2009

EXAMINATION OF THE DISCRIMINATIVE STIMULUS AND CROSS-TOLERANCE INDUCING PROPERTIES OF N-DESMETHYLCLOZAPINE IN C57BL/6 MICE.

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EXAMINATION OF THE DISCRIMINATIVE STIMULUS AND CROSS-TOLERANCE INDUCING PROPERTIES OF N-DESMETHYLCLOZAPINE IN C57BL/6 MICE.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

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May, 2009

Acknowledgement

There are many people to thank who helped me throughout the process of conducting and writing my thesis. First and foremost I would like to thank my mentor and thesis committee director Dr. Joseph H. Porter, for his guidance and patience throughout my first two years of graduate school at Virginia Commonwealth University. I would also like to thank my committee members Dr. Robert J. Hamm and Dr. John A. Rosecrans for their suggestions that led to improvement of my thesis. I must extend my gratitude to Dr. Robert E. Vann and Dr. Laura E. Wise for their support and advice. I owe many thanks to my fellow graduate students and friends Sarah Vunck, Matt Walentiny, Erin Wood, A. J. Kwilasz, and many others in Richmond and elsewhere. They have provided me with many insights and words of advice that contributed to the success of my work. My gratitude is extended to all of the undergraduate research technicians who donated their time and energy in the lab including Kevin Webster, William Renzulli and many others. I would also like to acknowledge my undergraduate research mentor and advisor Dr. David C. Jewett for teaching me the foundations behavioral pharmacology, and spurring my interest in science. My gratitude is also extended to my parents, Ron and Eileen Wiebelhaus and my brother, David Wiebelhaus and sister, Noelle Hansen for their endless support and willingness to listen. This work would not have been possible without all of their contributions.

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Abstract

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Due to its unique receptor binding profile and its relationship to clozapine, N-desmethylclozapine (NDMC) has been examined as a possible antipsychotic drug (APD). Clozapine has been trained as discriminative stimulus in our lab, but NDMC has not yet been established as a discriminative stimulus. In experiment 1, 12 C57BL/6 mice were trained to discriminate 10.0 mg/kg NDMC from VEH using a standard-two lever operant procedure to assess antipsychotic substitution. The typical APD clozapine fully substituted for NDMC at 2 doses tested (2.5 and 5.0 mg/kg), while typical APD haloperidol failed to substitute for NDMC. In Experiment 2, 11 mice were given repeated administration of

NDMC to assess cross-tolerance development to the discriminative stimulus of clozapine. NDMC was successfully trained as a discriminative stimulus and was also shown to induce cross-tolerance to clozapine's discriminative stimulus, indicating similar underlying pharmacological mechanisms of action between NDMC and clozapine.

Introduction

Schizophrenia

Schizophrenia is a devastating neurological disorder that affects roughly .07% of the world's population (Saha, Chant, Welham, & McGrath, 2005). It is a complicated disorder that affects many people indiscriminant of gender, culture, and social class, and commonly begins during early adulthood. Schizophrenia is a difficult prognosis to endure, having a mortality rate two to three times higher than the average person without schizophrenia. Not only do complications from the disorder and treatment harm schizophrenics over time, two thirds of people whose deaths are related to schizophrenia commit suicide (Auquier, Lancon, Rouillon, & Lader, 2007; Brown, 1997). The epidemiology of schizophrenia is unclear; there have been some studies that link schizophrenia with the presence of specific genes (Owen, Craddock, & O'Donovan, 2005), however it is apparent that the genetic component is not the only factor determining whether or not schizophrenia will develop. Monozygotic twins share roughly a 50% concordance rate of schizophrenia, which implies that environmental factors are also important for the development of the disorder (Owen et al., 2005).

Emil Kraepelin (1896) began constructing what is now the modern framework of schizophrenia. Kraepelin described the disorder as "dementia praecox" which means early dementia. Dementia is normally a condition that develops later in life; early dementia refers to the common early onset of schizophrenia. Kraepelin also developed a list of symptoms exhibited by people with the disorder. Dementia praecox was later termed schizophrenia by Eugene Bleuler (1911). The root of the word *schizophrenia* means "split

mind." The term refers to the disconnection between the mind and actions of someone afflicted with schizophrenia.

Symptoms

The symptoms of schizophrenia can be classified into two main categories: positive and negative (APA, 2000). The term positive symptoms refers to symptoms that exaggerate or distort normal functioning, while the term negative symptoms refers to the absence or reduction of normal functions (APA, 2000). Positive symptoms include hallucinations, disorganized thought and speech, delusions, catatonic behavior, and disorganized behavior.

Hallucinations are false perceptions of experiences involving distortion of the senses. Auditory hallucinations are the most common, but some people with schizophrenia have hallucinations that involve other or multiple senses. Disorganized thought is a very common feature of schizophrenia (APA, 2000) which is most noticeable when hearing a person with schizophrenia speak. Schizophrenics displaying disorganized thought and speech jump quickly from one topic to the next while making very vague, if not non-existent, associations. Delusions are false beliefs. People with schizophrenia display several different types of delusions. The most common form of delusion for people with schizophrenia to experience is delusions of persecution (APA, 2000). Delusions of persecution include thoughts that people are trying to single out and harm the person experiencing them (e.g. big brother is watching me; everyone's trying to get me). Referential delusions also occur as nonspecific things directed at person with schizophrenia (e.g. codes in advertisements directed toward the individual). Religious

delusions are also possible symptom (e.g. the person with schizophrenia claims to be a martyr). Another form of delusion endured by some schizophrenics is a delusion of grandeur (e.g. belief of being the king of the universe).

Disorganized behavior is behavior that does not make sense to an average person. Schizophrenics do not display clear purpose guiding their behavior. They dress themselves inappropriately based on the weather. They sometimes display loud outbursts in public for no apparent reason. These behaviors can severely impair a schizophrenic's ability to perform normal daily activities (APA, 2000). Catatonic motor behavior refers to a lack of reactivity to one's environment and is characterized by maintaining a rigid, uncomfortable position for an extremely long amount of time. Although disorganized thoughts and behaviors are considered positive symptoms in the *Diagnostic and Statistical Manual of Mental Disorders IV, TR* (DSM-IV-TR; APA, 2000), some research suggests that they have a different pathology than other positive symptoms (Arndt et al. 1991).

In addition to positive symptoms, people with schizophrenia are also subject to negative symptoms. Examples of negative symptoms include avolition, flat affect, anhedonia, and alogia. Avolition is characterized by the lack of desire, drive, or goal-motivated behavior. Flat affect is the reduction of the expression of emotions, sometimes apparent in people suffering from depression. Alogia in terms of schizophrenia is known as poverty of speech and refers to a general inability to engage in unprompted conversation. Instead of a normal flowing conversation, schizophrenics displaying alogia commonly will answer questions with one word answers, if at all. Anhedonia is defined as the inability to

receive pleasure from normally pleasurable experiences (e.g. no longer enjoy the taste of favorite foods).

Another symptom common in schizophrenia is cognitive impairment, and most schizophrenics have some degree of cognitive deficit (Meltzer, Thompson, Lee, & Ranjan, 1996). On average, people with schizophrenia perform in the lowest ten percent of the population on cognitive tests (Keefe, 2007). Cognitive impairment has historically been considered a negative symptom (loss of cognitive ability), but because of its unique effects, it has more recently started to be considered a separate category of symptoms. Schizophrenics may show severe cognitive deficits, including severe problems with executive functioning, working memory, verbal memory, verbal fluency and attention (Keefe, 2001) as well as moderate impairments in delayed recall, visuo-motor skills, and distractibility; and mild impairments in delayed recognition, perceptual skills, and IQ (Keefe, 2007). The degree of cognitive impairment in a schizophrenic's is very predictive of their functional outcome (Green, Kern, Braff, & Mintz, 2000; McEvoy, 2008). The MATRICS™ initiative (Measurement and Treatment Research to Improve Cognition in Schizophrenia) was established by the National Institute of Mental Health to encourage researchers to examine how we should address the issue of cognitive deficits in schizophrenia (Green, Nuechterlein, Gold, Barch, Cohen, Essock, Fenton, Frese, Goldberg, Heaton, Keefe, Kern, Kraemer, Stover, Weinberger, Zalcman, & Marder, 2004). Because of this initiative, achieving cognitive improvement for people with schizophrenia has become a target for researchers and will help drive the development of novel therapeutic agents that address that issue (Laughren & Levin, 2006; Meltzer & McGurk, 1999).

While there are a variety of symptoms displayed by people schizophrenics, they do not necessarily display all of the possible symptoms. Only a few of the listed symptoms need to be present to be diagnosed with schizophrenia. The DSM-IV-TR recognizes several different categories of schizophrenics, including paranoid, disorganized and catatonic subtypes.

Pharmacological Treatments for Schizophrenia

Until the early 1950s there were no effective treatments for schizophrenia, leaving no option for people with schizophrenia beyond being permanently institutionalized. In 1952, Henri Laborit was using promethazine, the original phenothiazine, during anesthesia. Later that year, other French researchers studied a second phenothiazine, chlorpromazine, to use in pre-operation patients because of its ability to produce conscious sedation and detachment from external stimuli, known as a neuroleptic state (Julien, 2004). Because of the tranquilizing effect it had on patients, chlorpromazine was classified as the first neuroleptic drug and introduced in 1954 as the first pharmacological treatment for schizophrenia. The term neuroleptic was used to describe what are now known as typical, classic, or first-generation antipsychotic drugs (APDs).

The emergence of chlorpromazine as a treatment for schizophrenia had an enormous effect on the mental health profession as a whole, as patients were able to be maintained on medication for a disorder that did not have any other effective treatments (Thompson, 1977). The treatment of schizophrenia with chlorpromazine made such an impact on the field of mental health that it also facilitated the beginnings of behavioral pharmacology (Thompson, 1977). While typical APDs were very beneficial to the

treatment of schizophrenics, they were only effective at treating the positive symptoms of the disorder, without addressing the negative symptoms or cognitive deficits associated with the disorder.

Following the success of chlorpromazine, several other antipsychotic medications were developed and marketed in the late 1950s and into the 1960s including reserpine, haloperidol, droperidol, loxapine, and molindone (Julien, 2004). These typical antipsychotics have similar mechanisms of action; they all reduce dopamine activity in the brain, primarily with antagonist activity at D_2 receptors (Julien, 2004). Dopamine antagonism contributes to the therapeutic effect of the medications, but it also is responsible for producing extrapyramidal motor side-effects (EPS) (Van Rossum, 1966; Carlsson & Lindqvist, 1963). EPS are debilitating side-effects that resemble the motor impairments found in patients with Parkinson's disease, including tremors and rigidity, involuntary movements, and akathesia (body restlessness). Because these side-effects often accompanied therapeutic doses of typical APDs, they were thought to be necessary components of treatment. As more drugs to treat schizophrenia were assessed, it became clear that it was possible to develop drugs with therapeutic efficacy that are not susceptible to producing EPS (Julien, 2004). The first drug developed that fit this profile was clozapine.

Clozapine

Clozapine was first developed by Wander Laboratories in 1959, as a member of a family of drugs being developed termed "…tricyclic antidepressants but with neuroleptic properties (Hippius, 1999)," but was not applied to the treatment of schizophrenia until the

early 1970s. Clozapine was first reported to have very similar properties to chlorpromazine in animals, and thus not very interesting, but because of some relevant behavioral properties that it displayed it was recommended to be tested in humans: Clozapine did not produce catalepsy similar to chlorpromazine and other antipsychotics, and it also increased the pain tolerance of laboratory animals (Crilly, 2007). Human tests produced favorable results, clozapine was shown to have antipsychotic properties, and it did not cause the undesirable side effects (EPS) that all other antipsychotic drugs produced (Crilly, 2007). Clozapine was introduced as an APD in Europe in 1974, but was soon pulled off the market after a small number of patients died because of complications related to agranulocytosis caused from taking the medication (Julien, 2004). Agranulocytosis is a condition that causes a reduction in circulating white blood cells in the bloodstream, which in turn causes susceptibility to disease and death. Despite this risk, clozapine remained of interest to researchers because of its therapeutic ability in patients with schizophrenia. A 1988 study by Kane and colleagues reported that 30% of treatment resistant schizophrenics showed therapeutic response to the medication, while only 4% of those treated with chlorpromazine responded (Kane, Honigfeld, Singer, & Meltzer, 1988). Because of the ability to treat patients resistant to other drugs, the reduced chance of EPS, and the discovery that the agranulocytosis, which only affects 0.8% of patients taking clozapine (Breier, Tran, Herrea, Tollefson, Bymaster, 2001), is reversible after discontinuation of the medication and can be monitored with regular blood testing, clozapine was reintroduced to the U.S. market in 1990 (Crill, 2007).

Clozapine is considered the first atypical antipsychotic drug (APD), which refers to its ability to treat both positive and negative psychotic symptoms without producing debilitating motor side effects such as EPS (Arnt & Skarsfeldt, 1998; Ellenbroek, 1993). Clozapine has high clinical efficacy, especially in treatment-resistant schizophrenic patients (Kane et al., 1988). Clozapine also has been shown to lower the risk of suicide in people with schizophrenia compared to classical anti-psychotic drugs, which is an important aspect of treatment, considering the increased risk of suicide in people with schizophrenia (Meltzer, 1998, Meltzer, 2001). Because of these reasons, CLZ is viewed as an atypical antipsychotic of great interest. It would be beneficial to develop a drug with similar mechanisms as CLZ, without the possible side-effect of agranulocytosis.

N-desmethylclozapine

Investigators have recently wondered whether or not N-desmethylclozapine (NDMC) contributes to the clinical efficacy of clozapine (CLZ). NDMC is the major active metabolite of CLZ and is found in humans treated with CLZ at plasma levels almost as high as CLZ itself (Flanagan, R., Yusufi, B., & Barnes, T., 2003). Though the metabolism of CLZ to NDMC varies greatly, as studies have cited NDMC recorded at levels between 20 and 150% of the parent compound (CLZ) (Bondesson & Lindstrom, 1988; Perry et al., 1991). Researchers studying the metabolism of CLZ, noted that NDMC is formed by the demethylation of CLZ by CYP3A4 and CYP1A2 isoforms of P450 enzymes (Eiermann et al., 1997; Olesen & Linnet, 2001). Many studies have shown that patients with higher ratios of NDMC to CLZ in the bloodstream show more reduction of both positive and negative symptoms of schizophrenia (Frazier, Cohen, Jacobsen, Grothe, Flood,

Baldessarini, Piscitelli, Kim & Rapoport, 2003; Mauri, Volonterie, Dell'Osso, Regispani, Papa, Baldi, & Bareggie 2003) and is predictive of cognitive improvement and a greater quality of life in patients with schizophrenia, suggesting an important contribution of NDMC to the clinical efficacy of CLZ (Weiner et al., 2004).

NDMC has been found to be active *in vivo* through examining the relationship between acute administration of NDMC and Fos protein expression in the forebrain of rats (Young, Meltzer, & Deutch, 1998). Fos protein expression was increased in the medial prefrontal cortex and nucleus accumbens but not in the dorsal striatum, indicating similar activity to that of its parent drug, CLZ (Young et al., 1998). NDMC has been shown to reverse hyperactivity induced by the dopamine receptor agonist amphetamine and the NMDA receptor antagonist MK-801, which are preclinical models of psychotic behavior (see Lameh, Burstein, Taylor, Weiner, Vanover, & Bonhaus, 2007). Early clinical trials with ACP-104 showed tolerability and safety at doses that showed signs of possible antipsychotic activity (Tamminga, Eamma, Ibraham, Jain, Taylor, Vanover, Hacksell, Brann, & van Kammen, 2006). NDMC (ACP-104) was recently found to be ineffective in treating psychotic symptoms compared to VEH during phase II clinical trials with conducted by ACADIA Pharmaceuticals, suggesting it may not be a likely stand-alone treatment for schizophrenia, but could be used in combination with other APDs (Reuters, 2008).

Like CLZ, NDMC has a diverse pharmacological profile, with affinity for many different receptors/receptor subtypes. The receptor pharmacology of NDMC is similar to that of CLZ in some ways, but quite different in others. These differences in drug-receptor

interactions may contribute to the overall therapeutic efficacy of CLZ. Overall, the contributions of NDMC to the therapeutic effects of CLZ are largely unknown. One important difference in NDMC relative to CLZ is that NDMC is a D_2 partial agonist. D_2 partial agonism has been hypothesized to be beneficial for the treatment of schizophrenia. Partial agonists can also act as agonists at presynaptic D_2 autoreceptors and function as antagonists at postsynaptic receptors (Lameh, et al., 2007). This is hypothesized to reduce dopamine activity in the brain more effectively than an antagonist alone, producing a more therapeutically effective treatment (Lameh et al., 2007).

While many typical and atypical antipsychotic drugs (APDs) are antagonists at D_2 receptors, D_2 partial agonism has been investigated more recently because of the advent of the partial D_2 agonist aripiprazole. Aripiprazole has shown to be effective at treating symptoms of schizophrenia without reduced motor side-effects (Lameh et al., 2007). Aripiprazole displays efficacy in treating both positive and negative symptoms of schizophrenia, as well as being tolerable to patients and safe to use (Potkin et al., 2003). The success of aripiprazole in the treatment of schizophrenia gives more credibility for the role of D_2 partial agonism, and thus NDMC, in treating schizophrenic symptoms.

Binding Affinities of Clozapine and N-desmethylclozapine

Clozapine has a very diverse binding profile, displaying an affinity for many different receptors including serotonergic, histaminergic, adrenergic, muscarinic receptors, opioid and others (Schotte, Janssen, Gommeren, Luyten, VanGompel, Lesage, DeLore, & Leysen, 1996). Some research has shown an important role of the serotonergic and

noradrenergic systems in the therapeutic efficacy of clozapine (Meltzer, Matsubra, & Lee, 1989).

Dopaminergic Receptor Activity

NDMC binds to dopamine receptor subtypes D_2, D_3, and D_4 (Lameh, Burstein, Taylor, Weiner, Vanover, & Bonhaus, 2007; Burstein, Ma, Wong, Gao, Pham, Knapp, Nash, Olsson, Davis, Hacksell, Weiner, & Brann, 2005). CLZ also binds to the aforementioned dopamine receptor subtypes. CLZ and NDMC are both antagonists at D_4 receptors (Lameh et al., 2007), but it is not known if D_4 receptor activation plays a role in the treatment of schizophrenia (Corrigan Gallen, Bonura, & Merchant, 2004). As shown in Table 1, CLZ shows different activity at D_2 and D_3 receptors than NDMC; while it is an antagonist or inverse agonist, NDMC is a partial agonist (Lameh et al., 2007).

Muscarinic Receptor Activity

NDMC has been found to bind to all muscarinic receptor subtypes (Weiner, Meltzer, Veinbergs, Donohue, Spalding, Smith, Mohell, Harvey, Lameh, Nash, Vanover, Olsson, Jayathilake, Lee, Levey, Hacksell, Burstein, Davis, & Brann, 2004). CLZ also binds to all 5 muscarinic receptor subtypes. Although both compounds bind to the receptors, differences in the actions of the drugs have been observed. The most obvious difference is seen at the M_1 receptor subtype. As shown in Table 2, NDMC is an efficacious partial or full agonist at M_1 receptors, while CLZ has been classified as having both antagonist and partial agonist properties at the receptor subtype (Sur, Mallorga, Wittmann, Jacobson, Pascarella, Williams, Brandish, Pettibone, Scolnick, & Conn, 2003; Davies, Compton-Toth, Hufeisen, Meltzer, & Roth, 2005). This partial agonistic action at

Table 1

RSAT assay (receptor selection and amplification technology) measuring agonist, antagonist, and inverse agonist activity of NDMC and CLZ at dopamine receptors

Compound	n-desmethylclozapine	clozapine
	Agonist	Agonist
Receptor	pEC50	pEC50
	%Efficacy	%Efficacy
D_2	7.8 ± 0.5	NA
	32 ± 9%	
D_3	7.3 ± 0.3 (49%)	NA
D_4	NA	NA
	Antagonist	Antagonist
Receptor	p*Ki*	p*Ki*
D_2	7.2 ± 0.1	7.1 ± 0.5
D_3	6.8 ± 0.4	7.1 ± 0.4
D_4	7.2 ± 0.5	8.1 ± 0.2
	Inverse Agonist	Inverse Agonist
Receptor	pIEC50	pIEC50
D_2	NA	7.1 ± 0.5
D_3	NA	7.6 ± 0.3
D_4	NA	NA

Agonist potency is expressed as pEC_{50} ($-\log EC_{50}$) and agonist efficacies were normalized in comparison to the efficacy of the dopamine agonist pergolide. Antagonist activity was measured in the presence of a fixed concentration of pergolide and are expressed as p*Ki* ($-\log Ki$). *Ki* values were determined using antagonist IC_{50} values by correcting for agonist concentration and pEC_{50} using the Cheng-Prusoff equation ($Ki = IC_{50}/[1+(\text{agonist})/EC_{50}\ \text{agonist}]$). Inverse agonist activity is shown as $pIEC_{50}$ ($-\log IEC_{50}$) and show potency of ligand in reversing constitutive activity of receptor without an agonist added. Data presented are means ± standard deviations. NA = no significant agonist activity up to 10 μM or no significant antagonist or inverse agonist activity up to 1 μM. This table is derived from data presented in previous papers (Burstein et al., 2005; Lameh et al., 2007).

