice with horseradish peroxide avidin-biotin complex (1:200 dilution; Vectastain Elite ABC Kit, Vector Laboratories) diluted in 0.02 M PBS. Immunolabled proteins were visualized with the aid of diaminobenzidine-based peroxide substrate (DAB Peroxidase Substrate Kit, Vector Laboratories) and mounted on gelatin-coated slides. Slides were air dried at room temperature, dehydrated in alcohol, cleared in xylene, and coverslipped with permount solution (Fisher Scientific, Fair Lawn, NJ, USA). Images of stained lesioned areas

were taken with a light microscope (Olympus CX41RF microscope, Japan; 4X)

and assessed for cell loss.

Statistical Analysis

During acquisition training there was no behavioral differences between

rats with sham lesions to either a- or p-dmCPu (no effect of Group and no Group

× Session interaction). Accordingly, they were combined into one sham group.

Thus, the 3 groups were: shams (n=17), a-dmCPu (n=13), and p-dmCPu (n=14).

Dipper entry rate prior to the first sucrose delivery, or equivalent time during

saline sessions, was used as a dependent measure. The effect of lesions on dipper

entry rates was first analyzed using omnibus 3 × 2 × 10 (Group × Drug × Session)

repeated measures ANOVA. Significant main effects were followed by separate 3

× 10 (Group × Session) ANOVAs for each drug condition (nicotine or saline).

Significant interactions were followed by the group mean comparisons to sham

controls (Tukey HSD).

In addition to the traditional group analysis described above, we used a

regression analysis to reveal the effect of individual lesion differences on the

acquisition of discrimination with the nicotine stimulus. This type of analysis

allows a better understanding of the role of independent measures on the

acquisition of discriminated learning with the nicotine stimulus. Because lesions

typically vary slightly in their position on the anterior-posterior axis, we used

individual Bregma position (based on the estimated center of the lesion) of each

lesion independent of the group assignment as a single continuous factor to

further investigate the nature of the effect. Using this approach, both a- and p-dmCPu groups were pooled together (n=27) and acquisition of goal-tracking CR was assessed as a factor of lesion placement on the anterior-posterior axis. Thus, difference in dipper entry rate prior to the first sucrose delivery, or equivalent time during no reward sessions, between each lesioned rat and a mean dipper entry rate of sham controls for each corresponding <u>nicotine</u> session was used a dependent measure. The difference score was calculated for each lesioned rat. The effect of lesion placement on this <u>difference</u> score was analyzed by fitting a linear model (Bregma × Session) and examining the fit using F-statistics. ANOVA of regression table followed regression analysis to determine significant predictor.

Results

Figure 5 shows the typical extent of the lesion sites for the a-dmCPu (A) and the p-dmCPu (C). Figure 5 also depicts variations of lesion placement and size (B and D). Although no volumetric analysis was performed, appropriate lesion placement was assessed by reconstructing NeuN stained lesions on the coronal atlas templates (Paxinos *et al*, 2007) and verifying that at least 75% of the lesion was localized to the predefined dorsomedial region. Lesions from all rats conformed to this criterion. Lesion placement on the anterior-posterior axis is depicted in Figure 6.
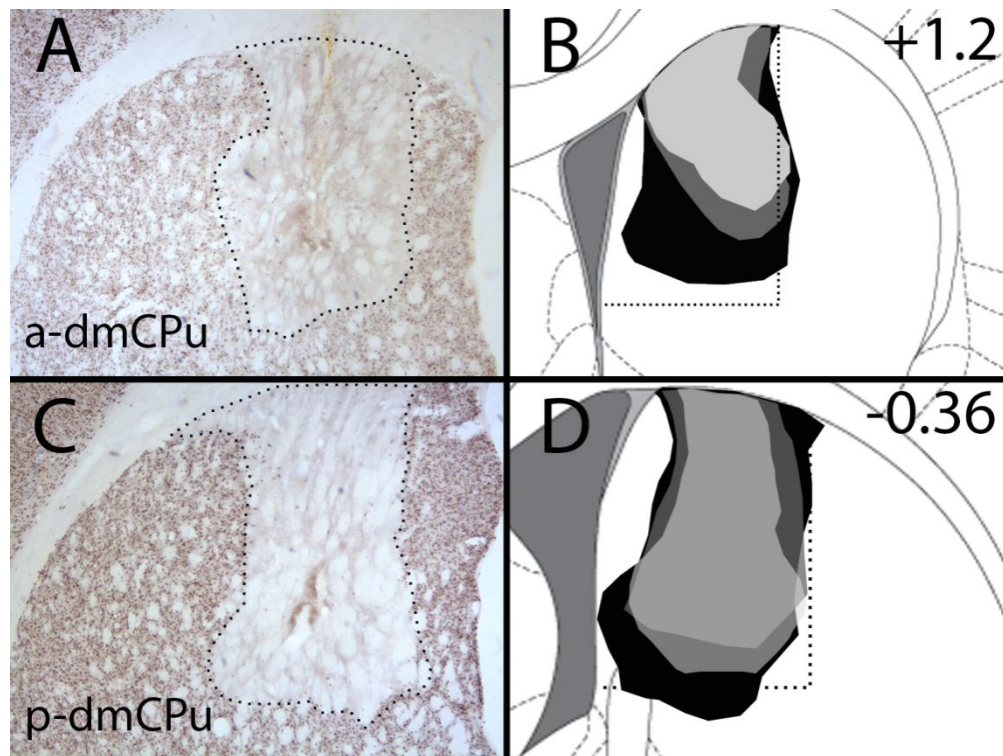
Figure 5. Photomicrographs of the representative NeuN stained (A) a-dmCPu and (C) p-dmCPu NMDA lesions. Dashed line traces the exact boundaries of the lesion sites. (B) Graphical illustration of the extent of lesions; black area represents largest extend of the damage and grey areas represent smaller lesion sites. Dashed line traces the arbitrarily predetermined dorsomedial target area and numbers indicate targeted Bregma position.
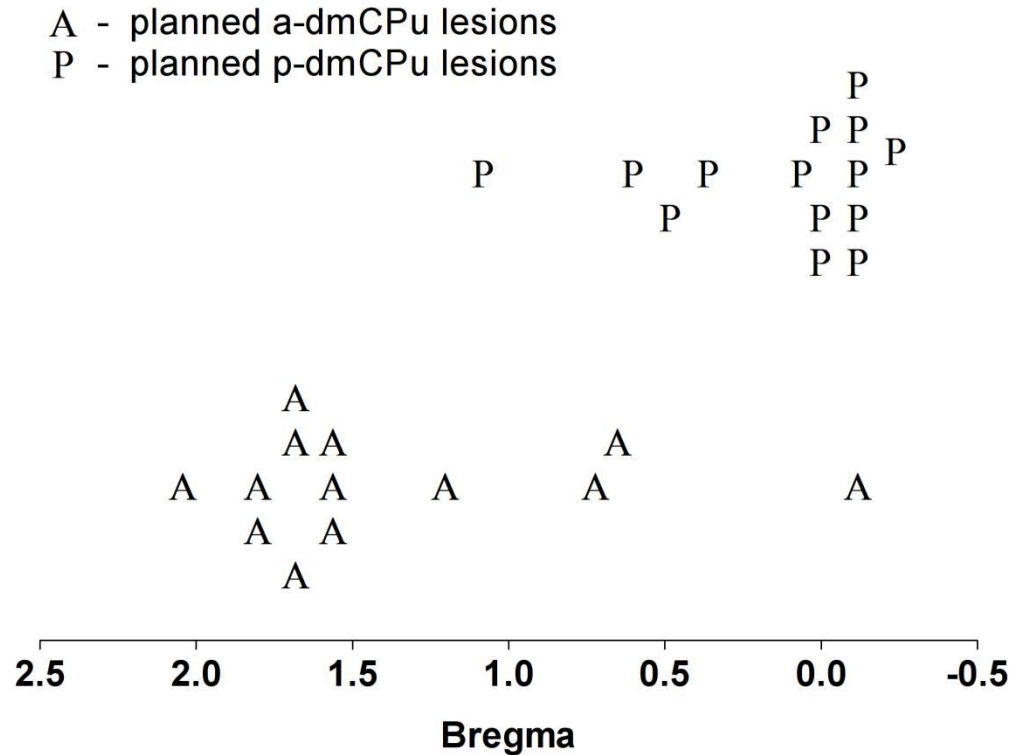
Figure 6. Distribution of planned anterior (A) and posterior (B) dmCPu lesions on a Bregma scale (Paxinos *et al*, 2007).

