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A COMPARISON OF THE DISCRIMINATIVE STIMULUS PROPERTIES OF THE ATYPICAL ANTIPSYCHOTIC CLOZAPINE AND THE GLUTAMATE AGONIST N-METHYL D-ASPARTATE IN C57BL/6 MICE.

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

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The glutamate system dysfunctions present in schizophrenia raise new questions about possible glutamatergic actions of the atypical antipsychotic clozapine. While clozapine has been shown to partially substitute for the discriminative stimulus of the glutamate agonist N-methyl D-aspartate (NMDA) in rats, NMDA discrimination has not previously been established in mice. The present study was designed to explore the possible role of NMDA activity in clozapine's discriminative stimulus. Two groups of C57BL/6 mice were trained to discriminate either 2.5 mg/kg CLZ from vehicle or 30 mg/kg NMDA from vehicle in a standard two-lever drug discrimination task. NMDA drug discrimination was successfully

established in C57BL/6 mice. While NMDA did not substitute for clozapine, clozapine partially substituted for NMDA at the 0.625 mg/kg dose, demonstrating an asymmetrical relationship between clozapine's and NMDA's discriminative stimuli. Dose combination tests further investigated this relationship. It was found that 0.625 mg/kg CLZ + 30 mg/kg NMDA produced partial substitution (61.82% DLR), while 0.625 mg/kg CLZ + 56 mg/kg NDMA full substitution (92.82% DLR) in CLZ-trained mice. In addition, combination testing with 10 mg/kg NMDA + 2.5 mg/kg CLZ and 10 mg/kg NMDA + 5.0 mg/kg CLZ produced full substitution in NMDA-trained mice ((80.04% DLR and 100% DLR, respectively). Finally, it was found that the α_1- adrenoreceptor antagonist prazosin fully substituted for both CLZ (3.0 mg/kg = 92.20% DLR) and NMDA (1.0 mg/kg = 98.77% DLR and 3.0 mg/kg = 99.62% DLR). These findings suggest that interactions between clozapine's and NMDA's discriminative stimuli may involve antagonism of α_1-adrenoreceptors, but further research of other mechanisms including serotonergic, histaminergic, and cholinergic receptor activity or metabolic interactions is needed. Finally, these initial finding suggest that that drugs active at glutamatergic receptors may have potential as therapeutic drugs for treatment of schizophrenia.

Introduction

Schizophrenia is a debilitating brain disorder that affects approximately 0.7% of the world's population (Saha, Chant, Welham, & McGrath, 2005). The disorder is complex with a range of symptoms that manifest diversely in the affected population. Schizophrenia occurs equally between the sexes and has an onset in early adulthood with rare cases occurring in late adolescence. While recent studies link specific genes to schizophrenia (Owen, Craddock, & O'Donovan, 2005) the genetic component is not absolute but rather may be an indicator of the predisposition for the disorder (Gottesman & Gould, 2003) suggesting that environmental and/or ecological factors may play an important role in the etiology of schizophrenia. Schizophrenia is a progressively deteriorating condition with a mortality rate of 2-3 times higher than the general population, with two-thirds of those excess deaths attributed to suicide (Auquier, Lancon, Rouillon, & Lader, 2007; Brown, 1997).

Characterizations of the contemporary symptomology of the disorder began with Emil Kraeplin (1896) who called the syndrome Dementia Praecox emphasizing the early adulthood onset of symptoms and the deterioration of thought processes. The term schizophrenia was coined by Eugene Bleuler (1911) from the Greek words *schizein* (which means split) and *phren* (which means mind), referring to the separation between mind and emotion.

Symptomology of Schizophrenia

According to the Diagnostic and Statistical Manual of Mental Disorders IV text revision (DSMIV-TR) the symptomology of schizophrenia is characterized by two broad symptom categories: positive (added or disproportionate occurrence with the disease) and negative (normal occurrence is deteriorated) symptoms. However, there is increasing research that supports adding cognitive dysfunction as another core set of symptoms (Goldman-Rakic, 1994; Joyce & Roiser, 2007; Keefe & Fenton, 2007). Positive symptoms include: delusions (flawed beliefs involving distortion of perception or experiences), hallucinations (including auditory, gustatory, visual, olfactory and tactile), disorganized speech (formal thought disorder), and grossly disorganized or catatonic behavior (self-monitoring of behavior). Furthermore these positive symptoms fall under two distinct dimensions, psychotic dimension and the disorganized dimension that may have separate neural basis. Hallucinations (most commonly auditory) and delusions fall under the psychotic dimension while disorganized speech and behavior fall under the disorganized dimension.

Delusions are non-logical beliefs held by the patient that cannot be changed even after the patient has been shown logical proof that their beliefs are not true. These delusions are frequently bizarre and can include beliefs that persons in movies or television are sending messages to them specifically. They can also believe that they are a famous historical figure or some special messiah (delusions of grandeur). Those with paranoid delusions believe that others are out to get them or persecute them in some way. Hallucinations are sensory experiences felt by the patient that are not able to be

experienced by anyone else. Hearing voices is widespread in the patient population, and these voices are most frequently negative in content and may comment on the person's behavior, converse between themselves (usually about the patient), or warn of impending doom or danger. These hallucinations may also include the other senses like seeing nonexistent persons or objects, feeling phantom touches, or even smelling odors not present. Another positive symptom is unusual thought processes. One striking form is disorganized thinking, the person would have great difficulty organizing their thoughts or connecting them in a logical way. Their speech may be jumbled and hard to understand. Another form is "thought blocking," in which the person stops abruptly in the middle of a thought. The patient would explain this by saying the thought had been snatched out of their head. Also the patient may make up words or "neologisms" that only make sense to them. Finally, positive symptoms also include disorganized or catatonic behavior. Patients are often clumsy or uncoordinated in their movements. They may also have involuntary movements like facial grimaces or muscle tics. They may also have difficulty initiating behaviors and in some cases cease movement altogether (catatonia). Catatonia is now extremely rare and occurred mostly before effective neuroleptics were available (Weder, Muralee, Penland, & Tampi, 2008).

Negative symptoms include flattened affect (a reduction in range and intensity of emotion), alogia (a reduction in the fluency and productivity of language and thought), anhedonia (an inability to feel pleasure), and avolition (lack of initiation of goal directed behavior). Flattened affect is a difficulty in expressing emotion in the correct facial and vocal context as well as an inability to control the intensity of that expression. A patient

may say they are very sad about something but laugh and smile while saying this. Patients may also have difficulty in expressing in words any idea or thought, and can resist verbal interaction even if initiated by others (alogia). Another negative symptom is the lack of pleasure or enjoyment that comes from everyday experiences (anhedonia).

Most patients with schizophrenia have some form of cognitive deficit (Meltzer, Thompson, Lee, & Ranjan, 1996). Cognitive symptoms of schizophrenia include severe impairments (2-3 standard deviations [SD] below mean) in verbal memory, executive functioning, vigilance, motor speed and verbal fluency. There are also moderate impairments (1-2 SD below the mean) in distractibility, delayed recall, visuo-motor skills, immediate memory span, and working memory. In addition there are mild impairments (0.5-1 SD below the mean) in perceptual skills, delayed recognition memory, confrontation naming, and verbal and full scale IQ (Keefe, 2007). These cognitive deficits are being recognized as an essential indicator of functional outcomes in those diagnosed with schizophrenia (McEvoy, 2008). The National Institute of Mental Health (NIMH) has recognized the importance of cognitive deficits in Schizophrenia and has established MATRICS™ (Measurement and Treatment Research to Improve Cognition in Schizophrenia) to better understand and approach diagnosis and treatment of cognitive deficits in Schizophrenia (Green et al., 2004).

Pharmacology and Pharmacotherapy of Schizophrenia

Pharmacological treatment using first generation or typical antipsychotics started with the development of chlorpromazine in the early 1950's. This revolutionized patient treatment as most patients before the availability of drug intervention were

institutionalized. The development of the phenothiazine chlorpromazine revolutionized the mental health industry and helped initiate the field of behavioral pharmacology (Thompson, 1977). Further development of typical antipsychotics, such as haloperidol (a butyropherone) relied heavily on Dopamine (DA) antagonist action, specifically postsynaptic blocking of the D_2 receptor subtype. This was further evidenced in their ability to cause extrapyramidal motor side-effects (EPS) (Carlsson & Lindqvist, 1963). At first these side-effects were seen as proof that the drugs were working. However, these motor tremors and slurred speech were eventually identified as undesirable side-effects. These first antipsychotic drugs functioned based on their ability to reduce dopaminergic activity in the mesolimbic and mesostriatal pathways. The pharmacological evidence of clinical efficacy was the basis for the Dopamine Hypothesis that was supported by numerous methods of study, from imaging to postmortem (Honey et al., 1999; Seeman, 1987). However, the pathology was more complex than simply a hyper-dopaminergic system. Currently this hypothesis has been refined to include more complex relationships between multiple DA pathways, and not just hyperactivity of DA. The revised hypothesis of DA in schizophrenia postulates that this disorder is associated with excess DA activity in the subcortical mesolimbic projections (associated with positive symptoms) together with hypoactivity in mesocortical DA projections to the prefrontal cortex (associated with negative symptoms including cognitive deficits) (Toda & Abi-Dargham, 2007).

Pharmacotherapy of schizophrenia remained tied to this theory until the development of clozapine, the first atypical APD. Clozapine was first synthesized by Wander Laboratories in 1958, but was not used as an antipsychotic until 1971. Shortly

after its introduction in Europe (1974) several patients died as a result of agranulocytosis and it was pulled from the market and other clinical trials suspended. Clozapine was not reintroduced until two trials in 1988 showed efficacy in treatment of refractory patients. The FDA approved clozapine for treatment of treatment resistant schizophrenia in 1990 (Hippius, 1999). Atypical antipsychotic drugs such as clozapine display a diverse mechanism of action at numerous receptors with less activity at DA receptors, as well as a reduced liability for extrapyramidal side effects at clinically prescribed doses compared to typical antipsychotics. Some have argued that one method of classification between typical and atypical antipsychotic drugs should be based on serotonergic activity (Meltzer, Matsubara, & Lee, 1989a, 1989b). However, some atypical antipsychotic drugs do not have serotonergic activity similar to clozapine, like aripiprazole that displays some partial agonism at D_2 and 5-HT_{1A} receptors with potent antagonism at 5-HT_{2A} receptors (Hirose et al., 2004; Taylor, 2003). Further characterization is needed to fully understand the mechanisms of action required for antipsychotic action.