Table 2

RSAT assay measuring agonist and antagonist activity of NDMC and CLZ at muscarinic receptors

Compound	n-desmethylclozapine	clozapine
	Agonist	Agonist
Receptor	pEC50	pEC50
	%Efficacy	%Efficacy
M_1	7.3 ± 0.1	7.7 ± 0.4
	72 ± 5%	24 ± 3%
M_2	6.5 ± .02	6.2 ± .01
	106 ± 19%	65 ± 8%
M_3	6.5 ± 0.2	NA
	27 ± 4%	
M_4	6.9 ± 0.2	7.4 ± 0.1
	87 ± 8%	57 ± 5%
M_5	7.6 ± 0.3	NA
	48 ± 6%	
	Antagonist	Antagonist
Receptor	p*Ki*	p*Ki*
M_1	NA	7.8 ± 0.2
M_2	NA	NA
M_3	6.8 ± 0.7	8.2 ± 0.2
M_4	NA	NA
M_5	NA	7.5 + 0.3

Agonist potency is expressed as pEC_{50} ($-\log EC_{50}$) and agonist efficacies were normalized in comparison to the efficacy of the muscarinic agonist carbachol. Antagonist activity was measured in the presence of a fixed concentration of carbachol and are expressed as p*Ki* ($-\log Ki$). *Ki* values were determined using antagonist IC_{50} values by correcting for agonist concentration and pEC_{50} using the Cheng-Prusoff equation ($Ki = IC_{50}/[1+(\text{agonist})/EC_{50}\ \text{agonist}]$). Data presented are means ± standard deviations. NA = no significant agonist activity up to 10 μM or no significant antagonist or inverse agonist activity up to 1 μM. This table is derived from data presented in previous papers (Weiner et al, 2004; Lameh et al., 2007).

the M_1 muscarinic receptor subtype is unique to most known APDs.

Serotonergic Receptor Activity

NDMC and CLZ both have a high affinity for 5-HT_{2A} receptors (Kongsamut, Roehr, Cai, Hartman, Weissensee, Kerman, Tang, & Sandrasagra, 1996). They are both classified as inverse agonists at that receptor subtype, as shown on Table 3. Because 5-HT_{2A} antagonism has been shown to modulate dopamine transmission, it has been hypothesized that 5-HT_{2A} antagonism or inverse agonism may block transmission at both $5HT_{2A}$ receptors and dopamine receptors (Kongsamut et al., 1996; Weiner et al., 2004). NDMC and CLZ both display high affinity for 5-HT_{2C} receptors and are inverse agonists in *in vitro* assays (Lameh et al., 2007). NDMC and CLZ are both partial agonists at 5-HT_{1A} receptors (Newman-Tancredi, Assié, Leduc, Ormiére, Danty & Cosi, 1998), which may offer benefits to the treatment of schizophrenia by reducing EPS, and alleviating negative symptoms (Lameh et al., 2007; Newman-Tancredi et al., 1998). NDMC and CLZ are both high affinity inverse agonists at 5-HT_6 and 5-HT_7 receptors, with similarly moderate potency (Weinter et al., 2004).

Histaminergic Receptor Activity

Both CLZ and NDMC are antagonists at H_1 receptors. This antagonism has been linked to the sedating properties of many APDs along with other effects such as weight gain. Although both CLZ and NDMC bind to H_1 receptors with similar affinity, CLZ displays up to 50 times more intrinsic activity than NDMC at those receptors as shown on Table 4 (Weiner et al., 2004). It is expected that NDMC could show less sedating effects *in vivo* because of this reduced activity at H_1 receptors.

Table 3

RSAT assay measuring agonist, antagonist, and inverse agonist activity of NDMC and CLZ at serotonin receptors

Compound	n-desmethylclozapine	clozapine
	Agonist	Agonist
Receptor	pEC50	pEC50
	%Efficacy	%Efficacy
5-HT_{1A}	6.3 ± 0.2	6.6 ± 0.4
	69 ± 7%	45 ± 10%
	Antagonist	Antagonist
Receptor	p*Ki*	p*Ki*
5-HT_{1A}	NA	NA
5-HT_{2A}	8.1 ± 0.3	8.0 ± .05
5-HT_{2C}	7.7 ± 0.2	7.4 ± 0.2
	Inverse Agonist	Inverse Agonist
Receptor	$pIEC_{50}$	$pIEC_{50}$
5-HT_{2A}	7.7 ± 0.4	7.9 ± 0.3
5-HT_{2C}	7.3 ± 0	6.7 ± 0.1
5-HT_6	6.9 ± 0.1	7.0 ± 0.2
5-HT_7	7.3 + 0.1	7.4 + 0.1

Agonist potency is expressed as pEC_{50} ($-\log EC_{50}$) and agonist efficacies were normalized in comparison to the efficacy of the serotonergic agonist 5-Carboxamidotryptamine. Antagonist activity was measured in the presence of a fixed concentration of 5-Carboxamidotryptamine and are expressed as p*Ki* ($-\log Ki$). *Ki* values were determined using antagonist IC_{50} values by correcting for agonist concentration and pEC_{50} using the Cheng-Prusoff equation ($Ki = IC_{50}/[1+(\text{agonist})/EC_{50}\ \text{agonist}]$). Inverse agonist activity is shown as $pIEC_{50}$ ($-\log IEC_{50}$) and show potency of ligand in reversing constitutive activity of receptor without an agonist added. Data presented are means ± standard deviations. This table is derived from data presented in previous papers (Weiner et al, 2001; Lameh et al., 2007).

Table 4

Activity of NDMC and CLZ at H_1 histamine receptors using RSAT, PI hydrolysis, and Binding Assays

Compound	n-desmethylclozapine	clozapine
Receptor/Assay		
RSAT		
	p*Ki*	p*Ki*
H_1	8.1 ± 0.2	9.8 ± 0.2
PI hydrolysis		
	p*Ki*	p*Ki*
H_1	8.4 ± 0.6	9.7 ± 0.4
Receptor Binding		
	p*Ki*	p*Ki*
H_1	8.3 ± 0.1	8.5 ± 0.2

Antagonist activity was measured in the presence of a fixed concentration of histaminergic agonist histamine and are expressed as p*Ki* (-log*Ki*). *Ki* values were determined using antagonist IC_{50} values by correcting for agonist concentration and pEC_{50} using the Cheng-Prusoff equation ($Ki=IC_{50}/[1+(\text{agonist})/EC_{50}\ \text{agonist}]$). Binding was measured using the radioligand [^{3}H]-pyrilamine. p*Ki* values were calculated using IC_{50} values from binding competition assays by correcting for the dissociation constant (*Kd*) and concentration of the radioligand used in each assay using the Cheng-Prusoff equation. Data are expressed as means ± standard deviations. This table is adapted from data presented in a previous study (Lameh et al., 2007).

Opioid Receptors

NDMC and CLZ show selective delta-opioid agonist activity using *in vitro* assays (Onali & Olianas, 2007). NDMC was found to have full agonist activity at the delta-opioid receptor subtype in human cloned receptors expressed in Chinese hamster ovary cells (Onali & Olianas, 2007). NDMC was reported to have 82% of full agonist efficacy at the delta opioid receptor, while CLZ only showed 33% efficacy (Onali & Olianas, 2007). Opioid receptor involvement in CLZ therapeutic action has not been thoroughly studied. Given that NDMC is a selective agonist at delta opioid receptors, they may be important to the therapeutic action of CLZ.

α-Adrenoreceptors

NDMC antagonist activity at α_{1A} adrenoreceptors creates orthostatic hypotension (Takata, Kurihara, Suzuki, Okubo, & Kato, 1999), which is side effect produced by many APDs. Both NDMC and CLZ have antagonistic properties at the α_{1A} adrenoreceptor (Lameh, 2007), but CLZ shows a five-fold higher affinity than NDMC as shown in Table 5. This lower affinity of NDMC could produce less potential side-effects if used as a treatment without CLZ.

Drug Discrimination

Drug discrimination is a valuable behavioral assay used in pharmacological research based on basic Pavlovian and Skinnerian principles of learning. Subjects are trained to discriminate the internal stimuli associated with a certain drug (training drug) from those associated with vehicle (saline-like or inactive solution). After subjects have been trained in a drug discrimination paradigm, usually using an operant response such as

Table 5

Activity of NDMC and CLZ at α_{1A} and α_{1B} Adrenoreceptors using RSAT, PI hydrolysis, and Binding Assays

Compound	n-desmethylclozapine	clozapine
Receptor/Assay		
RSAT		
	p*Ki*	p*Ki*
α_{1A}	7.2 ± 0.4	8.0 ± 0.2
α_{1B}	7.4 ± 0.3	8.1 ± 0.3
PI hydrolysis		
	p*Ki*	p*Ki*
α_{1A}	7.5 ± 0.4	8.7 ± 0.2
α_{1B}	8.5 ± 0.1	9.3 ± 0.2
Receptor Binding		
	p*Ki*	p*Ki*
α_{1A}	7.3 ± 0.1	8.1 ± 0.1

Antagonist activity was measured in the presence of a fixed concentration of adrenergic agonist phenylephrine and are expressed as p*Ki* (-log*Ki*). *Ki* values were determined using antagonist IC_{50} values by correcting for agonist concentration and pEC_{50} using the Cheng-Prusoff equation ($Ki=IC_{50}/[1+(\text{agonist})/EC_{50}\ \text{agonist}]$). Binding was measured using the radioligand [^{3}H]-prazosin. p*Ki* values were calculated using IC_{50} values from binding competition assays by correcting for the dissociation constant (*Kd*) and concentration of the radioligand used in each assay using the Cheng-Prusoff equation. Data are expressed as means ± standard deviations. This table is adapted from data presented in a previous study (Lameh et al., 2007).

lever pressing, nose-poking, or running a maze, they can recognize the specific interoceptive cues of different drugs and these drugs can serve as discriminative stimuli in drug discrimination paradigms to examine their pharmacological profiles (Overton, 1966; & Harris & Balster, 1971). After the animal has successfully acquired the discriminative stimulus, novel drugs can be assessed as to whether they will substitute for the training drug (Harris and Balster, 1971). Drugs with similar receptor binding profiles or drugs in the same pharmacological classes tend to substitute for each other in drug discrimination generalization tests (Harris & Balster, 1971; Brady & Balster, 1981). In animals trained to discriminate morphine, both heroin and methadone will substitute for discriminative stimulus of morphine (Overton and Batta, 1979).

In order to assess compounds using drug discrimination, they must exert centrally mediated effects. Drugs that do not cross the blood brain barrier are unable to be used as discriminative stimuli (Schuster & Balster, 1977). This concept was demonstrated when Schechter and Rosecrans showed that centrally acting, but not peripherally acting antagonists blocked the discriminative stimulus of nicotine (1971). Centrally administered drugs have also been found to serve as discriminative stimuli, showing the lack of necessity of peripheral activity.

Discriminative Stimulus Properties of Clozapine

Clozapine has been established as a discriminative stimulus in many species of animals including primates, rats, pigeons, and mice (Carey & Bergman, 1997; Goas & Boston, 1978; Wiley & Porter, 1992; Hoenicke, Vanecek, & Woods, 1992; & Philibin, Prus, Pehrson, & Porter, 2005). Clozapine has a diverse binding profile, but has been found

to serve as a reliable discriminative stimulus. Goas and Boston were the first investigators to establish clozapine as a discriminative stimulus in rats; training them to discriminate 6.0 mg/kg CLZ administered orally from vehicle using a two-lever operant procedure (Goas & Boston, 1978). They also trained chlorpromazine (2.0 mg/kg, oral administration) versus vehicle and chlorpromazine (2.0 mg/kg, oral administration) versus clozapine (6.0 mg/kg oral administration) (1978). They found that haloperidol substituted for chlorpromazine, but chlordiazepoxide failed to substitute in chlorpromazine discriminating subjects. None of the drugs assessed (haloperidol, chlorpromazine, chlordiazepoxide, atropine, or a combination of chlorpromazine and benztropine) fully substituted for clozapine in clozapine-trained subjects, suggesting a specificity of the discriminative stimuli of atypical antipsychotic drugs (Goas & Boston, 1978). Rats have also been trained to discriminate 20 mg/kg clozapine by route of intraperitoneal injection (i.p.) from 2.5 mg/kg haloperidol (i.p.) using a T-maze learning procedure (Overton, 1982).

Porter and colleagues established clozapine as a discriminative stimulus in rats using a 1.25 mg/kg dose (i.p.), and found that olanzapine, sertindole, and risperidone all fully substituted, and quetiapine, thioridazine, and haloperidol partially substituted for clozapine (Porter, Varvel, Vann, Philibin, & Wise, 2000). Remoxipride, chlorpromazine, and fluphenazine all failed to substitute reliably for clozapine. Prus and colleagues showed that the atypical APDs quetiapine, ziprasidone, and olanzapine, all substituted for clozapine in rats trained to discriminate clozapine (5.0 mg/kg) from vehicle, while sertindole and risperidone partially substituted for the CLZ discriminative stimulus (Prus, Philibin, Pehrson, Stephens, Cooper, Wise, & Porter, 2005). They also found that the

typical APD thioridazine produced full substitution, while typical APDs haloperidol, fluphenazine, and chlorpromazine failed to substitute. The divergence of results between these two studies shows the importance of training dose in assessing discriminative stimulus substitution.

Another example of training dose importance is related to the muscarinic receptor activity of clozapine. M_1 receptors have been found to be an important component of clozapine's discriminative stimulus (Goudie, Smith, Taylor, Taylor, & Tricklebank, 1998; Kelley & Porter, 1997; Nielsen, 1988; Prus, Baker, & Meltzer, 2004). Cross-generalization of clozapine and scopolamine, a M_1 antagonist, was reported by Kelly and Porter (1997) in rats trained to discriminate 5.0 mg/kg clozapine (i.p.) from vehicle and rats trained to discriminate 0.125 mg/kg (i.p.) scopolamine from vehicle. Goudie and colleagues confirmed that scopolamine fully substitutes for clozapine in 5.0 mg/kg trained rats (Goudie, et al., 1998). In a later study, rats were trained on 1.25 mg/kg clozapine (i.p.) and scopolamine failed to generalize to that discriminative stimulus (Porter et al., 2000). This suggests that rats trained on lower training doses (i.e. 1.25 versus 5.0 mg/kg, i.p.) may be more sensitive to the specific differences in discriminative stimulus properties of typical and atypical APDs and less sensitive to muscarinic cholinergic antagonism substituting for the discriminative stimulus of CLZ (Porter et al., 2000; Goudie & Taylor, 1998).

Serotonergic antagonism has been proposed as one of the underlying mechanisms of atypical APDs including clozapine (Meltzer et al., 1989), however it does not appear to be a necessary component of clozapine's discriminative stimulus in rats as 5-HT_{2A} receptor antagonists ketanserin and M100907 failed to substitute in subjects trained with 5.0 mg/kg

clozapine (i.p.) (Goudie et al., 1998). In rats trained with 0.16 mg/kg (i.p.) MDL100,907, a 5-HT_{2A} receptor antagonist, CLZ fully substituted for the training drug, showing asymmetrical generalization to the training drug (Dekeyne, Iob, & Millan, 2003). Rats trained to discriminate 5.0 mg/kg (i.p.) CLZ from VEH failed to substitute the 5-HT_{1A} receptor agonist, S-14506 and the 5-$HT_{2B/2C}$ receptor antagonist SB 200646A (Goudie et al., 1998). Combined antagonism of both 5-HT_2 and 5-HT_{1C} receptors using cyproheptadine, metergoline, mianserin, pizotifen, and fluperlapine, produced full substitution for the discriminative stimulus in pigeons trained to discriminate 1.0 mg/kg clozapine (i.m.) from saline (Hoenicke, et al., 1992). Pigeons failed to generalize the discriminative stimulus of clozapine to antagonists that were selective for 5-HT_2 over 5-HT_{1C} such as ketanserin, pirenperone, risperidone, and methiothepin (Hoenicke et al., 1992). The data reflected that antagonism of both receptor subtypes was necessary to produce full substitution for clozapine. The data produced in pigeon studies however, were not replicated in rats, as the 5-$HT_{2A/2B/2C}$ receptor antagonist, ritanserin did not substitute for clozapine in rats trained to discriminate 5.0 mg/kg clozapine (i.p.) from vehicle (Wiley and Porter, 1992).

The role of H_1 histaminergic antagonism in the discriminative stimulus of clozapine has also been investigated. The H_1 receptor antagonists, mepyramine, and pyrilamine failed to substitute in subjects trained with 5.0 mg/kg clozapine (i.p.) in rats (Goudie et al., 1998) and pigeons (i.m.) (Hoenicke et al., 1992) respectively. Kelly and Porter found that in rats trained to discriminate 5.0 mg/kg (i.p.) from vehicle, cyproheptadine, a H_1 receptor antagonist, 5-HT receptor antagonist, and M_1 receptor antagonist, fully substituted for the

clozapine discriminative stimulus (1997). Thus, H_1 appears to play a minor role of the discriminative stimulus of clozapine in rats. The discriminative stimulus of clozapine in clozapine trained rats (5.0 mg/kg i.p.) fails to generalize to drugs that display adrenergic antagonism (Kelly and Porter, 1997; Nielsen, 1988), while Goudie and colleagues found partial generalization of an α_1-adrenoreceptor antagonist, prazosin, and partial generalization (defined as 40% or above) of the α_2-adrenoreceptor antagonist, methoxyidazoxan (1998).

Discriminative Stimulus Properties of Clozapine in C57BL/6 mice

Clozapine has been established as a discriminative stimulus in C57BL/6 mice in a limited number of studies (Philibin, Prus, Pehrson, & Porter, 2005; Philibin, Walentiny, Vunck, Prus, Meltzer, & Porter, 2008). Mice trained to discriminate 2.5 mg/kg CLZ (s.c.) fully generalized the discriminative stimulus to olanzapine, risperidone, sertindole, ziprasidone, quetiapine, iloperidone, zotepine, and melperone (Philibin et al., 2005; Philibin et al., 2008). Both drugs displaying partial agonism at the D_2 receptor, aripiprazole and NDMC did not substitute for CLZ. Haloperidol, perphenazine, and fluphenazine all failed to substitute for the CLZ discriminative stimulus, while thioridazine and chlorpromazine did fully substitute for the discriminative stimulus showing some selectivity for atypical APD substitution over typical APD substitution (Philibin et al., 2008; Philibin et al., 2005). Chlorpromazine and thioridazine both have strong affinities for muscarinic and serotonergic receptors as compared to the other typical APDs examined which may indicate that those receptors play an important role in the discriminative stimulus of CLZ (Schotte et al., 1996). Selective ligand generalization tests produced

different results than previously obtained in other species. Ritanserin, a $5HT_{2A/2B/2C}$ antagonist and MDL 100,907, a 5-HT_{2A} antagonist, and prazosin, a α_1 antagonist, produced full substitution for the CLZ discriminative stimulus (Philibin et al., 2008). Scopolamine, the muscarinic antagonist produced partial substitution to CLZ, while amphetamine and fluoxetine did not substitute (Philibin et al., 2008).

Discriminative Stimulus Properties of N-desmethylclozapine

The discriminative stimulus properties of NDMC have not been established using NDMC as a training drug prior to this study, although other investigators have assessed NDMC in terms of generalization to other APDs (Prus, Pehrson, Philibin, Wood, Vunck, & Porter, 2008; Prus, Philibin, Pehrson, & Porter, 2006). Rats trained to discriminate 1.25 mg/kg CLZ from 5.0 mg/kg (i.p.) CLZ from VEH in a three-choice operant drug discrimination task failed to substitute NDMC for either training dose at doses of NDMC up to 8.0 mg/kg (i.p.), however the authors noted that 8.0 mg/kg NDMC failed to produce a significant decrease in response rate (Prus et al., 2006). Rats trained to discriminate 1.25 mg/kg CLZ (i.p.) failed to generalize NDMC to clozapine at doses up to 20 mg/kg (i.p.), although doses of 5.0 and 10.0 mg/kg NDMC did substitute when administered in combination with a small dose (.3125 mg/kg) of clozapine (Prus et al., 2008). Trihexyphenidyl, an M_1 receptor antagonist, failed to substitute for clozapine at any dose, but when combined with 5.0 mg/kg of NDMC, they produced partial substitution for clozapine, although response rate was significantly suppressed (Prus et al., 2008). These studies suggest that there may be a role of M_1 receptor antagonism mediating the discriminative stimulus properties of CLZ. It also suggests that NDMC may have a similar

binding profile to CLZ when combined with an M_1 antagonist, which could yield novel compounds to treat schizophrenia. Philibin and colleagues (2008) had similar findings in C57BL/6 mice trained to discriminate 2.5 mg/kg (s.c.) CLZ from VEH, showing that NDMC did not substitute for CLZ at doses up to 10.0 mg/kg, at which significant reduction of response rate was observed. When NDMC was combined with a .625 mg/kg (s.c.) dose of CLZ it produced full substitution at the 10.0 mg/kg (s.c.) dose of NDMC, however it was accompanied by a significant decrease in response rates (Philibin et al., 2008).

