THE SUBJECTIVE EFFECTS OF ETHANOL IN RHESUS MONKEYS: CHARACTERIZING THE ROLE OF THE NUCLEUS ACCUMBENS USING CHEMOGENETICS

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List of abbreviations

°C – degrees Celsius

5-HT – 5-hydroxytryptamine

AAV1 – adeno-associated virus, serotype 1

AC – anterior commissure

ANOVA – analysis of variance

AUC – area under the curve

BEC – blood ethanol concentration

Cd – caudate

Cmax – maximal concentration

CNO – clozapine-N-oxide, DREADD agonist

CNO-DMSO – commercially available form of CNO prepared in 10% DMSO

CNO-HCl – water soluble salt form of clozapine-N-oxide

CSF – cerebrospinal fluid

CNS – central nervous system

DMSO – dimethylsulfoxide

DREADD – Designer Receptors Exclusively Activated by Designer Drugs

ED50 – 50 percent effective dose

fMRI – functional magnetic resonance imaging

FR – fixed ratio

g/kg – grams per kilogram

GABA – gamma(γ)-aminobutyric acid

GAD – gadoteridol, a gadolinium-based contrast dye

GPCR – G-protein coupled receptor

hM3Dq – human muscarinic (type 3) DREADD receptor, (G_q) coupled

hM4Di – human muscarinic (type 4) DREADD receptor, ($G_{i/o}$) coupled

hr – hour

hSyn – human synapsin promotor for targeting neurons

i.g. – intragastric

i.m. – intramuscular

i.v. – intravenous

IC – internal capsule

IHC - immunohistochemistry

LC-MS/MS – liquid chromatography with tandem mass spectrometry

m – meter

mg/dl – milligrams per deciliter

mg/kg – milligrams per kilogram

mg/ml – milligrams per milliliter

min – minute

MK-801 – noncompetitive NMDA receptor antagonist (also called dizocilpine)

ml – milliliter

MRI – magnetic resonance imaging

MSN – medium spiny neuron

NAc – nucleus accumbens

ng/ml – nanogram per milliliter

NMDA – N-methyl-D-aspartate

Pre-Sx – before DREADD surgery

Post-Sx – after DREADD surgery

Put – putamen

RM ANOVA – repeated measure analysis of variance

sec – seconds

SN – substantia nigra

SNc – substantia nigra pars compacta

SNr – substantia nigra pars reticulata

Sx – surgery

Tmax – time of maximal concentration

μm – micrometer

v/v – volume by volume

VP – ventral pallidum

VTA – ventral tegmental area

w/v – weight per volume

β – beta, elimination rate constant

μl – microliter

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Contributions

Chapter 1: Sections 1.3, 1.4 and 1.5 from this chapter have been adapted from a published review on the topic (Allen et al., 2017).

Chapter 2: I conducted all studies in this chapter.

Chapter 3: I have conducted all experiments with CNO-HCl and analyzed the results presented in this chapter. The experiments with CNO-DMSO were conduced in Dr. Cuzon Carlson's lab by Tim Carlson and DCM staff at ONPRC. I conducted all data analyses of both CNO-DMSO and CNO-HCl.

Chapter 4: I conducted all behavioral experiments and surgeries described in this chapter, with mentorship from Drs. Grant and Cuzon Carlson. The tissue processing and IHC was done by Tim Carlson and myself. I conducted all tissue and data analysis.

Abstract

Alcoholism is a chronic, progressive disorder often characterized by patterns of binge drinking that leads to detrimental health consequences and a great economic burden on society. Alcohol (ethanol) is known to produce interoceptive (i.e., internally generated) effects that can be recognized and described by the user. These interoceptive effects have been associated with the perpetuation of binge drinking, suggesting that the way alcohol makes you feel may contribute to its abuse potential. Drug discrimination is a reliable *in vivo* behavioral pharmacological assay that can be used to characterize the receptor basis of ethanol's interoceptive effects, as measured through a behavioral output. This approach can identify the receptor basis of ethanol's effects at a particular dose to identify candidate receptor systems for targeted pharmacotherapy development. Previous work using this technique has established that ethanol is a stimulus complex made up of concurrent activity at multiple receptor systems in rodents and primates. Specifically, ethanol acts as a positive modulator at the $GABA_A$ receptor and an antagonist at the NMDA receptor, leading to an overall dampening of neuronal activity. However, the discriminative stimulus effects of ethanol have never been described in rhesus macaques. Thus, the first aim of this dissertation was to characterize the pharmacological basis of ethanol discrimination in rhesus macaques (Chapter 2). I found that $GABA_A$ receptor positive allosteric modulators pentobarbital and midazolam, and NMDA receptor antagonist MK-801 substituted for ethanol's discriminative stimulus effects in most subjects tested. These results are consistent with other macaque species, as well as humans, rodents, and pigeons.

While a great deal of research has been dedicated to alcohol's receptor basis in the brain, very little is known about the circuitry that underlies ethanol's interoceptive effects. Importantly, there are no published studies to date that have directly examined the brain circuitry that mediates ethanol's interoceptive effects in non-human primates. There is converging evidence from both rodents and humans suggesting that the nucleus accumbens (NAc) is involved in ethanol's interoceptive effects, and that these effects are composed of both

GABAergic and NMDA glutamatergic mechanisms. Rodent drug discrimination experiments have indicated that ethanol acts to inhibit activity of the NAc core through GABA and NMDA receptors to produce ethanol's discriminative stimulus effects. Thus, as a first step in mapping the circuitry related to the stimulus effects of ethanol intoxication, this dissertation utilized a chemogenetic strategy to directly inhibit neural activity within the NAc core in rhesus monkeys. Prior to application of chemogenetic approaches, a thorough pharmacokinetics study was conducted to examine the bioavailability of the chemogenetic actuator, clozapine-N-oxide (CNO), in the rhesus macaque (Chapter 3). I found that a water-soluble salt form on CNO demonstrated improved solubility and bioavailability when compared to the commercially available form and preparation. Lastly, in Chapter 4, chemogenetic inhibition of NAc neurons during ethanol discrimination was tested. The overall hypothesis was that ethanol acts to inhibit NAc activity to produce ethanol's discriminative stimulus effects. I found that inhibition of the NAc using chemogenetics produced mixed effects on ethanol discrimination, with some subjects demonstrating enhanced sensitivity to ethanol's discriminative stimulus effects, and others showing decreased sensitivity to ethanol's discriminative stimulus effects. However, individual variability was partially explained by the extent and localization chemogenetic receptor expression. Overall, these studies provide novel contributions to the fields of ethanol pharmacology, ethanol discrimination and circuitry, and chemogenetic approaches in non-human primates.

Chapter 1: Introduction

Sections 1.3, 1.4, and 1.5 of this chapter have been adapted from a published review on the topic (Allen et al., 2017).

Psychoactive drugs can elicit changes in the state of an individual, which can be identified and described by the user. The range of these effects is wide-reaching: extending from physiological changes such as increased heart rate to cognitive and psychological changes such as improved mood or impaired inhibitory control. The nature of these effects is interoceptive, meaning that they are generated internally, and contribute to a subjective drug experience. Subjective drug effects have been linked to abuse potential, suggesting that certain features of a drug effect can perpetuate continued use (Holdstock et al., 2000; Childs et al., 2011). The neural circuitry underlying subjective drug effects is not well understood, particularly in the primate brain. The goal of this dissertation is to begin to unravel the mechanisms by which alcohol produces specific, discriminable changes in the state of the user, with a focus on pharmacology and brain circuitry.

1.1. State-dependent learning

The study and theory of subjective drug effects in animals originated from the first studies describing state-dependent learning, which found that association of a conditioned stimulus with an unconditioned stimulus as measured by a conditioned response (CR) could not be transferred from one state to another (Overton 1964). In this case, “state” was defined by the presence or absence of a drug, and as such trained associations between the unconditioned and conditioned stimulus were not absolute and only expressed within the drug or nondrug state in which it was trained. The first published report on state-dependent learning trained the association between a simple muscle twitch reflex and the sound of a bell in dogs in the presence and absence of curare, a nicotinic receptor antagonist (Girden and Culler, 1937).

When conditioning took place under normal baseline conditions (i.e., nondrug state), the muscle twitch response was not found following curare administration (i.e., drug state), but returned if the drug was removed (Girden and Culler, 1937). This conditioning deficit could not be described simply by a sedative effect of the drug, however, because when both conditioning and testing took place following curare administration, the conditioned response was observed (Girden and Culler, 1937). Thus, Girden and Culler concluded that it was the transfer between a drug and non-drug state that was impaired, rather than conditioning in one state or another. From this initial result, it was hypothesized that there was a "dissociation of learning" from a drugged to a nondrugged state, such that conditioning could only be expressed under the same conditions that it was originally learned (Girden and Culler, 1937; Overton, 1964). Dissociative state-dependent learning experiments gained traction over the next several decades, expanding across several species from dogs (Girden and Culler, 1937), to rodents (Overton 1964; Overton 1966), monkeys (Bliss, 1972), and humans (Goodwin et al., 1969).

One limitation of the classical conditioning paradigms was that the expression of a dissociation of learning was based on whether a particular conditioned response was present or absent. Under these conditions, the absence of a response was difficult to interpret. It could either mean that there was a learning deficit or that a sedative effect of the drug was present (Overton, 1964; Overton, 1974). As a solution, Overton introduced the T-maze paradigm in state-dependent learning, in which rats were trained to run to one arm of the T-maze in the presence of a drug and the other arm in the absence of the drug to escape an unavoidable shock. By introducing a choice contingency which required a response on each trial, differential performance in state-dependent learning could be separated out from the response-decreasing sedative effects of the drug (reviewed in Overton, 1974). In these experiments, the association between the behavioral response (right or left turn on T-maze) and the avoidance of punishment (shock) was predicted by the presence of a drug. While these experiments were designed to improve the interpretability of state-dependent learning studies, the introduction of an operant

task (i.e., response → outcome learning) reframed these experiments to emphasize how the presence of a drug could differentially guide behavior. In the T-maze experiments, a correct turn (i.e. response) resulted in shock avoidance (i.e., outcome), and this sequence was predicted by the presence or absence of a drug cue. Thus, the drug cue served as a *discriminative stimulus*, which is defined as a stimulus that predicts the contigency between a response and outcome (either reinforcement or punishment).

1.2. Drug discrimination

The identification of drugs as discriminative stimuli generated new questions about the origin, nature, and specificity of drug cues and how they could differentially affect behavior, which led to the development of drug discrimination. In drug discrimination, the presence of a drug cue serves as a discriminative stimulus, which predicts that a given response will be reinforced (or punishment will be avoided) (Stolerman, 2014). Rather than emphasizing the conditioning aspects of the paradigm, training a drug as a discriminative stimulus serves as a behavioral tool, enabling a subject to report the presence or absence of a drug cue (Stolerman, 2014). Once the subject can reliably discriminate the drug, drug discrimination studies are designed to query the specific features of a drug's discriminative stimulus effects, also defined as the drug's *stimulus properties*. Discriminative stimuli can either be exteroceptive, meaning generated externally (such as lights or tones), or interoceptive, meaning generated internally. In drug discrimination, the presence of a drug generates interoceptive cues (or stimulus effects), which are trained as the discriminative stimulus. In addition, a secondary response is trained to be associated with the absence of that cue in order to differentiate between a cue response and the absence of a response (i.e., a discrimination) (Overton, 1974).

1.2.1. Receptor basis of stimulus properties of drugs

One of the first questions in drug discrimination was whether the discriminative stimulus effects were centrally or peripherally mediated, as a central mechanism for curare could not be

concluded due to its known activity at the neuromuscular junction (Girden and Culler, 1937). To address this question, pentobarbital was trained as a discriminative stimulus, which has selective activity within the central nervous system (CNS) (Overton, 1964). Rats in this experiment successfully acquired the discrimination and were able to use the interoceptive cues to make correct responses on the T-maze task (Overton, 1964). Once a central mechanism for discriminative stimulus effects was established, there was growing evidence that the stimulus properties of drugs were mediated by specific drug-receptor interactions.

The receptor basis of discriminative stimulus effects was established using substitution or transfer testing, which is still used in drug discrimination today (and throughout this dissertation). Substitution testing takes place following discrimination training after the subjects can reliably report the presence or absence of a drug stimulus with two discrete responses (i.e. right lever for drug, left lever for vehicle/water). Instead of the training drug or dose, a different drug and dose combination can be given, essentially asking the subject whether the stimulus properties of the test drug/dose are similar or dissimilar to the trained drug (i.e., will the trained stimulus response associations transfer to the new drug state). Using substitution tests, it was determined that the transfer of discriminative stimulus effects to a new drug was dependent on whether the two drugs had shared receptor pharmacology (Overton, 1966; Colpaert et al., 1975a,b). For example, Colpaert and colleagues trained the μ-opioid receptor agonist fentanyl as a discriminative stimulus and reported almost complete transfer to other μ-opioid receptor narcotics, such as morphine or fentatienil (Colpaert et al., 1975a,b). Importantly, non-narcotic drugs including barbiturates ($GABA_A$ receptor positive allosteric modulator), amphetamine (dopamine transporter inhibitor) and nicotine (nicotinic receptor agonist), which have no direct activity at the μ-opioid receptor, did not substitution for fentanyl (Colpaert et al., 1975a). The implications of this study are far-reaching, indicating that when a drug is trained as a discriminative stimulus, it is the specific stimulus properties of that drug that are learned, rather than a non-specific change from a non-drug state. The second finding that supported a receptor

mechanism was that stimulus properties appeared to be dose-dependent, such that the transfer of a discriminative stimulus was contingent upon the dose of each drug given, and this was consistently reported across many drug classes (Colpaert et al., 1980; Colpaert and Janssen, 1982; Overton 1977). Lastly, and perhaps most importantly, antagonism studies demonstrated that the discriminative stimulus effects of a drug could be blocked with a selective antagonist at the receptor that the trained drug acts through (Romano et al., 1981; Woolverton and Schuster, 1983). For example, in animals trained to discriminate morphine (μ-opioid agonist), administration of a μ-opioid receptor antagonist naloxone in combination with morphine results in impaired discrimination. Each of these findings is based on peripheral drug administration, but demonstrates a high level of specificity for a central mechanism of drug stimulus properties. However, later studies were able to use intracranial injection techniques to directly manipulate these receptors with local drug administration to further solidify the basis of stimulus properties in the brain (Wood et al., 1987; France et al., 1987; Hodge, 1994).

1.2.2. Methodological considerations in drug discrimination

As previously described, discriminative stimulus effects are highly specific to the receptor pharmacology of the drug (Colpaert et al., 1975a), but the degree to which that specificity can be measured using drug discrimination can depend on the training parameters used. One modifiable variable is the nature of the operant task that is required. Initially all studies were single trial two-choice T-maze experiments to escape footshock (Overton, 1964; Overton, 1974), receive a food pellet (Barry et al., 1965), or some combination of the two (Conger, 1951). However, under these conditions, training and testing sessions were conducted once daily, with only one trial to respond on the drug or non-drug arm of the maze. Thus, intermediate responses were not possible, restricting the specificity of the behavioral assay to only two binary responses. Subsequent two-choice variations, such as lever or key tasks, allowed for multiple responses over a longer period of time (up to 5 minutes) (Barry, 1968) and enabled increased variability of responding on the drug or non-drug lever by averaging across

the session time. In this task the number of responses on the drug lever could be divided by total number of responses over the session time to calculate a percentage score (Barry, 1968). Thus, discrimination of a drug cue was measured as a continuous variable, rather than a binary (Barry, 1968). The introduction of longer sessions also significantly improved the measurement of rate decreasing drug effects (Barry, 1968; Colpaert et al., 1975b).

In addition to the task parameters, the discrimination training parameters can have a significant impact on the specificity and interpretability of a drug discrimination study. The original design trained a specific drug stimulus against water (or saline/vehicle) in a two choice discrimination (drug vs. vehicle discrimination, Figure 1-1) (Overton, 1974). In this design, drugs with shared pharmacology to the training drug will substitute for the trained discriminative stimulus. However, the specificity of this assay can be increased further by training subjects to discriminate between two active drugs, rather than a single drug versus vehicle (i.e., drug vs. drug discrimination, Figure 1-1) (Overton, 1977; Overton, 1982). For example, pentobarbital will fully substitute for both phenobarbital and ethanol under drug vs. vehicle discrimination conditions, leading to the conclusion that they have similar discriminative stimulus effects (Overton, 1974; Overton, 1977). However, using drug vs. drug discrimination to train ethanol vs. pentobarbital, phenobarbital will only substitute for pentobarbital, highlighting the differences between ethanol and the barbiturates (Overton, 1977). Further, a pentobarbital vs. phenobarbital discrimination cannot be readily acquired at similar dose levels indicating that the drugs are not consistently discriminable, highlighting their shared pharmacology (Overton, 1977).

Drug vs. drug discrimination experiments indicated that multiple discriminative stimulus effects could be learned in a single task (Overton, 1982), which led to the introduction of the idea of compound discriminative stimulus effects. A compound stimulus (or stimulus complex) is a drug cue that is made up of activity at multiple receptor systems. Early on, it was hypothesized that even if a drug had activity at multiple sites, a discriminative stimulus relied on the presence

of all components, which could not be separated out (Colpaert et al., 1976). However, this hypothesis was tested and challenged by the introduction of AND and AND-OR discrimination paradigms (Stolerman et al., 1987; Stolerman and Mariathasan, 1990) and three-choice discriminations (Bowen et al., 1997) (Figure 1-1). First, AND discriminations trained a drug mixture vs. saline, where the components of the drug mixture were known to have distinct pharmacology such as pentobarbital ($GABA_A$) and amphetamine (dopamine) (Stolerman and Mariathasan, 1990). Under these conditions the two drug components were always presented together (i.e., AND). In substitution testing following AND discrimination training, either drug cue could be used for discrimination, indicating that these cues could be separated out from the compound cue of the drug mixture. Under these conditions, pentobarbital and amphetamine were termed "redundant," meaning that either cue alone was sufficient to substitute for the entire stimulus (Figure 1-1). However, in AND-OR discriminations, the compound stimulus was trained against either drug presented alone (pentobarbital AND amphetamine vs. pentobarbital OR amphetamine), and there was no vehicle condition. Under these conditions, neither drug alone will substitute for the compound cue of the drug mixture, and thus the discrimination is "conditional" on the presentation of all components together (Stolerman et al., 1987; Mariathasan and Stolerman, 1994; Mariathasan et al., 1997; Mariathasan et al., 1999a,b) (Figure 1-1).

A similar outcome can be achieved using three-choice discrimination, which trains the discrimination between a compound stimulus, one component of the complex, and vehicle (Bowen et al., 1997; Bowen and Grant, 1998). Under these conditions, the conditional or redundant nature of a particular stimulus within a compound cue can be determined. If separation of a component (Drug A) of a compound stimulus (Drug A+Drug B) to a separate lever disrupts the ability of another component of the complex to substitute (Drug B), then it can be concluded that the complex is "conditional" (Bowen et al., 1997; Bowen and Grant, 1998).

However, if Drug B is able to substitute for the compound cue in the three-choice discrimination, then the components can be termed “redundant” (Figure 1-1).

These studies of compound discriminative stimulus effects are of particular interest to the study of ethanol’s stimulus properties, which is the subject of this dissertation. Ethanol’s discriminative stimulus effects are mediated by concurrent activity at $GABA_A$, NMDA, and 5-HT receptor systems, which can act in parallel as discrete redundant or conditional cues, and thus it is an inherent stimulus complex (Grant, 1994; Grant 1999; Stolerman et al., 1999) (bottom panel, Figure 1-1). The details of the ethanol stimulus complex or compound cue will be described further in section 1.3, but it is important to highlight this key feature of the ethanol cue. The relative contribution of each of the stimulus components in the discriminative stimulus features of ethanol can be manipulated based on the training procedures (Bowen et al., 1997; Bowen and Grant, 1998; Porcu and Grant, 2004) and training history (Green and Grant, 1998).

	Training parameters			Substitution for stimulus effects of Drug A	
	Response 1		Response 2		
Drug vs. Vehicle Discrimination	Drug A	vs.	Vehicle	Drug A	
Drug vs. Drug Discrimination	Drug A	vs.	Drug B	Drug A	
AND Discrimination "redundant"	Drug A + Drug B	vs.	Vehicle	Drug A + Drug B	Drug A Drug B
AND-OR Discrimination "conditional"	Drug A + Drug B	vs.	Drug A OR Drug B	Drug A + Drug B	
3-choice Discrimination	Drug A + Drug B	vs. Drug A	vs. Veh	Drug A + Drug B	Drug B (only if redundant w/Drug A)
AND Discrimination "redundant"	$GABA_A$ + NMDA (Ethanol)	vs.	Vehicle	$GABA_A$ + NMDA	$GABA_A$ NMDA

Figure 1-1. Summary of drug discrimination training parameters. For each discrimination paradigm the training conditions are given in the center column. In the right column, the drug cue(s) that determine substitution for the trained stimulus (stimulus effects of Drug A) is indicated. As described in the text, drugs with shared pharmacology with the drug(s) indicated at the right will produce discriminative stimulus effects similar to the trained drug stimulus.

1.2.3. Relationship between discriminative stimulus effects and subjective drug effects

Over the last several decades, the relationship between discriminative stimulus effects and subjective drug effects has been discussed. In particular, this discussion has focused on relating the discriminative stimulus findings from animal research, with self-reported subjective drug effects in humans. These studies stated that since the discriminative stimulus effects in humans and animals were similar on the basis of drug class, then it could be concluded that the receptor pharmacology was shared across species (Schuster et al., 1981; Woolverton and Schuster, 1983; Schuster and Johanson, 1988). Further, by connecting self-report measures of "euphoria," an indirect connection could be made between stimulus properties and the reinforcing properties of the drugs (Schuster et al., 1981). However, these studies also recognized that even though some connection could be made between discriminative stimulus effects and subjective drug ratings, these terms were not necessarily interchangeable (Schuster and Johanson, 1988). For example, subjective effects encompassed valence and salience cues (such as drug liking) that were not necessary for successful discrimination. Moreover, a high dose stimulant could produce many unwanted and unpleasant effects to the user, which would qualitatively differentiate subjective drug ratings, but may not interfere with the discriminative stimulus features of the drug (Schuster and Johanson, 1988). Additionally, an inherently aversive substance such as lithium chloride can be trained in a discrimination task (Martin et al., 1990), suggesting that the stimulus features are not necessarily overlapping with the reinforcing features of a drug experience. These studies were mostly correlative until the work of Tom Kelly, who measured discriminative stimulus effects and subjective drug ratings in the same subjects (Kelly et al., 1997). In general, the conclusions from his work stated that discriminative stimulus effects were one component of the subjective drug experience that could be tied directly to a receptor mechanism to make conclusions about a drug's abuse potential (Kelly et al., 2003).

1.2.4. Drug discrimination as a behavioral pharmacology assay

Since the discriminative stimulus effects are based in pharmacology, drug discrimination is a behavioral pharmacological assay in which the receptor mechanisms mediating a drug cue can be assayed in awake, behaving animals. As such, many pharmacological principles can be applied to drug discrimination studies and substitution testing. Following drug discrimination training, a dose response curve can be constructed with the training drug. In all drug discrimination studies, in which more than one response is possible (i.e., two lever task as opposed to T-maze) a percentage of responses on the drug-appropriate lever can be calculated for each dose to construct a dose response curve (see Baseline curve in black, Figure 1-2). The percentage of responses on a given lever can be categorized into three groups: full drug substitution, partial substitution, or no substitution. The boundaries of full and partial substitution can vary across study, but in general >80% drug-appropriate responses are considered full substitution and <20% drug-appropriate responses is considered no substitution, with partial substitution between 20-80% (Figure 1-2). From each dose response curve, a 50% effective dose (ED_{50}) can be calculated, which can be used to compare across dose response curves.

From ED_{50} calculations, differences in drug potency can be measured. Lower ED_{50} indicates increased drug potency or an additive or agonist effect. Higher ED_{50} indicates decreased drug potency or an antagonist effect. Antagonist effects can also be observed in drug efficacy, which decreases the ability of a drug to produce full substitution (red dashed line, Figure 1-2).

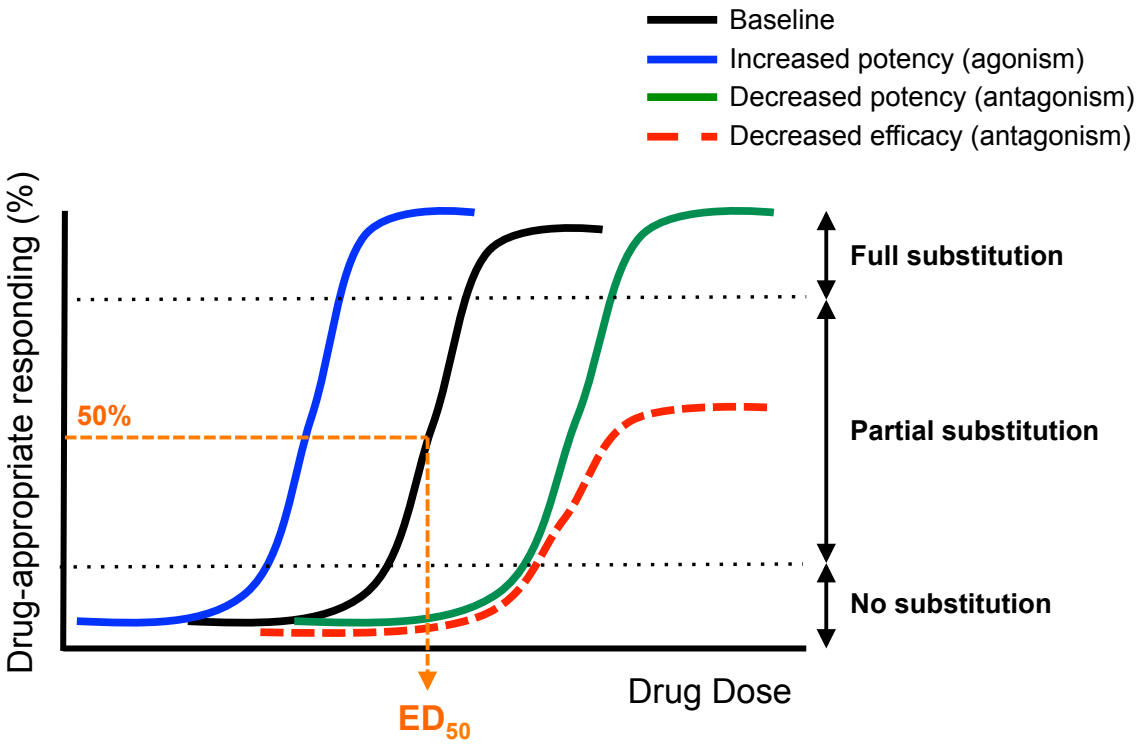

Figure 1-2. Pharmacology concepts in drug discrimination. Simple drug substitution curve is shown in the solid black trace. Drug dose (typically in log units) along the x-axis, and the percentage of drug-appropriate responding shown on the y-axis. Thresholds for full, partial, and no substitution are indicated with the horizontal dotted lines (typically at 20% and 80% for no substitution and full substitution respectively). From the dose response curve an ED_{50} can be calculated, representing 50% responding on the drug-appropriate lever. The ED_{50} can be used to compare across different drug substitution potencies or agonist (blue trace) and antagonist (green trace) effects. Decreases in efficacy can also be measured by a change in maximum drug-appropriate responding, such as antagonist effects that result in only partial substitution as maximal doses (red dashed trace).

1.3. Pharmacological basis of ethanol's stimulus effects: rodent studies

As previously mentioned, ethanol drug discrimination studies have established three primary receptor targets involved in ethanol's discriminative stimulus effects: $GABA_A$, NMDA, and 5-$HT_{1B/1C}$ systems (reviewed in Grant, 1999). There has also been some evidence for a secondary, modulatory role of both the opioid (Mhatre and Holloway, 2003; Middaugh et al., 1999; Middaugh et al., 2000; Shippenberg and Altshuler, 1985; Winter, 1975) and acetylcholine (Bienkowski and Kostowski, 1998; Ford et al., 2012; Ford et al., 2013; Korkosz et al., 2005; Le Foll and Goldberg, 2005) receptor systems, but there is no evidence of direct mediation of ethanol's discriminative stimulus effects at these receptor sites. Ethanol is known to act as a positive allosteric modulator at the $GABA_A$ receptor to increase chloride conductance through the channel and decrease cellular excitability (Figure 1-3; Lovinger and Roberto, 2013). Additionally, ethanol has antagonist activity at the NMDA glutamate receptor, which decreases cellular excitability by decreasing conductance for Na^+, Ca^{2+}, and K^+ (Figure 1-3). Lastly, ethanol has activity at several 5-HT receptor systems, but agonism at the 5-$HT_{1B/1C}$ receptor is most prominent in producing ethanol-like discriminative stimulus effects (Grant and Colombo, 1993c; Grant, 1994; Stolerman et al., 1999; Stolerman et al., 2011).

Somewhat unique to ethanol, the relative contribution of these stimulus components varies based on training dose magnitude, with $GABA_A$ receptors exerting greatest influence at low to moderate training doses (≤1.5 g/kg) and NMDA receptors playing a larger role at higher doses (≥1.5 g/kg) in rodents (primarily rats) (Stolerman et al., 2011; Grant and Colombo, 1993b; Colombo and Grant, 1992). Similarly, the 5-HT component of the ethanol stimulus complex is most prominent at low to moderate training doses (Grant and Colombo, 1993c). More recent work expands upon this foundation and emphasizes the selectivity of ethanol at different receptor subtypes and subunits by incorporating novel ligands.

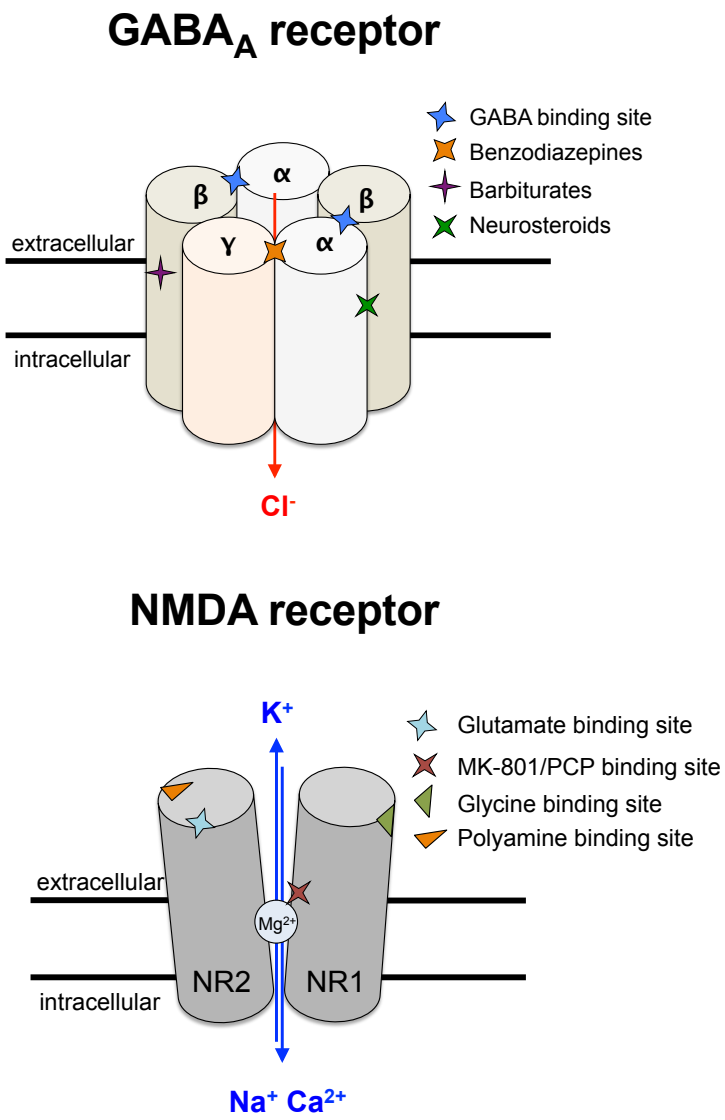

Figure 1-3. Diagram of $GABA_A$ and NMDA receptor structure and binding sites. $GABA_A$ diagram adapated from Seighart, 1995 and Rudolph and Mohler, 2004. NMDA receptor diagram adapted from Mori and Mishina, 1995, Paoletti and Neyton, 2007.

1.3.1. GABA

The $GABA_A$ receptor is a pentaheteromeric transmembrane receptor, classically made up of 2 alpha (α) subunits, 2 beta (β) subunits, and one gamma (γ) subunit (Figure 1-3) (McKernan and Whiting, 1996). There are 19 available subunits that are currently known: α1-6, β1-3, γ1-3, δ, ε, π, θ, and ρ1-3, with the 2α-2β-γ conformation making up 90-95% of the $GABA_A$ receptors (McKernan and Whiting, 1996). The delta (δ) subunit can substitute for the γ subunit in some receptor isoforms (McKernan and Whiting, 1996). The $GABA_A$ receptor has many distinct binding sites, which are of interest to ethanol discrimination. The GABA binding site resides between the α and β subunits, so there are two sites for GABA in the common conformation (Seighart, 1995; Rudolph and Mohler, 2004). Binding of GABA to the receptor results in channel opening and chloride ions move from the extracellular to the intracellular space, hyperpolarizing the cell (Seighart, 1995). Agonist muscimol is able to activate $GABA_A$ receptors in the absence of GABA at the GABA agonist binding site (Sieghart, 1995). There are also three distinct allosteric binding sites that correspond to drug classes: barbiturate, benzodiazepine, and neurosteroids. Each of these drugs act as positive allosteric modulators of the channel meaning that they can only increase chloride conductance when an agonist is bound. The localization of each of these binding sites on the channel is shown in Figure 1-3. Barbiturates bind primarily to residues on the β subunit to increase the duration of channel opening (Serafini et al., 2000). Benzodiazepines bind to residues at the α/γ intersection to increase the frequency of channel opening (Rudolph and Mohler, 2004). Neurosteroids act at the α and β subunits to increase $GABA_A$ conductance, and are particularly dependent on the specific residues in the alpha subunit (Hosie et al., 2006; Hosie et al., 2009) (Figure 1-3).

Consistent with ethanol's action as a positive allosteric modulator at the $GABA_A$ receptor, drugs in the benzodiazepine and barbiturate classes, with a similar mechanism to modulate chloride flow through the $GABA_A$ receptor consistently produce ethanol-like discriminative stimulus effects (reviewed in Grant, 1994). More recent work has expanded upon

these findings in two primary ways. First, the specific action of ethanol at $GABA_A$ receptors with distinct subunit compositions has been investigated using genetic knockouts and selective ligands. Second, the selective role of neurosteroid activity at the $GABA_A$ receptor has been tested, consistent with the action of neurosteroids as positive allosteric modulators at $GABA_A$ receptors, similar to the benzodiazepine and barbiturate drug classes.

Ethanol discrimination studies have primarily focused on isolating the role of α1-, α4/6-, and δ-subunit containing receptors. Specifically, zolpidem, an α1 subunit preferring benzodiazepine agonist, partially substitutes for ethanol in rats (Bienkowski et al., 1997), but does not produce ethanol-like stimulus effects in mice (Shannon et al., 2004), suggesting that activity at the α1 subunit is not sufficient to produce ethanol discriminative stimulus effects in rodents. Additionally, ethanol's action at α4/6-subunits has been investigated using Ro 15-4513, an inverse agonist at the benzodiazepine binding site, with some selectivity for the α4/6-subunits. While Ro 15-4513 successfully antagonizes the discriminative stimulus effects of benzodiazepines, the results are mixed for ethanol-trained rodents, with some studies showing antagonism of ethanol's discriminative effects (Rees and Balster, 1988; Gatto and Grant, 1997), and others showing no antagonism (Hiltunen and Jarbe, 1988; Middaugh et al., 1991). The mixed effects of Ro 15-4513 as an ethanol antagonist is likely due to the differences in training doses and routes of administration, suggesting that the prominence of the α4/6-subunits in ethanol discrimination is dependent on experimental parameters that might influence blood ethanol concentration (BEC). The δ-subunit of the $GABA_A$ receptor complex has also been isolated in ethanol discrimination using a constitutive δ-subunit knockout line of mice, and the results indicated that there were no differences in either the acquisition of ethanol discrimination or the substitution patterns of the $GABA_A$ receptor positive modulators compared to wild type mice (Shannon et al., 2004). Therefore the δ-subunit of $GABA_A$ receptors is not necessary for mediating ethanol-like discriminative stimulus effects or for the substitution of benzodiazepines, barbiturates or neurosteroids. The δ-subunit is thought to be an identifying feature of

extrasynaptic $GABA_A$ receptors that mediate tonic inhibitory currents and confer sensitivity to low doses of ethanol (Carver and Reddy, 2016; Farrant and Nusser, 2005), and thus these data suggest that either non-δ extrasynaptic or synaptic receptors associated with phasic inhibitory currents may be more prominent in producing the discriminative stimulus effects of ethanol.