Group Effects. The omnibus ANOVA on the dipper entry rates during acquisition of nicotine discrimination revealed a main effect of Group [F(2,36)=4.71, p<0.05], a main effect of Drug [nicotine or saline; F(1,360)=288.52, p<0.001], and a significant Drug × Session interaction [F(9,360)=34.26, p<0.001]. A separate ANOVA of responding on nicotine sessions (analysis of nicotine acquisition curves) revealed a main effect of Group [F(2,360)=13.98, p<0.001] and a main

effect of Session [F(9,360)=26.89, p<0.001]. There was no Group × Session

interaction. Overall, responding of rats with lesions to p-dmCPu was lower than

responding of sham controls (Tukey HSD tests; Figure 7A). In comparison,

responding of rats with lesions to a-dmCPu did not differ from shams (Tukey

HSD tests). This outcome indicates that p-dmCPu and not a-dmCPu is involved

in the acquisition of interoceptive conditioning with the nicotine stimulus.

Furthermore, analysis of responding on saline sessions (Figure 7B) revealed the

effects of Group [F(2,360)=7.54, p<0.001], and Session [F(9,360)=13.72,

p<0.001], but no significant interaction. Group mean comparisons revealed that,

overall, responding in p-dmCPu was lower than sham controls (Tukey HSD tests)

further implicating p-dmCPu in the acquisition of the discriminated learning with

nicotine stimulus. Responding of rats with lesions to a-dmCPu did not differ from

shams on saline sessions.
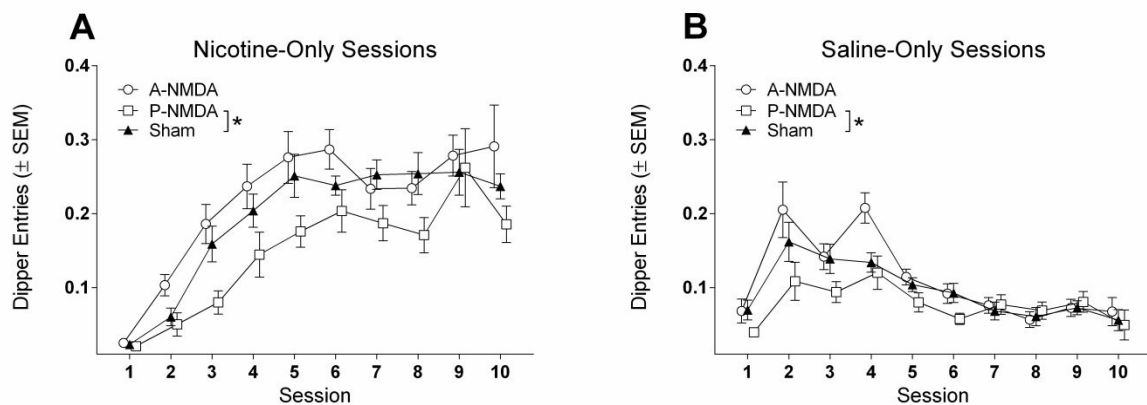
Figure 7. Nicotine (A) and saline (B) discrimination curves for groups of rats with NMDA lesions to a-dmCPu, p-dmCPu, and sham controls (sham).

Lesion Placement Effects. One of the inherent limitations associated with group

analysis is the minimization or exclusion of often important individual

differences. For example, the between-subjects or a group factor in Experiment 1

is the lesion placement (anterior vs. posterior dm-Cpu). Having lesion as a

between-subjects factor relies on the confidence of lesion placement at the

designated targets; with tighter group lesion clustering minimizing the error

variance. However, this type of experimental design often produces a greater

than anticipated distribution of the lesion placement on the anterior-posterior

axis. The variance on the anterior-posterior axis is often greater than the variance

on either lateral or ventral-dorsal axis because of a lack of definitive markers

across subjects on the Bregma scale. Bregma scale is originating at the Bregma

point on the skull (zero on the Bregma scale) where the coronal suture and the

sagital suture intersects. This intersection point is used as a landmark on the

anterior-posterior axis however its exact position in relation to the brain

structures often varies from subject to subject. Thus, this variation often

contributes to the greater than expected spread of the lesions on the anterior-

posterior axis. To use this variation to our advantage in this study, we

reconstructed each lesion placement on the Bregma scale and used the position

on this scale as one continuous variable instead of a between-subjects factor (i.e.,

lesion group). The creation of such a continuous variable allowed us to conduct

additional analyses and visualize individual behavioral differences as a factor of

lesion placement on the anterior-posterior axis.

Figure 8 shows the difference scores (see Statistical Analysis section for details) for each lesioned rat over the course of the training phase (8A) or plotted separately for each training session (8B). This aggregated data represents difference scores from every rat and every nicotine session which were further assessed using regression analysis. Regression analysis was used to test if lesion position on the anterior-posterior axis significantly predicted deviation of dipper entry rates from sham controls over the 10 training sessions. The results of regression indicated that Group and Session explained a significant proportion of variance in difference scores [R^2=0.18, $F(19,200)$=2.42, $p<0.01$]. ANOVA of regression table revealed main effect of Bregma [$F(1,200)$=26.49, $p<0.001$], no effect of Session, and no Bregma × Session interaction. Simplifying the model by removing non-significant factor (Session) revealed that lesion placement on the Bregma scale was a significant predictor of whether dipper entry rates would deviate from sham controls [β= 0.04, $t(218)$=5.12, $p<0.001$; Figure 8A]. Data plotted separately for each nicotine training session (8B) is presented for visual comparison only and was not a subject to by session analysis.
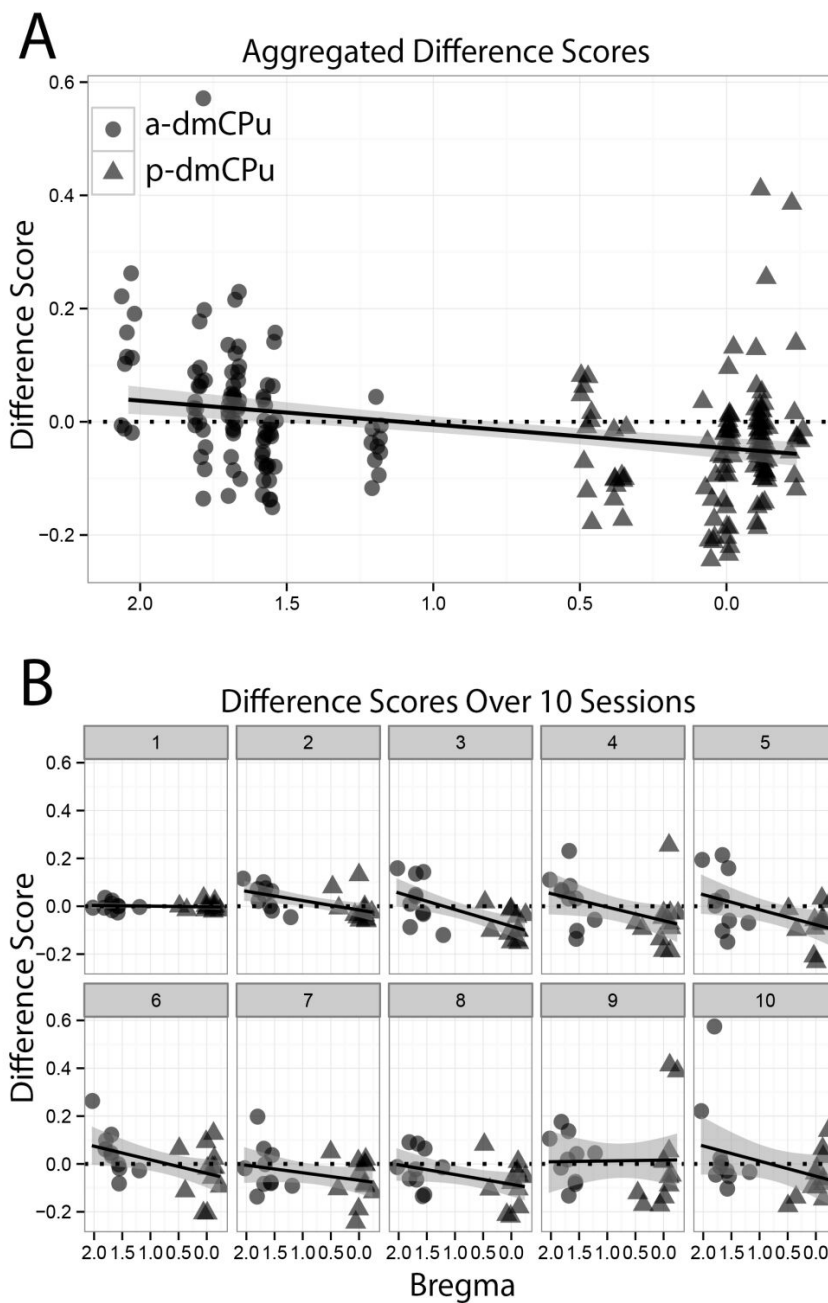
Figure 8. (A) Aggregated difference scores from all sessions and all lesioned rats. (B) Difference score from each lesioned rat for each training session. Dashed lines represent sham control like responding. Solid lines are fitted regression lines with a semi-transparent band representing a 95% confidence of fit interval.

