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Discriminative Stimulus Effects of Putative Antipsychotic Drugs

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By

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ABSTRACT

DISCRIMINATIVE STIMULUS EFFECTS OF PUTATIVE ANTIPSYCHOTIC DRUGS

By

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This study attempted to further explore the discriminative stimulus properties of antipsychotic drugs, by establishing the typical antipsychotic drug chlorpromazine, and the atypical antipsychotic drug clozapine as discriminative stimulus in two different groups of rats. The rats trained to discriminate chlorpromazine from vehicle failed to do so reliably, however nine of ten rats trained to discriminate 1.25 mg/kg clozapine from vehicle were able to acquire the discrimination in 19.1 sessions. The clozapine cue partially generalized (63.13% drug lever responding [SEM = ± 18.91]) to the antimalarial drug methylene blue at the 7.5 mg/kg dose, but not to the antimalarial quinacrine. This study found that the antimalarial drug methylene blue may share some pharmacological similarities or subjective effects with that of clozapine, and further studies into its antipsychotic value, if any, should be explored.

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This thesis follows the formatting prescribed by the *Publication Manual of the American Psychological Association* as well as the Department of Psychological Science.

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INTRODUCTION

Schizophrenia

Schizophrenia is a mental disorder that consists of dysfunction in thought processes and emotional regulation, and occurs in roughly 1% of the population (Simeone, Ward, Rotella, Collins, and Windisch, 2015). Schizophrenia is characterized by its symptomology. An individual may experience delusions, hallucinations, disorganized speech, and abnormal motor activity. These particular symptoms are referred to as positive symptoms, indicating that they are present. There are also negative symptoms, which indicate a lack of presence, such as social and emotional withdrawal, lack of motor response, and avolition (American Psychiatric Association, 2013).

History of Psychosis and Schizophrenia

Psychosis has been described in various texts from early in human history. The first known description of psychosis was found in religious scriptures of India around 1400 B.C.E., characterized by confusion and lack of self-control, but differed from confusion due to use of physiologically and psychoactive substances, or manic-depressive illness (Gottesman, 1991). Under the unitary theory of psychosis, all psychosis was considered symptomology of the same underlying spectrum of mental illness until 1889, when Emil Kraepelin was the first to formally characterize schizophrenia, which he referred to as dementia praecox, meaning precocial dementia. Kraepelin described dementia praecox as a combination of other conditions: catatonia; hebrephrenia; and dementia paranoia. This was also the first time that the disease was characterized to have a specific neuroanatomical pathology (Adityanjee, Aderibigbe, Theodoridis, & Vieweg 1999; Lavretsky, 2008). Kraepelin was among the first to differentiate

manic depressive illness and dementia praecox, which, while both shared similar symptomology, manic depressive illness main symptoms revolved around disordered mood, while dementia praecox symptoms were functional and social disability (Lavretsky, 2008). It was in 1911 when Eugene Bleuler first used the term schizophrenia, meaning "split mind". While Kraepelin believed that schizophrenia had a neurological pathology, Bleuler believed that it had a psychological pathology, and characterized schizophrenia by fundamental symptoms, common to all schizophrenia subtypes: cognitive disturbances; affective blunting; and ambivalence, and associative symptoms: hallucinations; delusions; and catatonia (Adiyanjee, et al., 1999; Lavretsky, 2008).

Kurt Schneider was one of the first researchers to look at schizophrenia longitudinally, and took issue with the definition of schizophrenia created by Kraepelin and Bleuler. Their definition could provide a diagnosis that was sometimes based on the psychological symptoms, and sometimes based on the progression of the disease, while Schneider's research showed that the course of the disease had a general sequence of clinical states, and should be diagnosed by the symptoms, as well as the progression (Hoenig, 1983). Schneider's list of symptoms would help improve interrater reliability of diagnosing schizophrenia, and his contributions are still used in diagnostic criteria today (Adiyanjee, et al., 1999).

Neurotransmitters Involved in Schizophrenia

The exact causes of schizophrenia remain unknown, although there are several neurotransmitters that are associated with the disease state (Lavretsky, 2008). Dopamine has been implicated to be associated with schizophrenia soon after discovery of antipsychotic drugs (APDs). Positron emission tomography (PET) and single photon computerized tomography (SPECT) scans of the brains of drug naïve individuals with schizophrenia have shown that there

is an increase in dopamine D_2 receptors in the striatum, as well as a decrease in the receptor affinity for D_2, with these differences becoming more pronounced with age (Laruelle, 1998). Other studies have found that changes in density of dopamine D_1 receptors in the prefrontal cortex that may be associated with the negative symptoms and cognitive deficits associated with schizophrenia (Howes & Kaspur, 2009).

Serotonin is another neurotransmitter implicated in the pathology of schizophrenia, which has been implicated since the discovery that the hallucinogenic drug, LSD, is a serotonin agonist (Lavretsky, 2009; Harrison, 1999). Some research has found evidence of excessive serotonin receptors in the anterior cingulate cortex, and the dorsolateral frontal lobe in the brains of individuals with schizophrenia (Gurevich & Joyce, 1997; Eggers, 2013). Additionally, many of the drugs used for the treatment of schizophrenia have a high affinity for serotonin receptors as antagonists and have demonstrated reduction in positive symptoms, and to a lesser effect, negative symptoms (Lavretsky, 2009). However, many of these drugs have action on multiple receptor sites, and antipsychotic efficacy may be a result of multiple mechanisms of action.

Glutamate is one of the primary excitatory neurotransmitters of vertebrate brains, which accounts for a majority of the synaptic connections in the brain (Meldrum, 2000). Drugs that cause dysfunction of glutamate transmission, such as PCP, ketamine, and MK-801, have been found to cause psychosis in humans, and cognitive impairments that resemble negative symptoms in preclinical models (van der Staay, Rutten, Erb, & Blokland, 2011). Glutamate binds to several receptor sites in the brain, such as the NMDA receptors. These receptors have been found to be dysfunctional in pre and post synaptic sites in individuals with schizophrenia (Moghaddam and Javitt, 2012). While in the dopamine model of schizophrenia, deficits are usually limited to the dorsolateral prefrontal cortex, and the striatum; the glutamate model of

schizophrenia shows deficits throughout cortical and subcortical brain regions (Javitt, 2009; Moghaddam and Javitt, 2012).

There are multiple neurotransmitters in dysfunction in the schizophrenic brain. Consequently, the most effective treatments for schizophrenia have been drugs that have multiple mechanisms of action. APDs are the primary treatment for schizophrenia. APDs fall into two different sub-classes: typical APDs, also known as first generation APDs; and atypical APDs, also known as second generation APDs.

The First Drug Treatments for Schizophrenia

Chlorpromazine is the first APD that was marketed for the treatment of schizophrenia. After the drug was developed in 1950, it was first used in combination with morphine to reduce the usage of an anesthetizer during surgery, where it was discovered that patients that had been treated with chlorpromazine experienced less preoperative and postoperative anxiety (Swazey, 1972). Chlorpromazine was found to have anxiolytic, antispasmodic, sedative, and antiemetic properties, which made it a useful as a surgical drug. Researchers would later discover its use in reducing agitation in individuals suffering from manic episodes (Swazey, 1972; Lopez-Monoz, et al. 2005).

Prior to the use of APDs, attempted pharmacological treatments for many mental illnesses such as schizophrenia included compounds that were often supported by mainly anecdotal accounts, and seldom addressed the core symptoms of the disorder. Treatments included use of barbiturates on belligerent patients during manic or otherwise agitated episodes to induce sleep; and hypoglycemic shock therapy, in which patients were given injections of insulin to induce diabetic coma, which could be reversed by glucose syrup. In addition to

hypoglycemic shock therapy, electroshock therapy was also used (Anderson, 2016). When the antipsychotic actions of chlorpromazine was first discovered, it brought on the development of several similar drugs, which were later characterized by their high binding affinity as antagonists to the dopamine D_2 receptors (Lehmann and Ban, 1997; Seeman, 2010). The use of chlorpromazine and other first generation ADPs were also found to cause movement disorders, known as extrapyramidal symptoms (EPS). These side effects include muscle spasms (dystonia); tremors, or jerky movements (tardive dyskinesia); restlessness (akathisisa); and general slowness of movement (bradykinesia) (Lieberman et al. 2005). Most of these movement disorders would appear rather quickly as a patient takes the drug, however tardive dyskinesia, a condition characterized by involuntary movements of the face and body, was only seen after being treated for several years, and continues even after the drug is terminated (Anderson, 2016).