The steroid binding site on $GABA_A$ receptors and its modulation by neurosteroids has received considerable attention because these endogenous compounds respond to stress and are implicated in a number of behavioral disorders (Paul and Purdy, 1992). Neurosteroids that act at $GABA_A$ receptors do so through binding sites that are distinct from the benzodiazepine and barbiturate sites (Figure 1-3), and the conformation of the steroid A-ring 3' and 5' carbon hydroxyl groups are key to receptor activation (see Chen et al., 2012). Select neurosteroids substitute for ethanol in rodents, including the reduced metabolites of progesterone (allopregnanolone or 3α,5α-P; pregnanolone or 3α,5β-P) and deoxycorticosterone (allotetrahydro-deoxycorticosterone or 3α,5α-THDOC) (Ator et al., 1993). Substitution was more prominent at a lower training dose (1 g/kg, i.g.) versus a higher one (2 g/kg, i.g.) (Bowen et al., 1999). The ethanol route of administration may also play a role in substitution patterns as 3β,5β-P has mixed effects in ethanol discriminations. 3β,5β-P produced no generalization with ethanol trained via an intraperitoneal route (Bowen et al., 1999) but produced complete substitution, as well as potentiation of the ethanol cue, when trained with an intragastric route (Ator et al., 1993; Ginsburg and Lamb, 2005). Finally, the neurosteroid substitution patterns for ethanol suggest sex differences in sensitivity. For example, in contrast to earlier studies in male rats (Ator et al., 1993; Bowen et al., 1999), female rats showed only partial substitution of allopregnanolone and pregnanolone for a 1 g/kg ethanol training dose (Helms et al., 2013). This latter finding is consistent with earlier work demonstrating that females were less sensitive to the modulatory effects of allopregnanolone on ethanol drinking behavior when compared to males (Finn et al., 2010). Collectively, these and other studies (e.g., Bienkowski and Kostowski, 1997) suggest that $GABA_A$ receptors that contain a neurosteroid binding site contribute to the discriminative

stimulus effects of ethanol. Similar to barbiturates and benzodiazepines, neurosteroids asymmetrically cross-substitute for ethanol, with only partial substitution when ethanol is substituted in pregnanolone-trained rats (Engel et al., 2001; Gerak et al., 2008; Vanover, 2000) and mice (Shannon et al., 2005). This asymmetrical cross-substitution likely reflects the inability of pregnanolone and related neurosteroids to encompass other aspects of the compound ethanol cue.

1.3.2. Glutamate

The NMDA glutamatergic receptor is also well established in contributing to the discriminative stimulus effects of ethanol, particularly at higher doses in rodents (Middaugh et al., 1999). The NMDA receptor is made up of four subunits, two similar NR1 and two NR2 (A-D) subunits with several known binding sites (Figure 1-3). The NMDA receptor is ligand-gated similar to the $GABA_A$ receptor, but a magnesium ion blocks the channel pore and must be displaced before the receptor channel can be opened (see Figure 1-3). Thus, the NMDA receptor is ligand gated and voltage-dependent, as depolarization is necessary for the removal of the Mg^{2+} block (Coan and Collingridge, 1985). Full activation of the channel requires binding of both glutamate (NR2 subunit) and glycine (NR1 subunit) in the agonist binding domain on the (Mori and Mishina, 1995; Paoletti and Neyton, 2007) (Figure 1-3). Consistent with ethanol's known action as an NMDA antagonist at the synapse (Lovinger and Roberto, 2013), drug discrimination studies have established that antagonism of the NMDA receptor produces ethanol-like discriminative effects. One of the earliest studies determined that the noncompetitive channel blocker dizocilpine (i.e., MK-801) fully substituted for ethanol in pigeons (Grant et al., 1991), and this finding has been replicated in rodents, including multiple strains of rats (Grant and Colombo, 1993b; Shelton and Balster, 1994; Hundt et al., 1998; Kotlinksa and Liljequist, 1997; Sanger, 1993; Schecter et al., 1993) and mice (Shannon et al., 2004; Shelton and Grant, 2002). Other NMDA channel blockers such as memantine, phencyclidine (PCP) and ketamine have yielded similar degrees of substitution for ethanol in rats (Grant and Colombo,

1993b; Hundt et al., 1998; Sanger, 1993). Often, however, substitution requires doses of the NMDA antagonists that also attenuate response rates (Shelton and Balster, 1994; Bienkowski et al., 1998) to the extent that full substitution by these compounds cannot be confirmed (Shelton, 2004).

In addition to the channel blocker site, multiple allosteric modulatory sites on the NMDA receptor have been examined, including the glycine and polyamine sites (see diagram in Figure 1-3). Overall, ligands for each of these other binding sites have been far less effective in producing ethanol-like stimulus effects, indicating that ethanol's action is most similar to the noncompetitive activity at the channel pore. Competitive antagonists at the glutamate site have substituted for ethanol's discriminative stimulus effects in some cases (CGS 19755) (Sanger, 1993), but have only partially substituted in other cases (CPPene, NPC-17742) (Shelton and Balster, 1994; Shelton, 2004). Similar results have been found with glycine site antagonists, with some ligands producing full substitution (L701,324) (Bienowski et al., 1998; Grant and Colombo, 1993b), and others not substituting at all (MRZ2-502 and MRZ2-576) (Hundt et al., 1998; Bienkowski et al., 1998). Lastly, polyamine binding site antagonists (eliprodil and arcaine) produce stimulus effects that do not substitute for ethanol (Hundt et al., 1998; Sanger, 1993). In conclusion, the contribution of the glutamate, glycine, and polyamine binding sites of the NMDA receptor appears minimal in ethanol discrimination, particularly when compared to the channel pore site. However, it is noteworthy that the aforementioned studies were all conducted in rats trained to discriminate a low to moderate dose of ethanol (i.e., 1 g/kg), and it is possible that inconsistent findings between studies may be partially attributable to the training dose studied, as previous work in rodents indicates that NMDA receptors contribute more predominantly to the ethanol stimulus at higher doses (> 1.5 g/kg) in rodents (Stolerman et al., 2011; Grant and Colombo, 1993a,b).

In addition to the NMDA receptor, recent studies have begun to examine the metabotropic glutamate receptor system (mGluR1, mGluR2/3, mGluR5) based on findings that

mGluR5 might modulate activity at the $GABA_A$ receptor (Besheer and Hodge, 2005). Selective mGluR5 antagonist MPEP antagonized the ethanol dose response function by decreasing the potency for ethanol to substitute for itself (Besheer and Hodge, 2005; Besheer et al., 2009; Besheer et al., 2006). An mGluR2/3 agonist also decreased the potency of ethanol discrimination (Cannady et al., 2011), but no effect was observed with any of the mGluR1 antagonists tested (Besheer et al., 2009). These studies have provided a novel pharmacological target for ethanol's discriminative stimulus effects, although it should be noted that these effects are modulatory in nature, and are not sufficient to produce ethanol-like effects on their own. Thus, the direct glutamatergic activity of ethanol remains primarily at the NMDA receptor.

1.3.3. Serotonin

The importance of serotonergic neurotransmission in ethanol discriminative stimulus effects was first reported with the observation that pretreatment with a tryptophan hydroxylase inhibitor (p-chlorophenylalanine; which depletes brain 5-HT) reduces compartment choice between ethanol and water to chance levels in rats studied within a shock avoidance-based discrimination paradigm (Schecter 1973). Since then, there have been several studies to manipulate levels of synaptic 5-HT, through enhancing 5-HT release (fenfluramine), a nonselective 5-HT receptor agonist (5-MeODMT), and selective serotonin uptake inhibitors (SSRIs; fluoxetine and paroxetine). In general, only SSRIs have produced ethanol-like discriminative stimulus effects (Maurel et al., 1997), but this may be mediated through a non-serotonergic mechanism via their augmentation of brain allopregnanolone levels (Pinna et al., 2006), which would be expected to exert positive modulation of $GABA_A$ receptors.

The first 5-HT receptor to be examined in an ethanol discrimination preparation was the $5\text{-}HT_3$ receptor (Lovinger, 1991), which is an ionotropic receptor and therefore from the same superfamily of receptors as the $GABA_A$ and NMDA receptors. Although studies in rats have found that a $5\text{-}HT_3$ receptor agonist (mCPBG) and antagonist (ICS 205-930) do not substitute for ethanol (Mhatre et al., 2001; Stefankski et al., 1996), there is some limited evidence in

pigeons that 5-HT_3 receptor antagonists (ICS 205-930 and MDL 72222) block the discriminative stimulus effects of low to moderate ethanol doses (Grant and Barrett, 1991). These data suggest that the contribution of 5-HT_3 receptors in producing discriminative stimulus effects of ethanol is likely minimal. This conclusion is also supported by data from transgenic mice that over-express 5-HT_3 receptors and show no differences in their ability to acquire an ethanol discrimination or in the substitution profiles with $GABA_A$ receptor positive modulators and a NMDA receptor antagonist when compared to wild-type mice (Shelton et al., 2004).

In contrast to nonselective or selective 5-HT_3 receptor agonists, there is sufficient evidence to indicate a role for agonism at metabotropic 5-HT receptor subtypes in ethanol discrimination. From an initial characterization of several 5-HT receptor agonists in rats, the only compound to yield full substitution for ethanol in rats was TFMPP, a relatively non-selective 5-HT_1 agonist with slightly greater affinity for the 1A isoform (Signs and Schechter, 1986). This finding with TFMPP was replicated in both male (Grant and Colombo, 1993c) and female (Helms et al., 2013) rats. Subsequent evaluations of multiple compounds with various 5-HT receptor agonist profiles in male rats revealed that CGS 12066B and CP 94,253 (both selective for 5-HT_{1B}) or mCPP and RU 24969 (both selective for 5-$HT_{1B/2C}$) fully substituted for ethanol (1 g/kg), whereas as 8-OH DPAT (5-HT_{1A}) and DOI (5-HT_{2A}) did not (Grant et al., 1997b; Maurel et al., 1998; Szeliga and Grant, 1998). A parallel set of antagonism studies used subtype selective antagonists to completely block the ethanol-like effects of CP 94,253 and mCPP (Maurel et al., 1998), leading to an overall conclusion that 5-HT_{1B} and 5-HT_{2C} receptors contribute to the ethanol cue. However, there are inconsistencies in the generalizability of 5-$HT_{1B/2C}$ agonists to substitute for ethanol across sex and species, as RU 24969 only partially substituted for ethanol in female rats (Helms et al., 2003) and mCPP did not substitute for ethanol in mice (Shelton et al., 2004). Refinement of receptor ligands with increased selectivity for 5-HT_1 and 5-HT_2 receptor isoforms (e.g., Gupta and Villalon, 2010; Jensen et al., 2010) coupled with a rapid expansion of novel ligand development for 5-HT_4 receptors, which also functions to regulate

neurotransmission in conjunction with 5-HT_1 and 5-HT_2 receptors (Bureau et al., 2010; Fink and Gothert, 2007), should prompt a fresh look at the involvement of metabotropic 5-HT receptors in modulating the discriminative stimulus effects of ethanol.

1.4. Pharmacological basis of ethanol discrimination in primates

Ethanol discrimination in monkeys has built upon findings from rodents in several key ways. In general, nearly all of the receptor targets of ethanol in monkeys have been taken from the rodent literature and are largely consistent across species. However, there are several important differences between the rodent and the monkey that may inform future clinical work, and shed light on potential limitations of smaller laboratory animals in ethanol discrimination. Non-human primate studies have focused on ethanol's action at the $GABA_A$ and NMDA receptors primarily, with some work on the opioid system. Additionally, non-human primate work has examined other biological variables that may contribute to ethanol's discriminative stimulus effects, such as sex (Grant et al., 2000; Grant et al., 2008a; Helms et al., 2009; Vivian et al., 2002), age (Helms and Grant, 2011), and menstrual cycle (Green et al., 1999a).

Ethanol's action at the $GABA_A$ receptor is highly selective in non-human primates. Specifically, studies in monkeys have examined subunit-selective ligands and antagonists at the $GABA_A$ receptor (Helms et al., 2009; Licata et al., 2010; Platt et al., 2005; Helms et al., 2008), as well as neurosteroid activity (Grant et al., 1996; Grant et al., 1997a; Green et al., 1999a; Grant et al., 2008a). Additionally, cross-generalization analysis was possible by studies that trained ethanol-like $GABA_A$ ligands and examined ethanol in substitution tests (Licata et al., 2010; Ator and Griffiths, 1997; Massey and Woolverton, 1994; McMahon and France, 2005). Similar to rodents, direct agonists at the $GABA_A$ receptor fail to produce ethanol discriminative stimulus effects, but positive allosteric modulators reliably substitute for ethanol (Grant et al., 2000). Specifically, positive modulators at the benzodiazepine and barbiturate binding sites produce the most robust ethanol-like effects (Grant et al., 2000). In contrast to rodents,

however, $GABA_A$ receptor modulators produce full substitution at low and high training doses (1.0-2.0 g/kg), rather than just predominantly at lower doses. Converging evidence from multiple studies suggests that α5 subunit-containing receptors are particularly important in ethanol's discriminative stimulus effects (Helms et al., 2009; Platt et al., 2005; Helms et al., 2008), as well as some contribution of the α1 and α2/3 subunits. Alpha-5 and alpha-1 selective agonists substitute for ethanol, but only inverse agonists selective for either α5 (L-655,708) and α5+α4/6 (Ro 15-4513) are able to antagonize ethanol's discriminative stimulus effects (Helms et al., 2009; Platt and Bano, 2011). Ro 15-4513 is also able to antagonize the substitution of benzodiazepines and barbiturates for ethanol, suggesting a shared action at the $GABA_A$ receptor subunit level (Vivian et al., 2002). Neurosteroids also selectively produce ethanol-like discriminative effects based on their pharmacological effect at the $GABA_A$ receptor. Specifically, 3-α-hydroxy metabolites of progesterone such as allopregnanolone and pregnanolone are positive modulators at the $GABA_A$ receptor and produce ethanol-like stimulus effects in male and female monkeys (Grant et al., 1996; Grant et al., 1997a; Grant et al., 2008a). However, 3-beta-hydroxy metabolites do not reliably substitute for ethanol at any training dose (Platt et al., 2005). Several studies in monkeys have trained $GABA_A$ ligands and tested ethanol for substitution. To summarize this work, ethanol only cross-substituted with pentobarbital (Massey and Woolverton, 1994), but did not substitute for midazolam (McMahon and France, 2005) or lorezepam (Ator and Griffiths, 1997). These data suggest that ethanol's discriminative stimulus effects in the monkey are more similar to barbiturates, as compared to benzodiazepines.

Ethanol's discriminative stimulus effects are also mediated by antagonist activity at the NMDA receptor, and may be modulated by the opioid system. Noncompetitive antagonists at the channel pore MK-801 (or dizocilpine) and phencyclidine (PCP) produce full substitution for ethanol in male and female monkeys, but (unlike rodents) ketamine has not produced full substitution (Vivian et al., 2002). NMDA antagonist substitution was most potent and efficacious at a lower training dose, which is also in contrast to studies in rodents suggesting a higher

ethanol training dose conferred greater NMDA antagonism substitution (Stolerman et al., 2001) (see Section 1.3). These data are consistent with rodent data in characterizing ethanol as a compound stimulus in the monkey, with activity at both $GABA_A$ and NMDA receptors. Further, there has been a limited attempt to characterize the role of mu and delta opioid receptors in mediating the ethanol cue in monkeys. This examination found that selective agonists at both the mu (i.e., morphine, fentanyl) and delta (i.e., SNC 80, SNC 162) receptors did not produce ethanol-like stimulus effects (Grant et al., 2000; Platt and Bano, 2011), indicating that the opioid system is likely not a primary target in ethanol's discriminative stimulus effects. However, non-selective antagonist naltrexone antagonized the ethanol dose-response relationship (Platt and Bano, 2011), suggesting that the opioid system may function as a modulator of the ethanol stimulus, adding to the complex basis of the ethanol cue.

Lastly, non-human primate studies have taken advantage of the overlapping physiology between humans and monkeys to examine biological variables that may contribute to ethanol's discriminative stimulus effects. Most notably, a few of the non-human primate studies have directly compared male and female subjects in the analysis of $GABA_A$ and NMDA receptor involvement in ethanol's discriminative stimulus effects (Grant et al., 2000; Vivian et al., 2002). Though there are small differences between male and female monkeys, in general the pharmacological basis of the ethanol cue is shared across the sexes. One exception relates to neurosteroid substitution for ethanol, which appears dependent on the phase of the menstrual cycle in female monkeys (Green et al., 1997). In the luteal phase when progesterone levels are high, allopregnanolone is more potent in its substitution for ethanol, consistent with greater levels of allopregnanolone in the plasma. Lastly, one study examined the effect of age on ethanol discriminative stimulus effects and determined that ethanol served as a relatively weaker stimulus in middle-aged monkeys, despite elevated blood ethanol concentrations relative to when the same monkeys were young adults (Helms and Grant, 2011). Additionally,

this study demonstrated that ethanol discrimination was persistent and demonstrated up to 3 years without any intermediate training (Helms and Grant, 2011).

1.4.1. Human studies

There are only five known reports of training ethanol as a discriminative stimulus in human subjects (Kelly et al., 1997; Duka et al., 1998; Duka et al., 1999; Jackson et al., 2001; Jackson et al., 2005) and one report of ethanol substitution in a nicotine-trained discrimination, in which it did not substitute (Perkins, 2009). These studies primarily demonstrated that ethanol can be trained with equal sensitivity in male and female subjects (Duka et al., 1999; Jackson et al., 2005), but the acquisition is sensitive to baseline weekly alcohol intake (Duka et al., 1999; Jackson et al., 2001) and ethanol generalization occurs in a dose-dependent manner (Kelly et al., 1997; Duka et al., 1998; Duka et al., 1999). The only study to test a compound other than ethanol examined the benzodiazepine lorazepam and found complete substitution (Jackson et al., 2001). Thus, the only receptor system directly tested and implicated in the basis of an ethanol discrimination in humans is the $GABA_A$ receptor system.

1.5. Neuroanatomical basis of ethanol discrimination

In the last 20 years, there have been only a handful of laboratories that have investigated the neuroanatomical basis of ethanol's discriminative stimulus effects exclusively in rodents. A majority of these studies are based on an initial finding that injection of muscimol ($GABA_A$ receptor agonist) directly in the nucleus accumbens (NAc) core produced full substitution for ethanol in the absence of ethanol (Hodge and Aiken, 1996; Hodge and Cox, 1998). These studies originally targeted the NAc core but there have been no studies to date that have directly tested the NAc shell specifically in ethanol discrimination. However, when rats are trained to discriminate ethanol from water, ethanol administration results in significant decreases in cFos immunoreactivity selectively within the NAc core, but not the shell (Besheer et al., 2008; Jaramillo et al., 2016), providing some evidence for regional specificity within the

NAc. Since the initial findings with muscimol, $GABA_A$ receptor positive allosteric modulators pentobarbital and allopregnanolone administered into the NAc core have also produced full ethanol substitution (Hodge and Aiken, 1996; Hodge and Cox, 1998; Hodge et al., 2001b). Interestingly, administration of the $GABA_A$ receptor antagonist bicuculline directly into the NAc core does not impair ethanol discrimination, suggesting that $GABA_A$ receptor activity within the NAc core is sufficient, but not necessary to produce ethanol's discriminative stimulus effects (Hodge and Aiken, 1996). In addition to $GABA_A$ agonists and positive modulators, the NMDA receptor antagonist MK-801 injected directly into the NAc core also produced full substitution for ethanol (Hodge and Cox, 1998). Lastly, injection of mGluR5 agonists are not sufficient to produce ethanol's discriminative stimulus effects alone, but injection of an mGluR5 antagonist into the NAc can decrease the potency of an ethanol discrimination (consistent with peripheral administration) (Besheer et al., 2009). Co-administration of muscimol and MK-801 in the NAc core resulted in enhanced potency of substitution compared to either drug alone (Hodge and Cox, 1998), suggesting that both components of the compound ethanol cue are mediated in some part by the NAc core.

In addition to the NAc core, regions that are known to project directly to the NAc have also been examined in ethanol discrimination. Specifically, the role of the amygdala (Hodge and Cox, 1998; Besheer et al., 2003), hippocampus (Hodge and Cox, 1998), prelimbic cortex (Hodge and Cox, 1998), medial prefrontal cortex (Jaramillo et al., 2016), insular cortex (Jaramillo et al., 2016; Jaramillo et al., 2017), and rhomboid thalamus (Jaramillo et al., 2016) have been examined using intracranial pharmacological manipulations. Interestingly, these brain areas appear to have some selectivity for whether they are involved primarily in the GABAergic or glutamatergic component of ethanol's stimulus properties. Specifically, direct $GABA_A$ receptor modulation in the amygdala produces ethanol-like effects, but there is no evidence for this brain region in the NMDA component (Hodge and Cox, 1998; Besheer et al., 2003). Conversely, NMDA receptor antagonist MK-801 in the prelimbic cortex and hippocampus

produced full ethanol substitution, but $GABA_A$ receptor agonists did not substitute (Hodge and Cox, 1998). The mPFC, insula and rhomboid thalamus have also been shown to contribute to the GABA component through pharmacological inactivation using a $GABA_A$+$GABA_B$ antagonist cocktail (Jaramillo et al., 2016). Lastly, the insular cortex has also been identified using a novel chemogenetic approach to selectively inactivate both the insula and the projection from the insula to the NAc (Jaramillo et al., 2017). The inactivation of the insula alone led to modest increases in ethanol discrimination potency (leftward shift), but the projection from insula to NAc led to more robust increases in ethanol potency (Jaramillo et al., 2017), providing further evidence for the critical involvement of the NAc core in ethanol's discriminative stimulus effects.

It is important to highlight that the NAc and regions that project to the NAc are the only circuits that have been tested in ethanol's discriminative stimulus effects. Additionally, among the regions tested, the NAc core was the only region in which infusion of both GABA and NMDA receptor ligands produced full ethanol substitution, indicating a unique contribution of this brain nucleus discrimination of the ethanol stimulus complex in rodents (Hodge and Cox, 1998). These findings are highly consistent with ethanol's known action to enhance $GABA_A$ receptor activity and inhibit NMDA receptor activity within the NAc in slice electrophysiology studies (Lovinger and Roberto, 2013; Nie et al., 1994; Nie et al., 2000). The potency of muscimol to substitute for ethanol was also highest in the NAc core (lower ED_{50}) relative to the amygdala (Hodge and Cox, 1998).

1.6. Nucleus accumbens circuitry

Based on the studies just described, the NAc (specifically NAc core) has been consistently implicated in mediating ethanol's discriminative stimulus effects in rodents. In addition, human functional imaging experiments (functional magnetic resonance imaging, fMRI) have reported that activity in the NAc is correlated with self-report measures of alcohol intoxication (Gilman et al., 2008; Gilman et al., 2012). Thus, there is converging, translational

evidence for the role of the NAc in ethanol's discriminative stimulus effects, providing evidence that it may be involved in ethanol discrimination in non-human primates.

The NAc is a subcortical nucleus within the striatum, which is the primary input structure of the basal ganglia. The primary function of the basal ganglia is to coordinate motor planning and execution through a series of cortical and subcortical feedback loops (Smith et al., 1998; Seasack and Grace, 2010; Haber 2003). The NAc is known to integrate information from both motor and limbic pathways, incorporating motivational information from the ventral midbrain with action planning in the cortex (Mogenson et al., 1980; Floresco, 2015). In this section, literature from both rodents and non-human primates (monkeys) will be reviewed, with an emphasis on the primate-specific findings. However, the anatomy and circuitry of the NAc is mostly consistent across species, so findings from primates are relevant to the rodent brain. The striatum is divided further into dorsal and ventral regions, with the nucleus accumbens residing in the ventral striatum in rats (Zaborszky et al., 1985) and primates (Haber and McFarland, 1999). The cellular composition of the striatum is fairly homogeneous between the dorsal and ventral striatum, and the boundary between the dorsal and ventral striatum are not clearly anatomically distinguishable (Meredith et al., 1996). The ventral striatum is distinguished from its dorsal counterpart based on a distinct pattern of calbindin D_{28K} expression, as well as differential expression of tyrosine hydroxylase (TH), acetylcholinesterase (AChE), and substance P (Voorn et al., 1994; Meredith et al., 1996; Haber and McFarland, 1999) The dorsal and ventral striatum are also more clearly defined by their inputs and outputs.

Within the NAc, there are at least two distinct nuclei, the core and shell regions of the NAc, which were first identified by expression of calbindin D_{28K} (calcium binding protein) in both rodents (Voorn et al., 1989) and primates (Meredith et al., 1996). The NAc core, as the name implies, lies more dorsal and lateral and is cylindrical in shape along the anterior-posterior axis and has the highest calbindin expression (Voorn et al., 1989; Meredith et al., 1996). The NAc shell lies medial to the core, and wraps around the core ventrally to create a crescent shape

(Heimer et al., 1991; Meredith et al., 1996). The shell region can be further subdivided based on calbindin immunoreactivity with the lateral and ventral portion of the shell (lateral shell) having greater calbindin expression than the medial portion of the shell (medial shell) (Voorn et al., 1989; Meredith et al., 1996; Groenewegen et al., 1999).

The NAc core and shell receive descending glutamatergic input from the medial prefrontal cortex (mPFC) (Haber et al., 1995; Groenewegen et al., 1999) and thalamus (Gimenez-Amaya et al., 1995; Groenewegen et al., 1999) (Figure 1-4), similar to the dorsal striatum. In macaques, there are also projections from the orbitofrontal cortex (OFC) (Haber et al., 1995). These cortico-striatal projections maintain strong topographical organization, with medial portions of the cortex and thalamus projecting to the NAc shell preferentially, and lateral portions projecting preferentially to the core in rodent and primates (Heimer et al., 1991; Meredith et al., 1996; Groenewegen et al., 1999; Haber and McFarland, 1999) (see Figure 1-4). The topographical organization leads to a series of feedback loops that can transmit information from the medial to lateral parts of the NAc to the medial and lateral cortex and thalamus (Seasack and Grace, 2010; Haber, 2003). In addition, the NAc also receives descending glutamatergic input from the insular cortex (Wright and Groenewegen, 1996), amygdala (Russchen et al., 1985; Groenewegen et al., 1999; Friedman et al., 2002), and hippocampus (Poletti and Creswell, 1977; Russchen et al., 1985; Groenewegen et al., 1999) (Figure 1-4). The projections from the insula, amygdala and hippocampus further distinguish the NAc from the dorsal striatum.

The topographical organization extends to the efferent projections from the NAc to the ventral pallidum (VP) and ventral midbrain (Figure 1-4). Specifically, the shell preferentially projects to the medial aspects of the VP and the core preferentially projects to the more lateral aspects (Selemon and Goldman, 1990; Hedreen and DeLong, 1991). In addition, the NAc shell projects primarily to the medial aspects of the ventral midbrain, specifically the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) (Heimer et al., 1991; Selemon and

Goldman, 1990; Lynd-Balta and Haber, 1994b). The NAc core projects preferentially to the SNc and substantia nigra pars reticulata (SNr). However, as with the thalamic and cortical projections, a series of feedback loops are present along this medial-lateral axis allowing for reciprocal connections across the entire NAc. The NAc shell also has projections to the lateral hypothalamus and bed nucleus of the stria terminalis (BNST) in the extended amygdala (Haber et al., 2000; Fudge and Haber, 2002) (Figure 1-4). These projections distinguish the shell from the rest of the striatum and have implicated this region as part of the extended amygdala (Fudge and Haber, 2002). In addition, the dopaminergic projections from the midbrain to the shell are more topographically restricted compared to the core, further distinguishing it from the rest of the striatum (Lynd-Balta and Haber, 1994a).

The primary projection neurons in the NAc (both core and shell regions) are GABAergic medium spiny neurons (MSNs), named for their medium soma size and spiny dendrites, make up about 90-95% of the cells in the NAc (Kawaguchi et al., 1995). Nucleus accumbens MSNs project primarily to the VTA/SN as part of the “direct” pathway, and to the VP as part of the “indirect” pathway. The direct and indirect pathways are defined by their projections to dopaminergic cells in the midbrain, with indirect pathway projections going from the VP to the subthalamic nuclei, before projecting back to the VTA/SN (Smith et al., 1998). These direct and indirect pathway projection neurons were originally defined as having distinct expression of dopamine receptors, with direct pathway neurons to the midbrain expressing primarily D1 receptors, and indirect pathway neurons expression dopamine D2 receptors (Gerfen et al., 1990; Lynd-Balta and Haber, 1994b). D1 receptors are G_s-coupled, so activation by dopamine leads to an increase in cellular excitability. Dopamine D2 receptors are $G_{i/o}$-coupled, so dopamine release onto these cells leads to a decrease in cellular excitability (Gerfen et al., 1990). The expression of D1 and D2 receptors appears to be predominantly segregated across distinct cell populations, with very few neurons co-expressing both receptor subtypes (LeMoine and Bloch, 1995). However, recent studies have found that both D1 and D2 receptor expressing

MSNs can project to the pallidum, overturning the idea that these two cell types have distinct connections (Kupchik et al., 2015). However, projections from the NAc to the midbrain appear to be primarily D1 expressing, maintaining the canonical organization (Kupchik et al., 2015).

In addition to the projection MSNs, there are also at least four classes of interneurons, each with a unique physiological and pharmacological profile (Groenewegen et al., 1999; Kawagugi et al., 1995; Sidibe and Smith, 1999). The first class of interneurons are cholinergic, and are identified by expression of choline acetyltransferase (ChAT) in rodents and primates (Kawaguchi et al., 1995; Sidibe and Smith, 1999). Interestingly, the size of cholinergic interneurons varies across the NAc core and shell regions, providing additional support for the distinction between these two structures (Brauer et al., 2000). In addition, there are at least three classes of GABAergic interneurons expressing either parvalbumin, somatostatin, neuropeptide Y, nitric oxide synthase, or calretinin (Kawaguchi et al., 1995; Sidibe and Smith, 1999).

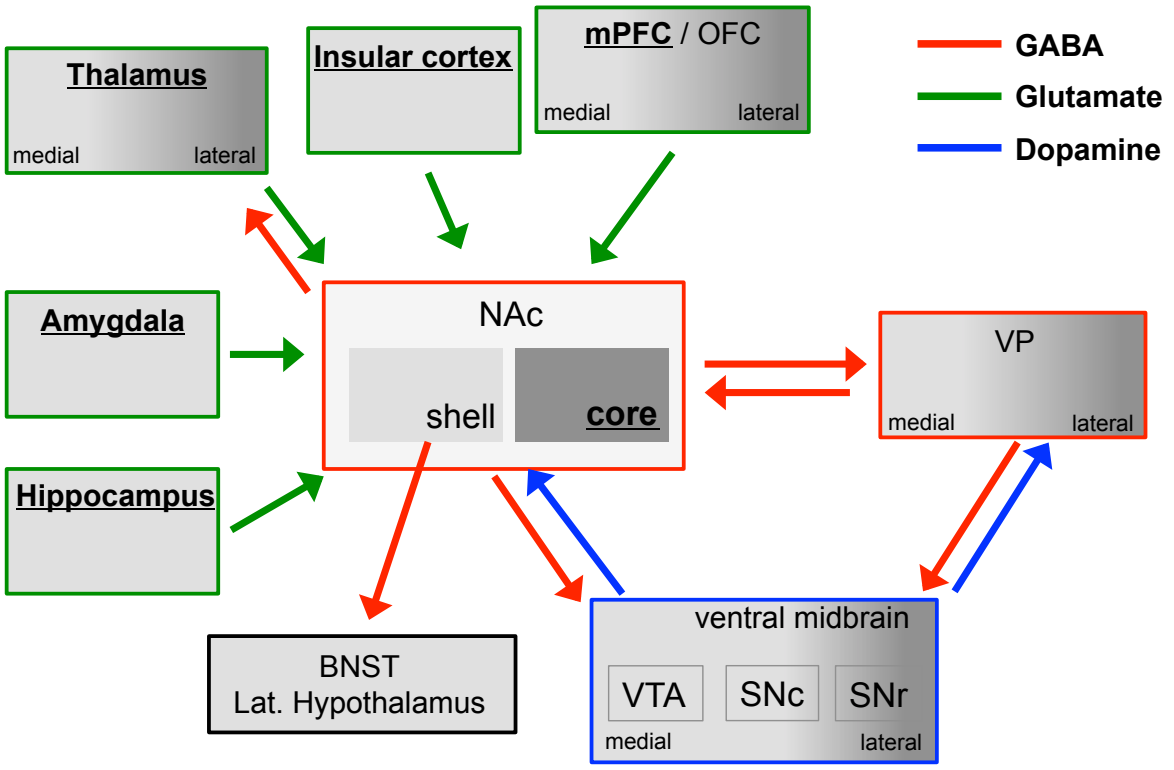

Figure 1-4. Nucleus accumbens circuitry. The major inputs and outputs of the NAc core and shell are indicated in this simplified diagram. Projections to and from the NAc maintain a topographic organization between NAc core and shell regions. Specifically, the NAc shell receives input predominantly from the medial portions of the thalamus, mPFC and OFC, and sends projections to medial portions of the ventral midbrain (primarily VTA) and medial VP. The NAc core receives input predominantly from the lateral portions of the thalamus, mPFC, and OFC, and sends projections to lateral aspects of the ventral midbrain (SNc and SNr) and lateral VP. This topographic organization is represented by a gradient, with the shell projections in light gray and core projections in darker gray. There are a reciprocal feedback loops across the medial-lateral gradient to connect the entire circuit. Regions that have been tested and found to be implicated in ethanol discrimination are underlined and bolded. Figure adapted from: Heimer et al., 1991; Meredith et al., 1996; Haber et al., 2000; Fudge and Haber, 2002; Haber 2003; Lynd-Balta and Haber, 1994a,b; Seasack and Grace, 2010

1.7. Chemogenetics in ethanol discrimination

Studies examining the neuroanatomical basis of ethanol discrimination have been almost exclusively conducted using site-specific pharmacological approaches, with the exception of one recent study that used chemogenetics (Jaramillo et al., 2017). Chemogenetics (or "chemical genetics") encompasses a range of tools in which a modified receptor can be delivered to and expressed in cells in the brain through a viral-mediated delivery system (Farrell and Roth, 2013; Sternson and Roth, 2014; Urban and Roth, 2015). The most widely used chemogenetic tool is Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). DREADDs are mutated G-protein-coupled receptors that have lost their affinity for their native ligand, and are instead activated by a designer actuator (Armbruster et al., 2007; Lee et al., 2014; Urban and Roth, 2015; Roth, 2016). One important feature of all DREADDs is that the designer actuators are peripherally bioavailable, allowing for reversible manipulation of specific cell populations without the need to maintain indwelling cannula (Armbruster et al., 2007; Urban and Roth, 2015; Roth 2016). Eliminating the need for cannula provides distinct advantages in non-human primate research, as it minimizes concern for repeated tissue damage with direct intracranial injections, thereby maximizing the longevity of non-human primates. This is particularly significant for drug discrimination experiments as training takes place over a long period of time with peripheral drug administration, and consistent methods are essential for isolating interoceptive drug cues (reviewed in Grant, 1999; Allen et al., 2017).

There are currently six different DREADD receptors available, each identified by the mutant receptor they were generated from or the signaling pathway they target: hM3Dq, hM4Di, $hM4D^{nrxn}$, GqS, β-Arr DREADD, and KORD (reviewed in Roth, 2016). The hM3Dq and hM4Di DREADDs are mutated human (h) muscarinic type 3 (M3) and type 4 (M4) DREADD receptors (D) that are G_q- or $G_{i/o}$-coupled, respectively (Armbruster et al., 2007; Roth, 2016). Activation of the hM3Dq DREADD leads to increased cellular excitability and activation of the hM4Di DREADDs leads to presynaptic inhibition and silencing, each through activation of the

respective G-protein cascades (Armbruster et al., 2007, Roth, 2016). The hM4D^{nrxn} DREADD is a variant on the hM4Di that was designed to selectively target and inhibit axons (Stachniak et al., 2014). The GqS is a mutated M3 adrenergic receptor that increases production of cAMP (Guettier et al., 2009), and the β-Arr DREADD is a mutated version of the hM3Dq DREADD that selectively activates the β-Arrestin pathway, as opposed to the G_q signaling cascade (Nakajima and Wess, 2012). Finally the KORD, or κ-opioid DREADD, is a mutated κ-opioid receptor that can lead to presynaptic inhibition and silencing (Vardy et al., 2015; Roth, 2016). Five out of the six available DREADDs (hM3Dq, hM4Di, hM4D^{nrxn}, GqS, β-Arr) are activated by clozapine-N-oxide (CNO), which is an inert metabolite of the pharmacologically active drug clozapine (Urban and Roth, 2015). The KORD DREADD is activated by salvinorin B (SalB), which is an inert mutant of the pharmacologically active drug salvinorin A.