Another way to visualize individual data obtained from lesioned rats in Experiment 1 is to use a heat map approach. With this approach, individual difference scores are plotted as colors and the variance is represented by the gradient value of single or multiple hues. We used this approach to visualize the variance of acquisition learning with nicotine stimulus as a factor of lesion placement on the Bregma scale. Figure 9 shows aggregated difference score for each lesioned rat, represented as a blue-white-red gradient color, which is mapped horizontally on the Bregma scale of a sagittal atlas plate (Figure 9).
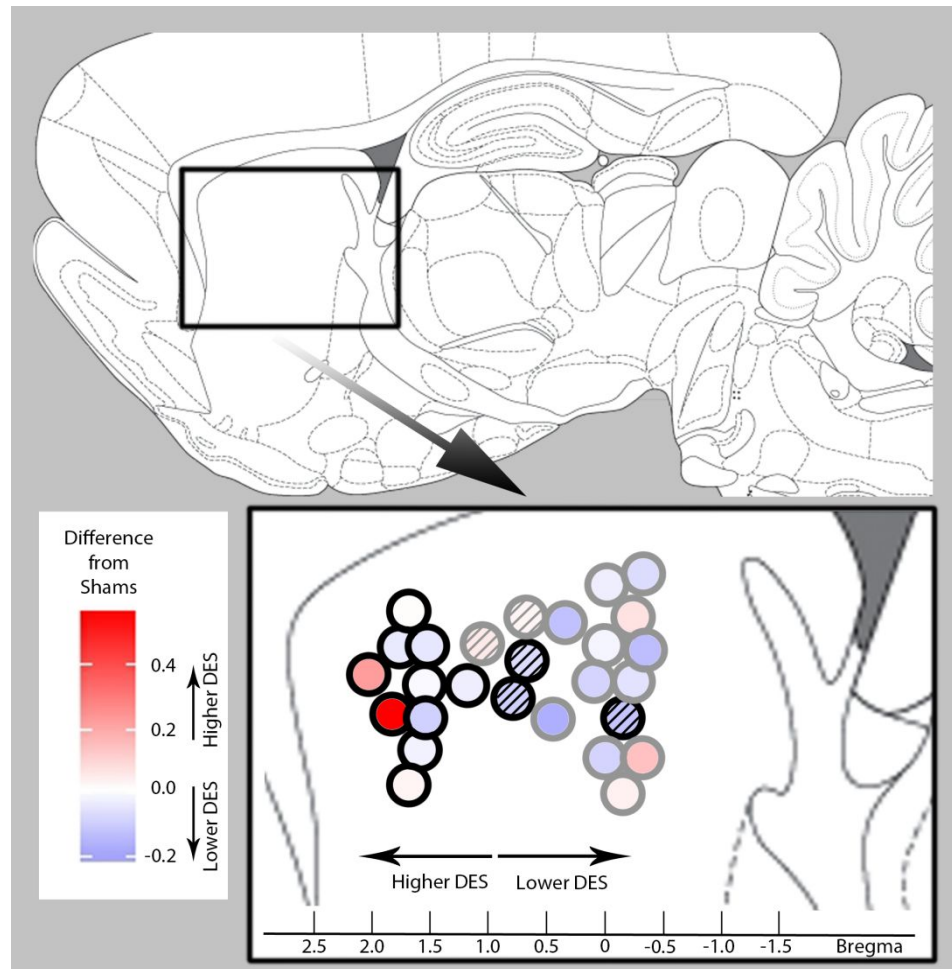
Figure 9. Heat map of the aggregated difference score for each lesioned rat. Each circle represents accurate lesion placement on anterior-posterior axis (Bregma). Position of circles representing lesion sites on the ventral-dorsal axis is not an accurate representation and was performed to better visualize each rat datum without obstruction. Black circles represent rats with lesions to a-dmCPu and gray circles represent rats with p-dmCPu lesions. Fill color of the circles indicates the magnitude of an aggregated over all training sessions difference score (see methods for details) from sham controls. White color represent control-like responding, red hue represents higher than control responding, and blue hue represents lower than control responding (consult color scale on left). Circles shaded with 45 degree lines represent rats removed from the group but not regression analysis.

Summary

All rats acquired the discrimination between nicotine and saline. Responding of sham controls replicates typical acquisition pattern of nicotine discrimination from our laboratory (Charntikov *et al*, 2012; Murray *et al*, 2007a; Murray *et al*, 2007b, 2011a). Dipper entry rates of rats with p-dmCPu lesions were overall lower than sham controls. Responding of rats with lesions to a-dmCPu did not differ from shams. These findings suggest that the p-dmCPu is involved in acquisition of learning with nicotine as an interoceptive conditioned stimulus. Our results parallel finding of Yin's (2005b) study where lesions to p-dmCPu slowed acquisition of instrumental lever training maintained by sucrose. This outcome suggests that at least early stages of Pavlovian and instrumental learning share common neural substrates (p-dmCPu).

CHAPTER 4

EXPERIMENT 2

THE INVOLVEMENT OF THE ANTERIOR OR POSTERIOR dmCPu IN THE

<u>EXPRESSION</u> OF THE NICOTINE CS EVOKED CR

Schultz and colleagues (1998; 2006) (1998, 2006) elegantly demonstrated that in monkeys neurons located within dorsal CPu regions can be activated by the conditioned stimuli previously associated with an appetitive reward. Furthermore, it appears that the dorsal CPu and not nucleus accumbens (Acb) mediates cue-activated drug-seeking in rats with chronic cocaine self-administration history (Vanderschuren and Everitt, 2005; Vanderschuren, 2005). During cocaine-seeking behavior maintained by the presentation of a light stimuli previously paired with cocaine (lever pressing results in light stimuli presentation on a schedule identical to self-administration but no cocaine is available), dopamine levels are elevated in the dorsal CPu, but not in the core or a shell of nucleus accumbens (Ito *et al*, 2000; Ito *et al*, 2002). Moreover, dopamine receptor blockade in the dorsal CPu, but not the AcbC, dose-dependently attenuates cocaine-seeking (Vanderschuren, 2005). These findings lend support to the hypothesis that as drug use progresses from the initial stages to the dependence state, the behavior depends less on nucleus accumbens and progressively more on dorsal CPu. This transition could be indicative of the role of the dorsal CPu in habitual stimulus-response processes (Berke and Hyman, 2000; Everitt *et al*, 2005; Tiffany, 1990; Vanderschuren, 2005). Whether or not

similar mechanisms are involved in the expression of CR evoked by a nicotine CS is unclear. As detailed earlier, dmCPu is involved in nicotine-evoked CR, but the evidence for this effect is only correlational (i.e., c-Fos expression) and it is unknown whether or not dmCPu directly mediates this CR evoked by the nicotine CS. Experiment 2 will answer this question by examining the role of the two distinct areas of dmCPu, either anterior or posterior, in the expression of CR evoked by the nicotine CS.

Procedures

All rats were initially trained for 28 days (14 paired sessions) to discriminate nicotine using the DGT task. The procedures used in this phase were identical to training in Experiment 1 and described in detail in the General Methods section. Following the training phase, rats were cannulated (a- or p-dmCPu; see General Methods for details) and given 7 days of post-surgery recovery. After recovery, rats were retrained for 10 days (5 paired sessions). Transient inactivation tests occurred after following this initial retraining with additional retraining sessions in between each test (see Figure 10 for experimental time-line). On the test day, rats in anterior and posterior groups were microinjected with either lidocaine or distilled water (see General Methods for details). Five minutes after intracranial microinjections rats were systemically injected with either nicotine or saline. Following systemic injection (5 min later), rats were placed in the conditioning chamber for a brief 4-min test during which dipper entries were recorded but sucrose was not available.
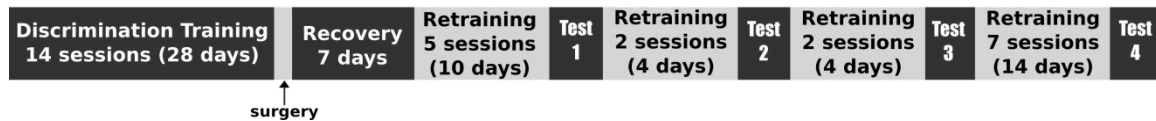
Figure 10. Procedural progression and the time line for the Experiment 2

Histology. The day after the last test, all rats were overdosed with sodium pentobarbital (150 mg/kg) and tissue was prepared for histological assessment as described in Experiment 1 (i.e., including perfusion, post-fixing, and sectioning). Brain sections with visible cannulae tracks were stained with thionin, dehydrated in alcohol, cleared in xylene, and coverslipped with permount solution (Fisher Scientific, Fair Lawn, NJ, USA). Images of sections with best representation of cannulae placement were taken with a light microscope (Olympus CX41RF microscope, Japan; 4X) and assessed for accuracy of placement.