Development of Cross-Tolerance to Clozapine

APDs have been shown to exert their full therapeutic effect only after chronic dosing of the drug (Kuhar and Joyce, 2003), furthermore APDs have differential effects during repeated dosing versus acute administration (Varvel, Vann, Wise, Philibin, & Porter, 2002) thus it is important that we assess the discriminative stimulus properties of clozapine before and after repeated administration of itself. Goudie and colleagues found that repeated administration of clozapine induced tolerance to its discriminative stimulus effects in rats, and that the tolerance was reversed after a period of drug abstinence, suggesting the cellular changes were pharmacodynamic (Goudie, Cooper, Cole, & Sumnall, 2007). After showing these pharmacodynamic changes following repeated clozapine administration, cyproheptadine was repeatedly administered to clozapine trained discrimination animals, to determine whether or not development of cross-tolerance to the clozapine discriminative stimulus would occur (Goudie et al., 2007). Cyproheptadine is an anti-allergy medication that interestingly has a similar binding profile to clozapine and

substitutes fully for clozapine without suppressing responding (Goudie et al., 2007). Repeated administration of cyproheptadine produced tolerance to the clozapine discriminative stimulus, suggesting similar pharmacodynamic changes as those produced by repeated administration of clozapine. In a separate study by Goudie and colleagues, rats trained to discriminate 5.0 mg/kg CLZ (i.p.) from VEH were then administered either JL13 or olanzapine for ten consecutive days following completion of an initial dose-effect curve. to assess development of cross-tolerance to the clozapine discriminative stimulus (Goudie Cole, & Sumnall, 2007). Both olanzapine and JL13 were found to substitute for CLZ at doses of 2.5 and 10.0 mg/kg respectively (Goudie et al., 2007). After treatment with olanzapine or JL13, there was a significant induction of cross-tolerance to the CLZ discriminative stimulus (Goudie et al., 2007), which was completely lost following 16 days of no drug administration, suggesting pharmacodynamic modulation of receptors mediating the CLZ discriminative stimulus. This development of cross-tolerance may be an indicator of pharmacologically specific receptor modulation, which may be an indicator of a therapeutically similar compound to CLZ following repeated administration.

Rationale

Although the CLZ discriminative stimulus failed to generalize to NDMC when NDMC was administered alone, it is important to assess whether or not NDMC can be trained as a discriminative stimulus, and if so, if CLZ will substitute for it (Prus et al., 2006; Prus et al., 2008). Understanding the discriminative stimuli of both NDMC and CLZ is important for understanding receptor mechanisms responsible for CLZs therapeutic effects, because NDMC is the major active metabolite of CLZ, and may be important for treatment efficacy. It is also important to assess whether or not mice trained to discriminate 10.0 mg/kg NDMC (s.c.) can differentiate between typical and atypical APDs, which is why haloperidol will also be tested for substitution.

Goudie and colleagues pointed out a major limitation of drug discrimination in the fact that it typically assesses only acute drug effects, rather than those associated with chronic administration of a drug as most APDs are used therapeutically (Goudie et al., 2007). We may be able to develop better *in vivo* assays to assess the effect of chronic drug administration. Goudie and colleagues have designed a way to study the tolerance inducing effects of different compounds on the discriminative stimulus of clozapine in rats (Goudie et al., 2007; Goudie et al., 2007). This present study will also assess the ability of NDMC to produce tolerance to the discriminative stimulus of 2.5 mg/kg CLZ in C57BL/6 mice, and whether or not those changes are pharmacodynamic in nature. Tolerance would be indicated by parallel shifts to the right in the dose-response curve for CLZ. Generation of reversible tolerance to the discriminative stimulus of CLZ would indicate that NDMC

produces pharmacodynamic changes similar to that of its parent drug, CLZ, and would suggest that similar receptor mediated changes are produced by the two compounds.

Methods

Experiment 1 NDMC Discrimination

Subjects Experiment 1

Twelve experimentally naïve, adult male C57/BL6NHsd-wild type mice (20-25g) obtained from Harlan Laboratories (Indianapolis, IN) were housed individually in clear plastic cages (Philibin, Prus, Pehrson, & Porter) with steel wire fitted tops and wood chip bedding (sanichips, Teklad, Madison, WI). Mice were transported daily (5-7 days per week) from the vivarium (12 hour light-dark cycle, lights on at 6 a.m.) to the laboratory where experimental training and testing sessions occurred. The vivarium temperature remained between 22 and 24 degrees Celsius. After one week of acclimation, the subjects were food deprived to 85-90% of their free feeding body weights and were maintained on a food restricted diet of standard rodent chow (Harlan Teklad Lab Diets, Teklad LM-485). Water was available *ad libitum* in the home cages. The *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy Press, 1996) was followed and the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU) approved the procedures that were used in the present study (IACUC Protocol AM 10284).

Drugs Experiment 1

N-desmethylclozapine (NDMC; provided by Herbert Y. Meltzer), Clozapine (CLZ; atypical antipsychotic drug; gift from Novartis, Hanover, NJ), haloperidol (HAL; typical antipsychotic drug; Sigma-Aldrich Chemical Company, St. Louis, MO) were dissolved in distilled water and a small quantity (approximately two drops per 50 ml) of 85% lactic acid.

All drugs were administered subcutaneously (Philibin et al.) with a volume of 10 ml/kg body weight with a 30 min pre-session injection time.

Apparatus Experiment 1

Testing was conducted in six standard computer-interfaced operant conditioning chambers (Model ENV-307A, Med Associates Inc., St. Albans, VT) each containing two retractable levers in the left and right positions (8 cm apart) on the front panel of the operant chamber. The levers extended 0.8 cm into the chamber and were positioned 2.5 cm above a grid floor constructed of parallel stainless steel rods. Centered between them was a recessed food trough into which a liquid dipper delivered 0.02 ml of sweetened-milk (by volume: 150 ml powdered milk, 150 ml sugar, and 500 ml water). The inner test chambers consisted of a 15 cm L X 11.5 cm D X 17.5 cm H area surrounded by an aluminum framed box with a single Plexiglas side door. Test chambers were housed in sound attenuating chambers equipped with ventilation fans. MED-PC software (Version 1.17, Med Associates Inc.) was used to control the operant sessions and record data.

Training Procedures

Magazine Training Experiment 1

Before beginning magazine training mice were exposed to sweetened milk in small bottle caps for 30 minutes in the home cage once a day for 2 days. Mice were magazine trained for 2 sessions. During the 15 minute magazine training sessions, no levers were available in the operant test chamber. Sweetened milk was delivered noncontingently on a fixed time 5 second (FT 5 sec) delivery schedule (i.e., a single presentation of sweetened milk was

delivered by raising the dipper cup every 5 seconds and holding in the up position for 3 seconds before filling the dipper again).

Lever-Press Training Experiment 1

Lever-press training began upon completion of magazine training. A single lever (the vehicle-paired lever) was extended inside the chamber. Each subject was placed in the operant chamber and trained to press the levers for 0.02 ml of sweetened milk on a fixed ratio one (FR1) schedule of reinforcement, in which the reinforcer was delivered after every lever press(dipper was available for 3 sec.). Subjects were trained to lever press on a single lever (i.e. the VEH-paired lever) until drug administration began. The position of the drug-associated lever (left vs. right) was counterbalanced between the groups of subjects to control for olfactory cues (Extance and Goudie 1981). The value of the FR was gradually increased over the next 6 sessions until FR10 was obtained. After response rates were consistently higher than 10 responses per minute drug discrimination training began.

NDMC Discrimination Training Experiment 1

Subjects were then injected daily with a vehicle solution (VEH) 30 min prior to each training session. The VEH-associated lever was extended in the test chamber and responding was reinforced according to an FR 10 schedule. After response rates were consistently above 10 responses per minute (RPM), subjects were administered 2.5 mg/kg NDMC injections 30 min prior to training sessions and were only presented with the NDMC-associated lever (opposite of the VEH-associated lever). Once response rates stabilized at over 10 responses per minute, two-lever training began. During two-lever training sessions both levers were extended in the operant chamber. The subjects were administered NDMC or VEH injections

according to a double alternation sequence (i.e., DDVVDDVV). On days when NDMC was administered, only responding on the NDMC associated lever was reinforced. On days when vehicle was administered, only responding on the VEH associated lever was reinforced. Responses on the incorrect lever reset the ratio requirement on the correct lever. Subjects received two-lever discrimination training until the training criteria were passed during 5 of 6 consecutive sessions.

NDMC Discrimination Training Criteria Experiment 1

Successful discrimination training was evaluated and assessed according to three criteria: (1) the first completed first fixed ratio (FFR) of the schedule (FR10) was executed on the appropriate lever (2) 80% or greater of the total responses made during the session occurred on the appropriate lever and (3) response rate for the session equaled or exceeded 10 RPM. Control vehicle and NDMC tests were administered and passed prior to generalization testing with all drugs. During control and test sessions, responses on both levers were reinforced according to the FR10 schedule and the FR requirement was reset when switching between levers occurred. The three training criteria also had to be met during the two consecutive training sessions immediately prior to all test sessions. Subjects were initially required to meet criteria 5 of 6 consecutive training sessions before beginning testing. In addition to these restrictions subjects were not tested unless criteria were met in the previous two training days. The two training days immediately preceding testing also had to include both a NDMC and a VEH day (i.e. either VD or DV passed immediately prior to test day).

NDMC Training Dose Adjustment Experiment 1

After 57 training sessions, 5 of 12 subjects met the training criteria with the 2.5 mg/kg training dose in a mean of 44.6 double-lever sessions (range = 17 - 57 sessions). Response rates and overall response percentage were generally acceptable; however the FFR requirement was usually the limiting factor. Because only 5 of 12 subjects met criteria, the training dose was increased to 5.0 mg/kg. Nine of twelve subjects obtained the discriminative stimulus of 5.0 mg/kg NDMC. Two subjects failed to meet training criteria after 57 sessions and were removed from the study; one subject was removed to begin 10.0 mg/kg discrimination training.

After completing the NDMC dose effect curve (0.625 - 10.0 mg/kg), it was apparent that subjects were failing to reliably generalize a training dose of NDMC to itself or a 10.0 mg/kg dose. A visual analysis of individual data revealed that test performance was dependent upon the training condition immediately prior to testing. When subjects were trained with NDMC prior to testing they responded appropriately, but when they were trained with VEH immediately prior to testing they responded more on the VEH appropriate lever. Subjects were then retested with the opposite condition on the day prior to testing from their first test with the 3 highest doses of the NDMC curve (2.5, 5.0 and 10.0 mg/kg) to compare the effect of previous training sessions within subjects. After observing this dependency upon previous test session, the training dose was increased to 10 mg/kg. The training criteria were also changed, requiring the mice to meet the training criteria for 7 of 8 consecutive training sessions before beginning testing.

NDMC Discrimination Testing Procedures Experiment 1

Subjects were required to pass the training criteria (5 of 6 or 7 of 8 appropriate training sessions) and pass both a NDMC and CLZ control test before beginning dose effect or generalization curves. Generalization or substitution testing occurred with a minimum of 2 days between tests and only when the subject passed the day immediately prior to testing. During 10.0 mg/kg training this criterion was changed and subjects were required to pass both a VEH and NDMC training session in the two training sessions prior to testing to assure the test subject was under stimulus control. After successful completion of vehicle and NDMC control tests, a generalization dose effect curve was determined for NDMC (1.25 – 20.0 mg/kg for 10.0 mg/kg trained mice). Substitution tests were then conducted with the atypical APD CLZ and the typical APD HAL. Control tests were performed between each new test drug with the training drug and vehicle to assess NDMC discriminative stimulus control.

Data Analysis Experiment 1

Med Associates Inc. software was used for all data recording during the experiments. During training and testing sessions, percent-drug lever responding (%DLR) was calculated by dividing the number of condition-appropriate responses (i.e. either NDMC or VEH lever) by the total number of responses made during the session and multiplying by 100. Responses per minute (RPM) was calculated by dividing the total responses made on both levers by 15 minutes. ED_{50} values (using 95% confidence intervals, C.I.) will be calculated for drugs that substitute for the training drug in %DLR curves (drugs with mean %DLR $\geq$ 80% are considered to fully substitute for training drug, drugs with mean %DLR between 60 and 80 are considered to partially substitute for training drug) using the least squares method of linear

regression, analyzing the linear portion of the dose effect curve. The %DLR was excluded from analysis for any mice if their response rate was less than 2 RPM, or if they failed to complete a FFR. All response rate data were included for data analysis. Repeated measures analysis of variance (ANOVA) was used to compare RPM for each dose of a given drug (GB-STAT software; Dynamic Microsystems, Inc., Silver Spring, MD). Significant ANOVA results ($p < 0.05$) will be investigated using Newman-Keuls post hoc tests. In Experiment 2, ED_{50} values and confidence intervals were examined to determine if ED_{50} values were significantly different from each other and to assess development of tolerance to CLZs discriminative stimulus.

Experiment 2 CLZ Discrimination: Evaluation of Cross-tolerance Induction Using NDMC

Subjects Experiment 2

Twelve experimentally naïve, adult male C57/BL6NHsd-wild type mice (20-25g) obtained from Harlan Laboratories (Indianapolis, IN) were housed and kept in an identical manner to those in Experiment 1.

Drugs Experiment 2

N-desmethylclozapine (NDMC; provided by Herbert Y. Meltzer) and Clozapine (CLZ; atypical antipsychotic drug; gift from Novartis, Hanover, NJ) were dissolved in distilled water and a small quantity (approximately two drops per 50 ml) of 85% lactic acid, sodium hydroxide was added to buffer the solution to near ph 7. All drugs were administered subcutaneously (Philibin et al.) at a volume of 10 ml/kg body weight with a 30 min pre-session injection time.

Apparatus Experiment 2

Six two-lever Med Associates mouse operant chambers were used exactly as described in Experiment 1.

Training Procedures Experiment 2

Magazine and lever press training were conducted as in Experiment 1. Drug discrimination training conditions were held constant except that subjects were only exposed to one training dose (10.0 mg/kg) of NDMC. After Experiment 1, it became clear that NDMC's discriminative stimulus may not be very robust so the training criteria were kept at 7 of 8 consecutive sessions passed. The three training appropriate criteria were kept the same as in Experiment 1 as during the 10.0 mg/kg training.

Twelve subjects began 10.0 mg/kg discrimination training, but one subject was removed from the study after becoming ill. After 47 training sessions, only 3 of 11 remaining subjects had met criteria for 7 of 8 consecutive sessions. While three subjects acquired the training criteria in a mean of 23.3 sessions with a range of 17to 30 sessions (SEM 3.76), eight of eleven subjects failed to establish 10.0 mg/kg NDMC as a discriminative stimulus after 48 double lever training sessions. Training was suspended and all subjects were given free access to food and water for 14 days.

Clozapine Discrimination & Assessment of Cross-tolerance induced by N-desmethylclozapine

New free-feeding weights were calculated for subjects, though if there new weight was lower, the original free-feeding weight was retained; subjects were reduced to 85-90% of their newly calculated free-feeding weights over a period of 7 days. Subjects then began discrimination training with 2.5 mg/kg CLZ training dose. The training procedures were

identical to those in Experiment 1 when 10.0 mg/kg was used for the training dose except for the training drug and dose used, and the training was conducted 7 days a week.

Clozapine Dose Effect Curve 1 (DEC 1) Experiment 2

After establishing CLZ as a discriminative stimulus, using procedures similar to those in Experiment 1 (i.e. 7 of 8 sessions appropriate, NDMC and CLZ control tests passed), subjects were trained for two sessions (VEH NDMC or NDMC VEH) and then a clozapine dose-effect curve was generated including doses of 0.3125, 0.625, 1.25, 2.5, and 5.0 mg/kg. Subjects were randomly assigned to complete the CLZ dose effect curve in either ascending or descending order of test doses. Each test session were separated by two training sessions (VEH, NDMC or NDMC VEH), and was not contingent upon training performance (Goudie et al., 2007 & Goudie et al., 2007). Thus, each subject will complete the CLZ dose effect curve in 15 sessions after passing both of their initial VEH and NDMC control tests. Upon completion of the CLZ dose-response curve (DEC 1), discrimination training was suspended and subjects were administered 10.0 mg/kg NDMC, twice daily (9:00 am and 6:00 pm) for 10 consecutive days (Goudie et al., 2006; & Goudie et al., 2007).

Clozapine Dose Effect Curve 2 (DEC 2) Experiment 2

Following ten days of NDMC administration mice were tested with three different doses (0.3125, 1.25 and 2.5 mg/kg) of CLZ over three consecutive days, producing a new dose effect curve. These tests were conducted on three consecutive days to prevent the loss of tolerance that could occur if there was training days in between tests (Goudie et al., 2006 & Goudie et al., 2007). Subjects were randomly assigned to complete dose-effect curves in ascending or descending order of test doses. Following completion of the second dose effect

curve, the mice will be given 16 days of drug abstinence to allow drug induced cellular changes to return to normal functioning (Goudie et al., 2007 & Goudie et al., 2007).

Clozapine Dose Effect Curve 3 (DEC 3) Experiment 2

Following the 10 days of no drug administration or training, mice were trained for two days, counterbalanced between two conditions (i.e. either VEH NDMC or NDMC VEH) before generating a CLZ dose effect curve using the same methods as in DEC 1. Each test will be separated by 2 training days. Subjects were assigned to complete dose-effect curves in ascending or descending order of test doses. Following completion of DEC 3, subjects were given 2 training days followed by testing 10.0 mg/kg CLZ, as this dose was added during DEC 2. After completion of the 10.0 mg/kg CLZ test, subjects completed both VEH and CLZ control points with two training days (including VEH and CLZ) before each test.

Data Analysis Experiment 2

Med Associates Inc. software was used for all data recording during the experiments. During training and testing sessions, percent-drug lever responding (%DLR) was calculated by dividing the number of condition-appropriate responses (i.e. either NDMC or VEH lever) by the total number of responses made during the session and multiplying by 100. Responses per minute (RPM) was calculated by dividing the total responses made on both levers by 15 minutes. ED_{50} values (using 95% confidence intervals, C.I.) will be calculated for drugs that substitute for the training drug in %DLR curves (drugs with mean %DLR $\geq$ 80% are considered to fully substitute for training drug, drugs with mean %DLR between 60 and 80 are considered to partially substitute for training drug) using the least squares method of linear regression, analyzing the linear portion of the dose effect curve. The %DLR was excluded

from analysis for any mice if their response rate was less than 2 RPM, or if they failed to complete a FFR. All response rate data were included for data analysis. Repeated measures analysis of variance (ANOVA) was used to compare RPM for each dose of a given drug (GB-STAT software; Dynamic Microsystems, Inc., Silver Spring, MD). Significant ANOVA results ($p < 0.05$) will be investigated using Newman-Keuls post hoc tests. In Experiment 2, ED_{50} values and confidence intervals were examined to determine if ED_{50} values were significantly different from each other and to assess development of tolerance to CLZs discriminative stimulus. GraphPad Prism 5 software was used to create figures as well as calculate regression lines and evaluate differences between dose effect curves.

Results

Experiment 1

2.5 And 5.0 mg/kg NDMC Acquisition

The mice began discrimination training using a 2.5 mg/kg training dose of NDMC; however, only 5 of 12 mice passed the training criteria (5 of 6 sessions) after 57 double-lever training sessions (Fig. 1) in a mean of 44.6 sessions (SEM 8.43) with a range 17-57 sessions. The training dose was increased to 5.0 mg/kg NDMC, and 9 subjects passed training criteria (5 of 6 sessions) in a mean of 15.7 sessions (SEM 2.73) with a range of 6 to 29 sessions (Fig. 2). Two mice were removed from the study after failing to pass criteria following 57 double-lever training sessions and the training dose was increased to 10.0 mg/kg for another mouse after 60 sessions of 5.0 mg/kg discrimination training.

5.0 mg/kg NDMC Generalization

These 9 mice completed a dose-effect curve including 0.625, 1.25, 1.77, 2.5, 5.0, and 10.0 mg/kg doses of NDMC. The generalization data produced by 5.0 mg/kg NDMC-trained mice revealed that none of the doses tested produced full substitution for 5.0 mg/kg NDMC including the training dose of 5.0 mg/kg (50.49% DLR) and 10.0 mg/kg (75.13% DLR), indicating a loss of stimulus control (Fig. 3). Visual inspection of the data suggested that the subjects' lever choice on test days was being influenced by the training condition on the day immediately prior to testing. To verify this, subjects were tested with doses of 2.5, 5.0, and 10.0 mg/kg NDMC with either VEH or NDMC training sessions immediately prior to testing. The subjects were tested with the opposite condition the day before testing to which they had been previously tested for these three doses. These data were then

plotted based on the training condition (either NDMC or VEH) of the day prior to testing. When a VEH training day preceded testing, the mice did not show full substitution for 2.5 mg/kg (35.59% DLR), 5.0 mg/kg (41.33% DLR), or 10.0 mg/kg (67.07% DLR) doses of NDMC (Fig. 4). In contrast, when a NDMC training session preceded testing (Fig. 5), doses of 2.5 mg/kg (93.03% DLR), 5.0 mg/kg (84.22% DLR), and 10.0 mg/kg (95.01% DLR) all produced full substitution for the training dose of 5.0 mg/kg NDMC. Thus, these results demonstrated that the 5.0 mg/kg dose of NDMC was not able to maintain a stable discriminative stimulus.

