BEHAVIORAL PHENOTYPING OF THE DISCRIMINATIVE STIMULUS PROPERTIES OF THE ATYPICAL ANTIPSYCHOTIC DRUG CLOZAPINE IN 129S2/HSV 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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Abstract

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The 129S2 inbred mouse strain is often used as a background strain in the production of genetically altered mice (i.e. knockout and transgenic mice). It is important to establish the behavioral phenotype of wild-type mice before making comparisons to genetically altered mice. Also, those comparisons can assist in the evaluation and interpretation of the *in vivo* effects of drugs. The drug discrimination assay measures the subjective effects of drugs and provides a measure of underlying neuropharmacological mechanisms responsible for the discriminative stimulus properties of drugs. The present study established the atypical antipsychotic drug clozapine as a discriminative stimulus in male 129S2 inbred mice and compared clozapine's discriminative stimulus properties in 129S2 mice to C57BL/6 and DBA/2 inbred mice. By comparing the discriminative stimulus properties between inbred strains of mice we hope to obtain a fuller picture of the underlying neuropharmacological mechanisms of antipsychotic drugs.

Behavioral Phenotyping of the Discriminative Stimulus Properties of the Atypical Antipsychotic Drug Clozapine in 129S2/HSV mice.

Schizophrenia is a severe and complex psychological disorder. It has been historically reported to affect approximately 1% of the world's population and onset typically occurs during late adolescence or early young adulthood in the early to mid-20s. The prognosis for a patient that develops schizophrenia is grim as most carry the disease with them their entire life. The early 1950s saw a revolution in the treatment of schizophrenic patients with the introduction of antipsychotic drugs; however, the disease has eluded a cure due in part to a murky etiology. While studies have shown a clear genetic component for schizophrenia, monozygotic twins only have a 50% concurrence rate for the disease implying that other factors such as neurodevelopmental events and/or environmental influences also play a strong role in the etiology of schizophrenia (Brown, 2011).

Etymology of schizophrenia

The term schizophrenia was first coined by Swiss physician Paul Eugen Bleuler at the 1908 meeting of the German Psychiatric Association in Berlin (Fusar-Poli & Politi, 2008). Bleuler introduced the term as more precise nomenclature for a series of symptoms that at the time was called dementia praecox, or young dementia. The term dementia praecox had been popularized by Emile Kraepelin, but Bleuler believed that the symptoms he had noticed were not a form of “dementia” but something more. Schizophrenia, Bleuler felt, was a more accurate word for the disorganization of thoughts that was prevalent with patients suffering from the disorder. However Beuler's choice has

led to modern day confusion among a majority of lay and medical professionals alike. The Grecian roots for the word skhizein and phren translate to “split mind”. Many unfamiliar with the field associate the word with someone who is suffering from a disorder now known as dissociative identity disorder, or split personality.

Symptomatology

The DSM-IV-TR (APA, 2000) characterizes schizophrenia with five main symptoms: delusions, hallucinations, disorganized speech, grossly abnormal psychomotor behavior, and negative symptoms. These symptoms can cause social skills and relations to deteriorate in the patient and may be noticed by trouble at work or with other interpersonal relationships. In childhood and adolescent patients the inability to acquire fully functional social skills can be an early indicator for schizophrenia. Other symptoms have been identified, however, and symptoms of schizophrenia are often classified in one of three categories: positive symptoms, negative symptoms, or cognitive deficits (APA, 2000).

Positive symptoms are characterized by a manifestation of behaviors not present in unaffected patients. Positive symptoms are the more recognizable manifestation of schizophrenia and when schizophrenics are portrayed in the media and popular culture the positive symptoms are most often emphasized. Hallucinations and delusions are the archetypal positive symptoms; however, disorganized thought, incoherent speech, and disorganized thinking also inhabit this class. Hallucinations are often auditory (e.g. the schizophrenic hears voices whispering constantly in their ear), but visual hallucinations

have also been reported, especially in children (David et al., 2011). Delusions are one of the other common positive symptoms of schizophrenia and can appear in different forms. Delusions of grandeur may lead the affected to believe that they are the reincarnation of a great leader, figure from history, or some form of powerful and omnipotent being. Delusions of persecution manifest with thoughts that someone is watching the schizophrenic constantly, be it a secret government agent, a shadowy organization, or even beings of a religious/supernatural nature. Disorganized thinking is considered another positive symptom of schizophrenia, and often manifests itself through disorganized speech sometimes referred to as "word salad". Noam Chomsky's famous phrase "Colorless green ideas sleep furiously" exemplifies the concept of word salad, while the sentence follows proper syntax for sentence construction it holds no logical weight. Finally other strange motor anomalies are classified as positive symptoms in the schizophrenic, including tracing patterns in the air or on a surface, holding a single pose for extended periods of time, or random frantic movement (APA 2000).

Negative symptoms of schizophrenia are not as prevalent in popular depictions of the disorder. This set of symptoms is characterized by a lack of behavior in the schizophrenic that is present in the unaffected population. Poverty of speech (alogia) and flat affect (a lack of emotional response), are two stereotypical negative symptoms. Apathy and avolition are two symptoms that define a patient's lack of motivation, avolition being distinct from apathy by a schizophrenic's desire to do a task but lacking the motivation to begin or initiate it. Asociality, an inability to empathize with other people, is one of the symptoms of schizophrenia that leads to a rapid deterioration of

social, familial, and work life for those who develop the disease. Finally anhedonia is used to describe the lack of pleasure schizophrenics feel when doing activities that normally bring them joy.

There has been some debate in the psychiatric community as to whether cognitive deficits should be included as a symptom of schizophrenia. Studies have shown that there is a correlation between decreased cognitive function and the schizophrenic's functional prognosis, yet the variety of factors that determine cognitive functioning leave the line between the two murky (Green, Kern, Braff, & Mintz, 2000). There is a strong body of research and reviews calling for cognitive impairment to be included in the *Diagnostics and Statistics Manual Edition Five,* the North American standard for classifying psychological disorders. Two of the main arguments for this distinction would be to raise awareness of cognitive dysfunction (Keefe & Fenton, 2007) hopefully leading to better treatment methods, and that the inclusion of cognitive deficits would help to distinguish schizophrenia from mood disorders (Keefe, 2008). However opponents of the inclusion of cognitive dysfunction claim that there is simply too much variability in that data to clearly associate specific cognitive symptoms with schizophrenia (Gold 2008).

Incidence and Prevalence

While what causes schizophrenia is still clouded in mystery, hypotheses range from genetic vulnerability to seasonality of birth. By studying the incidence, how many new cases are reported in a given time span, and prevalence, the proportion of the general population that have the disorder, researchers can start to identify areas to focus their

work to help develop treatment methods and discover the underlying cause of the disorder. Onset occurs most commonly in young adulthood and while those affected will struggle with the disease for the rest of their lives, recovery of a functional life is obtainable for many schizophrenics. Unfortunately, the burden of psychosis is too much for some to handle and suicide among schizophrenics is not uncommon, especially among those dealing with their first episode of the disorder (Caldwell & Gottesman, 1990).

While traditional estimates of the prevalence of schizophrenia have been reported to be 1% worldwide, recent research has suggested that this may be overestimating the number of people who have the disorder. In a review article of 158 studies incidence of schizophrenia was calculated to be 15.2 new cases annually per 100,000 people, less than .02% of the population. However this was the median rate of incidence with 10% and 90% quartiles ranging from 7.7 to 43.0 new cases annually per 100,000 people with studies more frequently reporting incidence rates above the median range (McGrath et al., 2004). However even at its highest estimate .04% of the population becoming schizophrenics is a relatively low rate, but with a life time struggle ahead for the majority of those diagnosed it is reasonable to see how prevalence rates can begin to compound.

Another review article analyzing prevalence of schizophrenia in 188 studies determined that the lifetime morbid risk, the number of people who will develop schizophrenia in their life time, was 7.2 per 1000 people, putting medial percentage of schizophrenia in the population at 0.72%, lower than the historically reported value of 1.0% prevalence. While median values for point, period, and life time prevalence varied

they were not significantly different from each other (Saha, Chant, Welham, & McGrath, 2005).

These studies on the incidence, how many new cases are reported in a given time span, and prevalence, the proportion of the general population that have the disorder, also reveal interesting demographic data on who in the population develops schizophrenia. Men have a higher incidence of the disorder, approximately 1.4 men will develop schizophrenia for every woman that does (McGarth et al. 2004). Interestingly there is no statistical difference in the prevalence of schizophrenia between the sexes, a median ratio of 1.11 men have this disorder for each woman that has it, possibly hinting at a difference in the course of the disease (Saha et al., 2005). Data were inconclusive or non-significant for differences between urban and rural dwelling schizophrenics. The review also reports a significantly increased incidence and prevalence of schizophrenia in migrant populations, median incidence in the migrant population is 4.6 new cases for every new case in native born population, and median prevalence reveals a ratio of 1.84 migrant schizophrenics for every native born schizophrenic (McGrath et al., 2004). The prevalence of schizophrenia also seems to be lower in less developed countries, with median rates of 2.62 per 1000 people. While rates in emerging economic countries are higher (median rates 4.69 per 1000 people) than developed countries (median 3.30 per 1000 people) the two are not significantly different (Saha et al., 2005).

Suicide among schizophrenics is another hidden problem with the disorder. While estimates for the prevalence of suicide have been reported as high as 10% (Phillips, Yang, Li, & Li, 2004) and 19.56% in affected patients 18-30 (Osborn, Levy, Nazareth, &

King, 2008) a life time risk of approximately 5% is the most accepted figure (Hor & Taylor, 2010). Unsurprisingly some of the major risk factors for suicide in schizophrenics is similar to those in the general population including mood disorder, recent loss, drug misuse, and previous attempts at suicide (Hawton, Sutton, Haw, Sinclair, & Deeks, 2005). More recently, a literature review also identified several illness related factors strongly associated with schizophrenia including depression, signs of physical illness, and increased positive symptoms of schizophrenia specifically hallucinations and delusions (Hor & Taylor, 2010).

Causes

What causes schizophrenia has been a point of interest ever since the discovery of the affliction. High concurrence rates in monozygotic twins as well as higher concurrence rates for closer relatives point to a genetic component of the disorder. However, fascinating case studies such as the Genain Quadruplets, four identical twins who all developed schizophrenia but in different severities (Mirsky & Quinn, 1988), show that development and environment still play a major role in a patient's prognosis.

Twin studies play an integral role in studying the genetic and environmental influences of schizophrenia. Monozygotic twins show a 45-60% concurrence rate for developing schizophrenia, compared to the 10-15% for dizygotic twins (Brown, 2011). Combined with the steady concurrence for the population worldwide it would seem that schizophrenia would be a prime candidate to be considered a genetic disorder. Most twin studies seem to support this idea; Borgwardt et al. (2010) reported that between

concordant and discordant monozygotic twins those with schizophrenia had similar decreases in grey matter volume, a trait common among all schizophrenics. A meta-analysis of twin studies reports that heredity (how much variation between subjects can be attributed to genomic differences) accounts for 73-90% of the variance of whether or not a patient develops schizophrenia while environment only accounts for 3%-19% (Sullivan, Kendler, & Neale, 2003); however, Brown et al. (2011) state that this estimate of the effect of environment is not completely correct and may downplay the influence of the environment.

Nonetheless, genetics remains an integral part of the question of who will develop schizophrenia and how severe it will be. With the advances in genetic screening and molecular genetics the search for specific genetic markers has swept the research community into a fervor. The vast number of potential subjects and vast amount of genetic variation in the world should make it easy to find a common genetic marker for many common psychological disorders; however, this "common disease, common variant" hypothesis has failed to produce convincing evidence that there is a single marker for schizophrenia (Gershon, Alliey-Rodriguez, & Liu, 2011) or other common psychological disorders. Still, genetic models and markers exist for schizophrenia, the leader being Disrupted in Schizophrenia 1 (DISC1). The *DISC1* gene encodes for a protein of the same name and appears to be important for many aspects of neuronal development. Translocation of this gene is what causes its disruption, and it is this disruption that is thought to cause predisposition to schizophrenia and other "common" psychological diseases. Transgenic animal models of *DISC1* have shown phenotypic

effects similar to those seen in human subjects in terms of cognitive and behavioral functioning as well as brain anatomy (Johnstone et al., 2011).

Schizophrenia seems to not only affect cognitive and social functioning but produces changes in brain structure as well. One important focus is the relationship between brain abnormalities and schizophrenia; do the abnormalities predispose someone to schizophrenia or does schizophrenia cause the brain to deteriorate as the patient continues to live? Studies have shown that magnetic resonance imaging (MRI) of first-episode schizophrenic patients shows similar brain abnormalities, specifically: enlarged ventricles, loss of overall brain volume, and decreased hippocampal mass (Vita, De Peri, Silenzi, & Dieci, 2006). While Vita et al. failed to show significant reduction in amygdala volume in first-episode patients, other studies (Lawrie & Abukmeil, 1998; Wright et al., 2000) have shown significant reductions in amygdala volume in chronic schizophrenic patients. Although more research needs to be done in the field, these findings suggest that some aspects of abnormal brain morphology are inherit to those who are predisposed to schizophrenia, while other changes in brain morphology may appear as a result of the disease. The idea of brain abnormalities in predisposed twins, regardless of actual affliction, are presented in a study that showed a phenotypic reduction in brain size across discordant twins affected with schizophrenia (van Haren et al., 2004) and decreases in grey matter volume across discordant twins (Borgwardt et al., 2010).