Statistical Analysis

DGT Training. Dipper entry rate prior to the first sucrose delivery, or equivalent time during no reward sessions was assessed using 2 × 14 (Drug × Session) repeated measures ANOVA (n=30). Significant interactions were followed by Bonferroni's multiple-comparisons tests.

DGT Retraining. Dipper entry rate prior to the first sucrose delivery, or equivalent time during no reward sessions was assessed by separate ANOVAs (2 × 5, 2 × 2, 2 × 2, and 2 × 7; Drug × Session; see Figure 10 for retraining time-line) for each lesion condition [a-dmCPu (n=15) or p-dmCPu(n=15)]. Significant interactions were followed by Bonferroni's multiple comparisons tests for each

session (nicotine vs. saline).

Transient dmCPu Inactivation Tests. To assess the effect of transient dmCPu inactivation separate ANOVA for each lesion condition (a- or p-dmCPu) were performed. Total dipper entries during brief 4-min tests were analyzed by 2 × 2 (Drug × Infusion) ANOVAs. Dipper entry means (nicotine vs. saline) for each Infusion condition (lidocaine or distilled water) were further analyzed by the planned Fisher's Least Significant Difference (LSD) tests.

Lesion Placement Effects. Similar to variation in lesion placement on the anterior-posterior axis observed in the Experiment 1, placement of guide cannulae and subsequently a placement of the injector tip varied from subject to subject (within the limitations of predefined a- or p-dmCPu) allowing us to capture this variation. Thus, the position of the injector on the Bregma scale (see rational for Lesion Placement Effects in Experiment 1) was used a continues variable instead of a two level between-subjects factor.

Similar to regression analysis of lesion placement in Experiment 2, the effect of lesion placement (a-dmCPu and p-dmCPu including) on nicotine-evoked or saline responding, following transient inactivation, was assessed using linear regression analysis. Total dipper entries per session was used as a dependent measure and lesion location on the saggital plane (Bregma) was used as a predictor. Regression outcomes were further analyzed using ANOVA to determine F-statistics and significance.

Results

Figure 11A-C represents cannula placement and the extent of vehicle and dye dispersion, 5 min following cannula withdrawal, from a preliminary dye dispersion study (see General Methods for details). Both anterior and posterior regions were tested for the die dispersion with results showing predominate (<80%) coverage of the predefined areas. Figure 11B-D shows representation of the predefined dorsomedial region (shaded grey) and the acceptable cannula placement within its boundaries. All rats had cannulae placement in the predefined dorsomedial areas.
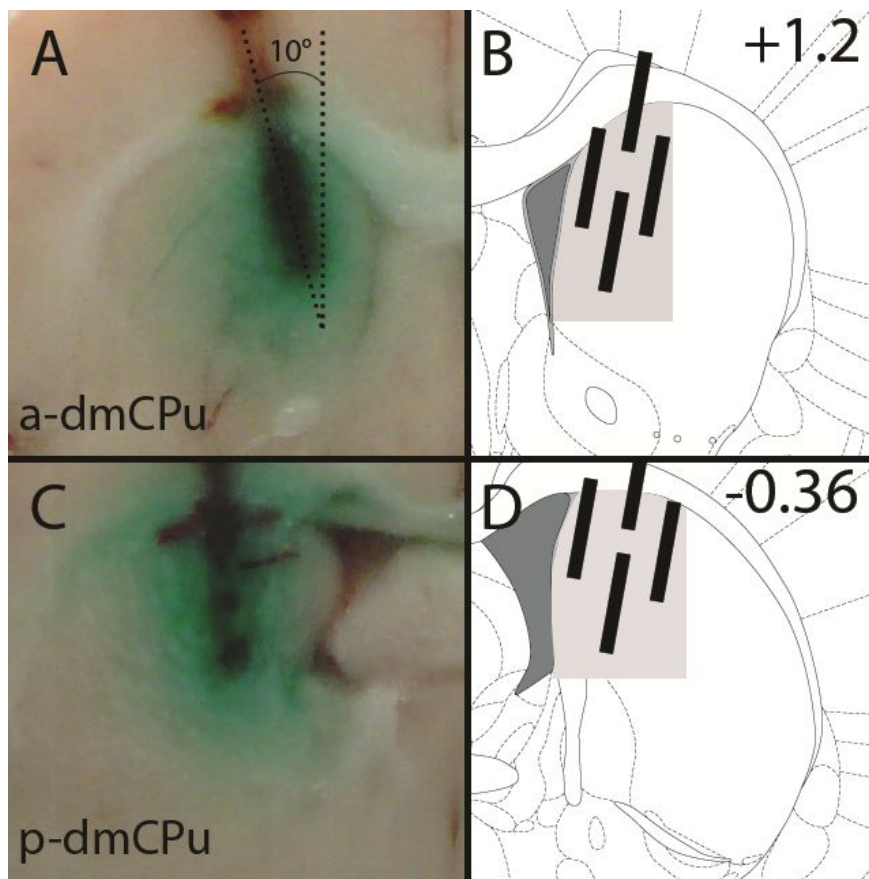
Figure 11. Photomicrographs of vehicle/dye dispersion in the (A) a-dmCPu or (C) p-dmCPu, 5 min following infusion. Dashed lines indicate 10° angle of cannulae placement for a- and p-dmCPu. (B and D) Graphical representation of the targeted area, shaded in grey, and the acceptable deviations of injector placement on the medial-lateral and ventral-dorsal axis. Numbers indicate targeted Bregma position.

DGT Training. Over the 14 sessions of nicotine discrimination training the analysis of dipper entry rates revealed significant main effects of Drug [$F_{(1,29)}=156.26$, $p<0.001$], Session [$F_{(13,377)}=10.70$, $p<0.001$], and significant Drug × Session interaction [$F_{(13,377)}=27.65$, $p<0.001$]. Responding on nicotine sessions 2 and 5-14 was higher than on corresponding saline sessions (Bonferroni's tests; Figure 12A).
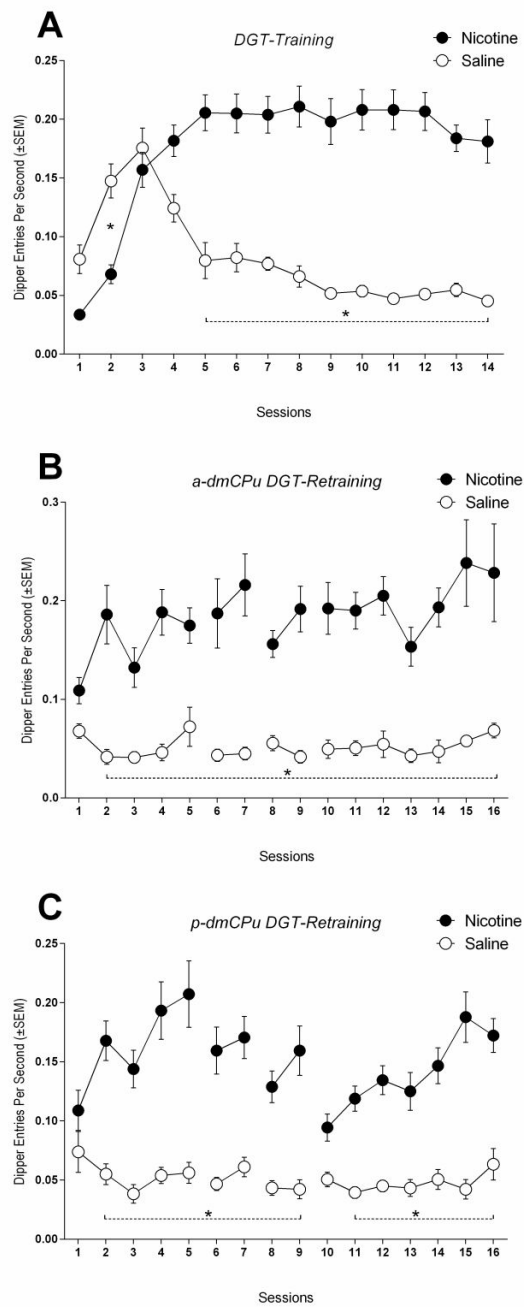
Figure 12. Dipper entry rates (±SEM) during initial training phase (A) and intermittent retraining following cannulae implantation for rats with a-dmCPu (B) and p-dmCPu (C) cannulae placements. *Denotes significant differences between corresponding saline and nicotine sessions.

a-dmCPu *condition: Retraining.* There was significant effect of Drug on retraining blocks 1-4, significant effect of Session on retraining block 1; their interaction was significant on blocks 1-3 (see Table 1 for main effects and interaction summaries). Over the 4 separate retraining blocks (Figure 12B) responding on nicotine sessions 2 to 16 was higher than on corresponding saline sessions (Bonferroni's tests). Thus, all rats were sufficiently retrained for each inactivation test.

p-dmCPu *condition: Retraining.* There was significant effects of Drug on retraining blocks 1 - 4, significant effect of Session on retraining blocks 1 and 4, and significant interaction on blocks 1 and 4 (see Table 1 for main effects and interaction summaries). Over the 4 separate retraining blocks (Figure 12C), responding on nicotine sessions 2-9 and 11-16 was higher than on corresponding saline sessions (Bonferroni's tests). Hence, rats with cannulae implanted into p-dmCPu were also sufficiently retrained for all 4 inactivation tests.