10.0 mg/kg NDMC Acquisition

The 9 mice (plus the subject that failed to complete 5.0 mg/kg discrimination training) began 10.0 mg/kg NDMC discrimination training after completing the 5.0 mg/kg NDMC generalization curve. Ten subjects passed the 10.0 mg/kg NDMC discriminative stimulus training criteria (7 of 8 sessions of criteria met) in a mean of 27.5 sessions (SEM 4.24) with a range of 14 to 58 sessions (Fig. 6).

10.0 mg/kg NDMC Generalization

NDMC was tested for substitution with doses 1.25, 2.5, 5.0, 7.1, 10.0, and 20.0 mg/kg. Doses of 10.0 mg/kg (83.95% DLR) and 20.0 mg/kg (86.92% DLR) NDMC fully substituted for the training dose of 10.0 mg/kg NDMC (Fig. 7), while no other doses substituted. Response rates were found to be significantly different ($F_{7, 63} = 2.94$, $p < .05$), however, Newman-Keuls post-hoc tests revealed there were no significant response rate differences as compared to VEH control rate for any dose tested. The NDMC ED_{50} = 6.29 mg/kg (95% C.I. = 4.72- 8.40 mg/kg). The 10.0 mg/kg NDMC generalization curve was

completed by eight subjects of the 10 that were trained. One subject failed to complete the curve before becoming ill and had to be removed from the study, so the animal's data were omitted; one subject failed to pass testing criteria and was removed from the study. After completing the generalization curve, the data were separated by the training condition (either NDMC or VEH) that preceded testing, and subjects were tested with doses of 10.0 mg/kg or 20.0 mg/kg NDMC with the opposite training condition given prior to testing. When subjects were trained with VEH prior to testing 10.0 mg/kg (73.16% DLR) produced partial substitution and 20.0 mg/kg (98.23% DLR) produced full substitution for 10.0 mg/kg NDMC (Fig. 8). When subjects were trained with NDMC prior to testing 10.0 mg/kg (62.66% DLR) produced partial substitution and 20.0 mg/kg (84.26% DLR) produced full substitution for 10.0 mg/kg NDMC (Fig. 9).

Clozapine Substitution Tests

Clozapine was tested for substitution with doses 0.15625, 0.625, 1.25, 2.5, and 5.0 mg/kg (Fig. 10). Six subjects completed the CLZ generalization curve; two of eight subjects included in the 10.0 mg/kg generalization curve became ill and were removed from the study before beginning the CLZ generalization curve. Clozapine produced full substitution at doses of 2.5 mg/kg (86.79% DLR) mg/kg and 5.0 mg/kg (94.82% DLR), however only 4 of 6 subjects responded at the 5.0 mg/kg dose of CLZ. Response rates were found to be significantly different ($F_{6, 41} = 2.51$, $p < 0.05$) from VEH control response rates, however a Newman-Keuls post-hoc tests revealed that there were no significant differences from VEH control response rate at any dose tested. Clozapine's ED_{50} = 0.94 mg/kg (95% C.I. = 0.61 – 1.46 mg/kg).

Haloperidol Substitution Tests

Haloperidol was tested for NDMC substitution with doses of 0.025, 0.05, 0.1, 0.2, and 0.4 mg/kg (Fig. 11). Haloperidol did not produce substitution for 10.0 mg/kg NDMC at any dose tested, with the highest mean drug lever percentage being 39.97% DLR when administered 0.05 mg/kg haloperidol. Response rates were significantly reduced ($F_{6, 34} = 37.07$, $p < 0.01$) as compared to VEH control response rates at all doses tested.

Experiment 2

10.0 mg/kg NDMC Acquisition

Twelve subjects began 10.0 mg/kg NDMC discrimination training. One mouse became ill during training and was removed from the study. Following 47 training sessions, only 3 of 11 remaining subjects acquired the discriminative stimulus of 10.0 mg/kg NDMC in a mean of 23.3 sessions with a range of 17-30 (SEM 3.76), while 8 of 11 subjects failed to pass training criteria. Subjects were given a 21 day drug washout period before beginning 2.5 mg/kg clozapine discrimination training.

2.5 mg/kg Clozapine Acquisition

After the training drug was changed to 2.5 mg/kg clozapine, the mice (N = 11) passed training criteria (7 of 8 days passed criteria) in a mean of 20.0 (SEM 2.71) double-lever training sessions with a range of 8 to 33 sessions (Fig. 12). One subject was removed from the study for failing to pass training criteria after 48 training sessions. Following 2.5 mg/kg clozapine acquisition, the mice completed VEH and CLZ control points and a CLZ generalization curve.

Clozapine Dose Effect Curve 1 (DEC 1)

Ten subjects began testing with CLZ doses of 0.3125, 0.625, 1.25, 2.5, and 5.0 mg/kg following completion of acceptable VEH and CLZ control points (≥ 80% DLR and 10.0 RPM). The dose response curve was completed with two training days (1 CLZ 1 VEH) in a counterbalanced order between each test dose and between subjects; the mice were randomly assigned to complete dose tests in an ascending or descending order. The clozapine dose of 1.25 mg/kg produced partial substitution (67.42% DLR), while 2.5 mg/kg (96.75% DLR) and 5.0 mg/kg (99.96% DLR) produced full substitution for 2.5 mg/kg CLZ (Fig. 13). Only 5 of 10 ten subjects responded at the 5.0 mg/kg dose of CLZ, and response rates were significantly ($F_{6, 69} = 11.80$, $p < 0.01$) different from VEH control response rates at that dose as revealed by a Newman-Keuls post-hoc test. The other tested doses of 0.3125 mg/kg (29.85% DLR), 0.625 mg/kg (49.26% DLR) did not produce substitution for 2.5 mg/kg CLZ. The clozapine ED_{50} = 0.63 mg/kg (95% C.I. = 0.49 – 0.80 mg/kg) for Dose Effect Curve 1.

Clozapine Dose Effect Curve 2 (DEC 2)

The mice began DEC 2 following 10 consecutive days of 10.0 mg/kg NDMC injections twice daily (9 AM and 6 PM). DEC 2 was completed without any training days in between tests, and subjects were randomly assigned to test doses in ascending or descending order. Subjects tested doses of 0.625, 1.25, 2.5, 5.0, and 10.0 mg/kg in 5 consecutive days (Fig. 14). The 10.0 mg/kg dose of CLZ fully substituted (86.96% DLR) for the 2.5 mg/kg CLZ discriminative stimulus, however only 5 of 10 subjects responded when administered 10.0 mg/kg, and response rates (mean = 13.36, SEM = 4.59) were

significantly ($F_{4, 49} = 12.35$, $p < 0.01$) lower than response rates for all other doses tested. The CLZ doses of 0.625 mg/kg (28.25% DLR), 1.25 mg/kg (31.52% DLR), 2.5 mg/kg (70.02% DLR), and 5.0 mg/kg (72.85% DLR) failed to fully substitute, however 2.5 mg/kg and 5.0 mg/kg NDMC produced partial substitution. The CLZ ED_{50} = 1.89 mg/kg (95% C.I. = 1.21 – 2.96 mg/kg) for DEC 2. Thus, there was a significant (p <0.05) right-ward shift for the CLZ DEC 2 as compared to DEC 1 as there was no overlap in the confidence intervals. Linear regression analysis revealed that the slopes of DEC 1 and DEC 2 were not significantly different ($F_{1, 6} = 0.63$, $p = 0.46$); however, the y-intercepts of DEC 1 and DEC 2 were significantly different ($F_{1, 7} = 27.75$, $p < 0.01$) from each other (Fig. 15).

Clozapine Dose Effect Curve 3 (DEC 3)

The mice began DEC 3 following 10 days of suspended training and no drug administration. DEC 3 was completed exactly in the same manner as DEC 1, and individual subjects were tested in the same dose order (ascending or descending) as they were in DEC 1. As shown in Fig. 16, subjects were tested with doses of 0.3125, 0.625, 1.25, 2.5, and 5.0 mg/kg, and all subjects were tested with 10.0 mg/kg CLZ upon completion of the other doses. Doses of 2.5 mg/kg (98.04% DLR) and 5.0 mg/kg (98.45% DLR) fully substituted for the discriminative stimulus associated with 2.5 mg/kg CLZ. None of the other tested doses substituted for 2.5 mg/kg CLZ, with the highest DLR reported at 47.47 % at the 1.25 mg/kg dose. The CLZ ED_{50} = 0.77 mg/kg (95% C.I. = 0.52 – 1.13 mg/kg) for DEC 3; thus, there was a significant left-ward shift for the DEC 3 curve as compared to DEC 2 and there was no overlap of the confidence intervals. Linear regression analysis revealed that the slopes of DEC 2 and DEC 3 were not significantly

different ($F_{1, 6} = 0.54$, $p = 0.49$) from each other; however, the y-intercepts of DEC 2 and DEC 3 were significantly different ($F_{1, 7} = 9.75$, $p < 0.05$) from each other (Fig. 17). DEC 3 and DEC 1 were not significantly different from each other (Fig. 18) as ED_{50} values and 95% confidence intervals for DEC 3 and DEC overlapped. Linear regression analysis revealed that the slopes of DEC 1 and DEC 3 were not significantly different ($F_{1, 6} = 0.03$, $p = 0.86$) from each other; the y-intercepts of DEC 1 and DEC 3 were also not significantly different ($F_{1, 7} = 0.82$, $p = 0.40$) from each other. The three dose-effect curves (DEC 1, DEC 2, and DEC 3) are displayed in Fig. 19, showing the right-ward shift following NDMC administration during DEC 2, and the left-ward shift of the DEC 3 curve following the 10 days of drug washout.

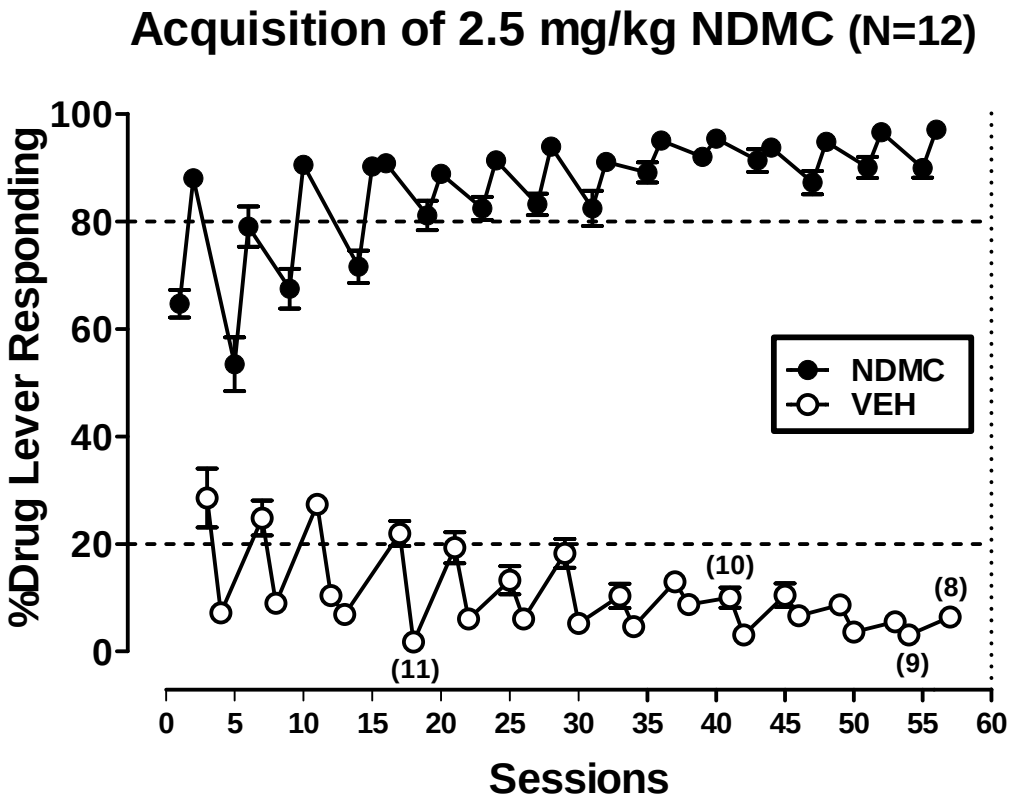

Figure 1. Acquisition of 2.5 mg/kg N-desmethylclozapine Discrimination

Figure 1. Acquisition of two-lever 2.5 mg/kg discrimination training criteria is shown. The mean percentage drug lever responding (± SEM) is shown for NDMC (*filled circles*) and VEH (*open circles*) two-lever training sessions. The area above the *dashed line* at 80% indicates group mean 2.5 mg/kg NDMC appropriate responding. As subjects passed training criteria (5 of 6 sessions) they were removed from the acquisition curve, which was indicated by decreasing N, shown in parenthesis.

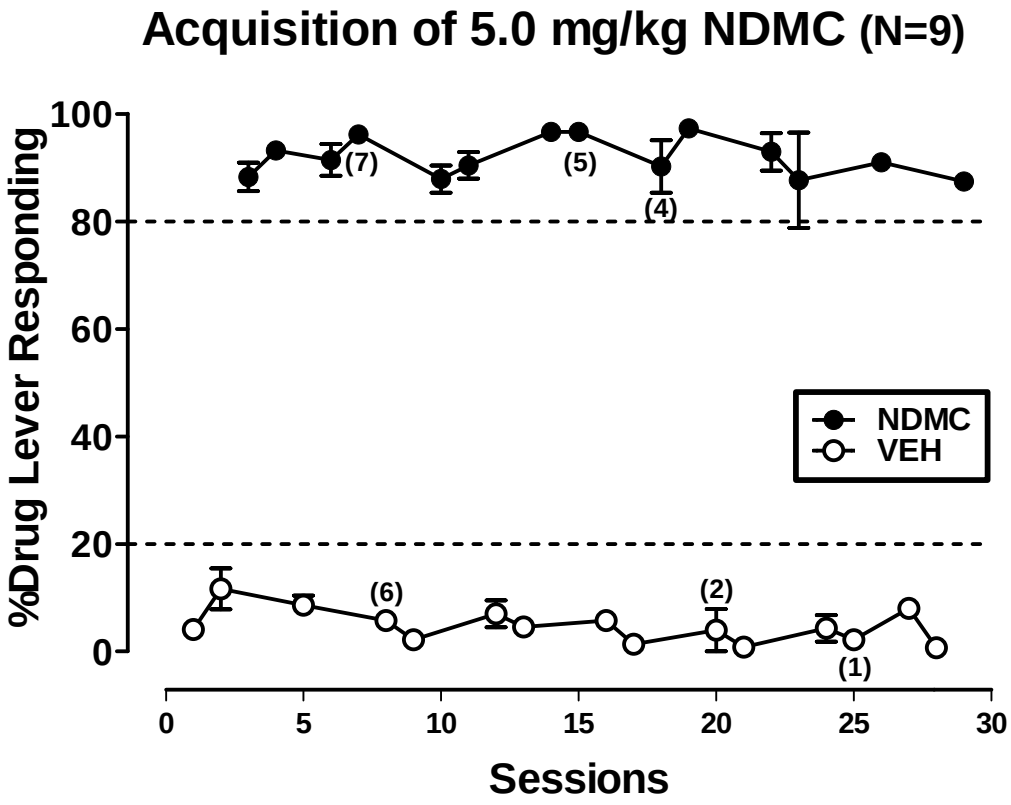

Figure 2. Acquisition of 5.0 mg/kg N-Desmethylclozapine Discrimination

Figure 2. Acquisition of two-lever 5.0 mg/kg NDMC discrimination training is shown. The mean percentage drug lever responding (± SEM) is shown for NDMC (*filled circles*) and VEH (*open circles*) two-lever training sessions. The area above the *dashed line* at 80% indicates group mean 2.5 mg/kg NDMC appropriate responding. As subjects passed training criteria (5 of 6 sessions) they were removed from the acquisition curve, which was indicated by decreasing N, shown in parenthesis.

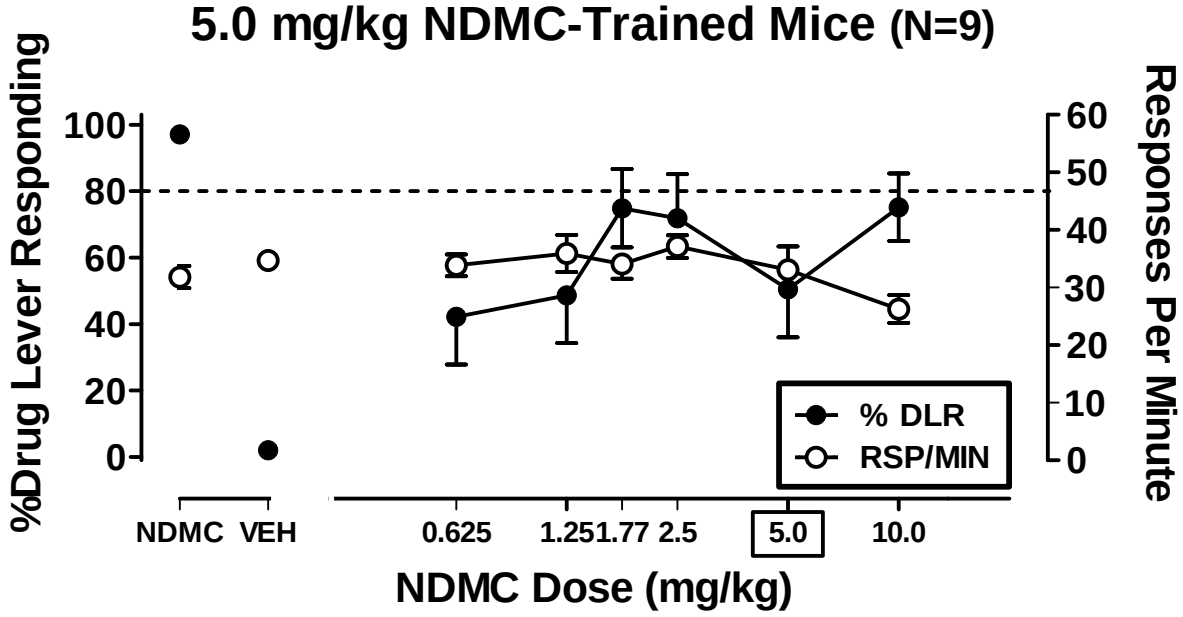

Figure 3. 5.0 mg/kg N-Desmethylclozapine Generalization Data

Figure 3. Generalization data for 5.0 mg/kg NDMC-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Control points were tested prior to testing other doses to confirm stimulus control. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

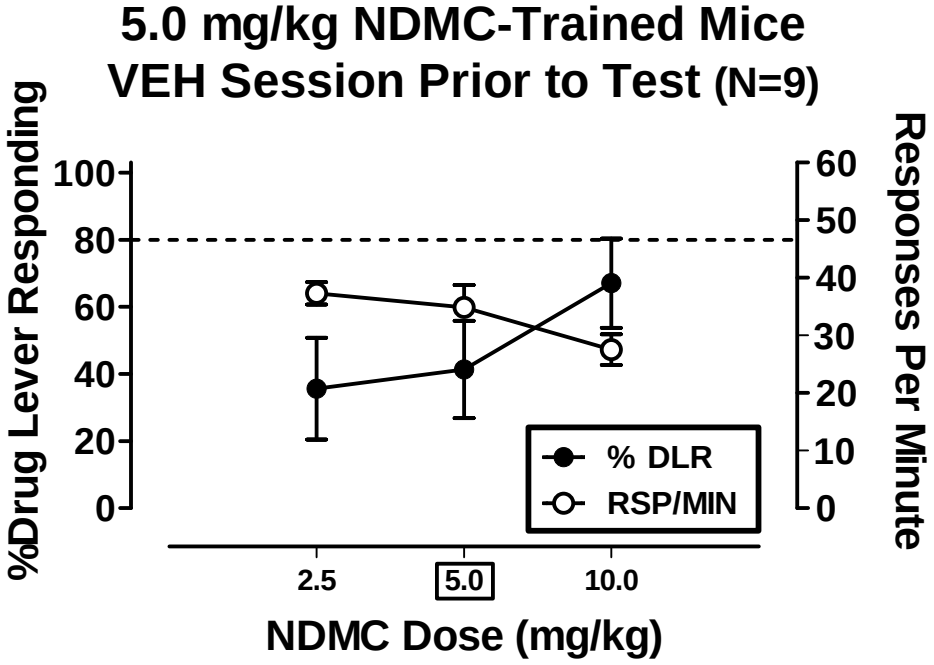

Figure 4. 5.0 mg/kg NDMC Generalization Data VEH Training Prior to Test

Figure 4. Generalization data for 5.0 mg/kg NDMC-trained mice including doses of 2.5, 5.0 and 10.0 mg/kg NDMC tested when VEH training session was conducted prior to test session including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