An interesting correlation has been drawn between prevalence of schizophrenia and the season of a patient's birth. A review article looking at 86 studies of birth season of schizophrenics identified a correlation between those born in the Winter-Spring,

specifically January-April, and an increased prevalence of schizophrenia (Torrey, Miller, Rawlings, & Yolken, 1997). Not only are birth rates of those who will develop schizophrenia higher in those months but this review also found there was a decrease in the number of births of schizophrenics in the opposing seasons (summer-fall). The winter-spring excess of schizophrenics also occurs in the southern hemisphere, even though the months for these seasons are reverse from the northern hemisphere. What causes this increased incidence of schizophrenia for the winter-spring months is still unclear. While theories range from seasonal variations of infection and external toxins to procreation habits of parents of schizophrenics, research for each hypothesis seems to be contradictive or nonsignificant. The more likely story, as with most things, is that there are multiple factors at play, which will exacerbate other factors eventually leading to the excess birth rates during these months (Torrey et al., 1997).

Exposure to specific viral infections has also been suspected as a possible risk factor for schizophrenia. Prenatal infection of many diseases has been shown to cause brain abnormalities, mental retardation, and learning disabilities (Brown & Derkits, 2010) making investigation for schizophrenia a relatively easy choice. Recently research in this area has shifted from studying prevalence in the wake of infection epidemics to birth-cohort longitudinal studies. Influenza, toxoplasma gondii, herpes simplex type 2, and certain cytokines have been shown to increase the prevalence of schizophrenia as compared to nonaffected controls. Again, the question is raised whether each disease has a specific causal link between infection and development of schizophrenia or are there more common factors involved, i.e. does prenatal infection simply lead to a more

vulnerable fetus? Preclinical studies looking at animal models of prenatal infection have shown that pregnant mice infected with influenza have shown abnormal behavior in a variety of assays commonly used as animal models of schizophrenic behaviors (Shi, Fatemi, Sidwell, & Patterson, 2003) similar results have been shown with agents that mimic viral infection without actually causing an infection (Brown & Derkits, 2010). Gene-environment interactions may also play a role in infectious disease and schizophrenia though no clear causal link has been identified.

Today many researchers believe that schizophrenia has a 'two-hit' model of infection. First proposed in Bayer (1999) this 'two-hit' model suggests that neither genetic vulnerability nor environmental factors alone are enough to bring about the disease. A genetic predisposition or prenatal environmental event disrupts neural development in some way, the 'first-hit', which establishes an increased vulnerability for a second hit later in life (Maynard et al. 2001). The idea that both genetics and environment play a summative role has been reported in clinical data and presented in animal models. Further examination of this 'two hit' model in animals can also differentiate the influence of different genes of interest in human schizophrenia. If two genes produce different behavioral phenotypes under the same environmental influence then they may have different importance for schizophrenia.

Treatment History

While Kraepelin and Bleuler pioneered the definition of schizophrenia, historical medical texts and accounts can trace disorders that have symptoms similar to

schizophrenia across multiple cultures and time periods. Even as far back as 1500 B.C.E. the Book of Hearts, part of an ancient Egyptian medical scroll the Ebers Papyrus, describes cognitive dysfunction similar to that of schizophrenia (Kyziridis, 2005). Most of these early descriptions draw the cause of mental anguish back to some supernatural cause, possession by a daemon or the wrath of a displeased god, and treatments ranged from prayer and sacrifices, an attempt to appease the wronged god, to more dangerous methods such as drilling holes in the patients head to exorcise the trapped daemon.

Supernatural influences maintained a firm grasp on the origin and explanation of mental disorders throughout the middle ages. This did not completely hinder the development of diagnosis and treatment of mental disorder though, as early medical scholars and physicians began to distinguish mental disease from bodily disease as early as the 6th century C.E. (Kyziridis, 2005). By the end of the middle ages mental disorders, usually under the broad term insanity, were a distinct class of disease. However treatment was far from what it is today. In the early 14th century the first insane asylums began to appear in Europe but most asylums at this time were simply places to hold patients, not treat them. The "treatments" that were practiced in these asylums were often crude, ineffective, and dangerous; ranging from being restrained in a chair for days on end to more farfetched procedures like trepanning, an ancient surgical technique where a hole was drilled in the patients head to exorcise the daemons trapped within. By the late 1700s a movement had started, as William Battie noted in *Treatise on Madness* "Madness, therefore, like most other morbid cases, rejects all general methods, e.g. bleeding, blisters, caustics, rough cathartics, the gummes and faetid anti-hysterics, opium, mineral waters, cold bathing and vomits" (Morris, 2008). What had become standard practice for

treatment of disease, mental or otherwise, was not working and a new protocol was needed. In the late 18th century pioneers of psychiatric treatment had begun to emerge and called for better management of the mentally ill. Phillipe Pinel, William Tuke, and others began to open reform asylums that used more humane methods to manage the mentally ill. The success of these methods caught on quickly and by the mid-19th century asylums began to celebrate institutions free of the restraints and barbaric treatments of the past (Kyziridis, 2005)

In the early 1950s a monumental breakthrough in the management of schizophrenia came with the first pharmacological treatment- chlorpromazine. Originally developed for use as an anesthetic during surgery, French surgeon Henri Laborit was among the first to notice how the drug produced tranquilizing effects without sedation; and he began to postulate about its antipsychotic application (Stip, 2002). While these behavioral effects were being noticed the underlying pharmacological mechanism was still a complete mystery to the early prescribers of neuroleptics. It would take over ten years before results were published showing that blockade of dopamine receptors was the main mechanism of action for what became the first generation antipsychotics (Carlsson & Lindqvist, 1963). Chlorpromazine is classified as a typical or first generation, antipsychotic and shares this nomenclature with other early antipsychotics such as haloperidol and thioridazine. As with chlorpromazine, these drugs' main mechanism of action is through blockade of dopamine receptors, specifically D_2 and D_3 receptors (Tajima, Fernandez, Lopez-Ibor, Carrasco, & Diaz-Marsa, 2009). While these first generation antipsychotics represented a major advancement for the treatment and management of schizophrenia, they were not without their drawbacks. In general, typical

antipsychotic drugs work well to reduce the positive symptoms of schizophrenia, but they are marginally effective, at best, at alleviating negative symptoms and cognitive deficits that are common in schizophrenics (Tajima et al., 2009). These drugs also have a long list of side effects, the most prevalent among them being extrapyramidal motor side effects (EPS), which are classified as Parkinsonian like tremors and other small, repetitive motor movements. These EPS effects were thought to be predictive of clinical efficacy in the early days of treatment- the stronger or more pronounced the EPS the more effective the drug was thought to be. This theory turned out to not only be wrong but dangerous, since strong EPS became a desired quality when dosing early generation antipsychotics (Weiden, 2007).

In the same decade that chlorpromazine came on the market another drug that would again revolutionize the treatment of schizophrenia came out of the laboratory setting. Clozapine, sometimes hailed as the gold standard of "atypical" antipsychotics, was first synthesized in 1958 by a small Swiss laboratory (Wander Laboratories). While early testing of clozapine was meet with mixed results, including a lack of motor side effects, in 1966 Hanns Hippius continued the clinical trials started by Wander just 7 years earlier and found that clozapine effectively treated psychotic symptoms without the expected EPS effects (Ramachandraiah, Subramaniam, & Tancer, 2009). Clozapine's efficacy at alleviating the positive and negative symptoms, combined with its lack of EPS effects, helped clozapine gain momentum until misfortune struck in the mid-1970s. In 1975 Griffith and Saameli reported in Lancet that sixteen Finnish patients who had been given clozapine had developed agranulocytosis, an acute drop in white blood cell count, resulting in nine deaths (Griffith & Saameli, 1975). This caused the Finnish government

to quickly pull clozapine from the market, and other European countries soon followed suit. Although it had been pulled from most markets, research on this new antipsychotic continued. In 1988 a study was published showing that patients who did not respond well to typical antipsychotic drugs showed a significant improvement after treatment with clozapine. Patients who had been given clozapine treatment displayed significant improvements in Clinical Global Impressions, Brief Psychiatric Rating Scale, Nurses' Observation Scale for Inpatient Evaluation - all scales that measure the severity of a patient's symptoms or quality of life (Kane, Honigfeld, Singer, & Meltzer, 1988). A number of clozapine "clones", drugs with similar binding profiles or chemical structures, began to appear in the 1990s. Drugs such as olanzapine, quetiapine, and risperidone were more efficacious and caused less side effects, especially EPS, than their typical predecessors; although, except for clozapine, none of the newer drugs seemed to be more efficacious than any other (Ramachandraiah et al., 2009). While clozapine is still reserved for use in patients resistant to other forms of treatment, its superior efficacy for treating the symptoms of schizophrenia and decreased motor side effects leaves it as one of the most effective treatments for schizophrenia.

Clozapine

Clozapine began a new era of pharmacological treatment in schizophrenia. The drugs that would later fill out the ranks of the atypical antipsychotics mimic, in some way, its structure and receptor binding profile. While classification of antipsychotics is often characterized by presence or severity of EPS effects (Meltzer, 2000), most atypical antipsychotics share a mechanism of action that differs from the mechanism of most

typical antipsychotics. Most typical antipsychotics work through antagonism of D_2/D_3 receptors with strong receptor binding affinity (Creese, Burt, & Snyder, 1976). This dopamine receptor antagonism is also thought to be the pharmacological source of many of the side effects in first generation drugs (Meltzer & Stahl, 1976). Atypical antipsychotics bind to dopamine D_2 and D_3 receptors, although their affinity for D_2 and D_3 receptors is lowered as compared typical antipsychotics, while the ratio of binding to 5-HT_2 receptor subtypes relative to dopamine binding is greater than in typical antipsychotics (Meltzer, Matsubara, & Lee, 1989). Specifically it is thought that the inverse agonist action of 5-HT_{2A} in combination with weak D_2/D_3 antagonism as well as 5-HT_{2A} antagonism causes atypical antipsychotics to be more efficacious and more tolerable than typical antipsychotic drugs. (Meltzer & Massey, 2011).

Behavioral Phenotyping

Behavioral phenotyping is the study of how genetic differences between organisms affect the organisms' behavior. While integral to finding behavioral differences between genetically altered animals and their background control strains, it can also be used to examine differences between inbreed strains of the same species. Behavioral phenotyping can also be used to draw similarities from studies using different strains of the same species. Although the C57/BL6 mouse has become the "poster child" for rodents, specifically mice, used in behavioral research it is not perfect for all behavioral models. Testing done with other popular strains, including DBA/2 and 129 substrains (129S, 129T, 129P) can draw correlates to other research done with C57BL/6 mice. The C57BL/6 and 129 strains are commonly used as background strains when producing knockout mice. Examining how each of these strains performs on a specific

task, as well as the B6129 hybrid strain, can give important insights into the behavior of any knockout mice produced using C57 and 129 inbred strains as the parent or background strain by providing a behavioral baseline free of genetic manipulation. Review articles have examined a large number of inbred mouse strain using a variety of different behavioral measures, however the battery of behavioral assays and strains or genes of interest change from area to area (Crawley et al., 1997; Hossain, Wong, & Simpson, 2004). Also, behavior is highly susceptible to subtle environmental changes from lab to lab leading some to question the validity of some results found through behavioral phenotyping (Crabbe, Wahlsten, & Dudek, 1999).

Behavioral phenotyping of inbred strains can also highlight the importance of genetics, metabolism, and receptor expression on behavior. In particular drug discrimination studies with clozapine using different inbred strains and knockout animals can lead to insights about the underlying receptor mechanisms that account for the discriminative stimulus properties of a drug. Clozapine discrimination has already been established in C57BL/6 mice and DBA/2 mice. Both strains were able to acquire the discrimination with a training dose of 2.5 mg/kg and ED_{50} values for clozapine were similar in both strains, (ED_{50} = 1.19 mg/kg (95% CI = 1.09-1.30 mg/kg) for C57BL/6 (Philibin et al., 2009) and ED_{50} = 1.30 (95% CI = 1.178-1.443 mg/kg) for DBA/2 (Porter, Walentiny, Philibin, Vunck, & Crabbe, 2008)). While antagonism of α_1 and 5-HT_2 receptors are important in the discriminative stimulus properties of clozapine in C57BL/6 mice, none of the tested selective antagonists substituted for clozapine in the DBA/2 strain, implying a compound cue in the DBA/2 strain (Porter et al., 2008) (see Table 1).

Drug Discrimination with Antipsychotic Drugs

Drug discrimination is a preclinical assay where animals are trained to make a specific operant response depending on the presession treatment condition the animal received. Drug discrimination with antipsychotics has been established in a variety of species including pigeons (Hoenicke, Vanecek, & Woods, 1992), rodents (Goudie, Smith, Taylor, Taylor, & Tricklebank, 1998; Philibin, Prus, Pehrson, & Porter, 2005), and non-human primates (Carey & Bergman, 1997). Drug discrimination studies with antipsychotics have also been shown to be resistant to small methodological changes, as response to training drugs and test drugs are fairly consistent despite differences in pre-session injection times, schedule of reinforcement, type of reinforcer, and route of injections across studies (Porter & Prus, 2009).