The pharmacology of the DREADD receptor and the pharmacokinetics of the activating ligand contribute to the time course that the DREADDs are activated (Roth, 2016). CNO is active after peripheral administration in rodents and primates on the scale of several hours (Roth, 2016), and the DREADDs are expected to remain active as long as CNO is present (Armbruster et al., 2007; Urban and Roth, 2015; Roth, 2016). The onset of DREADD activation is expected to match CNO pharmacokinetics, within 30-60 minutes of CNO administration (Armbruster et al., 2007; Roth, 2016). SalB however is quickly absorbed and distributed, so KORD DREADDs are expected to be active within a few minutes of SalB administration, and remain active for up to one hour (Vardy et al., 2015).

1.8. Dissertation studies and hypothesis

The overall goal of the dissertation is to begin to uncover the neuroanatomical basis of ethanol's stimulus properties in the rhesus macaque. Despite the longstanding use of rhesus macaques in ethanol self-administration experiments (Winger and Woods, 1973; Kornet et al., 1990; Grant et al., 2008b), the discriminative stimulus effects of ethanol have never been tested

in this species. Long-term ethanol self-administration experiments have indicated that rhesus monkeys can model many aspects of AUD in human patients, particularly the individual variability to become a heavy drinker (Grant et al., 2008b; Baker et al., 2014; Baker et al., 2017), age of onset of drinking (Helms et al., 2014b), adrenal response to long term drinking (Helms et al., 2014a), and response to repeated abstinence periods (Allen et al., 2018). Identifying the pharmacological basis of ethanol's discriminative stimulus effects in rhesus monkeys can help identify candidate receptor systems for the development of targeted pharmacotherapies (Grant, 1999). In particular, the extensive characterization of the behavioral phenotype of rhesus monkeys during and following long term alcohol drinking can be combined with the pharmacology of ethanol's discriminative stimulus effects for development of AUD treatment. Importantly, there is evidence for species differences in the discriminative stimulus effects of ethanol, emphasizing the importance of characterizing the discriminative stimulus effects in the rhesus macaque (Allen et al., 2017).

Thus, the first aim of this dissertation (Chapter 2) was to characterize the receptor basis of ethanol in rhesus monkeys. The dose of ethanol selected was 1.0 g/kg for two reasons: 1) this dose of ethanol is expected to lead to blood ethanol concentrations (BECs) of approximately 80mg/dl, which is the legal limit of intoxication (NIAAA, 2015), and 2) previous studies in cynomolgus macaques indicated that both $GABA_A$ and NMDA components of the ethanol cue are active at this dose (Grant et al., 2000; Vivian et al., 2002). Additionally, the time course of ethanol absorption and elimination in rhesus monkeys was determined in a complete pharmacokinetic study in this dissertation, which had only been reported in cynomolgus macaques previously (Green et al., 1998). $GABA_A$ receptor positive allosteric modulators pentobarbital and midazolam and the noncompetitive NMDA receptor antagonist MK-801 (or dizocilpine) were tested for substitution for 1.0 g/kg ethanol. The use of two $GABA_A$ receptor ligands allowed for improved specificity of the GABAergic component of the ethanol stimulus complex, as barbiturates and benzodiazepines have distinct binding profiles at the $GABA_A$

receptor. Specifically, the barbiturate binding site is on the β subunit of the $GABA_A$ receptor and enhances GABA activity through increasing the duration of channel opening (Serafini et al., 2000). The benzodiazepine binding site is at the α and γ subunits (Sigel and Buhr, 1997) and binding at this site increases the frequency of GABA channel opening (Rudolph and Mohler, 2004). MK-801 was the primary NMDA antagonist examined in ethanol discrimination because it is an NMDA receptor channel blocker that had the highest efficacy of the NMDA ligands previously tested in cynomolgus macaques (Vivian et al., 2002).

In the next section of this dissertation, a novel chemogenetic approach was used to directly test the role of the nucleus accumbens in ethanol discrimination in rhesus monkeys (Chapter 4). There have been no studies to date prior to this dissertation work that have examined a specific brain nucleus in ethanol discrimination in any primate species, so these studies represent a novel area of research. These experiments contribute to just the small handful of published reports using chemogenetics in monkeys (Eldridge et al., 2016; Nagai et al., 2016; Grayson et al., 2016) and the single published study using chemogenetics in a behavioral paradigm in monkeys (Eldridge et al., 2016). Prior to application of chemogenetics to ethanol discrimination, the pharmacokinetics of the primary DREADD actuator (CNO) was examined in these subjects (Chapter 3).

Based on the rodent drug discrimination experiments detailed in section 1.5, it was hypothesized that ethanol acts within the NAc core at $GABA_A$ and NMDA receptors to produce the discriminative stimulus effects of ethanol in the rhesus macaque (Hodge and Cox, 1998). Positive allosteric modulation of the $GABA_A$ receptor and antagonism at the NMDA receptor results in an overall decrease in neuronal excitability, which leads to a decrease in GABA release at projection targets, with the VP and VTA/SN being the most prominent (see Figure 1-5a). Since the NAc projections are GABAergic, the result of ethanol's action is disinhibition of the NAc projection sites, leading to increased excitability of the post-synaptic cells within the VP and VTA. In order to test this hypothesis using chemogenetics, the hM4Di DREADD receptor

was targeted to the NAc core. The hM4Di inhibitory DREADD was selected over the KORD DREADD due to the longer time course, which would ensure that DREADD activation could be maintained throughout the entire behavioral session (Vardy et al., 2015). I hypothesized that activation of hM4Di DREADDs would enhance the inhibitory effect of ethanol on NAc neurons, leading to a potentiation of ethanol's discriminative stimulus effects (Figure 1-5b). This hypothesis can be extended to either $GABA_A$ or NMDA substitution for ethanol, each contributing to a decrease in NAc excitability, and thus activation of hM4Di receptors in the NAc was expected to increase the potency of substitution (Figure 1-5b, right panel). The DREADD experiments took place over and 8-10 month period, and thus will not only inform the field of ethanol's discriminative stimulus effects, but also the durability of chemogenetics over a long term study in non-human primates. A detailed timeline is presented in Table 1-1, and highlights the extensive, longitudinal nature of this dissertation in characterizing the stimulus effects of ethanol.

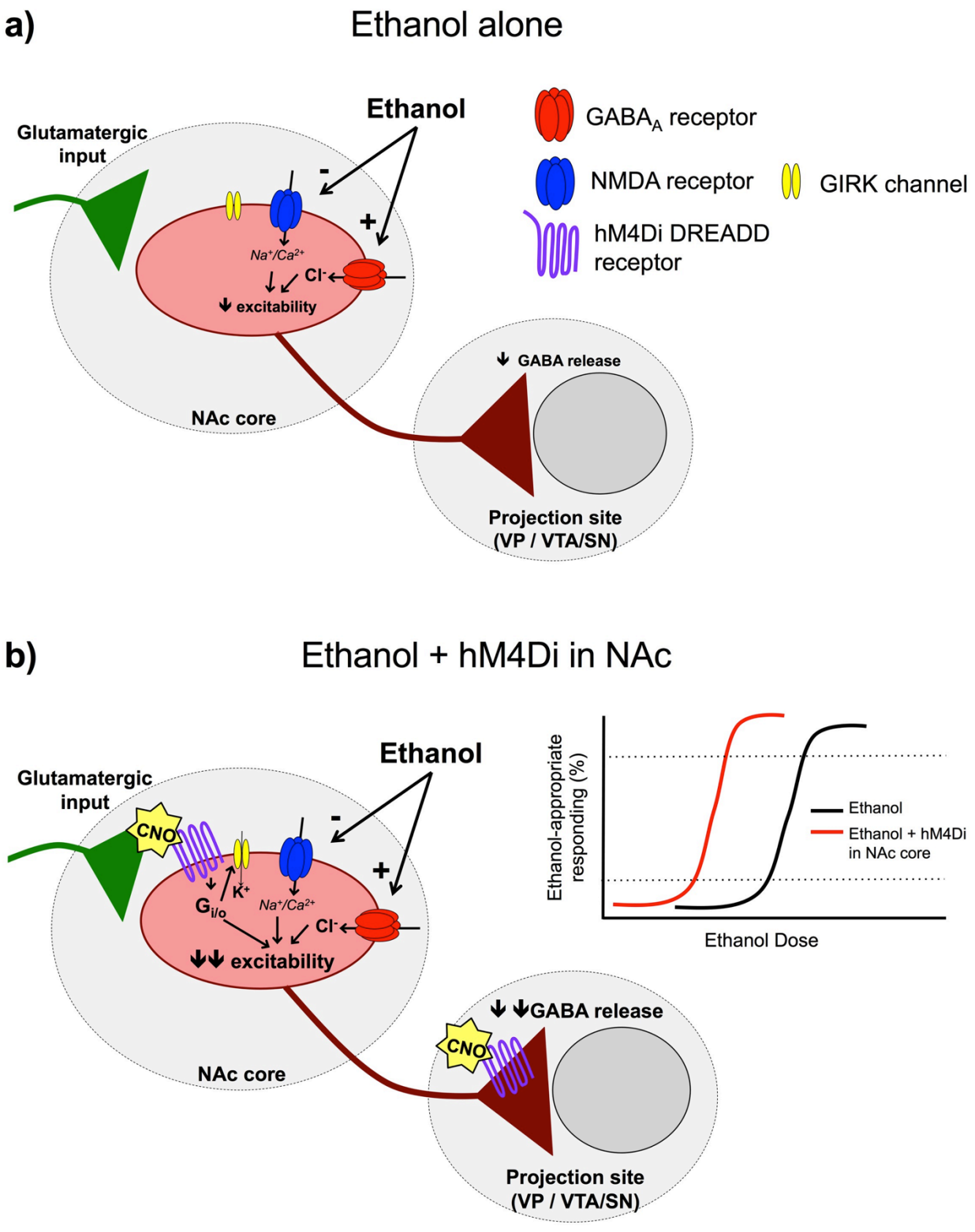

Figure 1-5. Primary hypothesis. a) Simplified diagram of ethanol's action within the NAc core to inhibit NMDA receptor activity and enhance $GABA_A$ receptor activity to decrease cellular

excitability of GABAergic projection neurons. The result of ethanol's action is a decrease NAc core excitability and a decrease in GABA release onto projection targets including the VP and ventral midbrain (VTA and SN). Substitution for ethanol by $GABA_A$ positive modulators or NMDA antagonists can occur through direct action on each of these receptors in the absence of ethanol. b) Research hypothesis: Injection of hM4Di inhibitor DREADD receptors into the NAc core will lead to expression on the cell bodies within the NAc core and axonal projections at downstream targets. Activation of hM4Di receptors by CNO activates $G_{i/o}$ downstream targets to activate GIRK channels and inhibit the production of cAMP to reduce cellular excitability. Additionally, GABA release onto downstream targets will be further reduced by hM4Di activation. The hypothesized effect of hM4Di activation is to enhance the potency of ethanol's discriminative stimulus effects, resulting in leftward shifts in the dose response curve (shown on the right).

Timeline	Duration	Experiment	Dissertation chapter
Jul 2015 - Aug 2016	11-14 months	Training -Procedural training -Discrimination training	*Chapters 2 & 4
Feb 2016	< 1 month	Ethanol PK study	*Chapter 2
Jun 2016 - Apr 2017	12-14 months	Subsitution testing -Ethanol (n=8) -Morphine (n=8) -PB, MDZ, MK-801 (n=5-7)	*Chapter 2
Dec-Jan 2017	1 month	CNO PK Study	*Chapter 3
Jan/Mar 2017	< 1 month	Pre-Sx CNO tests	
Feb/Apr 2017	< 1 month	**DREADD Surgery**	
Mar-Jul 2017	1-2 months	Re-establish EtOH discrimination	
Mar-Nov 2017	6-8 months	DREADD testing -Ethanol (n=7) -PB, MDZ (n=6-7) -MK-801 (n=1)	*Chapter 4
Nov-Dec 2017	< 1 month	Necropsy & Tissue collection	

Table 1-1. Experimental timeline overview. Experiments from all chapters were conducted longitudinally in the same eight subjects. EtOH= ethanol; PK = pharmacokinetics; Sx= surgery; PB= pentobarbital; MDZ=midazolam.

Chapter 2: Characterizing the pharmacokinetics and discriminative stimulus effects of ethanol in the rhesus monkey

2.1. Introduction

Alcohol intoxication is highly prevalent with an estimated 26.9% of adults in the United States having had a binge-drinking episode in the last month (NIAAA, 2015). Binge drinking is a pattern of alcohol intake (approximately 4-5 drinks per occasion) that results in a blood alcohol level of greater than 0.08%, or 80 mg/dl, which can lead to significant cognitive and motor impairments. Impairments associated with binge drinking contribute to 75% of the total economic burden of alcohol-related costs totaling $249 billion in the US (NIAAA, 2015). Despite the wide-ranging and costly effects of alcohol intoxication, there are limited successful, targeted treatments for alcohol use and alcohol use disorder (AUD) (Jonas et al., 2014), necessitating continued development of robust translational animal models.

Interoceptive drug effects are strongly based in receptor pharmacology and can be queried through a behavioral output using a drug discrimination procedure (Grant 1999). Using this approach, previous studies have established that ethanol is a stimulus complex with concurrent activity at multiple receptor systems (Grant 1999; Green and Grant, 1998; Stolerman et al., 1999; Stolerman and Olufsen, 2001). Specifically, in drug discrimination, interoceptive drug cues are trained as discriminative stimuli, enabling an animal to reliably report the presence of a specific drug cue through a discrete behavioral response (Stolerman, 2014). Ethanol's discriminative stimulus properties are primarily mediated by activity at the $GABA_A$ and NMDA receptor systems. Specifically, stimulus effects of ethanol are most strongly associated with positive modulatory action at the $GABA_A$ receptor (Overton 1977; Ator et al., 1993; Grant et al., 2000; Platt et al., 2005; Grant et al., 2008a) and non-competitive antagonism at the NMDA receptor (Balster et al., 1992; Grant and Colombo, 1993b; Vivian et al., 2002). The relative contribution of each of these receptor systems to ethanol's discriminative stimulus effects is

dependent on ethanol training dose in rodents, with lower ethanol doses being most similar to $GABA_A$ receptor positive modulators, and higher ethanol doses being most similar to NMDA receptor antagonists (Stolerman et al., 2011). However, this dose relationship does not directly translate to macaque monkeys, with $GABA_A$ receptor positive modulation and NMDA receptor antagonism remaining prominent at both low and high ethanol doses in cynomolgus macaques (Grant et al., 2000; Grant et al., 2008a; Vivian et al., 2002; Allen et al., 2017).

There is evidence that the alcohol's subjective effects (i.e., interoceptive effects) can perpetuate continued drinking, indicating that the way alcohol makes you feel may contribute to its abuse potential (Holdstock et al., 2000; Kelly et al., 2003; Childs et al., 2011). Thus, investigation of the mechanisms that mediate alcohol's interoceptive effects can inform our understanding of alcohol's actions in the brain to improve targeting strategies for potential pharmacotherapies. Rhesus monkeys have been used as a translational model of alcohol use and alcohol use disorders for several decades, and have been demonstrated to voluntarily self-administer alcohol to intoxication (Winger and Woods, 1973; Kornet et al., 1990; Grant et al., 2008b). Despite the increased use of this species for alcohol research, there have been no published studies to date characterizing the receptor basis of the discriminative stimulus effects of ethanol in this species. Given the number of species differences between rodent and cynomolgus macaque that have been reported (reviewed in Allen et al., 2017), it is essential to characterize species-specific stimulus effects.

In addition to limited understanding of ethanol's discriminative stimulus effects in rhesus macaques, there have also been no published studies to date characterizing the absorption and elimination rate of ethanol in this species. These data are essential to understanding the dose-dependent receptor basis of alcohol through drug discrimination, but also inform our understanding of outcomes associated with binge-level alcohol consumption. Thus, in order to improve our understanding of alcohol receptor pharmacology and improve translatability of alcohol-related research in rhesus monkeys, the current study examined alcohol

pharmacokinetics and discriminative stimulus effects of a moderate (1.0 g/kg) dose of ethanol. This dose was selected as it was expected to result in blood alcohol levels of ~80 mg/dl (Green et al., 1999b), which translates to legal intoxication in humans. Additionally, other non-human primate species (cynomolgus macaque and squirrel monkeys) have demonstrated $GABA_A$ and NMDA receptor substitution at this training dose, allowing us to compare across species within both receptor systems (Platt et al. 2005; Grant et al., 2000; Vivian et al., 2002).

2.2. Materials and Methods

2.2.1. Animals

Eight experimentally-naïve late adolescent male rhesus monkeys (*Macaca mullata*) were used in the current study (3.9-4.2 years old, 5.5-7.6 kg at assignment), and the experiments took place over 28 months (6.3-6.6 years old, 7.8-10.2 kg at end). This age range was selected for this study based on a population analysis from our laboratory that found exposure to alcohol during late adolescence and early adulthood represents the highest risk for developing a heavy drinking phenotype in rhesus (Helms et al., 2014b). All monkeys were born and raised at the Oregon National Primate Research Center (ONPRC; Beaverton, OR) and confirmed to not have common parents or grandparents. They were housed in stainless steel one-over-one cages (32 x 28 x 32 in) that were attached along the vertical axis into quad (2 x 2) cages to allow for side-by-side pair housing. All monkeys lived in a single housing room and were pair-housed at all times, except for during behavioral testing and feeding (3-4 hours/day). The housing room was temperature (20–22°C) and humidity (65%) controlled, with a 12-hr on/off light cycle (lights on at 7AM). All monkeys had visual, auditory, and olfactory contact with other members of the study. Monkeys were weighed weekly without sedation and were monitored throughout the experiment by veterinary staff. All procedures were conducted in accordance with NIH and the Guide on the Care and Use of Laboratory Animals and approved by the IACUC at ONPRC.

2.2.2. Behavioral testing apparatus

Discrimination training and testing sessions were conducted 4-6 days/week in four ventilated, sound attenuating operant chambers (1.50×0.74×0.76m; Med Associates, Inc., St. Albans, VT) in a behavioral suite down the hall from the housing room. Each chamber had an operant panel (0.48×0.69 m) equipped with two retractable levers, three lights (red, amber, green) above each lever, and a centrally located white light above a food magazine. The red and green lights were not active during discrimination training and testing, and only the center amber light was illuminated when the associated lever was available (Figure 2-1). Two house lights and a fan were located in the top rear of the chamber. The panel was accessible from a primate chair (1.17×0.61×0.61 m; Plas Labs, Lansing, MI) that had a food magazine tray. One-gram banana flavored pellets (Bio-Serv) were delivered through vinyl tubing attached to a feeder located outside the chamber, and a corresponding stimulus light was illuminated (Figure 2-1). All events were programmed and recorded by LabView (version 4.0.1., National Instruments, Austin, TX) connected to a computer interface (Med Associates, Fairfax, VT) attached Mac computer.

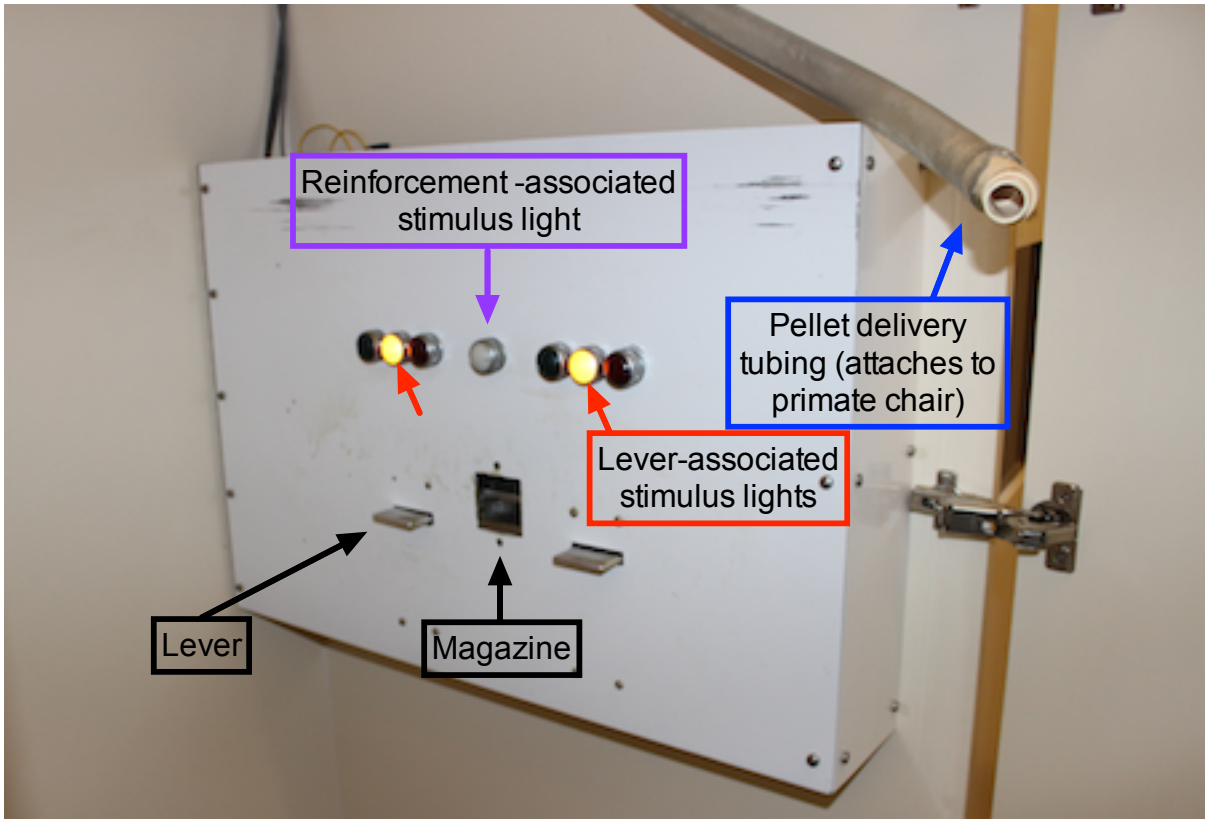

Figure 2-1. Operant panel for discrimination sessions. When each lever was extended, its associated stimulus light (red arrows) was illuminated indicating it was available. After successful completion of the terminal fixed ratio (FR), a pellet was delivered either through tubing attached to the primate chair (blue arrow) or through the magazine (only one subject had pellets delivered to the magazine throughout the experiment). When the pellet was delivered the reinforcement associated stimulus light was illuminated (purple arrow).

2.2.3. Procedural training

Upon arrival to the laboratory, all monkeys were trained with positive reinforcement (fruit and seeds) to sit in a primate chair with the guided pole-and-collar technique. The monkeys were then transported in chairs down the hallway to the behavioral suites where all testing occurred. There were four primate chairs and four testing chambers so behavioral training took place in two groups of four at the same time each morning (8AM and 10:30AM). Responding on the lever was established by differential positive reinforcement of successive approximations of the monkey's hand toward the lever, and eventually downward pressure on the lever to engage the spring mechanism. Once the behavior was acquired, training sessions took place once per day, initially with only one of the two levers extended into the chamber beginning with a fixed ratio 1 (FR1) schedule. Once responding was stable and consistent on both levers, the FR was escalated on an individual basis to a terminal FR that accounted for individual variability in response rate. Terminal FRs were selected that resulted in delivery of 25 reinforcers in approximately 10 minutes, so that the session length was consistent across subjects, which provided experimental control over blood ethanol concentration (BEC) of the trained ethanol stimulus across subjects. Additionally, targeting 10 minutes allowed for significant changes in response rate to be measured, since sessions timed out at 30 minutes. Following panel training, monkeys were trained to accept a nasogastric infant feeding tube (5 French, 36" length), which was measured to the 10^{th} rib for accurate placement into the stomach of each monkey. An endoscopy of two monkeys indicated that 14-16" depth placement from the nostril reached the stomach in adult male rhesus monkeys. During this time, monkeys were also trained to comply with awake venipuncture for blood collection from the medial saphenous vein to determine BECs following testing.

2.2.4. Ethanol pharmacokinetics

Following training to accept the nasogastric tube, but before training the discrimination (less than 7 alcohol administrations per monkey), ethanol (20% w/v ethanol in water, i.g.) was

administered through a nasogastric gavage and repeated blood samples (20µl from medial saphenous vein) were taken for BEC analysis over a 5-hour period to capture the absorption and elimination phases. Ethanol administration and blood sampling were performed without sedation following an overnight fast, and at least 72 hours after the last ethanol administration. Two doses of ethanol were tested at least 2 weeks apart: 0.5 g/kg (n=4) and 1.0 g/kg (n=5, same 4 subjects, plus an additional monkey). Blood samples were collected at the following time points following ethanol administration: 15 min, 30 min, 45 min, 60 min, 75 min, 90 min, 120 min, 180 min, 240 min, and 300 min.

2.2.5. Discrimination training

Next, all monkeys were trained to discriminate 1.0 g/kg ethanol (1.0 g/kg, 20% w/v in water) from water (equivalent volume to 1.0 g/kg ethanol) with a 60 minute pre-treatment interval. During both ethanol and water gavages, a flavored 1-gram pellet (Bio-Serv) was given halfway through the injection and immediately after to mask any taste cues. The animal was then immediately placed in a darkened operant chamber for a programmed 60-minute pretreatment time, after which the house lights turned on, two levers were extended into the chamber and associate stimulus lights (amber) turned on, signaling the start of the session (Figure 2-1). Sessions ended when 25 pellets were earned under the terminal FR, or at 30 minutes, whichever came first. Terminal FRs ranged from FR20 to FR110 over the course of the experiment. In general the terminal FRs were fixed, but were occasionally adjusted over the course of the experiment to account for changes in response rate. For the first 5 training sessions, water was administered, only the water-appropriate lever was extended, and completion of the terminal FR resulted in pellet delivery (forced choice procedure). The same conditions were repeated for the next five sessions, except ethanol was administered before the session, and only the ethanol-appropriate lever was extended and associated with pellet delivery. Lever assignments associated with ethanol (left or right) were counterbalanced across animals. For the remaining training sessions, both levers were extended into the chamber and

ethanol and water were administered on a double-alternating schedule (e.g., 2 water days followed by 2 ethanol days, and so on). Successful completion of the terminal FR on the condition-appropriate lever resulted in the delivery of a 1 g banana-flavored food pellet. Responding on the inappropriate lever reset the FR requirement and was not reinforced (punishment contingency). Discrimination training was complete once the monkeys met the following criteria for 5 consecutive sessions: 1) ≥90% of total session responding must be on the condition-appropriate lever, and 2) ≥70% of the first FR responses must be on the condition-appropriate lever.

2.2.6. Substitution Testing

Test sessions were identical to training sessions, with two key differences: 1) successful completion of consecutive FR requirement on either lever resulted in the delivery of a banana pellet, and 2) the route of administration varied based on the drug administered. Both levers were reinforced to ensure that reinforcement did not influence the responding during test sessions without introducing an extinction contingency. The pretreatment time was kept constant at 60 minutes following drug administration to maintain consistent experimental parameters, eliminating the possibility that different pretreatment time could be incorporated to a responding strategy. Additionally, all test drugs were expected to be active at 60 minutes. In general, test sessions occurred 1-2 days/week, with training sessions on the intervening days. If performance on a training day did not meet criteria, then training sessions were continued until criteria was met for 3 consecutive sessions. All drug and dose combinations were conducted on a single day (single dosing procedure) and each test dose was double determined, counterbalancing for the training session on the day prior to each test. Negative control tests (morphine and muscimol) were not double determined.

An ethanol dose-response determination was first determined for all monkeys (0.0-2.0 g/kg), beginning with the training dose (1.0 g/kg). Selected doses of morphine (μ-opioid receptor agonist, 0.01-1.7 mg/kg; i.m.) were tested next, followed by pentobarbital (barbiturate, $GABA_A$

receptor positive allosteric modulator, 0.56-10.0 mg/kg; i.g.), midazolam (benzodiazepine, $GABA_A$ receptor positive allosteric modulator 0.30-5.6 mg/kg; i.g.), muscimol ($GABA_A$ receptor agonist, 0.3-0.56 mg/kg; i.m.) and MK-801 (NMDA receptor antagonist, 0.003-0.10 mg/kg; i.m.). In general, a new drug was not introduced until the all doses of the previous drug were tested. For each drug, testing began at an intermediate dose and then escalated incrementally until a dose was found that either substituted fully (≥80% ethanol-appropriate responding) or was demonstrated to be behaviorally active (decreased response rate to <65% baseline was the most common measure of behavioral activity). Lower doses were also tested until a dose was found that did not produce substitution (≤20% ethanol-appropriate responding). In some cases, higher doses of morphine did not produce a significant effect on response rate during the session, but did produce a significant increase in scratching (operationally defined as 3-4 fold increase in scratching compared to water session) to verify a behaviorally active dose was given. Additionally, not all subjects showed a decrease in response rate following muscimol, but did vomit during or after the session. If side effects were observed, dose levels were not increased further. All tests maintained the same 60 min pretreatment time. For i.m. test sessions, monkeys first received a water gavage to match the training procedures.

These monkeys were part of the larger experimental study of this dissertation that included surgery for DREADD injections into the nucleus accumbens. For 7 out of 8 monkeys, tests for ethanol, morphine, pentobarbital, and midazolam took place prior to surgery. For all but one monkey, MK-801 tests took place after surgery. All muscimol tests took place after surgery, and for one monkey, all testing was conducted after surgery. Surgery did not have any effect on ethanol discrimination so these data are collapsed in the present analysis (data presented in Chapter 4).

In addition to blood samples to determine ethanol pharmacokinetics, 20µl blood samples were also collected immediately following ethanol test sessions (approx. 75 minutes following ethanol administration) for BEC analysis. All BEC samples were collected in a capillary tube and

diluted with 500µl sterile water, placed in airtight containers and stored at − 4°C until assayed using headspace gas chromatography (Agilent Technologies, Santa Clara, CA). Samples were analyzed using linear regression against a standard curve that included 25, 50, 100, 200, and 400 mg/dl.

2.2.7. Drugs

In general, all drugs were prepared fresh on the morning of the test session or the night before. Ethanol (95%) was diluted to 20% w/v in water for doses ≤ 1.0 g/kg, and to 25% and 30% for 1.5g/kg and 2.0g/kg tests respectively, leading to gavage volumes between 30-50ml for all doses tested. Pentobarbital was purchased in prepared form (Nembutal, 50 mg/ml), and midazolam hydrochloride (Sigma Aldrich) was diluted in saline to 3 mg/ml. Both drugs were administered through the nasogastric gavage, followed by a gavage of water up to the training dose volume (30-45ml). Morphine maleate salt (Sigma Aldrich) was diluted in saline to 5 mg/ml, and muscimol (Tocris) was diluted in saline to 7 mg/ml (doses were based on the salt form of the drug). All i.m. drug preparations were less than 3ml, and if the injection volume exceeded 1ml, it was given across two injection sites. Vehicle injections matched the maximum drug volume given. All drugs administered i.m. (pentobarbital, morphine, muscimol) were filtered through a 20µm millipore filter into a sterile vial prior to administration.

2.2.8. Data analysis

Data from the BEC time course were used for three analyses: 1) determination of peak BEC, 2) time to peak, and 3) calculation of elimination rates (β). Elimination rates were calculated using the linear portion of each elimination curve (0.5 g/kg ethanol: 90-180 min; 1.0 g/kg ethanol: 120-300 min). These time points were put into a linear regression and the slope of the line for each monkey was used to calculate an individual elimination rate per hour. Elimination rates were then averaged across the group for between subjects and within-subjects comparisons.

Following each session, the percentage of ethanol-appropriate responding and response rate (responses/second) were calculated for each subject. In cases where substitution was double-determined, the ethanol-appropriate responses and response rates were averaged for the two sessions before further analyses and served as the primary dependent variables. Full substitution was defined as ≥80% responding on the ethanol-appropriate lever and no substitution was defined as ≤20% ethanol-appropriate responding. Partial substitution was between 21-79% ethanol-appropriate responding. For all dose response curves that reached full substitution, the ED_{50} (50% effective dose) was calculated using linear interpolation with the two doses that encompassed the 50% effect. ED_{50} was then used in paired t-tests or RM ANOVAs comparing drug potency.

Baseline response rates were calculated as a rolling average of three water sessions prior to, or at the beginning of, a new dose response determination for the given drug. These baseline response rates were compared to water or saline (i.g. or i.m. routes, respectively) and were tested for equivalency (data shown in Chapter 4, Figure 4-8). This method accounted for variance in a single subject's response rate over the duration of the experiment (28 months, shown in Table 1-1, page 40).

2.3. Results

2.3.1. Ethanol time course

Under fasted conditions, peak BEC following 1.0 g/kg ethanol (i.g.) was 86 ± 6 mg/dl (range: 80-95 mg/dl) and occurred between 75-90 minutes following ethanol gavage (87 ± 6.7 min). Peak BEC following 0.5 g/kg ethanol (i.g.) was 34 ± 5 mg/dl (range: 28-40 mg/dl) and occurred at variable time points between 45-90 min following ethanol administration across monkeys (67.5 ± 19.4 min). The effect of dose on elimination rate was compared using a paired t-test of the four subjects that were tested at both dose levels. There was no significant effect of dose on elimination rate ($t(3)=1.6$, $p=0.2$) and the group averages were 14.8 ± 1.7 mg/dl/hr following 0.5

g/kg ethanol (n=4), and 14.7 ± 3.6 mg/dl/hr following 1.0 g/kg ethanol (n=5). The time to peak and peak BEC recorded was used to define our discrimination training parameters. We selected a 60 minute pretreatment interval following a 1.0 g/kg ethanol gavage to capture the final rising phase of BEC between 70-83 mg/dl during the 30 minute testing period (Figure 2-2).

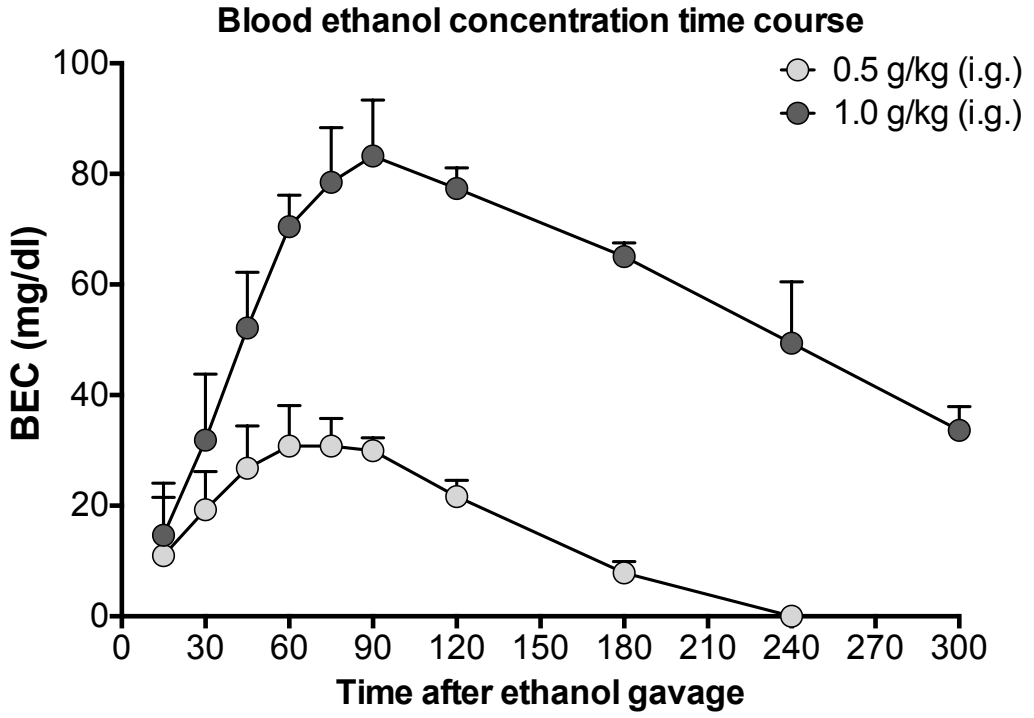

Figure 2-2. Blood ethanol concentration (BEC) time course following 0.5 g/kg (n=4) and 1.0 g/kg (n=5) ethanol gavage (i.g.). Four subjects were tested at both doses, and one additional subject was included in the 1.0 g/kg ethanol group. All data are plotted as mean ± SD.