Table 1. Statistical summaries from DGT retraining phases in Experiment 2.

Retraining	Main Effects		Interaction
Block	Drug	Session	
a-dmCPu			
Retraining-1	**F(1,14)=60.52, p<0.001**	**F(4,56)=5.24, p<0.001**	**F(4,56)=5.24, p<0.001**
Retraining-2	**F(1,14)=23.71, p<0.001**	F(1,14)=4.27, p=0.05	**F(1,14)=5.86, p<0.05**
Retraining-3	**F(1,14)=66.49, p<0.001**	F(1,14)=1.09, p=0.31	**F(1,14)=7.91, p<0.05**
Retraining-4	**F(1,14)=44.01, p<0.001**	F(6,84)=2.88, p=0.05	F(6,84)=1.30, p=0.28
p-dmCPu			
Retraining-1	**F(1,14)=54.38, p<0.001**	**F(4,56)=5.14, p<0.01**	**F(4,56)=5.49, p<0.01**
Retraining-2	**F(1,14)=34.94, p<0.001**	F(1,14)=2.51, p=0.13	F(1,14)=0.007, p=0.9
Retraining-3	**F(1,14)=37.45, p<0.001**	F(1,14)=1.88, p=0.19	F(1,14)=3.19, p=0.09
Retraining-4	**F(1,14)=60.22, p<0.001**	**F(6,84)=8.04, p<0.001**	**F(6,84)=5.7, p<0.001**

Significant effects are in bold.

___***a-dmCPu*** *condition: Transient Inactivation.*__ Data from all 4 tests were combined into one dataset for this analysis. There was significant main effect of Drug [$F(1,14)=8.40$, $p<0.5$], no effect of Infusion [$F(1,14)=2.87$, $p=0.11$], and a significant Drug × Infusion interaction [$F(1,14)=6.00$, $p<0.05$]. Following distilled water infusion (control), nicotine-evoked responding was higher than responding after saline injections; there was no effect of cannula implantation

and vehicle infusion on the expression of discriminated learning with nicotine stimulus (Bonferroni's tests; Figure 13A). Lidocaine infusions into a-dmCPu evoked nicotine-like responding following saline injections, but did not affect nicotine-evoked responding; dipper entries following saline injection were higher after infusions of lidocane than after infusions of distilled water (Bonferroni's tests, Figure 12A).

__p-dmCPu__ condition: Transient Inactivation. Data for this analysis were aggregated from 4 separate inactivation tests. There were significant main effects of Drug [$F_{(1,14)}=10.07$, $p<0.01$] and Infusion [$F_{(1,14)}=10.88$, $p<0.01$], as well as significant Drug × Infusion interaction [$F_{(1,14)}=5.03$, $p<0.05$]. There was no effect of cannula implantation or vehicle infusion on the expression of discrimination performance with nicotine stimulus as nicotine-evoked responding was higher than responding after saline injections following distilled water (control) infusions into p-dmCPu (Bonferroni's tests; Figure 13B). Lidocaine infusions into p-dmCPu attenuated nicotine-evoked responding (compare nicotine responding following distilled water or lidocaine infusions; Bonferroni's tests; Figure 13B). Responding on nicotine test following lidocaine infusion was not statistically different from responding on saline test following lidocaine infusion (Bonferroni's tests, Figure 13B).
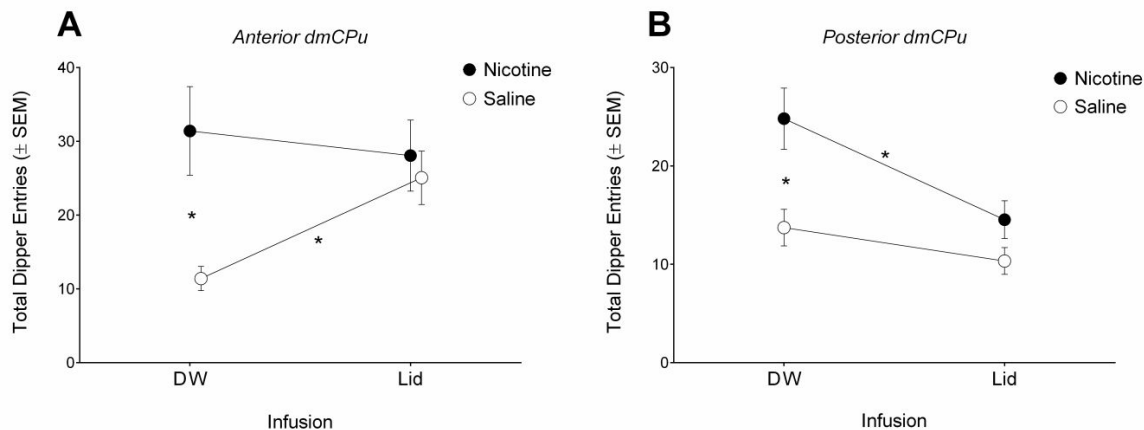
Figure 13. Mean (±SEM) number of total dipper entries during nicotine or saline 4-min test following either distilled water (DW) or lidocaine (Lid) infusion into (A) a-dmCPu or (B) p-dmCPu.

Lesion Placement Effects. Regression analysis was used to test if a position of transient inactivation area (Bregma point) on the anterior-posterior axis significantly predicted nicotine-evoked or saline-maintained responding. To ascertain the effect of Bregma on the nicotine-evoked responding, data from all rats (a- and p-dmCPu) tested with nicotine following lidocaine infusions were aggregated for the regression analysis. The results of regression indicated that a significant proportion of variance in nicotine-evoked responding was explained by the position of lidocaine infusion site on the Bregma scale [R^2=0.19, $F_{(1,28)}$=6.59, $p<0.05$; Figure 14A]. Thus, lesion placement on Bregma scale was a significant predictor of magnitude of nicotine-evoked dipper entries in lesioned rats with progressively decreased responding from anterior to posterior sub-regions of dmCPu [β= 6.20, $t(28)$=2.56, $p<0.05$; Figure 14A].

To ascertain the effect of Bregma on the saline-maintained responding, data from all rats (a- and p-dmCPu) tested with saline following <u>lidocaine infusions</u> were aggregated for the regression analysis. The results of regression analysis showed that a significant proportion of variance in saline-maintained responding can be explained by the lesion placement on the Bregma scale [R^2=0.28, $F(1,28)$=11.18, $p<0.01$; Figure 14B]. Hence, the position of infusion site on the Bregma scale (anterior vs. posterior) significantly predicted magnitude of saline-maintained response in lesioned rats; progressively increased responding from p-dmCPu to a-dmCPu [β= 6.26, $t(28)$=3.34, $p<0.01$; Figure 14B]
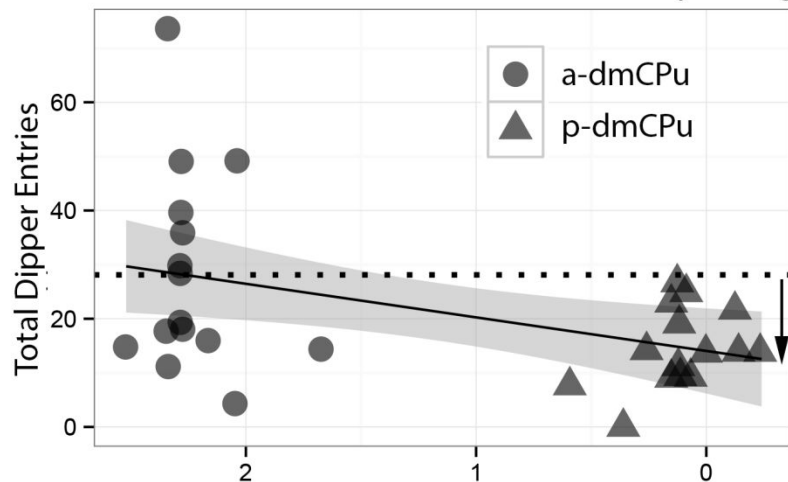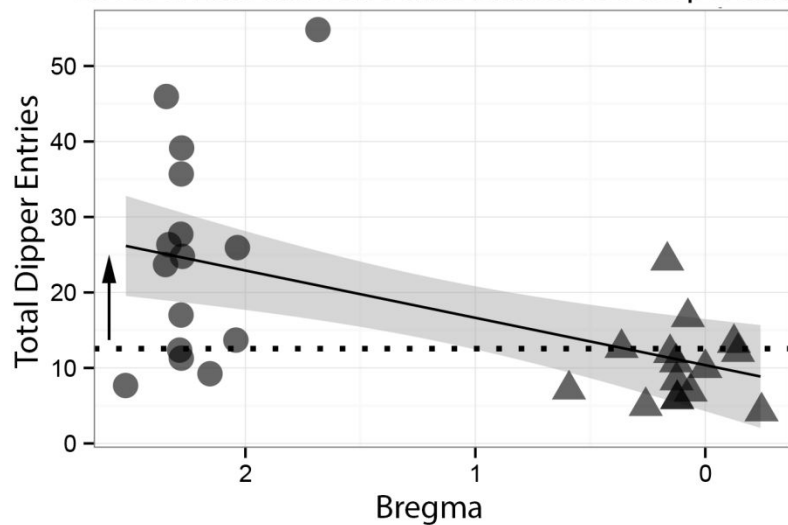
Figure 14. (A) Total dipper entries from all rats during <u>nicotine</u> test following transient inactivation. (B) Total dipper entries from all rats during a <u>saline</u> test following transient inactivation. Arrows indicate the direction of change from the control – the average responding (dashed line) on a nicotine (A) or saline (B) sessions following distilled water infusion.