Drug discrimination is used to determine which neurochemical mechanisms play an important role in the discriminative properties of the training drug. The idea that action at a specific neurotransmitter receptor can produce discernible changes in how the animal feels (i.e. subjective effects) is central to the drug discrimination procedure. Drug discrimination allows researchers to explore the pharmacological effects of drugs *in vivo* and can uncover important behavioral effects that molecular assays are unable to detect.

Table 1

Comparison of selective ligands tested in C57BL/6 and DBA/2 animals in a clozapine drug discrimination assay.

Shows substitution testing of selective ligands tested in both C57 and DBA inbreed strains of mice trained to discriminate 2.5mg/kg clozapine from vehicle. FULL: Full substitution with percent drug lever responding ≥ 80%, PARTIAL: Partial with substitution percent drug lever responding ≥ 60%, NO: No substitution with percent drug lever responding < 60%. Data presented is compiled from (Philibin et al., 2009; Porter et al., 2008)

Selective Ligands Tested	C57BL/6 mice	DBA/2 mice
Ritanserin (5-$HT_{2A/2B/2C}$ antagonist)	FULL	NO
Scopolamine (muscarinic antagonist)	PARTIAL	PARTIAL
Prazosin (α_1 adrenergic antagonist)	FULL	NO
Pyrilamine (H_1 antagonist)	NO	NO
Amphetamine (dopamine agonist)	NO	NO

Nearly a decade after chlorpromazine was introduced the first drug discrimination studies using antipsychotics began to appear. Chlorpromazine was used as a training drug for many of these early studies and while training doses, types of reward, and subsequent results varied between these early studies, the studies showed that antipsychotic drugs could be established as discriminative stimuli in this procedure. Stewart (1962) using 4.0 mg/kg chlorpromazine versus vehicle, showed that stimulus control could be established and maintained in a three-compartment shock avoidance chamber. Twelve years later chlorpromazine (1.0 mg/kg) versus vehicle was again established in rats using a two-lever operant task, in which the animals received a food reinforcer for correct responses and a shock for incorrect responses (Barry, Steenberg, Manian, & Buckley, 1974).

Compared to atypical antipsychotics most typical antipsychotics have a relatively limited binding profile. High affinity at the dopamine D_2 receptor family is one of the main similarities of receptor binding in typical antipsychotics. Atypical antipsychotics have a higher affinity at 5-HT_{2A} receptors relative to D_2 receptors. D_2 receptor blockade is thought to be important for the therapeutic action of both typical and atypical antipsychotics (Seeman & Tallerico, 1999), while action at 5-HT_{2A} receptors is believed to be responsible for the lowered risk of EPS seen in atypical antipsychotics as the ratio of D_2 and 5-HT_{2A} binding is higher in atypical antipsychotics while maintaining D_2 receptor affinity is similar (Meltzer, 2002). Although binding data can give us insight to binding affinity of specific receptor subtypes, it does not tell us about agonism or antagonism; thus, knowing the binding of a drug alone can only give us clues to the mechanisms of action of a drug.

Clozapine is the prototypical atypical antipsychotic, which makes it a prime target for drug discrimination studies. With a superior clinical efficacy and diverse binding profile understanding the discriminative stimulus properties of clozapine may provide important information about the psychopharmacology of schizophrenia and its treatment. While clozapine's discriminative cue has been established in different animal models what mediates its cue differs from species to species and even within strain. The discriminative cue in pigeons is mediated by 5-$HT_{2A/2C}$ antagonism (Hoenicke et al., 1992). While antagonism of 5-HT receptors plays a role in clozapine's discriminative stimulus for C57BL/6 mice, 5-HT_{2A} along with α-$_1$ adrenoceptors are the main mechanisms responsible for clozapine's cue in that mouse strain (Philibin et al., 2005; Philibin et al., 2009) but not in DBA/2 mice who did not demonstrate substitution for any selected ligands (Porter et al., 2008). Interestingly multiple studies have identified cholinergic antagonism of M_1 receptors as the primary cue mediating clozapine's discriminative cue in Wistar and Sprague-Dawley rats (Goudie et al., 1998; Kelley & Porter, 1997; Nielsen, 1988) .

Mutant Mice – Knock Out and Transgenic mice

With the undeniable evidence that schizophrenia has a genetic component to it, the development of mutant animal models has allowed for the study of the genetic variability in both the treatment and prevention of schizophrenia by allowing how specific genetic manipulations change development of schizophrenic like symptoms and what drugs can be used to treat these behavioral abnormalities. While no one single gene has been identified as causing schizophrenia, a number of candidate genes have been identified and it is likely that a number of genes play a small, summative role in the

development of the disorder (Picchioni & Murray, 2007). Mutant animal models have also helped in preclinical trials for new treatment drugs by giving researchers animals that have cognitive deficits or neurological morphology closer to what is seen in humans with schizophrenia.

The ability to genetically manipulate the mouse genome to delete genes of interest was done first by a trio of cancer researchers Mario R. Capecchi, Martin J. Evans, and Oliver Smithies, in 1989 for which they were awarded the 2007 Nobel Prize in medicine (Beckman, 2008). Since then an entire industry for the production and distribution of genetically altered mice has been developed, placing specific models of behavior and the ability to study genes of interest in the hands of researchers. Two genes in particular have garnered great attention from the research community in the study and understanding of schizophrenia: *NGR1*, and *DISC1*.

DISC1 (disrupted in schizophrenia 1) was originally found in a large Scottish family in 1970 and has since been shown to play a role in the development of multiple mood disorders, including schizophrenia (Blackwood et al., 2001). Researchers found that *DISC1* knockout animals display animal analogs of schizophrenic behavior (Hikida et al., 2007) as well as changes in brain morphology similar to those seen in human patients (Ellison-Wright, Glahn, Laird, Thelen, & Bullmore, 2008). Bolstered by similarities in both human and animal studies *DISC1* is one of the genetic targets of schizophrenia that has received a large deal of attention from the research community.

NRG1 (neuregulin 1) is a gene that in humans is heavily involved with neurodevelopment, particularly in aspects that have been tied to the brain abnormalities seen in schizophrenia specifically serotonin and dopamine receptor expression and

monoamine transporters (Mei & Xiong, 2008). While complete knockout of the *NRG1* gene is lethal (animals that are homozygous knockouts cannot survive without this gene), +/- heterozygous knockouts have increased levels of dopamine receptors in the prefrontal cortex (Stefansson et al., 2002). Research has shown a strong positive correlation with polymorphisms of *NRG1* and susceptibility to schizophrenia (Li, Collier, & He 2006) and while this research helps to unify a number of different leads on the cause of schizophrenia, including dysregulation of multiple neurotransmitter systems, it is far from the final answer to a genetic cause of schizophrenia.

Rationale

Clozapine is the prototypical second generation (atypical) antipsychotic. Its unique and diverse binding profile paired with its clinical superiority over other atypical and typical antipsychotics could lead to better understanding of the neuropharmacological mechanisms important for the treatment of schizophrenia and how to improve the quality of life for those afflicted through management of the disease. With alleviation of both positive and negative symptoms in schizophrenia, a severely reduced presence of EPS, and the ability to alleviate symptoms in patients who have shown resistance to other antipsychotics clozapine's clinical superiority as an antipsychotic is clear.

Drug discrimination is a powerful behavioral assay that allows researchers to examine the *in vivo* subjective effects of a drug and to determine what receptor mechanisms *in vivo* mediate a drug's discriminative stimulus properties. Examining the difference in discriminative cues between species and strains can help to explain how differences in brain morphology, receptor availability, metabolism, and other factors can

change the discriminative cue of a drug and may help lead to more specific treatments for those who have schizophrenia.

The present study used drug discrimination to examine mechanisms of action of clozapine in 129S2/HSv mice and how these mechanisms differ from C57BL/6 and DBA/2 inbred mouse strains. Clozapine is a second generation, atypical antipsychotic drug developed in 1958. To date no drug discrimination studies have used clozapine as a training drug in the 129S2 strain. As such, this research is an original preclinical study in the effort to investigate the discriminative stimulus properties of clozapine in 129S2 inbred mice.

There are four objective of this study: first, to establish clozapine as a discriminative stimulus in a standard two-lever drug discrimination procedure in 129S2/HSv mice; second, to test typical and atypical antipsychotic drugs to see if they share any discriminative stimulus properties with clozapine; third to test selective ligands to determine the underlying pharmacological mechanisms mediating the discriminative stimulus properties of clozapine in 129S2/HSv mice; and fourth, to compare these findings to previous studies that used C57BL/6 and DBA2 mice to determine how clozapine's discriminative cue compares between these three inbred strains of mice.

Methods

Subjects

Seventeen adult male 129S2/SvHsd inbred mice weighing between 20-30 g (Harlan Laboratories, Indianapolis, IN) were used for this study. Mice were individually

housed in clear plastic cages (18 x 29 x 13 cm) with fitted steel wire tops and cornhusk bedding. They were moved daily (6 to 7 days each week) from a temperature controlled vivarium (22-24° C) under a 12h light/dark cycle (0600/1800 hours) to the laboratory where testing occurred. All research was conducted in accordance with the Institutional Animal Care and use Committee at Virginia Commonwealth University, which approved all procedures. After a one week habituation period animals were food restricted and maintained at 85-90% free feeding body weight on standard rodent chow (Harlan Teklad Lab Diets, Teklad LM-485). Water was available ad libitum in home cages.

Apparatus

Drug discrimination experiments were conducted in six standard computer-interfaced mouse operant conditioning chambers (Model ENV-307A; Med Associates, St. Albans, VT, USA), with two retractable levers positioned on the left and right positions equidistantly (8 cm apart) on the front wall. The levers extended 0.8 cm into the chamber and were positioned 2.5 cm above a grid floor constructed of parallel stainless steel bars, measuring 0.3 cm in diameter. A recessed well in which a liquid dipper would deliver 0.02 ml of sweetened milk (by volume 150 ml sugar, 150 ml powdered non-fat milk, and 500 ml water) was positioned between the two levers-. The inner area of the test chamber measured 15 x 11.5 x 17.5 cm and was surrounded by an aluminum chassis box with a Plexiglas back wall, 2 aluminum side walls, and a single Plexiglas door. Test chambers were housed in a sound attenuated cubicle (Model ENV-022; Med Associates). Experimental events and data collection during these experiments were controlled by Med-PC for Windows software (Med Associates Inc. version 1.0). Unless otherwise

noted the dipper that delivered the milk was raised and available to the animal in the operant chamber for three seconds before descending back into the trough where the sweetened milk liquid reinforcer was kept.

Drugs

Clozapine (gift from Novartis, Hanover, NJ, USA), haloperidol, scopolamine and pyrilamine (Sigma, St. Louis, MO, USA), olanzapine (gift from Eli Lilly, Indianapolis, Indiana, USA), ritanserin (Research Biochemicals International, Natick, Mississippi, USA), iloperidone (gift from HY Meltzer), aripiprazole (and M100907 (gift from Lundbeck, Copenhagen, Denmark) were dissolved in distilled water with two to three drops of lactic acid and pH balanced with sodium hydroxide (all drugs had a pH balance close to 7.0). Chlorpromazine, amphetamine, prazosin (Sigma), thioridazine (Novartis), and ziprasidone (ziprasidone mesylate, Roerig, Division of Pfizer, New York, USA) were dissolved in deionized water and pH balanced with sodium hydroxide (all drugs had a pH balance close to 7.0). Drugs were administrated subcutaneously (s.c.) at a volume of 10 ml/kg body weight with a 30-min presession injection time. All doses refer to the salt (HCl) form of the drugs.

Procedures

Magazine training. The mice were placed in an operant chamber for fifteen minutes. No levers were extended and mice were given access to a sweetened milk liquid reinforcer every 10 sec; the reinforcer would be available for 5 sec. During magazine training reinforcers were presented regardless of the animals' behavior.

FR training. After 3 days of magazine training, mice began single lever fixed ratio (FR) training. Only the vehicle lever was presented and animals were given access to a liquid reinforcer every time they pressed the lever, i.e. a FR 1 schedule of reinforcement. Training sessions (15 min) were conducted daily, six days a week. The FR requirement was gradually increased over 18 sessions until a stable response rate at FR 10 was achieved. On average the mice achieved FR 10 after 12.2 days (Range 11-18).

Errorless training. Once all mice had reached a stable response rate at FR 10 they began errorless vehicle training. Animals received an injection of vehicle (deionized water with 3 drops of lactic acid (~.01 ml) per 50 ml deionized water, pH balanced to 7.0 with sodium hydroxide). Test sessions were 15 minutes long with only the vehicle lever available. All animals received 6 days of errorless vehicle training before moving to errorless drug training. During errorless drug training, the mice were given an injection of the training dose of clozapine and placed in the operant chamber for a 15 minute session. Only the drug lever was presented and animals were again under the FR 10 schedule of reinforcement. The drug and vehicle lever positions were counterbalanced between groups to control for olfactory cues (Extance and Goudie 1981). In order to provide a comparison to clozapine drug discrimination in C57BL/6 mice (Philibin et al. 2005), a 2.5 mg/kg training dose of clozapine was initially used; however this dose was abandoned after 8 sessions due to continued, severe rate suppressant effects in the mice. The training dose was lowered to 1.25 mg/kg and response rates for all animals increased to acceptable levels. Errorless clozapine training continued for an average of 9.7 days (range 8-15 days).