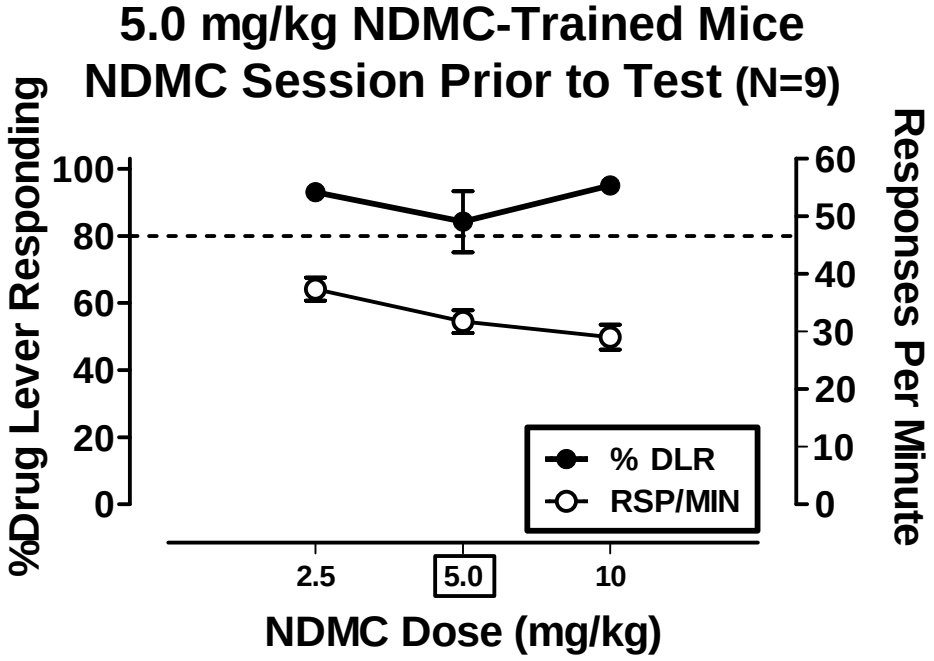

Figure 5. 5.0 mg/kg NDMC Generalization Data NDMC Training Prior to Test

Figure 5. Generalization data for 5.0 mg/kg NDMC-trained mice including doses of 2.5, 5.0 and 10.0 mg/kg NDMC tested when NDMC training session was conducted prior to test session including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

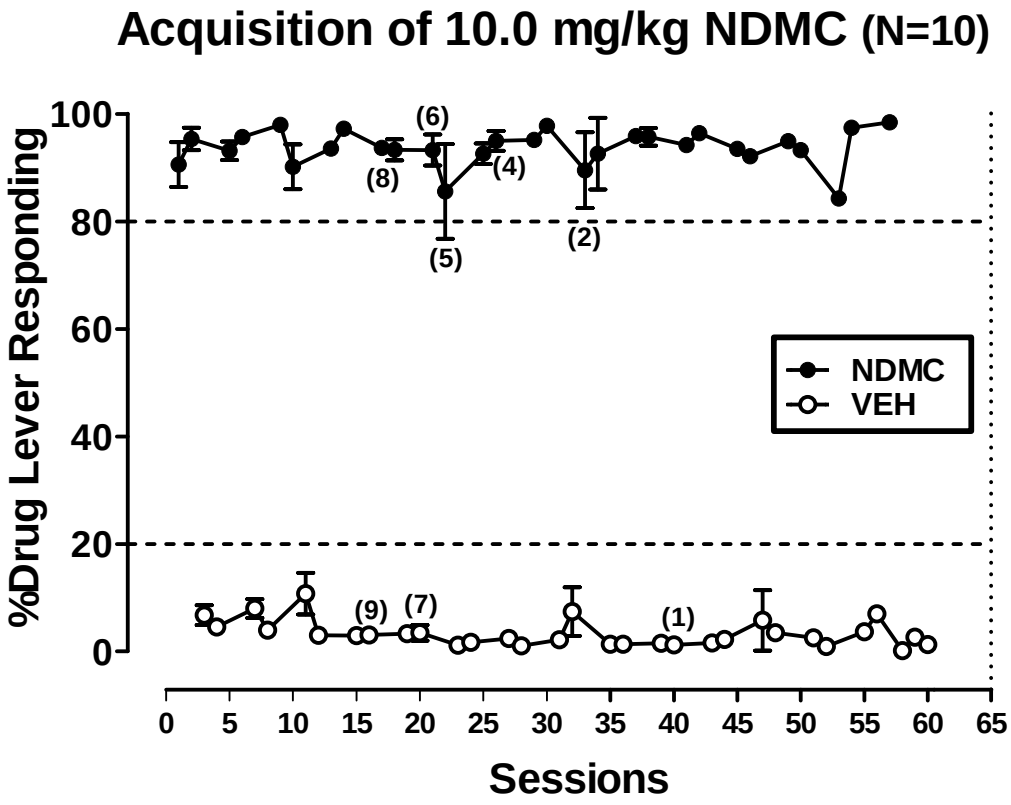

Figure 6. Aquistion of 10.0 mg/kg N-desmethylclozapine Discrimination

Figure 6. Acquisition of two-lever 10.0 mg/kg NDMC discrimination training criteria is shown. The mean percentage drug lever responding (± SEM) is shown for NDMC (*filled circles*) and VEH (*open circles*) two-lever training sessions. The area above the *dashed line* at 80% indicates group mean 10.0 mg/kg NDMC appropriate responding. As subjects passed training criteria (7 of 8 sessions) they were removed from the acquisition curve, which was indicated by decreasing N, shown in parenthesis.

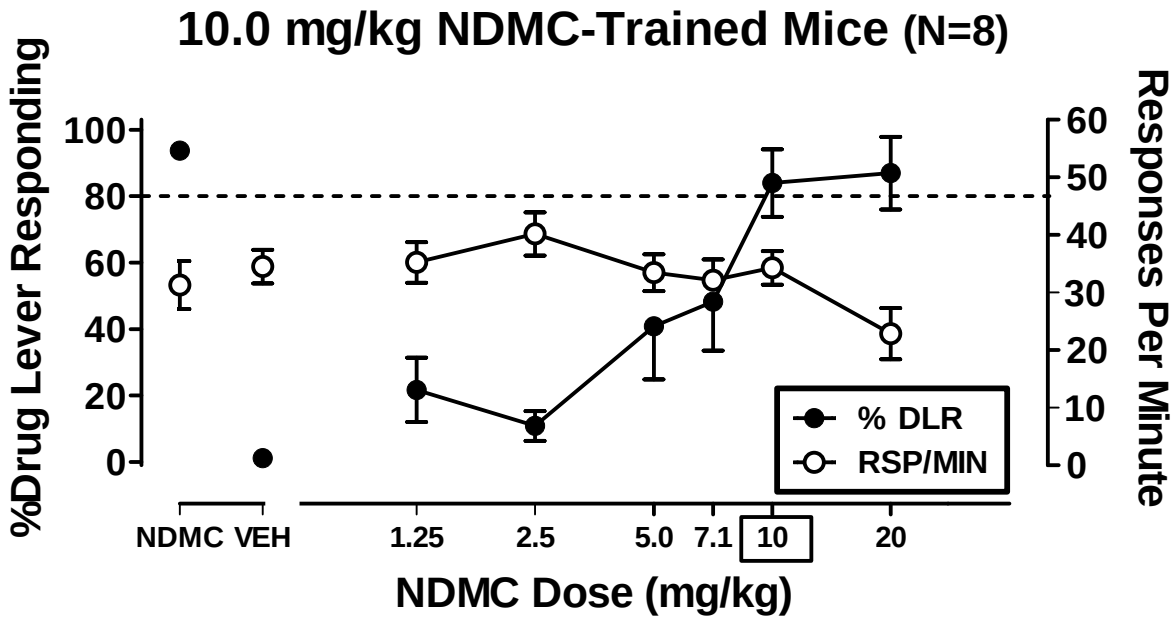

Figure 7. 10.0 mg/kg N-desmethylclozapine Generalization Data

Figure 7. Generalization data for 10.0 mg/kg NDMC-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Control points were tested prior to testing other doses to confirm stimulus control. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

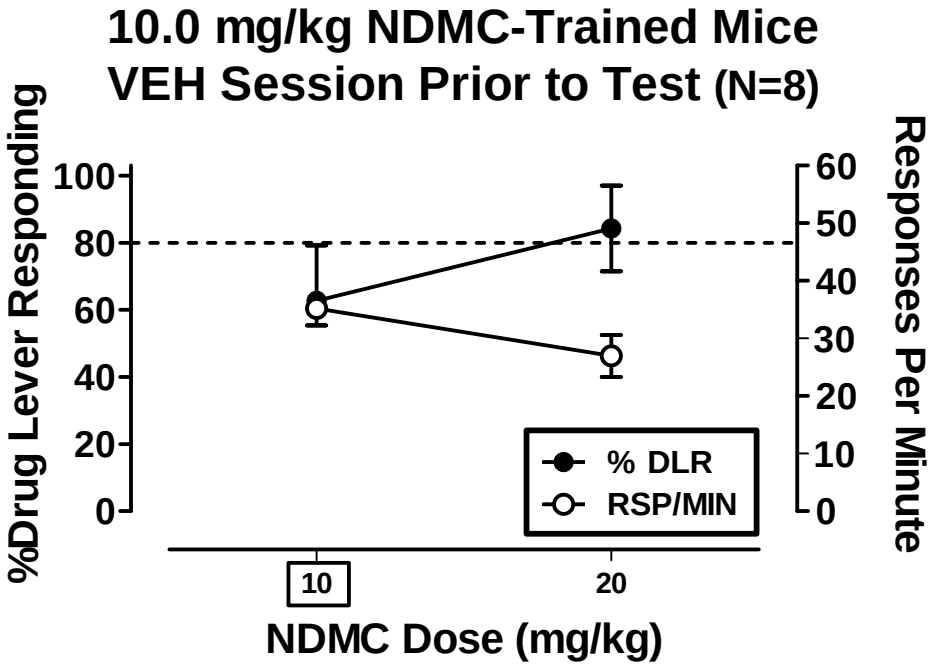

Figure 8. 10.0 mg/kg NDMC Generalization Data VEH Training Prior to Test

Figure 8. Generalization data for 10.0 mg/kg NDMC-trained mice including doses of 10.0 and 20.0 mg/kg NDMC tested when VEH training session was conducted prior to test session including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

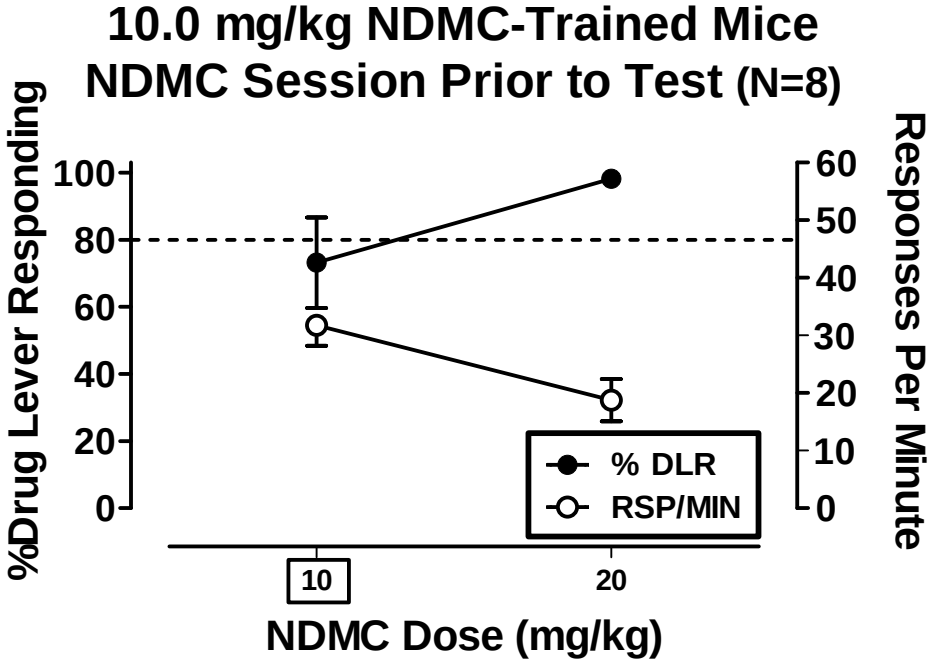

Figure 9. 10.0 mg/kg NDMC Generalization Data NDMC Training Prior to Test

Figure 9. Generalization data for 10.0 mg/kg NDMC-trained mice including doses of 10.0 and 20.0 mg/kg NDMC tested when NDMC training session was conducted prior to test session including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

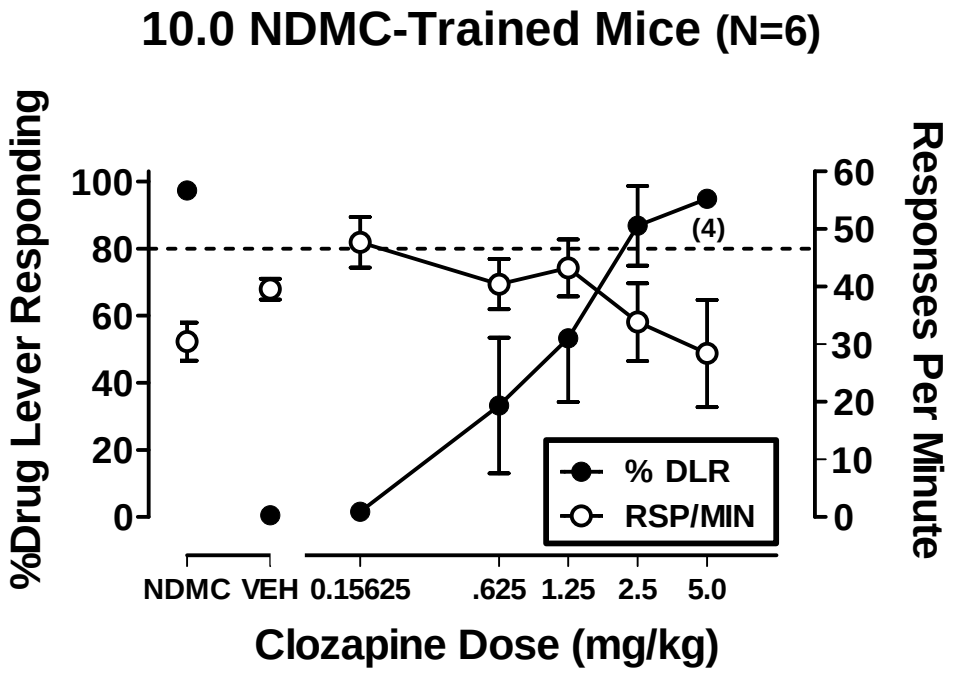

Figure 10. Clozapine Substitution Data for 10.0 mg/kg NDMC-Trained Mice

Figure 10. Clozapine substitution data in 10.0 mg/kg NDMC-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute, which was indicated by noting the number of subjects to complete a particular data point in parenthesis next to that data point. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p <$ .01).

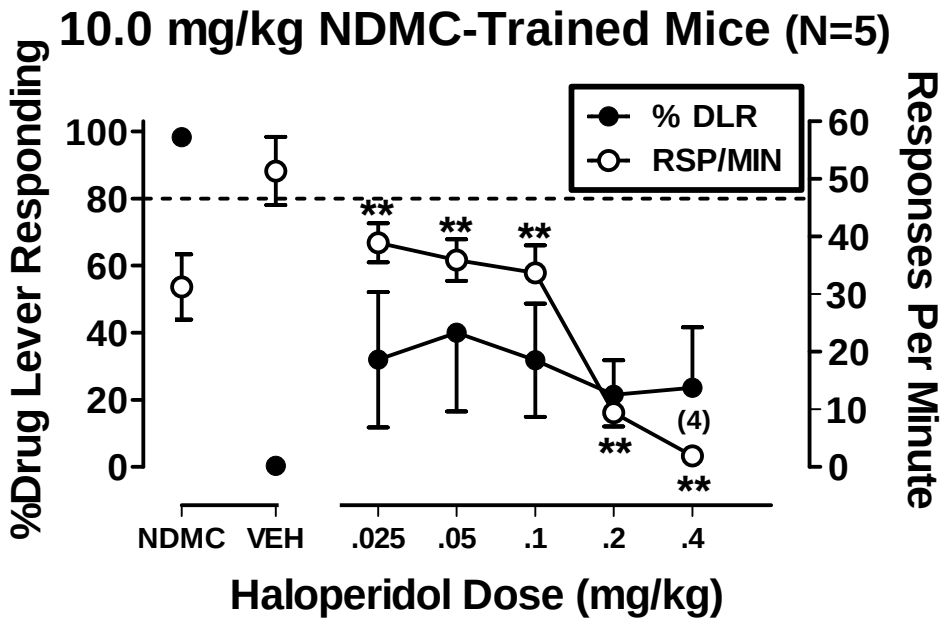

Figure 11. Haloperidol Substitution Data for 10.0 mg/kg NDMC-Trained Mice

Figure 11. Haloperidol substitution data in 10.0 mg/kg NDMC-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' spercentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute, which was indicated by noting the number of subjects to complete a particular data point in parenthesis next to that data point. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p <$ .01).

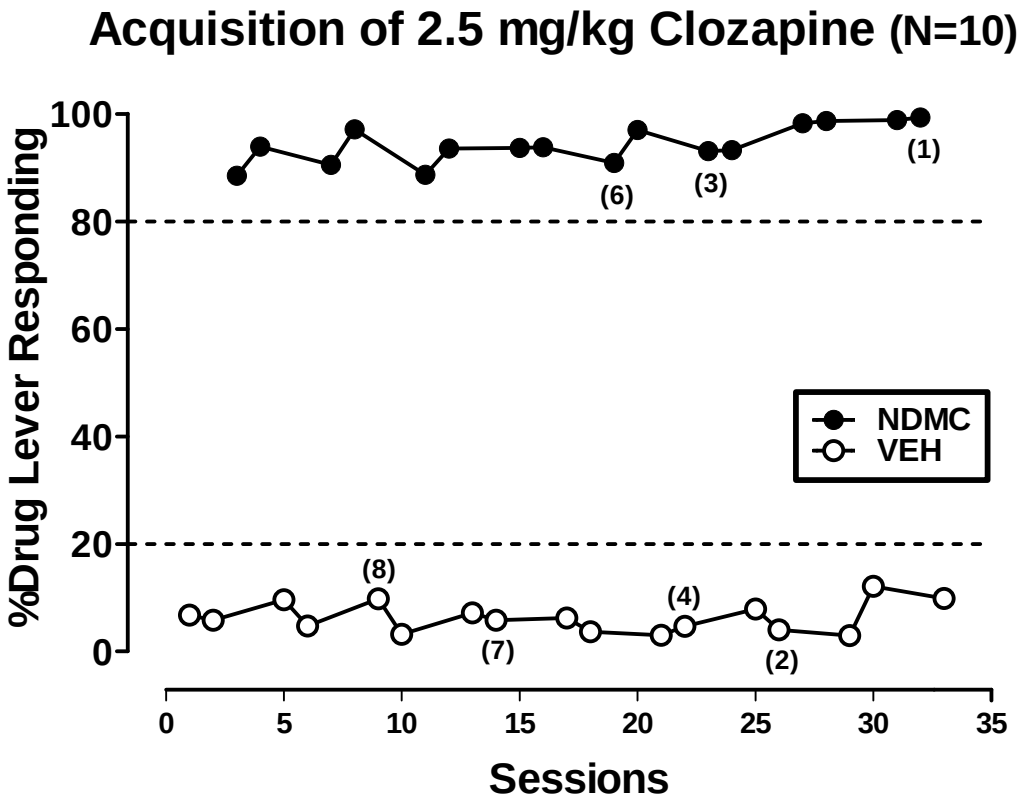

Figure 12. Acquisition of 2.5 mg/kg Clozapine Discrimination

Figure 12. Acquisition of two-lever 2.5 mg/kg discrimination training criteria is shown. The mean percentage drug lever responding (± SEM) is shown for NDMC (*filled circles*) and VEH (*open circles*) two-lever training sessions. The area above the *dashed line* at 80% indicates group mean 2.5 mg/kg NDMC appropriate responding. As subjects passed training criteria (7 of 8 sessions) they were removed from the acquisition curve, which was indicated by decreasing N, shown in parenthesis.