When clozapine was first developed in 1959, it was found to have very similar neuroleptic effects to that of chlorpromazine, yet did not show the same involuntary movements that were thought at the time to be an unavoidable component of APD treatment, which is why it later became referred to as an atypical antipsychotic (Crilly, 2007). While atypical APDs can cause these extrapyramidal symptoms, they do so with far less frequency than typical antipsychotics. With select drugs, this may be due to their high binding affinity for the dopamine D_2 receptors (Kapur and Remington, 2001). However, there are several atypical antipsychotics that do have high binding affinity for dopamine that do not exhibit these symptoms with the same frequency as the first generation antipsychotics. Research has suggested instead, that the reason ADPs show less extrapyramidal symptoms is due to their complex receptor binding profiles (Meltzer, 2004). Although, atypical APDs are less likely to cause movement disorders, they often carry risk of weight gain, new-onset type II diabetes, and metabolic dysfunction

(Baptista, Kin, Beaulieu, and de Baptista, 2002). Clozapine, like many other atypical antipsychotics, has a higher binding affinity to several serotonin receptors, in addition to affinity for dopaminergic, histaminic, adrenergic, and cholinergic receptors. (Goudie, Cooper, Cole, and Sumnall, 2007).

Chlorpromazine, in addition to many other typical APDs, is derived from the organic compound phenothiazine, which has a history in the development of a number of different drugs (Lopez-Monoz, et. al 2005). The first phenothiazine compound synthesized was methylene blue, which had originally been used as a textile dye, before the discovery of its antimalarial properties (Shen, 1999). In addition to methylene blue, there are many other compounds derived from phenothiazine that have medical uses, aside from antipsychotics, yet are no longer used due to their low therapeutic window. These compounds have been used as antihistamines (promazine; promethazine), trypanocidals (trypan red), antihelmintics (phenothiazine), and antiparkinsonians (diethazine) (Swayze, 1972). With such slight variations in the chemical structure of these phenothiazine based compounds, they have shown a wide variety of uses in medicine over the years.

The discovery of antipsychotics has improved the lives of those afflicted with mental illness, yet it still comes at a great financial cost. People with schizophrenia deal with direct costs of: hospitalization; follow up treatment; care givers; and drugs, as well as indirect costs related to loss of productivity due to managing their symptoms. In the United States, the direct and indirect costs of the disease have been growing steadily over the past years (Tajima-Pozo, de Castro Oller, Lewczuk, and Montanes-Rada, 2015), with direct and indirect costs totaling over $62.7 billion as of 2002, with about 8% of that total being the cost of drugs (McEvoy, 2007). In the areas developing world, making older, typical APDs available in the place of atypicals can

significantly lower the cost of treatment, as well as repurposing old drugs that may have antipsychotic efficacy (Chisholm, et al. 2008).

Before the Discovery of Chlorpromazine

While there are many drugs that are currently available for the treatment of schizophrenia, the cost may be especially high for those in the developing world (Chisholm, et al., 2008). Some preclinical trials have shown that there are other, more cost effective drugs that may work in place of current APDs (Goudie, et al., 2007), and that there are many similar compounds that have therapeutic value for a variety of ailments (Swayze, 1972). While the results may be modest, there are many other antihistamines and phenothiazine compounds that could be evaluated for their therapeutic effects in the treatment of schizophrenia and other psychotic disorders.

Methylene blue is a phenothiazine compound that was originally used as a dye in textiles, and became the first fully synthetic drug used in medicine (Howland, 2016). Methylene blue was found effective in treating the symptoms of malaria, in addition to having several other medicinal uses (Healy, 2002), such as reducing the symptoms of manic depressive psychosis (Narsapur, and Naylor, 1983). More recently, it has been found to have some anxiolytic and antidepressant effects (Eroglu, and Caglayan, 1997). Due to the calming and anxiolytic effects that methylene blue may share with other phenothiazine compounds, such as chlorpromazine, there may be some value in evaluating it against current APDs.

Several other phenothiazine derived drugs are still currently used in medicine, mostly in the treatment of malaria, but also used as antiprotozoals, and antihelmintics, such as quinacrine. While quinacrine is quite similar in structure to methylene blue and chlorpromazine, it does not

share many of their sedative or agitation reducing features. Antimalarials that share features with quinine, in that they cause many psychiatric features similar to psychosis and cognitive deficits (Nevin & Croft, 2016). Quinacrine has also been shown to decrease sensitivity to prepulse inhibition (PPI) testing in rats, similar to that of patients with schizophrenia, who have issues with sensorimotor gating (Lee, Farooqui, Dawe, Burgunder, & Ong, 2009). Quinacrine would likely be able to serve as a negative control for a comparison to the subjective effects of APDs against other drugs in the antimalarial class.

Drug Discrimination

Drug discrimination is an operant conditioning procedure in which an organism is trained to discriminate between the subjective effects of a drug from the solution in which it is delivered, known as the vehicle. When using non-human animals for this procedure in an operant chamber, the animal is taught to respond a certain way after given a drug in order to receive a reinforcement, usually food. When the animal feels the subjective effects of the drug, it knows to respond by pressing the drug appropriate lever to receive food, and when the animal is given the vehicle that serves as the placebo, it will respond by pressing the non-drug lever for reinforcement. Once an animal is trained to respond to the subjective effects of one drug, the animal is then given novel drugs to examine if they will respond in a similar way while under the subjective effects of the novel drug. If they respond in a similar manner, it may be likely that the two drugs have similar pharmacological activity (Young, James, and Rosencrans, 2009). In this way, drug discrimination assays can be used as a tool to determine the mechanisms of action of novel drugs.

The panoply of receptor actions by clozapine, as noted earlier, appear to engender a compound discriminative cue as studied in a drug discrimination procedure (Kelley & Porter,

1997; Prus, et al., 2016). When looking at other drugs that have a similar compound cue, it was found that the antihistamine cyproheptadine had a similar binding profile to clozapine. When these drugs were evaluated for their subjective effects in a drug discrimination trial, a dose of cyproheptadine at 40 mg/kg fully substituted for a dose of clozapine in rats at 5 mg/kg (Kelley & Porter, 1997; Goudie, et al. 2007). Although, this was a preclinical study, showing similarity of these drugs in rats, it shows that drugs with similar receptor binding profiles can have similar effects.

Several studies have established chlorpromazine as a discriminative cue in rats (Stewart, 1962; Overton, 1982; Porter et al., 1998; Porter et al., 2004). Porter et al. (1999) successfully trained six rats to discriminate a 1.0 mg/kg IP dose of chlorpromazine from its vehicle in a two lever discrimination task. When the rats were tested against various APDs, the atypical APDs clozapine and olanzapine, fully substituted for chlorpromazine, as well as the typical APD thioridazine, while the atypical antipsychotic raclopride, and typical antipsychotic haloperidol only partially substituted for chlorpromazine, showing the chlorpromazine does share discriminative stimulus properties with some atypical and typical APDs (Porter et al., 1999).

Clozapine has also been shown to be able to be able to form a discriminative cue (Goudie, & Taylor, 1998; Porter, Varvel, Vaan, Philibin, & Wise, 2000; Porter, et al., 2005; Prus, et al., 2005; Porter, & Prus, 2009). Prus et al. (2005) had trained nine rats to discriminate 5.0 mg/kg of clozapine from vehicle in a two lever discrimination task. The atypical antipsychotics olanzapine, quetiapine, and ziprasidone, as well as the typical APDs thioridazine fully substituted for clozapine, while there was partial substitution for the atypical APDs risperidone, and sertindole, while typical antipsychotics chlorpromazine, fluphenazine, and haloperidol did not substitute for clozapine. While many typical antipsychotics have been found to generalize to

chlorpromazine (Porter, et al., 1999), and many atypicals have been found to generalize to clozapine (Prus, et al. 2005), there has been less research looking at completely novel drugs with calming properties to see how they generalize to current APDs that are available.

RATIONALE

It has been close to 70 years since the discovery of chlorpromazine, and the therapeutic effects of modern ADPs have not made substantial gains in therapeutic efficacy. Therefore, an investigation into early compounds with putative APD-like effects is warranted. In particular, few studies have re-evaluated such compounds in direct comparison to established APDs in assays with strong predictive validity for determining mechanism of action, such as the drug discrimination procedure. Current APDs can be evaluated for their subjective effects through the use of drug discrimination procedures. The purpose of the present study is to evaluate the subjective effects of the antimalarial drugs methylene blue and quinacrine, which are known to have similarity in chemical structure, in rats trained to discriminate the APDs chlorpromazine, and clozapine to see if the test drugs show similarity in their subjective effects to that of the current APDs by way of stimulus generalization. If these drugs do generalize to current antipsychotic medication, it may indicate that there is therapeutic value in further evaluating these drugs as antipsychotic alternatives.