Two lever acquisition training. All animals were placed on a double alternation injection schedule with two days of vehicle followed by two days of clozapine and repeated (VEH, VEH, CLZ, CLZ, VEH, VEH etc.). Both levers were present during this stage of the procedure; however, only responses on the condition-appropriate lever were reinforced. Any response made on the opposite lever reset the FR 10 counter to 0. In order for a mouse to pass a training day it had to meet three criteria: (1) complete the first FR on the condition-appropriate lever, (2) at least 80% of total responses made were on the condition appropriate lever, and (3) at least ten responses per minute were made. Animals were required to meet training criteria for 5 of 6 days to pass the acquisition phase of the study. All animals meet training criteria in an average of 21.2 days (range 6 to 33 days).

Testing. Once animals meet the training criteria, generalization and substitution testing began. Drug testing was conducted approximately two times per week with at least two training days in between. In order to be eligible for testing mice were required to pass both a clozapine and vehicle training day consecutively; however, they could be passed in either order. Before the clozapine generalization curve and subsequent dose response curves were conducted, clozapine and vehicle control tests had to be passed. During these control tests animals received an injection of 1.25 mg/kg clozapine or vehicle and both levers were reinforced on the FR 10 schedule (switching levers prior to completing the FR 10 requirement reset the counter for the opposite lever). To pass a control test animals were required to meet the training criteria (correct first FR, 80% or greater condition-appropriate responding, and response rates equal to or greater than 10 responses per minute). If an animal failed a control test, it was placed back on the double

alternation training schedule and the next time it was available to test it would be retested at that control point.

Data analysis. The number of lever presses on the drug lever divided by the total number of lever presses (% Drug Lever Responding), the average number of responses per minute, and the lever that the animal pressed ten times consecutively (First Fixed Ratio) were recorded for each session. ED_{50} values were calculated for each drug dose effect curve that fully substituted (average percent drug lever responding >80%) for the training drug clozapine. ED_{50} values were calculated using the least squares method of linear regression with the linear portion of the dose effect curve. A repeated measures analysis of variance (ANOVA) comparing mean response rates for each dose was performed for each drug (GB-STAT software; Dynamic Microsystems, Inc., Silver Spring, MD). Significant ANOVAs were followed by a Dunnett's post-hoc test ($p < 0.05$). Animals were required to receive one reinforcer or have response rates equal to or greater than 2.0 responses per minute to have percent drug lever responding (%DLR) included in the group data.

Results

Acquisition

The results of the acquisition training for the mice successfully trained to discriminate 1.25 mg/kg clozapine from vehicle are shown in Figure 1. Seventeen of the nineteen mice reached training criteria in an average of 21.2 days (SEM $\pm$ 8.5) with a range of 6-33 days. One mouse became ill during single lever training and another animal

failed to develop tolerance to the rate suppressant effects of clozapine and both were removed from the study.

Clozapine Generalization Curve

Mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the clozapine generalization curve (1.25 mg/kg training dose) are shown in Figure 2. Generalization testing yielded an ED_{50} = 0.5026 mg/kg (95% C. I. 0.3812 – 0.6627 mg/kg). Full generalization to clozapine's discriminative cue was attained at 1.25 mg/kg, 1.77 mg/kg, and 2.5 mg/kg with a significant suppression ($F_{7, 105} = 6.86$, $p < .001$) of response rates at the 2.5 mg/kg dose (the %DLR data were not included for 1 mouse whose RPM fell below 2.0 RPM. Four mice were tested at a dose of 5.0 mg/kg clozapine; however, this dose was abandoned as responding was completely suppressed for all 4 animals.

Clozapine Time Course

Time course data shown in Figure 3 demonstrated that the 1.25 mg/kg training dose of clozapine produced full responding on the drug-paired lever only at the 30 minute post s.c. injection time point. Partial clozapine substitution was seen at 15 minutes post s.c. injection (average drug lever responding = 66.1%). At 60 minutes post s.c. injection drug-lever responding dropped to 39.1% and at 120 minutes post injection drug-lever responding decreased to vehicle-level responding with only 6.2% drug-lever responding. Mean response rates were stable across all time points.

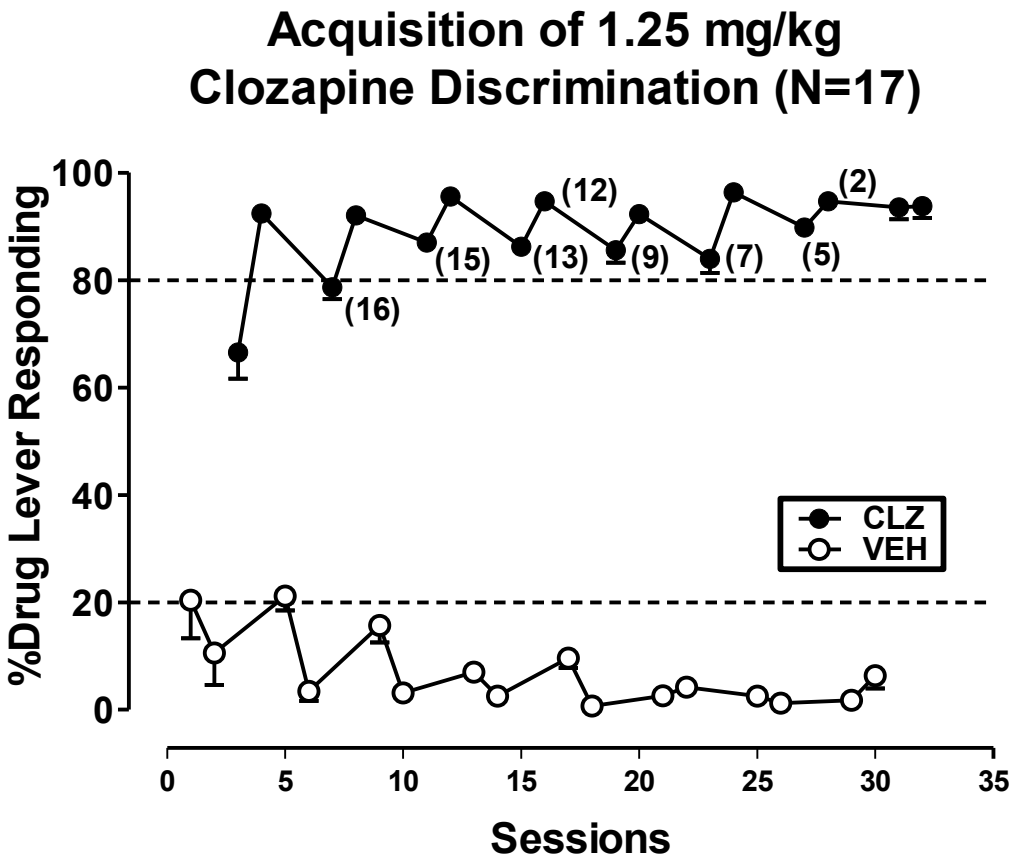

Figure 1. Acquisition of Clozapine Discrimination

Acquisition of two-lever drug discrimination is shown for 1.25 mg/kg clozapine training dose. Mean percent drug lever responses ($\pm$ SEM) are presented separately for drug injections (*closed circles*) and vehicle injections (*open circles*). The *dashed line* at 80% indicates drug-appropriate responding and the *dashed line* at 20% indicated vehicle-appropriate responding. As the mice met the training criteria, they were removed from the curves (the numbers in parenthesis indicate the number of remaining mice).

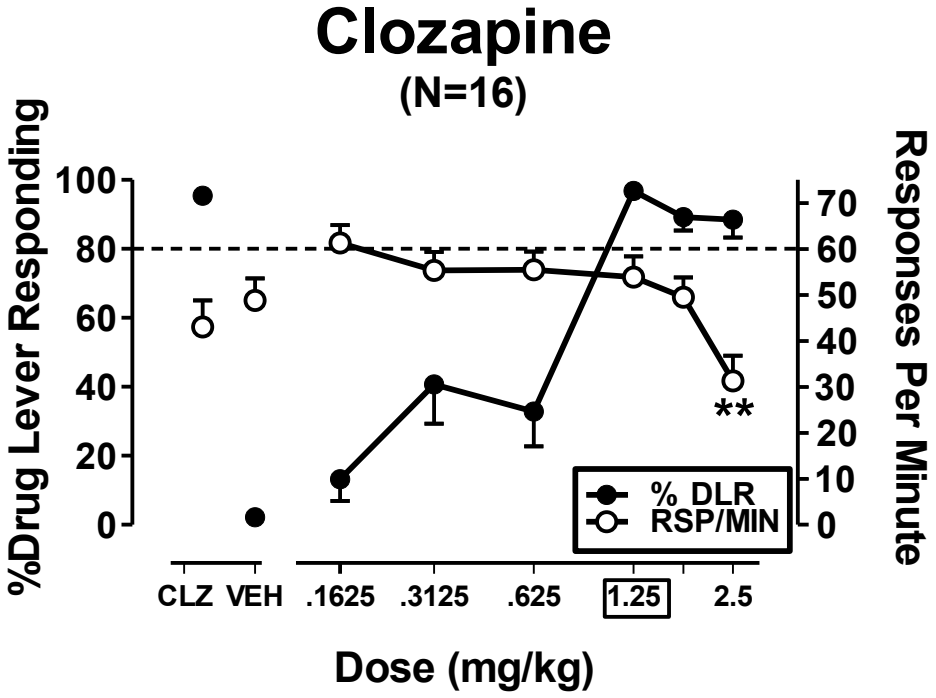

Figure 2. Clozapine Generalization Curve

Mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) are shown for the atypical antipsychotic clozapine generalization curve (1.25 mg/kg clozapine training dose) in a two-lever drug discrimination procedure. The *dashed line* at 80% drug lever responding (DLR) indicates full generalization to the training drug. Prior to generalization testing, control test sessions were conducted with both clozapine (1.25 mg/kg) and vehicle. The data for mice with response rates lower than two responses per minute were not included in the %DLR data. For the response rate data, significant differences from vehicle are indicated by asterisks *(* $P < 0.05$, ** $P < 0.01$)*

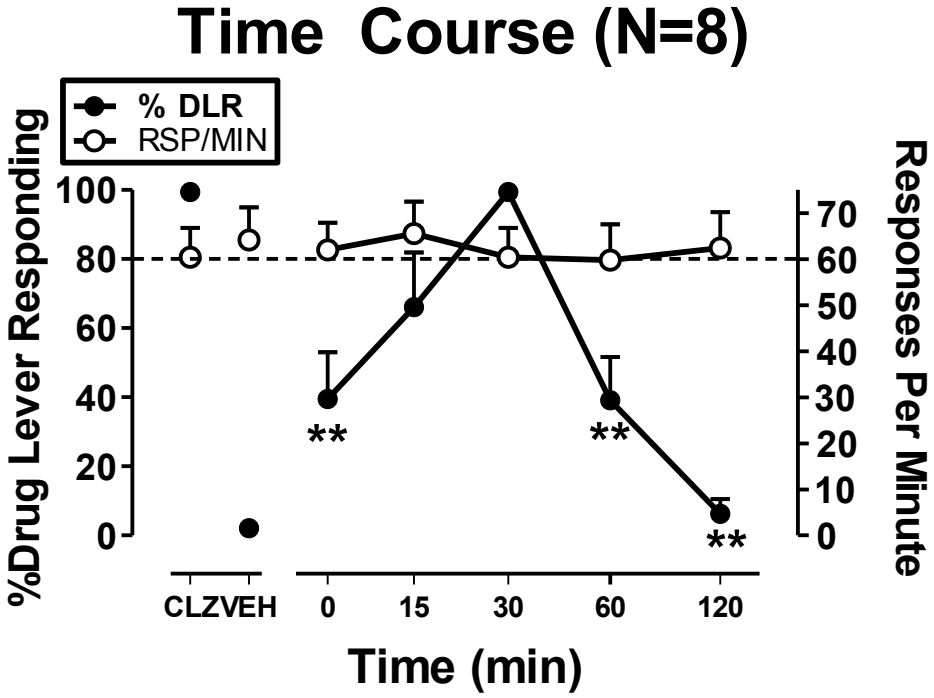

Figure 3. Clozapine Discrimination Time Course

Time course data are shown for 0, 15, 30, 60, and 120 min presession s.c. injection times for the 1.25 mg/kg training dose of clozapine. For percent drug lever responding, significant differences from the presession injection time (30 min) are indicated by asterisks (** P<.001). There were no significant differences for response rates.

Olanzapine Substitution

The atypical antipsychotic olanzapine produced full substitution for clozapine (Figure 4) at 0.25 mg/kg (95.6% DLR) with a significant reduction in response rates at both the 0.125 and 0.25 mg/kg doses ($F_{5,30} = 37.11$, $P < .001$) Generalization testing with olanzapine yielded an $ED_{50} =$ 0.03774 mg/kg (95% CI 0.02553 – 0.05580 mg/kg).