2.3.2. Ethanol discrimination and substitution

All monkeys successfully acquired the discrimination in 81 ± 21 sessions (±SD, range: 52-114 sessions, n=8). A representative acquisition curve is shown in Figure 2-3. Following training, responding on the ethanol-lever increased as a function of ethanol dose ($F(4,28)=270.3$, $p<0.0001$), with only two monkeys showing partial substitution of 0.5 g/kg ethanol (Fig. 2-4a; $ED_{50}=0.7 \pm 0.1$ g/kg). All monkeys showed generalization of higher test doses (1.5-2.0 g/kg) to the 1.0 g/kg ethanol training dose. BEC following the testing session also increased as a function of ethanol dose (Fig. 2-4b; $F(3,21)=52.9$, $p<0.0001$). There was no effect of ethanol dose on response rate (Table 2-1; $F(4,28)=1.4$, $p=0.26$). In order to confirm the specificity of the discrimination for ethanol-like stimulus effects, morphine substitution tests were conducted. As described in Chapter 1, ethanol does not have any direct activity at the μ-opioid receptor, so including morphine (μ-opioid agonist) substitution tests will confirm that the ethanol discrimination is specific for ethanol-like discriminative stimulus effects. Substitution of morphine would indicate that the ethanol discrimination was not successfully acquired and generalized to stimulus properties separate from ethanol. In all eight monkeys, morphine did not substitute for ethanol in any of the eight monkeys tested (mean percent ethanol-appropriate responding below 2%), even at doses that were behaviorally active (Table 2-2).

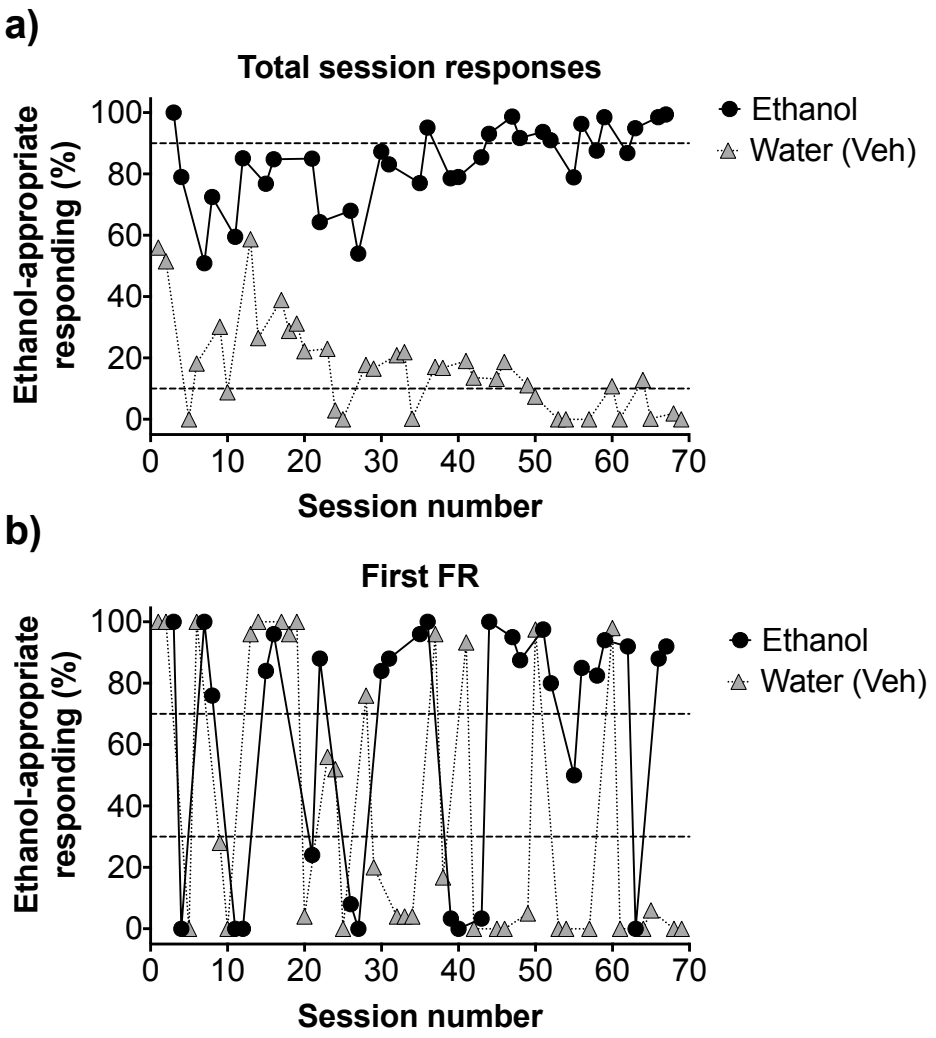

Figure 2-3. Representative discrimination training acquisition curve for a single subject. a) Total session ethanol-appropriate responding for ethanol (black circles) and water (grey triangles) discrimination training sessions for a single subject (monkey 7). Dotted lines at 10% and 90% represent discrimination criteria for water and ethanol sessions respectively. 10% ethanol-appropriate responding during water sessions corresponds to 90% water-appropriate responding and vice versa. b) First FR percentage ethanol-appropriate responding for ethanol and water sessions. Dotted lines at 30% and 70% represent the criteria for the first FR responses. Monkeys must meet both criteria for 5 consecutive sessions before discrimination testing can begin. This subject met both criteria in 69 sessions.

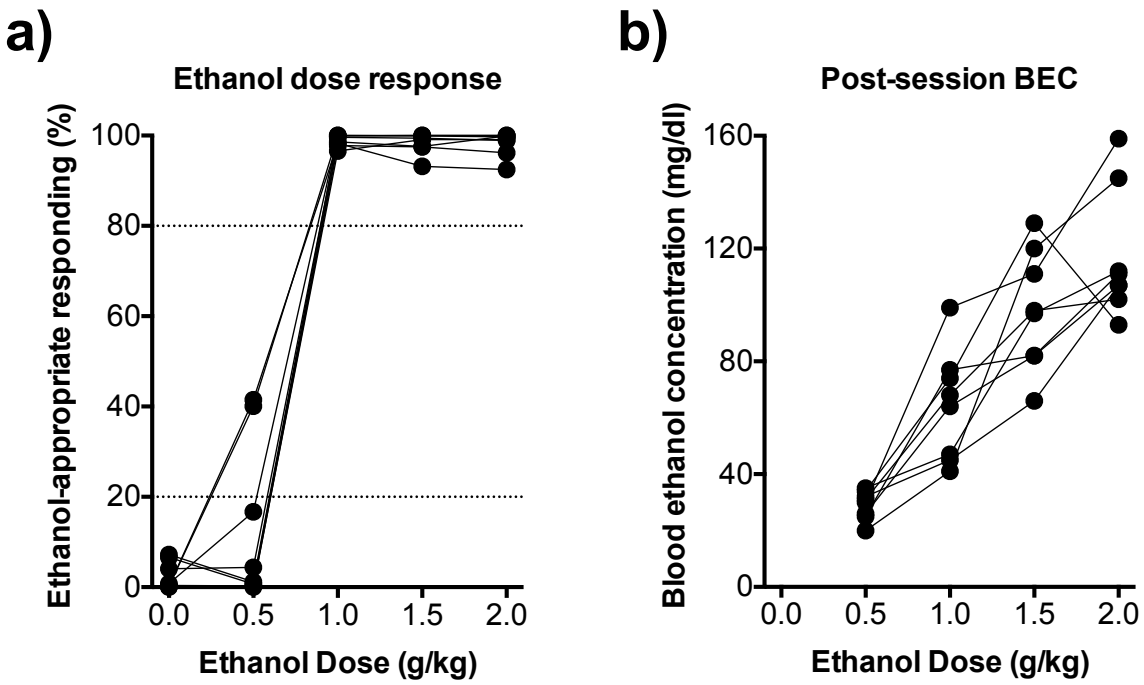

Figure 2-4. Ethanol dose response function and post-session BECs. a) Ethanol dose response curves plotted for each individual subject. Each data point represents an average for each subject (double determination, n=8). Dotted lines represent the threshold for full substitution (≥80% on ethanol-appropriate lever) and no substitution (≤20% on ethanol-appropriate lever). The area between the dotted lines indicates partial substitution. b) BEC samples taken immediately following the test session, between 70-90 min post-ethanol administration for each monkey (single determination).

2.3.3. $GABA_A$ and NMDA substitution

Pentobarbital produced dose-dependent increases in ethanol-appropriate responding in 7 out of 8 subjects following i.g. administration (Figure 2-5a, left panel). The ED_{50} for pentobarbital (i.g.) substitution was 3.7±1.6 mg/kg and there was no significant effect of dose on response rate relative to baseline (Table 2-1; $F(5,11)=2.6$, $p=0.09$). A subset of subjects (n=3) were tested with pentobarbital (i.m.) and the ED_{50} was 3.8±0.7 mg/kg (Figure 2-5a, right panel) and was not significantly different from the i.g. route of administration ($p=0.9$). Midazolam fully substituted for ethanol in six out of seven subjects tested (ED_{50} = 1.6 ± 0.4 mg/kg; Figure 2-5b) and did not have a significant group effect on response rate ($F(5,11)=1.9$, $p=0.2$; Table 2-1). Muscimol substituted for ethanol in 1 out of 4 subjects tested (% ethanol-appropriate responding; Table 2-2). MK-801 produced full substitution in 4 out of 5 subjects, with one subject showing partial substitution (44-54%, Figure 2-5c) at doses that significantly lowered response rate (MK-801 ED_{50} = 0.017 ± 0.009 mg/kg; Table 2-1). The potency ranking of substitution in these subjects was MK-801 (0.017 mg/kg) > midazolam (1.6 mg/kg) >pentobarbital (3.7 mg/kg) > ethanol (700 mg/kg, or 0.7 g/kg) (Figure 2-5d). Interestingly neither pentobarbital or midazolam substituted for ethanol in one subject, whereas there was full generalization of MK-801 to ethanol in this subject. These data suggest that the NMDA component of the ethanol cue was more prominent in this subject. Pentobarbital did not have a significant effect on response rate in the dose range tested. Midazolam decreased response rates below 60% of baseline in 1 out of 7 subjects, and MK-801 decreased response rates in 3 out of 5 monkeys tested (Table 2-2).

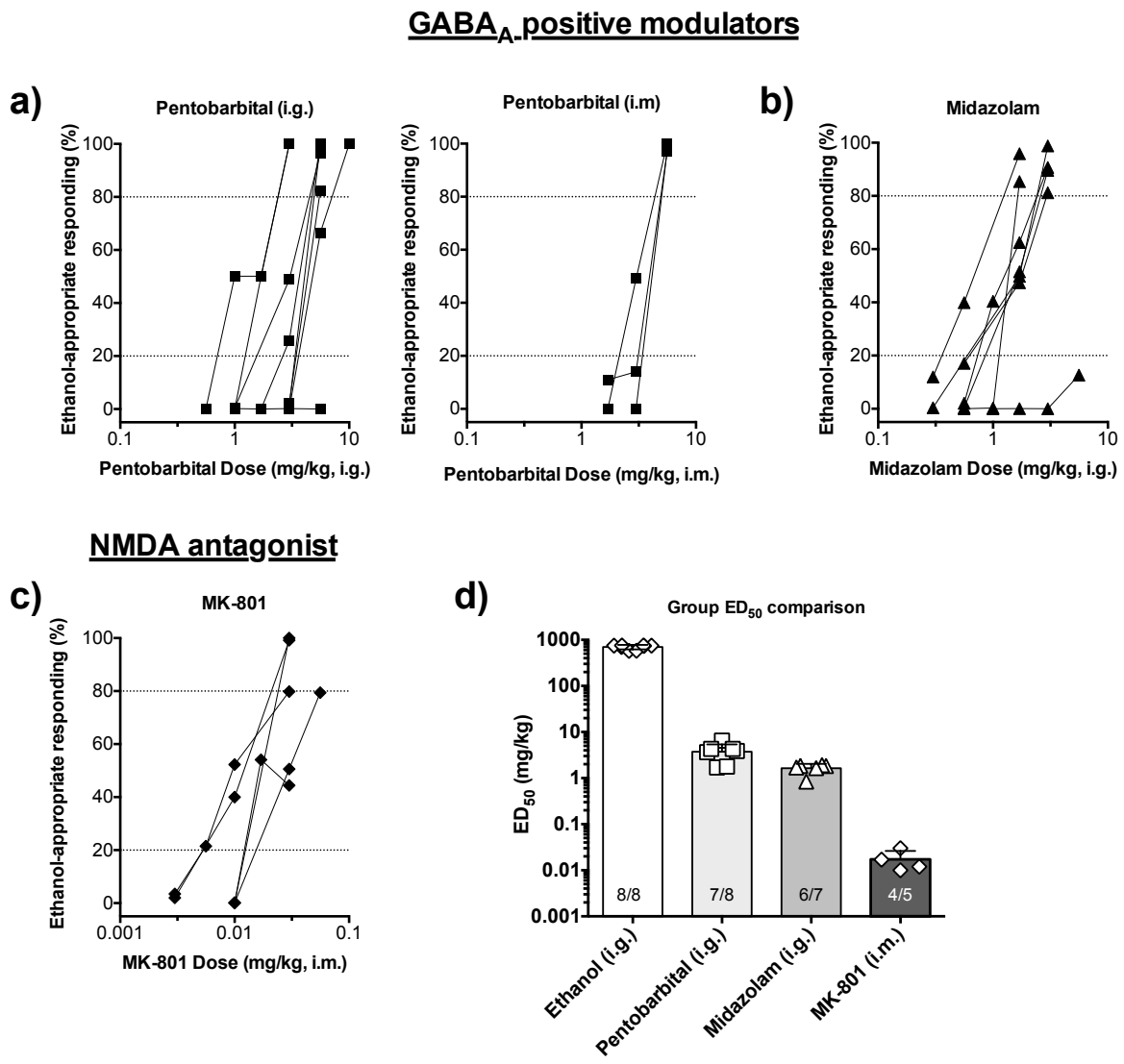

Figure 2-5. GABA$_A$ and NMDA ligand substitution profiles. a-c) Dose response curves are plotted for individual subjects for pentobarbital (i.g., left panel, i.m., right panel), midazolam and MK-801. Each data point is an average value for each subject (double determination). Dotted lines represent the threshold for full substitution (>80% on ethanol-appropriate lever) and no substitution (<20% on ethanol-appropriate lever). The area between the lines represents partial substitution. d) Average ED$_{50}$ values for each test drug to compare drug potency. Each data point is an individual monkey, and the bar graphs are the group mean ± *SD*. The numbers at the bottom of the bar graphs indicate the proportion of subjects tested that had full substitution with the specific drug.

Table 2-1. Response rates during test sessions (*mean* ± *SD*)

Test Drug	Dose (mg/kg)	Resp. rate (% baseline)	# of subjects
Ethanol	Water	100 ± 6	8
	500	102 ± 22	8
	1000	119 ± 38	8
	1500	128 ± 70	8
	2000	122 ± 58	8
Pentobarbital	0.56	130	1
	1.0	135 ± 28	4
	1.7	136 ± 63	4
	3.0	139 ± 80	8
	5.6	119 ± 32	6
	10.0	78	1
Midazolam	0.3	79 ± 15	2
	0.56	115 ± 26	5
	1.0	141 ± 69	5
	1.7	129 ± 59	6
	3.0	73 ± 16	4
	5.6	74	1
MK-801	Saline	93 ± 24	5
	0.003	114 ± 16	2
	0.0056	147	1
	0.01	105 ± 17	5
	0.017	107	1
	0.03	74 ± 21	5
	0.056	53	1

Table 2-2. Morphine and muscimol substitution and response rates. (*mean* ± *SD*)

Test Drug	Dose (mg/kg)	% Ethanol responses	Full substitution	Resp. rate (% saline)	Beh. active dose[1]	# of subjects
Morphine (n=8)	0.1	1.7 ± 2.4	0/2	93 ± 31	0/2	2
	0.3	0.8 ± 1.8	0/8	77 ± 28	2/8	8
	1.0	0.3 ± 0.3	0/6	67 ± 32	5/6	6
	1.7	0.0	0/1	84	1/1	1
Muscimol (n=4)	0.3	26 ± 46	1/4	79 ± 15	3/4	4
	0.56	0.1 ± 0.2	0/3	59 ± 27	3/3	3

[1]Number of monkeys in which a given dose demonstrated to be behaviorally active.

2.4. Discussion

The current study is the first study to our knowledge to 1) determine the pharmacokinetic time course of ethanol and 2) train an ethanol vs. water discrimination in rhesus monkeys. These studies fill an important gap on the psychopharmacology of alcohol in the rhesus monkey, which have been used as a translational model of alcohol drinking and alcohol use disorders (Grant et al., 2008b; Baker et al., 2014; Jimenez and Grant, 2017; Allen et al., 2017). Our pharmacokinetic data indicate that the time to peak BEC following 1.0 g/kg (i.g.) is approximately 90 minutes in male rhesus monkeys, which is 30 minutes longer than was previously reported in the cynomolgus macaque (Green et al., 1999b). However, the previous experiment did not measure BEC at any point between 60 and 120 minutes following ethanol administration, and thus, differences in time to peak may be related to sampling resolution. The magnitude of peak BEC was not significantly different however, at 86 mg% for male cynomolgus macaques (Green et al., 1999b) and 86 mg% in male rhesus macaques reported here. For 0.5 g/kg ethanol, time to peak BEC was highly variable, ranging from 45-90 minutes even under fasted conditions. For both doses tested in the current experiment, time to peak was slower for rhesus macaques relative to reports in human subjects, which found that time to peak BEC for 0.5 g/kg and 1.0 g/kg was ~30 min faster than the times reported here (29 and 52 minutes to peak for 0.5 and 1.0 g/kg respectively) (Dubowski, 1985; Zorzano and Herrera, 1990). For the elimination phase of the pharmacokinetic time course, the current data support zero order kinetics in that the elimination rate was nearly identical at both doses tested. The elimination rates in male rhesus monkeys reported here (~15mg/dl/hr) are somewhat faster than rates reported previously in cynomolgus monkeys (20-30 mg/dl/hr) (Green et al., 1999b). Relative to the clinical literature, however, young adult rhesus monkeys appear to eliminate alcohol at slightly faster rates compared to a sample of healthy adults, which have been reported between 8-17 mg/dl/hr for both men and women (Taylor et al., 1996; Baraona et al., 2001). Overall, our data indicate that the pharmacokinetic time course of ethanol in rhesus monkeys is similar to

reports in human literature, providing additional support for the translational strengths of the rhesus monkey in alcohol research.

The current study demonstrated that rhesus monkeys reliably learned to discriminate a moderate dose of ethanol from water in a two-choice discrimination task. There is a previous report of ethanol being used as a test drug for substitution in a midazolam versus water discrimination in rhesus monkeys (McMahon and France, 2005), but it has never been established as a training drug. Mean sessions to criteria was lower for this cohort relative to the cynomolgus macaque (average of 81 sessions to criteria for rhesus versus 137 sessions for cynomolgus macaques) (Grant et al., 2000), but similar to squirrel monkeys trained to discriminate 1.0 g/kg ethanol intravenously (82 sessions, Platt et al., 2005). Importantly, tests with morphine established that the ethanol discrimination was specific for the ethanol cue, as the μ-opioid receptor system is not directly involved in producing the discriminative stimulus effects of ethanol in primates (Platt and Bano, 2010).

In general, our findings that $GABA_A$ receptor positive modulators and NMDA receptor antagonists are both sufficient to produce ethanol discriminative stimulus effects are consistent with previous reports in Old and New World monkeys (Grant et al., 2000; Grant et. al., 1999; Helms et al., 2011; Vivian et al., 2002; Platt et al., 2005). However, there were several cases that appear to be a departure from previous reports. There was one monkey in the current group in which muscimol produced full substitution for ethanol, which has not been reported in monkeys or rodents following peripheral muscimol administration (Shelton and Balster, 1994; Grant et al., 2000). The only case in which muscimol has substituted for ethanol is when it was directly administered into specific brain nuclei associated with ethanol discrimination in rats (Hodge and Cox, 1996; Hodge et al., 1998). Additionally, there was one subject that did not demonstrate any ethanol substitution for either pentobarbital or midazolam, indicating that the GABAergic component of the ethanol cue was not prominent in this subject. Interestingly, however, MK-801 fully substituted for ethanol in this monkey, suggesting that the glutamatergic

cue was guiding the discrimination. This finding is contrary to several other published reports on ethanol discrimination in cynomolgus macaques that have concluded that the GABAergic component is more prominent in macaques relative to rodents across several training doses (Grant et al., 2000; Grant et al., 2008a; Stolerman et al., 2011; Allen et al., 2017). One explanation is the relatively large sample size (8 subjects relative to groups of 4 monkeys in previous studies) allowed this individual difference to be captured. Importantly, these subjects were assigned to the study from a genetically heterogeneous population confirmed to not have any common grandparents, which may provide a basis for individual differences in ethanol's stimulus effects. Further studies with additional subjects are necessary to confirm these differences are representative of the rhesus population.

In conclusion, data presented here demonstrate that ethanol's discriminative stimulus effects in rhesus monkeys are largely consistent with reports in many other species including pigeons (Grant and Barrett, 1991), rats (Shelton and Balster, 1994), mice (Shelton and Grant, 2002), squirrel monkeys (Platt et al., 2005), cynomolgus macaques (Grant et al., 1999; Grant et al., 2000; Vivian et al., 2002; Helms et al., 2011), and humans (Duka et al., 1998) (for review: Grant 2003, Stolerman et al., 2011; Allen et al., 2017). Additionally, we have conducted a thorough ethanol pharmacokinetics time course with 15-min blood sampling intervals during the rising phase of BEC to capture the time and value of peak BEC following low and moderate ethanol doses. The rate of ethanol elimination is within the range of reports in human subjects, providing face validity for future research on alcohol self-administration in rhesus macaques. One limitation of the current experiment is the inclusion of only male subjects, as there are known differences between males and females in ethanol pharmacokinetics (Zorzano and Herrera, 1990; Green et al., 1999b; Baraona et al., 2001) and sex-specific effects in females related to different phases of the menstrual cycle (Grant et al., 1996). Future studies examining these variables in female rhesus macaques would allow for complete cross-species and cross-sex comparisons in translational alcohol research.

Chapter 3: The pharmacokinetics of clozapine-n-oxide in rhesus monkeys

3.1. Introduction

The development and use of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) in neuroscience research has allowed for a rapid expansion in our ability to identify the cell types and circuits that are involved in a wide range of behaviors, from simple sensory and motor processes, to feeding, to complex cognitive behaviors such as drug addiction (reviewed in Urban and Roth, 2015; Roth, 2016). One of the primary advantages of DREADDs is the ability to manipulate particular brain regions *in vivo* without the need to maintain an indwelling cannula and repeatedly damage surrounding tissue with direct intracranial injections. While this technology was rapidly acquired throughout the rodent literature, it has only been utilized in a small number of non-human primate studies, all published within the last two years (Eldridge et al., 2016; Nagai et al., 2016; Grayson et al., 2016; Galvan et al., 2017). However, the relatively non-invasive nature of DREADDs, combined with the high degree of cell-type specificity through targeted viral approaches, makes it a strong candidate for application to behavioral studies in non-human primates. One of the main advantages of non-human primate studies is their longevity, so utilizing DREADDs to dissect out the circuit mechanisms will maximize the potential of the non-human primate model through repeated within-subjects testing.

However, when translating DREADD techniques between rodent and non-human primate research (in this case rhesus macaques), there are several considerations, particularly with the administration of the activating ligand clozapine-N-oxide (CNO). CNO is a metabolite of clozapine, and metabolism of clozapine occurs through the cytochrome P450 system in the liver, primarily at the CYP1A2 isoform in humans (Doude van Troostwijk, 2003). When administering CNO directly, conversion to clozapine occurs rapidly in vivo and was originally reported in guinea pigs and humans (Jann et al., 1994). The production of clozapine from CNO

in this report has spurred follow up experiments in macaques and rodents to determine if clozapine is also present after CNO dosing, and these studies have confirmed the same low levels of clozapine in plasma when CNO is administered (Raper et al., 2017; Gomez et al., 2017). Since clozapine is pharmacologically active at several different receptors (primarily dopamine, serotonin and adrenergic systems; Bymaster et al., 1996; Selent et al., 2008), it is important to quantify the extent of clozapine in the plasma and CSF following CNO administration. Additionally, CNO is not water soluble, and typically DMSO is required to reach necessary concentrations of the drug. This presents a problem with DREADD work in macaques, because the injection volumes must be very large in order to accommodate a safe concentration of DMSO (Eldridge et al., 2016). Thus, in order to address some of these concerns, a thorough pharmacokinetics profile of the commercially available form of CNO, as well as a new salt form of CNO (CNO-HCl) that has improved water solubility has been conducted in a group of rhesus monkeys[1]. One advantage of the macaque is that pharmacokinetic characterization of drug bioavailability is possible within-subjects since blood and cerebrospinal fluid (CSF) can be sampled in large enough volumes repeatedly for detection of CNO and clozapine. There has been one full report on the pharmacokinetics of CNO in rhesus monkeys (Raper et al, 2017), but none have used a water-soluble salt form of CNO. Raper and colleagues concluded that the amount of CNO that reaches the CSF is limited with the commercially available CNO, calling into question the mechanism of DREADD activation in monkeys (Raper et al., 2017). However, the bioavailability of CNO-HCl is not known, so it is important to characterize the distribution of CNO and its metabolites before application to DREADD research. In the context of the dissertation, the data presented in this chapter will

[1] It is important to highlight that the water-soluble salt form of CNO used here was converted by a collaborator at Mount Sinai and is distinct from the newly available water soluble form of CNO through Tocris Bioscience. The version of this salt from Tocris has 2 HCl molecules per CNO molecule, whereas the CNO-HCl used here just has one HCl per CNO, which may influence purity and stability (unpublished correspondence, Dr. Jian Jin).

serve as a foundation for applying DREADDs (specifically hM4Di inhibitory receptors) to the drug discrimination studies previously described in Chapter 2.

3.2. Materials and methods

3.2.1. Animals and experimental overview

Seven of the eight monkeys described in Chapter 2 were used in the following experiments, in addition to two female rhesus monkeys (5-7 years old) and three male rhesus monkeys (7-10 years old) from ONPRC. All monkeys were housed under the same temperature, humidity and light-controlled conditions that were previously described (Section 2.2.1). The two female monkeys were used for all intramuscular experiments with the commercially available form of CNO prepared in DMSO (CNO-DMSO). The three additional male monkeys from ONPRC were used for the intravenous CNO-DMSO experiments. The male monkeys described in Chapter 2 were used for all studies with the water-soluble salt form of CNO (CNO-HCl group) (see Timeline, Table 1-1, page 40). These two studies were conducted separately. Specifically, the CNO-DMSO experiments were conducted by Dr. Cuzon Carlson's laboratory (ONPRC, Beaverton, OR) and are included in this dissertation for direct comparison to CNO-HCl. All procedures were conducted in accordance with NIH and the Guide on the Care and Use of Laboratory Animals and approved by the IACUC at ONPRC.

3.2.2. CNO preparation

All drugs were prepared fresh on the morning of each experiment. CNO and CNO-HCl were stored at room temperature, protected from light, and in the desiccator. Storage in the desiccator was essential for CNO-HCl as the drug is highly hygroscopic.

CNO-DMSO

CNO (MW: 342.82) was obtained from several commercial and institutional sources (Tocris, NIH RAIDD, Toronto Research Chemicals). Prior to injection, CNO was initially suspended in a minimal volume of DMSO (Sigma) at concentrations up to 100mg/ml. Saline (0.9%) was added

to the CNO and DMSO solution to achieve 10% v/v DMSO in the final solution (concentration range: 5-8 mg/ml). The drug was then passed through a 20μm millipore filter into a sterile vial before being pulled into individual sterile syringes for administration. Throughout the rest of the dissertation, this drug preparation of CNO will be referred to as CNO-DMSO.

CNO-HCl

The same stock of CNO described above was converted to CNO-HCl (MW: 379.29) in the laboratory of Dr. Jian Jin (Icahn School of Medicine at Mount Sinai, New York, NY). The CNO-HCl was dissolved in sterile saline (0.9%) to achieve a concentration of between 30-46 mg/ml. 46 mg/ml was the initial concentration, but due to inconsistency in solubility, it was decreased to 30 mg/ml for the remaining doses. As with the CNO in DMSO preparation, this saline mixture was then passed through a 20μm millipore filter into a sterile vial before being drawn into sterile syringes for administration.

3.2.3. CNO dosing / Blood and CSF collection

Prior to all pharmacokinetics studies, monkeys were fasted overnight beginning at 4pm on the day prior to dosing and blood and CSF sampling. Water access was not restricted.

CNO-DMSO

On separate days, with at least 1 week in between, CNO-DMSO was administered in the home cage at 3.0 mg/kg (i.m.), 5.0 mg/kg (i.m.), and 7.0 mg/kg (i.m.). In this dose range, injection volumes were up to 8 ml and were administered over 4 injection sites to maintain single injection volumes of less than 2 ml per ONPRC guidelines (upper legs and upper arms). Monkeys were then trained to enter a bleeding tower using a jump box for transfer to assist with blood collection. In some instances, animals were administered midazolam and/or ketamine to mildly sedate as necessary for safe blood collection. Approximately 1 ml of blood was collected into EDTA vacutainers from the femoral vein at the following times after CNO injection: 60, 90, 120, 150, 180 and 240 minutes. At the 120-min time point, monkeys were fully sedated with Zolazepam (0.04mg/kg) for CSF collection, so blood was also collected under sedation for this

time point. Previous studies have indicated that there is no significant effect of sedation on CNO pharmacokinetics in macaques (Raper et al., 2017). For CSF collection, monkeys were positioned in lateral recumbency with head held in flexion. A sterile prep of the posterior neck was performed, and a 0.5-1.0ml sample of CSF was obtained via percutaneous cisternal access with a 23-gauge needle. Monkeys were recovered for the subsequent blood sampling time points.

In addition, 6.5 mg/kg CNO was administered intravenously (i.v.) under 1% isofluorane anesthesia during an MRI experiment that is not included in this dissertation. At 45 or 60 minutes following drug administration, CSF was collected as described above, followed by blood collection from the femoral vein under sedation. Both samples were collected within 5 minutes, and each monkey only had CSF and blood collected at a single time point (2 monkeys at 45 min, one at 60 min). The 45 and 60-min time points were collapsed for analysis.

Blood samples were kept on ice and then centrifuged at 3000 rpm at 4°C for 20 minutes in an Eppendorf 581OR centrifuge. Plasma was transferred to screw top freezer tubes (Starstedt) and stored at -80°C until ready for assay. CSF was transferred directly to storage at -80°C until ready for assay. If contaminating blood was present in the CSF sample, it was centrifuged at 1000 rpm at 4°C for 5 minutes to pellet red blood cells. The supernatant was then collected and transferred to screw top freezer tubes and stored at -80°C for further analysis.

CNO-HCl

Male rhesus monkeys were trained to comply with pole-and-collar training, as previously described (Chapter 2, section 2.2.3, page 46), and were guided into primate chairs for drug administration and blood collection. Sedation was not used for any of these experiments. Similar to the above protocol, on separate days at least 1 week apart, CNO-HCl was administered at 3.0 mg/kg (i.m.), 5.6 mg/kg (i.m.), and 10.0 mg/kg (i.m.). Injection volumes were up to 3 ml, and when the volume exceeded 2 ml it was split across two injection sites. Blood samples (1-2 ml)

were then collected from the femoral vein into EDTA tubes at the following time points: 30, 60, 90, and 240 min.

In addition, 5.6 mg/kg CNO-HCl was administered i.v. under 1% isofluorane anesthesia during an MRI experiment that is not included in this dissertation. At 30 minutes following drug administration, CSF was collected in the same manner described above, followed by blood collection from the femoral vein under sedation. Both samples were collected within 5 minutes.

Blood samples were kept on ice until centrifuged at 3000 rpm for 15 minutes at 4°C. Plasma and CSF were then transferred to a -80°C freezer until ready for assay. CSF samples from the CNO-HCl experiments were not used if blood was present in the sample.

3.2.4. CNO and clozapine assay

For assay, all samples (CSF and plasma) were transported to the OHSU Bioanalytical Shared Resource/Pharmacokinetics Core (Portland, OR). The CNO and clozapine assay was developed from the method described by Wohlfarth and colleagues (Wohlfarth et al., 2011). Briefly, plasma and CSF samples were prepared for liquid chromatography with tandem mass spectrometry (LC-MS/MS) by transferring the following into a 16 x 125 glass tube: 500µl of the sample (blood/CSF), 5µl of internal standard (100 ng/ml clozapine-d_8 in methanol), 1 ml sodium carbonate (100g/L in water), and 3 ml ethyl acetate. Samples were then vortexed for 30 sec, centrifuged at 2000g to separate the phases, and the ethyl acetate layer was transferred to a 13x100 glass tube. The solvent was evaporated using a speed vaccum, reconstituted in 100µl of methanol, and filtered through a 0.22µm filter and transferred to the LC-MS/MS autosampler. The samples were then analyzed using an ABSciex 4000 QTRAP hybrid/triple quadrupole linear ion trap mass spectrometer (Framingham, MA) with electrospray ionization in positive mode. Instrument control and data were acquired and analyzed using Analyst 1.6.2 software. The lower limit of quantification was 0.05 ng/ml for clozapine and clozapine N-oxide from plasma

and CSF. The slopes of standard curves for each analyte were the same when prepared from CSF or plasma.

3.2.5. Data analysis

For every blood and CSF sample, a percentage of the amount of clozapine to CNO was calculated (% clozapine/CNO). Using time points that were common to both CNO-DMSO and CNO-HCl studies (60, 90, and 240 min), area under the curve (AUC) and peak concentration (C_{max}) were calculated for plasma samples, which served as the primary dependent variables for further analyses. Time to peak (T_{max}) was also reported, but was only based on the three time points listed above. One-way repeated measures ANOVAs were conducted separately for CNO-DMSO and CNO-HCl groups to determine the effect of dose administered on plasma concentration of CNO, clozapine, and % clozapine/CNO. Next, differences in plasma concentration of CNO, clozapine and % clozapine/CNO were compared between CNO-DMSO and CNO-HCl using the results from 3.0 mg/kg, which was common to both drug groups. To account for differences in sample size, unpaired Welch's t-test's were used for across group analyses (CNO-DMSO: n=2; CNO-HCl: n=6).

CNO, clozapine and the % clozapine/CNO were directly compared between i.m and i.v. routes of administration for the CNO-HCl group (n=6) using a paired Student's t-test. Additional comparisons were made between the relative amount of CNO in the plasma as compared to the CSF between the CNO-DMSO and CNO-HCl groups (one-way ANOVA).

3.3. Results

3.3.1. Plasma pharmacokinetics – CNO-DMSO and CNO-HCl

CNO-DMSO and CNO-HCl both resulted in measurable increases in plasma levels of both CNO and clozapine (Figure 3-1a-d). In general plasma CNO reached peak concentrations between 30-90 min, then decreased as a function of time, though still detectable at 4 hours following drug administration. Clozapine levels however, rose slowly over the 4-hour period

(Figure 3-1a,c) with peak concentrations either at 90 or 240 min. Plasma concentrations from 60, 90, and 240 min following intramuscular injection were used for dose comparisons and to compare across the two forms of the drug. Mean peak concentrations (C_{max}) of CNO in the plasma following CNO-DMSO after 3.0 mg/kg, 5.0 mg/kg, and 7.0 mg/kg were 226 ng/ml, 415 ng/ml, and 595 ng/ml respectively, but the variance also increased greatly with dose (Table 3-1). A RM ANOVA of plasma CNO AUC by dose was not significant, likely due to high variability (p=0.24). In the CNO-HCl group, plasma CNO C_{max} was 1471 ng/ml, 1932 ng/ml, and 3710 ng/ml a after 3.0 mg/kg, 5.6 mg/kg and 10.0 mg/kg, respectively. There was a trend level dose-dependent increase in AUC with CNO-HCl (CNO-HCl AUC: F(2,10)=3.46, p=0.07), which was also accompanied by increased variance between monkeys (Table 3-1). For both CNO-DMSO and CNO-HCl groups, the standard deviation of plasma CNO was over 50% of the mean at the highest doses, reflecting the high individual variability.