MATERIALS AND METHODS

Animals

Twenty male Sprague-Dawley rats were used for this study. Ten of which were trained to discriminate chlorpromazine at 1.0 mg/kg from vehicle (Group 1), and the other ten of which were trained to discriminate clozapine at 1.25 mg/kg from vehicle (Group 2). The rats were individually housed in a temperature and humidity controlled room, on a 12 hour light to dark cycle, with the lights on from 5:00 AM to 5:00 PM. Rats were put on restricted feeding to achieve 85% free feeding weight. Water was available ad-lib in their home cages. Rats were fed following daily sessions that occurred around the same time every day.

Apparatus

Eight standard rat operant chambers were used (ENV-008-VP, MED Associates, St. Albans, VT) contained in sound attenuating cabinets for this study (ENV-018MD, MED Associates, St. Albans, VT). Cabinets were equipped with fans for masking noise and ventilation, and all equipment was controlled by MED-PC IV software. The operant chambers (30 cm long × 24 cm wide × 29 cm high) were constructed of a Plexiglas top and side door panels, and other walls and components made of stainless steel. A concealed light bulb located near the top of the operant chamber provided illumination during all training and test sessions. Two retractable levers (drug and vehicle levers) were located on either side of a food hopper centered on the stainless steel wall of the chamber. Food reinforcement consisted of 45 mg food pellets (Dustless Precision Pellets, Rodent Grain-Based Diet, Bio-Serv, Flemington, NJ)

Drugs

Generalization testing was conducted with chlorpromazine hydrochloride (0.0625; 0.125; 0.5; 1.0; 2.0; and 4.0 mg/kg), clozapine (0.15625; 0.3125; 0.625; 1.25; 2.5; and 5.0 mg/kg); methylene blue – tetramethylthionine chloride (7.5; 15.0; 30.0; and 60.0 mg/kg); quinacrine dihydrochloride (1.0; 10.0; and 30.0 mg/kg) All drug doses were based on previous literature (Al Asmari, Al Sadoon, Obaid, Yesunayagam; & Tariq, 2017; Overton, 1982; Porter, et al., 1999; ; Porter, Varvel, Vann, Philibin, & Wise, 2000; Oz, Lorke, Hasan, & Petroianu, 2011). All drugs will be purchased from Sigma-Aldrich, St. Loius, MO, except clozapine which was purchased from Ascent Scientific, Bristol, UK. The salt form of chlorpromazine, methylene blue, and quinacrine was used, while the base form of clozapine was used. All drugs were dissolved in water with a few drops of lactic acid and sodium hydroxide to raise the pH of the solution above 5.5. All drugs were administered intraperitoneally (IP) at a volume of 1.0 ml/kg of body weight, with the exception of chlorpromazine also being administered subcutaneously (SC) after the first 30 training sessions (see below). All drugs were administered 30 minutes before test sessions (Overton, 1982), unless otherwise noted.

Magazine Training

Rats were placed in the operant chamber in which food was delivered to them every 60 seconds (fixed time 60), with no levers available. This was intended to train the rats to associate the sound of the food hopper with the availability food reward. This occurred over one session.

Lever Press Training

During lever press training, the center lever was available, while the left and right levers were retracted, as they were used later when they were paired with the drug and vehicle. For

every lever press the rat makes, it was given a food pellet as a reinforcement (Fixed Ratio-1). The session concluded when the rat received 30 food pellets, or when 15 minutes elapsed. As the rats learned the lever pressing behavior, the fixed ratio to receive the food reinforcement was increased until the rats reliably lever pressed 30 times to receive a food pellet (Fixed ratio-30).

Single Lever Training

During single lever training, the rats were be given an intraperitoneal (IP) injection of either their respective training drug, 1.0 mg/kg chlorpromazine for group 1, and 1.25 mg/kg clozapine the group 2, or vehicle (consisting of deionized water with a few drops of lactic acid and sodium hydroxide) in it thirty minutes prior to their training session. The rats had one of the left or right levers assigned to the drug condition and the opposite lever assigned to the vehicle condition. The lever assignment was counterbalanced among the rats. The rats were tested on a single-double alternating design, on the drug (D), and vehicle (V) for training sessions (D-V-D-D-V-V-D-V). After the animals successfully received 30 food pellets on an FR-30 for seven of the eight sessions, they moved on to discrimination training.

Two Lever Training

During two lever discrimination training, the rats were injected with either their respective training drug or vehicle, and presented with the right and left lever both available. The two lever training continued to use a single-double alternation design. These trials used an FR-30, in which if the rat pressed the incorrect lever, the count on the condition-appropriate lever would reset, and the rat had to press 30 more times in order to receive a food reinforcement. The rats had to complete the following criteria for five of six consecutive sessions: the first completed fixed ratio requirement must be on the condition appropriate lever; the rat must have

had a response rate of 0.08 responses per second and, and the rat must have completed 80% of their responding on the condition appropriate lever during the given session. These conditions continued throughout the course of the study to make sure that the rats retain discriminability among the conditions.

During the course of the two lever training, the group 1 had to undergo several phase changes, as during the first 30 sessions, nine out of the ten rats would fail to discriminate each session, At session 32, the training dose was increased to 2.0 mg/kg via IP based on the dose used by Goas and Boston (1978), however this appeared to make no difference in the ability for the rats to discriminate. At session 52, the route of administration was changed from IP to SC, but all but two rats were completely rate suppressed by this change. At session 54, the dose was lowered to 1.0 mg/kg via SC. The rats were still performing below 5 responses per minute, and so at session 62, the dose was lowered to 0.5 mg/kg, as this dose has still found to partially substitute for the 1.0 mg/kg dose in previous literature (Porter, et al., 1999).

Substitution Testing

When the rats had successfully completed two training sessions, a test session was given. The rat was given one of the test drugs via IP or SC injection, and the rat was put in the operant chamber with a two lever choice after waiting for the appropriate pretreatment time for the drug. The first FR30 completed on a lever (with a press to the opposite lever resetting the counter) resulted in the end of the session. The length of the session depended on how long it took the rat to complete an FR-30, or the test session would end when 15 minutes had passed, regardless of whether or not they had completed an FR-30. Doses of drugs began with the drug vehicle and were subsequently tested in an escalating order.

Data Analysis

The percent drug lever responding for drug and non-drug levers, the responses per minute (RPM), and the lever on which the first FR-30 schedule were completed and recorded for each training and test sessions. Percent drug appropriate responding and RPM was reported as means and standard error of the mean (SEM) in dose-response curves. Full substitution was defined as 80% or greater drug appropriate responding; and partial substitution was defined as above 60% drug appropriate responding and below 80% drug appropriate responding. As the drug discrimination procedure was designed to evaluate the novel compounds for the subjective effects of their respective training drugs, the drugs were assessed on whether they met criteria for full, partial, or no substitution, as opposed to statistical significance, to determine the percent of training drug appropriate responding (Glennon, and Young, 2011). Drugs that produced full substitution had the ED_{50} value calculated for the dose response curve using a least-squares linear regression analysis. One factor repeated measures analysis of variance (ANOVA) tests were conducted to look at changes in the response rates, with Dunnett's multiple comparison tests conducted to identify significant differences in the changes among the response rates between vehicle and each dosage of the drug tested. Rate comparisons were calculated using Prism Graphpad 8.1.2 (San Diego, CA).

RESULTS

Group 1: Chlorpromazine Drug Discrimination Training

Out of the ten rats trained to discriminate 1.0 mg/kg administered via IP injection chlorpromazine from its vehicle, two were able to meet the training criteria. After a month of additional training, all ten rats had the dose increased to 2.0 mg/kg via IP injection, in which one more rat was able to meet training criteria. Chlorpromazine administration was then changed from 30 minutes of pretreatment time to 60 minutes of pretreatment time, which had no effect on performance. Many of the rats began to exhibit rate suppressing effects of the 2.0 mg/kg dose, but did not do so with consistency, experiencing rate suppression on the drug one day, but not the next. The route of administration was subsequently changed from an IP injection to an SC injection. All rats began to succumb to rate suppressing effects at this time, so the dose was once again lowered to 1.0 mg/kg, administered via SC. The rats continued to experience rate suppression at the 1.0 mg/kg dose, so the dose was once again lowered to 0.5 mg/kg via SC injection. After being acclimated to the 0.5 mg/kg SC dose, five more rats made discrimination criteria. Including the five phase changes, eight of the ten rats made criteria in 55.1 sessions (SEM = ± 12.2). Two rats failed to make discrimination criteria and were excluded from the study (See Figure 1).