Glutamate Hypothesis of Schizophrenia

The dominant hypothesis in schizophrenia as previously mentioned is the Dopamine Hypothesis, and current pharmacotherapies have reflected this emphasis. Recently however, more research (Coyle, 2006; Goff & Coyle, 2001; Ibrahim et al., 2000; Meador-Woodruff & Healy, 2000; G. Tsai & Coyle, 2002) has focused on the role of glutamate (GLU) in schizophrenia and a Glutamate Hypothesis is now gaining research attention. Some reasoning GLU has been explored is due to the ability of NMDA antagonists such as ketamine and phencyclidine to induce psychotic-like positive, negative,

and cognitive symptoms in non-schizophrenic humans (Itil, Keskiner, Kiremitci, & Holden, 1967; Krystal et al., 1994; Luby, Cohen, Rosenbaum, Gottlieb, & Kelley, 1959). An additional reason is that GLU is the most common excitatory cortical neurotransmitter. Due to the specific dysfunctions of the disorder, the GLU pathways are most likely involved in the pathology of the disease (Carlsson, Hansson, Waters, & Carlsson, 1997). Multiple subtypes of glutamate receptors are involved in the pathology of schizophrenia. Many deficits, according to this hypothesis, are explained by the disproportionate sensitivity of a specific subpopulation of NMDA receptors, located on cortico-limbic GABAergic interneurons, to NMDA antagonists (Coyle, Tsai, & Goff, 2003; G. Tsai & Coyle, 2002), which is supported by neurophysiologic studies and the cortical disinhibition seen upon the administration of ketamine at subanesthetic doses (Krystal et al., 1994). This cortical disinhibition is also supported by imaging studies (Weinberger & Gallhofer, 1997). Additional reasoning behind this hypothesis is the interaction of DA and GLU neurons in diverse brain areas. Some of those interactions are inhibitory DA action on GLU release, GLU stimulation of neurons that inhibit DA release, and GLU excitatory action on neurons that DA inhibits. Antipsychotics that are DA antagonists indirectly increase GLU levels (Collier & Li, 2003; Lang, Puls, Muller, Strutz-Seebohm, & Gallinat, 2007; Mehler-Wex & Renner, 2008; Mehler-Wex, Riederer, & Gerlach, 2006; Moghaddam, Adams, Verma, & Daly, 1997; Stone, Morrison, & Pilowsky, 2007).

Drug Discrimination

Drugs can function in a variety of ways to control behavior, discriminative, reinforcing, punishing, and unconditioned stimuli (Schuster & Balster, 1977). Drugs have unique

interoceptive effects that can be used as discriminative stimuli in an operant paradigm to distinguish different aspects of a drug's pharmacological profile (Overton, 1966). Overton (1966) defined operant drug discrimination as when the specific feeling that occurs in a lab animal's internal environment is used to cue them to make a certain operant response. In drug discrimination with rodents the interoceptive effects involved in a certain stimulus are often associated with an action like pushing a lever or making a directional choice in a maze. In the operant version of drug discrimination a drug at a specific dose is associated with one operant lever (training drug) and vehicle (an inactive substance) is associated with another lever. This allows the researcher to interpret the responses on those levers during testing as being like the training drug or not being like the training drug (Harris & Balster, 1971). Determining dose effects, generalization curves, or stimulus gradients are common characterizations that this paradigm is used for. Clozapine drug discrimination has been established in many animal species including rats (Goas & Boston, 1978; Prus, Baker, & Meltzer, 2004), monkeys (Carey & Bergman, 1997a), pigeons (Hoenicke, Vanecek, & Woods, 1992) and most recently mice (Philibin, Prus, Pehrson, & Porter, 2005).

NMDA drug discrimination has been conducted in rats (Amrick & Bennett, 1987; Arnt, Sanchez, Lenz, Madsen, & Krogsgaard-Larsen, 1995; Balster, 1989; Willetts & Balster, 1989) and in pigeons and non-human primates (Baron, Butelman, & Woods, 1993). Drug discrimination has been used with ligands that act selectively on specific receptors to further classify the pharmacological properties of a drug's stimulus effects such as onset and duration of activity, structure-activity relations, mechanism of action,

tolerance and withdrawal, activity of metabolites, identification and development of potential antagonists and similarity of effect to other agents. Drugs that belong to the same class substitute for each other in the drug discrimination paradigm, for instance amphetamine and cathinone cross-generalize to each other's stimulus cues (Stolerman & D'Mello, 1981). This implies that compounds that share discriminative stimulus properties with the training drug could have similar pharmacological actions (Brady & Balster, 1981). This has led to the use of drug discrimination in the study of drugs of abuse in an effort to look for the action of addiction and for developing new drug treatments (Holtzman, 1990). Drug discrimination is also used as a preclinical assay to study existing drug treatments and to aid in the development of new therapeutic drugs for many other psychological diseases including schizophrenia. Previous studies have used clozapine drug discrimination to compare the discriminative effects of clozapine to other atypical antipsychotic drugs (Goas & Boston, 1978; Overton, 1982; Philibin et al., 2005; Porter, Varvel, Vann, Philibin, & Wise, 2000; Prus et al., 2004; Prus, Philibin, Pehrson, & Porter, 2005, 2006). In understanding the effects of clozapine in relation to other atypical antipsychotic drugs we may gain key insights into the mechanisms of action required for antipsychotic action as well as possibly identify receptor subtype targets for future drug development (Ortmann et al., 1986).

Discriminative Stimulus of Clozapine

Clozapine is often referred to as the gold standard of atypical antipsychotic drugs due to its superior clinical efficacy. Research suggests that the cue is complex and may require multiple coordinated receptor actions. The precise receptor subtypes necessary to the cue

have yet to be determined, which may be due in part to its multi-receptor binding profile (i.e., high binding affinity for serotonergic, dopaminergic, adrenergic, muscarinic and histaminergic receptors).

One of the first drug discrimination studies to use clozapine as a training drug in a two lever operant paradigm was by Goas and Boston (1978). They trained two different groups of rats, one to discriminate 6.0 mg/kg clozapine (oral) from saline. Several classes of compounds chlorpromazine, haloperidol, chlordiazepoxide and atropine failed to substitute for clozapine. A second group of rats was trained to discriminate 8.0 mg/kg clozapine from 4.25 mg/kg chlorpromazine (both oral), and haloperidol substituted for chlorpromazine (Goas & Boston, 1978). Using a t-maze paradigm instead of two-lever operant, Overton (1982) trained rats to discriminate 20 mg/kg clozapine from 2.5 mg/kg haloperidol (both intraperitoneal). However, in using a t-maze only choice can be recorded with no gradation of dose or partial substitution (Overton, 1982).

In pigeons trained to discriminate 1.0 mg/kg clozapine (intramuscular), antagonists of 5-HT_{2A} and 5-HT_{2C} receptors (cyproheptadine, metergoline, fluperlapine, mianserin and pizotifen) substituted for the CLZ cue in all animals (Hoenicke et al., 1992). However, these results may be species specific as they have not been repeated in rats. Previous research from our lab in rats trained to discriminate clozapine vs. vehicle (J. L. Wiley & Porter, 1992) and clozapine vs. haloperidol (Wiley & Porter, 1993), all delivered intraperitoneally, showed ritanserin, a 5-$HT_{2A/B/C}$ antagonist failed to substitute for clozapine. However, in C57BL/6 mice trained to discriminate 2.5 mg/kg (subcutaneous)

clozapine from vehicle, serotonin $(5\text{-HT})_{2A/2B/2C}$ antagonist ritanserin fully substituted for CLZ with an ED_{50} = 2.08 mg/kg (Philibin et al., 2005).

Dopamine blockade alone by specific ligands does not engender clozapine responding in rats as evidenced by the failure of the dopamine D_1 receptor antagonist SCH 23390 to substitute for clozapine in rats (Franklin & Tang, 1994; Goudie, Smith, Taylor, Taylor, & Tricklebank, 1998; Porter, Villanueva, & Rosecrans, 1999) and in pigeons (Hoenicke et al., 1992). Clozapine responding is also not engendered by D_2 antagonists (Browne & Koe, 1982; Franklin & Tang, 1994; Goas & Boston, 1978; Goudie et al., 1998; Porter et al., 1999; Tang, Franklin, Himes, Smith, & Tenbrink, 1997; Villanueva, Arezo, & Rosecrans, 1992; J. Wiley & Porter, 1993) or in squirrel monkeys (Carey & Bergman, 1997b) nor by D_4 and D_3 antagonists in rats (Goudie et al., 2001; Goudie et al., 1998). In C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine (subcutaneous) from vehicle the DA_2 antagonist and typical antipsychotic haloperidol did not substitute for clozapine, producing a maximum of 51.6% DLR at the 0.2 mg/kg dose (subcutaneous) of haloperidol (Philibin et al., 2005).

Many atypical antipsychotics generalize to the clozapine cue as is evidenced by previous research from our lab that shows olanzapine, quetiapine and ziprasidone fully substitute in rats trained to discriminate 5.0 mg/kg clozapine vs. vehicle delivered intraperitoneal (Prus et al., 2005). Typical antipsychotics chlorpromazine, fluphenazine and perphenazine did not substitute, while the atypical antipsychotics risperidone and sertindole produced partial substitution for clozapine with 60-79% clozapine-lever responding (Prus et al., 2005). However, it should be noted that in rats trained to

discriminate 1.25 mg/kg clozapine vs. vehicle (intraperitoneal) sertindole and risperidone fully substituted for the clozapine cue (Porter et al., 2000). In C57BL/6 mice trained to discriminate 2.5 mg/kg (subcutaneous) from vehicle olanzapine produced full substitution for CLZ at the 1.0 mg/kg (87.3% DLR) and 2.0 mg/kg (86.7% DLR) doses. Risperidone also fully substituted for CLZ at two doses with 86.1% DLR at 0.25 mg/kg and 95.0% DLR at 0.50 mg/kg. Ziprasidone produced full substitution for CLZ at the 1.0 mg/kg (83.3% DLR) and 2.0 mg/kg (93.6% DLR) doses (Philibin et al., 2005).

Pyrilamine, an H_1 histaminergic antagonist, fails to substitute for clozapine in rats, intraperitoneal (Goudie et al. 1998) or pigeons, intramuscular (Hoenicke et al. 1992). But when H_1 antagonists that also have antagonist properties at multiple 5-HT and muscarinic sites (promethazine and cyproheptadine) are tested they fully substitute for clozapine in rats, intraperitoneal (Kelley & Porter, 1997). Adrenergic antagonists fail to substitute clozapine in rats, intraperitoneal (Goudie et al., 1998; Kelley & Porter, 1997; Nielsen, 1988) pigeons, intramuscular (Hoenicke et al. 1992), but not in C57BL/6 mice, subcutaneous (Porter, Walentiny, Philibin, Vunck, & Crabbe, 2008).

Discriminative Stimulus of NMDA

The discriminative properties of NMDA have been explored in rats (Amrick & Bennett, 1987; Willetts & Balster, 1989), and in pigeons and non-human primates (Baron et al., 1993) but not in mice. Many NMDA antagonists have been characterized via drug discrimination as well (Balster, 1989; Holter, Danysz, & Spanagel, 2000; Hundt, Danysz, Holter, & Spanagel, 1998; Koek, 1999; Koek, Woods, & Colpaert, 1990; Medvedev, Dravolina, & Bespalov, 1998; Willetts, Bobelis, & Balster, 1989). A study by Amrick and

Bennett (1987) found that rats learned to discriminate 30 mg/kg NMDA from saline in approximately 45 sessions, with a 60% successful training rate. The generalization curve was dose dependant with an ED_{50} value = 13.6 mg/kg (intraperitoneal delivery). The specific NMDA receptor antagonist, 3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), intraperitoneal, blocked NMDA-induced discriminative stimuli confirming that the NMDA cue was mediated by activation of NMDA receptors (Amrick & Bennett, 1987). In a second comparison study it was found that ketamine and dexoxadrol, both NMDA antagonists, blocked the stimulus properties of NMDA without behavioral interference (Bennett, Bernard, & Amrick, 1988).