Plasma clozapine concentrations (ng/ml) were dose-dependent in both CNO-DMSO and CNO-HCl groups, as measured by AUC values across 60, 90, and 240 minutes post-injection [CNO-DMSO: F(2,2,)=137.0, p=0.007; CNO-HCl: F(2,10)=31.2, p<0.0001] (Figure 3-1d; Table 3-1). In the CNO-DMSO group, peak concentrations of clozapine were 8.7 ng/ml, 16.4 ng/ml, 22.6 ng/ml following 3.0 mg/kg, 5.0 mg/kg, and 7.0 mg/kg. Variance was lower between monkeys than was observed for plasma CNO, with standard deviations of less than 13% of the mean. For CNO-HCl, peak clozapine was 15 ng/ml, 27.9 ng/ml, and 39.0 ng/ml for 3.0 mg/kg, 5.6 mg/kg, and 10.0 mg/kg respectively, with standard deviations up to 21% of the mean (Table 3-1).

The relative concentration of clozapine compared to CNO (% clozapine/CNO) was calculated as an index of the extent of the conversion from CNO to clozapine. In general, the percentage of clozapine/CNO increased as a function of time, consistent with decreasing CNO concentrations and relatively stable clozapine concentrations (Figure 3-1a, c, e; Table 3-1). This

effect was not dose-dependent in either CNO-DMSO or CNO-HCl groups (AUC: CNO-DMSO: p=0.39; CNO-HCl: p=0.66; Figure 3-1f).

3.3.2. Pharmacokinetic comparison between CNO-DMSO and CNO-HCl

The results from 3.0 mg/kg doses of CNO-DMSO and CNO-HCl were used for direct comparison between the two drug preparations. In general, CNO-HCl was associated with significantly higher plasma levels of CNO compared to CNO-DMSO [t(5.03)=3.91, p=0.01] and clozapine [t(5.01)=5.12, p=0.004] as measure by AUC (Figure 3-1b, d). The difference in plasma CNO levels between the two drug preparations was non-overlapping as well, with peak CNO concentrations in the CNO-DMSO group between 200-250 ng/ml, but peak levels in the CNO-HCl group between 1000-4000 ng/ml. In the CNO-HCl group, half of the monkeys (3/6) had peak plasma CNO concentration at 30 minutes following drug administration. However, these values were not included in the statistical comparison since this time point was not available for the CNO-DMSO group. When looking at only common time points, peak CNO concentrations were at 60 min for both drug groups. As shown in Table 3-1, CNO-HCl resulted in plasma CNO levels between 540-2280 ng/ml, whereas CNO-DMSO resulted in plasma CNO between 200-215 ng/ml. Plasma clozapine levels at 60-minutes following drug administration for the CNO-DMSO group ranged from 8-9 ng/ml, and from 8-26 ng/ml for the CNO-HCl group. Thus, despite a 2-10 fold more plasma CNO in the CNO-HCl group, there was only up to a 3-fold increase in plasma clozapine (though 5 out of 6 monkeys had plasma clozapine concentrations below 15 ng/ml in the CNO-HCl group). This result is best reflected in the percentage of clozapine/CNO depicted in Figure 3-1e,f. At 60 minutes following drug administration, the percentage of clozapine/CNO after the CNO-DMSO preparation ranged from 3.2-5.0%, and from 0.6-1.5% in the CNO-HCl preparation. As previously mentioned, this effect was not dose-dependent, but was dependent on the drug preparation when collapsed across dose [AUC, t(7.02)=3.59, p=0.009] (Figure 3-1f).

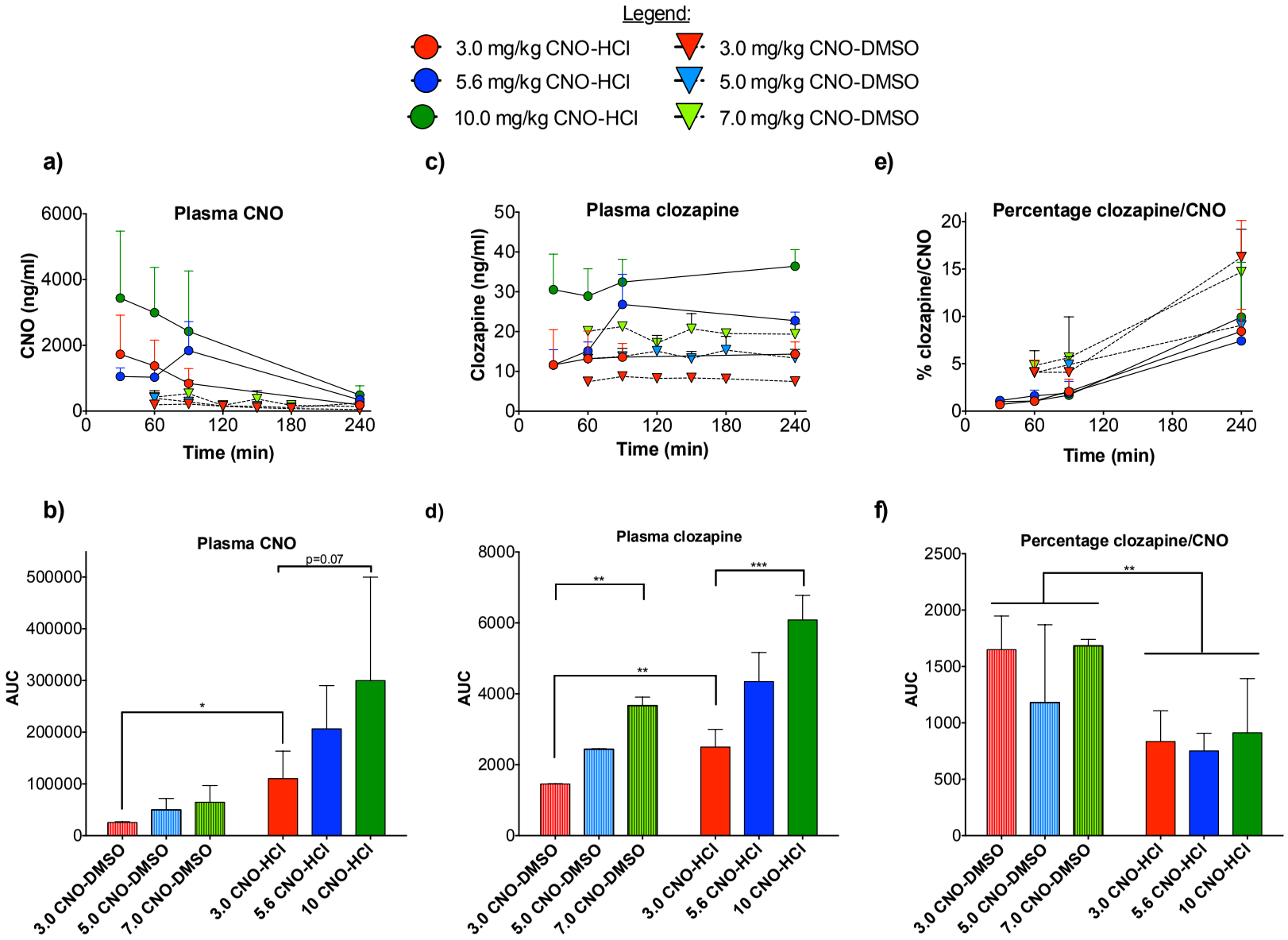
Legend:
3.0 mg/kg CNO-HCl
5.6 mg/kg CNO-HCl
10.0 mg/kg CNO-HCl
3.0 mg/kg CNO-DMSO
5.0 mg/kg CNO-DMSO
7.0 mg/kg CNO-DMSO
a)
Plasma CNO
CNO (ng/ml)
Time (min)
c)
Plasma clozapine
Clozapine (ng/ml)
Time (min)
e)
Percentage clozapine/CNO
% clozapine/CNO
Time (min)
b)
Plasma CNO
AUC
p=0.07
*
d)
Plasma clozapine
AUC
**
**
***
f)
Percentage clozapine/CNO
AUC
**
3.0 CNO-DMSO
5.0 CNO-DMSO
7.0 CNO-DMSO
3.0 CNO-HCl
5.6 CNO-HCl
10 CNO-HCl

Figure 3-1. Plasma pharmacokinetics of CNO-DMSO and CNO-HCl. a, c, e) 4-hour time courses of plasma CNO (a), plasma clozapine (c) and the plasma percentage of clozapine/CNO (e).CNO-DMSO: n=2; CNO-HCl: n=6. b, d, f) Data from the 60, 90, and 240 time points were used to calculate area under the curve values to directly compare across drug dose and preparation. The AUC data are presented in panels b, d, and f. All data are presented as mean ± SD. *p<0.05; **p<0.01; ***p<0.001.

Table 3-1. Pharmacokinetic parameters following intramuscular injection of CNO-DMSO or CNO-HCl. Data are presented as mean ± SD.

Analyte	Drug/dose	*Peak (C_{max}) ng/ml	*Time to peak (min)	*AUC / 10^3 ng/ml×min
Plasma CNO				
	CNO-DMSO			
	3.0 mg/kg	226 ± 20	75 ± 21	25 ± 2
	5.0 mg/kg	415 ± 197	75 ± 21	50 ± 22
	7.0 mg/kg	595 ± 308	75 ± 21	65 ± 32
	CNO-HCl			
	3.0 mg/kg	1471 ± 586	65 ± 12	110 ± 53
	5.6 mg/kg	1932 ± 704	85 ± 12	207 ± 84
	10 mg/kg	3710 ± 1938	75 ± 16	300 ± 200
Plasma clozapine				
	CNO-DMSO			
	3.0 mg/kg	8.7 ± 0.4	90 ± 0	1.5 ± 0.007
	5.0 mg/kg	16.4 ± 2.1	165 ± 106	2.4 ± 0.014
	7.0 mg/kg	22.6 ± 1.2	90 ± 0	3.7 ± 0.2
	CNO-HCl			
	3.0 mg/kg	15 ± 2.9	160 ± 88	2.5 ± 0.5
	5.6 mg/kg	27.9 ± 5.7	140 ± 77	4.3 ± 0.8
	10 mg/kg	39.0 ± 4.4	190 ± 77	6.1 ± 0.7
% Clozapine:CNO				
	CNO-DMSO			
	3.0 mg/kg	16.2 ± 3.9	240 ± 0	1.6 ± 0.3
	5.0 mg/kg	9.4 ± 7.2	240 ± 0	1.2 ± 0.7
	7.0 mg/kg	14.7 ± 4.5	240 ± 0	1.7 ± 0.06
	CNO-HCl			
	3.0 mg/kg	8.4 ± 2.3	240 ± 0	0.8 ± 0.3
	5.6 mg/kg	7.4 ± 2.2	165 ± 106	0.8 ± 0.2
	10 mg/kg	9.9 ± 5.8	240 ± 0	0.9 ± 0.5

*Only data from sampling time points that were common to both forms of the drug were included in these calculations (60, 90, and 240 min post-drug administration).

3.3.3. Route of administration – CNO-HCl

A separate study was conducted to measure the amount of CNO and clozapine in the CSF following CNO-HCl administration as a part of an MRI experiment in which CNO-HCl was administered during the MRI scan. In order to facilitate drug administration during the MRI, CNO-HCl was administered intravenously through a catheter attached to a syringe pump in the adjacent MRI operator room. Thus, prior to CSF analysis, plasma pharmacokinetics of CNO-HCl following both i.m. and i.v. routes of administration were compared. All samples were collected 30 minutes after 5.6 mg/kg CNO-HCl administration. Plasma CNO was modestly higher at 30 minutes after intravenous administration (mean of differences: 875 ng/ml) but this did not reach statistical significance ($t(5)=2.12$, $p=0.087$) (Figure 3-2a). Interestingly, the relative amount of clozapine to CNO (% clozapine/CNO) was significantly lower in the plasma after i.v. administration ($t(5)=3.46$, $p=0.018$) (Figure 3-2c), even though the absolute amount of both CNO and clozapine ($t(5)=1.5$, $p=0.20$) was not statistically different (Figure 3-2b).

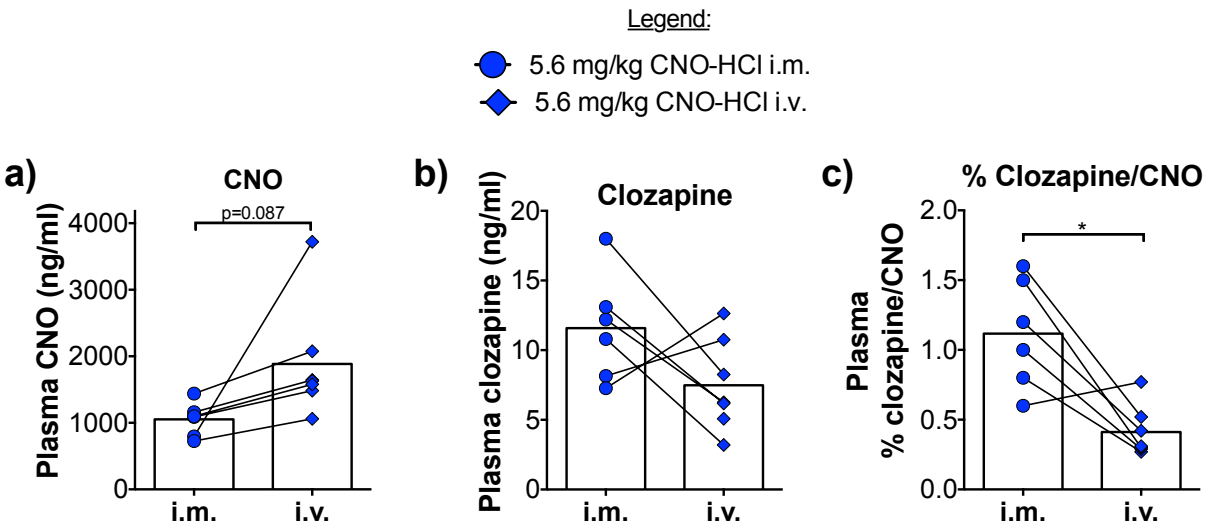

Figure 3-2. Pharmacokinetics of i.m. and i.v. routes of administration for CNO-HCl (5.6 mg/kg). a) Plasma CNO (ng/ml), b) plasma clozapine (ng/ml), and c) percent clozapine to CNO in plasma (%) following 5.6 mg/kg CNO-HCl in male rhesus monkeys (within-subjects). All data points represent a single subject, and bars represent group means. In a few cases, plasma concentrations were double-determined for a single subject, in which chase they were averages before inclusion in this analysis.Intramuscular injections took place under awake conditions, and intravenous injections were under 1% isofluorane anesthesia. *p<0.05.

3.3.4. CSF pharmacokinetics – CNO-DMSO and CNO-HCl

CNO-DMSO (6.5 mg/kg) and CNO-HCl (5.6 mg/kg) were both administered intravenously under sedation and CSF samples were collected between 30-60 minutes for CNO and clozapine assay. For the CNO-DMSO group, blood and CSF were taken at 45 or 60 minutes after 6.5 mg/kg (i.v.) administration, but collapsed for analysis (Figure 3-3a). For CNO-HCl in saline, blood and CSF samples were taken at 30 minutes following 5.6 mg/kg (i.v.). (Figure 3-3b). CSF concentrations of CNO in the CNO-DMSO group were between 17-21 ng/ml (mean 19 ng/ml, equivalent to 55nM) and CSF concentrations of clozapine were between 1.2-3.8 ng/ml (mean: 2.0 ng/ml, equivalent to 6.2 nM) (Figure 3-3a).The percentage of clozapine/CNO in the CSF in the CNO-DMSO group ranged from 5.6-22.5% (mean 11.2%) (Figure 3-3a). In the CNO-HCl group, CSF CNO concentrations were between 38-109 ng/ml (mean: 67 ng/ml, equivalent to 196 nM) and clozapine concentrations ranged from 0.4-0.9 ng/ml (mean: 0.6 ng/ml, equivalent to 1.9 nM) (Figure 3-3b). The percentage of clozapine/CNO in the CSF in the CNO-HCl group ranged from 0.5-1.6% (Mean: 1.0%) (Figure 3-3b).

In order to examine the distribution of CNO from the plasma to the CSF, a relative ratio of CNO measured in the CSF and plasma was calculated for all doses in which both samples were collected at a single time point (Figure 3-3c). For the intramuscular CNO-DMSO samples, there was no effect of CNO dose on the ratio of CNO in the CSF/plasma ($F(2,2)=2.5$, $p=0.29$), so dose was not included as a factor in the analysis. When comparing across all available samples, there were no significant group differences ($F(4,11)=0.85$, $p=0.52$) (Figure 3-3c). The relative amount of CNO in the CSF was between 1-7% of plasma CNO across all doses, routes, and preparations presented (Figure 3-3c).

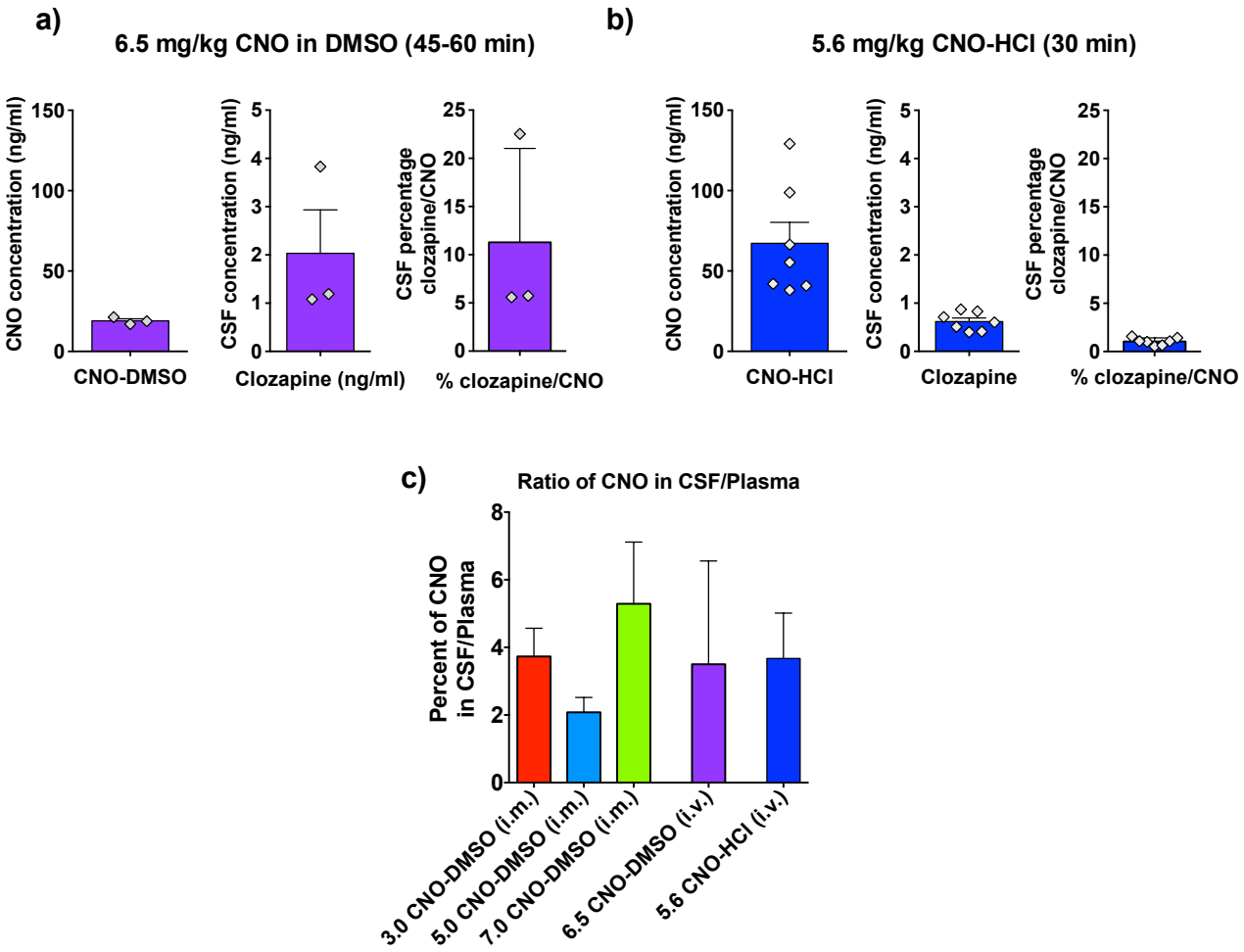

Figure 3-3. CSF concentrations of CNO and clozapine following i.v. drug administration. a) CSF concentrations of CNO, clozapine, and the relative % of clozapine to CNO 45-60 minutes after 6.5 mg/kg CNO-DMSO, i.v., n=3. b) CSF concentrations of CNO, clozapine, and the relative % of clozapine to CNO 30 minutes after 5.6 mg/kg CNO-HCl, i.v., n=7. c) Percent of CNO measured in the CSF to plasma following 3.0, 5.0, 7.0 mg/kg CNO-DMSO i.m., 6.5. mg/kg CNO-DMSO, i.v., and 5.6 mg/kg CNO-HCl i.v.

3.4. Discussion

The data presented here represent the first report on the pharmacokinetics of a water-soluble salt form of CNO, significantly expanding the limited existing literature on CNO pharmacokinetics in the rhesus monkey (Eldridge et al., 2016; Nagai et al.2016; Raper et al., 2017). The most significant finding is that there is an increase in the solubility and bioavailability of CNO when prepared as a salt (CNO-HCl), rather than preparing as a suspension in DMSO (CNO-DMSO). From a methodological perspective, eliminating the need for DMSO is expected to reduce the discomfort of animal subjects and translatability of the research. In particular, although it was previously thought that DMSO concentrations of less than 10% could be well-tolerated, recent studies have shown that even at concentrations as low a 2-4% (v/v), DMSO can induce apoptosis through inhibition of mitochondrial respiration (Galvao et al., 2014). Additionally, DMSO has a long half-life of 16 hours in rhesus monkeys and is not eliminated fully for 72 hours after administration (Layman and Jacob, 1985), prolonging the toxic effects, as well as limiting the frequency of repeated testing. This slow rate of elimination is in contrast to mice, which show almost complete elimination of DMSO by 8 hours after administration (Kaye et al., 1983), therefore limiting the use of DMSO is of particular interest in larger animals.

The current data set highlight several important similarities and differences between CNO-DMSO and CNO-HCl that may improve our understanding about the mechanisms involved in the absorption and distribution of CNO and clozapine. Several features of the pharmacokinetic results are common to both CNO-DMSO and CNO-HCl. First, the individual variability in the plasma concentrations of CNO was high, with standard deviations up to 50% of the mean concentrations at the highest doses in both forms. This finding is similar to the clinical literature, which reports large differences in plasma clozapine levels across patients given the same dosing regimen (Oleson, 1998; Chang et al., 1998; Chetty and Murray, 2007). For application to DREADD studies, this finding suggests the importance of taking blood samples to determine the circulating amount of CNO following a given dose in individual monkeys. Some of

the individual variance in CNO pharmacokinetics can be explained by the rate and extent of the metabolism between clozapine and its metabolites, including CNO (Chang et al, 1998). The relative amount of clozapine varied between monkeys, and was not dose-dependent, consistent with zero order kinetics of this pathway through the P450 system (Chang et al., 1998). Additionally, the relative time course of CNO and clozapine concentrations in plasma were consistent across both forms of the drug, such that CNO levels peaked within 90 minutes, but clozapine levels remained constant or even rising over the 4 hour sampling window, consistent with earlier reports in humans (Chang et al., 1998). Lastly, one feature common to both forms of CNO was that the amount of CNO in the plasma and CSF was consistent across all doses and routes of administration (Figure 3-3c). This is particularly important for future DREADD research, as it indicates that an approximate value of the CSF concentration can be reliably estimated from plasma CNO concentrations, providing further support to the importance of measuring plasma CNO when possible.

In addition to the similarities, there were also several differences between CNO-DMSO and CNO-HCl. Most notably, the absolute values of plasma CNO concentrations were 4-5 times higher on average in the CNO-HCl group compared to the CNO-DMSO group when the same doses were administered (Figure 3-1b; Table 3-1). While the mechanism of this difference is not yet known, it appears to occur during the absorption of the drug following intramuscular injection. One hypothesis is that the presence of DMSO, while improving the solubility of CNO is impairing the transport across biological membranes from the muscle into the blood stream. However, the highly permissible structure of DMSO and ability to diffuse quickly and efficiently across compartments makes this somewhat unlikely (Rammler and Zaffaroni, 1967; Brayton, 1986). Additionally, the ratio of CNO in the CSF/plasma was consistent between the CNO-DMSO and CNO-HCl groups (Figure 3-3c), suggesting similar distribution of CNO from the plasma to the brain. Another hypothesis is that the CNO-HCl solution is more stable, since CNO in DMSO often precipitated out of suspension, while the CNO-HCl remained in solution for up to

8 hours. However, futher analyses of the chemical properties of CNO-DMSO versus CNO-HCl are necessary, such as the stability at different pH levels in order to full characterize the pharmacokinetic profile in each of the distribution compartments.

The levels of clozapine reported in the CSF in the CNO-HCl group were below the levels reported to activate muscarinic DREADDs in culture (Armbruster et al., 2007; Gomez et al., 2017). In DREADD research, the primary concern with the presence of clozapine is that it will have off-target effects on any one of its known receptor targets, including the D_1, D_2, D_4, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_3 receptors (Bymaster et al., 1996). One other important finding from the current study is the low levels of clozapine found in both the plasma and CSF. Specifically, at 30 minutes when CNO concentrations are high, clozapine in the CSF was less than 3 nM for all subjects (< 1 ng/ml; 3.08 conversion factor from ng/ml to nM). However, binding studies in in vitro rat brain tissue have shown that clozapine binding affinities exceed this low level of clozapine found in the CSF. Clozapine had the highest affinity for the D_4, 5-HT_{2A}, and 5-HT_{2C} receptors with K_i values in the 10-30 nM range, moderate affinities for D_1, D_2, and 5-HT_3 in the 65-125 nM range, and the lowest affinities for the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors in the 750-1200 nM range (Bymaster et al., 1996). While receptor-binding studies are informative, the translatability of these data to functional activity are limited. Most of the research on clozapine activity has come from serum levels of clozapine following treatment in schizophrenics. These studies commonly report a threshold level of 350-400 ng/ml for effectiveness of clozapine in most patients (Potkin et al., 1994; Spina et al., 2000, Olesen, 1998). However, more detailed analyses have identified some patients that show a response at “sub-threshold” clozapine concentrations of approximately 150 ng/ml (Olesen, 1998). One study used positron emission topography (PET) to correlate plasma concentrations with receptor occupancy and found that plasma clozapine concentrations of 140 ng/ml (~430 nM) were associated with 80-90% receptor occupancy at 5-HT_2 receptors (Nordstrom et al., 1995), far exceeding the levels of clozapine reported in either plasma or CSF in this study.

Finally, it is essential to note the relatively high concentrations of CNO present in the CSF 30 minutes after drug administration (100-400 nM). Based on the time-response curves also presented (Figure 3-1a; Table 3-1), it is expected that plasma CNO levels would continue to rise for another 30-60 minutes, leading to a similar increase in CSF concentrations of CNO. The absolute magnitude of CNO in the CSF is significantly improved in the CNO-HCl group, in comparison to our own findings with CNO in DMSO, as well as earlier reports with CNO in DMSO in rhesus monkeys (Raper et al., 2017). At CNO concentrations > 100 nM, it is expected that hM3D and hM4D DREADD receptor should be activated, leading to functional changes that can excite or inhibit cellular activity (Armbruster et al, 2007). The most recent pharmacokinetics study in rhesus monkeys found that CSF levels of CNO were significantly lower than what is reported here, and used cell culture experiments to demonstrate that CNO is acting as a substrate for the efflux protein Pgp, which was inhibiting distribution across the blood brain barrier (Raper et al., 2017). However, by increasing the solubility of CNO we have greatly improved the pharmacokinetic profile by increasing CNO concentrations in the brain, and decreasing the relative amount of clozapine, particularly within 2 hours after drug administration when behavioral experiments typically occur.

Based on the CSF results, the overall conclusions from this study is that with water soluble CNO-HCl, there are large enough volumes of CNO getting to the brain to activate hM4D and hM3D DREADD receptors. In addition, the concentrations of clozapine in the CSF are very low, and are not expected to activate receptors based on preliminary data (Armbruster et al., 2007). The study presented here also focused on the early time points as they relate to the discrimination tasks described in the next chapter, confirming that CNO levels are high throughout the time course of behavioral studies. In conclusion, these data are counter to earlier studies that claim limited CNS bioavailability of CNO, which may be related to the water soluble salt preparation. Further studies are necessary to determine the mechanism of this difference.

As described previously, the overall goal of this dissertation is to begin to characterize the neural circuitry involved in mediating the discriminative stimulus effects of ethanol in non-human primates. The methodological approach of the dissertation is to utilize chemogenetics (specifically DREADDs) to directly manipulate neuronal activity, but the application of this technique to non-human primate studies has been limited (Eldridge et al., 2016; Grayson et al., 2016; Nagai et al., 2016). Additionally, there have been two reports in the last year, one in monkeys and another in mice, that have questioned the bioavailability of the most common chemogenetic ligand, CNO, specifically in crossing the blood brain barrier (Raper et al, 2017; Gomez et al., 2017). Thus, prior to applying DREADD techniques to a study of ethanol's discriminative stimulus effects in rhesus monkeys, a pharmacokinetics study was conducted in this species as detailed in this chapter. The conclusions of these experiments were that utilization of CNO-HCl has improved bioavailability and was measured in the CSF in meaningful concentrations to activate DREADD receptors *in vivo*.

Chapter 4: Chemogenetic modulation of the nucleus accumbens in ethanol discrimination

4.1. Introduction

The receptor basis of ethanol discrimination has been studied for decades, but relatively little is known about the brain circuitry underlying the subjective effects of ethanol. The entirety of this literature has been conducted in rodents, as there are no published studies to date examining the role of a specific brain nucleus in non-human primates. A close examination of the rodent drug discrimination literature on this topic strongly implicates the nucleus accumbens (NAc) core (Hodge and Aiken, 1996; Hodge and Cox, 1998; Hodge et al., 2001b; Besheer et al., 2003; Besheer et al., 2008; Jaramillo et al., 2016; Jaramillo et al., 2017). $GABA_A$ receptor agonist muscimol and NMDA receptor antagonist MK-801 have the highest potency (lower ED_{50}) to substitute for ethanol when injected directly into the NAc core, relative to the amygdala, prelimbic cortex and hippocampus (Hodge and Cox, 1998). Additionally, the NAc core was the only brain nucleus in which both $GABA_A$ and NMDA receptor drugs were sufficient to produce ethanol-like discriminative stimulus effects, and a combination of the two drugs enhanced the potency of the substitution relative to a single drug alone (Hodge and Cox, 1998).

Beyond the drug discrimination literature, the NAc core in particular has been implicated in cue-learning encoding, particularly in the context of reinforcement learning and motivation (Ambroggi et al., 2011; Saunders et al., 2013; West and Carelli, 2016; Stefanik et al., 2012), providing additional evidence of potential NAc core involvement in interoceptive cue discrimination. Recent neuroimaging studies in humans have indicated that activity within the NAc (core and shell resolution not possible) is highly correlated with self-report measures of alcohol intoxication in humans, providing additional translational evidence of NAc involvement in ethanol's interoceptive effects (Gilman et al., 2008; Gilman et al., 2012; Seo and Sinha, 2014). In addition to the drug discrimination data described above, electrophysiological studies of

neurons in the NAc suggest that moderate doses of ethanol enhance $GABA_A$ inhibitory neurotransmission (Nie et al., 2000) and decrease glutamatergic excitatory transmission (Nie et al., 1994), consistent with an overall dampening of cellular activity. Lastly, following ethanol discrimination training sessions, cFos activity is selectively decreased in the NAc core when compared to water training sessions (Besheer et al., 2008; Jaramillo et al., 2016).

Thus, there is strong evidence from drug discrimination, human neuroimaging, and slice electrophysiology, that ethanol acts as a positive modulator at $GABA_A$ receptors and antagonist at NMDA receptors within the NAc core to produce ethanol's discriminative stimulus effects (see Figure 1-5a, page 38). The overall result of increased $GABA_A$ receptor conductance and decreased NMDA receptor conductance is an overall decrease in neuronal excitability, which leads to a disinhibition of downstream projection targets, particularly the VP and ventral midbrain (VTA/SN) (Figure 1-5a, page 38). The aim of this final research chapter was to translate these findings to the non-human primate (specifically rhesus macaque) and directly test the involvement of the NAc core in ethanol's discriminative stimulus effects.

In order to test this hypothesis, a chemogenetic approach was employed (DREADDs), which afforded several key advantages over traditional site-specific pharmacological approaches. Repeated testing was possible without the need to maintain an indwelling cannula or risk excessive tissue damage. One of the key strengths of both drug discrimination experiments and non-human primate studies are the extensive longitudinal designs in which each animal can serve as its own control. Thus, application of chemogenetics to a non-human primate drug discrimination experiment maximized the utility of both the behavioral paradigm and animal model.

The leading hypothesis was that chemogenetic inhibition of the NAc core would enhance the potency of ethanol's discriminative stimulus effects, resulting in leftward shifts in the ethanol dose response curve (Figure 1-5b, right panel, page 38). Thus, hM4Di inhibitory DREADDs were injected into the NAc core for direct neuronal inhibition and silencing in ethanol

discrimination (Armbruster et al., 2007; Roth, 2016). The hypothesis was that activation of hM4Di receptors would lead to activation of GIRK channels (G-protein coupled inward rectifying potassium channels) and activation of $G_{i/o}$ signaling pathways (decreased cAMP-dependent activity) to decrease neuronal activity. Thus, ethanol and hM4Di receptors were expected to have an additive effect to shift the ethanol dose response curve to the left (Figure 1-5b, page 38).

Given the novelty of this experimental design and limited number of studies with chemogenetics in non-human primates, the results of Chapters 2 and 3 served as a strong foundation for the experimental design of this study. Specifically, findings from Chapter 2 guided the substitution dose response curves conducted and informed the drug doses for each subject. There was one monkey in which neither pentobarbital or midazolam substituted for ethanol's discriminative stimulus effects, so the NMDA receptor antagonist MK-801 was tested for this subject in combination with hM4Di activation. For the remaining subjects, $GABA_A$ receptor substitution was selected for examination in conjunction with chemogenetic NAc inhibition because full substitution was found in a majority of subjects, but also the range of doses tested did not produce any appreciable rate decreasing effects (Table 2-1, page 58). Thus, shifts in the dose response curve could be found in either direction without confounding non-specific behavioral effects (i.e., sedation). The rationale for testing the effect of NAc inhibition of GABA or NMDA substitution was to identify the receptor basis of the involvement of the NAc in the ethanol discrimination. Additionally, results from Chapter 3 determined the time course of CNO-HCl administration relative to discrimination test sessions, and informed CNO-HCl dose determinations.

4.2. Materials and methods

4.2.1. Animals

The same eight male monkeys described in Chapter 2 were used in the following experiments. The current experiments took place immediately following the experiments described in Chapter 2.

4.2.2. Experimental timeline and design

All monkeys were first trained on the discrimination task and run through a series of substitution tests (Chapter 2) and a series of CNO-HCl challenges to characterize the pharmacokinetic time course before surgery (Chapter 3) (Table 1-1, page 40). Next, CNO-HCl was tested in the discrimination to capture any non-specific effects of CNO-HCl prior to DREADD insertion. Following these experiments, hM4Di inhibitory DREADDs (n=7) were stereotaxically injected into the bilateral NAc core, using an MRI-guided approach. One subject had hM3Dq excitatory DREADDs injected into the NAc core, but this surgery took place prior to discrimination training. Monkeys were given 7-10 days to recover from surgery, and DREADD testing did not begin until at least 4 weeks following surgery to allow for viral expression. During this period, all monkeys resumed daily discrimination training until training criteria was re-established, and the ethanol dose response curve was re-determined without any DREADD manipulations. Once DREADD receptors were expressed and ethanol dose response curves were re-established, substitution tests were conducted with CNO-HCl to activate DREADD receptors. First, DREADD activation was applied to ethanol dose response curves (n=8), followed by pentobarbital (n=6), midazolam (n=5) and MK-801 (n=1). Blood samples were collected following DREADD test sessions with ethanol for CNO and clozapine assay.

The primary outcome of these experiments is the effect of hM4Di or hM3Dq receptor manipulation on ethanol discrimination and the substitution of $GABA_A$ and NMDA receptor ligands for ethanol. However, a number of control experiments were also conducted. First, the effect CNO-HCl administration on ethanol and water discrimination was determined prior to

DREADD injection surgery. Second, for every dose response curve conducted with CNO-HCl to activate DREADD receptors, a matched dose response curve was conducted without DREADD manipulation. These dose response curves were previously conducted in the studies in Chapter 2, so a direct comparison could be made between dose response curves before and after surgery (with up to 1 year of training and testing between the two determinations). Lastly, blood samples taken after ethanol DREADD test sessions allowed for the examination of any potential pharmacokinetic interactions between ethanol and CNO.