Group 1: Chlorpromazine Generalization Testing

Only one of the eight rats that made criteria had completed the chlorpromazine generalization testing, therefore it was not possible to depict a generalization curve based on the averages of the drug discrimination testing for this group.

Group 2: Clozapine Drug Discrimination Training

Out of the 10 rats that were trained to discriminate 1.25 mg/kg clozapine from its vehicle, nine rats were able to meet the training criteria within a mean of 19.1 sessions (SEM = ± 2.6). One rat failed to meet discrimination criteria and was removed from the study (See Figure 2).

Group 2: Clozapine Generalization Testing

For the nine rats tested clozapine produced full generalization, 80% drug lever responding or above, for clozapine at the training dose of 1.25 mg/kg (ED_{50} = 0.5094 mg/kg). Partial generalization, between 60 and 80% drug lever responding, occurred to the 0.3125 mg/kg (75.00% [SEM = ± 15.43]), and 2.5 mg/kg doses (76.07% [SEM = ± 14.42]). A significant decrease in response rate compared to vehicle occurred at the 5.0 mg/kg dose ($F [6, 48] = 4.84$, $p < 0.001$) (See Figure 3).

Group 2: Chlorpromazine Generalization Testing

In the four rats tested partial generalization for clozapine occurred with a 2.0 mg/kg (78.75% [SEM = ± 22.05]), and 4.0 mg/kg (77.55% [SEM = ± 12.36]) doses of chlorpromazine. The tests had also indicated that 3 out of 4 rats had full generalization at the 2.0 mg/kg dose, while only two of them exhibited full generalization at the 4.0 mg/kg dose. There was a significant difference in responding relative to vehicle chlorpromazine ($F [6, 18] = 3.502$, $p = 0.0179$), however a post-hoc analysis did not reveal differences between each dose and vehicle (See Figure 4).

Group 2: Methylene Blue Generalization Testing

In the five rats tested partial generalization for clozapine occurred at the 7.5 mg/kg (63.96%, [SEM = ±18.91]) dose of methylene blue. Among the tests, only one rat displayed full generalization at the 7.5 mg/kg dose. There was no significant change in responding relative to vehicle at any of the doses of methylene blue ($F [3, 12] = 2.125$, $p = 0.1504$). However, rate suppression occurred for one rat, and in different rats at each of the three doses that were tested (See Figure 5).

Group 2: Quinacrine Generalization Testing

In the three rats tested, quinacrine did not fully or partially substitute for the 1.25 mg/kg clozapine training dose, with the highest drug lever responding at the 30 mg/kg dose (43.77% [SEM = ± 19.57]). There was no significant change in responding at any of the doses tested ($F [3, 12] = 1.313$, $p = 0.3157$) (See Figure 6).

DISCUSSION

Group 1: Chlorpromazine Generalization

The rats from group one were successfully able to establish chlorpromazine as a discriminative stimulus, which is supported by previous literature (Stewart, 1962; Porter, et al., 1999; Porter, et al., 2005), but only after several phase changes. Eight of ten rats acquired the discrimination at a mean 55.1 training sessions at the 0.5 mg/kg SC dose, which was much longer that the 29.7 training sessions used by Porter et al. (1999) at the 1.0 mg/kg IP dose. There first phase change could have failed due to increasing the time, as opposed to shortening the time, as in Overton (1982), in which the pretreatment time for rats given 2.0 and 4.0 mg/kg chlorpromazine to discriminate a T-maze was only 20 minutes. This is highly unlikely, as the rats could be seen succumbing to the rate suppressing effects of the 2.0 mg/kg via SC of chlorpromazine within the first ten minutes of their pretreatment time in the present study.

The route of administration might have accounted for this. Some drugs that are taken in through the digestive system, whether it be orally, or intraperitoneally, may be subject to first pass metabolism, and have their active metabolites degrade in the gut or liver before being able to exerting their psychoactive effect. Certain drugs are more susceptible to first pass metabolism, chlorpromazine has been found to be less stable in plasma concentrations in humans that have taken the drug orally, compared to intramuscular (IM) injection (Dahl & Standjord, 1977), which shows that the route of administration in administering chlorpromazine can affect biological availability. Additionally, chronic administration can be an issue, as an organism may become tolerant to drug effects over time. Rats given increasing doses of chlorpromazine over a 40 week period have been able to take up to 200 mg/kg via IM (Boyd, 1960), while other studies have shown that the lethal dose in 50% of rats (LD_{50}) for chlorpromazine for other routes of

administration is 142 mg/kg orally; 75 mg/kg SC; 58 mg/kg IP; and 23 mg/kg IV (Lewis & Sax, 2004). While Lewis and Sax (2004) did not include the LD_{50} for IM dosages, 200 mg/kg IM is still much higher than even the highest LD_{50} of chlorpromazine at 142 mg/kg orally. The rats of this study tolerating a dose of chlorpromazine at the 2.0 mg/kg level is not very likely, as if they had developed a tolerance, they would not likely be able to discriminate at the lower 0.5 mg/kg dose.

There is evidence in the literature for chlorpromazine, among other typical APDs not being easily established as a discriminative stimulus. Harris and Balster (1971) were unable to get rats to discriminate chlorpromazine at the 1.0 mg/kg via IP dosage in a two lever operant chamber. McElroy, Stimmel, and O'Donnell (1978) were able to establish the typical APD haloperidol at a discriminative stimulus in rats in a mean of 45 training sessions, while Donahue, Webster, Hillhouse, Oliveira, and Porter (2019) were able to establish haloperidol as a discriminative stimulus in mice in a mean of about 31 training sessions at the 0.05 mg/kg SC dose. However, the mice of this study eventually failed to retrain discriminability. As haloperidol has been show to generalize in chlorpromazine trained rats (Porter, et al., 1999), as well chlorpromazine being generalized in haloperidol trained rats (McElroy, et al., 1978), indicates that these two drugs likely have a similarity in pharmacological action, while Donahue, et al. (2019), shows that haloperidol does not display a particularly strong discriminative stimulus, in which discriminability may be difficult to maintain. The results may be species, drug, or even route of administration specific, however, the previous literature does show there have been difficulties establishing typical antipsychotics as discriminative stimuli, and this may be due to a failure to retain discriminability, which should be further explored.

Another likely factor contributing to the difficulty in establish typical antipsychotics as discriminative stimulus is the antagonism of dopamine receptors. Porter et al. (1999), was not able to get rats to discriminate 2.0 mg/kg, due to the rats responding below five per minute during initial two lever training, which is why the dose was reduced to 1.0 mg/kg. Additionally, the study had showed that at the 1.0 and 2.0 mg/kg doses, there was a significant decrease in response rate compared to vehicle. Donahue et al. (2019) showed similar results in the discrimination of haloperidol in mice. The training dose of 0.05 mg/kg did not any significant changes in response rate, the 0.1 and 0.2 mg/kg doses showed significant rate suppression compared to that of vehicle. Previous studies were able to establish dosages of chlorpromazine as discriminative stimulus as high as 4.0 mg/kg (Overton, 1982; Stewart, 1962), although the discrimination procedures in these studies used a shock avoidance discrimination procedure. These studies may show that shock avoidance is a more salient reinforcement than a food reward, but do not necessarily show that using a higher dose is more salient in discrimination.

Group 2: Clozapine Generalization

The rats from group two were successfully able to establish clozapine as a discriminative stimulus, which is supported by previous literature at the 5.0 mg/kg dose (Kelley and Porter, 1997; Goudie and Taylor, 1998; Prus, et al., 2004; Goudie, et al. 2007), and the 1.25 mg/kg dose (Porter, et al., 2000; Prus, et al., 2004; Prus, et al. 2006), which is more selective for atypical antipsychotics. Nine out of ten rats were able to acquire the two lever discrimination for the 1.25 mg/kg dose in a mean of 19.1 training sessions, which is faster than Prus, et al. (2004), which had achieved discrimination in about 55 sessions, as well as slightly faster than Porter, et al., which achieved discrimination in about 28 sessions. The discriminative stimulus was dose

dependent with an ED_{50} of 0.5094 mg/kg, and full generalization for only the training dose of 1.25 mg/kg.