Grech, Lunn, & Balster (1995) trained rats to discriminate 30 mg/kg NMDA, via intraperitoneal delivery, from saline and investigated the discriminative stimulus properties of a number of excitatory amino acids in this assay. LY 285265 produced full substitution at a dose of 0.3 mg/kg (> 95% NMDA-lever responding) in all subjects, with an ED_{50} value of 0.17 mg/kg. Complete rate suppression occurred at the next highest dose tested 1.0 mg/kg (Grech, Lunn, & Balster, 1995). Generalization testing with L-glutamate (30-560 mg/kg) was also completed and resulted in partial substitution for NMDA, producing a maximum of 59% NMDA-lever responding. Partial substitution was also observed with monosodium glutamate (100-3000 mg/kg), which produced a maximum of 49% NMDA-lever responding, with partial rate suppression at the 3000 mg/kg dose. Higher dose testing was not completed in all animals after two deaths at the 6000 mg/kg dose. L-cysteine failed to substitute for NMDA with a maximum of 33% NMDA-lever responding. L-cysteine at l000 mg/kg produced rate suppressant effects. L-Homocysteic acid (100-1500

mg/kg) achieved partial substitution for NMDA, producing maximum values of 61-67% NMDA-lever responding at doses of 1000 and 560 mg/kg, respectively. Rate suppression occurred at the 1500 mg/kg dose. Kainic acid (0.1-3 mg/kg) was tested in five subjects, and produced <40% NMDA-lever responding at a single dose of 1 mg/kg, three subjects were tested at a 5.6 mg/kg dose which generated lethality so no further testing was completed (Grech et al., 1995). Many of the amino acid compounds did not produce behavioral effects even up to lethal doses. L-Aspartate (30-300 mg/kg) also failed to substitute for NMDA, producing <17% NMDA-lever responding. No rate suppressant effects were observed.

The stimulus effects of competitive vs. noncompetitive antagonists of NMDA differed in a study in which rats were trained to discriminate 30 mg/kg NMDA from saline, with an ED_{50} of 17.1 mg/kg (Willetts & Balster, 1989). PCP and (+)-NANM (noncompetitive antagonists) failed to antagonize the cue even at doses that affected response rates. MK-801, pentobarbital, and diazepam mildly antagonized NMDA responding but did not fully block NMDA's discriminative cue even at rate disruptive doses (Willetts & Balster, 1989). The competitive antagonists NPP and NPC12626 completely antagonized the NMDA cue and when NPP and NPC 12626 (as well as CGS 19755) were tested alone they surprisingly produced NMDA like responding at close to generalization levels though only in four of the eight animals (Willetts & Balster, 1989). In a later study (Balster, Grech, & Bobelis, 1992) ethanol failed to antagonize NMDA stimulus effects though it had been shown to produce similar stimulus effects as other NMDA antagonists (Balster et al., 1992).

In rats trained to discriminate 20 mg/kg pentobarbital, intraperitoneal, stimulus properties of NMDA antagonists were evaluated. The competitive N-methyl-D-aspartate (NMDA) antagonist 3−[(±)–2-carboxypiperazin-4-yl] propyl-1-phosphonic acid (CPP) substituted for pentobarbital. The uncompetitive NMDA antagonist, dizocilpine (MK-801) partially substituted for pentobarbital (Willetts, Tokarz, & Balster, 1991). Additional characterizations of the affects of competitive and uncompetitive antagonists on the stimulus effects of NMDA have been studied in rats trained to discriminate 40 mg/kg NMDA, intraperitoneal, from saline (Koek et al., 1990). The findings were similar to those of Amrick & Bennett (1987) as CPP and CGS 19755 blocked the NMDA cue. Similar to findings in the Willets and Balster (1989) study CGS 19755 produced NMDA responding when tested alone. Additionally ketamine (in contrast to previous studies) did not fully block the NMDA cue, while PCP and MK-801 partially blocked the cue as in previous studies (Koek et al., 1990).

Swiss-Webster mice were trained to discriminate the uncompetitive NMDA receptor antagonist, dizocilpine 0.17 mg/kg, subcutaneous, from saline in a T-maze. Several uncompetitive antagonists substituted for dizocilpine: TCP, SKF 10,047, dextrorphan, and PCP. While, competitive NMDA antagonists CGS 19755, NPC 17742, (+/-)CPP and LY 233536 also substituted for dizocilpine suggesting similarities in the stimulus properties of competitive and noncompetitive NMDA antagonists (Geter-Douglass & Witkin, 1997).

In a study by Baron and Woods (1989) Pigeons were trained to discriminate 0.64 mg/kg (intramuscular) of phencyclidine (an uncompetitive NMDA antagonist) vs. vehicle.

Other uncompetitive antagonists MK-801, etoxadrol, and dexoxadrol generalized to the phencyclidine cue while competitive antagonists AP-5 and AP-7 did not generalize (Baron & Woods, 1989).

However, not all NMDA drug discrimination studies have found clear pharmacological specificity. Baron et al. (1993) trained both pigeons (intramuscular) and monkeys (subcutaneous) to discriminate NMDA (5.6 mg/kg NMDA for both) from saline. Similarly to previous studies PCP failed to fully antagonize NMDA, but unlike previous studies CGS 19755 did not engender NMDA responding. However, kainite, AMPA, morphine, pentobarbital, and d-amphetamine generalized to the NMDA cue in at least half of the pigeons. As these drugs are from several different drug classes, this suggests that perhaps this particular study fell short of establishing pharmacological specificity in the drug discrimination (Baron et al., 1993).

A study involving the specificity of NMDA drug discrimination (Grech, Willetts, & Balster, 1993) in which rats were trained to discriminate 30 mg/kg NDMA, intraperitoneal, from saline sought to clarify drug class differences in this assay. Various pharmacological agents from many drug classes were tested. Caffeine and amphetamine, both stimulants, did not substitute for NMDA. Additionally, morphine (an opiate), pentylenetetrazol (an epileptegenic) and picrotoxin (a GABA antagonist) did not substitute for NMDA. The cholinergic drugs nicotine (nicotinic agonist), physostigmine (a reversible cholinesterase inhibitor), arecoline (muscarinic agonist) and mecamylamine (nicotinic antagonist), produced only low partial NMDA responding and only at doses that had rate suppressing effects (Grech et al., 1993).

Comparisons of the AMPA agonist ATPA to NMDA in a drug discrimination study with two groups of rats trained to discriminate 40mg/kg NMDA or 5 mg/kg ATPA, both intraperitoneal, from saline found that the two drugs did not generalize to each other. The NMDA agonists (RS)-tetrazol-5-yl-glycine and AMAA both generalized to the NMDA cue in rats trained to discriminate 40 mg/kg NMDA, intraperitoneal (Arnt et al., 1995)

Compounds that bind to the glycine modulatory site on the NMDA receptor also have been evaluated via drug discrimination. The two novel quinoxalinedone glutamatergic antagonists ACEA-1011 and ACEA-1021 that have *in vitro* selectivity for the glycine modulatory site fail to block the NMDA stimulus cue in rats trained to discriminate 30 mg/kg NMDA, intraperitoneal, from saline (Balster et al., 1995). Also, in a study investigating lead exposure effects on stimulus properties of NMDA rats were trained to discriminate 30 mg/kg NMDA, intraperitoneal, after exposure to 0, 50 or 150 ppm lead acetate in drinking water post-weaning (Cory-Slechta, Pokora, & Johnson, 1996). The D_2 antagonist spiperone achieved full substitution and the D_1 antagonist SCH23390 produced partial substitution (Cory-Slechta et al., 1996).

In a study investigating α_1-adrenoreceptors one group of rats were trained to discriminate 30 mg/kg NMDA, intraperitoneal, from saline. The atypical antipsychotics clozapine and sertindole both engender partial substitution. The α_1-adrenoreceptor antagonists prazosin and WB 4101 fully substitute for NMDA (Arnt, 1997).

Rationale

Although the Dopamine Hypothesis remains dominant in the explanations of schizophrenia; it does not fully explain the complexities of the disorder. In addition to abnormalities in brain DA systems there are abnormalities in glutamatergic systems, which have led to the Glutamate Hypothesis. The interplay between dopamine and glutamate systems suggested by recent research implies that a combination of the two hypotheses may be a more accurate portrayal of the disorder. Therefore it is important to understand the interactions between these two systems. One approach to understanding these interactions on a behavioral level is drug discrimination.

Previous research in this lab (Kelley & Porter, 1997) found that NMDA failed to substitute for clozapine in rats. However, when a T-maze drug discrimination task was used, NMDA substituted for clozapine (Schmidt & Volz, 1992). Arnt (1997) in research with α_1 antagonists showed partial substitution of clozapine and full substitution of prazosin, an α_1-adrenoresptor antagonist, in 30 mg/kg NMDA-trained rats, even though neither clozapine or prazosin has been shown to have an affinity for NMDA receptors. In clozapine (1.25 and 5.0 mg/kg) trained rats prazosin does not substitute for the clozapine cue (Prus et al., 2006). However, recently published data from this lab (Porter et al., 2008) showed that prazosin produced full substitution in C57BL/6 mice trained to discriminate 2.5 mg/kg CLZ. This suggests that α_1-adrenoresptor antagonism may play a role in the stimulus cue of NMDA in C57BL/6 mice. Although some NMDA antagonists have been

trained as discriminative stimuli in mice, NMDA has never been trained as the discriminative stimulus in mice. I propose to train two groups of C57BL/6 mice, one to discriminate NMDA from vehicle and another to discriminate CLZ from vehicle. Generalization curves will be generated for both training drugs in both groups to ascertain if there is any cross-generalization between CLZ and NMDA. Further comparisons between the two compounds using selective ligands will elucidate the similarities and differences between the discriminative stimulus properties of NMDA and CLZ.

Methods

Animals

Twenty four adult male C57BL/6NHsd wild-type mice (Harlan, Indianapolis, IN) weighing 20-25g were utilized as subjects. The mice were acclimatized to normal lab handling and free-feeding weights were obtained for fourteen days. Then the mice were removed from free food and maintained at 85% of their free feeding body weights via restricted food (water was available *ad libitum* in the home cages). The food provided in home cages was from Harlan Teklad Lab Diets (Teklad LM-485, Madison, WI) and bedding was sanichips (Teklad, Madison, WI). The mice were housed individually in a temperature-controlled vivarium at 22-24 degrees Celsius with a 12 hour light/dark cycle (lights on at 0600h and off at 1800h) and transported daily (0830h) to the laboratory where they remained until procedures were complete for the day (1100h). Research was performed in agreement with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2003) and all procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University (IACUC Protocol AM10284). The initial number of twenty four mice was chosen to ensure that after initial acquisition at least twenty mice had acquired the task. As the design was within-subjects, each mouse served as its own control. A power analysis (power = 0.8, alpha = 0.05) indicated that 8-10 mice per group were sufficient to detect significant treatment effects.