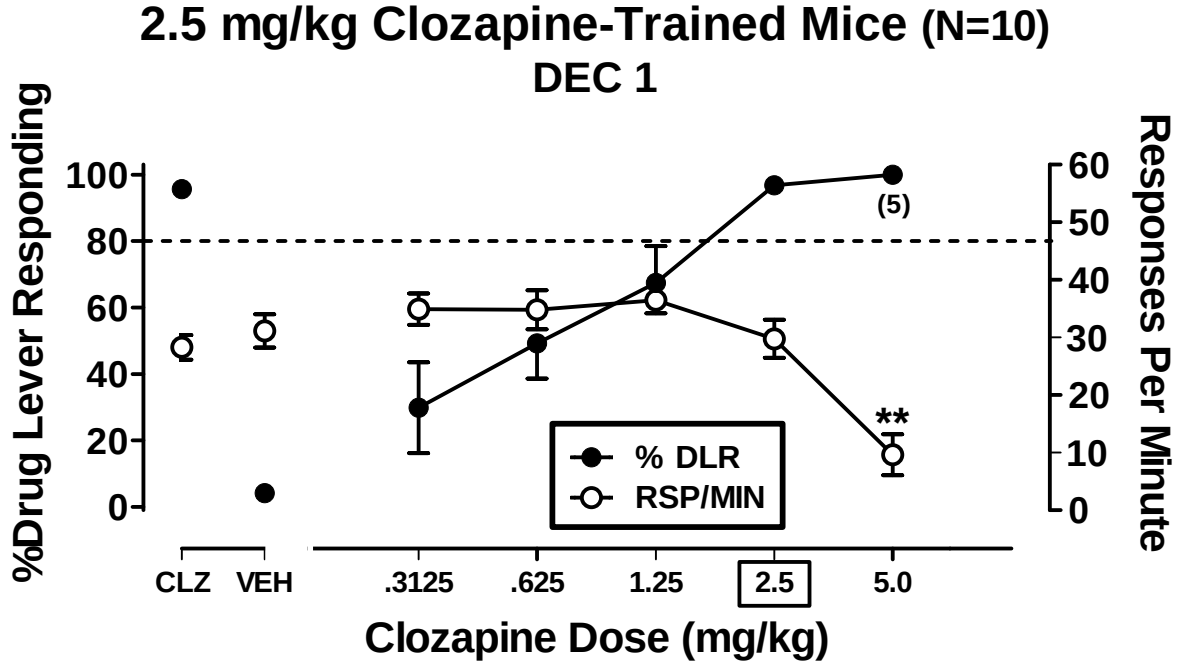

Figure 13. 2.5 mg/kg Clozapine Generalization Data: DEC 1

Figure 13. Generalization data (DEC 1) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Control points were tested prior to testing other doses to confirm stimulus control. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

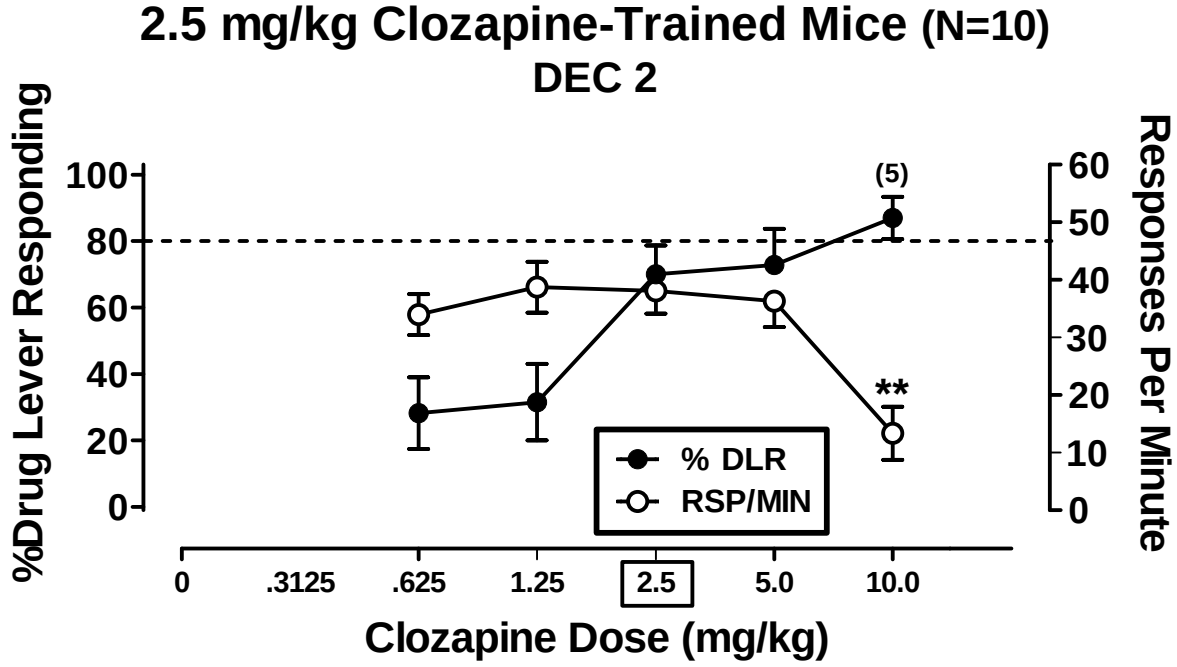

Figure 14. 2.5 mg/kg Clozapine Generalization Data: DEC 2

Figure 14. Generalization data (DEC 2) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

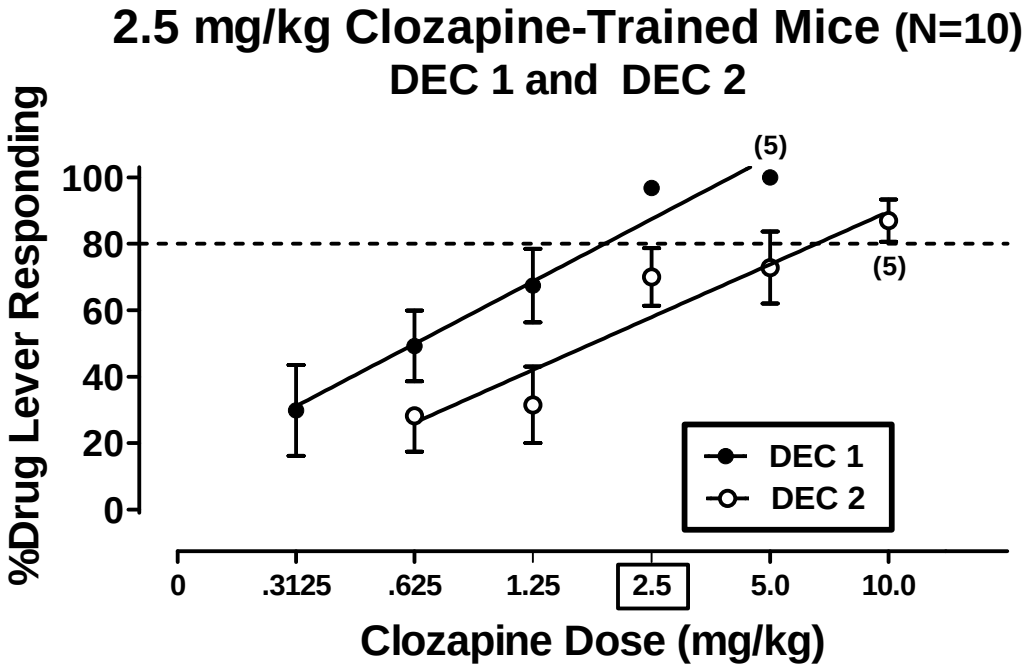

Figure 15. 2.5 mg/kg CLZ DEC 1 and DEC 2 With Regression Lines

Figure 15. Generalization data (DEC 1 and DEC 2) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted to the y-axis and dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Linear regression revealed that the slopes between the lines were not significantly different. The elevations of the lines however, were found to be significantly different indicating a significant right-ward shift of the dose-response curve in DEC 2.

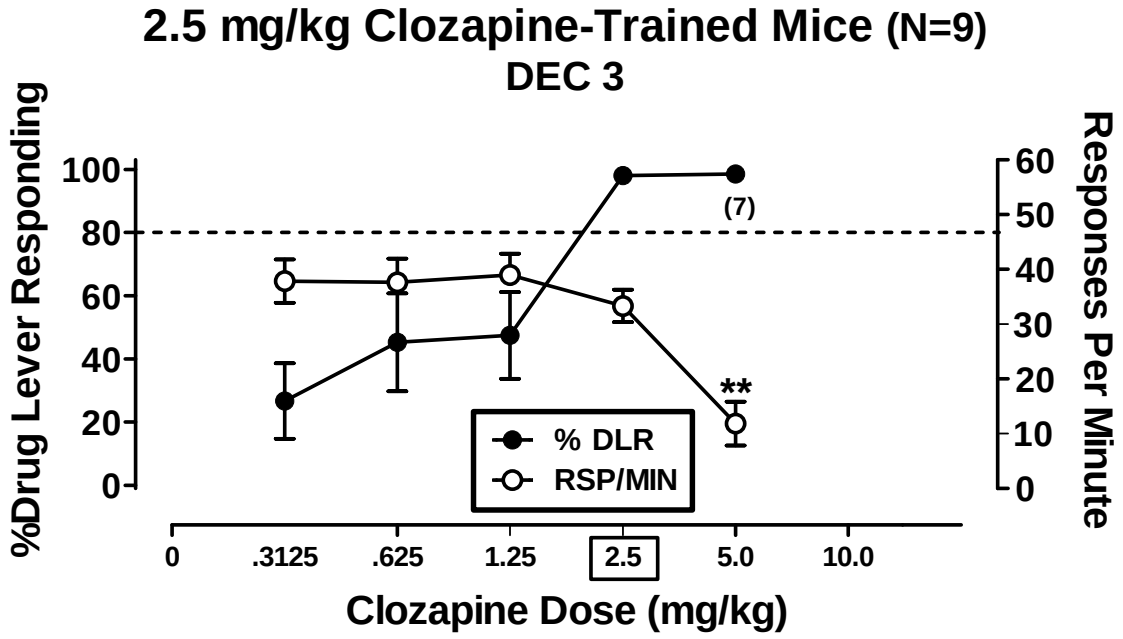

Figure 16. 2.5 mg/kg Clozapine Generalization Data: DEC 3

Figure 16. Generalization data (DEC 3) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) (*filled circles*) plotted on the left y-axis and mean response rate, shown as responses per minute (± SEM) (*open circles*) plotted to the right y-axis. Dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Significant decreases in response rate are noted by asterisks (* $p < 0.05$, ** $p < .01$).

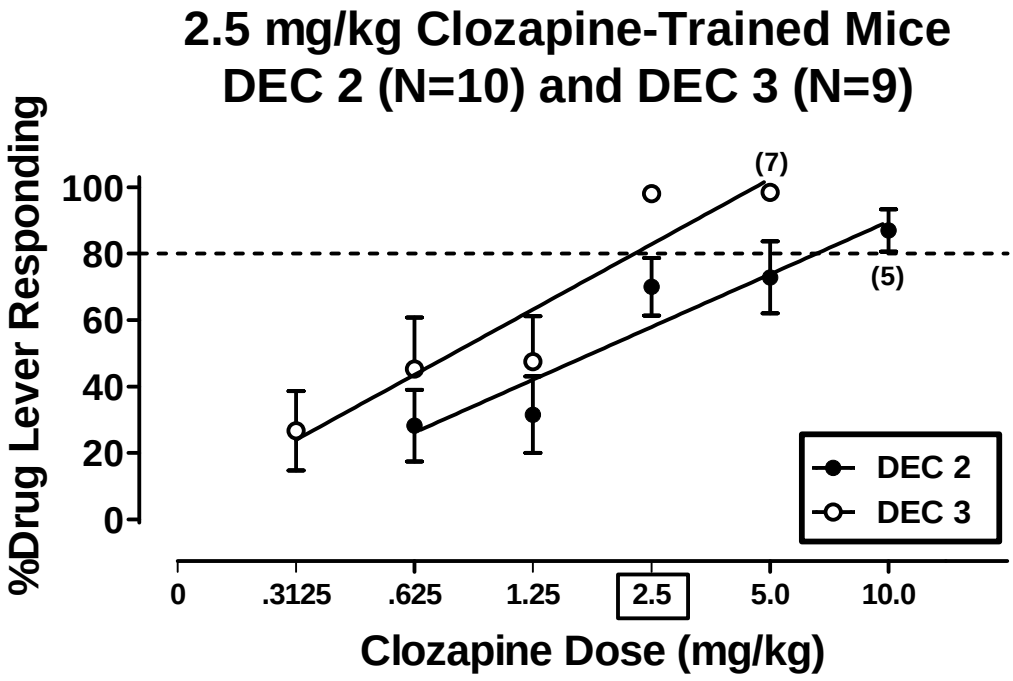

Figure 17. 2.5 mg/kg CLZ DEC 2 and DEC 3 With Regression Lines

Figure 17. Generalization data (DEC 1 and DEC 2) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) plotted on the y-axis and dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Linear regression revealed that the slopes between the lines were not significantly different. The elevations of the lines however, were found to be significantly different indicating a significant right-ward shift of the dose-response curve in DEC 2.

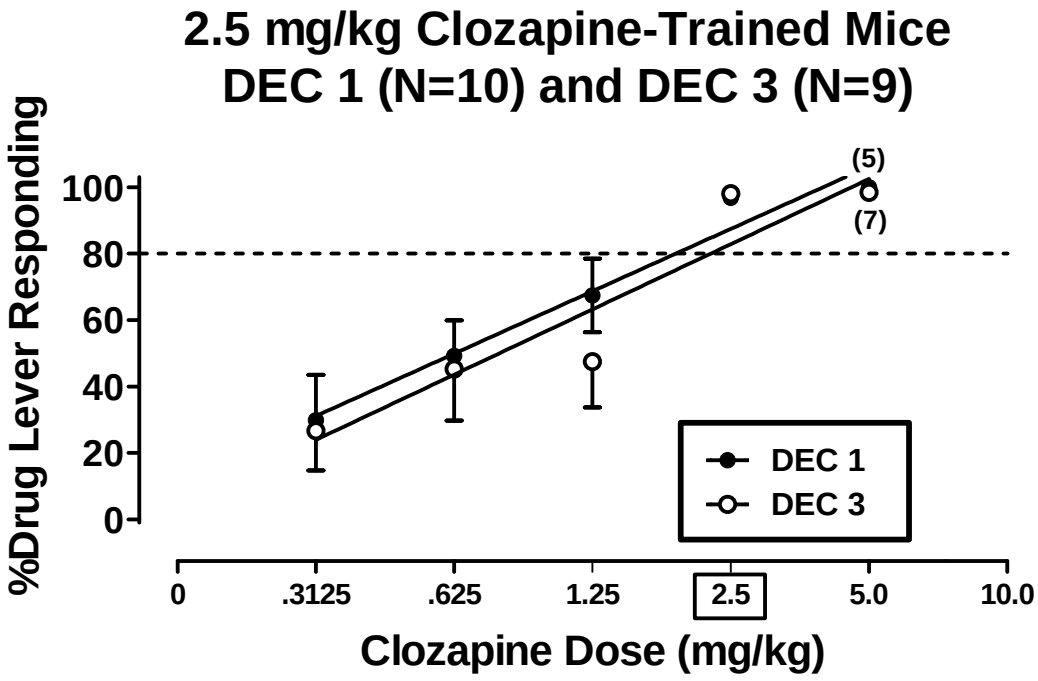

Figure 18. 2.5 mg/kg CLZ DEC 1 and DEC 3 With Regression Lines

Figure 18. Generalization data (DEC 1 and DEC 3) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) (± SEM) plotted on the y-axis and dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Linear regression revealed that the slopes between the lines were not significantly different. The elevations of the lines were also not significantly different indicating a significant right-ward shift of the dose-response curve in DEC 2.

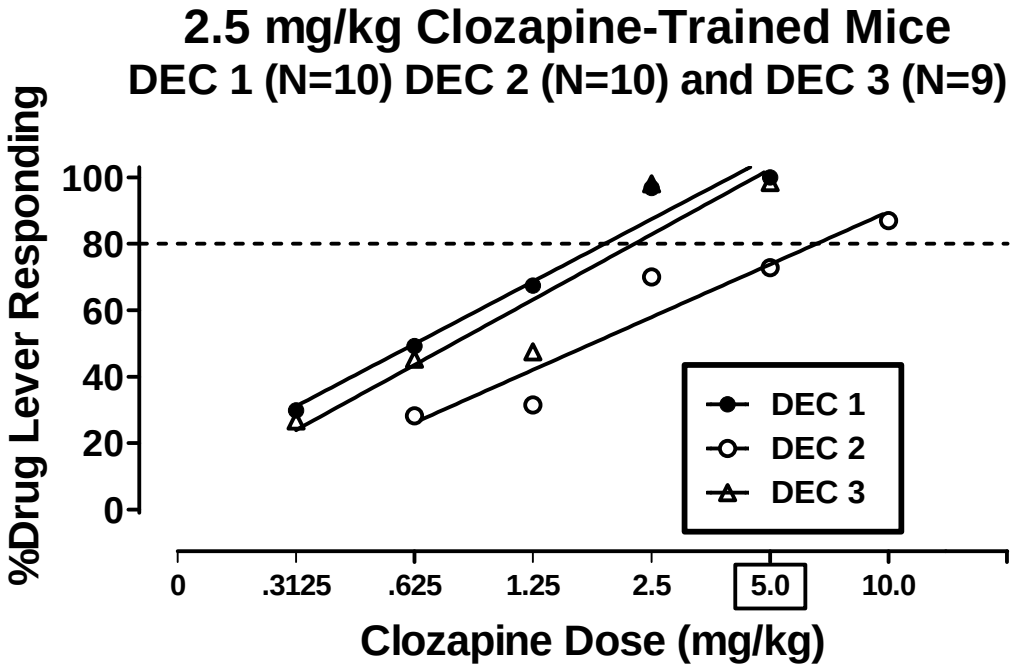

Figure 19. 2.5 mg/kg CLZ DEC 1, DEC 2 and DEC 3 With Regression Lines

Figure 19. Generalization data (DEC 1 DEC 2, and DEC 3) for 2.5 mg/kg clozapine-trained mice is shown including mean drug lever percentage choice (% DLR) plotted on the y-axis and dose is plotted on the X axis. Data points (% DLR) that fall above the *dashed line* at 80% DLR are considered to fully substitute for the training drug/dose. Subjects' percentage drug lever responding data was omitted from analysis if individual response rate was less than 2.0 responses per minute. Linear regression revealed that the slopes between the lines were not significantly different. The elevations of the lines were also not significantly different indicating a significant right-ward shift of the dose-response curve in DEC 2.

Discussion

The present study set out to examine the discriminative stimulus properties of N-desmethylclozapine in C57BL/6 mice, by establishing NDMC as a discriminative stimulus using a two-lever operant procedure, and to evaluate substitution tests of other antipsychotic compounds. To our knowledge, N-desmethylclozapine has not been established as a discriminative stimulus in any species of animal. We chose to establish this stimulus in mice in order to compare results with clozapine-trained mice, and to provide the possibility of studying transgenic and knockout mice in the future. N-desmethylclozapine does not substitute for clozapine in CLZ-trained mice (Prus et al., 2008; Philibin et al., 2009), so our first question to ask after establishing NDMC as a discriminative stimulus was whether or not CLZ will substitute for its major active metabolite, NDMC. Following that assessment the mice were tested with haloperidol and aripiprazole.

Experiment 1

Subjects began drug discrimination training with a 2.5 mg/kg dose of NDMC. Following 57 double-lever training sessions only 5 of 12 subjects had met the training criteria set forth for drug discrimination. The first training dose used had been based on the training dose previously used with the parent drug clozapine (2.5 mg/kg) and also the lowest dose tested for CLZ substitution used in studies assessing the same strain of mouse (Philibin et al., 2009). Philibin and colleagues found that 2.5 mg/kg NDMC failed to substitute for CLZ and also failed to produce significant rate suppression (2009). Following 57 training sessions with less than half of subjects meeting criteria in the present

study, it became obvious that 2.5 mg/kg NDMC was not readily discriminated by C57BL/6 mice. Inspection of the data in Figure 1 suggests that subjects were discriminating NDMC after roughly 22 sessions, but Figure 1, and other acquisition figures in the paper display percent drug lever responding, which was only one of three training criteria that must be passed before testing can begin. The criterion that was most often missed by subjects was the first fixed ratio (FFR) of the session. The mice readily learned to switch levers after completing a ratio, in this case FR 10, which was not reinforced with sweetened milk. Thus, mice rarely missed total lever percentage, or had response rates of less than 10.0 responses per minute after habituating to the procedure of drug discrimination training.

The acquisition of 5.0 mg/kg NDMC was much more rapid and successful, as all 9 remaining subjects in the study acquired the discriminative stimulus in a mean of 15.7 double-lever training sessions with a range of 6 to 29 sessions as shown in Figure 2. The data shown in Figure 2 show that the subjects were discriminating based on overall DLR percentage and appropriate response rates on session 1, but again, FFR was the limiting criteria to begin substitution testing.

Following acquisition of discrimination training with 5.0 mg/kg NDMC a dose effect curve was generated as shown in Figure 3. The data produced for the NDMC generalization curve looked normal until animals were tested with the 5.0 mg/kg training dose and the 10.0 mg/kg dose of NDMC. The mice failed to show full or partial substitution (50.49% DLR) with 5.0 mg/kg NDMC, which was the training dose, and 10.0 mg/kg NDMC only produced partial substitution (75.13% DLR). This revealed a fading or loss of the discriminative stimulus over time. Subjects were tested with doses of 2.5, 5.0,

and 10.0 mg/kg NDMC with either a NDMC or VEH training session the day prior to testing (the opposite condition to which each subject had been tested on before). When the results were plotted as a function of the training condition (see Figure 4), it was found that the mice failed to substitute at doses of 2.5 mg/kg (35.5% DLR) and 5.0 mg/kg (41.33% DLR) NDMC and only showed partial substitution (67.07% DLR) with 10.0 mg/kg NDMC when a VEH training session preceded the test session. Subjects fully substituted with all three doses tested (93.03%, 84.22%, and 95.01% respectively) when a NDMC training session immediately preceded the test session (see Figure 5). This indicated that 5.0 mg/kg NDMC was not serving as an effective discriminative stimulus.

Thus, the training dose of NDMC was increased to 10.0 mg/kg after subjects failed to retain discrimination of the 5.0 mg/kg NDMC training dose. The training criteria were also changed, as subjects were required to pass all 3 criteria for 7 of 8 consecutive double-lever training sessions before beginning testing. Also, after beginning testing, subjects were required to pass both a VEH and a NDMC training session consecutively immediately prior to testing in order to ensure that the animals were maintaining stimulus control. The higher dose of 10.0 mg/kg NDMC was able to be successfully trained in a mean of 27.5 sessions with a range of 14 to 58 sessions (Fig. 6). The dose effect curve revealed that both 10.0 and 20.0 mg/kg NDMC fully substituted for the training dose (83.95% and 86.92% DLR respectively) without producing significant rate suppression as shown in Figure 6. This generalization profile was different from that of clozapine, as CLZ produced significant rate suppression at 1 $\log_2$ step above the training dose.