Summary

All rats were successfully trained to discriminate nicotine stimulus from saline as evident by significantly higher responding on nicotine days starting from session 5 (Figure 12A). Following surgeries, and prior to each test, all rats were retrained to baseline levels of nicotine-evoked responding (Figure 12B-C). Reversible inactivation of a-dmCPu evoked nicotine-like responding following saline administration. Because CPu is a major inhibitory structure, with efferent GABAergic projections to globus pallidus (the indirect pathway) and substantia nigra (the direct pathway), it seems that inactivation of a-dmCPu disinhibited responding that otherwise controlled by the nicotine stimulus. Therefore it appears that a-dmCPu is not directly involved in the expression of nicotine-evoked responding but rather is involved in inhibiting context-evoked responding when nicotine stimulus is not present. In contrast, inactivation of p-dmCPu attenuated nicotine-evoked responding. Therefore it seems that there is a functional dissociation between a- and p-dmCPu in the expression of conditioned responding with nicotine as a conditioned stimulus. While a-dmCPu is inhibiting context evoked responding, intact function of p-dmCPu is needed for the expression of nicotine-evoked responding.

CHAPTER 5

GENERAL DISCUSSION

Introduction to Discussion

Previous studies have established that dorsal caudate-putamen is critically involved in goal-directed and habitual learning (Charntikov *et al*, 2012; Corbit and Janak, 2010; Corbit *et al*, 2012; Murray *et al*, 2012; Yin *et al*, 2004; Yin *et al*, 2005b). Caudate-putamen is a heterogeneous nucleus which can be divided into several functional domains based on their anatomical connections and behavior-specific involvement. For example, while the dorsomedial region of CPu is involved in goal-directed actions, the dorsolateral region of CPu facilitates the development of habits (Charntikov *et al*, 2012; Yin *et al*, 2004, 2005a; Yin *et al*, 2006; Yin *et al*, 2005b). On the other hand, the posterior portion of the dmCPu is involved in early stages of instrumental learning, while the anterior portion of the dmCPu facilitates expression of well-established instrumental behaviors (Murray *et al*, 2012; Yin *et al*, 2005b). Furthermore, dorsal CPu is involved in other aspects of associative learning such as latent inhibition (Jeanblanc *et al*, 2003), early stages of sequence learning (Hikosaka *et al*, 1999), and reward encoding in primates (Samejima *et al*, 2005; Schultz, 1998; Schultz, 2006). In addition to this existing body of literature, we recently reported that dmCPu was involved in associative learning with the nicotine stimulus. That is, when nicotine (the CS) was paired with intermittent access to sucrose (the US) in the DGT task it comes

to evoke a goal-tracking response associated with elevated c-Fos activity in the

dmCPu (Charntikov *et al*, 2012). Because elevated expression of sub-cellular c-

Fos protein provides only a correlational account of regional involvement in the

behavior of interest, the goal of the current dissertation was to further investigate

functional involvement of dmCPu in acquisition and expression of associative

learning with the nicotine stimulus.

Summary of Experiment 1

Experiment 1 was designed to investigate the role of dmCPu in the

acquisition of learning with the nicotine stimulus. Because dmCPu is a large

heterogeneous area with distinct connections to a- and p-dmCPu (Kelley *et al*,

1982), we decided to assess both of these subregions for their involvement in

initial stages of learning with the nicotine stimulus. To accomplish this goal,

before all behavioral manipulations, rats received permanent excitotoxic lesions

to either a- or p-dmCPu. Prior to Experiment 1, a series of pilot studies were

conducted to determine the most suitable parameters (stereotaxic coordinates,

infusion volume, infusion speed, and vehicle among others) that would result in

sufficient neuron destruction (above 80%) within the predetermined areas. The

results of this preliminary work confirmed that excitotoxic lesions induced by

NMDA injections under the chosen parameters produce near complete neuron

destruction as visualized by the NeuN anti-neuron specific antibody staining (see

Figure 5A-C). Following recovery from lesion surgeries, rats were trained with

nicotine as an excitatory interoceptive stimulus using the DGT task described above and the effect of lesions on the acquisition of this task was assessed over the subsequent 10 training sessions (see Figure 7).

To elucidate a role of a- or p-dmCPu in the acquisition of learning with nicotine stimulus we utilized multiple levels of assessment including analysis of group effects, regression analysis of lesion placement effects, and visual assessment of effects using a heat map approach. The analysis of group effects revealed that the lesions to p- but not a-dmCPu blunted acquisition of learning with nicotine stimulus as overall responding with lesions to p-dmCPu was lower than controls over the duration of the training phase. To further investigate this effect, and to confirm the results of the group analysis, we analyzed individual differences in lesion placement and their effect on acquisition learning. Thus, instead of group as a categorical variable we used lesion placement on the anatomical Bregma scale as one continuous independent variable. The dependent variable, in this way of assessment, was a difference score, which was computed by subtracting the mean dipper entry rate of sham controls from the dipper entry of each lesioned subject for each corresponding nicotine session (for details see Methods section of Experiment 1). The corresponding dataset, with individual variance in acquisition learning as dependent variable and lesion placement on the Bregma scale as the independent variable, was assessed using regression analysis. This alternative way of assessment confirmed that p-dmCPu was

involved in the early stages of learning with nicotine stimulus and also provided additional validation of our group analysis findings.

This visualization of the effects using the heat map approach allowed for alternative data assessment using multiple variables on one all-encompassing plot. The heat map that was constructed for visual assessment of the results from Experiment 1 and combined a lesion placement variable on x-axis and a difference score that was visualized as a gradient from blue to white to red. In this heat map plot, the overall deficits in responding were visualized in blue shades, control like responding in white shades, and higher than control responding in red shades (see Figure 9). Representing data in this fashion allows for a simplified way of identifying of regional effects critical for the behavior of interest and, in our example, allows for alternative assessment of a role of dmCPu in learning with the nicotine stimulus as a factor of lesion placement on the anterior-posterior axis. The heat map represented in Figure 9 confirms the role of p-dmCPu in acquisition of learning with nicotine stimulus using yet another alternative mean of comparison.

Results of Experiment 1 confirm the importance of p-dmCPu for the acquisition of interoceptive learning with the nicotine stimulus. Pretraining lesions of p-dmCPu blunted acquisiton of learning with appetitive nicotine stimulus which was revealed through multiple methods of assessment. Although limited, previous reports corroborate the importance of p-dmCPu in the early

stages of associative learning in rodents. For example, reports from studies investigating a role of p-dmCPu in acquisition of instrumental learning show that inactivation of this area impairs acquisition of response-outcome association like instrumental responding for food reward (Corbit *et al*, 2010; Yin *et al*, 2005a) or cue-induced cocaine-seeking under second-order schedule of reinforcement (Murray *et al*, 2012). Lesions to p-dmCPu also impair acquisition of stimulus-outcome association using a classical conditioning training protocol (Corbit *et al*, 2010). In this task, exteroceptive auditory stimulus was paired with a food reward and after a period of training food seeking following an auditory conditioned stimulus served a measure of associative learning. This converging evidence indicates that p-dmCPu may be involved in a broad range of associative learning processes including, as demonstrated by our study, a polymodal pharmacological stimulus like nicotine.