4.2.3. Behavioral testing apparatus

Described in detail in Chapter 2 (section 2.2.2, page 43)

4.2.4. Procedural training and discrimination training

Described in detail in Chapter 2 (section 2.2.3. and 2.2.5, pages 46 and 47)

4.2.5. Viral constructs and preparation

Plasmids (pAAV-hSyn-hM3Dq-mCherry and pAAV-hSyn-hM4Di-mCherry) were obtained from Addgene and packaged into an adeno-associated virus serotype 1 (AAV1) by the ONPRC viral vector core. The AAV1 serotype was chosen because it provides the appropriate expression and spread in the striatum of macaque monkeys, targets mostly neurons and is trafficked to projection areas of the original target site (Dodiya et al., 2010; McBride et al., 2011). The human synapsin 1 promoter (hSyn) was selected because it is neuron-specific and has been shown to support long-term expression of AAV plasmids (Kugler et al, 2003). Prepared viral vectors were diluted to 1e12 viral genomes/microliter (vg/µl) in AAV storage buffer (Dulbecco's phosphate-buffered saline, 35nM NaCl, 5% glycerol). Immediately before surgery an equivalent of 1:250 dilution of 2mmol gadoteridol (gadolinium-based contrast dye, GAD) was added to the prepared virus for all seven hM4Di surgeries for visualization of the injection site after surgery (4µl of 0.5mmol GAD was added to a 250µl aliquot of virus). For the excitatory hM3Dq DREADD monkey, GAD was not added to the prepared virus. The viral titers were kept on ice and away from light until injection in the surgery suite.

4.2.6. MRI-guided stereotaxic surgery

In preparation for stereotaxic surgery, monkeys were sedated with 10 mg/kg ketamine (i.m.) and transported to the MRI suite where they were intubated and maintained on 1-3% isofluorane for the duration of the procedure. Monkeys were positioned and secured in an MRI-compatible stereotaxic frame (Crist Instrument, Hagerston, MD) and transported into the magnet (Siemens 3T Trio). A T1-weighed MP-RAGE scan (9 minutes) was collected with a surface coil for determination of stereotaxic coordinates (with 0.5 mm resolution) for each individual monkey. MR images of the brain on the coronal axis were used to determine bilateral coordinates targeting the nucleus accumbens, biasing toward the NAc core (Figure 4-1). The coordinates were aimed toward the dorsal portion of the NAc, at the tip of the internal capsule for diffusion throughout the NAc core without causing damage from the needle tract (Figure 4-1). Additionally, care was taken to avoid the lateral ventricles as well as any visible blood vessels in determination of stereotaxic coordinates. Visual identification of the ear bars on the MR images in conjunction with an AP zeroing plate (Kopf Instruments, Tujunga, CA) were used for determination of AP coordinates. ML coordinates were determined from the sagittal sulcus and DV coordinates were determined from the surface of the brain, both of which could be visualized during surgery (Figure 4-1).

Immediately following the scan, the monkeys were maintained under anesthesia with 1-3% isofluorane and transported to the sterile surgical suite. A staff veterinarian at ONPRC then performed a single craniotomy to allow access to the bilateral NAc. A 100µl Hamilton syringe was used to inject 30-50µl/hemisphere (single injection per side) to the nucleus accumbens using convection-enhanced delivery (increasing from 1.0µl/min to 3.0µl/min for a total of 50µl per hemisphere) (McBride et al., 2011). Each injection took approximately 30 minutes. The needle was left in place for 5 minutes before retracting the syringe to allow for diffusion from the injection site. At the end of the injections, the incision site was closed using sterile sutures by the ONPRC veterinary staff.

Following the surgery, monkeys were transported back to the MRI suite under sedation for a final scan to visualize the injection site with the GAD contrast. T1-weighted MP-RAGE scans (4 scans total for improved signal to noise) were acquired with 0.5mm resolution. Finally, monkeys were transported back to their home cage for recovery and maintained on standard pain medication for 3-5 days. Each monkey was given at least 7 days of rest after surgery, in which no behavioral tests were performed, though most monkeys recovered quickly and returned to normal eating patterns within 1-3 days.

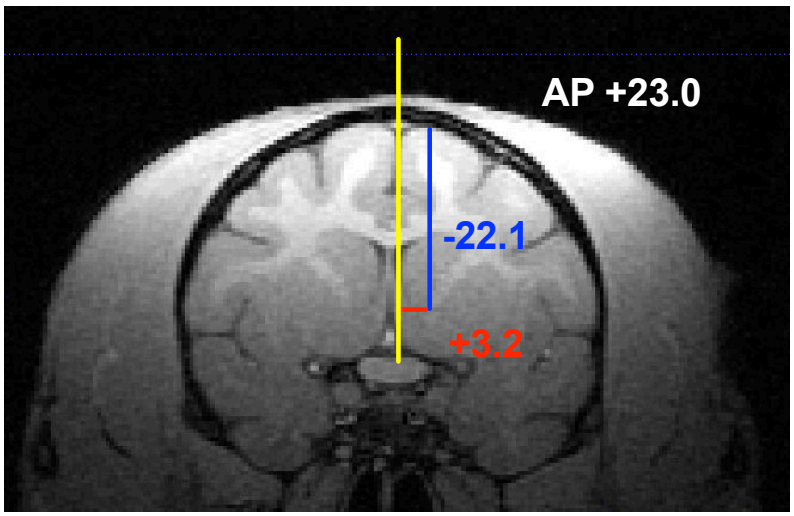

Figure 4-1. Representative image of MRI-guided determination of stereotaxic surgery coordinates targeting the dorsal end of the NAc core (right side, monkey 6). Midline at the saggital sinus shown in yellow. AP coordinate determined from ear bars. ML coordinate in red, DV coordinate in blue.

4.2.7. Substitution testing / DREADD testing

Substitution testing sessions were procedurally similar to the test sessions described in Chapter 2 (section 2.2.6, page 48). Before DREADD surgery, 10 mg/kg CNO-HCl (i.m.) was tested for substitution to determine if CNO alone interfered with the discrimination in the absence of DREADD receptors. CNO-HCl (i.m.) was administered immediately following a water gavage 60 minutes prior to session start (n=2), based on the preliminary data from CNO-DMSO that peak plasma concentrations of CNO were at 60 minutes after administration (Figure 3-1a, page 71). In addition to testing CNO-HCl alone, substitution tests were also conducted with CNO-HCl in combination with the training dose of ethanol (1.0 g/kg, i.g.). For these tests, 10 mg/kg CNO-HCl was administered intramuscularly 30 min prior to ethanol gavage, and 90 minutes prior to session start (n=5) (see Figure 4-2). This time course was selected to so that CNO was present to activate DREADD receptors throughout the entire rising phase of BEC based on the CNO-HCl time courses presented in the Chapter 3 (Figure 3-1a, page 71). In addition, administering CNO-HCl 30 minutes before the gavage kept the 60 min ethanol pretreatment consistent across all tests, so the monkeys did not have to be removed from their testing chambers for drug administration. For all remaining DREADD testing described in this chapter, this time course was used (Figure 4-2).

Following surgery, monkeys were returned to discrimination training after one week of rest and recovery, and to ensure that all monkeys were no longer being given pain management medication. Discrimination training was continued until performance on both water and ethanol training sessions was above criteria (>90% total session condition-appropriate responses and >70% condition-appropriate responses on first FR). However, six out of seven monkeys reached criteria on the first session after surgery, and the final monkey reached criteria within 5 sessions (Figure 4-3). These data highlight the stability of drug discrimination, in that performance is not detectibly affected by major surgery.

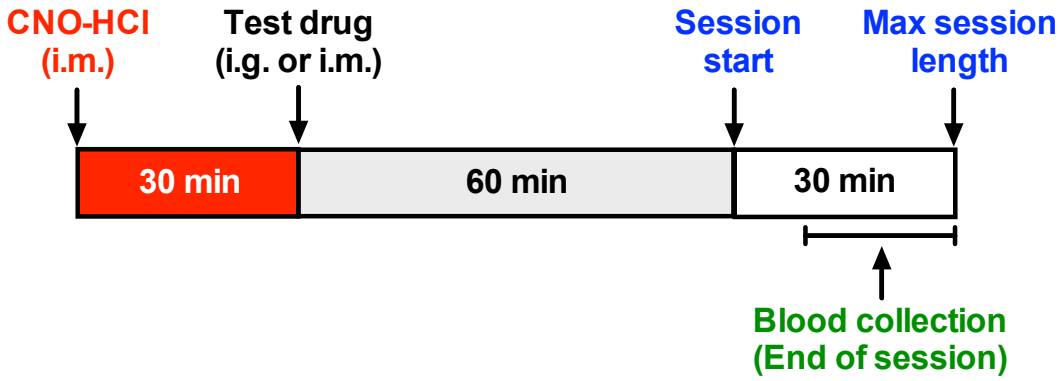

Figure 4-2. CNO discrimination test session experimental timeline. CNO-HCl and test drugs were administered in a procedural room adjacent to the testing room that housed the operant chambers. After drug administrations, monkeys were placed in darkened operant chambers until the next drug administration or session start. Blood was collected immediately after the session ended.

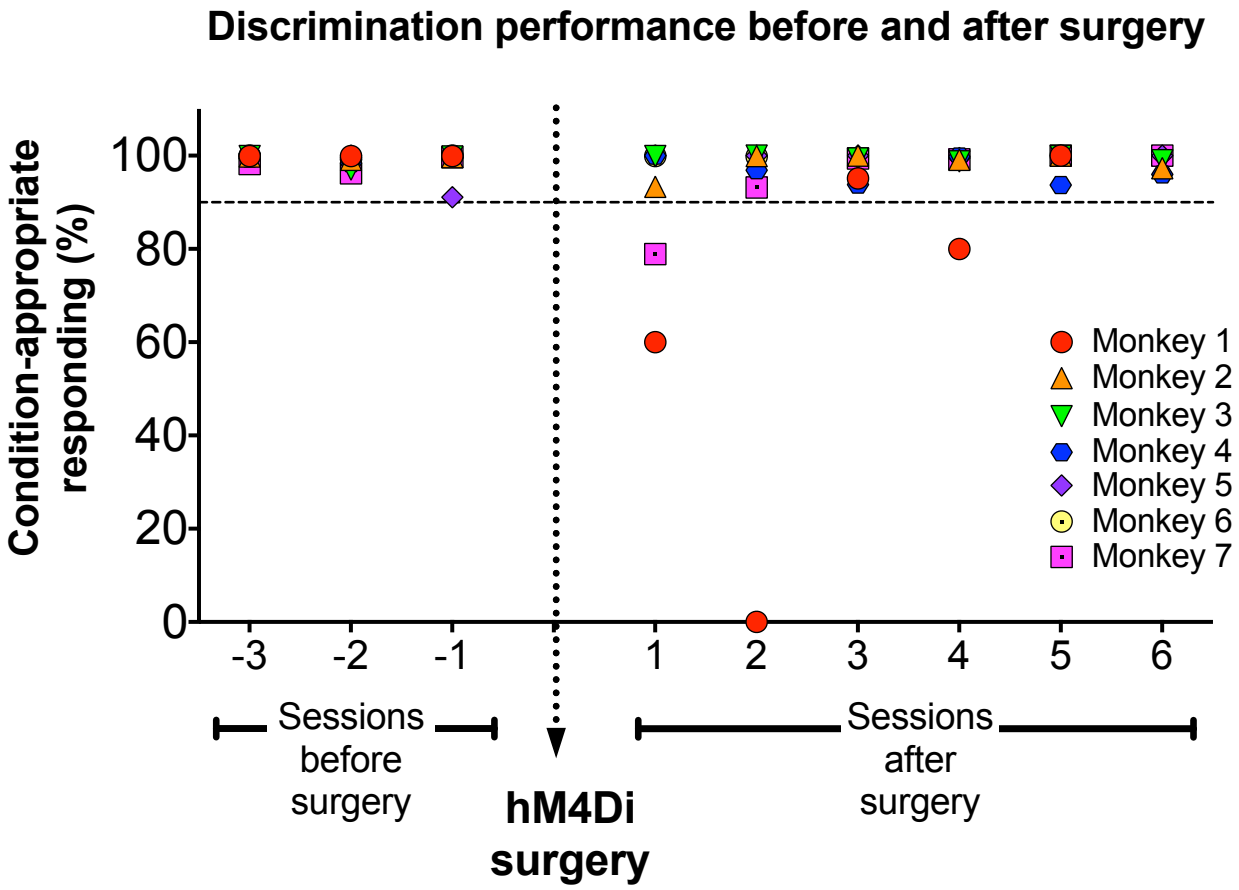

Figure 4-3. Discrimination performance before and after surgery. Training sessions immediately before and after surgery are plotted, with each monkey as an individual data point and color. Both ethanol and water sessions are included, so condition-appropriate responses are plotted on the y-axis according to the pretreatment condition. Inhibitory DREADD (hM4Di) surgery is indicated by the vertical dotted line and arrow. The horizontal dotted line indicates the discrimination training criteria (>90% condition-appropriate responding).

Following surgery and recovery, ethanol dose response curves (0.0-1.5 g/kg) were re-determined for each monkey without any DREADD manipulation. Due to the longitudinal nature of the experiment, up to 1 year had passed since the previous ethanol dose response curve was determined, so these tests were included to make sure that changes in ethanol substitution were due to DREADD activation, rather than an effect of time, and up to 1 year of additional daily discrimination training (for detailed individual timelines, see Table 4-4). Intermediate doses (0.25-0.5 g/kg) were double-determined, counter balancing for the training session on the day prior, but water, 1.0 g/kg ethanol, and 1.5 g/kg ethanol tests were not (replicate of data in Chapter 2, Figure 2-4a, page 46). These dose response curves (Post-surgery, or Post-Sx) were used as baseline measurements for DREADD activation test sessions.

After a minimum of 4 weeks to allow for DREADD expression, DREADD receptor manipulations began. All monkeys began DREADD testing with 5.6 mg/kg (i.m.) CNO-HCl administered 30 minutes prior to either water or 1.0 g/kg ethanol administration (i.e., the training conditions), which corresponded to 90 minutes before the session start time (Figure 4-2). In a pilot experiment measuring inhibitory DREADD receptor activation using blood oxygen level dependent (BOLD) MRI, a plasma CNO concentration of 870 ng/ml was associated with decreased neuronal activity consistent with inhibitory DREADD activation (data not shown). Thus, at the start of the study, 5.6 mg/kg CNO-HCl was selected to target this plasma CNO concentration throughout the ethanol pretreatment time (30 minutes after CNO-HCl administration) and discrimination testing session (session start 90 minutes after CNO-HCl administration) (Figure 4-2). The pharmacokinetics study in Chapter 3 indicated the plasma CNO levels after 5.6 mg/kg CNO-HCl were above 1000 ng/ml at the 30 and 90 minute sampling time points after drug administration (Fig. 3-1a, page 71; Table 3-1, page 73).

If a rate decreasing effect of CNO-HCl was observed, the dose of CNO-HCl was decreased until the response rate effect was no longer present. Initially, in cases where a behavioral effect was not observed with 5.6 mg/kg CNO-HCl, the dose was increased up to 10

mg/kg. However, several subjects were tested with 10 mg/kg CNO-HCl and there was no change in behavior from 5.6 mg/kg. Due to the high plasma levels shown in Chapter 3, the remaining tests were conducted with only the 5.6 mg/kg CNO-HCl dose. Once a final dose of CNO-HCl was selected, ethanol dose response curves were conducted as described previously. All tests with CNO-HCl and ethanol combinations were double determined, counterbalancing for the training session on the day before (water or ethanol). At the end of the session, blood samples (1-2ml) were taken from the femoral vein for assay of CNO and clozapine concentrations in plasma, as described in Section 3.2.3 (Figure 4-2). Additionally, 20µl blood samples were collected from the medial saphenous vein for BEC assay (Section 2.2.6, page 49).

Following the ethanol substitution tests, hM4Di activation was tested in combination with pentobarbital (i.g., n=6), midazolam (i.g., n=5), and MK-801 (i.m., n=1). The subject that received hM3Dq receptors was not tested for $GABA_A$ or NMDA receptor ligand substitution for ethanol. Most subjects were tested only with the GABAergic drugs based on results from Chapter 2, and one subject was tested with MK-801 as it was the only drug that produced full substitution before surgery. For each drug, the dose response curves from Chapter 2 were used as a reference for determining doses that produced full, partial and no substitution. However, it quickly became apparent that the ED_{50} for substitution had shifted in many subjects, likely due to the amount of time between test sessions. Thus, for each dose response curve, the test drug was first tested alone to find a range of doses that capture full, partial, or no substitution (in quarter log unit increments) (Post-surgery). These same doses were tested with CNO-HCl pretreatment 30 min prior to drug administration. This procedure allowed for direct comparison of substitution curves with and without hM4Di activation, without the confound of 12-18 months of discrimination training and testing (see Table 4-4). As described previously, doses were not escalated once a rate decreasing effect was observed (<65% baseline). Also, for all test sessions with an i.m. route of administration, monkeys first received a water gavage to match

the training procedures. Blood samples were not taken following $GABA_A$ or NMDA substitution tests.

4.2.9. CNO and clozapine assays

Described in Chapter 3, section 3.2.4, page 67.

4.2.10. Drugs

Ethanol, pentobarbital, midazolam, and MK-801 were prepared as described in Chapter 2 (section 2.2.7, page 49-50). CNO-HCl was prepared at 30 mg/ml as described in Chapter 3 (section 3.2.2, page 64-65)

4.2.11. Necropsy and tissue collection

Monkeys were sedated with 15 mg/kg ketamine (i.m.) and transported to the necropsy suite where an overdose of Nembutal (25 mg/kg, i.v.) was administered. Immediately following, ice cold artificial cerebrospinal fluid (aCSF) was perfused through the ascending aorta and the brain was removed using a bone saw. The brain was transported to the lab bench in aCSF where it was placed in a brain matrix for sectioning (TedPella Inc., Redding, CA). Beginning at the rostral tip of the temporal poles, the tissue was blocked into 4-6 mm blocks along the coronal axis as previously described (Daunais et al., 2010; Davenport et al., 2013). The size of the brain blocks were determined based on MRI images to best capture the NAc, VP and VTA for immunohistochemistry. Three smaller sections were collected containing the three areas of interest: the NAc, VP, and ventral midbrain (VTA/SN) (see Figure 4-4). Up to 2mm from these blocks were collected for slice electrophysiology and the remaining sections were transferred to 4% paraformaldehyde for 48 hours before transfer to 30% sucrose.

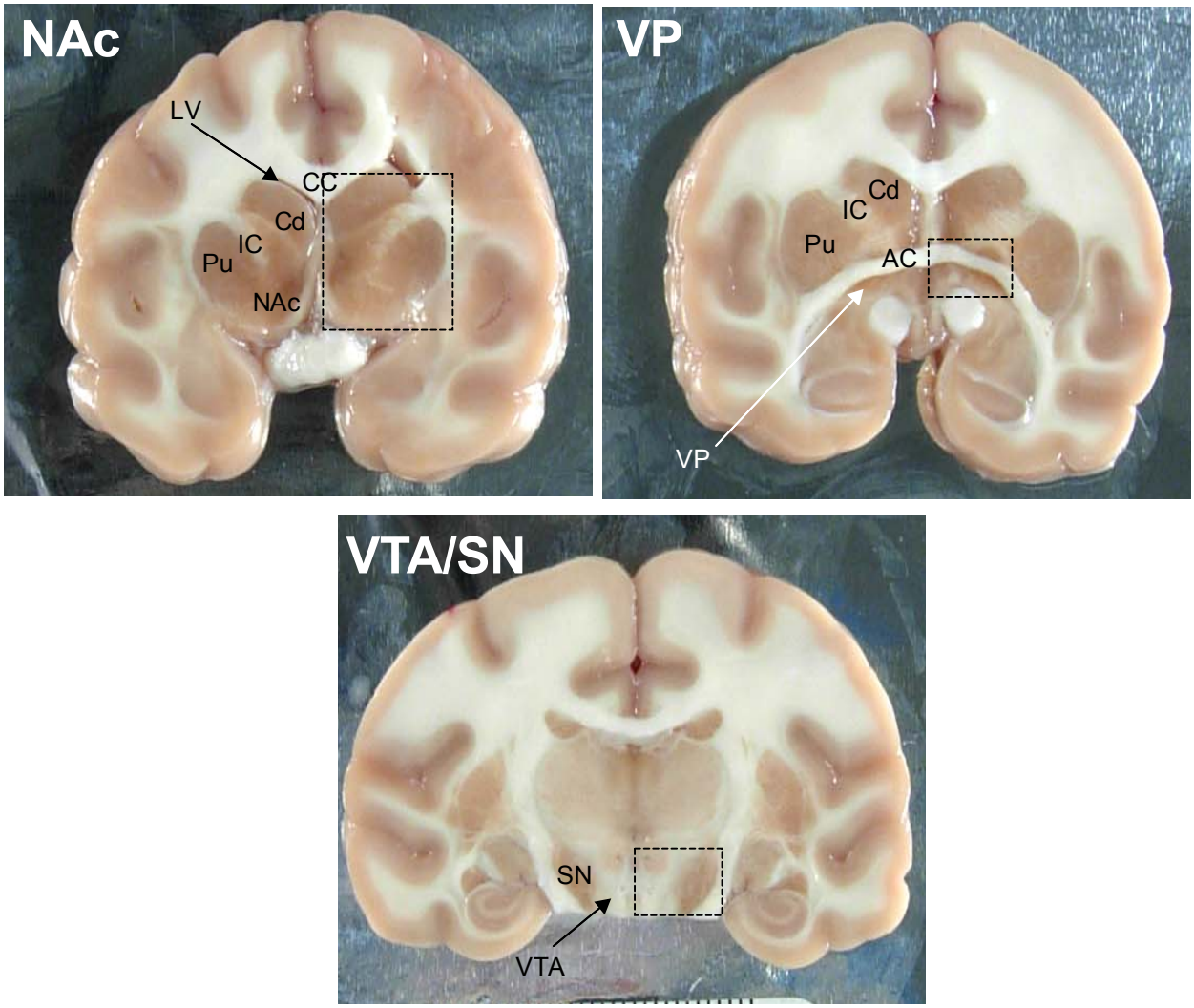

Figure 4-4. Representative brain blocks of the primary regions of interest taken at necropsy. For the NAc, the entire striatum was dissected out (top left). For the VP (top right) a block was dissected that contained the anterior commissure as a reference region. For the VTA/SN, each block contained the SN to the midline, encompassing the VTA. All regions were collected bilaterally along the dotted lines indicated. AC = anterior commissure; Cd = caudate; CC = corpus callosum; IC = internal capsule; LV = lateral ventricle; Pu = putamen; SN = substantia nigra.

4.2.12. Immunohistochemistry

Brain blocks were sectioned (40µm) using a sliding microtome (Thermo Scientific HM-430) and stored in a cryoprotectant solution (phosphate-buffered saline (PBS) containing 30% sucrose, 30% ethylene glycol, 0.2% sodium azide). When ready for immunohistochemical analysis, sections were washed first in PBS containing 0.2% Triton-X100 (EMD TX1568) (PBS-T) at room temperature for at least 10 minutes. To quench endogenous peroxidase activity, sections were incubated in Bloxall (Vector Labs, SP-6000) for 10 min at room temperature, followed by a PBS-T wash. Sections were then incubated in 10% goat serum (v/v in PBS-T, Lampire) overnight at 4°C. The primary antibody (rabbit anti-mCherry, Abcam Ab167453) was then added in a 1:250-1:300 dilution (3.33-4ug/ml) in PBS-T containing 2.5% goat serum and incubated overnight at 4°C. Tissue was washed again in PBS-T then incubated in the secondary antibody (1:200 of biotinylated goat-anti-rabbit IgG, Vector Labs PK-4001) for either 3 hours at room temperature or overnight at 4°C. Tissue was washed again in PBS-T before being incubated in an avidin-biotinylated peroxidase ABC solution (Vector Labs, PK-4001) for 30 minutes at room temperature. Sections were washed three times for at least 5 minutes in PBS-T. Finally, immunohistochemical staining was revealed using a diaminobenzidine (DAB) peroxidase substrate containing nickel for 6 minutes before mounting onto slides. Slides were air-dried overnight and counterstained with Gill's Formula Hematoxylin (Vector Labs, H-3401) for 5 minutes, before dehydrating and clearing through a series of increasing ethanol and xylene incubations. Slides were coverslipped with Cytoseal 60 (Richard Allan Scientific, 8310-4) as the mounting media. Sections from each brain region were run in parallel as a without primary control to assess non-specific staining.

Due to the inherent variability in the placement of the blades used for brain blocking, and the sections that were removed for electrophysiology, a detailed analysis of virus expression using mCherry was not possible. Thus, for the immunohistochemical analysis, sections from

both sides of the brain were visually inspected for the extent of mCherry expression from each of the brain areas available (NAc, caudate, putamen, VP, VTA, and SN).

4.2.13. MRI image analysis for determination of injection site

Structural MP-RAGE scans with GAD contrast following surgery were analyzed for approximate localization of injection sites. First, the four MP-RAGE scans were registered using rigid body affine registration (Advanced Normalization Tools, ANTs), then averaged together using FSL for improved signal to noise ratio. The images had a resolution of 0.5 mm^3. The image contrast was manually adjusted to identify the tip of the injector syringe as visualized in Figure 4-5a. From sagittal images, the coronal plane where the injection took place could be identified. In all cases, the coronal plane identified by this method was within 0.5mm (one coronal section) of the peak brightness. The injection site image could then be viewed in the coronal plane (Figure 4-5b) and spread of contrast in the AP direction was calculated (Table 4-1).

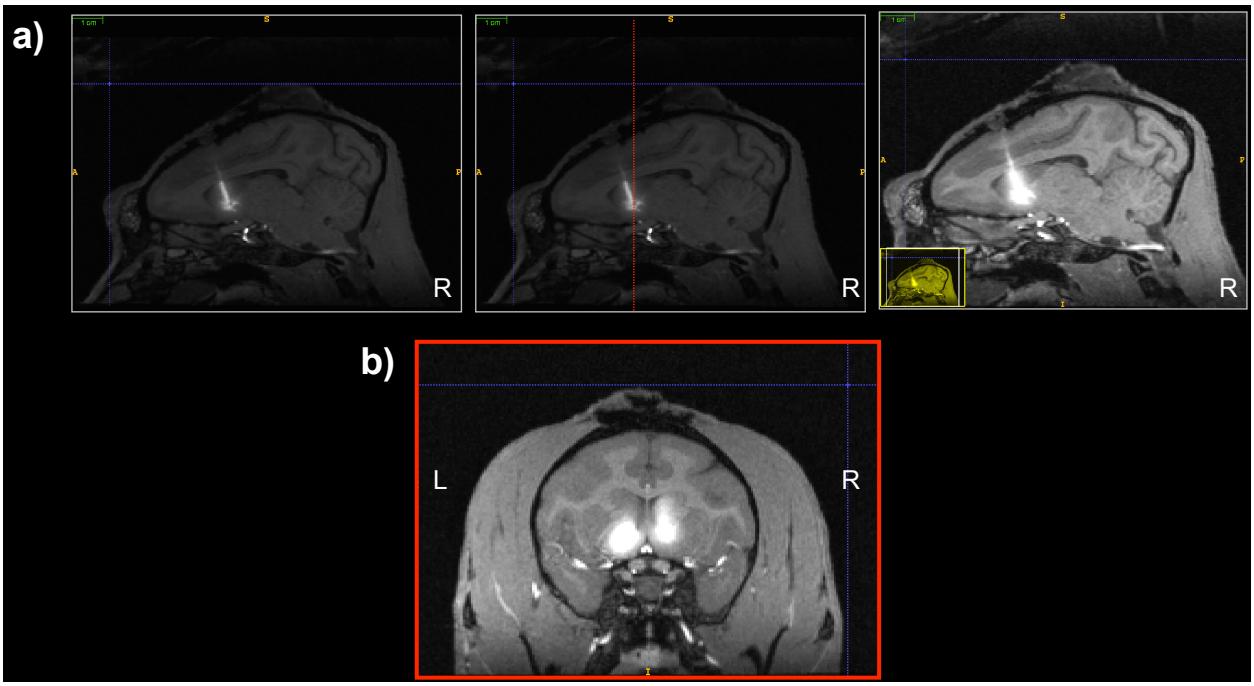

Figure 4-5. Identification of injection site using GAD contrast MRI. a) Sagittal images at low and high contrast (left to right) depicts visualization of needle tract, indicating injection site. b) Coronal section at the level indicated from sagittal visualization of injection site (see red crosshair in middle panel in a. Images shown are from Monkey 6. R=right, L=left.

Table 4-1. Surgery records and MRI determination of GAD contrast spread

Monkey	DREADD	Side	Inj. vol (µl)	Injection coordinates (mm)			GAD AP spread (mm)
				+AP	±ML	-DV	
1 (035)	hM4Di	R	50	24.5	4.5	23.3	9
		L[x]	50	24.5	5.1	23.5	5.5
2 (052)	hM4Di	R	50	25.0	3.3	25.0	6.5
		L[x]	50	24.3[y]	4.3	23.6	6.5
3 (272)	hM4Di	R	50	25.5	4.6	24.7	9.5
		L[x]	50	25.5	5.7	24.7	9.5
4 (283)	hM4Di	R[x]	50	25.0	4.6	25.2	8.5
		L	50	25.0	4.4	26.5	9
5 (332)	hM4Di	R	40	26.0	4.4	23.8	9
		L[x]	40	26.0	4.4	23.9	8
6 (570)	hM4Di	R[x]	30	23.0	3.2	22.1	9
		L	30	23.5	5.1	22.6	8
7 (722)	hM4Di	R	50	21.5	4.1	24.1	8
		L[x]	50	21.5	4.8	24.3	6.5
8 (165)	hM3Dq	R[x]	50	23.5	3.3	23.7	n/a
		L	50	23.5	2.5	25.0	n/a
						Mean:	8.0±1.3 mm

[x]Hemisphere injected first
[y]Coordinate was adjusted 0.2mm during surgery to avoid a blood vessel

4.2.14. Data analysis

The initial behavioral data processing to calculate the percent ethanol-appropriate responding, percent response rate, and ED_{50} values were the same as described in Chapter 2 (section 2.2.8, page 50). For comparison of substitution profiles before and after surgery, and in the presence and absence of CNO-HCl, paired t-tests were conduced on the ED_{50} values wherever possible. Response rates were input into a two-way repeated measures mixed effects model that allowed for missing data points. Pharmacokinetics data were also analyzed in two-way repeated-measures mixed effects models. ANOVAs and t-tests were conducted in GraphPad Prism 6, and mixed effects models were conducted using the nlme package in R (version 3.1.2).

4.3. Results

4.3.1. Confirmation of injection site using GAD contrast MRI and IHC

Following stereotaxic surgery to insert hM4Di receptors in the NAc, the MRI images from each monkey confirmed that the surgery was successful and that the virus was bilaterally infused into the NAc target. From this preliminary analysis, all injections appeared to target the NAc with the exception of monkey 7, who had the peak of GAD spread more posterior than the others, and crossing the anterior commissure (Figure 4-6). Direct comparison of GAD contrast images to the immunohistochemical results at the end of the experiment indicated that the extent of the GAD spread was not representative of the DREADD receptor expression, as indexed by mCherry expression (Figure 4-7a). In the GAD-enhanced MRI images, the apparent spread was throughout the entire ventral striatum, and even extending beyond the boundaries of the brain (Figure 4-7a). However, in this same subject, the mCherry reporter indicated that the hM4Di expression was restricted to a relatively small area within the NAc core (Figure 4-7a, monkey 3). Despite the limited utility of the GAD spread in localizing DREADD expression, these contrast scans appeared fairly accurate in targeting the site of the injection as depicted in

Figure 4-5. Additionally, the coronal plane depicted in Figure 4-6 for each monkey (injection site along AP axis) was consistent with the immunohistochemical identification of the injection site (Table 4-2).

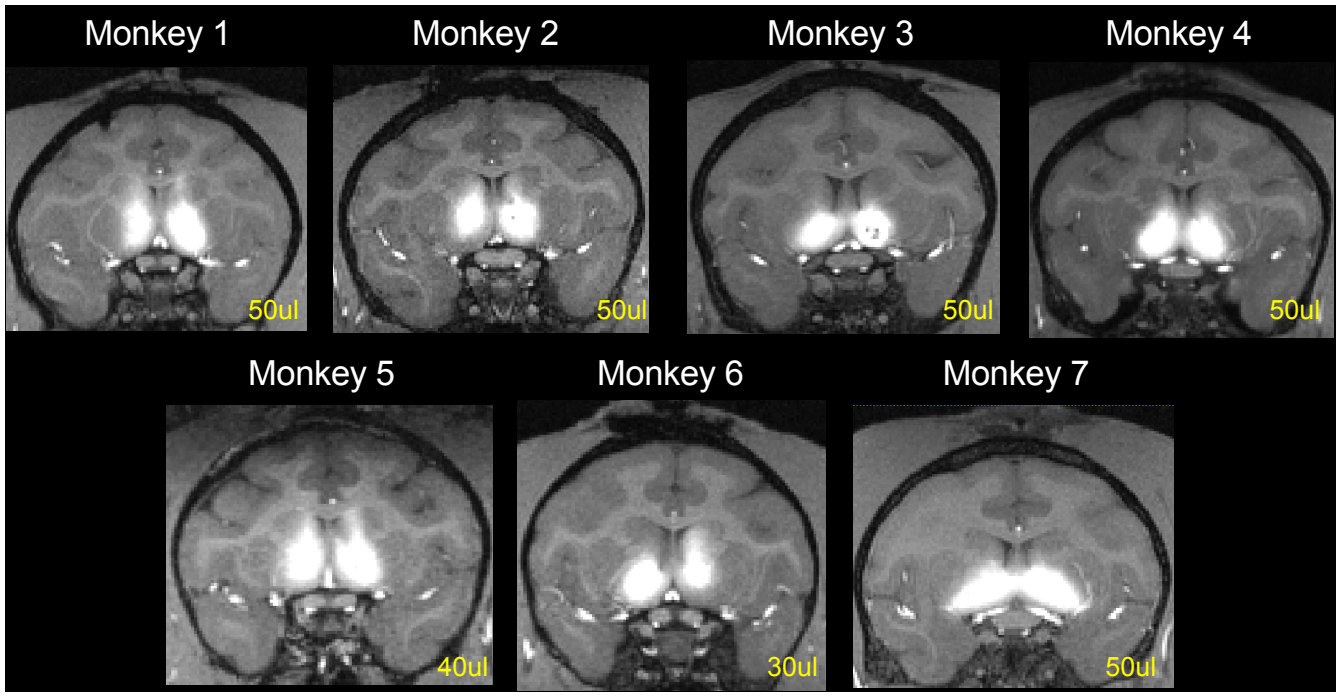

Figure 4-6. Coronal plane of injection site for each of the hM4Di DREADD injection surgeries to the bilateral NAc. Each image represents the peak intensity of GAD contrast for each subject, which is expected to reflect the injection plane. The injection volume is given for each subject and was the same volume bilaterally.

4.3.2. Chemogenetic inhibition (hM4Di) of NAc neurons in ethanol discrimination

At the end of the experiment, all seven monkeys demonstrated mCherry positive cells within the NAc and so all animals were included in the group analysis (see Table 4-2 for injection site and IHC summary). Individual differences in the spread of the DREADD expression were present and are examined in detail in the next section. Ethanol dose response curves were re-determined in the presence of CNO-HCl to determine the effect of chemogenetic inhibition of NAc neurons on ethanol's discriminative stimulus effects (hM4Di group). For six out of seven monkeys, 5.6 mg/kg CNO-HCl was selected for DREADD discrimination testing, but one monkey was given a lower dose of CNO-HCl (1.7 mg/kg), due to significant rate decreasing effect of higher doses (3.0 and 5.6 mg/kg decreased response rate to <5% of baseline response rates). The rate decreasing effects of CNO-HCl were not present in this subject before DREADD expression even at a higher dose of CNO-HCl (10 mg/kg) in combination with 1.0 g/kg ethanol. All seven monkeys were collapsed for group analyses.