After completion of the NDMC dose effect curve, subjects were re-tested with the highest doses of the curve, 10.0 and 20.0 mg/kg, but again with the opposite preceding training condition to which they had been tested on before. It revealed that 10.0 mg/kg NDMC failed to fully substitute with VEH training prior to testing (62.66% DLR) and with NDMC training prior to testing (73.16% DLR) as shown in Figure 7. The highest dose tested of 20.0 mg/kg NDMC fully substituted under both conditions (84.26% and 98.23% respectively) as shown in Figure 8. This observation revealed that there still may be a tolerance or fading problem developing with the discriminative stimulus training dose of 10.0 mg/kg NDMC. It also suggests that NDMC has a weak discriminative stimulus overall, and is difficult to establish as a training drug. This shifting discriminative stimulus or loss of discrimination over time indicated that NDMC may be difficult to study using drug discrimination. It requires precautions to be taken including implementing stringent training and testing criteria to ensure good stimulus control.

The first compound tested for substitution was the parent drug of N-desmethylclozapine, clozapine, as NDMC did not substitute in CLZ-trained mice (Philibin et al., 2009). We expected that CLZ might substitute in 10.0 mg/kg NDMC trained-mice even though NDMC itself didn't substitute for CLZ in C57BL/6 mice, as a combination dose of 0.625 mg/kg CLZ plus 10.0 mg/kg NDMC fully substituted for CLZ, although it also significantly reduced response rates (Philibin et al., 2009). A combination dose of CLZ (0.3125 mg/kg) plus NDMC (5.0 or 10.0 mg/kg) also substituted for CLZ in 1.25 mg/kg CLZ-trained rats, and the 5.0 mg/kg dose combined with the 0.3125 dose of CLZ fully substituted without producing significant rate suppression (Prus et al., 2009). In the

present study, clozapine was found to fully substitute (86.79% and 94.82% DLR) at the two highest doses (2.5 and 5.0 mg/kg) tested, and interestingly, both doses that fully substituted failed to produce a significant reduction in response rate as compared to VEH control response rates as shown in Figure 9. Even though, 5.0 mg/kg did not produce a significant reduction of response rates, 2 of the 6 subjects tested failed to respond when given 5.0 mg/kg, which is a common effect seen in 2.5 mg/kg CLZ-trained mice when tested with a 5.0 mg/kg dose. The subjects that responded produced such a high response rate that the mean produced was not significantly different from the VEH control response rate. Because the response rates of subjects that responded were so high, it suggested that NDMC may play a role in providing protection from CLZ's rate-reducing effects at doses higher than the training dose. This generalization of the NDMC discriminative stimulus to CLZ, along with lack of CLZ generalization to NDMC displays an asymmetrical generalization between the two compounds, which has been found in other drug discrimination studies including studies of MDMA and cocaine, ephedrine and methamphetamine, and alcohol and NMDA antagonists, (Bondareva, Young, & Glennon, 2002; Khorana, Pullagurla, Young, & Glennon, 2003; Bondareva, Wesoloska, Dukat, Lee, Young, & Glennon, 2005; Butelman, Baron, & Woods, 1993) .

It was interesting that clozapine was found to fully substitute for its metabolite, NDMC, given that NDMC didn't substitute for CLZ at any dose in past studies; however, this was somewhat expected because CLZ is rapidly metabolized to NDMC, therefore it may be an important component of CLZ's discriminative stimulus. This asymmetrical generalization is most likely due to differences in receptor activity at M_1 muscarinic and/or

D_2 dopaminergic receptors. A study by Prus and colleagues has shown that NDMC administered in combination with trihexyphenidyl, an M_1 receptor agonist, full clozapine substitution was produced, although it was accompanied by significant rate-suppression (Prus et al., 2008). This shows M_1 antagonism may play a role in CLZ's discriminative stimulus, although this has only been shown in rats, not mice (Philibin 2005; 2009). It makes sense that CLZ will substitute for NDMC as CLZ is rapidly metabolized to NDMC in the mouse and may possibly contribute to substitution for the NDMC discriminative stimulus. NDMC and CLZ also show difference at D_2 receptors, with NDMC being a partial agonist, and CLZ a D_2 antagonist, but since CLZ is metabolized into NDMC after administration, it makes sense that CLZ substitutes for NDMC.

Haloperidol failed to substitute for NDMC at any dose tested (0.025, 0.05, 0.1, 0.2, and 0.4 mg/kg), but significant response rate reductions were observed at all doses as compared to VEH response rates. A visual analysis of Figure 10 reveals that the VEH response rate was the highest observed in any dose effect or generalization curve generated in the present study. The two highest doses of haloperidol (0.2 and 0.4 mg/kg) appeared to reduce response rates markedly more than the lower doses. This confirms past research from our lab and others that show typical APDs such as haloperidol fail to substitute for the atypical APD, CLZ (Goas & Boston, 1978; Porter et al., 2000; Porter et al., 2008 & Goudie et al., 1998).

After some training dose adjustments NDMC was able to be trained and maintained as a discriminative stimulus, although continuous assessment of stimulus control was required in order to properly conduct substitution tests in subjects. Generation of the dose-

effect curve revealed a dose-dependent relationship for NDMC and drug lever response percentage, which is important when assessing drugs as discriminative stimuli. It is important to establish NDMC as a discriminative stimulus, as it has been found to contribute to the clinical efficacy of CLZ, and its properties need to be further investigated (Flanagan et al., 2003). NDMC may also be important for cognition, as patients with higher ratios of NDMC to CLZ show more cognitive improvement (Weiner et al., 2004). Understanding the similarities and differences between CLZ and NDMC *in vivo* may lead investigators to develop new strategies in clinical treatment. Establishing NDMC as a discriminative stimulus also provides further evidence that it is behaviorally active in the brain, as shown by studies assessing Fos protein expression in the prefrontal cortex of rats and by studies showing NDMC's ability to reverse both MK-801 and amphetamine induced hyperactivity which is a preclinical model of psychotic behavior that can be reversed by APDs (Young et al., 1998; see Lameh et al., 2007).

Experiment 2

In Experiment 1, it was observed that during CLZ substitution testing there appeared to be some tolerance development to the rate-suppressing effects of CLZ; thus it was hypothesized that there might be a cross-tolerance between CLZ and its major metabolite N-desmethylclozapine. To assess this possible cross-tolerance, a method similar to that of a pair of studies conducted by Goudie and colleagues was employed (Goudie et al., 2007a; Goudie et al., 2007b). A group of mice (N = 11) was trained to discriminate 2.5 mg/kg CLZ from VEH and a CLZ dose effect curve was determined. Following this first dose effect curve (DEC 1), the subjects were administered 10.0 mg/kg NDMC twice daily

for 10 days. A second CLZ dose response curve was generated following 10 days of repeated NDMC administration. The regression lines for the two dose response curves remained parallel, but there was a significant right-ward shift in the dose response curve indicating the development of cross-tolerance between NDMC and CLZ (Fig 15).

In Experiment 2 subjects were first trained with 10.0 mg/kg NDMC using methods similar methods to those used in Experiment 1 when 10.0 mg/kg NDMC was trained as a discriminative stimulus. In Experiment 1, the subjects that achieved training criteria for 10.0 mg/kg NDMC had an extensive, and complicated training history that involved increasing the training dose 3 times throughout the course of the experiment. This training history may have impacted training of the 10.0 mg/kg NDMC dose and subsequent substitution tests (Stolerman & White, 1996). In Experiment 2, subjects had no training or drug history before beginning 10.0 mg/kg NDMC training. Drug-naïve mice were used in order to determine if training history affected the acquisition of 10.0 mg/kg NDMC discrimination in Experiment 1. The majority of the mice (8 of 11) were unable to pass the discrimination training criteria after 57 training sessions. The three subjects that were able to pass the criteria did so in a mean of 23.3 double-lever training sessions with a range of 17 to 30 sessions.

Since only 3 subjects were able to discriminate 10.0 mg/kg NDMC from VEH reliably, NDMC discrimination training was suspended and the mice were given 14 days of free access to food followed by 7 days to re-establish food deprivation before training to discriminate 2.5 mg/kg clozapine from VEH began. The acquisition of the training criteria for 2.5 mg/kg CLZ discrimination, shown in Figure 11, was similar to that displayed in

Figure 2 for 5.0 mg/kg NDMC. The acquisition curve is flat due to the past training history of discrimination training with 10.0 mg/kg NDMC. As discussed earlier, the subjects were easily able to pass 2 of the 3 criteria at the beginning of discrimination training, but failure to pass the FFR criterion was the most common criteria to miss. All 11 subjects passed the training criteria in a mean of 20.0 double-lever training sessions, with a range of 8 to 33 sessions. The only change made to the discrimination training procedure was that subjects were trained or tested 7 days a week for the entire experiment. This minimized variability on test and training days and maintained a high level of stimulus control over the 2.5 mg/kg CLZ discriminative stimulus.

The dose effect curve generated initially (DEC 1) produced an ED_{50} for CLZ dose that was not found to be significantly different than another group of C57BL/6 mice being currently trained in another project (Philibin et al., 2005). DEC 1 shows full substitution with doses of 2.5 and 5.0 mg/kg CLZ for the training dose of 2.5 mg/kg CLZ; however, there was significant response rate reduction as compared to VEH response rates at the 5.0 mg/kg dose. Only 5 of the 11 subjects responded at that dose, which was comparable to past data from our lab. This provides support that the CLZ discrimination acquisition was not hindered by the behavioral history of 10.0 mg/kg NDMC training. Upon completion of DEC 1, the mice were given 10 days of 10.0 mg/kg NDMC injections twice daily.

DEC 2 was generated immediately following the 10 days of 10.0 mg/kg NDMC administration. The dose effect curve was also generated with no training days in between tests, to prevent the loss of any tolerance induced by NDMC administration; this was a replication of Goudie's DEC 2 as it was thought that it was more important to preserve the

tolerance that may have developed over the 10 days of dosing than assessing stimulus control, as that would be assessed in the final dose effect curve (DEC 3). The lowest dose (0.3125 mg/kg) of CLZ was not tested in DEC 2, to minimize chances of losing tolerance during testing, similar to Goudie's methods, which did not test the lowest dose in the curve following repeated drug administrations (DEC 2). The first subject was tested with doses of CLZ in an ascending order and 5.0 mg/kg CLZ failed to produce substitution for the training dose of 2.5 mg/kg CLZ, and also failed to significantly reduce response rates. Following this observation, a high dose of 10.0 mg/kg CLZ was also tested in all subjects to assess its ability to produce 2.5 mg/kg CLZ substitution. The ED_{50} generated from the DEC 2 data was found to be significantly different than the DEC 1 ED_{50} and the dose-effect curve was significantly shifted right-ward, indicating tolerance, or lessening of the discriminative stimulus. The tolerance was shown for both DLR percentage and in response rate suppression. During DEC 1, 5.0 mg/kg CLZ produced significant response rate suppression, which was not seen in DEC 2; however, 10.0 mg/kg CLZ produced significant rate suppression in DEC 2.

Following DEC 2, training was suspended for 10 consecutive days without drug treatment. On day 11, subjects were trained for 2 days including both a CLZ and VEH training condition, and then completed the final dose-effect curve (DEC 3). DEC 3 was conducted exactly as DEC 1 for each individual subject; each test was separated by 2 training days, and subjects were assigned to ascending or descending dose test order based on the order tested during DEC 1. The ED_{50} completed during DEC 3 was not significantly different from the ED_{50} calculated for DEC 1; however, it was significantly different from

the ED_{50} calculated for DEC 2, showing a significant left-ward shift for DEC 3 as compared to DEC 2. This significant shift was confirmed using linear regression analysis, indicating a loss of the tolerance displayed in DEC 2.

Thus, N-desmethylclozapine induced cross-tolerance to clozapine's discriminative stimulus, suggesting that NDMC's discriminative stimulus properties share some similarities to its parent drug CLZ. However, despite this ability to produce cross-tolerance to CLZ's discriminative stimulus, NDMC does not substitute for CLZ in mice or rats trained to discriminate CLZ from VEH at any dose tested, despite producing significant rate suppression (Philibin et al., 2009; Prus et al., 2009). CLZ substituted for NDMC in mice that were trained to discriminate NDMC from VEH in the present study. These results support findings from *in vitro* studies suggesting that NDMC and CLZ show many similarities in their receptor binding, but with a couple of important differences at M_1 muscarinic and D_2 dopamine receptors (Lameh et al., 2007).

Assessing the induction of cross-tolerance to clozapine is important, because APDs are almost always administered repeatedly over time, rather than given only acutely; In addition to that, therapeutic effects produced by administration of CLZ are usually not apparent until after repeated use of CLZ. This indicates that long-term receptor based or metabolic changes may take place during CLZ treatment that contribute to or create the therapeutic effects seen in patients using clozapine. Since long-term administration is the typical situation encountered by patients prescribed CLZ, it is important that we assess the preclinical effects of CLZ after repeated administration as well. One way to examine effects of a given drug over time is to examine its effects on generalization of a

discriminative stimulus. Cross-tolerance assessment can be used to examine similarities in drugs administered over long periods of time, and one way to assess cross-tolerance to CLZ is to examine effects of repeated administration of a drug to a subject who is trained to discriminate CLZ from VEH. If repeated administrations of a certain drug produce a right-ward shift in the dose-response curve, it indicates cross-tolerance development. The current study displayed cross-tolerance development both through a significant right-ward shift in the dose-response curve, and also potentiation of previously suppressed response rates. This indicates that possible neuroadaptive or metabolic changes take place during NDMC administration, which are similar to those shown during CLZ administration.

Goudie and colleagues have shown that cross-tolerance induction to a discriminative stimulus is relatively pharmacologically specific, as JL13, olanzapine, and clozapine all induce cross-tolerance to the CLZ discriminative stimulus, but chlordiazepoxide failed to induce tolerance (Goudie et al., 2007a & Goudie et al., 2007b). However, this also points to the fact that drugs with similar receptor mechanisms to CLZ may induce cross-tolerance to the discriminative stimulus, but still fail to possess any antipsychotic efficacy in the clinic, such as cyproheptadine, which lacks D_2 binding affinity. It is important to interpret results cautiously given the fact that cyproheptadine produces a false positive in terms of screening for APDs using this cross-tolerance development procedure; however, it may still be used to test for pharmacological similarities of drugs.

Future Studies

We are currently testing aripiprazole for substitution in 10.0 mg/kg NDMC-trained mice. Due to its similarity in D_2 dopamine receptor activity, aripiprazole may have similar discriminative stimulus properties to NDMC, indicating the importance of D_2 partial agonism in the discriminative stimuli of both compounds. Substitution of aripiprazole for NDMC may indicate that NDMC should be further investigated as a potential APD as aripiprazole was recently approved by the FDA for treatment of schizophrenia, and is the first APD to have D_2 partial agonism. We are also currently training aripiprazole as a discriminative stimulus in C57BL/6 mice in order to further investigate the discriminative stimulus properties of aripiprazole. After testing clozapine and haloperidol for substitution, NDMC will be assessed in the assay. The generalization results between these two studies will help to determine how similar the two compounds are, and specific differences can be assessed be using selective agonists and antagonists to separate mechanisms mediating the discriminative stimuli. Future studies assessing the discriminative stimulus of NDMC using selective agonists and antagonists would be helpful to discern the specific differences between the discriminative stimuli of N-desmethylclozapine and clozapine.

Conclusion

Drug Discrimination is a useful preclinical model used to assess drug receptor activity *in vivo*. Clozapine drug discrimination has been an important tool to discern similarities and differences among various APDs in terms of receptor activation. Establishing NDMC, CLZ's major metabolite, as a discriminative stimulus will help define the mechanistic differences between the two compounds. These studies may help the

development of novel APDs with therapeutic effects similar to CLZ but with fewer undesirable side-effects. Assessment of cross-tolerance development is also a method to compare discriminative stimulus effects of different drugs, which utilizes longer term administration of a drug to more accurately portray human APD use.

Although NDMC is clearly similar to CLZ in many ways, and is important for the clinical effects of clozapine, it is not exactly the same as CLZ, and does not have the clinical efficacy of its parent drug either. N-desmethylclozapine has recently endured some negative results in phase II clinical trials indicating it may lack antipsychotic efficacy as a stand-alone drug treatment for schizophrenia (Reuters, 2008). NDMC has shown promise in some pre-clinical studies and is likely important for CLZ's mechanism of action, so it is still possible that NDMC may have some value as an "add on" to existing or forthcoming treatments for schizophrenia due to its similar receptor binding profile to clozapine, and it is still being investigated.

List of References

APA. (2000). *Diagnostic and Statistical Manual of Mental Disorders, 4th ed., text revision*. Washington, D.C.: American Psychiatric Press, Inc.

Arndt, S., Alliger, R. J., & Andreasen, N. C. (1991). The distinction of positive and negative symptoms. The failure of a two-dimensional model. *Br J Psychiatry, 158*, 317-322.

Arnt, J., & Skarsfeldt, T. (1998). Do novel antipsychotics have similar pharmacological characteristics? A review of the evidence. *Neuropsychopharmacology, 18*(2), 63-101.

Auquier, P., Lancon, C., Rouillon, F., & Lader, M. (2007). Mortality in schizophrenia. *Pharmacoepidemiol Drug Saf, 16*(12), 1308-1312.

Becker, H. C., & Baros, A. M. (2006). Effect of duration and pattern of chronic ethanol exposure on tolerance to the discriminative stimulus effects of ethanol in C57BL/6J mice. *J Pharmacol Exp Ther, 319*(2), 871-878.

Bleuler, E. (1911). Dementia Praecox oder die Gruppe der Schizofrenien. In G. Aschaffenburg (Ed.), *Handbuch der Psychiatrie*. Leipzig and Vienna.

Bliss, C. (1967). *Statistics in Biology*. New York: McGraw-Hill.

Bondareva, T., Wesolowska, A., Dukat, M., Lee, M., Young, R., & Glennon, R. A. (2005). S(+)- and R(-)N-methyl-1-(3,4-methylenedioxyphenyl)-2-aminopropane (MDMA) as discriminative stimuli: effect of cocaine. *Pharmacol Biochem Behav, 82*(3), 531-538.

Bondareva, T. S., Young, R., & Glennon, R. A. (2002). Central stimulants as discriminative stimuli. Asymmetric generalization between (-)ephedrine and S(+)methamphetamine. *Pharmacol Biochem Behav, 74*(1), 157-162.

Bondesson, U., & Lindstrom, L. H. (1988). Determination of clozapine and its N-demethylated metabolite in plasma by use of gas chromatography-mass spectrometry with single ion detection. *Psychopharmacology (Berl), 95*(4), 472-475.

Breier, A. (2001). Introduction: a new era in the pharmacotherapy of psychotic disorders. *J Clin Psychiatry, 62 Suppl 2*, 3-5.

Brown, S. (1997). Excess mortality of schizophrenia. A meta-analysis. *Br J Psychiatry, 171*, 502-508.

Burstein, E. S., Ma, J., Wong, S., Gao, Y., Pham, E., Knapp, A. E., et al. (2005). Intrinsic efficacy of antipsychotics at human D2, D3, and D4 dopamine receptors: identification of the clozapine metabolite N-desmethylclozapine as a D2/D3 partial agonist. *J Pharmacol Exp Ther, 315*(3), 1278-1287.

Butelman, E. R., Baron, S. P., & Woods, J. H. (1993). Ethanol effects in pigeons trained to discriminate MK-801, PCP or CGS-19755. *Behav Pharmacol, 4*(1), 57-60.

Carey, G. J., & Bergman, J. (1997). Discriminative-stimulus effects of clozapine in squirrel monkeys: comparison with conventional and novel antipsychotic drugs. *Psychopharmacology (Berl), 132*(3), 261-269.

Carlsson, A., & Lindqvist, M. (1963). Effect of Chlorpromazine or Haloperidol on Formation of 3methoxytyramine and Normetanephrine in Mouse Brain. *Acta Pharmacol Toxicol (Copenh), 20*, 140-144.

Chakos, M., Lieberman, J., Hoffman, E., Bradford, D., & Sheitman, B. (2001). Effectiveness of second-generation antipsychotics in patients with treatment-resistant schizophrenia: a review and meta-analysis of randomized trials. *Am J Psychiatry, 158*(4), 518-526.

Clarke, M. C., Harley, M., & Cannon, M. (2006). The role of obstetric events in schizophrenia. *Schizophr Bull, 32*(1), 3-8.

Colpaert, F. C., & Balster, R. L. (Eds.). (1988). *Transduction mechanisms of drug stimuli*. Berlin: Springer-Verlag.

Colquhoun, D. (1971). *Lectures on Biostatistics: an Introduction to Statistics with Applications in Biology and Medicine*. Oxford: Clarendon Press.

Corrigan, M. H., Gallen, C. C., Bonura, M. L., & Merchant, K. M. (2004). Effectiveness of the selective D4 antagonist sonepiprazole in schizophrenia: a placebo-controlled trial. *Biol Psychiatry, 55*(5), 445-451.