Summary of Experiment 2

Experiment 2 was designed to elucidate the role of dmCPu in the expression of well established conditioned response to the nicotine stimulus. To accomplish this goal, rats were first trained with nicotine as an excitatory conditioned stimulus and following the training phase were subsequently cannulated to allow for delivery of a transient lesioning agent (lidocane) into either a- or p-dmCPu on the test day. The advantage of using lidocaine instead of specific receptor blockers, like dopamine antagonist a-flupenthixol (Murray *et al*, 2012;

Vanderschuren, 2005), GABA agonists muscimol (Yin *et al*, 2005a) or muscimol/baclofen combination (Corbit and Janak, 2007; Corbit *et al*, 2010; Corbit *et al*, 2012), is its reduced selectivity. Lidocaine indiscriminately inactivates all neurons within its site of action by blocking sodium channels which in turn effectively blocks the occurrence of action potentials (Ritchie, 1979; Tehovnik *et al*, 1997). This transient effect is quite similar to the widely used permanent electrolytic lesioning approach as it renders all affected neurons, including those with passing axons, effectively shut down. Lidocaine is also highly effective at inactivating neurons and with high concentration used in our study (100 μg/0.5 μl/side) functionally blocks more than 90% of neurons within sites comparable in size to a- and p-dmCPu (Hiranita *et al*, 2006; Kantak *et al*, 2011; Sandkuhler *et al*, 1984; Sandkuhler *et al*, 1987; Tehovnik *et al*, 1997). Accordingly, these lidocaine characteristics make it a suitable inactivation agent for investigating a gross regional involvement in the behaviors of interest.

Experiment 2 found differential involvement of the anterior and posterior sub regions of dmCPu in the expression of the nicotine-evoked responding. Interestingly, the reversible inactivation of a-dmCPu evoked nicotine-like responding following saline administration. Because CPu is a major inhibitory structure, with efferent GABAergic projections to GPe (the indirect pathway) and GPi/SNr (the direct pathway; see Figure 4), it seems that inactivation of a-dmCPu disinhibited responding that otherwise was controlled (evoked) by the nicotine stimulus. Therefore, it appears that a-dmCPu is not directly involved in the

expression of well established nicotine-evoked responding but rather is involved in inhibition of context evoked responding when nicotine stimulus is not present (see below for more). In contrast, inactivation of p-dmCPu attenuated established nicotine-evoked responding which parallels the results of Yin et al., (2005) study. In that study, inactivation of the p-dmCPu reduced rat's sensitivity to the devaluation or degradation of reward following a period of instrumental learning with food as a reward. Therefore, our data pattern suggests a functional dissociation between a- and p-dmCPu in the control of well established responding to the nicotine stimulus where a-dmCPu is involved in inhibition of context induced motor responding while the p-dmCPu is involved in activation of goal-tracking behavior when nicotine stimulus is detected.

One of the most interesting finds of our study is the context-induced disinhibition of established conditioned goal-tracking responding following transient a-dmCPu inactivation. Recall that on the test day, following lidocaine infusion into the a-dmCPu, rats that were trained to discriminate nicotine stimulus in the DGT task showed elevated goal-tracking response following saline treatment that was comparable in the magnitude to that of the nicotine stimulus. We interpret this effect as a context induced disinhibition of the conditioned responding that otherwise would be inhibited given the intact functioning of the a-dmCPu. Because on the saline test day rats with inactivated a-dmCPu responded in a nicotine-like fashion, this responding was likely triggered either by a) the lesion evoking nicotine-like stimulus effect or by b) some other stimulus

(see below) capable of evoking nicotine-like responding without involving a-dmCPu. Based on previous research investigating nicotine's stimulus effects it is unlikely that dmCPu, which is involved in inhibiting other principle areas (GPe, STN) involved in motor control, capable of inducing nicotine-like state without nicotine being administered to the rat (Shoaib *et al*, 1996; Stolerman and Shoaib, 1991). Furthermore, what is known about the involvement of dmCPu in learned behaviors centers around its effect in controlling learned motor responses (Hikosaka, 2007; McHaffie *et al*, 2005; Nambu, 2008). Change in these motor responses is taken to represent learning or plasticity where new information, whether exteroceptive or interoceptive, facilitates that change. Therefore, a more probable alternative explanation is that the nicotine like responding of lesioned rats following a saline injection was evoked by the other stimulus or stimuli utilizing circuitry independent of a-dmCPu and it is likely that the test chamber itself (i.e., the context) served as that stimulus.

Test chamber or the context is an important part the DGT learning task. All rats reinforced with sucrose exclusively in the test chamber and at least in the early acquisition stage test chamber reliably predicts (50% of the time) sucrose reinforcement as evident by the elevation of goal-tracking on early saline and nicotine sessions (see Sessions 1-3 of Figure 15). This early learning about the context and reinforcement is gradually inhibited (see decline in responding on saline sessions 4-9; Figure 15) as nicotine becomes to provide superior information about the reinforcement availability (nicotine paired with sucrose

100% of the time). Therefore, based on the outcome of Experiment 2, it seems that in the later sessions (4-14; Figure 15), when nicotine is not detected, the context-evoked responding was inhibited whereas on nicotine sessions goal-tracking was disinhibited by the a-dmCPu. This mechanism is also supported by the main principles by which dmCPu mediates learned motor behaviors (Chevalier and Deniau, 1990; Grillner *et al*, 2005; Hauber, 1998; Nambu, 2008).

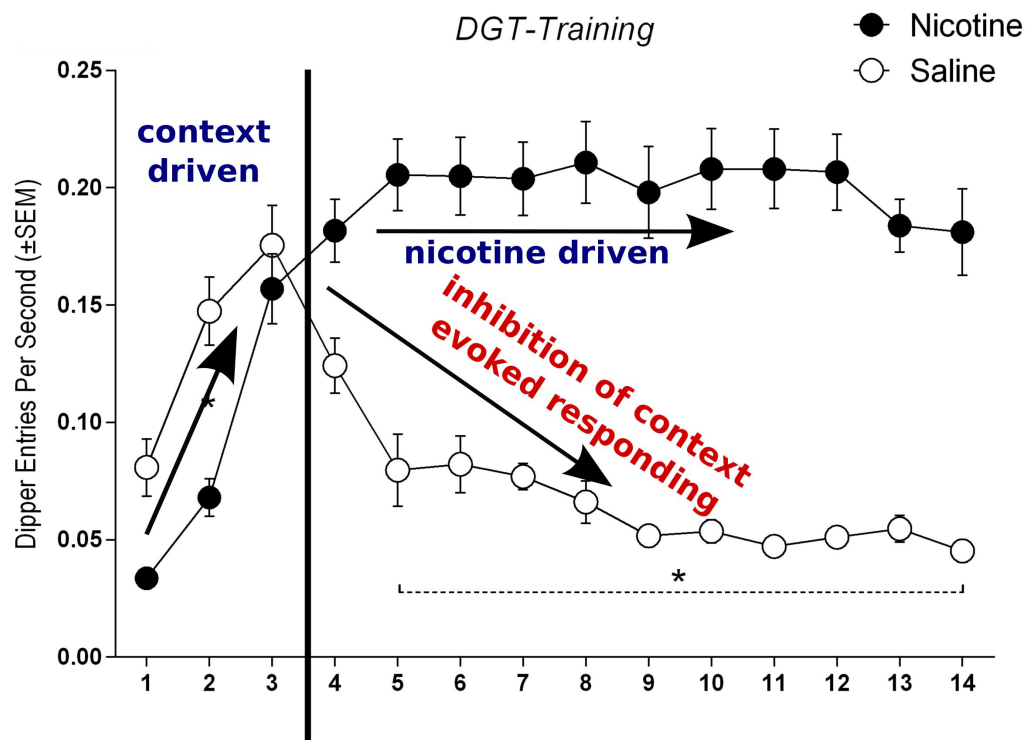Figure 15. Dipper entry rates (±SEM) during initial training phase of Experiment 2. Arrows indicate hypothesized control of responding initially by the context (sessions 1-3) which is gradually overtaken by the nicotine stimulus at the later sessions with simultaneous inhibition of the context evoked responding (sessions 4-14).*Denotes significant differences between corresponding saline and nicotine sessions

Caudate-putamen plays an important role in initiation of goal directed

behaviors. Caudate-putamen is a principle input structure of the basal ganglia, a

main assembly of nuclei that governs motor behaviors, receiving excitatory input

from the cerebral cortex and thalamic nuclei (Graybiel, 1995; Marin *et al*, 1998;

Nambu, 2008). Pallidum is the principal output structure of the basal ganglia

(Grillner *et al*, 2005; Hauber, 1998). Pallidum is an assembly of nuclei including

substantia nigra pars reticulata (SNr), globus pallidus pars interna (GPi), and

ventral pallidum (VP). Pallidal projections innervate critical motor areas such as

thalamus, superior colliculus, mesencephalic locomotor region, pedunculopontin

nucleus, and brainstem (Grillner *et al*, 2005). Inhibitory medium spiny neurons

are the main cells (95%) forming caudate-putamen. These neurons release GABA

at their terminals and project either a) directly to pallidum which in turn inhibit

motor areas (thalamus, superior colliculus) or b) indirectly to the pallidum via

the globus pallidus pars externa (GPe) that disinhibits the excitatory subthalamic

(SN) neurons projecting to the pallidum (see Figure 4). The inhibitory neurons

projecting directly to the pallidum form a direct pathway and are virtually silent

in the resting state but upon dopaminergic activation inhibit pallidum and thus

disinhibit motor areas (DeLong, 1990; Wilson and Kawaguchi, 1996). Other

populations of striatal inhibitory output neurons comprise the indirect pathway

and when activated inhibit globus pallidus pars externa which in turn disinhibits

excitatory subthalamic neurons innervating pallidum thus effectively applying a

brake (inhibition) on the motor areas and hence motor behaviors (for review on this topic see, Grillner *et al*, 2005; Nambu, 2008).