Apparatus

The experiments were conducted in six standard sound attenuating operant chambers (Model BNV-307A, Med Associates, St. Albans, VT). Experimental sessions and data collection were controlled by Med-PC for Windows software (version 1.17; Med Associates). The internal operant chamber (15 cm L X 11.5 cm D X 17.5 cm H) had a stainless steel grid floor with Plexiglas walls and roof and a stainless steel intelligence panel. The operant test chambers contained two retractable levers (0.8 cm when extended and located 2.5cm above the grid floor) that were placed equidistantly from a recessed well (centered on the intelligence panel) where a liquid dipper could be accessed. The liquid dipper delivered 0.02 mL of sweetened reconstituted milk (25% powdered non-fat dry milk, 25% sugar, 50% tap water).

Drugs

Clozapine (gift from Novartis, Hanover, NJ) and NMDA (gift from Lundbeck DK, Copenhagen–Valby, Denmark) and prazosin (purchased from Sigma Aldrich; St. Louis, MO) were dissolved in 50 mL deionized water with two drops of 85% lactic acid. The vehicle (VEH) solution consisted of 50 mL deionized water plus two drops of 85% lactic acid. Injections were administered subcutaneously (SC) 30 minutes prior to test sessions at a volume of 10 ml/kg body weight.

Procedure

Single Lever Training. Subjects were trained to press the VEH lever on a Fixed Ratio 1 (FR 1; every 1 lever press resulted in the delivery of a reinforcer) food reinforcement schedule for the sweetened milk reinforcer in 15 minute operant sessions. To facilitate bar press training one drop of milk was placed directly on the lever at the beginning of the session. Once bar

pressing was established, the FR schedule was gradually increased (1 FR per day) to FR 10. Subjects were randomly assigned to either the CLZ group (N = 12) or to the NMDA group (N = 12) and the operant chamber assignments for the two groups were counterbalanced. The mice were trained with single-lever errorless training with only a single lever extended into the test chamber. The mice were first trained with VEH injections (only the VEH-appropriate lever was present). After response rates stabilized on the VEH lever, the mice received training with drug injections (either 2.5 mg/kg CLZ or 30 mg/kg NMDA) and were trained on the opposite (i.e. DRUG) lever. The DRUG-lever location was counterbalanced between groups to avoid possible confounding by olfactory cues (Extance & Goudie, 1981). Tolerance to rate suppression effects of the training drugs was seen after approximately one week (mice were allowed to recover to VEH levels of responding).

Drug Discrimination Training. Following single-lever training, the mice then received their training drug (2.5mg/kg CLZ or 30mg/kg NMDA) or VEH on a double-alternation schedule (DDVVDDVV) with both levers present in the test chambers. Only responses on the correct lever were reinforced and a response on the non-appropriate lever during a string of responses reset the FR counter on the correct lever; for example, if the animal received a DRUG injection they were rewarded only for pressing the lever that they had previously been trained to associate with DRUG. In order to pass discrimination training, the mice had to meet three criteria in 5 of 6 consecutive training days: 1) completion of the first FR (FFR) on the condition-appropriate lever; 2) a minimum response rate of 10 responses per minute (RPM) during each session; and 3) 80% or greater responding on the condition-appropriate lever.

Generalization and Substitution Testing. Once drug discrimination training was successfully completed, subjects moved on to generalization testing with the training drugs. During Test Sessions, both levers were reinforced, but as before, switching levers during a string of responses reset the FR counter. Test Sessions was generally conducted on Wednesdays and Saturdays but could occur on any day that the subject met testing criteria. The testing criteria included a minimum of two training days (had to include both a DRUG and a VEH training session) between tests and each animal was required to pass two consecutive days of training (one of each condition) before testing. The double alternation training schedule was maintained between Test Sessions. Both training DRUG and VEH control tests were established before each drug dose response curve in order to determine that the training drugs were maintaining good stimulus control. All housing, training and testing conditions were identical to those used by Philibin et al. (2005).

After generalization dose response curves were obtained for the training drugs, cross-generalization testing for the two training drugs was conducted to determine if they would substitute for the other training drug. Since it was found that the two training drugs did not cross-generalize to each other, combination testing was conducted. A dose for NMDA and for CLZ that engendered less than 35% DLR in the generalization curves was chosen (10 mg/kg for NMDA and 0.625 mg/kg for CLZ). Then the dose response curves for each drug were re-determined in combination with either the low dose of NMDA or CLZ. A single selective ligand was tested, the α_1-adrenoreceptor antagonist, prazosin. Further testing with other compounds was not completed.

Data Analysis. For all sessions, the Med-PC software was programmed to calculate the percent drug-lever responding (%DLR) by counting the total lever responses on the condition appropriate lever, dividing that by the total number of responses on both levers, and then multiplying that by 100. Responses per minute (RPM) were calculated by taking the total number responses on both levers and dividing by fifteen. A subject's %DLR was excluded from data analysis if they did not complete an FFR (i.e. received a reinforcer) or if their RPM < 2. However, all response rates were included in the calculation of the RPM data, even if it was 0 (i.e. no responses during the session). Full substitution to the drug cue was defined as $\geq$ 80% DLR. Partial substitution to the drug cue was defined as $\geq$ 60% and < 80% DLR. No substitution to the drug cue was defined as $\leq$ 60% DLR. Effective $dose_{50}$ (ED_{50}) values were calculated for the linear portion of the dose response curve for any drug that produced full substitution (Goldstein, 1964). Values for ED_{50} were calculated using least squares linear regression analysis, followed by calculation of confidence limits as by Bliss in Statistics in Biology (1967). For each drug tested a repeated-measures analysis of variance (ANOVA) compared RPM at each dose to the vehicle control point for that drug (GB-STAT software, Version 10; Dynamic Microsystems, Inc., Silver Spring, MD). Significant ANOVAs were followed by Newmen-Keuls post-hoc tests ($p < 0.05$).

Results

Acquisition

Ten of the original twelve mice were successfully trained at 2.5 mg/kg CLZ (Fig.1); two mice were removed from the study due to an inability to gain tolerance to the rate-suppressing effects of the drug (40 days total without showing tolerance). The average number of sessions until CLZ animals met criteria was 25.2 (SEM ±10.9) with a range of 10-39 sessions which was consistent with previous results from our lab (Philibin et al., 2005). NMDA was successfully and reliably trained at a dose of 30mg/kg in all twelve C57BL/6 mice (Fig. 2). The average number of sessions to completion of criteria was 27.3 with a range of 14-56 (SEM ± 11.4) sessions. The number of sessions to reach criteria did not differ significantly between the two drugs (t_{20}= 0.44, p= 0.67).

Generalization

For clozapine's dose effect curve, the training dose of 2.5 mg/kg (96.68%DLR) and the 5.0 mg/kg (100%DLR) dose produced full generalization (Fig. 3). The highest dose 5 mg/kg produced profound rate suppression as only two of the ten animals responded ($F_{7, 79}$ = 16.14, $p < 0.0001$). Clozapine in this curve had an ED_{50} = 0.92 mg/kg (95% C.I. = 0.66-1.29 mg/kg).

The dose effect curve for NMDA was completed by eleven animals. One animal was removed after consistently failing to complete control points over a period of four weeks. The training dose of 30 mg/kg as well as the 56 mg/kg dose engendered full

generalization as illustrated in Figure 4. Drug lever responding for these two doses was 96.9% and 97.0% respectively. Significant rate suppression occurred at the 56 mg/kg dose ($F_{5, 65} = 12.15$, $p < 0.0001$). The ED_{50} was calculated at 10.8 mg/kg (95%C.I= 7.69-15.16 mg/kg) for the NMDA generalization curve.

Cross generalization

Cross generalization testing was then completed for both CLZ-trained mice (Fig. 5) and NMDA-trained mice (Fig. 6). The NMDA dose response curve was completed by nine CLZ-trained animals as one animal was removed due to illness. NMDA failed to produce either full or partial substitution at any dose in the CLZ-trained mice with the 56 mg/kg dose producing a maximum of 25.08% DLR. The two highest doses tested (30 and 56 mg/kg) both engendered significant rate suppression ($F_{5, 53} = 9.47$, $p < 0.0001$).

The CLZ dose response curve was completed by nine NMDA-trained mice, as two of the NMDA mice were removed from the study because of illness. An intermediate dose of CLZ (0.625 mg/kg) produced partial substitution (61.72% DLR). Significant rate suppression occurred at the 5.0 mg/kg dose of CLZ ($F_{7, 95} = 11.87$, $p < 0.0001$).

Combination Testing

Combination testing was conducted to see if either drug could potentiate the discriminative stimulus effects of the other. A low non-generalizing dose of CLZ 0.625 mg/kg was given in combination with the range of NMDA doses (Fig. 7) to nine CLZ trained mice, producing both partial substitution and full substitution. The 0.625 mg/kg

dose of CLZ produced a maximum of 33.03 % DLR when administered alone during the initial generalization curve, this increased slightly (45.09%) when administered with VEH in the combination curve. As the dose of NMDA given with the 0.625 mg/kg CLZ increased the %DLR increased. The combination of 0.625 mg/kg CLZ + 30 mg/kg NMDA achieved partial substitution (61.82% DLR) and with the combination dose of 0.625 mg/kg CLZ + 56 mg/kg NDMA full substitution (92.82% DLR) occurred. Significant rate suppression ($F_{6, 62} = 3.18$, $p = 0.0104$) was produced by the 56 mg/kg NMDA + 0.625 mg/kg CLZ combination dose although all animals responded. The ED_{50} calculated for this combination curve was 5.66 mg/kg NMDA + 0.625 mg/kg CLZ (95% C.I. = 2.15-14.89 mg/kg).

A low, non-generalizing dose of NMDA (10mg/kg) was given to eight NDMA trained mice in combination with the range of CLZ doses (Fig. 8). The 10 mg/kg dose produced a maximum of 31.56 %DLR when given to the NMDA-trained animals alone. When given in combination with the CLZ the %DLR increased as the CLZ dose increased, full substitution occurred in the 10 mg/kg NMDA + 2.5 mg/kg CLZ (80.04% DLR) and for the 10 mg/kg NMDA + 5.0 mg/kg CLZ (100% DLR) dose combinations. Rate suppression was significant at the 10 mg/kg NMDA + 5.0 mg/kg CLZ combination with only a single animal responding ($F_{6, 55} = 24.46$, $p < 0.0001$). The ED_{50} calculated for the combination curve was 0.76 mg/kg CLZ +10 mg/kg NMDA (95% C.I. = 0.37-1.58 mg/kg).

Prazosin Generalization

The prazosin dose response curve for the CLZ-trained animals is presented in Figure 9. All nine of the CLZ-trained mice completed prazosin testing. Prazosin produced

full substitution for CLZ at a dose of 3.0 mg/kg (92.20% DLR). Significant rate suppression was observed at both the 1.73 mg/kg and 3.0 mg/kg doses ($F_{6, 62} = 7.64$, $p < 0.0001$). The prazosin ED_{50} in CLZ-trained animals was 1.50 mg/kg (95% C.I. =1.21-1.87 mg/kg).