Aripiprazole Substitution

The atypical antipsychotic aripiprazole (Figure 5) did not substitute for clozapine at any of the tested doses (1.25 – 10.0 mg/kg) and maximum %DLR was seen at the 5.0 mg/kg dose (44.5% DLR). All doses of aripiprazole produced significant rate suppression ($F_{4,20} = 6.14$, $p = .002$).

Ziprasidone Substitution

The atypical antipsychotic ziprasidone (Figure 6) did not produce substitution to clozapine at any of the tested doses (0.25 – 8.0 mg/kg) with maximum clozapine-appropriate responding at 8.0 mg/kg (47.9% DLR). Ziprasidone did not produce any significant changes in response rates ($F_{6,30} = 1.57$, $p = .191$)

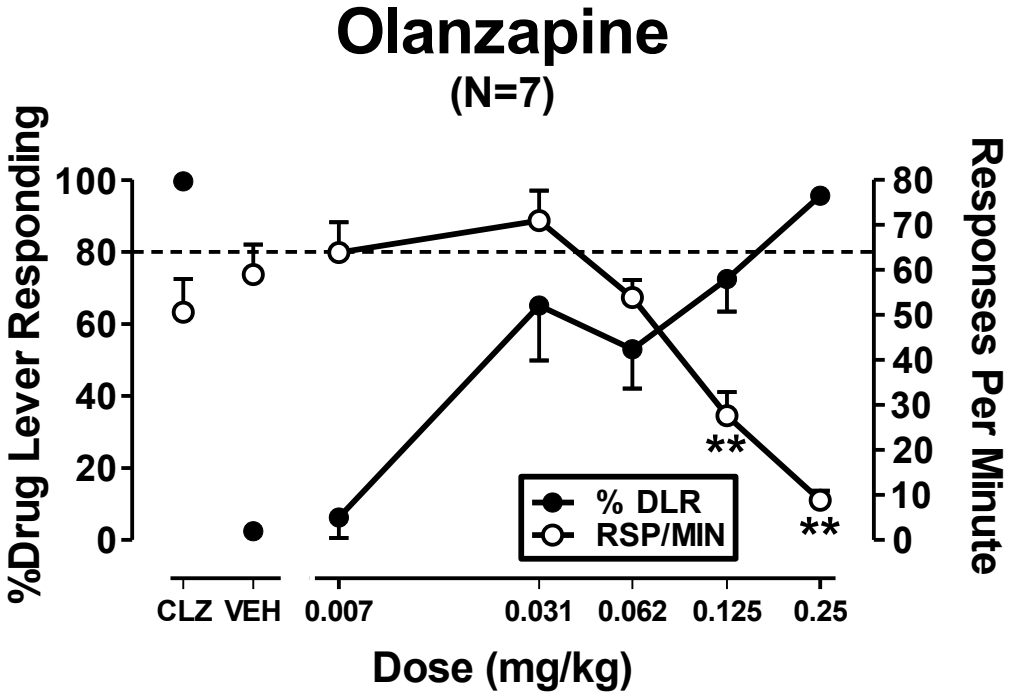

Figure 4. Olanzapine Substitution Curve

Mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) are shown for the atypical antipsychotic olanzapine substitution curve. All other details are the same as Figure 2.

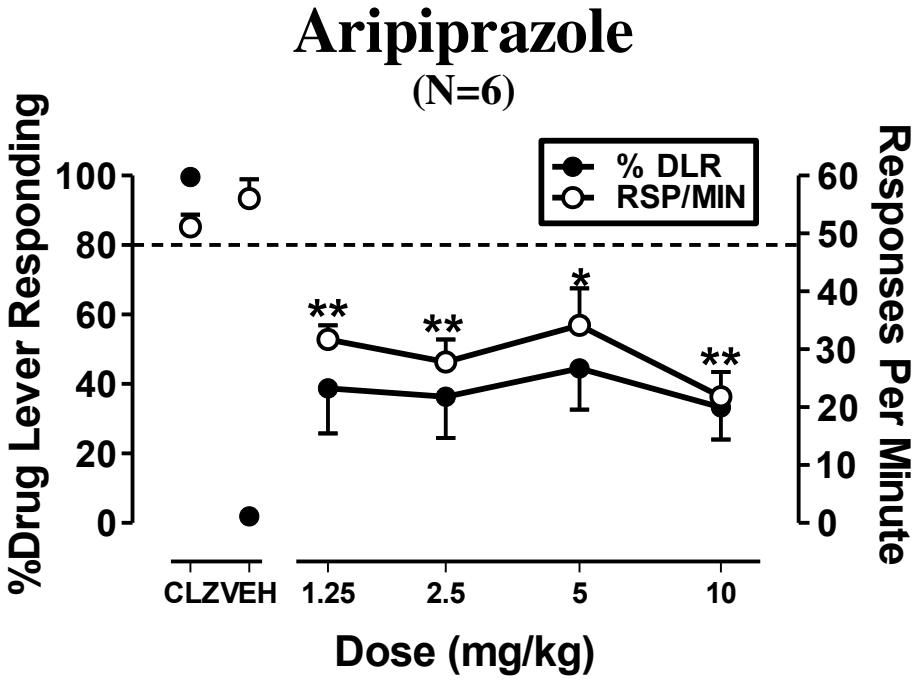

Figure 5 Aripiprazole Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the atypical antipsychotic aripiprazole substitution curve. All other details are the same as Figure 2.

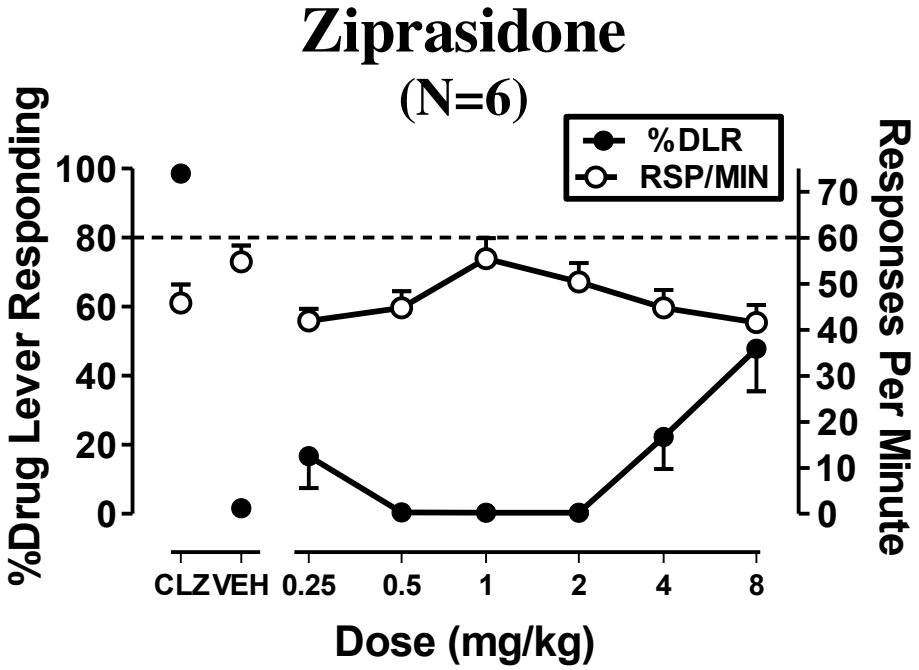

Figure 6 Ziprasidone Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the atypical antipsychotic ziprasidone substitution curve. All other details are the same as Figure 2.

Iloperidone Substitution

The atypical antipsychotic iloperidone (Figure 7) produced full substitution at 0.2 mg/kg (84.6% DLR) and high partial substitution at 0.4 mg/kg (75.8% DLR). Generalization testing revealed an ED_{50} = 0.0947 mg/kg (95% CI = 0.0608 – 0.1456 mg/kg). While both 0.2 and 0.4 mg/kg doses produced significant rate suppression as compared to vehicle ($F_{5,35}$ = 27.23, p <.001), the effects at 0.4 mg/kg were stronger as only 3 of the 7 animals had response rates over 2.0 RPM.

Haloperidol Substitution

The typical antipsychotic haloperidol did not fully substitute for clozapine (see Figure 8) at any of the tested doses. Partial substitution was seen at 0.2 mg/kg (66.0% DLR) and 0.4 mg/kg (66.4% DLR) doses of haloperidol. Rates were significantly suppressed at by the 0.1, 0.2 and 0.4 mg/kg doses ($F(5,245) = 1944.6$, $p < .001$)

Chlorpromazine Substitution

The typical antipsychotic chlorpromazine (Figure 9) produced partial substitution at 0.25 mg/kg (72.5% DLR), but no substitution at 0.125 or 0.5 mg/kg. The 0.25mg/kg dose produced a significant suppression of response rates ($F_{3,24}$ = 6.56, p = .003). Three mice were tested at 1.0 mg/kg, but responding was completely suppressed so testing of that dose was abandoned.

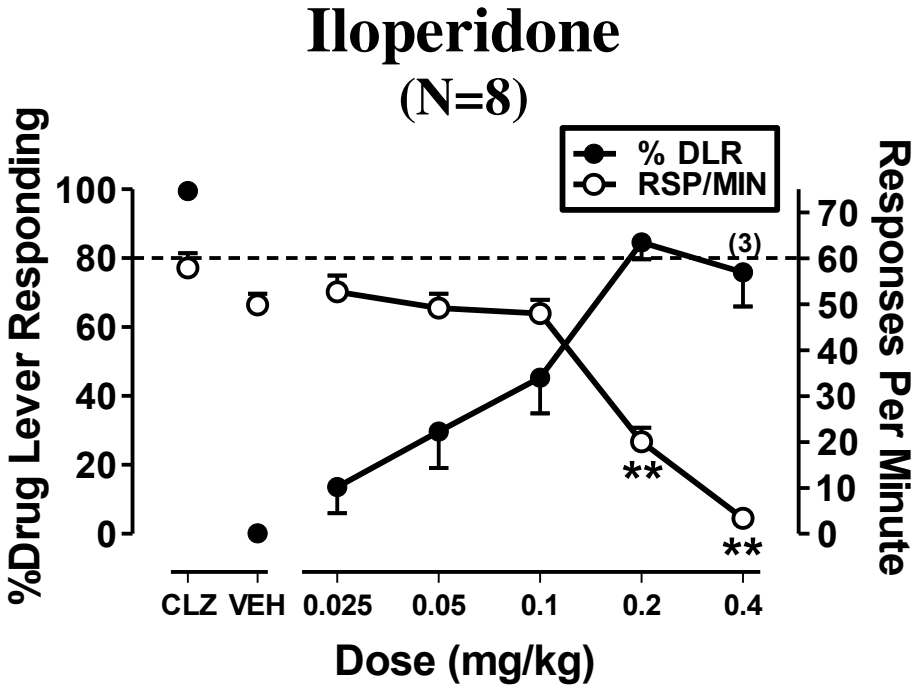

Figure 7 Iloperidone Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the atypical antipsychotic iloperidone substitution curve. All other details are the same as Figure 2.

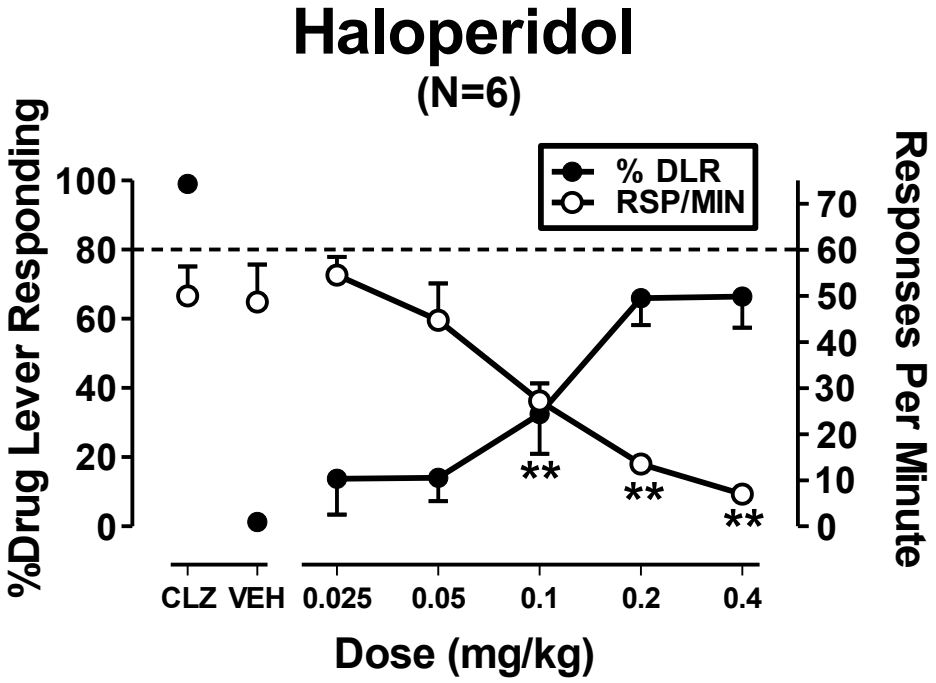

Figure 8. Haloperidol Substitution Curve

Mean percent drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the typical antipsychotic haloperidol substitution curve. All other details are the same as Figure 2