Group 2: Chlorpromazine

Chlorpromazine was found to partially substitute for clozapine at the 2.0 and 4.0 mg/kg dosages in the four of the rats that were tested. This does not follow along with previous research for clozapine with a training dose of 1.25 mg/kg, as Porter, et al. (2000) found that chlorpromazine did not produce substitution in clozapine-trained rats, although that study did not include a 2.0 mg/kg dose, and there was rate suppressing effects for all seven rats in that study at the 4.0 mg/kg dose of chlorpromazine. In the current study, there is a significant difference in responding when the clozapine trained rats were given chlorpromazine, but post-hoc analysis did not find that it occurred at any specific doses.

Drug discrimination studies that have used 5.0 mg/kg of clozapine as the training drug, which has shown to be less sensitive to the differences between typical and atypical antipsychotics (Goudie and Taylor, 1998; Prus, et al., 2005), also found that chlorpromazine does not substitute for clozapine. Other studies have shown that clozapine will produce substitution in rats trained on chlorpromazine (Porter, et al. 1999), yet the substitution for clozapine trained rats testing chlorpromazine has not been symmetrical (Porter, et al., 2000; Prus, et al, 2005).

These previous studies had both looked at two lever discrimination for clozapine trained rats, however, there is some evidence for rats substituting clozapine. In a three lever discrimination task, Porter et al. (2005) was able to get rats to discriminate 1.0 mg/kg chlorpromazine versus 5.0 mg/kg clozapine versus vehicle. This study found that chlorpromazine at the 4.0 mg/kg dose did partially generalized to clozapine.

Group 2: Methylene blue

In the five rats that were tested, methylene blue was found to partially substitute at 7.5 mg/kg for clozapine for four of the rats. While five of the rats had tested for methylene blue, three of experienced rate disrupting effects, and each of them had experienced it at a different dose. This study may be the first time methylene blue has been used a testing compound in a discriminative stimulus assay, but it's not the first time that methylene blue has been compared to other APDs: Klamer, Engel, and Svensson (2004) had found that methylene blue at 50 mg/kg and 100 mg/kg dosages via IP reversed PPI of the acoustic startle response caused by phencyclidine in mice.

Methylene blue has also been explored for anxiolytic and antidepressant properties. In rats that were subjected to an elevated plus maze, a preclinical assay designed to measure anxiety by watching how much time an animal spends in the open sections of the maze or the closed section of the maze, it was found the methylene blue had dose dependent effects. At low doses, 7.5 mg/kg, 15 mg/kg, and 30 mg/kg via intravenous (IV) injection, the rats had spent a significantly more time in the open arms compared to baseline, which indicates these doses likely have anxiolytic properties, yet at 60 mg/kg via IV, the rats spent significantly more time in the enclosed arms compared to baseline, which may indicate anxiogenic effects. Additionally, 60 mg/kg was found to cause a significant decrease in locomotor activity (Eroglu & Caglayan, 1997). During the course of the current study, two of the rats were given a 60 mg/kg test sessions, but failed to respond when put in their operant chamber, so that dose was not given to the remaining rats. Due to the low dose of 7.5 mg/kg partially substituting for clozapine, it may be worth exploring even lower doses to see it increases drug lever responding, in addition to seeing if there are still selective rate disrupting effects for lower doses for some of the rats.

While there are many other APDs that generalize for clozapine in rats, there is also evidence for full substitution by the antihistamines cyproheptadine and promethazine, and the antidepressants, amitriptyline and mianserin for clozapine at the 5.0 mg/kg dose (Kelley & Porter, 1997). The tricyclic antidepressant imipramine has been found in some studies to partially substitute for clozapine in rats (Kelley & Porter, 1997), and not at all in other studies (Nielson, 1988). Methylene blue does have some antidepressant and anxiolytic properties (Eroglu & Çaglayan, 1997; Oz, et al., 2011), and despite being used in medicine for 100 years, little is known about the receptor binding profile of methylene blue, aside from it likely binding to $GABA_A$ receptors (Chen, Liu, Yang, Dillon, & Huang, 2017). The discriminative stimulus of clozapine relies on its complex binding profile which acts on a range of receptors. Previous drug discrimination studies have indicated that clozapine's discriminative stimulus properties can be mediated by muscarinic, $alpha_1$ andrenergic, or dopaminergic D_2 antagonism (Porter, Prus, Vann, & Varvel, 2005), and more recently studies have implicated serotonergic 5-HT_{2A} inverse agonism, and dopaminergic D_4 antagonism (Prus, et al., 2016). This complex receptor binding affinity is likely what it has in common with the various antidepressants and antihistamines that have substituted for it by the animals of the previous drug discriminations, and possibly what it shares in common with methylene blue as well.

Group 2: Quinacrine

In the four rats tested, quinacrine was not found to substitute for clozapine. Quinacrine falls under two chemical families, the phenothiazines, of which it shares three heterocyclic rings, and its antihelmintic properties (Swazey, 1974), and the quinolines, which looks chemically similar to phenothiazine but has two heterocyclic rings instead of three, which it shares antimalarial properties with, and includes other drugs such as quinine, chloroquine, and

mefloquine. The quinoline antimalarials have been found to be linked to depression, confused states, insomnia, anxiety, and delusions (Nevin & Croft, 2016). Quinacrine decreasing sensitivity to PPI in rats does help to provide further evidence to suggest that, aside from methylene blue, antimalarial drugs do not appear to have antipsychotic efficacy (Lee, et al., 2009).

Malaria is a disease that is characterized by fever, loss of energy, emesis, and in more extreme cases, seizures and coma. Malariotherapy had also been used as a treatment for schizophrenia, among other conditions, by use of fever, seizures, and coma, as treatments for psychosis, in the pre-chlorpromazine era (Freitas, et al., 2014). Malriotherapy had been so effective at treating individuals with neurosyphilis and schizophrenia, Julius Wagner-Jauregg had won the 1927 Nobel Prize in medicine for developing the treatment (Tsay, 2013). To say that malaria induced sedative like effects, while quinacrine and other quinoline antimalarials reduce the effects of Malaria by inducing agitation, would be a gross oversimplification of the treatment of the disease, but one that follows along the logic of early scientific merit.

Limitations

This study was limited by several factors. Despite chlorpromazine being described as having a robust discriminative stimulus, the drug was not able to be established as a discriminative stimulus without great difficulty, or reliability. It cannot be ruled out that the effects of several phase changes likely affected how the rats will generalize novel drugs. The clozapine trained rats despite having more readily acquired the discrimination, had their share of difficulties as well, such as the chlorpromazine partially substituting at the higher doses for clozapine, despite not having a precedent for that in previous literature in a two lever discrimination procedure. This illustrates how the drug discrimination paradigm is affected by individual components of a compound cue, and while it may be useful for exploring similarities

in the subjective effects of drugs due to similarity in pharmacological profile, but does not elucidate the specific mechanism of action of these drugs. Drug discrimination can be useful in determining behavioral effects of drugs, however there is not yet it has not been determined the link between the discriminative stimulus effects of antipsychotic drugs, and their therapeutic efficacy.

Methylene blue having a partial generalization to clozapine is of interest in this particular study, it should also be noted that to confirm that these drugs do indeed have similar receptor activity, positive and negative controls should be used to confirm the findings. Using another atypical antipsychotic, such as olanzapine that has previously been shown to generalize to clozapine (Prus, et al., 2005), would confirm that drug lever responses are due to similarity of receptor activity. A negative control could be established by a drug with known pharmacological dissimilarity to clozapine, such as amphetamine, which has been shown to have its discriminative stimulus effects antagonized by clozapine, among other antipsychotics (Nielsen & Jepsen, 1985) would help to confirm that lever pressing on drug lever was not due to dissimilarity from the control condition.

Finally, if a drug such as methylene blue is indeed found to have therapeutic effects in the treatment of schizophrenia, or other illnesses, it may not be of much value to patients that experience the drugs unpleasant subjective effects. Individuals with schizophrenia report higher levels of dissatisfaction and perceived adverse effects with typical antipsychotics compared to that of atypical antipsychotics (Hellewell, 2002). This should serve as a reminder to the point that new drug treatment should not be worse than the illness.