The prazosin dose response curve for the NMDA-trained animals is presented in Figure 10 and included five mice. One animal became ill and did not complete testing for one dose (as noted in Fig. 10 legend). Very high partial substitution (79.16% DLR) was achieved at the 0.3 mg/kg PRZ dose. Full substitution was achieved at the 1.0 mg/kg (98.77% DLR) and 3.0 mg/kg (99.62% DLR) PRZ doses. No significant rate suppression occurred at any of the tested doses of prazosin. Prazosin produced an $ED_{50} = 0.67$ mg/kg (95% C.I. = 0.025-1.78 mg/kg) in the NMDA-trained mice.

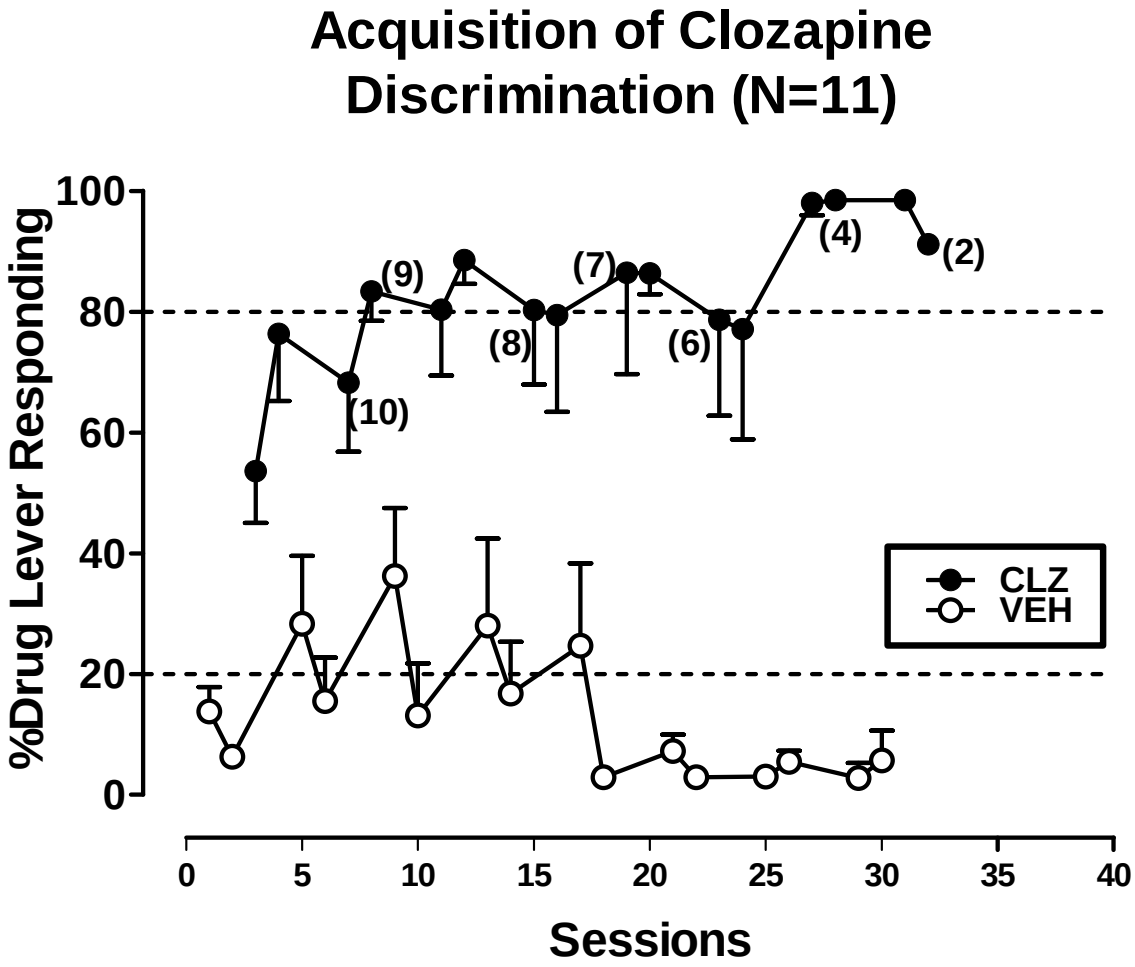

Figure 1. Acquisition of Clozapine Discrimination

Figure 1. Acquisition of two-lever drug discrimination for the 2.5 mg/kg clozapine training dose is shown. The mean percentage of drug lever responding (± SEM) is presented for both drug injection (*filled circles*) and for vehicle injection (*open circles*). The area below the *dashed line* at 20% indicates vehicle appropriate responding and the area above the *dashed line* at 80% indicates drug appropriate responding. Animals that had achieved criteria were removed from the plot as indicated by the numbers in parenthesis.

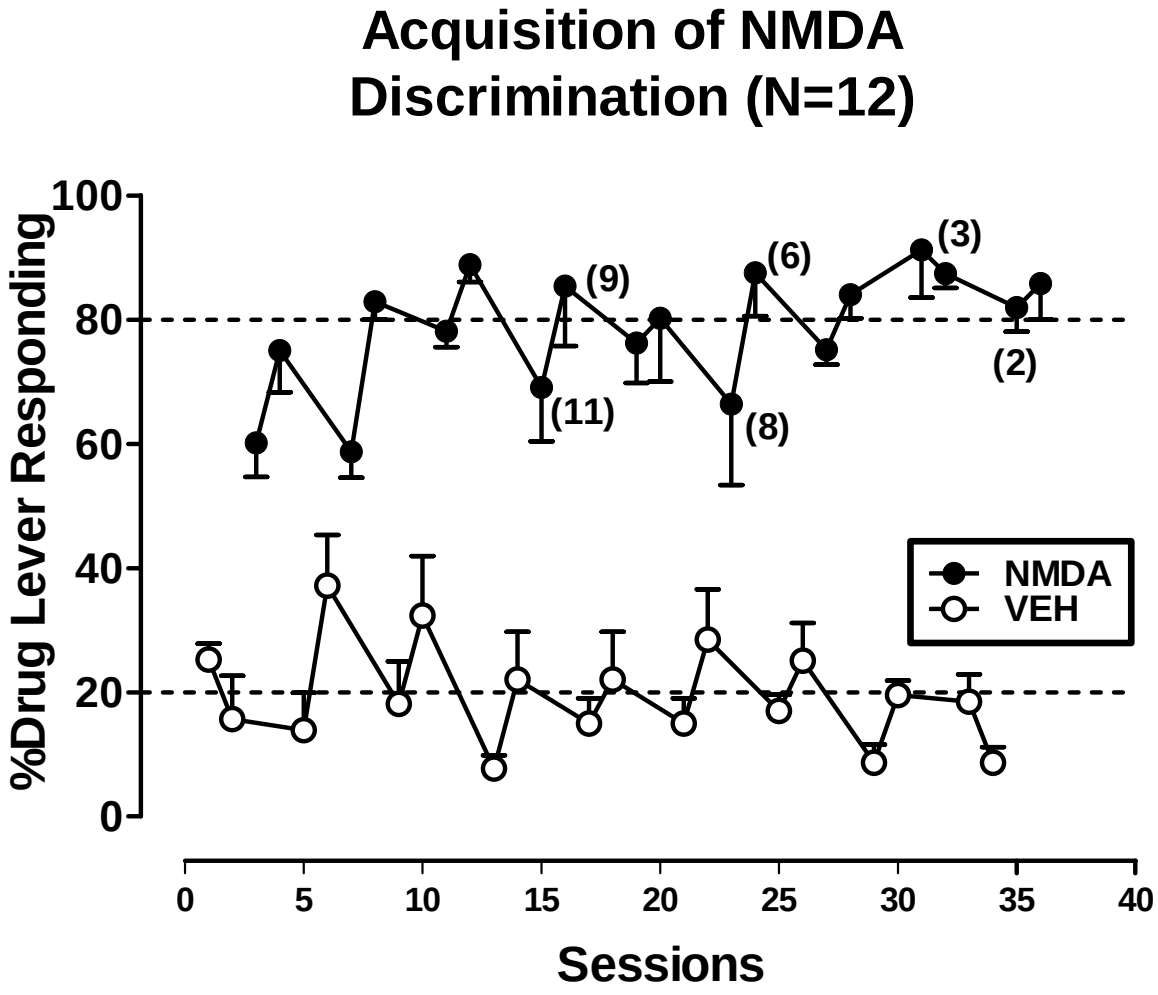

Figure 2. Acquisition of NMDA Discrimination

Figure 2. Acquisition of two-lever drug discrimination for the 30 mg/kg NMDA training dose is shown. The mean percentage of drug lever responding (± SEM) is presented for both drug injection (*filled circles*) and for vehicle injection (*open circles*). The area below the *dashed line* at 20% indicates vehicle appropriate responding and the area above the *dashed line* at 80% indicates drug appropriate responding. Animals that had achieved criteria were removed from the plot as indicated by the numbers in parenthesis.

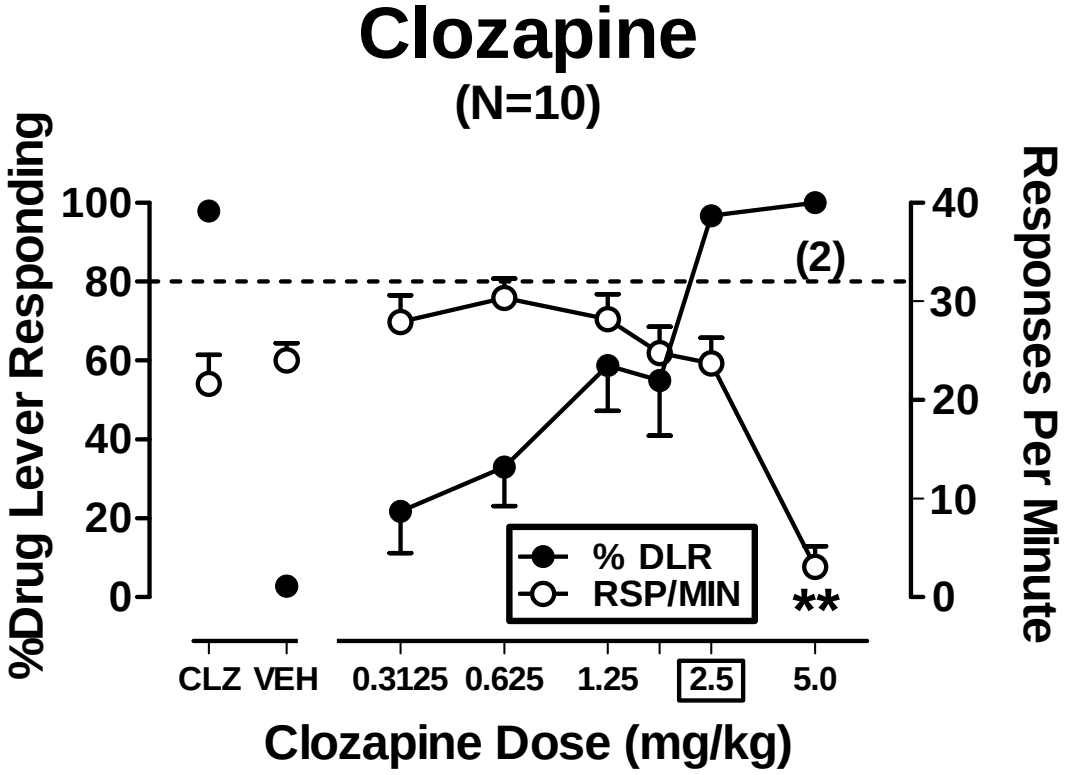

Figure 3. Clozapine Generalization Graph

Figure 3. The generalization data for the clozapine 2.5 mg/kg training dose curve is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