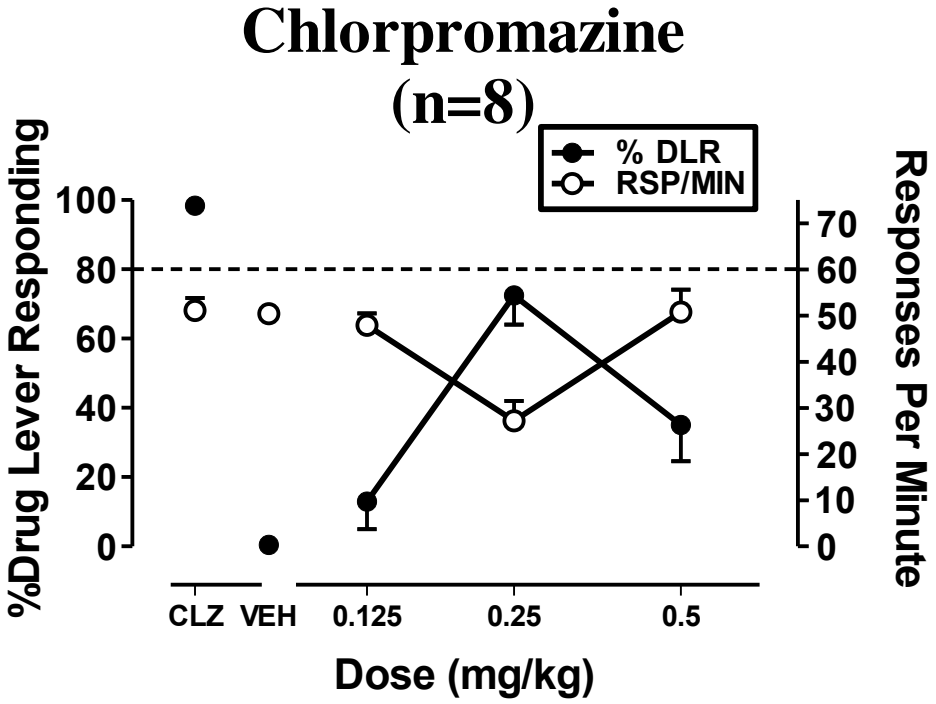

Figure 9 Chlorpromazine Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the typical antipsychotic chlorpromazine substitution curve. All other details are the same as Figure 2.

Thioridazine Substitution

The typical antipsychotic thioridazine (Figure 10) produced full substitution for clozapine at 16.0 mg/kg (93.7% DLR) and generalization testing revealed an ED_{50} = 2.71 mg/kg (95% CI 1.65 - 4.46 mg/kg). The 8.0 and 16.0 mg/kg doses produced a small, but significant suppression of response rates as compared to vehicle ($F_{4,20}$ = 5.21, p = .005)

Pyrilamine Substitution

The histaminergic H_1 antagonist pyrilamine (Figure 11) did not substitute for clozapine at any of the tested doses (5.0 mg/kg – 28.3 mg/kg) never generating more than 16.8% drug-lever responding. Response rates were significantly reduced by the 28.3 mg/kg dose of pyrilamine ($F_{4,20}$ = 6.84, p = .001). Four animals were tested at 40.0 mg/kg pyrilamine however all animals were completely rate suppressed and testing at this dose was abandoned.

Prazosin Substitution

The adrenergic α_1 antagonist prazosin (Figure 12) fully substituted for clozapine at the 10.0 mg/kg dose (83.4% DLR). Generalization testing yielded an ED_{50} = 1.1427 mg/kg (95% CI 0.70669 – 1.84784 mg/kg). Response rates were significantly reduced at 1.0, 3.0, and 10.0 mg/kg doses, as compared to vehicle ($F_{6,42}$ = 11.63, p < .001).

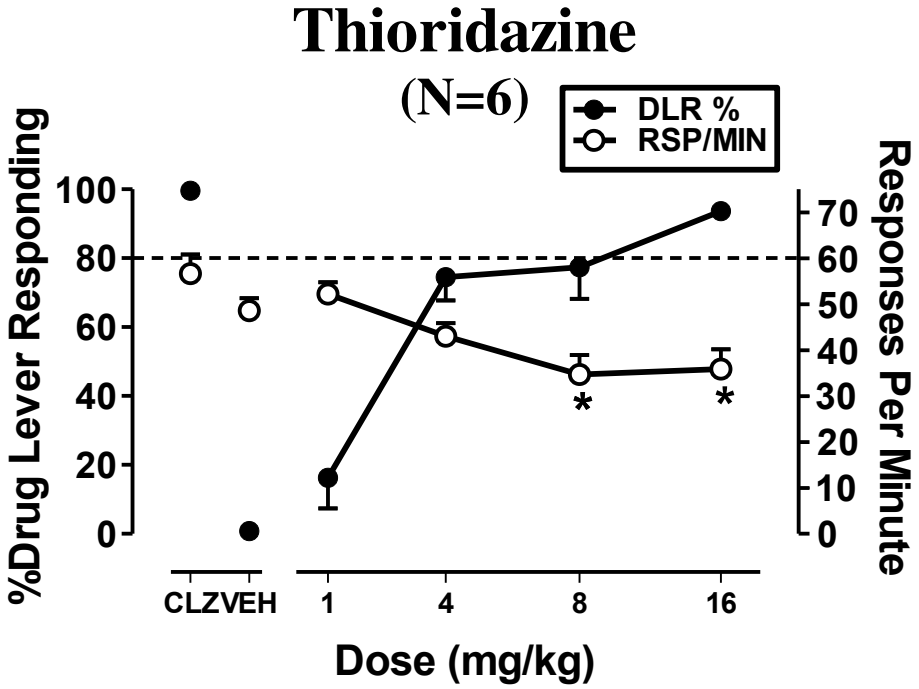

Figure 10 Thioridizine Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the typical antipsychotic thioridazine substitution curve. All other details are the same as Figure 2.

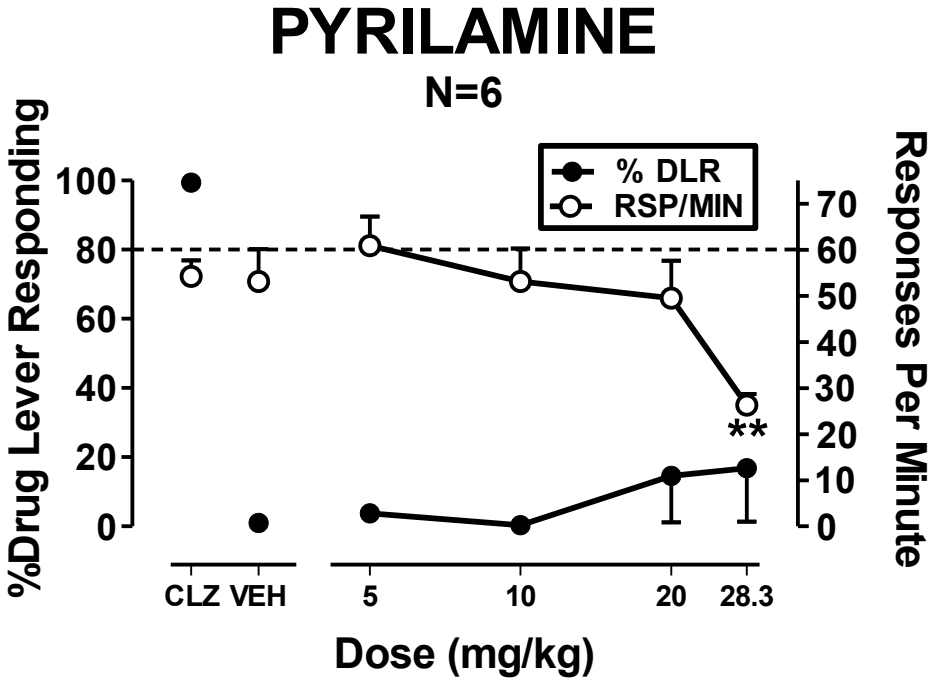

Figure 11. Pyrilamine Substitution Curve

Mean percent drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the histaminergic (H_1) antagonist pyrilamine substitution curve. All other details are the same as Figure 2.

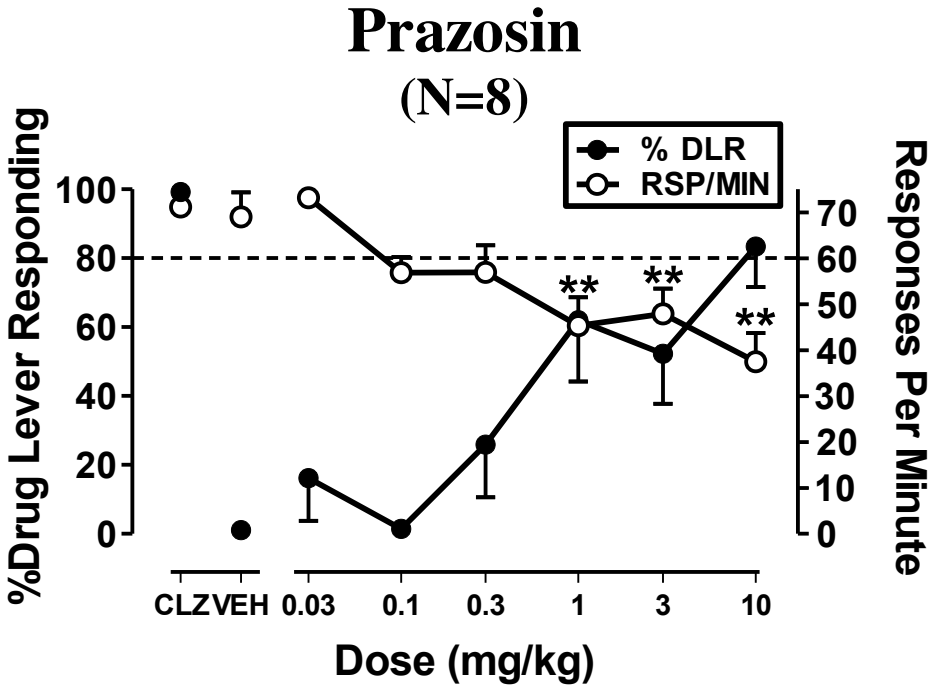

Figure 12. Prazosin Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the adrenergic α_1 antagonist prazosin substitution curve. All other details are the same as Figure 2.

Scopolamine Substitution

The cholinergic muscarinic antagonist scopolamine (Figure 13) did not fully substitute for clozapine; however, partial substitution was achieved at 8.0 mg/kg (68.0% DLR). Significant rate suppression was seen at the 8.0 mg/kg dose ($F_{5,35} = 4.55$, p = .003).

Amphetamine Substitution

The dopamine agonist amphetamine (Figure 14) did not substitute for clozapine at any of the tested doses (0.25 – 2.0 mg/kg) with maximum clozapine-lever responding reaching 20.53%. All doses produced significant rate suppression ($F_{4,20} = 16.18$, p < .001) as compared to vehicle rates of response.

Ritanserin Substitution

The 5-HT_2 antagonist ritanserin (Figure 15) did not substitute for clozapine at any of the tested doses (1.0 – 16.0 mg/kg) with maximum clozapine lever responding reaching 57.4%. Ritanserin did not produce any significant changes in response rates.

M100907 Substitution

The selective 5-HT_{2A} antagonist M100907 (Figure 16) did not produce full substitution for clozapine, although partial substitution was evident at the 3.0 and 5.6 mg/kg doses (69.1% DLR and 69.3% DLR, respectively). M100907 did not produce any significant changes in response rates ($F_{5,30} = 1.60$, p =.190). 10.0 mg/kg M100907 was tested in three animals but all were completely rate suppressed and testing at this dose was abandoned.

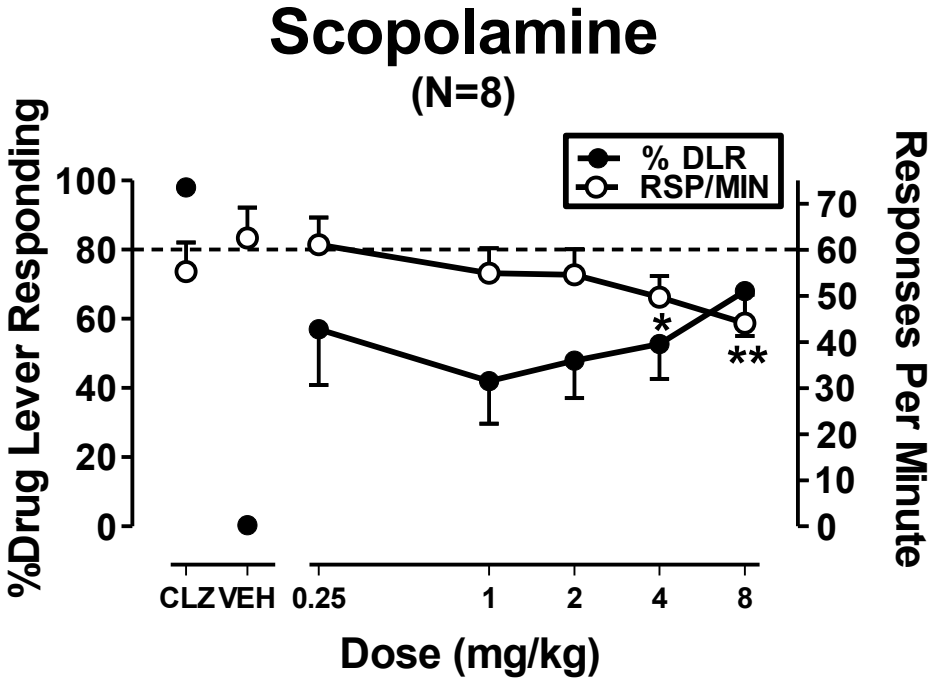

Figure 13 Scopolamine Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the cholinergic muscarinic antagonist scoplamine substitution curve. All other details are the same as Figure 2.