The effect of hM4Di receptor activation with CNO-HCl pretreatment in the ethanol discrimination varied across individual monkeys. When collapsed across the group there was no significant change in the dose response curve or ED_{50} (Figure 4-7b,c), but the individual subject's data indicated that there were three categorical responses to hM4Di activation. Two out of seven monkeys (29%) had a significant decrease in ethanol ED_{50} (leftward shift, increased ethanol sensitivity), consistent with the initial hypothesis (Figure 4-7c, shown in green). One of these two subjects received the lower dose of CNO-HCl (monkey 5), providing support of an increased sensitivity to DREADD manipulation in this subject. In three out of seven monkeys (43%), hM4Di activation with CNO-HCl significantly increased the ethanol ED_{50} (rightward shift, decreased ethanol sensitivity) (Figure 4-7c, shown in blue). The remaining two out of seven monkeys (29%) did not demonstrate any changes in ethanol discrimination potency with hM4Di activation by CNO-HCl.

In addition to the effect of hM4Di on ethanol discrimination, response rates were also examined. There was no effect of hM4Di activation by CNO-HCl on response rate during ethanol substitution testing (p=0.8), but there was an effect of ethanol to increase response rates (F(4,38)=3.2, p=0.02) (Figure 4-8d).

The one monkey that had hM3Dq excitatory DREADDs injected in the NAc core was also tested in ethanol discrimination with CNO-HCl (monkey 8; Table 4-1). However, for this subject, only the training doses were tested (water and 1.0g/kg) in combination with hM3Dq activation. There were no significant changes in either ethanol discrimination potency or response rate in this subject. The surgery for this subject took place approximately 18 months prior to necropsy, and at necropsy it appeared that there was minimal evidence of mCherry expression (Table 4-2). In addition, the electrophysiological analysis from this subject indicated that the DREADDs may not have been active at the time of sacrifice, which was approximately 6 months after test sessions were conducted (data not included in dissertation).

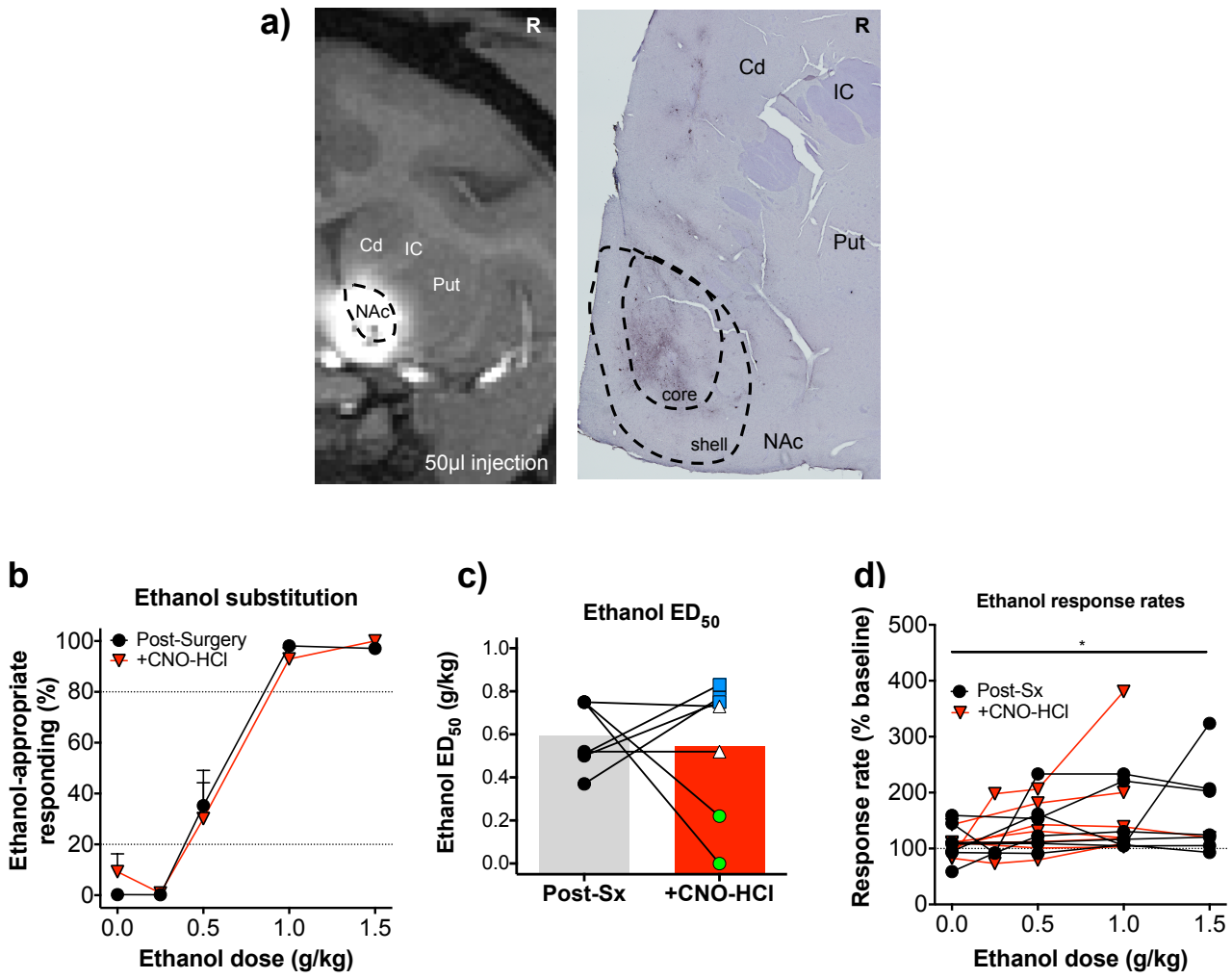

Figure 4-7. Chemogenetic inhibition of NAc in ethanol discrimination. a) Representative images (right hemisphere, monkey 3) of GAD contrast after DREADD surgery to localize the injection site (left) and immunohistochemical analysis of hM4Di receptor expression (right). The entire NAc is outlined in the left image and the NAc core and shell are indicated on the right. b) Ethanol dose response curves in combination with CNO-HCl pretreatment to activate hM4Di receptors (+CNO-HCl, red), as compared to dose response curves with ethanol alone after surgery (Post-Sx, black). Data presented as mean ± SD. c) Ethanol ED_{50} values calculated from dose response curves shown in panel b. Monkeys that demonstrated a decrease in ethanol ED_{50} with hM4Di activation are shown in green, monkeys that had an increased ethanol ED_{50} are shown in blue, and no change are in white. d) Response rates during ethanol test sessions

with and without hM4Di activation with CNO-HCl (+CNO-HCl, red) as compared to Post-Sx ethanol tests (Post-surgery, black). Each data point represents an average of two test sessions for each subject, counterbalancing for the training session on the day prior. *p<0.05, main effect of ethanol dose.

4.3.3. Individual differences in hM4Di expression and ethanol discrimination

In order to better understand differences in the behavioral effect of hM4Di activation on ethanol discrimination, the spread of hM4Di expression was examined. As previously mentioned, there were three categorical behavioral effects: 1) increased ethanol potency (monkeys 3 and 5), 2) decreased ethanol potency (monkeys 1, 2, 6), and 3) no change (monkeys 4 and 7). The individual dose response curves for monkey 3 (leftward shift), monkey 1 (rightward shift), and monkey 4 (no change) are shown in Figure 4-8a,d,g. The IHC analysis indicated that there was significant variability in the extent of hM4Di expression in the NAc core, as well the VP, which lies just posterior to the NAc. Based on previous studies using AAV1 in rhesus monkeys, significant spread across synapses was not predicted (McBride et al., 2011). Thus, mCherry positive cell bodies outside of the NAc may indicate spread of the injection volume to neighboring sites at the time of surgery. For monkey 3, hM4Di expression was dense within the NAc core and is not observed in the shell region (Figure 4-8b). The expression of hM4Di within the VP of this subject was relatively sparse, with only a small number of labeled cell bodies (Figure 4-8c). Thus, the results from this subject indicate that inhibition of NAc core enhanced ethanol discrimination potency in this subject. For monkey 1 (Figure 4-8d-f), the opposite behavioral effect was observed, and was accompanied by dense expression of mCherry in the VP (Figure 4-8f). The mCherry expression in the NAc (Figure 4-8e) was limited to the most dorsal portion of the NAc core and the density of labeled cell bodies was much less than observed in the VP. The differential expression pattern with denser staining in the VP was also observed in monkey 6, who also had a rightward shift in the ethanol dose response curve (Table 4-2).

There was also one subject (monkey 4) that had no effect of hM4Di receptor activation on ethanol discrimination (Figure 4-8g) despite hM4Di receptor expression in the NAc core (Figure 4-8h). Unfortunately, sections VP was not available for this subject. Monkey 7 also did not have any behavioral effect of hM4Di activation on ethanol discrimination, but the needle

placement during surgery targeted the anterior commissure, just posterior to the NAc (Table 4-2).

Sections from the VTA and SN were collected for IHC analysis to determine the extent of hM4Di expression down the projection neurons. In all subjects, a similar pattern of mCherry signal was observed as shown in Figure 4-9 (Table 4-2). A lighter pattern of staining was found resembling cell bodies and fibers of passage are labeled as well (indicated by black arrows). This pattern of expression is distinct from the dark cell bodies in Figure 4-8, but was not present in the no primary antibody control sections.

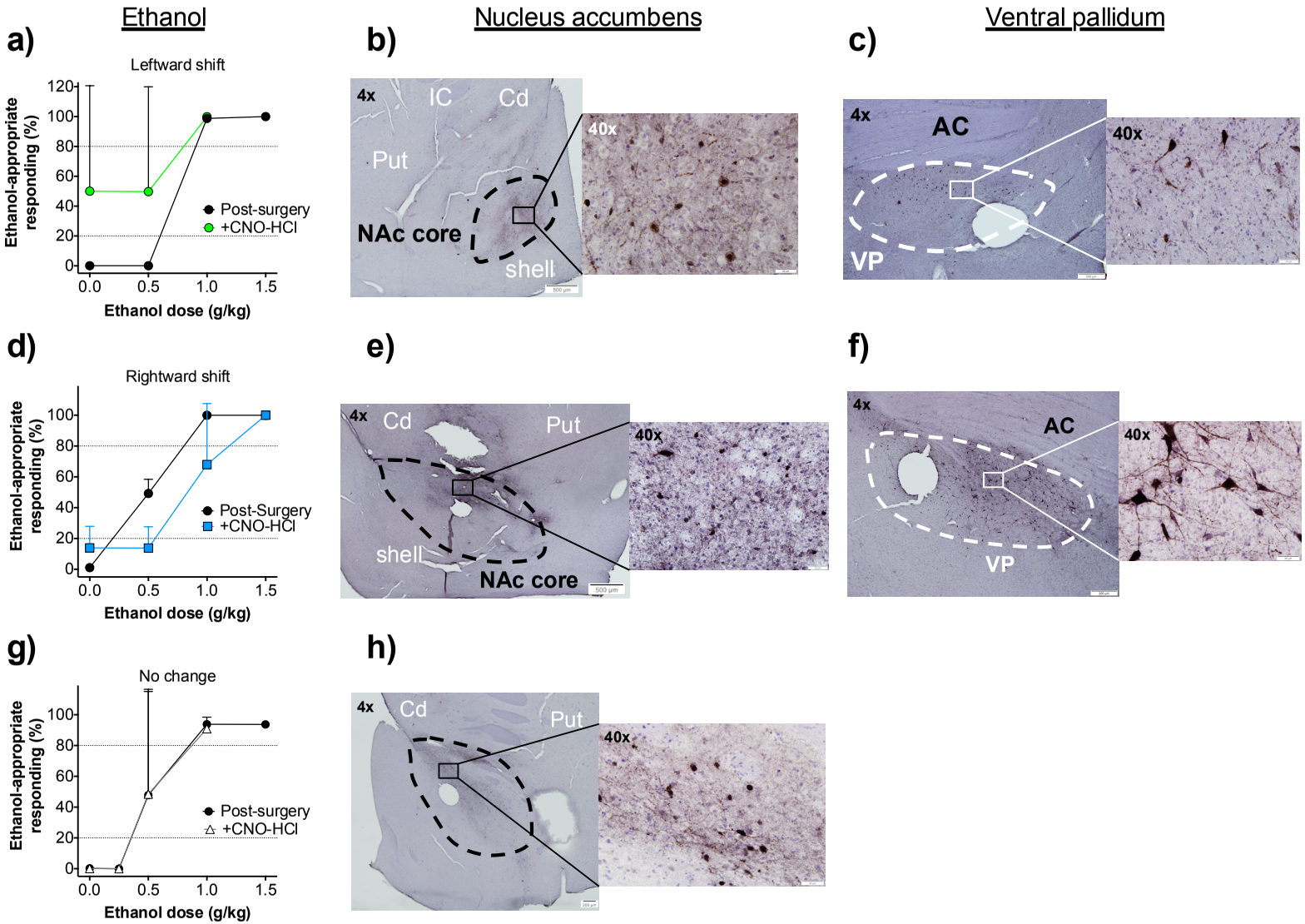
Ethanol
Nucleus accumbens
Ventral pallidum
a)
Leftward shift
Ethanol-appropriate responding (%)
Post-surgery
+CNO-HCl
Ethanol dose (g/kg)
b)
4x
IC
Cd
Put
NAc core
shell
40x
c)
4x
AC
VP
40x
d)
Rightward shift
Ethanol-appropriate responding (%)
Post-Surgery
+CNO-HCl
Ethanol dose (g/kg)
e)
4x
Cd
Put
shell
NAc core
40x
f)
4x
AC
VP
40x
g)
No change
Ethanol-appropriate responding (%)
Post-surgery
+CNO-HCl
Ethanol dose (g/kg)
h)
4x
Cd
Put
40x

Figure 4-8. Individual differences in hM4Di (mCherry) expression**.** Representative examples of each categorical behavioral effect of hM4Di activation on ethanol discrimination is depicted alongside IHC results from the NAc and VP. Monkey 3 is shown in panels a-c, monkey 1 in panels d-f, and monkey 4 in g-h.

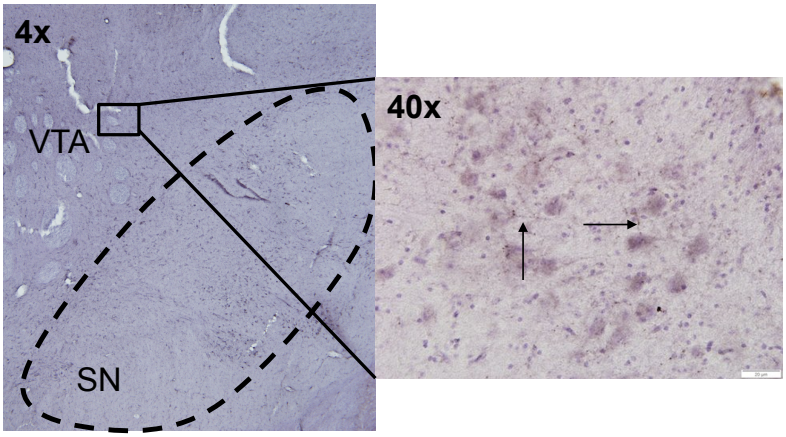

Figure 4-9. Representative image of hM4Di (mCherry) expression in the ventral midbrain. a) Sections from monkey 1 with light mCherry expression in the VTA. Arrows indicate labeled fibers of passage.

Table 4-2. Summary of mCherry immunohistochemistry

Monk #	DREADD	EtOH discrim effect	Injection site	mCherry+ cell bodies		
				NAc	VP	VTA /SN
1*	hM4Di	↑	NAc core	+	++	light
2	hM4Di	↑	Cd/NAc core	+	+	light
3*	hM4Di	↓	NAc core	++	+	light
4*	hM4Di	No Δ	NAc	++	n/a	light
5	hM4Di	↓	Not visualized	+	+	light
6	hM4Di	↑	NAc core	+	++	light
7	hM4Di	No Δ	AC	+	++	light
8	hM3Dq	No Δ	No clear expression			

+: sparse staining; **++**: dense staining; light: cell bodies visualized but much lighter than at injection site; n/a: tissue not available; NAc=nucleus accumbens; VP=ventral pallidum; VTA= ventral tegmental area; SN=substantia nigra,

*Data shown in Figure 4-8

↑: increased ED_{50}; ↓: decreased ED_{50}; No Δ: no change in ED_{50}

4.3.4. NAc modulation of GABA and NMDA substitution tests

The effect of hM4Di receptor activation on the substitution of the $GABA_A$ receptor positive modulators pentobarbital and midazolam and NMDA receptor antagonist MK-801 for ethanol were also examined. Only one monkey was tested for changes in potency of MK-801 to substitute for ethanol based on data from Chapter 2 that indicated that neither of the two $GABA_A$ receptor positive modulators tested substituted for 1.0 g/kg ethanol (Figure 2-5, page 57). Five monkeys were tested with pentobarbital and midazolam in series, and one monkey was tested with only pentobarbital (no midazolam) (pentobarbital group: n=6; midazolam: n=5). Similar to ethanol substitution, the effect of hM4Di receptor activation by CNO-HCl on $GABA_A$ and NMDA receptor ligand substitution for ethanol varied across subjects. For pentobarbital and midazolam, there was no significant group effect of hM4Di activation by CNO-HCl on substitution dose response curves (Figure 4-10a,d) as measured by a group comparison of ED_{50} (pentobarbital: $t(5)=1.6$, $p=0.3$; midazolam: $t(4)=0.4$, $p=0.7$) Figure 4-10b,e).

On the individual level, hM4Di activation decreased the potency of pentobarbital substitution (leftward shifts) in two out of six monkeys (33%) (representative subject shown in Figure 4-11b, monkey 1). Interestingly, both subjects that had leftward shifts in the pentobarbital dose response curve with the hM4Di activation had rightward shifts in the ethanol dose response curves with the same dose of CNO-HCl (Figure 4-11b; Table 4-3). For midazolam, one out of five monkeys (20%) had a decrease in the midazolam substitution potency (leftward shift) with hM4Di activation (monkey 3, Figure 4-11a), and one out of five monkeys (20%) had an increase in the midazolam substitution potency for ethanol with hM4Di activation (rightward shift). For both of these subjects, the direction of the shift in midazolam potency was consistent with the direction of the effect of hM4Di activation on ethanol substitution (Figure 4-11a; Table 4-3). For the one monkey tested for the effect of hM4Di activation on MK-801 substitution for ethanol, there was a significant decrease in MK-801 potency (rightward shift) with hM4Di activation by CNO-HCl (Post-surgery ED_{50}: 0.004 mg/kg; +CNO-HCl ED_{50}: 0.008 mg/kg Figure

4-10g). The direction of this effect was consistent with a rightward shift in this subject following hM4Di activation with ethanol (Table 4-3). For the two monkeys that did not have any effect of hM4Di activation on ethanol discrimination (monkeys 4 and 7), there were no effects on either pentobarbital or midazolam substitution (Figure 4-11c; Table 4-3)

Response rates for $GABA_A$ and NMDA receptor ligand substitution curves in combination with CNO-HCl to activate hM4Di receptors were also examined (Figure 4-10c,f,h). There was a significant main effect of CNO-HCl (hM4Di activation) to increase response rate when given in combination with pentobarbital (F(1,11)=5.0, p=0.048), but not midazolam (F(1,15)=0.3 p=0.6) or MK-801 (n=1, no statistical analysis). Additionally, there was a main effect of pentobarbital dose to increase response rate when collapsed across test condition (with and without CNO-HCl) (F(5,11)=5.9, p=0.007), similar to the effect of ethanol on response rate in Figure 4-8c. Midazolam had the opposite effect on response rate, as there was a significant decrease in response rate with increasing doses of midazolam across both testing conditions (with and without CNO-HCl) (F(4,15)=3.46, p=0.03). DREADD activation by CNO-HCl did not differentially affect response rates in either pentobarbital or midazolam dose response determinations, as the interaction terms were not significant in either analysis. There was no evidence of an effect of CNO-HCl on response rate with MK-801 but this was not statistically tested (Figure 4-10h).

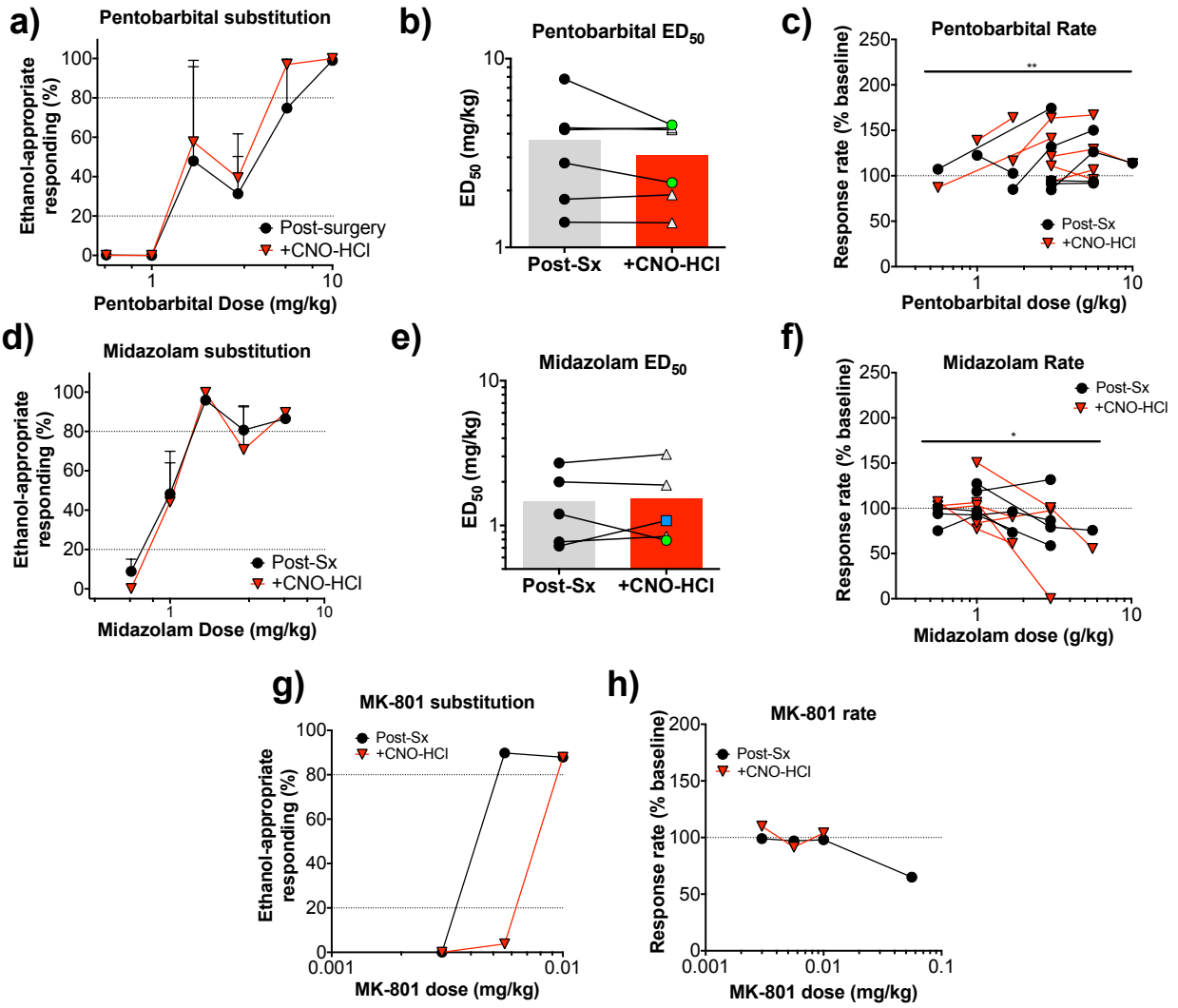

Figure 4-10. Chemogenetic inhibition of NAc on $GABA_A$ and NMDA receptor ligand substitution. a,d,g) Effect of hM4Di activation on a) pentobarbital dose response curves, n=6, d) midazolam dose response curve, n=5, and e) MK-801 dose response curves, n=1. b,e) ED_{50} values for pentobarbital and midazolam substitution after surgery (Post-Sx) and following CNO-HCl pretreatment to activated hM4Di receptors. Individual data points represent individual subjects, mean values are represented by the bar graphs. Monkeys that had decreaed ED_{50} values are in green, increased ED_{50} are in blue, and no change are in white. c,f,h) Response rates for hM4Di testing with pentobarbital, midazolam, and MK-801 normalized to baseline response rates. Each

connected line represents a single subject. Horizontal dotted line at 100 represents 100% of baseline responding, or no change from baseline. *p<0.05, **p<0.01, main effect of pentobarbital or midazolam dose on response rate.

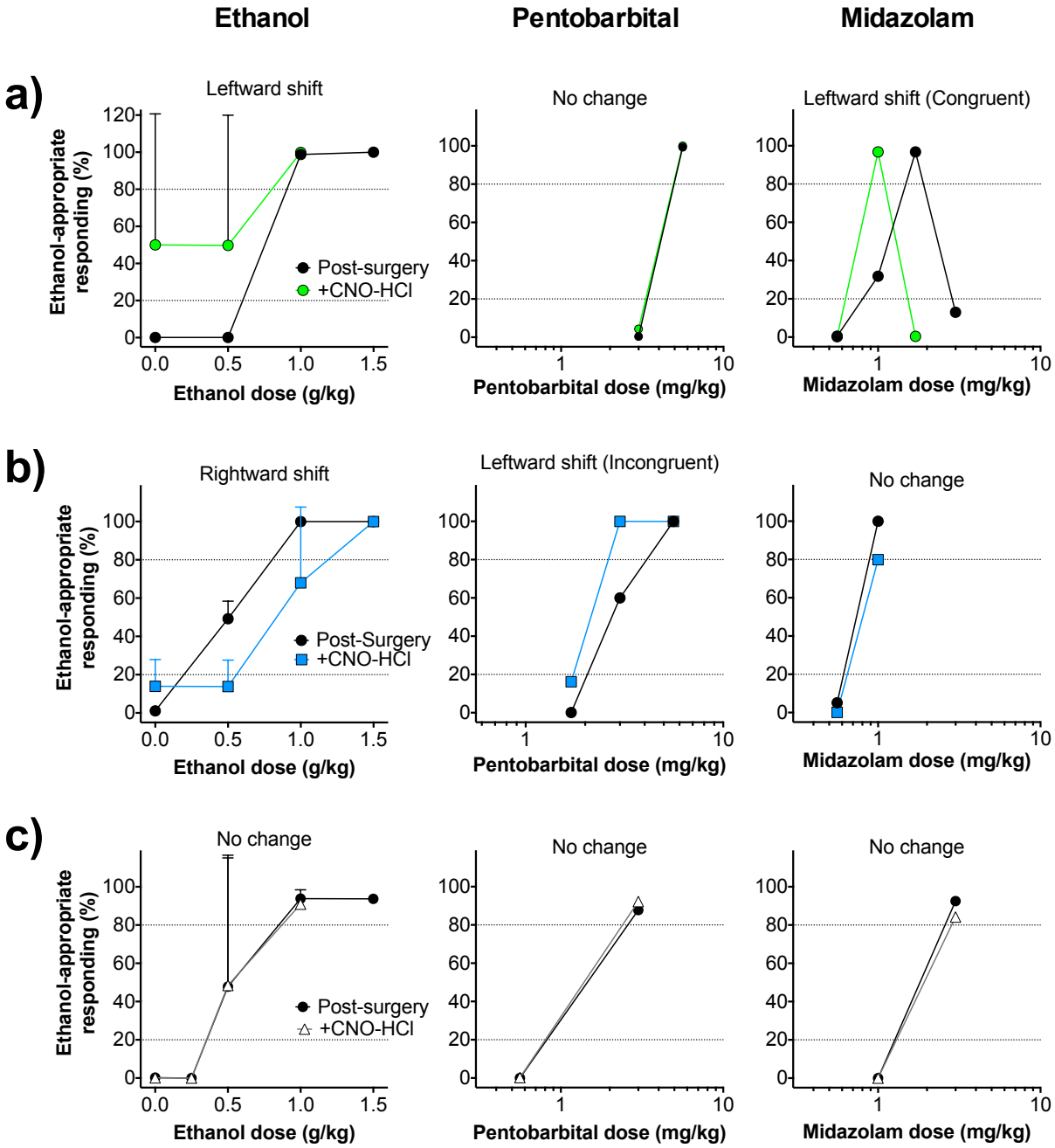

Figure 4-11. Individual differences in hM4Di activation on $GABA_A$ substitution. Dose response curves for individual monkeys are plotted for comparison between ethanol and $GABA_A$ receptor positive modulators. a) Monkey 3; b) Monkey 1; c) Monkey 4.

Table 4-3. Summary of behavioral results.

Monk #	DREADD	CNO Dose (mg/kg)	Ethanol substitution + CNO pretreatment (Δ ED_{50})				Ethanol + Sub
			EtOH	PB	MDZ	MK	
1	hM4Di	5.6	↑	↓	No Δ	-	Incongruent
2	hM4Di	5.6	↑	↓	↑	-	Incongruent
3	hM4Di	5.6	↓	No Δ	↓	-	Congruent
4	hM4Di	5.6	No Δ	No Δ	No Δ	-	No effect
5	hM4Di	1.7	↓	No Δ	-	-	EtOH only
6	hM4Di	5.6	↑	-	-	↑	Congruent
7	hM4Di	5.6	No Δ	No Δ	No Δ	-	No effect
8	hM3Dq	5.6	No Δ	-	-	-	No effect

↑: increased ED_{50}; ↓: decreased ED_{50}; No Δ: no change in ED_{50}; - : not tested

4.3.5. Control experiments

CNO-HCl testing before surgery

Before surgery, 10 mg/kg CNO-HCl (i.m.) was tested in the discrimination task in combination with 1.0 g/kg ethanol (n=2) or water (n=5) to confirm that CNO-HCl was not behaviorally active in the absence of hM4Di or hM3Dq receptors. These results were directly compared to saline tests conducted on different days. In the absence of DREADD receptors, CNO-HCl did not produce any ethanol-like discriminative stimulus effects, as indicated by responding almost exclusively on the water-appropriate lever (Figure 4-12a), that was not different from saline (p=0.42). Additionally, CNO-HCl did not interfere with the ethanol's discriminative stimulus effects, as all monkeys responded on the ethanol-appropriate lever following 1.0 g/kg ethanol, and was not different from saline pretreatment tests (p=0.50) (Figure 4-12a). Lastly, response rates in the water or 1.0 g/kg ethanol conditions were not statistically different from saline test sessions (water: p=0.24; ethanol: p=0.62) (Figure 4-12b).

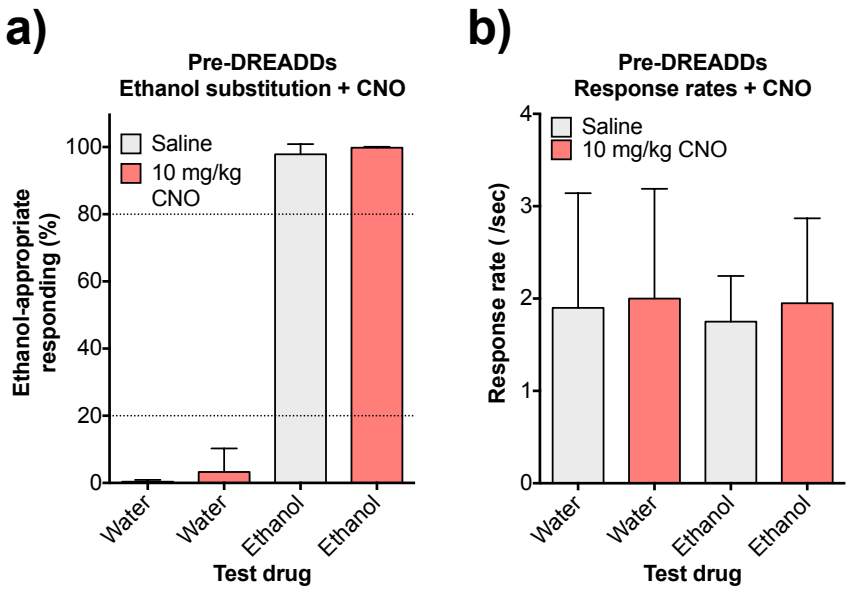

Figure 4-12. Effect of CNO-HCl on ethanol discrimination prior to DREADD insertion. a) Ethanol-appropriate responding following water (n=5) and 1.0 g/kg ethanol test sessions (n=2) in combination with saline i.m., (grey bars) and 10 mg/kg CNO-HCl i.m., (red bars). b) Average response rates (responses/sec) following water and ethanol test sessions in combination with saline (grey bars) and 10 mg/kg CNO-HCl (red bars). All data are shown as mean ± SD.

Substitution testing before and after surgery

After surgery, ethanol dose response curves were first re-determined prior to CNO testing (8-12 months after initial ethanol testing, Table 4-4). Overall, there were no group differences in ethanol substitution (Ethanol ED_{50}: $t(6)=1.5$, $p=0.19$), but there were some changes within individual monkeys (Figure 4-13a). Blood samples were taken after test sessions and BEC was not significantly different post-surgery ($F(1,6)=4.3$, $p=0.08$) (Figure 4-13b). Based on the individual differences in ethanol sensitivity after surgery, all dose response curves were re-established immediately prior to CNO testing to ensure that effects of CNO were compared to a recent dose response determination. Similar to the ethanol ED_{50}, there were no group differences in pentobarbital ($t(5)=0.4$, $p=0.7$) or midazolam ED_{50} values ($t(4)=0.4$, $p=0.7$), but there were within-subjects increases or decreases in sensitivity to both $GABA_A$ receptor positive modulators (Figure 4-13c). The one monkey that did not have any substitution for pentobarbital or midazolam (Chapter 2) was tested using MK-801 and demonstrated similar changes in discrimination potency (Figure 4-13c).

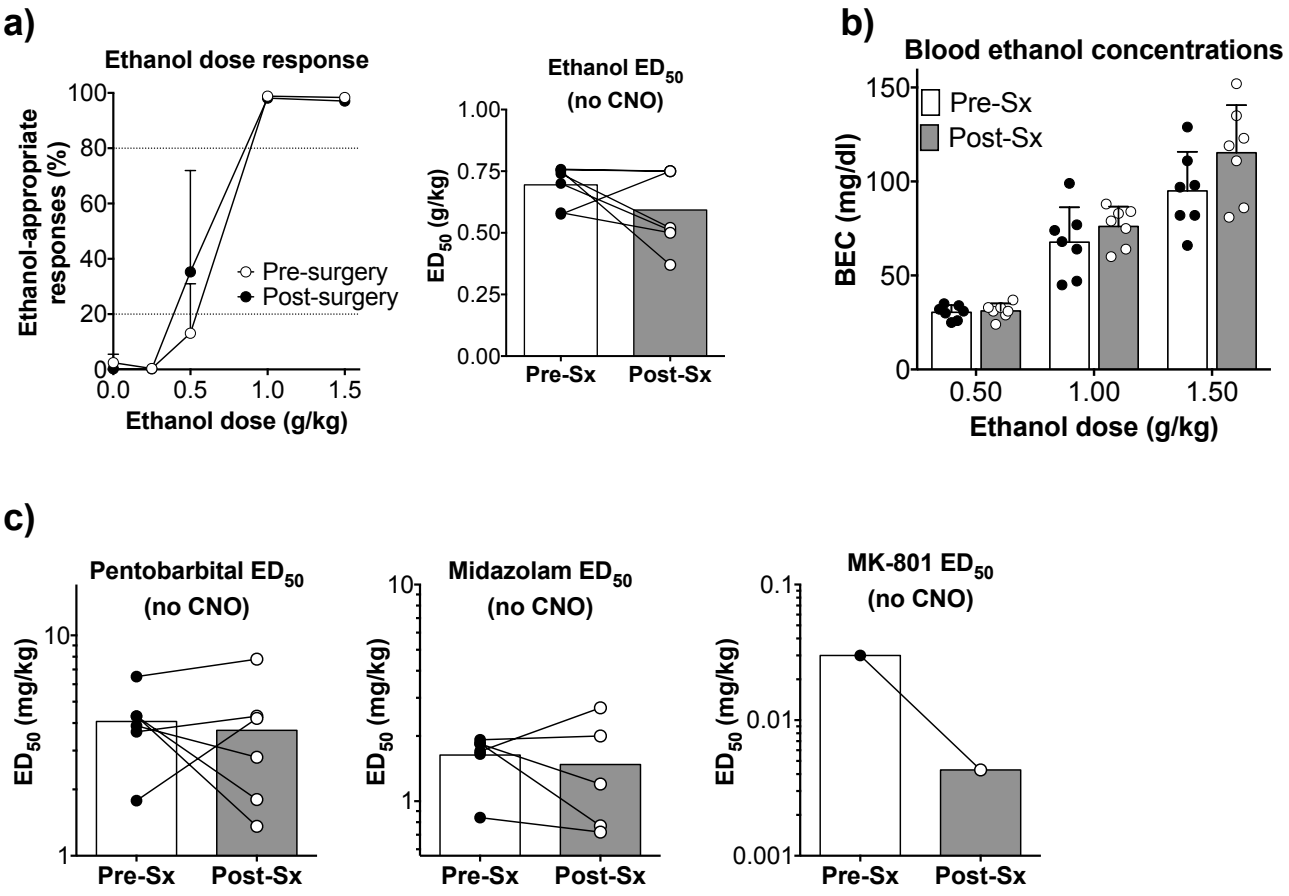

Figure 4-13. Comparison of ethanol substitution before surgery and after surgery. a) Average ethanol dose response curves (left panel, mean ± SD) for all monkeys that had hM4Di receptors injected before and after surgery. ED_{50} values calculated from dose response curves before (Pre-Sx) and after surgery (Post-Sx). Each data point represents a single subject, and bar graphs indicate the group mean (n=7). b) BEC values during discrimination testing before and after surgery for ethanol tests sessions (0.5-1.5 g/kg, i.g.). All samples were taken immediately after the session ended, approximately 75 min after ethanol administration. These BEC values correspond to the dose response curves shown in panel a). c) Pentobarbital (i.g.), midazolam (i.g.) and MK-801 (i.m.) ED_{50} values for each subject before and after surgery. All bar graphs data are presented as mean ± SD. Pre-Sx: pre-surgery; Post-Sx: post-surgery.