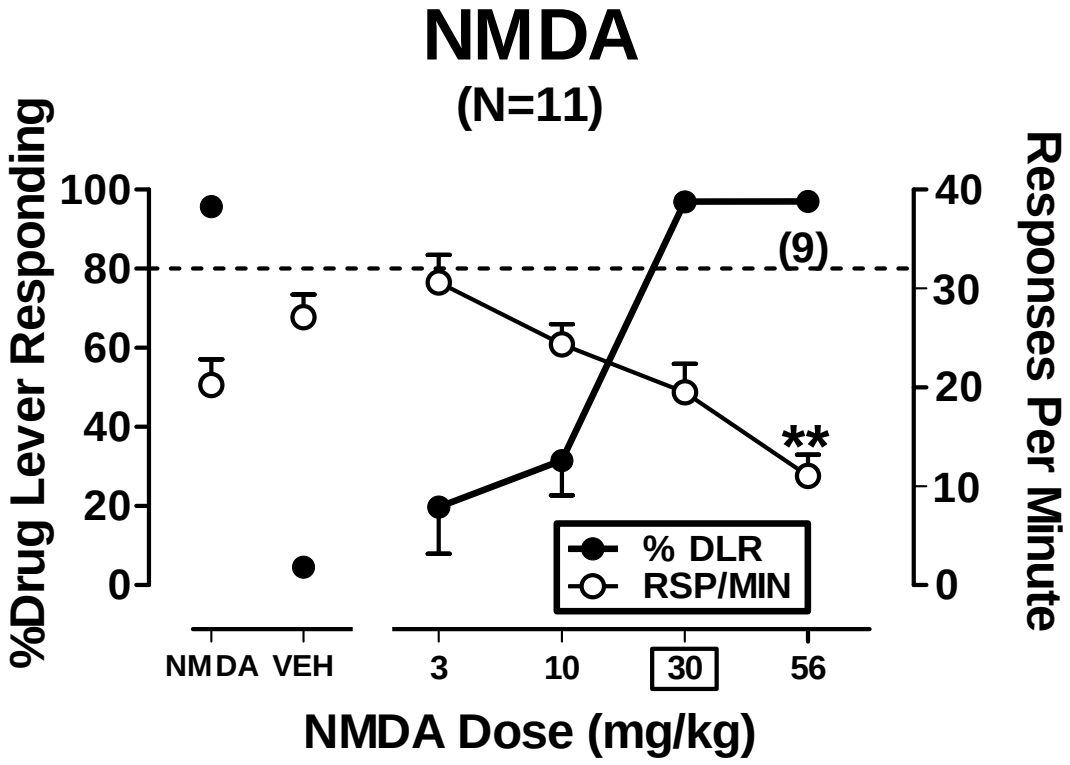

Figure 4. NMDA Generalization Graph

Figure 4. The generalization data for the NMDA 30 mg/kg training dose curve is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

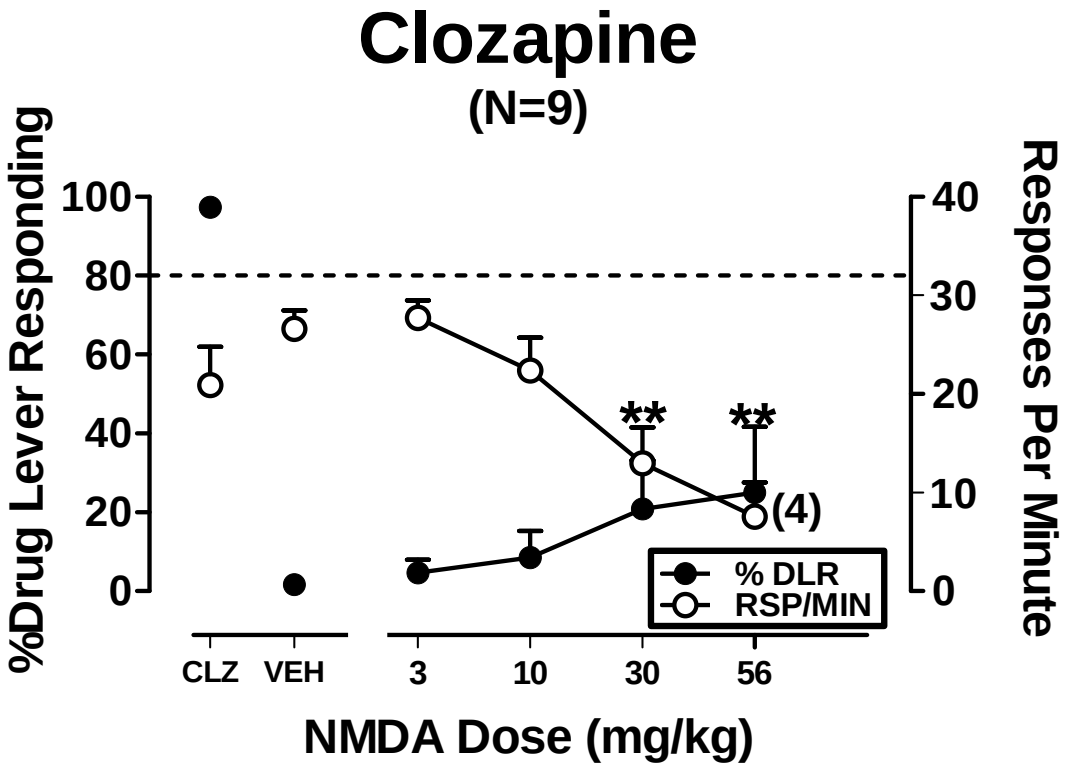

Figure 5. Clozapine Cross-generalization With NMDA

Figure 5. The cross-generalization data for the clozapine 2.5 mg/kg trained animals NMDA dose curve is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

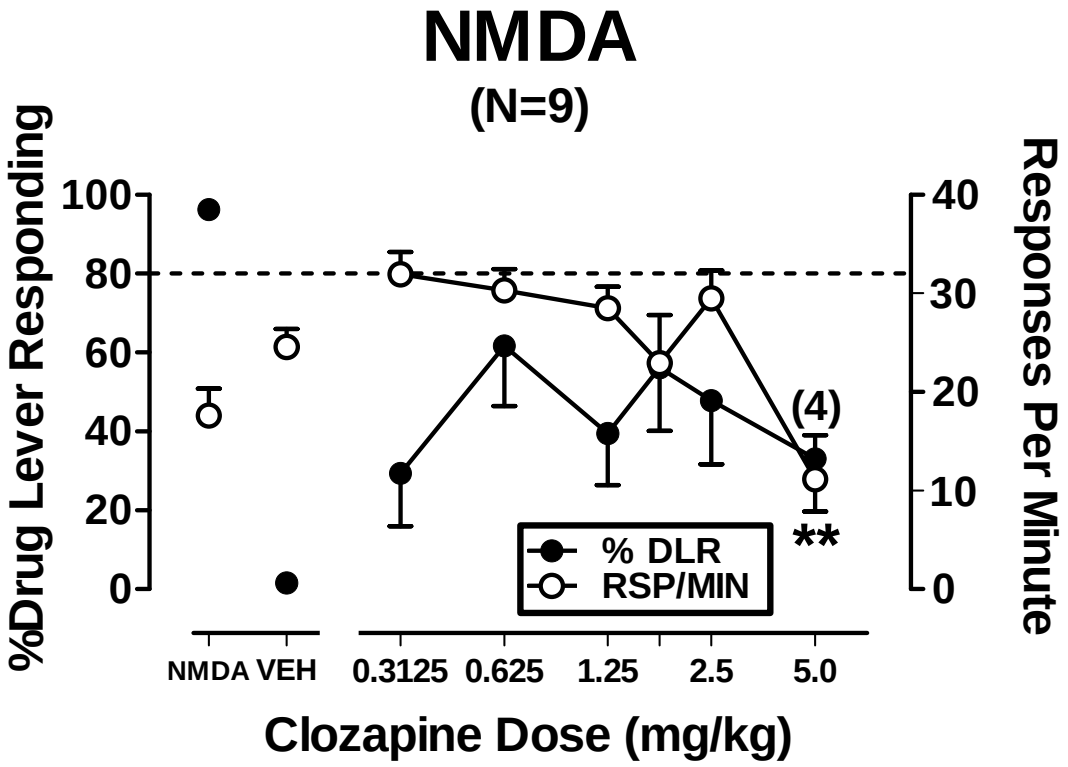

Figure 6. NMDA Cross-generalization With Clozapine

Figure 6. The cross-generalization data for the 30 mg/kg trained animals clozapine dose curve is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

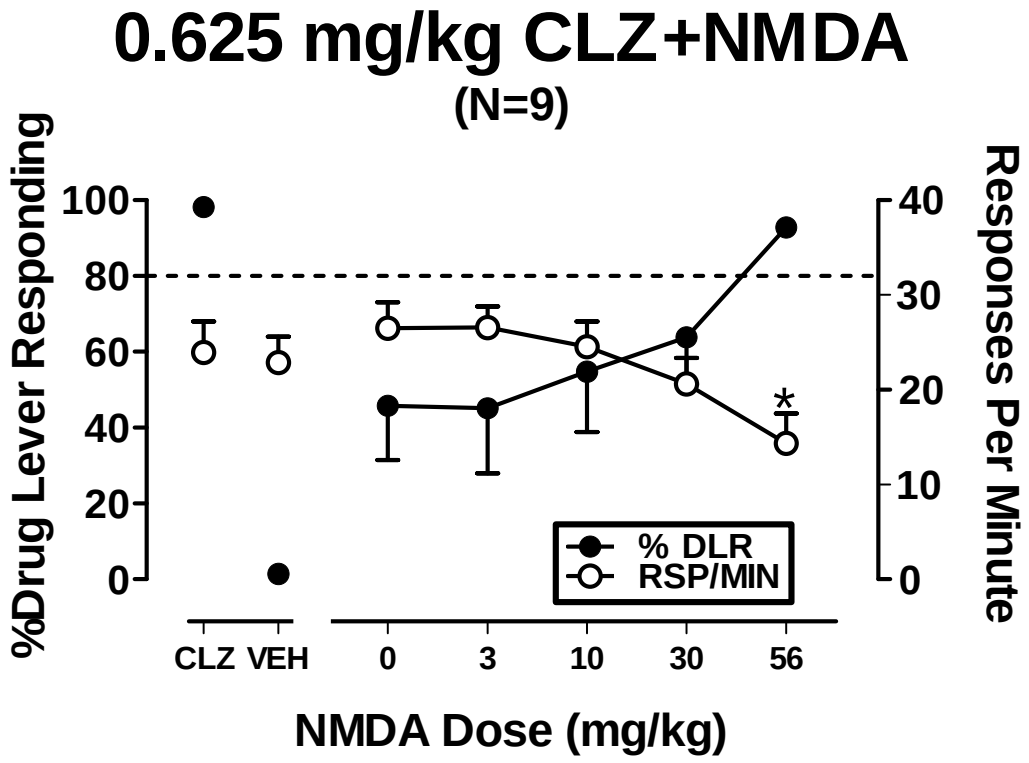

Figure 7. Low Dose Clozapine In Combination With NMDA

Figure 7. The generalization data for the 2.5 mg/kg CLZ trained animals given 0.625 mg/kg CLZ in addition to NMDA is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