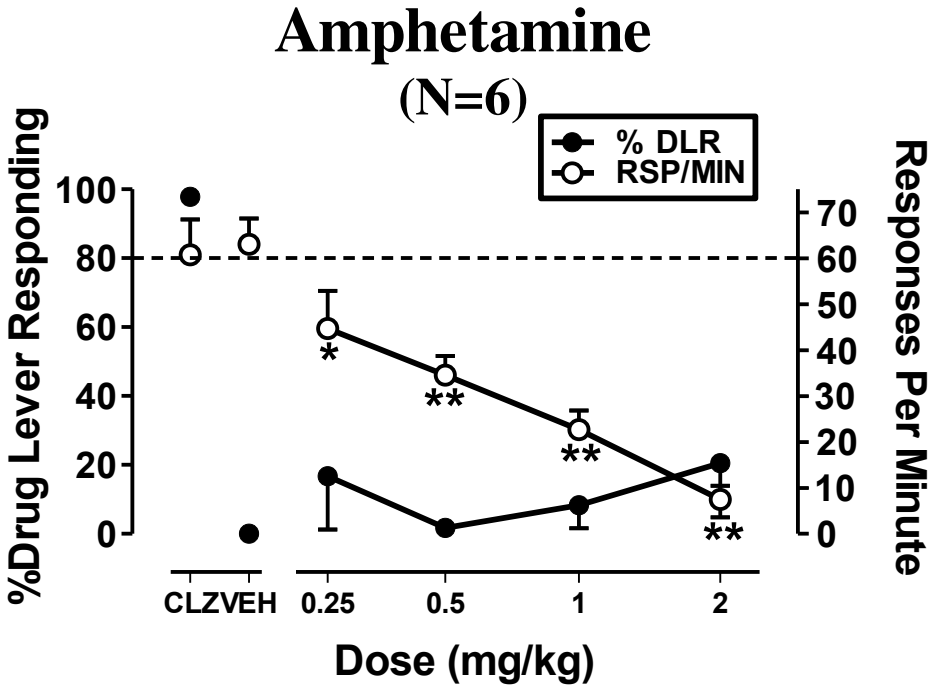

Figure 14 Amphetamine Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the dopamine agonist amphetamine substitution curve. All other details are the same as Figure 2.

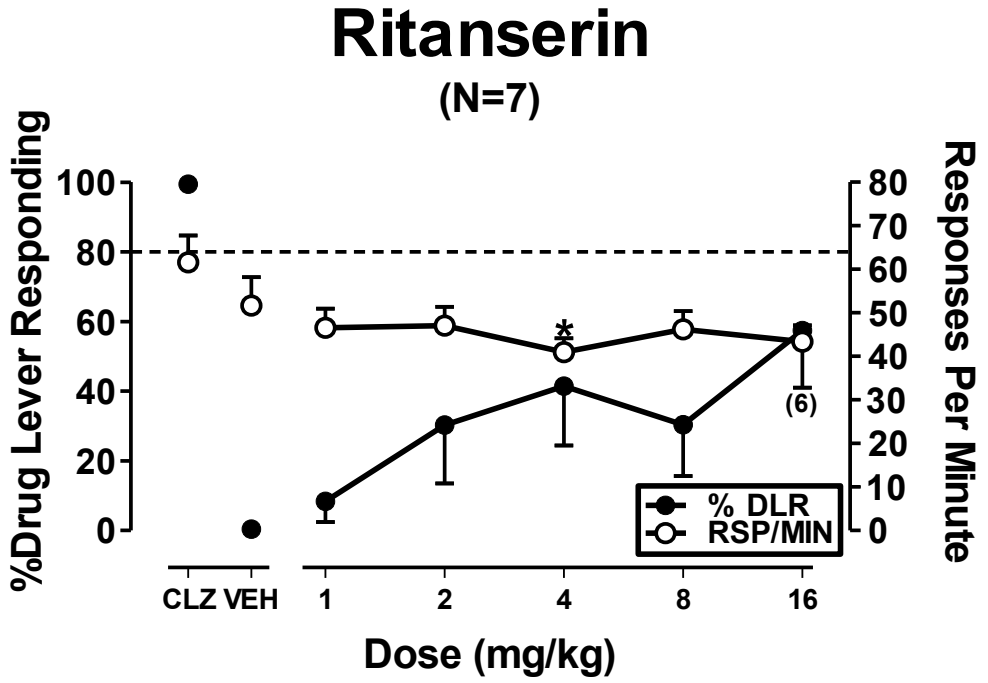

Figure 15 Ritanserin Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the 5-HT_2 antagonist ritanserin substitution curve. All other details are the same as Figure 2.

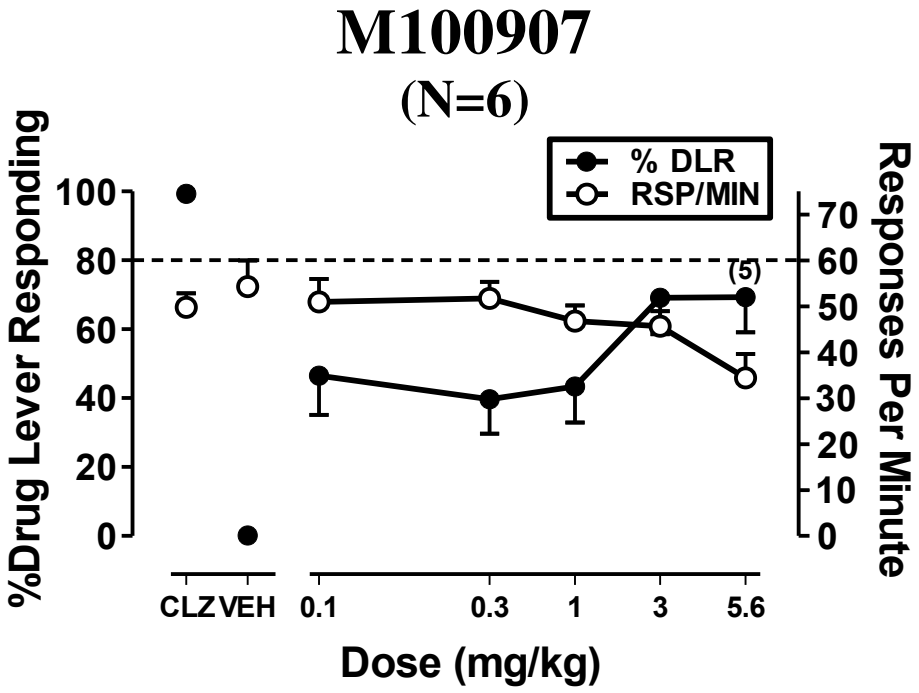

Figure 16 M100-907 Substitution Curve

Mean percentage drug-lever responding (± SEM) and mean responses per minute (± SEM) are shown for the 5-HT_{2A} antagonist M100907 substitution curve. All other details are the same as Figure 2.

Discussion

Clozapine as a discriminative stimulus in 129S2/SvHsd mice

The current study demonstrated that clozapine can successfully be trained in the 129S2/SvHsd inbred mouse strain, continuing to expand on the data characterizing the robust discriminative stimulus properties of clozapine, and how differences in genotype and receptor expression can affect this cue. Clozapine's discriminative stimulus has been established in several species of animals; including rats (Kelley & Porter, 1997; Millan et al., 1999; Prus, Philibin, Pehrson, & Porter, 2005), pigeons (Hoenicke et al., 1992), squirrel monkeys (Carey & Bergman, 1997), C57BL/6 inbred mice (Philibin et al., 2005; Philibin et al., 2009) and DBA/2 inbred mice (Porter et al., 2008). While 129S2 mice readily acquired the discriminative stimulus cue of clozapine, the average number of training sessions for an animal to successfully pass 5 of 6 training days was significantly longer than for C57 and DBA mice. Also the 129 mice did not initially develop tolerance to 2.5 mg/kg of clozapine (used as the training dose in C57BL/6 and DBA/2 mice) requiring the training dose to be lowered to 1.25 mg/kg.

Clozapine Tolerance

The first and most surprising behavioral difference seen in 129S2 mice was the delayed development of tolerance to clozapine's rate suppressant effects. 129S2 mice did not initially develop tolerance to 2.5 mg/kg clozapine, showing complete rate suppression after 8 days of chronic administration. Although clozapine (as well as other antipsychotic drugs) is known to have rate suppressant effects, tolerance to the suppressant effects of

clozapine develops relatively rapidly after chronic administration (Varvel, Vann, Wise, Philibin, & Porter, 2002). After a one-week washout period 1.25 mg/kg clozapine was administered and the 129S2 mice were able to develop tolerance to the rate suppressant effects of clozapine at this dose. Interestingly, when mice were tested with 2.5 mg/kg clozapine in the clozapine generalization curve rate suppressant effects had somewhat recovered, although response rates were still significantly less than vehicle rates (see figure 2). This shows that the 129S2 inbred strain was able to develop some tolerance to the rate suppressant effects of clozapine, but at a delayed rate.

Differences in how mouse strains metabolize clozapine could help to explain these differences in development of tolerance. P450 cytochrome is a family of endogenous enzymes that is the largest contributor of drug metabolism and bioactivation (Guengerich, 2008). Examining differences in enzyme expression between mouse strains could help to explain the differences between the 129, C57, and DBA inbred mouse strains. Clozapine is mainly metabolized by the liver enzyme CYP1A2 while CYP2C19, CYP2D6 and CYP3A4 play a lesser role in its metabolism (Urichuk, Prior, Dursun, & Baker, 2008). Examination of liver enzyme expression in 5 inbred mouse strains, C57BL/6 and 129/SvJ included, found no significant difference between strains in expression or activity of cytochrome P450 enzyme family, including phenacetin O-deethylation which was used as a marker for CYP1A2 activity (Löfgren, Hagbjörk, Ekman, Fransson-Steen, & Terelius, 2004). The lack of differences in the metabolizing enzymes for clozapine should yield similar time courses between C57BL/6 and 129S2. However, the time course for 1.25 mg/kg clozapine in 129S2 mice (see figure 3) showed that the mice only fully substituted at the 30 minute presession injection time (the

injection time the animals were trained at). By 60 minutes enough of the drug had left the animal's system that drug lever responding was significantly reduced. This stands in contrast to clozapine's time course in C57BL/6 mice that showed clozapine-like responding at 15, 30, and 60 minutes post injection time (Philibin et al., 2005). These data suggest that differences in metabolism of clozapine cannot be used to explain the reduced tolerance to clozapine seen in the 129S2 inbred mouse strain.

5HT Antagonism

While it is not the only factor that differentiates atypical from typical antipsychotic drugs, atypical antipsychotics possess a higher ratio of 5-HT_{2A} binding to DA_2 binding as compared to typical antipsychotics with the exception of aripiprazole (Meltzer et al., 1989) (see table 2) and amisulpride (Schoemaker et al., 1997) . Of the atypical antipsychotics tested in the present study olanzapine and iloperidone engendered clozapine-like responding while ziprasidone did not. Antagonism at 5-HT receptors has been shown to be important for clozapine's discriminative cue in pigeons (Hoenicke et al., 1992) and in C57BL/6 mice (Philibin et al., 2005; Philibin et al., 2009). The mixed $5HT_{2A/2B/2C}$ antagonist ritanserin did not substitute for clozapine in the present study and the selective 5-HT_{2A} antagonist M100907 only engendered partial substitution for clozapine. These data suggest that antagonism of 5-HT_2 receptors is not as important for the discriminative stimulus properties of clozapine in 129S2 mice as it is in C57BL/6 mice. While M100907 has not been tested in DBA/2 mice, the mixed $5HT_{2A/2B/2C}$ antagonist ritanserin did not substitute (maximal clozapine drug lever responding of only 18.49%) and examination of individual animal data showed that no mice displayed partial

substitution (>60% drug lever responding) (Porter et al., 2008) further supporting the idea that for DBA/2 and 129S2 mice, antagonism of 5-HT receptors is not important for clozapine's discriminative cue.

Cholinergic Muscarinic Antagonism

The muscarinic antagonist scopolamine produced partial substation for clozapine in the 129S2 inbred strain. While partial substitution with scopolamine was evident in both C57 and DBA mice, partial substation for clozapine in 129S2 mice was not seen until a much higher dose of scopolamine (8.0 mg/kg) was tested (as compared to 2.0 mg/kg and 1.0 mg/kg respectively in the C57 and DBA mice). K_i binding data (See table 2) for iloperidone and ziprasidone shows a relatively weak affinity for muscarinic receptors and their substitution for clozapine coupled with the inability of the selective muscarinic antagonist scopolamine to substitute suggests that antagonism of muscarinic receptors does not play an important role in the discriminative cue of clozapine in 129S2 mice.