Table 4-4. Discrimination testing timelines. Cumulative number of sessions conducted after discrimination criteria were met for each subject (≥90% condition-appropriate responding over total session and ≥70% condition-appropriate responding on the first FR for 5 consecutive session). Sessions to criteria are also presented at the top of the table. Tests were conducted 1-2 times per week, unless training criteria was not met between sessions.

	Monk1	Monk2	Monk3	Monk4	Monk5	Monk6	Monk7
Sessions to criteria	69	104	52	67	114	77	69
Cumulative sessions to complete each dose response curve (reset after criteria was reached)							
EtOH	49	51	42	33	36	71	69
MOR	68	60/87[A]	62	38/73[A]	50	78	82
PB (i.m.)	75	-	105	-	-	-	90
PB (i.g.)	95	70	160[B]	70	104	101/122[A]	101
MDZ	125	85	134[B]	91	127	131	138
MK-801	-	-	-	-	-	159	-
CNO-HCl	145	105	175	100	134	178	143
DREADD Surgery (*Discrimination sessions prior to surgery)*							
Testing only	148	109	175	104	134	178	145
+Training	217	223	227	171	248	255	214
EtOH	176	127	195	122	163	194	160
EtOH + CNO	209	183	238	176	216	218	202
PB + CNO	223	215	251	201/237 [A]	240	-	219
MDZ + CNO	250	234	276	231	-	-	236
MK801 + CNO	-	-	-	-	-	286	-
Muscimol	265	-	298[B]	-	-	-	256
MK801	284	258	285[B]	-	-	-	275

[A]Additional tests conducted after dose response curve was completed
[B]Dose response curves were conducted in reverse order from the order given in the table
- Dose response curve not conducted
EtOH = ethanol; MOR = morphine; PB = pentobarbital; MDZ = midazolam

Baseline and saline response rates

In order to calculate changes in response rate during discrimination testing, response rates were calculated for each test session and compared to a baseline response rate. Baseline response rates were calculated by averaging the last three water sessions prior to test sessions for each drug combination. This method was selected because occasionally inherent response rates would change within subject that was not related to the drug administered, so comparison to a fixed baseline did not reflect drug-specific rate effects. However, for DREADD testing, CNO-HCl was administered with an additional pretreatment interval (30 min before ethanol/drug administration), followed by the 60-min standard pretreatment time (Figure 4-2). Thus, it was important to test whether modifying the pretreatment schedule (additional 30 min pretreatment time) affected response rates, to confirm that water training sessions remained a valid baseline for DREADD testing.

After DREADD surgery, both saline and CNO-HCl were administered 30 min before water gavage and compared to baseline response rates calculated immediately prior to these test sessions. The dose of CNO-HCl that was selected for DREADD testing for each monkey was used in this comparison (5.6 mg/kg, i.m., n=6; 1.7 mg/kg, i.m., n=1). A one-way ANOVA indicated that there was no effect of either saline pretreatment or DREADD activation by CNO-HCl on response rate compared to baseline ($F(2,12)=0.2$, $p=0.8$) (Figure 4-14). Given this result, baseline response rates are used to determine the effect of CNO-HCl in combination with test drugs throughout the rest of the chapter.

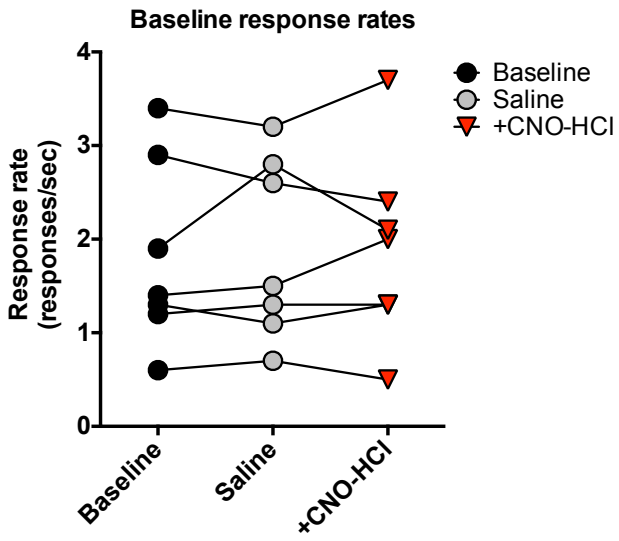

Figure 4-14. Baseline response rate comparison after DREADD surgery. 'Baseline' refers to a rolling response rate calculated from the average of three water training sessions prior to the test session. Saline (i.m.) and CNO-HCl (i.m.) were administered 30 min prior to water gavage (Figure 4-2). Each data point represents a single monkey, each with hM4Di receptors in the NAc (n=7).

Ethanol and CNO pharmacokinetics

Since both ethanol and CNO are metabolized through the cytochrome P450 system in the liver, potential drug interactions were characterized. Blood samples were collected immediately after CNO-HCl + ethanol test sessions for CNO and clozapine assay, approximately 100-110 minutes after CNO-HCl administration (Figure 4-15). CNO-HCl doses ranging from 1.7 mg/kg to 10 mg/kg were available in the presence and absence of varying ethanol doses (0-1.0 g/kg). Due to the small number of samples at 1.7 mg/kg and 3.0 mg/kg CNO-HCl doses (1-2 samples), only 5.6 mg/kg and 10 mg/kg were included in pharmacokinetics analyses, though all data are plotted in Figure 4-15. There was no effect of ethanol dose on plasma CNO concentration (ng/ml) in the dose range tested ($F(3,17)=1.1$, $p=0.4$) (Figure 4-15a), but plasma CNO did increase with CNO-HCl dose ($F(1,17)=20.44$, $p=0.003$). Similarly, plasma clozapine (ng/ml) and percent clozapine/CNO did not vary across ethanol dose conditions ($p=0.6$ and 0.3, respectively) (Figure 4-15b,c), but there was an effect of CNO-HCl dose on both clozapine ($F(1,17)=38.9$, $p<0.0001$) and %clozapine/CNO ($F(1,17)=6.6$, $p=0.02$). Additionally, there was no effect of CNO-HCl on BEC ($F(1,6)=0.1$, $p=0.4$) (Figure 4-15d).

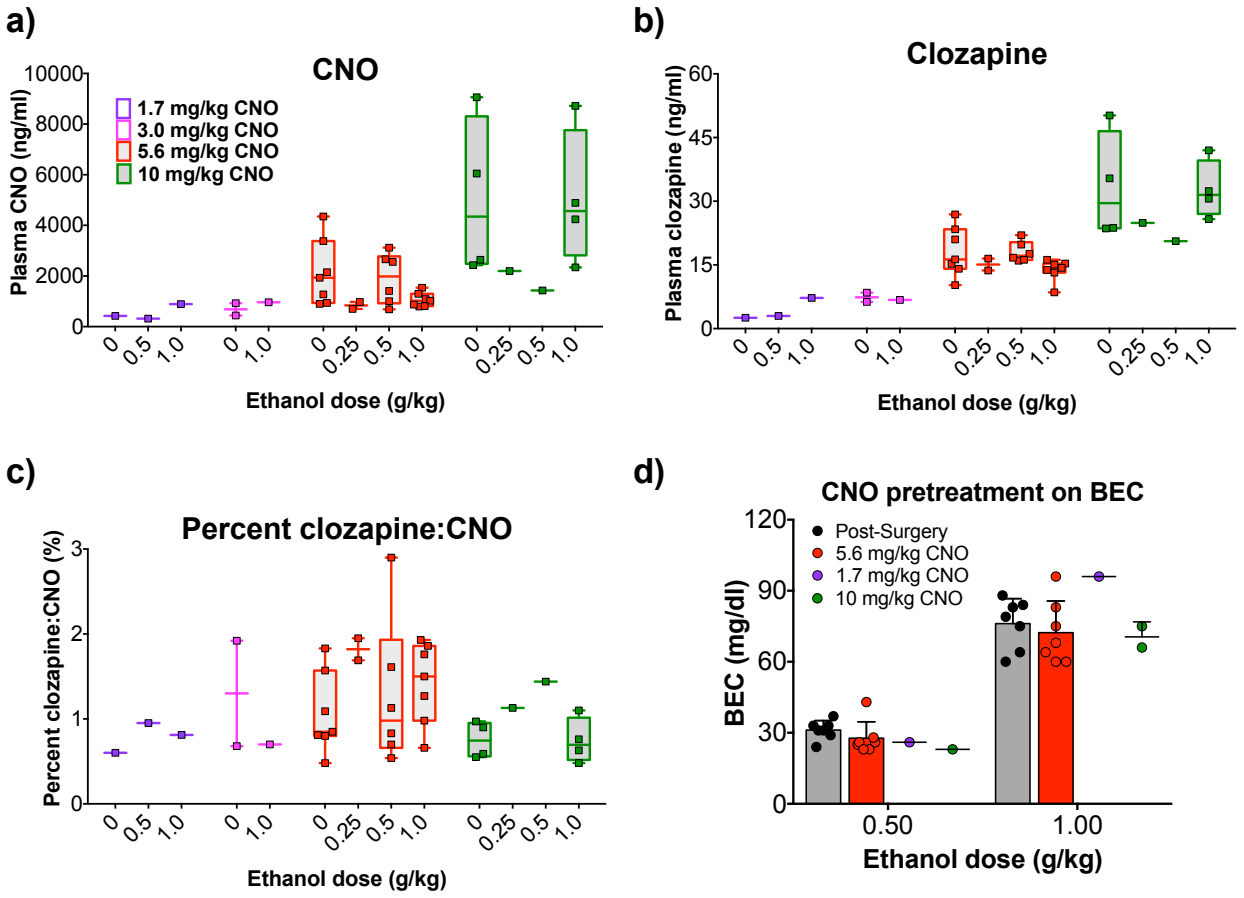

Figure 4-15. CNO and ethanol pharmacokinetics following discrimination testing. a) Plasma CNO (ng/ml), b) plasma clozapine (ng/ml), and c) percent clozapine/CNO following discrimination test sessions, presented as box plots. Blood samples were collected approximately 100-120 minutes following CNO-HCl administration, and 70-90 minutes following ethanol administration. All data points represent single subjects with tests after various doses of CNO-HCl (i.m.) and ethanol (i.g.). In cases where a data point was double determined, an average for that subject was taken from the dose combination before being entered into this graph. d) BEC concentrations following discrimination testing. All data points represent single subjects, bar graphs are mean +/- SD.

4.4. Discussion

The data presented here represent the most extensive behavioral characterization of chemogenetic manipulation in a non-human primate species, as there is only one other published report in a group of two rhesus monkeys (Eldridge et al., 2016). Additionally, this is the first report to examine the role of a targeted brain nucleus in the discriminative stimulus effects of ethanol in primates. The original hypothesis that inhibition of the NAc core through activation of hM4Di DREADD receptors by CNO-HCl would enhance the potency of ethanol's discriminative stimulus effects was supported in a subset of subjects (2/7, 29%), but 3/7 monkeys (43%) demonstrated the opposite effect with decreased ethanol potency following hM4Di activation. The individual variability in the expression of the DREADDs may have contributed to some of the variability in the behavioral results.

4.4.1. DREADD expression

There were two primary methods by which the injection sites were localized: gadolinium-based MRI contrast dye and immunohistochemical analysis for mCherry expression. In general, it appears that the MRI images are fairly accurate at localizing the needle track and injection site, but limited at describing the spread of the virus (Figure 4-6,7a). The GAD contrast images showed more extensive spread along the dorsal-ventral (DV) and medial-lateral (ML) axes compared with the IHC results (Figure 4-7a). The IHC results indicate that the injection spread primarily posterior from the point of injection, as indicated by all subjects that had some expression of mCherry in the VP, which lies just posterior to our target site, the NAc core. Careful examination of another study in rhesus monkeys with the same AAV1 vector, found that there was limited spread of the viral injections anterior to the injection site (McBride et al., 2011). This is an important methodological note, particularly when targeting a small brain nucleus. In the current study, the relative proximity of the NAc and its downstream target, the VP, was particularly significant due to the strong connectivity between these two regions in the NAc circuitry (see Figure 1-4, page 32). There was evidence for expression of hM4Di receptors along

the projection axons, as visualized in the VTA and SN (Figure 4-9), but it still remains a question as to what led to the cell-like structures that were visualized in all subjects. Interpretation of the projection targets in the VP was confounded by the cell body expression found here, though fibers were also labeled consistent with previous studies (McBride et al., 2011).

Another feature of the mCherry expression was some positive cells were localized along the needle tract into the caudate (Figure 4-7a). This pattern was also apparent in the GAD contrast MRI images (Figure 4-5). Following the injections, a 5-minute wait time was employed to allow for diffusion of the virus into the brain region. However, a longer wait time may be necessary, particularly in a deep structure like the NAc. This may represent a difference from the rodent brain, due to a significant increase in overall brain volume (Watson et al., 2006). Lastly, the duration of our behavioral studies (8-10 months from surgery to necropsy for hM4Di group), and robust mCherry expression at tissue collection, highlight the stability over long-term behavioral experiments. However, the one subject that had hM3Dq DREADD injected 18 months prior to necropsy showed very little mCherry expression at necropsy. It is difficult to draw conclusions since a different DREADD was used and GAD contrast images were not available, but this may be preliminary evidence of the maximum longevity of the mCherry reporter.

4.4.2. NAc circuitry in ethanol discrimination

The initial hypothesis that the discriminative stimulus effects of ethanol are mediated in part by ethanol's actions to decrease cellular excitability of neurons within the NAc core was strongly supported by the rodent literature using direct pharmacological manipulations (Hodge and Aiken, 1996; Hodge and Cox, 1998; Hodge et al., 2001b; Besheer et al., 2003; Besheer et al., 2008; Jaramillo et al., 2016). There is one study that was published recently that applied chemogenetics to an ethanol discrimination in rats, and found that inhibition of the insular cortex or select inhibition of projection neurons from the insula to NAc enhanced the potency of the ethanol discrimination (Jaramillo et al., 2017). The cortical projection from the insula is

excitatory (glutamatergic), so inhibition of this project would result in a decrease in the excitability of the NAc, consistent with the current hypothesis and the two monkeys that demonstrated this effect (monkey 3 and 5, Table 4-3). From a circuitry perspective, inhibition of the NAc would lead to a decreased inhibitory signal to its projections (decreased GABA) in the VP and VTA. Thus we would expect that inactivation of the NAc would lead to increased activity in both the VP and VTA (Kourrich et al., 2015; Figure 1-4, page 32). The viral spread beyond the NAc was most consistently observed in the VP in about half of subjects, which can explain the variance in the ethanol substitution. Direct inhibition of VP neurons would shunt the disinhibition of the GABA projection from the NAc and result in an opposite effect to decrease the potency of the ethanol discrimination (monkey 1, 2, 6; Table 4-3). For reference, see hypothesis diagram in Figure 1-5, page 38.

In addition to the specific effects of CNO on the ethanol discrimination, it is important to highlight that there was no effect of CNO-HCl on the discrimination in any subject prior to DREADD insertion. There was also no significant interaction between CNO-HCl and ethanol pharmacokinetics, as measured by plasma concentrations of both drugs. These data suggest that the results from chemogenetic manipulations in ethanol discrimination were not confounded by non-specific effects of CNO on ethanol discrimination, consistent with the rodent study that has also utilized hM4Di DREADDs in ethanol discrimination (Jaramillo et al., 2017).

4.4.3. NAc circuitry on GABA and NDMA substitution for ethanol

The overall findings from the effect of chemogenetic inhibition of the NAc circuitry on GABA and NMDA substitution for ethanol were variable, with only a few subjects demonstrating selective modulation of a subset of the $GABA_A$ receptor ligands tested and occasionally in opposite directions. There are several explanations for the variance. First, ethanol is a stimulus complex with concurrent activity and multiple receptor systems (Grant, 1999; Stolerman and Olufsen, 2001), suggesting that different components of the cue determine successful discrimination based on the training parameters (Bowen et al., 1997; Stolerman and Olufsen,

2000; Stolerman and Olufsen, 2001). Single drug substitution testing isolates a single component of the ethanol cue and asks the subject whether that stimulus is similar or dissimilar to the trained cue. For example, midazolam substitution for 1.0 g/kg ethanol indicates that the ethanol stimulus effects at that dose (and pretreatment interval) are similar to positive modulatory activity at the benzodiazepines binding site, most commonly at the $GABA_A$ α subunit (Rudolph and Mohler, 2004; Tan et al., 2011). Thus, in the context of the NAc circuitry, if inhibition of the NAc enhanced the potency of midazolam to substitute for ethanol, but not for pentobarbital (such as in monkey 3; Table 4-3), it might be concluded that the NAc contribution to the ethanol stimulus complex is most similar to benzodiazepine activity. It may also be concluded that the pentobarbital-like features of the stimulus complex may not be NAc mediated, but instead are produced by other brain areas. One interesting case study in this data set is the subject in which neither $GABA_A$ receptor positive allosteric modulator substituted for ethanol, but the NMDA receptor antagonist produced full substitution. Additionally, neuronal inhibition by hM4Di activation resulted in a consistent rightward shift in the ethanol and MK-801 dose response curves. In this subject, it is possible that since the GABAergic component of ethanol was not learned in the discrimination, only the glutamatergic component was available, and thus led to more consistent effects between ethanol itself and drug substitution. Perhaps in other subjects NAc inhibition was not as effective because the other components of the cue could make up for any changes from the DREADD manipulation (see Figure 1-1, page 9).

4.4.4. NAc circuitry in other behaviors

Beyond the scope of ethanol discrimination, the NAc core has been extensively characterized in motivated behaviors, particularly in the context of reward prediction and behavioral inhibition (Ambroggi et al., 2008; Ambroggi et al., 2011; Meyer and Bucci, 2016). More recently, the VP has been described as instrumental to the encoding of the salience of a discriminative stimulus and is associated with reward seeking following cue presentation (Richard et al., 2016). In the context of this literature, it was unknown whether chemogenetic

inhibition of the NAc (and VP, given the expression data) would globally disrupt task performance. However, no such deficit was present, with the exception of one monkey that required a lower dose of CNO. Many of the studies that have directly tested the role of the NAc in cue-learning have done so in the context of drug seeking following limited training under extinction conditions (Di Ciano and Everitt, 2001; Chaudhri et al., 2010; Ambroggi et al., 2011). Thus, it is possible that the results found in these studies reflected the acquisition of a cue association with an operant response, or were specific to a drug seeking context (Chaudhri et al., 2010). The current data suggest that after extensive training (up to 2 years) to a specific discriminative stimulus, responding is highly stable even following chemogenetic inactivation of the NAc and VP circuit. Thus, the conclusions from this study extend beyond ethanol discrimination and suggest that DREADD manipulation can be successfully applied to behavioral pharmacology studies in rhesus monkeys.

Chapter 5: General Discussion

5.1. Scope of dissertation and main findings

The overarching goal of this dissertation was to begin to unravel the neuroanatomical basis of the discriminative stimulus effects of ethanol in rhesus monkeys. In addition to querying the pharmacology underlying ethanol discrimination in this species, this dissertation is the first study to my knowledge that has directly tested the involvement of a discrete brain nucleus in ethanol's stimulus effects in a non-human primate. Finally, the studies described herein are the most extensive behavioral, structural and functional characterization of a chemogenetic manipulation in monkeys to date. Thus, the results from these experiments contribute novel findings to the fields of behavioral pharmacology, chemogenetics, and the greater effort of translating novel techniques and results from small animals to primates. The studies presented in Chapters 2 and 3 serve as foundational studies to support the primary research questions addressed in Chapter 4.

Several fundamental questions about the pharmacology of alcohol in rhesus monkeys had not been addressed prior to this dissertation. The rhesus macaque has been used for many years to understand many aspects of AUD and addiction, and has been shown to voluntarily self-administer alcohol to intoxication (Winger and Woods, 1973; Kornet et al., 1990; Williams et al., 2004; Grant et al., 2008b; Rüedi-Bettschen et al., 2013; Baker et al., 2014; Baker et al., 2017). Thus, an investigation into the blood alcohol pharmacokinetics and discriminative stimulus effects fills a gap in the literature and improves the understanding of the mechanisms of alcohol in the brain of this species for future development of targeted pharmacotherapies. Prior to these experiments, most studies on alcohol pharmacokinetics and discriminative stimulus effects of ethanol in non-human primate research had been done in cynomolgus macaques or squirrel monkeys (Grant et al., 1996; Grant et al., 2000; Vivian et al., 2002; Platt et al., 2005; Grant et al., 2008a; Helms et al., 2011; Platt and Bano, 2011). The takeaway from

these experiments was that ethanol pharmacology is largely conserved across species, with stimulus effects of ethanol at both $GABA_A$ and NMDA receptors. Ethanol absorption and elimination rates in male rhesus monkeys were also largely consistent with the cynomolgus macaque (slightly slower rate of both absorption and elimination in rhesus) and human literature.

In Chapter 3 and 4, a chemogenetic approach was introduced to characterize the role of the nucleus accumbens core in ethanol discrimination in rhesus monkeys. The overall findings of Chapter 3 indicate that CNO-HCl is peripherally bioavailable and reaches the brain at concentrations that are expected to activate hM4Di and hM3Di DREADD receptors (Figure 3-3, page 77) (Armbruster et al., 2007). This result was critical particularly in the context of recent reports indicating that CNO did not readily cross the blood brain barrier in monkeys (Raper et el., 2017). In Chapter 4, DREADDs were expressed in the NAc to query the role of this brain region in ethanol's discriminative stimulus effects. The main discrimination effects were found on ethanol itself rather than $GABA_A$ or NMDA receptor ligand substitution, indicating the NAc is more involved in the discrimination of the entire ethanol stimulus complex, as opposed to individual pharmacological components (i.e., $GABA_A$ receptor positive modulatory activity alone).

5.2. Pharmacological specificity of ethanol's stimulus effects in rhesus monkeys

As described in Chapter 1 (section 1.2.1, page 4), the stimulus effects of psychoactive drugs are mediated by the receptor systems at which the drug is known to act (Colpaert et al., 1975a). In the case of ethanol, the stimulus properties are best described as a compound stimulus, with concurrent activity at multiple receptor systems, specifically $GABA_A$, NMDA and 5-HT (reviewed in Grant, 1999). Though the role of serotonin was not examined here, the data from Chapter 2 are consistent with the literature, and indicate that both positive modulation of the $GABA_A$ receptor and noncompetive antagonism of the NMDA receptor contribute to

ethanol's discriminative stimulus effects in rhesus monkeys. In the literature, one consistent feature of this pharmacological profile is the preferential activity of ethanol for ionotropic rather than metabotropic receptors (Grant, 1994). The one exception to this is the 5-HT_1 receptor class, at which agonism has produced full substitution for ethanol in rats (Grant and Colombo, 1993c), however this was not replicated in cynomolgus monkeys (Helms et al., unpublished). The two other classes of metabotropic G-protein coupled receptors that have been implicated in ethanol's stimulus effects are mGluRs and the opioid receptor family. Substitution testing with mGluR and opioid ligands for ethanol have been modulatory in nature, resulting in changes in the potency of ethanol discrimination, but were not sufficient to substitute for ethanol on their own (Besheer and Hodge, 2005; Besheer et al., 2006; Besheer et al., 2009; Cannady et al., 2011; Mhatre and Holloway, 2003; Middaugh et al., 2000; Platt and Bano, 2011). Additionally, in cynomolgus macaques, 5-HT_1 agonists were able to increase the sensitivity of ethanol's discriminative stimulus effects in the presence of ethanol, but were not sufficient to substitute for ethanol alone (Helms et al., unpublished).

The basis of the ethanol stimulus at ionotropic and metabotropic receptors is particularly important in understanding the chemogenetic manipulations employed here. The hM4Di and hM3Dq receptors are mutated muscarinic receptors that inhibit cellular excitability through G-proteins in the presence of CNO (Ambruster et al., 2007; Roth, 2016). The effect of activating hM4Di receptors within the NAc circuit produced three distinct outcomes: increased potency of ethanol discrimination (29% of monkeys), decreased potency (43%), or no change in ethanol discrimination (29%). There was only one subject in which DREADD receptor activation alone was sufficient to produce ethanol-like stimulus effects, and this was only observed in 50% of test sessions (Monkey 3; Figure 4-7a; Figure 4-8b-c; Table 4-2). Thus, the overall effect of DREADD manipulation in either direction appears to be modulatory, altering only the potency of the ethanol discrimination, consistent with the modulatory effects of other metabotropic receptors in ethanol discriminative stimulus effects. The limitation of a small sample size in the dissertation

(and in most non-human primate behavioral research) makes it particularly important to address the negative data as directly as the positive. While differences in the extent and localization of hM4Di receptor expression may explain some variance, another possibility remains that the downstream effects of DREADD activation were not pharmacologically similar enough to ethanol in some subjects. The experimental evidence to support the hypothesis that inhibition of NAc core neurons would produce ethanol-like stimulus effects was largely based on rodent studies that injected $GABA_A$ and NMDA receptor ligands directly into the NAc core (Hodge and Aiken, 1996; Hodge and Cox, 1998). The hypothesis was that a similar effect would be found if those neurons were inactivated by DREADDs, but it remains possible that specific pharmacology is essential for ethanol discrimination. Additional evidence for this specificity was provided by the majority (75%) of subjects in which muscimol did not substitute for ethanol, despite activity at the same receptor as pentobarbital and midazolam (Table 2-2, page 58). One simple way to confirm this hypothesis would be directly administer the same ligands (pentobarbital, midazolam, MK-801) into the NAc, but those experiments would require the maintenance of indwelling cannula, and the number of possible tests for a single subject would be limited.

5.3. Evidence of distributed network mediating the stimulus effects of ethanol

The general finding that inhibition of the NAc circuitry increased or decreased the potency of the ethanol discrimination rather than changes in efficacy can also be interpreted in the context of brain circuitry. Prior to the experiments here, it was not known whether inhibition of the NAc would be sufficient to produce full ethanol substitution in the absence of any ethanol. Alternatively, it was possible that inhibition of the NAc would fully antagonize the training dose of ethanol, despite similar circulating blood alcohol levels. Either of these results would indicate the NAc is a final common pathway for ethanol's discriminative stimulus effects in rhesus monkeys. However, these predictions do not describe the results found here. Instead, modulatory effects

were found (as detailed above) and suggest that the basis of ethanol's stimulus effects is likely mediated by a distributed network of brain areas. The only experimental evidence for which other brain regions might be playing a role come from the same set of original studies from Hodge and colleagues (Hodge and Cox, 1998; Besheer et al., 2005; Besheer et al., 2008), and indicate a role for the amygdala, hippocampus, and prelimibic cortex. More recently, the direct projection from the insular cortex to the NAc was implicated in ethanol discrimination in rats, as well as the rhomboid thalamic nucleus (Jaramillo et al., 2016; Jaramillo et al., 2017). In addition to discrimination studies in rats, there have also been a handful of human studies that have given alcohol during a functional MRI (fMRI) scan to try to determine which brain areas are involved in subjective drug effects. While discrimination has not been directly tested in the scanner, subjective ratings of intoxication (self-report visual analog scale) have strongly implicated the ventral striatum, including the NAc (Gilman et al., 2008; Gilman et al., 2012; Seo and Sinha, 2014). However, there are also differences in brain activity in the anterior cingulate cortex and the parahippocampal gyrus (Gilman et al., 2008), which match the data from the rodent ethanol discriminations. However, in fMRI it is difficult to isolate the discriminative stimulus effects of alcohol from the reinforcing effects of the drug. Thus, one approach to address some of these unknown questions would be to train participants to an alcohol discrimination and measure neuronal activation under vehicle (water) conditions as compared to a training dose of ethanol, similar to the animal operant paradigm. MRI scanning sessions could be up to 30 minutes and would be able to capture a specific blood alcohol level in that window, while allowing for enough trials to increase signal to noise in fMRI.

5.4. Advantages and considerations of using chemogenetics in drug discrimination

Throughout the dissertation, the small number of published studies that have examined chemogenetics in non-human primates has been emphasized. Chemogenetics, specifically DREADDs, has enabled the direct, reversible manipulation of select populations of cells within

the brain through mutated designer receptors and a viral delivery strategy. In the context of translating a relatively new technique based in pharmacology to non-human primates (or any new species), drug discrimination offers several key advantages. First, as established throughout the dissertation, testing the effect of DREADDs on discrimination ensures that any positive behavioral effects observed are specific to the trained stimulus cue. In the field of behavioral drug addiction research, it can be difficult to fully explain the mechanisms at play when a particular manipulation decreased drug taking or drug seeking behavior. Drug discrimination however, remains strongly rooted in psychopharmacology, and thus a difference in discrimination potency and efficacy can be linked to the receptor-mediated events that dictate the stimulus features of the trained drug and dose. Secondly, as a behavioral pharmacology assay, drug discrimination allows for dose-dependent DREADD effects to be measured (i.e., CNO dose response curves). This approach was initially applied to the ethanol discrimination studies in Chapter 4, but no consistent dose-dependent effects were observed. However, the potential remains for measuring the effect of incremental increases in the activation of the DREADDs based on the dose of CNO administered to improve the sensitivity of behavioral studies to detect differences with increasing or decreasing activity of a target nucleus. Early evidence for this concept has been presented in a positron emission topography study that was able to measure dose-dependent increases in clozapine binding at DREADD receptors in the macaque brain (Nagai et al., 2016).

In addition to the advantages, this dissertation highlighted several important considerations when applying a new technique to drug discrimination. The primary consideration is the difficulty of interpreting negative data in the absence of a reliable positive control test. For the 7 monkeys that were injected with hM4Di receptors, 6 of them had reliable injections into the NAc core, but not all of these subjects demonstrated shifts in the ethanol dose response curve with CNO pretreatment. The CNO pharmacokinetics study confirmed that CNO was in fact getting to the brain at meaningful concentrations, eliminating an earlier hypothesis

that perhaps CNO was not centrally active (Raper et al., 2017; Gomez et al., 2017). However, it still remains a question how to best interpret a negative finding given the relatively few number of subjects and potential for individual variance. One example from the current dataset is the one monkey that did not have any $GABA_A$ receptor substitution for ethanol, but the NMDA receptor antagonist MK-801 produced full substitution. This finding was not based in the literature, and had NMDA drugs not been tested, the conclusions from this finding would have been limited. A similar case is seen with the chemogenetic manipulation of NAc neurons on ethanol discrimination. The introduction of a positive control experiment to confirm that the DREADDs are functional, that enough CNO is given and in the brain region that was targeted, would greatly improve the interpretability of these results.

The best task for measuring selective inhibition of the NAc can be best determined from lesion and pharmacological inactivation studies. Lesions of the NAc core specifically has been linked to impulsivity, particularly in a delayed discounting task (Cardinal et al., 2001). Pharmacological inactivation of the NAc core in rats in a discriminative stimulus task has found that nonspecific responding on the inactive lever or when no cue was present increased, perhaps also suggesting an increase in impulsive behavior (Yun et al., 2004). NAc core inactivation also decreased the selective responding on the food-reinforced lever (Ambroggi et al., 2011). In monkeys (cynomolgus macaques), NAc lesions resulted in ‘disorganized’ behavior, which included repetitive action that may be similar to the inactive lever presses observed in rodents, as well as a general increase in locomotor activity (Stern and Passingham, 1994; Stern and Passingham, 1996). From this handful of studies, it is apparent that the behavioral correlates of NAc inhibition depend on the behavioral task being employed. However, positive control experiments for neural manipulations would improve the interpretability of these studies, particularly in a monkey model where confirmation of injection site and infection rate may be far removed in time from the behavioral studies.

5.5. Understanding stimulus effects in the context of alcohol use disorders

Considering the current findings with rhesus monkeys in the context of the larger body of literature on ethanol's stimulus effects, it is readily apparent that the basis of the ethanol discrimination is highly conserved across species (reviewed in Allen et al., 2017). The translatability of the stimulus effects of ethanol is particularly relevant when considering the larger goal of an examination of alcohol's effect on the brain and behavior, which is to improve therapeutic approaches and treatment outcomes. One outstanding unanswered question from the drug discrimination literature over the past several decades has been how to directly connect discriminative stimulus effects to both the subjective drug experience and the reinforcing effects of the drug. As described by Schuster and Johanson, a direct correlation between the stimulus effects and subjective effects cannot be made based on the limited salience and valence features necessary for discrimination (Schuster and Johanson, 1988). One first step to connecting drug discrimination to alcohol reinforcement has been to compare the stimulus effects of experimenter-administered ethanol (i.e., gavage) with self-administered ethanol. These studies have shown that the same GABAergic and glutamatergic ligands produce full substitution for self-administered ethanol, following discrimination training with experimentally-administered ethanol (Hodge et al., 2001a; Besheer et al., 2006). However, relating findings under these training and testing conditions (trained to discriminate gavaged ethanol, tested with self-administration) to naturalistic drug self-administration conditions must be done with caution. There is evidence that extensive exposure to one cue or stimulus can influence the discrimination of subsequent similar cues, suggesting that the trained stimulus (gavaged ethanol) may limit the ability to detect subtle features after self-administration (Young et al., 1981; Green and Grant, 1998; Stolerman and Olufsen, 2001). Another difficulty with assessing the stimulus effects of self-administered drugs in animal models is the lack of control over dose and timing. Without the precision of a gavage, training may be impaired by variable rates of drug intake that can lead to significant differences in circulating drug concentrations. A

discussion on the limitations of directly translating discriminative stimulus effects to self-administration here serves to caution a direct connection between discrimination and reinforcement. Instead, drug discrimination procedures can provide other information that can be useful in developing targeted pharmacotherapy. The dose and timing specificity of drug discrimination, particularly in the case of ethanol where dose and time can result in distinct pharmacological properties, can inform the complex, dynamic state of an animal over different phases of a drinking episode.

5.6. Conclusions and future directions

The studies in this dissertation provide novel data to the field of alcohol discrimination, as well as the new field of chemogenetics, particularly in non-human primates. The data provided on ethanol discrimination here are consistent with the highly translational nature of ethanol's discriminative stimulus effects and consistent action at both $GABA_A$ and NMDA receptors. Evidence for a pharmacologically-specific, distributed network involved in mediating ethanol discrimination in monkeys was provided, but future work is necessary to determine which other brain areas might be involved. The application of fMRI approaches in human subjects may be able to elucidate the circuitry involved in discriminative stimulus effects by tracking activity in the whole brain, while still maintaining experimental control over drug dosing. The studies from this dissertation also provide a strong foundation for future applications of DREADD technique to non-human primates, particularly in the alcohol field. These data indicate the need to transition from DMSO preparations of CNO to water-soluble counterparts and to measure CSF concentrations of CNO wherever possible. Future studies are needed to investigate the mechanism by which CNO-HCl has significantly increased bioavailability relative to CNO-DMSO. Overall, DREADDs remain a powerful tool for dissecting the neural mechanisms of the discriminative stimulus effects of ethanol, and the studies here indicate that CNO does not introduce non-specific confounds when given in combination with ethanol (no

rate decreasing effects or pharmacokinetic interactions). As the chemogenetic techniques develop, with multiple DREADD receptors each with their own ligands (hM3Dq/hM4Di and KORD DREADDs), there will be increased opportunity to investigate the interaction between multiple brain areas in the behavioral pharmacology of ethanol, a technique that has yet to be applied to non-human primates (Vardy et al., 2015). Further, the development of cell-type specific promoters will enable circuit analysis in the monkey and can be applied to drug discrimination studies for specific circuit mapping of stimulus effects (Urban and Roth, 2015). These studies will improve our understanding about the mechanisms of alcohol in the brain to improve potential treatment approaches and outcomes.

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