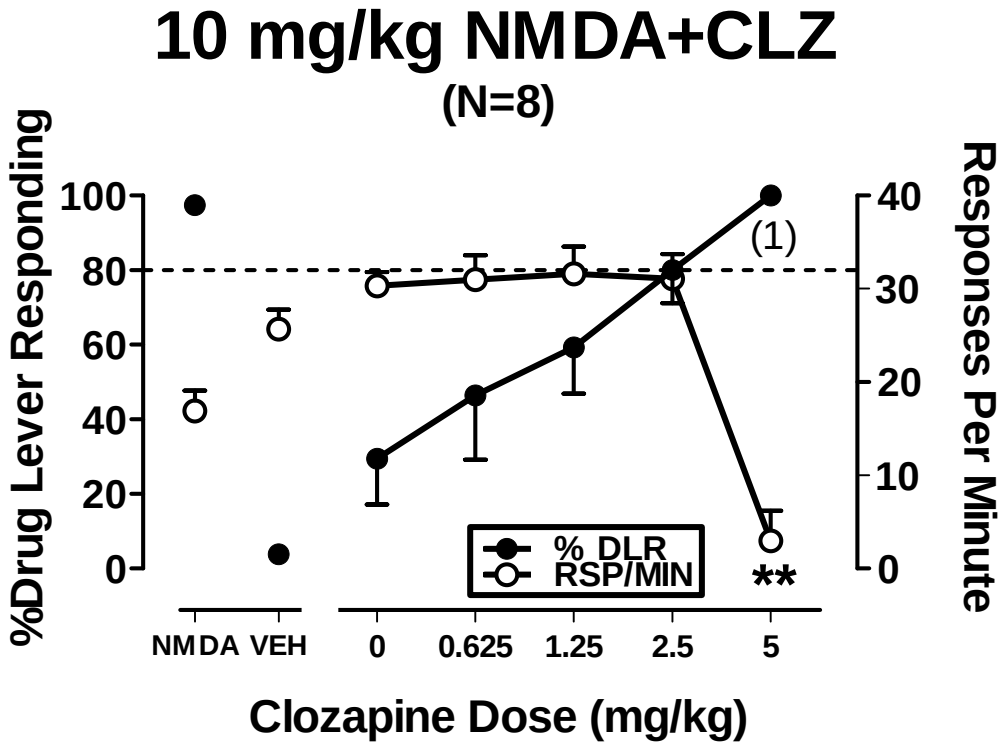

Figure 8. Low Dose NMDA In Combination With Clozapine

Figure 8. The generalization data for the 30 mg/kg NDMA trained animals given 10 mg/kg NDMA in addition to CLZ is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

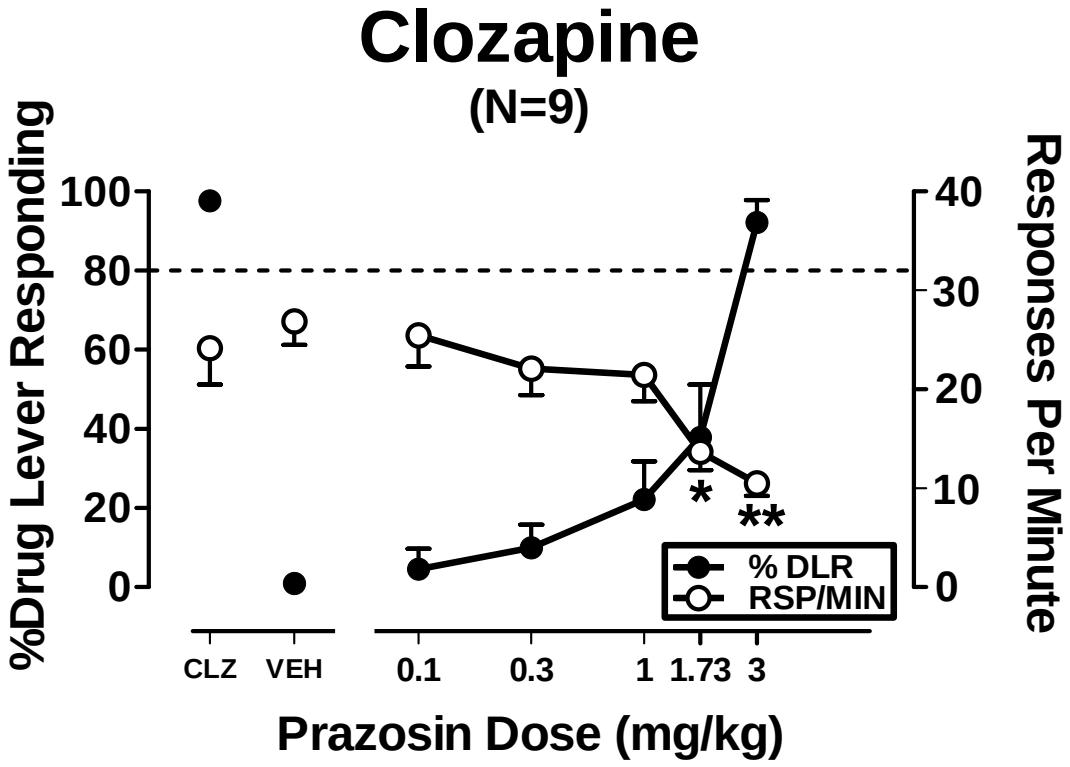

Figure 9. Prazosin Generalization In Clozapine Trained Animals

Figure 9. The Prazosin generalization data for the 2.5 mg/kg CLZ trained animals is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

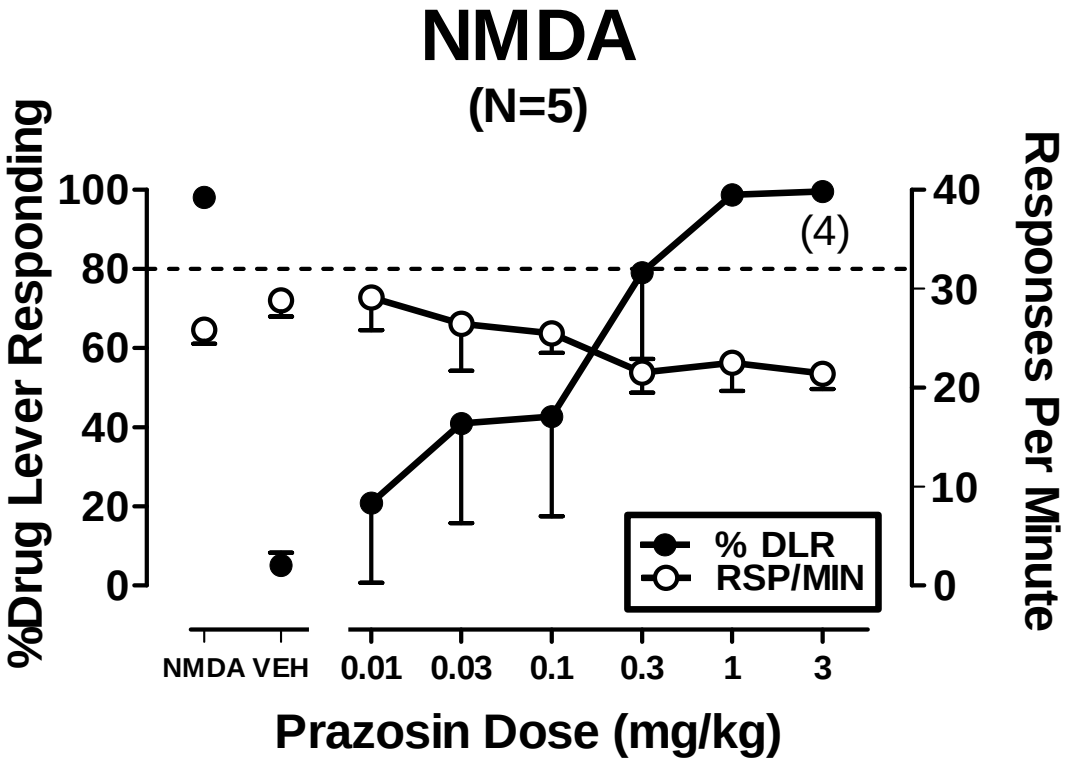

Figure 10. Prazosin Generalization In NMDA Trained Animals

Figure 10. The Prazosin generalization data for the 30 mg/kg NMDA trained animals is shown including mean percentage (± SEM) drug-lever responding (*filled circles*) and the mean (± SEM) responses per minute (*open circles*). The *dashed line* at 80% indicates full generalization to the training drug. Prior to testing control test sessions were conducted for both clozapine and vehicle. Mice with response rates of lower than two responses per minute were not included in the percentage drug lever responding. Significant decreases in response rate are noted by asterisks (* $p<0.05$, ** $p<0.01$).

Discussion

The current study replicates previous findings from this lab that clozapine has a robust and reliably trainable discriminative stimulus. The majority of C57BL/6 mice successfully reached criteria (10 of 12) at the training dose of 2.5 mg/kg. The average number of sessions until clozapine-trained animals met criteria was 25.2 with a range of 10-39 sessions, which was consistent with previous studies from our lab in which C57BL/6 mice reached training criteria in an average of 35.6 sessions with a range of 15-52 sessions (Philibin et al., 2009) or 14.8 sessions with a range of 6-34 sessions (Philibin et al., 2009). The generalization results in the present study for clozapine were also similar to previous studies completed in this lab with an ED_{50} = 0.92 mg/kg (95% C.I. = 0.66-1.29 mg/kg), which was slightly lower than the earlier cohorts of clozapine trained mice with ED_{50}s of 1.14 mg/kg and 1.19 mg/kg, respectively (Philibin et al., 2005; Philibin et al., 2009). The two highest doses (2.5 and 5.0 mg/kg) of clozapine administered produced full generalization.

The current study also showed that NMDA has a sufficiently robust discriminative stimulus to successfully establish two-lever drug discrimination in C57BL/6 mice at 30 mg/kg. The complete group of 12 C57BL/6 mice successfully reached criteria at the training dose 30 mg/kg. The average number of sessions to reach training criteria was 27.3 with a range of 14-56 sessions. The generalization results for NMDA-trained C57BL/6 mice were similar to NMDA-trained rats in the number of sessions required to reach

criteria (*M*=30) but they had a much lower ED_{50} = 10.8 mg/kg (95%C.I= 7.69-15.16 mg/kg) as compared to rats ED_{50} = 17.1 (Willetts & Balster, 1989). The two highest doses (30 and 56 mg/kg) of NMDA produced full generalization. The number of sessions to reach criteria did not differ significantly between the two training drugs, clozapine and NMDA.