Alpha Adrenergic Antagonism

The selective alpha adrenergic antagonist prazosin produced full substitution for clozapine although, like scopolamine, the dose that engendered full substitution in 129S2 mice (10.0 mg/kg) was much higher than the dose that fully substituted for clozapine in C57BL/6 mice (2.8 mg/kg) (Philibin et al., 2009). K_i values (see table 2) for iloperidone reveal a stronger binding affinity for alpha adrenergic receptors than for ziprasidone

Table 2.

Dissociation rate constants for typical and atypical antipsychotic drugs

Dissociation Rate Constants (K_i, nM) are shown for antipsychotic drugs at selected neurotransmitter receptor subtypes (from Schotte et al. 1996 except where indicated). These values should be used for general comparisons only since the species, conditions, tissues and assays varied among the studies.

Species *(except where indicated)*:	RAT	RAT	RAT	RAT	GUINEA PIG
Tissue *(except where indicated)*:	Frontal Cortex	Striatum	Striatum	Total Cortex	Cerebellum
ATYPICAL APDs	**5-HT_{2A}**	**D_2**	**M**	**α_1**	**H_1**
Clozapine	3.3	150.0	34	23.0	2.1
Olanzapine	1.9	17.0	26	60.0	3.5
Ziprasidone	0.31	9.7	5,000	12.0	110.0
Iloperidone[1]	0.2	3.3	6,000	0.31	12.3
Aripiprazole[2]	8.7	3.3	6,780 (M_1)	25.7 (α_{1A})	25.1
TYPICAL APDs					
Haloperidol	25.0	1.4	4,670	19.0	730.0
Chlorpromazine	3.3[5]	1.2[6]	378[7]	14.0[7]	9.0[4]
Thioridazine	6.3[6]	7.9[6]	18[4]	5.0[4]	16.0[4]

5-HT_{2A} = serotonin 5-HT_{2A} receptors; D_2 = dopamine D_2 receptors; M = cholinergic muscarinic receptors; α_1 = α_1-adrenoceptors, H_1 = histamine H_1 receptors; Ki = equilibrium dissociation constant of the competitive inhibitor; K_D = dissociation equilibrium constant

[1]Richelson and Souder 2000 (human brain, K_D); [2]Shapiro et al. 2003 (human cloned); [4]Richelson and Nelson 1984 (human brain, K_D); [5]Leysen et al.. 1982 (rat cortex); [6]Roth et al. 1995 (rat brain); [7]Hals et al. 1986 (rat brain);

suggesting that antagonism of these receptors may be responsible, at least in part, for clozapine's discriminative stimulus properties in 129S2 mice.

Training Dose

The ED_{50} values for all drugs that produced full substitution for clozapine were lower in 129S2 mice than C57 and DBA mice (see table 3). The most obvious explanation for this finding is the lower clozapine training dose used in the 129 mice, as decreased training dose has been shown to lower ED_{50} values for substituting drugs (Stolerman, Childs, Ford, & Grant, 2011). The ED_{50} values for the atypical antipsychotics that fully substituted for clozapine (clozapine, olanzapine, and iloperidone) were roughly half the ED_{50} values for those drugs than what was seen in the C57 and DBA mice (iloperidone was not tested in DBA mice). An interesting contrast to this idea, however, lies in the drugs that did not fully substitute for clozapine.

All three strains of mice showed partial substitution for scopolamine although 129S2 mice did not show substitution until a much higher dose (C57 at 2.0 mg/kg, DBA at 1.0 mg/kg, and 129 at 8.0 mg/kg). One possible explanation for this increased dose for drugs substituting for clozapine may be receptor expression; however with little to no published articles on receptor population or expression in 129S2 mice inferences from behavioral data must be made. Scopolamine is known to disrupt attention in the Five-Choice Serial Reaction Time Test (5CSRTT) as well as increase omissions and response latency at higher doses. However, scopolamine produces lower response latencies and a lower number of omissions in 5CSRTT in 129S2 mice than in DBA and C57 mice, suggesting that 129S2 mice are less sensitive to the cognitive disruption that scopolamine

produces in the task (Pattij et al., 2007). It has also been shown that M_1 receptor knock-out mice (M_1R -/-) have a hyperactive phenotype as compared to their wild type littermates (Miyakawa, Yamada, Duttaroy, & Wess, 2001). In our lab 129S2 mice displayed significantly lower level of locomotor activity compared to C57, DBA, and Balb/c inbred mouse strains (unpublished data) and Rogers et al. (1999) reported hypoactivity in 129 mice as compared to C57 and DBA. These findings imply that M_1 receptor expression in 129S2 may be higher than the C57 and DBA inbred strains; this could also help to explain why 129 mice in the present study were not as susceptible to scopolamine's rate suppressant effects.

The only atypical antipsychotic with discordant substitution and levels of rate suppression between the three strains was ziprasidone. Both DBA and C57 produced significant rate suppression at 2.0 mg/kg scopolamine while 129S2 mice were tested up to 8.0 mg/kg without any significant rate suppression. C57BL/6 mice showed full substitution for clozapine with ziprasidone while DBA/2 mice produced partial substitution and 129S/2 mice did not substitute (See Table 3). Ziprasidone's diverse binding profile makes it harder to pinpoint exactly why this is although K_i data suggest a weaker affinity for muscarinic and H_1 histaminergic receptors (as compared to atypical antipsychotics that substitute for clozapine) that may play a role in this discordant substitution and rate suppression profile.

Interspecies/Intraspecies Comparisons

While differences in clozapine's discriminative stimulus properties between species have been established, differences within species and between strains may tell us

Table 3. *Comparison of 129S2, C57BL/6, and DBA/2 substitution and generalization tests in clozapine drug discrimination*

Results of generalization and substitution testing in C57BL/6, DBA/2, and 129S2/Hsv mice trained to discriminate clozapine from vehicle in two-lever drug discrimination from the present study, Philibin et al (2005), Philibin et al (2009), and Porter et al (2008). ED_{50} values are shown for those drugs that fully substituted for clozapine (i.e. ≥ 80% clozapine-lever responding; dashes indicate that the drug did not fully substitute for clozapine). The maximum % clozapine-lever responding is shown for all drugs tested. All drugs were administered s.c.

Drug	ED_{50} (mg/kg)/Max % DLR 129S2	ED_{50} (mg/kg)/Max % DLR C57BL/6	ED_{50} (mg/kg)/Max % DLR DBA/2
Atypical Antipsychotic Drugs			
CLOZAPINE	0.50 / 96.8%	1.19 / 97.4%	1.30 / 99.5%
ARIPIPRAZOLE	--- / 44.5%	--- / 41.2%	--- / 37.5%
ILOPERIDONE	0.09 / 84.6%	0.19 / 89.8%	Not Tested
OLANZAPINE	0.04 / 95.6%	0.24 / 87.3%	0.74 / 84.2%
ZIPRASIDONE	--- / 47.9%	0.27 / 93.6%	--- / 62.8%
Typical Antipsychotic Drugs			
CHLORPROMAZINE	--- / 72.5%	1.37 / 94.5%	1.51 / 82.0%
HALOPERIDOL	--- / 66.0%	--- / 51.6%	--- / 68.2%
THIORIDAZINE	2.71 / 93.7%	5.85 / 97.5%	6.81 / 90.8%
Selective Ligands			
AMPHETAMINE (DA agonist)	--- / 20.5%	--- / 8.1%	--- / 5.5%
M100907 (5-HT_{2A} antagonist)	--- / 69.3%	1.95 / 87.6%	Not Tested
PRAZOSIN (α_1-adrenoceptor antagonist)	1.14 / 83.4%	1.68 / 92.0%	--- / 20.5%
PYRILAMINE (H_1 histaminergic antagonist)	--- / 16.8%	--- / 38.9%	--- / 50.0%
RITANSERIN (5-$HT_{2A/2B/2C}$ antagonist)	--- / 57.4%	2.08 / 94.5%	--- / 18.5%
SCOPOLAMINE (muscarinic antagonist)	--- / 68.0%	--- / 62.3%	--- / 69.2%

Full substitution for clozapine = ≥ 80% clozapine-lever responding
Partial substitution for clozapine = ≥ 60% to < 80% clozapine-lever responding
No substitution for clozapine = < 60% clozapine-lever responding

more about the importance of different mechanisms for the discriminative stimulus properties of clozapine and other antipsychotic drugs. While many studies examining clozapine discrimination with rats report muscarinic antagonism as the main mechanism of action for the discriminative cue (Goudie et al., 1998; Kelley & Porter, 1997; Millan et al., 1999) , studies with pigeons and C57BL/6 mice suggest that 5-HT_{2A} antagonism is important for clozapine's discriminative stimulus properties (Hoenicke et al., 1992; Philibin et al., 2005) and that antagonism of alpha 1 adrenoceptors also plays a role in clozapine's discriminative cue in C57 and 129 mice. Even though the testing of selective antagonists have suggested neuropharmacological mechanisms that are important for clozapine's discriminative cue, a complete and definitive answer to the underlying mechanism(s) of action for clozapine discriminative stimulus properties remains elusive (see reviews by (Goudie & Smith, 1999; Porter & Prus, 2009) and a compound discriminative cue is the most likely scenario.

The data from (Porter et al., 2008) looking at clozapine discrimination in DBA/2 mice seems to embody this idea of a compound discriminative cue, as none of the selective ligands tested fully substituted for clozapine. Stolerman and colleges (Stolerman, Rauch, & Norris, 1987) examined the discriminative stimulus properties of a compound cue by using a mixture of two pharmacologically independent agents as the training drug. Rats were trained to discriminate a mixture of 0.2 mg/kg nicotine and 0.4 midazolam. Generalization curves were obtained with both nicotine and midazolam and each produced high drug lever responding for the compound mixture. While they reported that each component of the mixture was likely perceived separately, as antagonism of nicotine and midazalam separately did not block the cue and neither drug

substituted for the other. However the data from DBA/2 discrimination of clozapine does not follow this pattern. The drugs used in (Stolerman et al, 1987) were a mixture of different pharmacological agents, and in that study rats substituted the individual components of the drug. Clozapine, while having a diverse binding profile, is not considered a "mixture" of the different receptors antagonists but a single compound affecting different systems. If clozapine were to be thought of as a "mixture" of different receptor antagonists then one of the selective antagonists should have produced substitution. Thus DBA/2's discriminative cue is either mediated by a receptor antagonist that was not tested in the study or it is a complex cue, mediated by a specific mix of receptor antagonists and not, as Stolerman saw in his rats, parts of a whole.

Training dose of the drug may also change what is important for the pharmacological mechanisms that mediate clozapine's discriminative stimulus. While antagonism of muscarinic receptors has been shown to be important in rats trained to discriminate clozapine from vehicle (Goudie et al., 1998; Kelley & Porter, 1997; Nielsen, 1988) a study by Prus, Philibin, Pehrson, and Porter (2006) in which rats trained to discriminate 5.0 mg/kg and 1.25 mg/kg clozapine from vehicle in a three lever drug discrimination procedure showed that scopolamine, a muscarinic antagonist, only produced partial substitution on the 5.0 mg/kg clozapine lever and did not substitute for clozapine on the 1.25 mg/kg lever. However, if the percent clozapine-lever responding on both drug levers were combined, scopolamine engendered full substitution for clozapine. The lack of substitution on a single clozapine dose lever may suggest that the mechanisms mediating clozapine-like responding may be different between the two doses. In the present study, the 129S2 mice were trained at a lower training dose than was

used in previous studies for C57 and DBA mice. This difference in training dose may account for some of the differences observed between these strains.

Future Studies

Studying the response of inbred strains of mice in the drug discrimination paradigm helps to establish an important behavioral phenotyping baseline from which we can compare transgenic and knockout strains. Using knockout mouse strains can help to further pinpoint the importance of different receptor systems for clozapine's discriminative cue and for other antipsychotic drugs. For example, a 5-HT_{2A} knockout mouse in a C57BL/6 background could help to determine the importance of 5-HT_{2A} antagonism in clozapine's cue. If 5-HT_{2A} knockout mice are able to be trained to discriminate clozapine from vehicle that would suggest that alpha adrenergic antagonism is sufficient for establishing and maintaining clozapine's discriminative stimulus in the absence of 5-HT_{2A} antagonism. Testing other selective ligands as well as other antipsychotic drugs may also help to identify other putative targets for clozapine's discriminative cue. Examining the phenotypic response to these drugs and further knowledge of differences in receptor expression between mouse strains can help to uncover the mechanism(s) of action of antipsychotic drugs and give us further insight to the neuropharmacological mechanisms for drugs used in the treatment of schizophrenia.

Conclusion

Continued examination of the phenotypic expression of clozapine's drug discrimination can help to determine the neuropharmacological underpinnings of

antipsychotic drugs and help us to make inferences about the mechanisms of action for the development of drugs used to treat schizophrenia. Clozapine drug discrimination continues to be an important preclinical assay for novel antipsychotic drugs that have similar mechanisms as clozapine and that lack EPS effects. By building on the knowledge of how both inbred and genetically manipulated mouse strains differ we can also develop better models to screen and explore the mechanisms of novel antipsychotic drugs as well as other drugs. While the discriminative stimulus properties of clozapine are robust in both mice and rats, its relationship to the therapeutic efficacy or adverse side effects of clozapine remain to be determined.

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Vita

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