Future research

This study has several findings that could be useful in the development of future research. Methylene blue partially substituting for clozapine is noteworthy, as there are a number of different experiments that can be performed to further explore antipsychotic, antidepressant, or anxiolytic value of methylene blue. Additionally, it may be of interest to examine the psychoactive effects of the different antimalarial drugs, as their mechanism of action is not well understood, and if quinacrine or other quinoline antimalarials do exert adverse psychiatric effects that may be worth exploring as a putative deficit inducer.

Conclusions

The present study sought to further explore the discriminative stimulus properties of current APDs. Despite being well characterized in the literature, antipsychotic medicine is far from perfect, as current antipsychotic medicine only treats symptoms of schizophrenia, and asymmetrically treats positive symptoms more so than negative symptoms. Many individuals that suffer from schizophrenia also struggle with the side effects of the medications that they have to take. Finding the right medication for each individual can also be quite difficult, as not all antipsychotics work effectively in all individuals. It is important for us to find the treatment for the right person. The use of methylene blue, may not be right treatment for all people who experience psychosis, but it may have therapeutic value for some. The findings of this study suggest methylene blue may exhibit antipsychotic-like effects, further research is needed in models that are specifically predictive of antipsychotic activity.

Until a treatment is discovered that is more effective than the drugs that we have available today, there should be a continued effort to find new drugs, and drug discrimination can be an effective tool for exploring the pharmacological action of new drugs.

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APPENDIX A

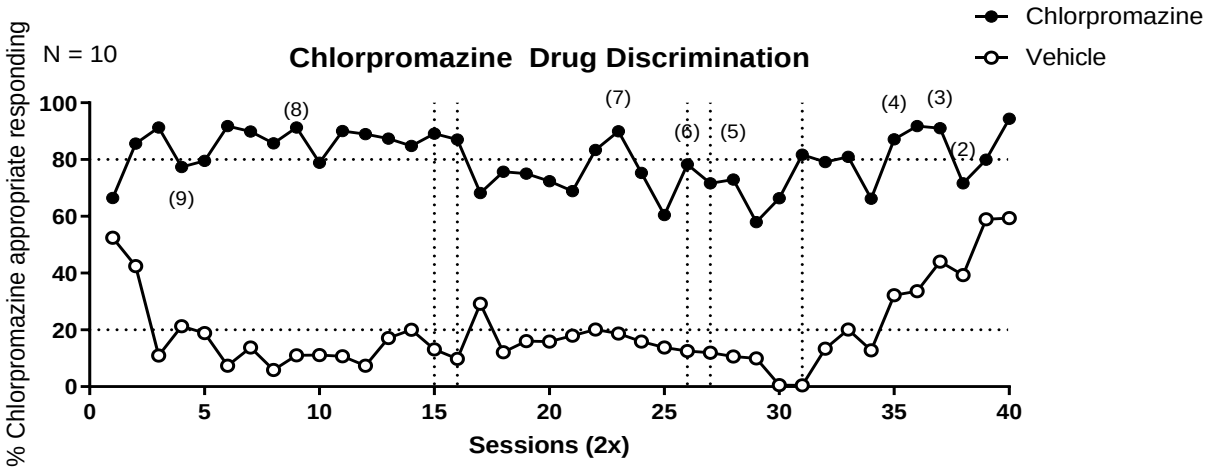

Figure 1: Mean % chlorpromazine lever responding for two lever training in rats trained to discriminate chlorpromazine from vehicle. The x-axis indicating the number of sessions for the drug and vehicle testing, and the y-axis indicates the percent of drug lever responding. The number in parenthesis indicates the number of rats that had yet to meet criteria, otherwise the number of rats is equal to N. The dotted lines across the x-axis indicating phase changes: the first one indicating change in training dose from 1.0 mg/kg via IP to 2.0 mg/kg via IP; second indicating change in route of administration from IP to SC; third indicating change from 2.0 mg/kg via SC to 1.0 mg/kg via SC; and fourth indicating 1.0 mg/kg SC to 0.5 mg/kg SC.

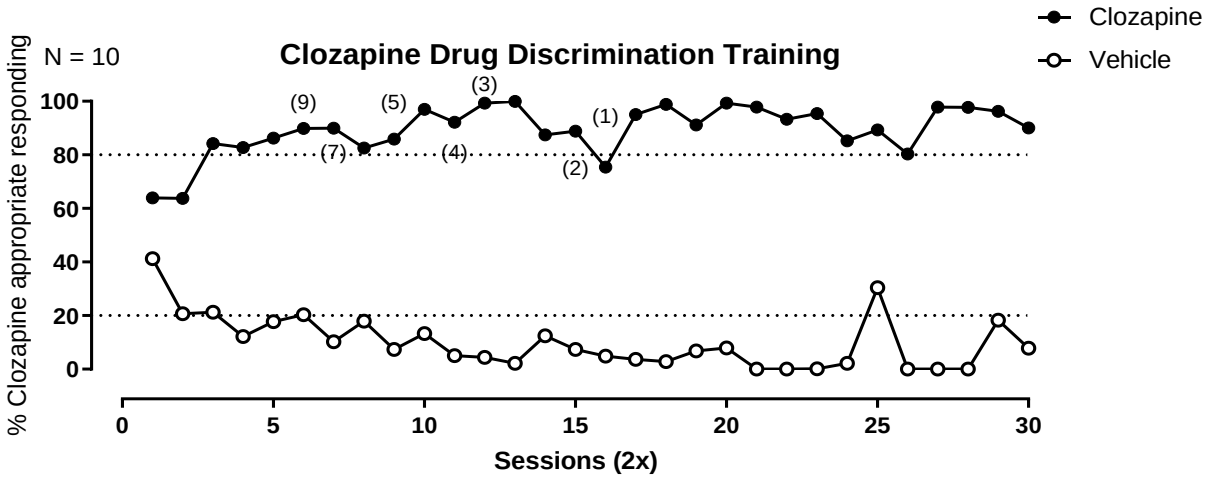

Figure 2: Mean % clozapine lever responding for two lever training in rats trained to discriminate 1.25 mg/kg clozapine from vehicle. The x-axis indicating the number of sessions for the drug and vehicle testing, and the y-axis indicates the percent of drug lever responding. The number in parenthesis indicates the number of rats that had yet to meet criteria, otherwise the number of rats is equal to N.