When cross-generalization testing was completed, an asymmetrical relationship between the two training drugs was revealed. In the clozapine-trained animals NMDA failed to produce clozapine-appropriate responding more than 25% of the time even at doses that produced significant rate suppression. In the NMDA-trained animals, however, clozapine produced partial substitution (above 60%) at the 0.625 mg/kg dose. This demonstrated an asymmetrical relationship between clozapine and NMDA in terms of their discriminative stimuli.

In subsequent testing, a low, non-generalizing dose of clozapine (0.625 mg/kg) was administered in combination with NMDA in the clozapine-trained animals to evaluate if this combination would result in a dose dependant shift of the curve. The combination testing produced a leftward shift in the curve. The highest drug lever responding engendered by the 0.625 mg/kg dose of clozapine when administered alone was only 33%; however, in combination with doses of NMDA the drug lever responding increased and with the two highest doses partial (61.82%) and full substitution (92.82%) for clozapine was achieved

A similar leftward shift was obtained in the NMDA-trained animals with the combination of a low dose of NMDA plus several clozapine doses. The non-generalizing

dose (10 mg/kg) of NMDA produced only 31% NMDA-lever responding when administered alone, but in combination with doses of clozapine the NMDA-lever responding increased at all doses and full substitution (80% and 100%) was obtained at the two highest doses of clozapine (2.5 and 5.0 mg/kg). This potentiation of the NMDA discriminative stimulus provides further evidence of the interaction of these two drugs through some intermediate action as there is no evidence that clozapine acts directly on the NMDA receptor complex.

Interestingly, the α_1 adrenoreceptor antagonist prazosin produced full substitution in both the clozapine- and the NMDA-trained animals. The intermediate dose (1.0 mg/kg) produced full substitution in the NMDA-trained animals, while the high dose of prazosin (3.0 mg/kg) produced full substitution in both NMDA-trained and clozapine-trained animals. This replicates findings from our lab that 2.8 mg/kg prazosin fully substitutes for 2.5 mg/kg clozapine in C57BL/6 mice (Philibin et al., 2009). These results also are similar to previous findings in 30 mg/kg NMDA-trained rats in which the α_1 adrenoreceptor antagonists prazosin and WB4101 fully substituted for NMDA (Arnt, Sanchez, Lenz, Madsen, & Krogsgaard-Larsen, 1997).

Clozapine has a complex receptor profile that has actions at serotonergic, dopaminergic, adrenergic, cholinergic, and histaminergic receptors. Previous research regarding the discriminative stimulus properties of clozapine in C57BL/6 mice has revealed that antagonism of serotonergic receptors and α_1 adrenoreceptors is an important part of clozapine's discriminative stimulus cue (Philibin et al., 2009). When tested in NMDA-trained C57BL/6 mice, the α_1 adrenoreceptor antagonist prazosin fully substituted

for NMDA. In previous research in rats the antipsychotic spiperone, which has serotonergic and dopaminergic activity, also fully substituted for NMDA (Cory-Slechta et al., 1996). This indicates that although NMDA is a specific agonist at its endogenous site on the NMDA receptor, activation of other receptors can mimic the subjective effects of NMDA in the drug discrimination procedure.

This relationship may be due to an interaction between NMDA, serotonin and α_1 adrenoreceptor systems. It has been shown that systemic administration of phencyclidine and ketamine (non-competitive NMDA antagonists) increases serotonin efflux in the medial prefrontal cortex of rats. Blockade of this enhanced serotonin efflux was achieved by the atypical antipsychotics clozapine and olanzapine and by prazosin, but not by haloperidol (Amargos-Bosch, Lopez-Gil, Artigas, & Adell, 2006). Serotonin efflux resulting from microdialysis of cirazoline, an α_1 adrenoreceptor agonist, in the medial prefrontal cortex can be reversed by clozapine, olanzapine and prazosin and by the typical antipsychotics chlorpromazine and haloperidol (Amargos-Bosch, Adell, Bortolozzi, & Artigas, 2003). This suggests that α_1 adrenoreceptor activity results in modulation of serotonin in the medial prefrontal cortex of rats whether induced by serotonin agonists or NMDA antagonists, and may be part of the therapeutic action of clozapine.

Additional aspects of clozapine that are of interest in its relationship to NMDA involve dopamine functioning in response to adrenergic and NMDA receptor actions. Clozapine's unique efficacy in refractory patients, cognitive improvement, and reduction of suicidality has been argued to be due to its higher ratio of serotonin vs. dopamine occupancy (Meltzer et al., 1989b; Meltzer & McGurk, 1999; Meltzer et al., 1996). Current

research suggests that while that ratio is important, adrenergic action should also be studied further as a possible component of clozapine's therapeutic action. The α_1 adrenoreceptor antagonist prazosin and the α_2 adrenoreceptor antagonist idazoxan when administered with D_2 antagonists or haloperidol in a condition avoidance paradigm (CAR) significantly enhanced the suppression of CAR activity without increasing catalepsy in the D_2 antagonists and decreased catalepsy significantly with haloperidol (Svensson, 2000; Wadenberg, Wiker, & Svensson, 2007). Prazosin also stimulates dopamine neurons in the ventral tegmental area (VTA) of rats but suppresses activation of mesolimbic sub-cortical DA neurons (Svensson, 2000, 2003). These findings suggest that α_1 adrenoreceptor antagonists may modulate dopaminergic function in certain brain areas, one of the key functions of antipsychotic drugs. However, there are many other receptors and neurotransmitter systems that are activated by the administration of clozapine (muscarinic, histaminergic, etc.) and their contributions to the therapeutic effects of the drug are not yet fully understood.

Recent attention in treatment development and genetic modeling in schizophrenia research has focused on the glycine-binding site of the NMDA receptor complex. Glycine site agonists $_D$-serine, $_D$-alanine and glycine have repeatedly been shown to improve negative symptoms. However, when administered with clozapine (which is known to show clinical improvement of negative symptoms), they do not show statistically increased effectiveness on negative symptoms (Shim, Hammonds, & Kee, 2008). This suggests that the receptor action produced by administration of these drugs may already be present in the complex receptor actions of clozapine. Glycine site activation also has been shown to

improve cognition in several animal models (Andersen & Pouzet, 2004; Shim et al., 2008). Genetically induced reduction in NMDA receptor glycine affinity in C57BL/6 mice produces $GRIN^{D481N}$ mice (Labrie, Lipina, & Roder, 2008) and these genetically modified mice show unusually prolonged latent inhibition, deficits in social approach, and reduced reactivity to spatial change. In these mice used to model the negative and cognitive symptoms present in schizophrenia, treatment with $_D$-serine and clozapine reversed the abnormalities (Labrie et al., 2008). This finding suggests a role for the glycine site both in regard to the symptoms of schizophrenia and to the functionality of clozapine and also suggests that glycine agonists may have therapeutic efficacy for the treatment of schizophrenia.

The glycine site also is important as it may be the actual site of clozapine's action on the NMDA receptor complex. Some recent *in vivo* electrophysiological data suggests that clozapine's activation of dopamine neurons in the VTA of rats depends on the level of endogenous kynurenic acid, an NMDA glycine site antagonist (Schwieler, Linderholm, Nilsson-Todd, Erhardt, & Engberg, 2008). Clozapine given intravenously increased the firing of dopamine neurons in the VTA unless rats were treated with indomethicin, a cox_1 inhibitor that increased brain levels of endogenous kynurenic acid. When rats were treated with indomethicin, clozapine's excitatory action was reversed into an inhibitory one. Conversely, when a cox_2 inhibitor was given and kynurenic acid levels decreased, the excitatory action of clozapine was potentiated (Schwieler et al., 2008). Further, the activation of dopamine neurons by clozapine mirrors the activation seen after administration of L-701,324, a site specific glycine antagonist, or by increased levels of

kynurenic acid (Erhardt & Engberg, 2002; Schwieler, Engberg, & Erhardt, 2004). This may signify that although clozapine has D_2 receptor affinity (though lower than typical antipsychotics) its actions on dopaminergic neurons in some brain areas are through modulation of the glutamate system via NMDA receptor activity.

In the current study clozapine produced partial substitution in NMDA-trained animals when administered alone. Clozapine's diverse receptor binding profile suggests that actions at one or more receptor sites may contribute to this partial substitution (i.e., at $5HT_{2A}$ receptors, action at α_1 adrenoreceptors, and indirect action at the NMDA glycine receptor site). Also, the fact that clozapine binds to receptors that have been shown to interact with NMDA suggests that it may be activity at one or more of these receptors that prevented clozapine from fully substituting for NMDA. NMDA also failed to substitute in the clozapine-trained mice, illustrating that agonism at the glutamate site is insufficient to mimic the complex cue of clozapine. Further study with other select ligands is needed to investigate these complex relationships.

The α_1 adrenoreceptor antagonist prazosin also achieved full substitution in both training groups. This replicates previous research in C57BL/6 mice trained to discriminate clozapine (Philibin et al., 2009), and replicates previous research in rats trained to discriminate NMDA (Arnt, 1997). Thus, clozapine's complex cue in C57BL/6 mice appears to be partially mediated by α_1 adrenoreceptor antagonism. Similarly, NMDA also appears to have a compound discriminative stimulus that includes antagonism at α_1 adrenoreceptors.

Serotonergic antagonism, specifically at $5HT_{2A}$ receptors, has been shown previously in clozapine-trained C57BL/6 mice to be a significant part of clozapine's cue as the antagonist ritanserin fully substituted for clozapine (Philibin et al., 2009). Previous research in NMDA-trained rats has shown that the antipsychotic spiperone, which has antagonistic activity at $5HT_{2A}$ and at D_2, fully substitutes for NMDA. This additional similarity has not yet been explored in C57BL/6 mice and it would be of interest to investigate whether this similarity is shared in mice, as well as rats, by testing ritanserin in NMDA-trained mice.

The modulating effect of kynurenic acid on the activation of dopamine by clozapine is also of interest. It would be interesting to see if clozapine's complex cue can be blocked by the administration of glycine site agonists. It would also be of interest to investigate whether site specific agonists substitute for NMDA's discriminative cue, even thought they have different activation sites on the NMDA receptor complex. Since NMDA antagonists PCP and Ketamine produce psychotic-like symptoms in humans, the effects of NMDA antagonists on clozapine's cue would also be of interest.

In human trials the results of adjunctive treatment with NMDA agonists have had mixed results. Treatment with moderate doses of D-serine (30mg/kg/ day) combined with typical antipsychotics improved negative and, to a lesser degree, positive and cognitive symptoms without an increase in side effects (G. Tsai, Yang, Chung, Lange, & Coyle, 1998). However, when given in conjunction with clozapine there was no increase in therapeutic effect (G. E. Tsai et al., 1999). Use of other agonists at the glycine site like D-cycloserine worsened negative symptoms when given with clozapine (Goff et al., 1999;

Javitt, 2002). The mixed results of glycine site modulators in clinical application underlines the importance of a greater preclinical knowledge of the interactions between the NMDA receptor and clozapine.

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