Creese, I., & Snyder, S. H. (1978). Behavioral and biochemical properties of the dopamine receptor. In M. A. Lipton, A. Dimascio & K. F. Killam (Eds.), *Psychopharmacology: A generation of progress* (pp. 377-388). New York: Raven Press.

Crilly, J. (2007). The history of clozapine and its emergence in the US market: a review and analysis. *Hist Psychiatry, 18*(1), 39-60.

Crow, T. J. (1985). The two-syndrome concept: origins and current status. *Schizophr Bull, 11*(3), 471-486.

Davies, M. A., Compton-Toth, B. A., Hufeisen, S. J., Meltzer, H. Y., & Roth, B. L. (2005). The highly efficacious actions of N-desmethylclozapine at muscarinic receptors are unique and not a common property of either typical or atypical antipsychotic drugs: is M1 agonism a pre-requisite for mimicking clozapine's actions? *Psychopharmacology (Berl), 178*(4), 451-460.

Dekeyne, A., Iob, L., & Millan, M. J. (2003). Generalization of clozapine as compared to other antipsychotic agents to a discriminative stimulus elicited by the serotonin (5-HT)2A antagonist, MDL100,907. *Neuropharmacology, 44*(5), 604-615.

Eiermann, B., Engel, G., Johansson, I., Zanger, U. M., & Bertilsson, L. (1997). The involvement of CYP1A2 and CYP3A4 in the metabolism of clozapine. *Br J Clin Pharmacol, 44*(5), 439-446.

Ellenbroek, B. A. (1993). Treatment of schizophrenia: a clinical and preclinical evaluation of neuroleptic drugs. *Pharmacol Ther, 57*(1), 1-78.

Extance, K., & Goudie, A. J. (1981). Inter-animal olfactory cues in operant drug discrimination procedures in rats. *Psychopharmacology (Berl), 73*(4), 363-371.

Flanagan, R. J., Yusufi, B., & Barnes, T. R. (2003). Comparability of whole-blood and plasma clozapine and norclozapine concentrations. *Br J Clin Pharmacol, 56*(1), 135-138.

Frazier, J. A., Cohen, L. G., Jacobsen, L., Grothe, D., Flood, J., Baldessarini, R. J., et al. (2003). Clozapine pharmacokinetics in children and adolescents with childhood-onset schizophrenia. *J Clin Psychopharmacol, 23*(1), 87-91.

Geyer, M. A., Swerdlow, N. R., Mansbach, R. S., & Braff, D. L. (1990). Startle response models of sensorimotor gating and habituation deficits in schizophrenia. *Brain Res Bull, 25*(3), 485-498.

Goas, J. A., & Boston, J. E., Jr. (1978). Discriminative stimulus properties of clozapine and chlorpromazine. *Pharmacol Biochem Behav, 8*(3), 235-241.

Goldstein, A. (1964). *Biostatistics: An introductory text*. New York: McMillan Company.

Goudie, A., & Taylor, A. (1998). Comparative characterisation of the discriminative stimulus properties of clozapine and other antipsychotics in rats. *Psychopharmacology (Berl), 135*(4), 392-400.

Goudie, A. J., Cole, J. C., & Sumnall, H. R. (2007). Olanzapine and JL13 induce cross-tolerance to the clozapine discriminative stimulus in rats. *Behav Pharmacol, 18*(1), 9-17.

Goudie, A. J., Cooper, G. D., Cole, J. C., & Sumnall, H. R. (2007). Cyproheptadine resembles clozapine in vivo following both acute and chronic administration in rats. *J Psychopharmacol, 21*(2), 179-190.

Goudie, A. J., Smith, J. A., Taylor, A., Taylor, M. A., & Tricklebank, M. D. (1998). Discriminative stimulus properties of the atypical neuroleptic clozapine in rats: tests with subtype selective receptor ligands. *Behav Pharmacol, 9*(8), 699-710.

Green, M. F., Kern, R. S., Braff, D. L., & Mintz, J. (2000). Neurocognitive deficits and functional outcome in schizophrenia: are we measuring the "right stuff"? *Schizophr Bull, 26*(1), 119-136.

Green, M. F., Nuechterlein, K. H., Gold, J. M., Barch, D. M., Cohen, J., Essock, S., et al. (2004). Approaching a consensus cognitive battery for clinical trials in schizophrenia: the NIMH-MATRICS conference to select cognitive domains and test criteria. *Biol Psychiatry, 56*(5), 301-307.

Harris, R., & Balster, R. L. (1971). An analysis of the function of drugs in the stimulus control of operant behavior. In T. Thompson & R. Pickens (Eds.), *Stimulus Properties of Drugs*. New York: Meredith.

Hippius, H. (1999). A historical perspective of clozapine. *J Clin Psychiatry, 60 Suppl 12*, 22-23.

Hoenicke, E. M., Vanecek, S. A., & Woods, J. H. (1992). The discriminative stimulus effects of clozapine in pigeons: involvement of 5-hydroxytryptamine1C and 5-hydroxytryptamine2 receptors. *J Pharmacol Exp Ther, 263*(1), 276-284.

Julien, R. M. (2004). *A Primer of Drug Action* (10th ed.). New York: Worth.

Kane, J. M., Honigfeld, G., Singer, J., & Meltzer, H. (1988). Clozapine in treatment-resistant schizophrenics. *Psychopharmacol Bull, 24*(1), 62-67.

Kapur, S., & Seeman, P. (2000). Antipsychotic agents differ in how fast they come off the dopamine D2 receptors. Implications for atypical antipsychotic action. *J Psychiatry Neurosci, 25*(2), 161-166.

Keefe, R. S. (2007). Cognitive deficits in patients with schizophrenia: effects and treatment. *J Clin Psychiatry, 68 Suppl 14*, 8-13.

Keefe, R. S., & Fenton, W. S. (2007). How should DSM-V criteria for schizophrenia include cognitive impairment? *Schizophr Bull, 33*(4), 912-920.

Keefe, R. S. E. (2001). Neurocognition. In A. Breier, P. V. Tran, J. M. Herrea, G. D. Tollefson & F. Bymaster (Eds.), *Current Issues in the Pharmacology of Schizophrenia*. Philadelphia: Lippincott Williams & Wilkins.

Kelley, B. M., & Porter, J. H. (1997). The role of muscarinic cholinergic receptors in the discriminative stimulus properties of clozapine in rats. *Pharmacol Biochem Behav, 57*(4), 707-719.

Khorana, N., Pullagurla, M. R., Young, R., & Glennon, R. A. (2004). Comparison of the discriminative stimulus effects of 3,4-methylenedioxymethamphetamine (MDMA) and cocaine: asymmetric generalization. *Drug Alcohol Depend, 74*(3), 281-287.

Kongsamut, S., Roehr, J. E., Cai, J., Hartman, H. B., Weissensee, P., Kerman, L. L., et al. (1996). Iloperidone binding to human and rat dopamine and 5-HT receptors. *Eur J Pharmacol, 317*(2-3), 417-423.

Kraepelin, E. (1896). *Psychiatrie, Ein Lehrbuch fur Studierende und Arzte*. Leipzig: Johann Ambrosius Barth.

Kuhar, M. J., & Joyce, A. R. (2003). Is the onset of psychoactive drug effects compatible with a protein-synthesis mechanism? *Neuropsychopharmacology, 28 Suppl 1*, S94-97.

Lameh, J., Burstein, E. S., Taylor, E., Weiner, D. M., Vanover, K. E., & Bonhaus, D. W. (2007). Pharmacology of N-desmethylclozapine. *Pharmacol Ther, 115*(2), 223-231.

Laughren, T., & Levin, R. (2006). Food and Drug Administration perspective on negative symptoms in schizophrenia as a target for a drug treatment claim. *Schizophr Bull, 32*(2), 220-222.

Leucht, S., Wahlbeck, K., Hamann, J., & Kissling, W. (2003). New generation antipsychotics versus low-potency conventional antipsychotics: a systematic review and meta-analysis. *Lancet, 361*(9369), 1581-1589.

Lieberman, J. A. (2006). Comparative effectiveness of antipsychotic drugs. A commentary on: Cost Utility Of The Latest Antipsychotic Drugs In Schizophrenia Study (CUtLASS 1) and Clinical Antipsychotic Trials Of Intervention Effectiveness (CATIE). *Arch Gen Psychiatry, 63*(10), 1069-1072.

Mauri, M. C., Volonteri, L. S., Dell'Osso, B., Regispani, F., Papa, P., Baldi, M., et al. (2003). Predictors of clinical outcome in schizophrenic patients responding to clozapine. *J Clin Psychopharmacol, 23*(6), 660-664.

McElroy, J. F., Stimmel, J. J., & O'Donnell, J. M. (1989). Discriminative stimulus properties of haloperidol. *Drug Development Research, 18*, 47-55.

McEvoy, J. P. (2008). Functional outcomes in schizophrenia. *J Clin Psychiatry, 69 Suppl 3*, 20-24.

McNeil, T. F., Cantor-Graae, E., & Ismail, B. (2000). Obstetric complications and congenital malformation in schizophrenia. *Brain Res Brain Res Rev, 31*(2-3), 166-178.

Meltzer, H. Y. (1992). The mechanism of action of clozapine in relation to its clinical advantages. In H. Y. Meltzer (Ed.), *Novel Antipsychotic Drugs*. New York: Raven Press.

Meltzer, H. Y. (1998). Suicide in schizophrenia: risk factors and clozapine treatment. *J Clin Psychiatry, 59 Suppl 3*, 15-20.

Meltzer, H. Y. (2001). Treatment of suicidality in schizophrenia. *Ann N Y Acad Sci, 932*, 44-58; discussion 58-60.

Meltzer, H. Y., Alphs, L., Green, A. I., Altamura, A. C., Anand, R., Bertoldi, A., et al. (2003). Clozapine treatment for suicidality in schizophrenia: International Suicide Prevention Trial (InterSePT). *Arch Gen Psychiatry, 60*(1), 82-91.

Meltzer, H. Y., & Luchins, D. J. (1984). Effect of clozapine in severe tardive dyskinesia: a case report. *J Clin Psychopharmacol, 4*(5), 286-287.

Meltzer, H. Y., Matsubara, S., & Lee, J. C. (1989). Classification of typical and atypical antipsychotic drugs on the basis of dopamine D-1, D-2 and serotonin2 pKi values. *J Pharmacol Exp Ther, 251*(1), 238-246.

Meltzer, H. Y., & McGurk, S. R. (1999). The effects of clozapine, risperidone, and olanzapine on cognitive function in schizophrenia. *Schizophr Bull, 25*(2), 233-255.

Meltzer, H. Y., Thompson, P. A., Lee, M. A., & Ranjan, R. (1996). Neuropsychologic deficits in schizophrenia: relation to social function and effect of antipsychotic drug treatment. *Neuropsychopharmacology, 14*(3 Suppl), 27S-33S.

Millan, M. J., Brocco, M., Gobert, A., Schreiber, R., & Dekeyne, A. (1999). S-16924 [(R)-2-[1-[2-(2,3-dihydro-benzo[1,4]dioxin-5-yloxy)-ethyl]- pyrrolidin-3yl]-1-(4-fluorophenyl)-ethanone], a novel, potential antipsychotic with marked serotonin1A agonist properties: III. Anxiolytic actions in comparison with clozapine and haloperidol. *J Pharmacol Exp Ther, 288*(3), 1002-1014.

Millan, M. J., Iob, L., Peglion, J. L., & Dekeyne, A. (2007). Discriminative stimulus properties of S32504, a novel D3/D2 receptor agonist and antiparkinson agent, in rats: attenuation by the antipsychotics, aripiprazole, bifeprunox, N-desmethylclozapine, and by selective antagonists at dopamine D2 but not D3 receptors. *Psychopharmacology (Berl), 191*(3), 767-782.

Natesan, S., Reckless, G. E., Barlow, K. B., Nobrega, J. N., & Kapur, S. (2007). Evaluation of N-desmethylclozapine as a potential antipsychotic--preclinical studies. *Neuropsychopharmacology, 32*(7), 1540-1549.

Newman-Tancredi, A., Gavaudan, S., Conte, C., Chaput, C., Touzard, M., Verriele, L., et al. (1998). Agonist and antagonist actions of antipsychotic agents at 5-HT1A receptors: a [35S]GTPgammaS binding study. *Eur J Pharmacol, 355*(2-3), 245-256.

Nielsen, E. B. (1988). Cholinergic mediation of the discriminative stimulus properties of clozapine. *Psychopharmacology (Berl), 94*(1), 115-118.

Olesen, O. V., & Linnet, K. (2001). Contributions of five human cytochrome P450 isoforms to the N-demethylation of clozapine in vitro at low and high concentrations. *J Clin Pharmacol, 41*(8), 823-832.

Onali, P., & Olianas, M. C. (2007). N-Desmethylclozapine, a major clozapine metabolite, acts as a selective and efficacious delta-opioid agonist at recombinant and native receptors. *Neuropsychopharmacology, 32*(4), 773-785.

Overton, D. A. (1966). State-dependent learning produced by depressant and atropine-like drugs. *Psychopharmacologia, 10*(1), 6-31.

Overton, D. A. (1982). Comparison of the degree of discriminability of various drugs using the T-maze drug discrimination paradigm. *Psychopharmacology (Berl), 76*(4), 385-395.

Overton, D. A., & Batta, S. K. (1979). Investigation of narcotics and antitussives using drug discrimination techniques. *J Pharmacol Exp Ther, 211*(2), 401-408.

Owen, M. J., Craddock, N., & O'Donovan, M. C. (2005). Schizophrenia: genes at last? *Trends Genet, 21*(9), 518-525.

Perry, P. J., Miller, D. D., Arndt, S. V., & Cadoret, R. J. (1991). Clozapine and norclozapine plasma concentrations and clinical response of treatment-refractory schizophrenic patients. *Am J Psychiatry, 148*(2), 231-235.

Philibin, S. D., Prus, A. J., Pehrson, A. L., & Porter, J. H. (2005). Serotonin receptor mechanisms mediate the discriminative stimulus properties of the atypical antipsychotic clozapine in C57BL/6 mice. *Psychopharmacology (Berl), 180*(1), 49-56.

Philibin, S. D., Walentiny, D. M., Vunck, S. A., Prus, A. J., Meltzer, H. Y., & Porter, J. H. (2009). Further characterization of the discriminative stimulus properties of the atypical antipsychotic drug clozapine in C57BL/6 mice: role of 5-HT(2A) serotonergic and alpha (1) adrenergic antagonism. *Psychopharmacology (Berl), 203*(2), 303-315.

Porter, J. H., Covington, H. E., III, Varvel, S. A., Vann, R. E., & Warren, T. A. (1998). Chlorpromazine as a discriminative stimulus in rats: generalization to typical and atypical antipsychotics. *Drug Development Research, 48*, 38-44.

Porter, J. H., Varvel, S. A., Vann, R. E., Philibin, S. D., & Wise, L. E. (2000). Clozapine discrimination with a low training dose distinguishes atypical from typical antipsychotic drugs in rats. *Psychopharmacology (Berl), 149*(2), 189-193.

Prus, A. J., Baker, L. E., & Meltzer, H. Y. (2004). Discriminative stimulus properties of 1.25 and 5.0 mg/kg doses of clozapine in rats: examination of the role of dopamine, serotonin, and muscarinic receptor mechanisms. *Pharmacol Biochem Behav, 77*(2), 199-208.

Prus, A. J., Pehrson, A. L., Philibin, S. D., Wood, J. T., Vunck, S. A., & Porter, J. H. (2009). The role of M(1) muscarinic cholinergic receptors in the discriminative stimulus properties of N-desmethylclozapine and the atypical antipsychotic drug clozapine in rats. *Psychopharmacology (Berl), 203*(2), 295-301.

Prus, A. J., Philibin, S. D., Pehrson, A. L., & Porter, J. H. (2005). Generalization to atypical antipsychotic drugs depends on training dose in rats trained to discriminate 1.25 mg/kg clozapine versus 5.0 mg/kg clozapine versus vehicle in a three-choice drug discrimination task. *Behav Pharmacol, 16*(7), 511-520.

Prus, A. J., Philibin, S. D., Pehrson, A. L., & Porter, J. H. (2006). Discriminative stimulus properties of the atypical antipsychotic drug clozapine in rats trained to discriminate 1.25 mg/kg clozapine vs. 5.0 mg/kg clozapine vs. vehicle. *Behav Pharmacol, 17*(2), 185-194.

Prus, A. J., Philibin, S. D., Pehrson, A. L., Stephens, C. L., Cooper, R. N., Wise, L. E., et al. (2005). Generalization testing with atypical and typical antipsychotic drugs in rats trained to discriminate 5.0 mg/kg clozapine from vehicle in a two-choice drug discrimination task *Drug Development Research, 64*, 55-65.

Rosenheck, R. A., Leslie, D. L., Sindelar, J., Miller, E. A., Lin, H., Stroup, T. S., et al. (2006). Cost-effectiveness of second-generation antipsychotics and perphenazine in a randomized trial of treatment for chronic schizophrenia. *Am J Psychiatry, 163*(12), 2080-2089.

Saha, S., Chant, D., Welham, J., & McGrath, J. (2005). A systematic review of the prevalence of schizophrenia. *PLoS Med, 2*(5), e141.

Schechter, M. D., & Rosecrans, J. A. (1971). C.N.S. effect of nicotine as the discriminative stimulus for the rat in a T-maze. *Life Sci I, 10*(14), 821-832.

Schotte, A., Janssen, P. F., Gommeren, W., Luyten, W. H., Van Gompel, P., Lesage, A. S., et al. (1996). Risperidone compared with new and reference antipsychotic drugs: in vitro and in vivo receptor binding. *Psychopharmacology (Berl), 124*(1-2), 57-73.

Schuster, C. R., & Balster, R. L. (1977). The discriminative stimulus properties of drugs. In T. Thompson & P. B. Dews (Eds.), *Advances in Behavioral Pharmacology* (Vol. 1, pp. 85-138).

Seeman, P., Lee, T., Chau-Wong, M., & Wong, K. (1976). Antipsychotic drug doses and neuroleptic/dopamine receptors. *Nature, 261*(5562), 717-719.

Shannon, H. E., & Holtzman, S. G. (1977). Discriminative effects of morphine administered intracerebrally in the rat. *Life Sci, 21*(4), 585-594.

Stewart, J. (1962). Differential responses based on the physiological consequences of pharmacological agents. *Psychopharmacologia, 3*, 132-138.

Sur, C., Mallorga, P. J., Wittmann, M., Jacobson, M. A., Pascarella, D., Williams, J. B., et al. (2003). N-desmethylclozapine, an allosteric agonist at muscarinic 1 receptor, potentiates N-methyl-D-aspartate receptor activity. *Proc Natl Acad Sci U S A, 100*(23), 13674-13679.

Takata, Y., Kurihara, J., Yoda, T., Suzuki, S., Matsuoka, Y., Okubo, Y., et al. (2001). KT3-671, an angiotensin AT1 receptor antagonist, attenuates vascular but not cardiac responses to sympathetic nerve stimulation in pithed rats. *J Cardiovasc Pharmacol, 37*(4), 427-436.

Thompson, T. (Ed.). (1977). *Advances in Behavioral Pharmacology* (Vol. 1). New York: Academic Press.

Toda, M., & Abi-Dargham, A. (2007). Dopamine hypothesis of schizophrenia: making sense of it all. *Curr Psychiatry Rep, 9*(4), 329-336.

van Rossum, J. M. (1966). The significance of dopamine-receptor blockade for the mechanism of action of neuroleptic drugs. *Arch Int Pharmacodyn Ther, 160*(2), 492-494.

Varvel, S. A., Vann, R. E., Wise, L. E., Philibin, S. D., & Porter, J. H. (2002). Effects of antipsychotic drugs on operant responding after acute and repeated administration. *Psychopharmacology (Berl), 160*(2), 182-191.

Walker, E. F., Harvey, P. D., & Perlman, D. (1988). The positive/negative symptom distinction in psychoses. A replication and extension of previous findings. *J Nerv Ment Dis, 176*(6), 359-363.

Weiner, D. M., Burstein, E. S., Nash, N., Croston, G. E., Currier, E. A., Vanover, K. E., et al. (2001). 5-hydroxytryptamine2A receptor inverse agonists as antipsychotics. *J Pharmacol Exp Ther, 299*(1), 268-276.

Weiner, D. M., Meltzer, H. Y., Veinbergs, I., Donohue, E. M., Spalding, T. A., Smith, T. T., et al. (2004). The role of M1 muscarinic receptor agonism of N-desmethylclozapine in the unique clinical effects of clozapine. *Psychopharmacology (Berl), 177*(1-2), 207-216.

Wiley, J. L., & Porter, J. H. (1992). Serotonergic drugs do not substitute for clozapine in clozapine-trained rats in a two-lever drug discrimination procedure. *Pharmacol Biochem Behav, 43*(3), 961-965.

Wiley, J. L., & Porter, J. H. (1993). Effects of serotonergic drugs in rats trained to discriminate clozapine from haloperidol. *Bulletin of the Psychonomic Society, 31*, 94-96.

Zeidman, H., Oscherwitz, M., & Addario, D. (1975). Amphetamine psychosis or paranoid schizophrenia? *West J Med, 122*(5), 394-405.

Vita

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