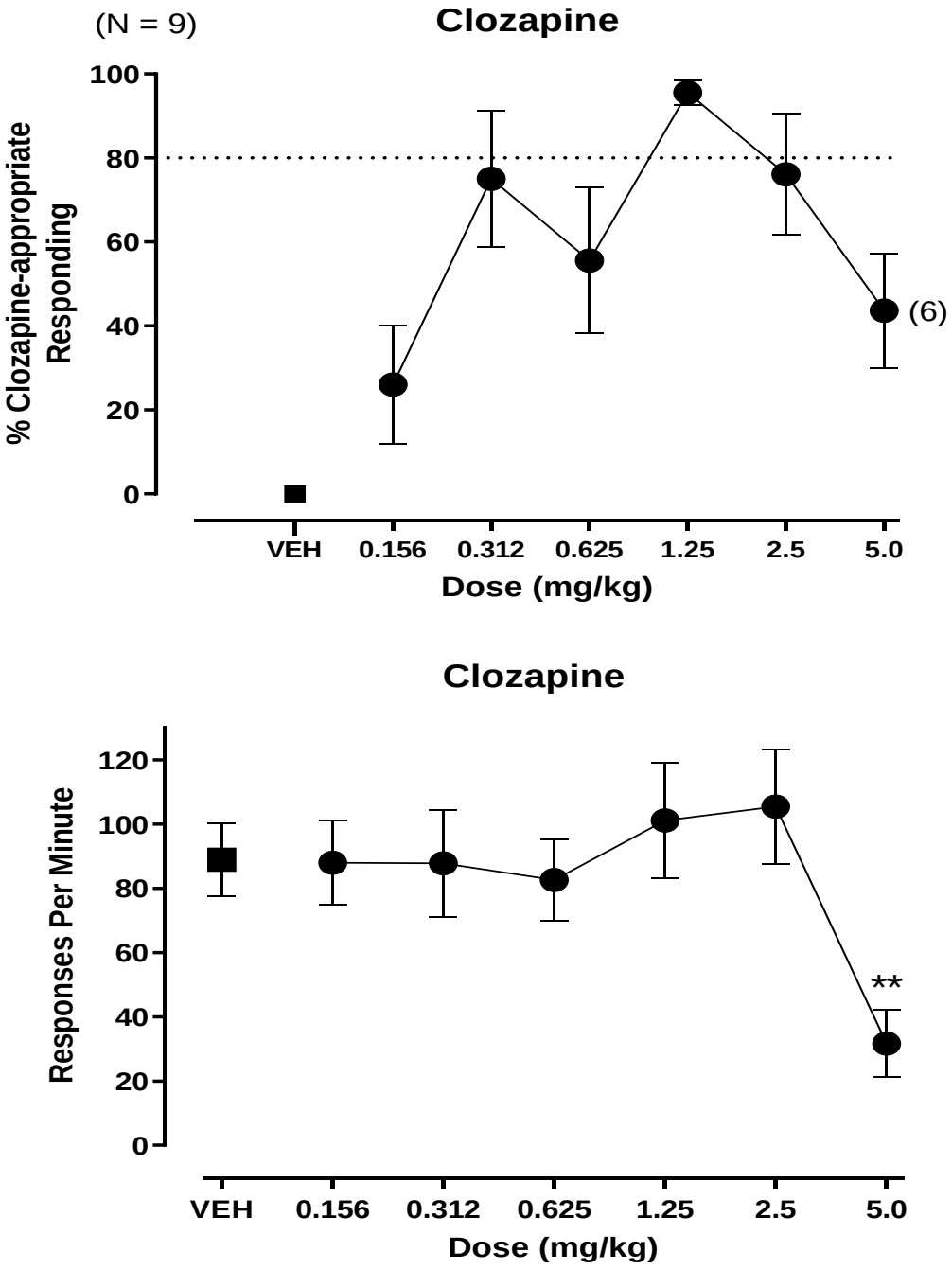

Figure 3: Generalization results for clozapine in rats trained to discriminate 1.25 mg/kg clozapine from vehicle in a two lever drug discrimination task. Mean percent drug lever responding is shown in the upper panel, and mean responses per second are shown in the lower panel. The dashed line at 80% indicates full generalization to the drug lever. Prior to generalization testing, control tests were conducted with the clozapine training dose and vehicle. Rats with response rates below 5 responses per minute in a given test session were not included in the % drug lever data.

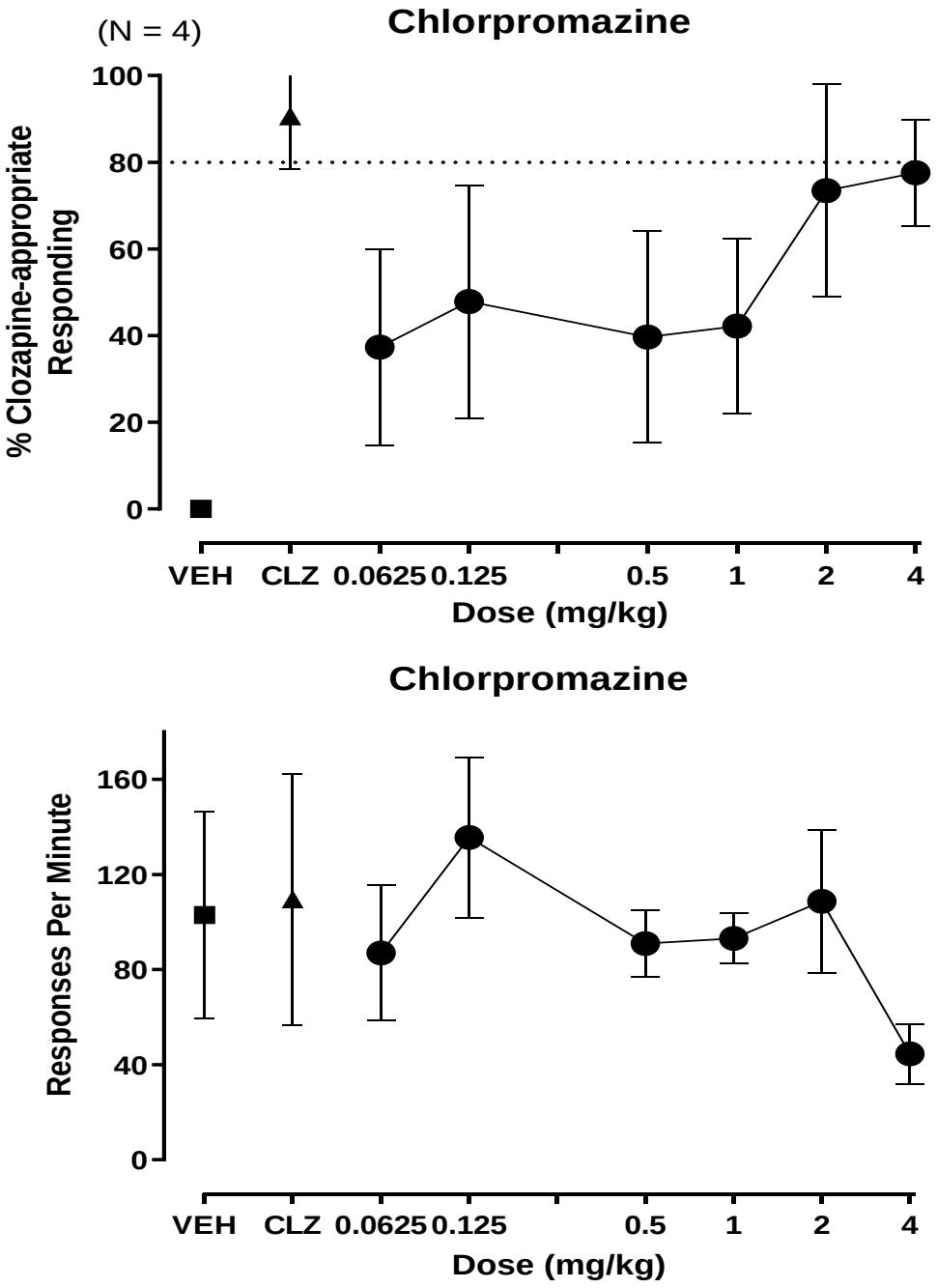

Figure 4: Generalization results for chlorpromazine in rats trained to discriminate 1.25 mg/kg clozapine from vehicle in a two lever drug discrimination task. Mean percent drug lever responding is shown in the upper panel, and mean responses per second are shown in the lower panel. The dashed line at 80% indicates full generalization to the drug lever. Prior to generalization testing, control tests were conducted with the chlorpromazine training dose and vehicle. Rats with response rates below 5 responses per minute in a given test session were not included in the % drug lever data.