Taking previous arguments into consideration, the outcome of Exeriment 2 suggests that the expression of nicotine controlled goal-tracking response is governed by the delicate balance in activity of a direct and indirect pathways. Lesions to p-dmCPu inhibited the expression of nicotine evoked goal-tracking suggesting inactivation of the direct pathway. Activation of the direct pathway, which otherwise is tonically inactive, is needed to disinhibit thalamus and thus disinhibit goal-tracking response (Figure 16). Because the indirect pathway is tonically active and provides inhibition of learned motor responses it is not plausible that inactivating its efferent projections can facilitate this effect. On the other hand, our findings suggest that transient lesions to a-dmCPu inhibited activity of the neurons forming the indirect pathway which manifested itself in disinhibition of context induced goal-tracking responding (Figure 16). Removing the inhibition from globus pallidus pars externa, through transient lesion of the a-dmCPu, renders the "braking" mechanism impaired, or disinhibits motor areas (thalamus and superior colliculus). Because the goal-tracking was observed without nicotine administration, and thus without nicotine's interoceptive stimulus effects, some other stimulus seems to be involved in the activation of this goal tracking response. The most plausible candidate that evoked goal-tracking in the absence of nicotine stimulus was the chamber itself which was paired with sucrose 50% of the time and seed to be involved in enhancing goal-

tracking responding in the beginning of training phase (Figure 15, sessions 1-3). Therefore, the expression of goal-tracking responding evoked by the nicotine stimulus seems be reliant on the balance in activity of both the direct and the indirect pathways (Figure 16). Importantly, these findings suggest that the p-dmCPu sends efferent projections to GPi/SNr via the direct pathway whereas a-dmCPu interacts with GPi/SNr complex via the indirect pathway. Further studies will need to confirm this hypothesis.
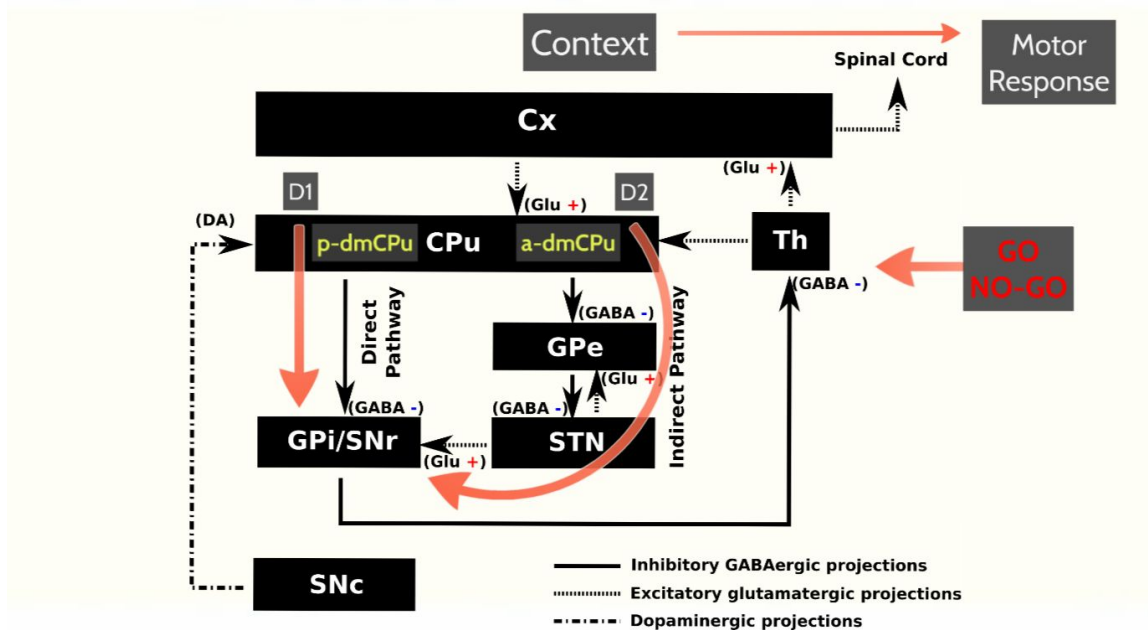
Figure 16. Graphical representation of circuitry in the basal ganglia. Cx, cerebral cortex; CPu, caudate putamen; GPe, external segment of globus pallidus; GPi, internal segment of globus pallidus; SNc, substantia nigra pars compacta, SNr, substantia nigra pars reticulata, Th, thalamus. Arrows indicate critical nodes of the basal ganglia involved in expression of nicotine evoked goal-tracking response (partially adapted from Nambu, 2008).

Conclusion

Dorsal CPu is a critical part of the basal ganglia, which have been shown to be involved in regulation of a variety of mechanisms including sensory, motor, and learning. Basal ganglia receives dopaminergic input from substantia nigra pars compacta which in turn regulates the activity of the direct and indirect pathways through dopaminergic activity at the D1 and D2 receptors. In addition to the nigrostriatal input, basal ganglia also receives input from various areas of the cerebral cortex with some of the most prominent being the medial prefrontal cortex, orbitorfrontal cortex, and sensory-motor cortex. Basal ganglia also outputs primarily back to the cerebral cortex (frontal lobe) via the thalamus, thus, effectively forming a cortico-basal ganglia loop. This looped architecture is conducive to prioritizing and evaluating complex inputs and returning the solution back to the origin (Gurney *et al*, 2001). Based on this architecture, it seems that basal ganglia, after receiving simultaneous and potentially incompatible inputs from the cerebral cortices, is in a position to compute the most appropriate outcome and to subsequently provide the solution, via the output, back to the originating areas (Gurney *et al*, 2001; McHaffie *et al*, 2005).

The discriminated goal-tracking task is a complex task involving all three aforementioned systems - sensory, motor, and learning. Sensory system is involved in discerning the contextual stimuli (visual, tactile, olfactory, and auditory), the interoceptive stimuli, and is involved in the initial stages of reward

detection (gustatory system). Motor system is required to locomote about the chamber in search of a reward and to subsequently acquire the reward by entering the dipper compartment and consuming the sucrose. Finally, the neural plasticity, or learning, is required to consolidate sensory input with the motor output into one efficient neural program that would establish the most beneficial behavioral output in a presence of stimuli associated with reward (S-O association). Because dorsal CPu is a critical part of this sensori-motor-learning mechanism, it is it is not entirely surprising that it is a position to to regulate various aspects of associative learning including learning with interoceptive stimuli like nicotine. However, what is not entirely clear is which aspects of sensori-motor-learning mechanism are regulated by the dorsal CPu and what kind of plastic changes they may be associated with.

Though using lesioning approach is a useful tool in the early investigation stages of neural networks, like in the experiments presented in this study, it lacks mechanistic specificity needed to fully understand the role of an area in the behavior of interest. Therefore, it is unclear whether lesions to dmCPu hindered mechanisms associated with learning, sensory, motor or their combination. To further understand the role of dmCPu in learning with the nicotine stimulus more studies need to be conducted using recently developed precision manipulation techniques. These recently developed techniques (e.g., optogenetics, DREADDs) allow excitation or inhibition of neurons by stimulating synthetic receptors (genetically modified or introduced via viral vectors) specifically

designed to be activated by either light or non-endogenous synthetic ligands

(Deisseroth and Schnitzer, 2013; Rogan and Roth, 2011). The use of these

techniques would allow a much greater understanding of a role of basal ganglia in

associative learning processes with nicotine stimulus. To do that, there is a need

to a) identify population of dmCPu neurons involved in learning with nicotine, b)

identify their afferent and efferent connections, c) use non-destructive neuron

specific manipulation techniques (e.g., DREADDs/optogenetics) to test the

involvement of previously identified neuronal ensembles and networks in the

different stages of associative learning with nicotine stimulus. Although more

research needs to be done to fully understand the role of basal ganglia in learned

behaviors associated with nicotine stimulus, the results presented in this

dissertation provide an important initial step to achieve this understanding.

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