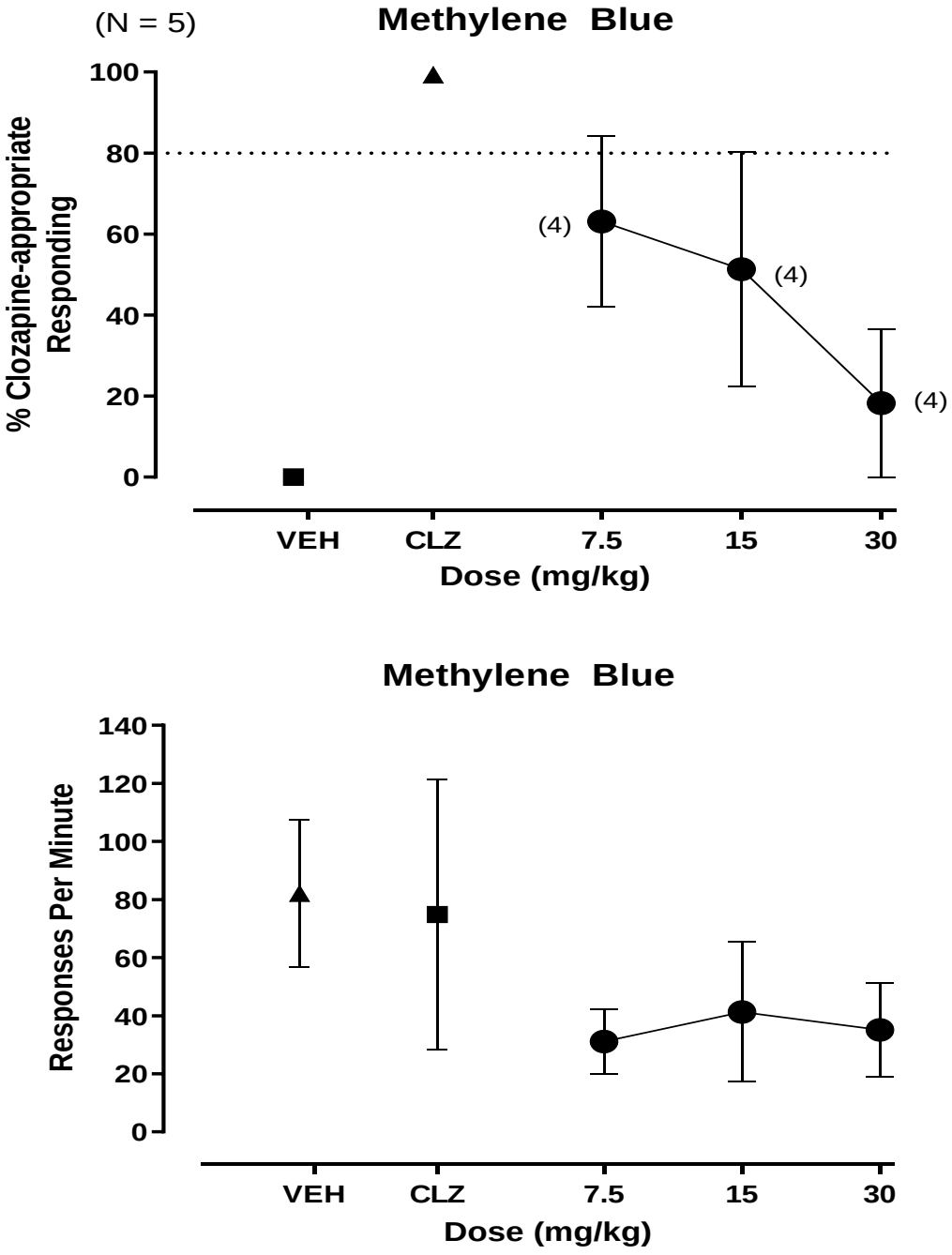

Figure 5: Generalization results for methylene blue in rats trained to discriminate 1.25 mg/kg clozapine from vehicle in a two lever drug discrimination task. Mean percent drug lever responding is shown in the upper panel, and mean responses per second are shown in the lower panel. The dashed line at 80% indicates full generalization to the drug lever. Prior to generalization testing, control tests were conducted with the methylene blue training dose and vehicle. Rats with response rates below 5 responses per minute in a given test session were not included in the % drug lever data.

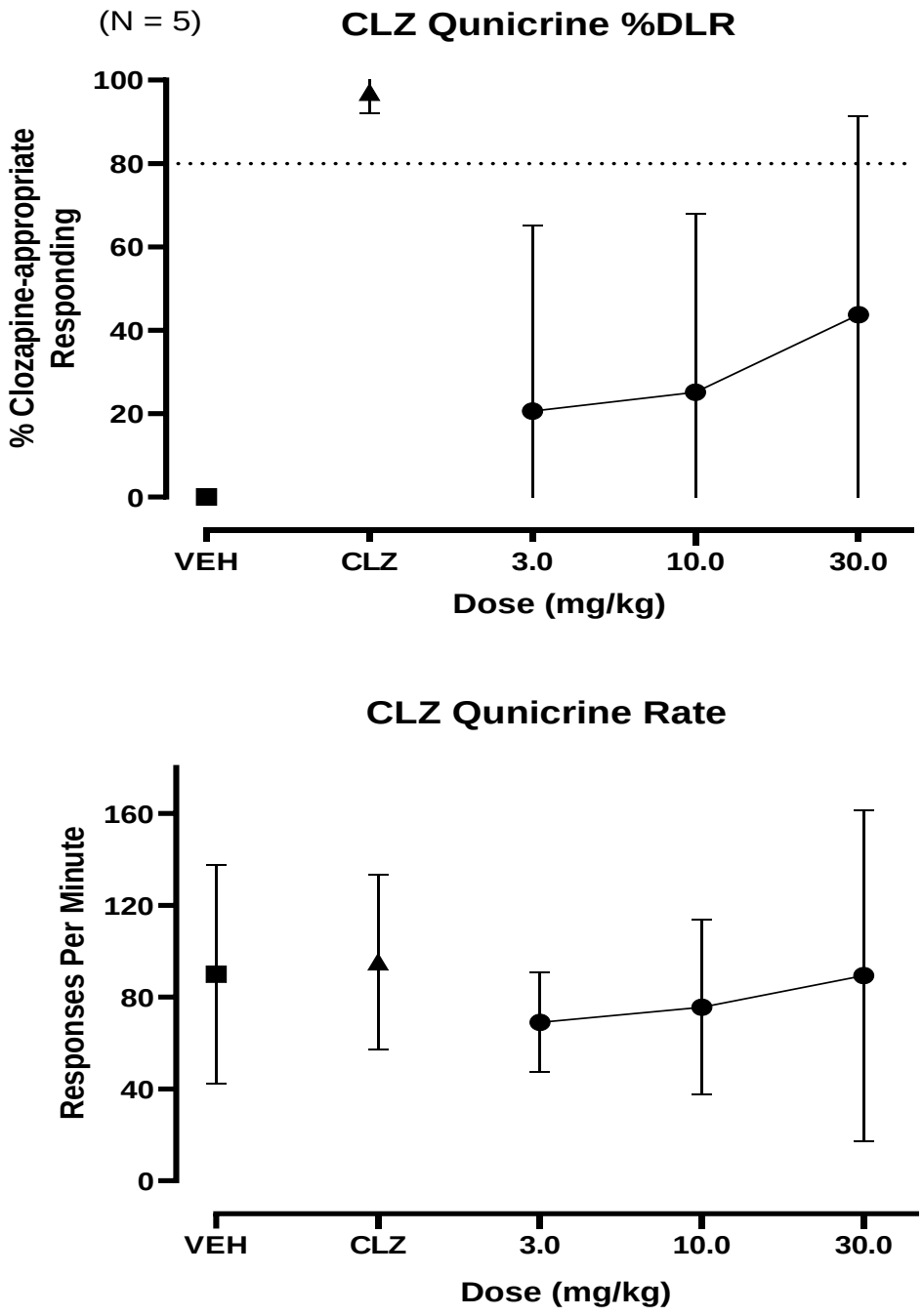

Figure 6: Generalization results for quinacrine in rats trained to discriminate 1.25 mg/kg clozapine from vehicle in a two lever drug discrimination task. Mean percent drug lever responding is shown in the upper panel, and mean responses per second are shown in the lower panel. The dashed line at 80% indicates full generalization to the drug lever. Prior to generalization testing, control tests were conducted with the quinacrine training dose and vehicle. Rats with response rates below 5 responses per minute in a given test session were not included in the % drug lever data.

APPENDIX B

Institutional Animal Care and Use Committee Approval Form

SIGNATURE PAGE

IACUC #: 350 **PROPOSAL TITLE:** Behavioral effects of classical and novel antipsychotics

III. ACKNOWLEDGEMENT BY PRINCIPAL INVESTIGATOR

I acknowledge responsibility for this project. I have read the Northern Michigan University Principles for the Care and Use of Laboratory Animals and certify that this project will be conducted in compliance with those principles. I assure that I will obtain Institutional Animal Care and Use Committee approval prior to significant changes in the protocol. I assure that this project does not unnecessarily duplicate previous research or instructional projects. I assure that students, staff and faculty on the project are qualified or will be trained to conduct the project in a humane, safe, and scientific manner.

Signature: _____ 02/07/2019

Principal Investigator Date

IV. APPROVAL OF SCIENTIFIC MERIT (to be completed by the Department Head)

Before the project is initiated, it must be reviewed and approved on the basis of its scientific merit.

☐Review conducted by external agency.

☐Governmental Agency: Please specify the reviewing agency or board Federal agency (e.g., NIH, NSF, USDA, etc.) and evidence of approval

☐Nongovernmental agency (e.g., University review, specify if other):

☐Departmental Review: I assure that this project has been reviewed and approved for scientific or instructional merit by:

☐Expert reviewer (Name)

☐Departmental Committee Review (Committee Name and Chairperson):

☐Other (Describe):

Signature: _____

2/07/2019 **Department Head/Other Authorized Departmental Designee**

Date

V. REVIEWED AND APPROVED BY THE IACUC

Signature: _____ 02/07/2019

Institutional Animal Care and Use Committee Chair Date

Signature: _____ 02/07/2019

Institutional Animal Care and Use Officer Date

Following action on this application, copies of approval or denial letters will be sent to the applicant, Department Head, and appropriate College Dean.