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CHARACTERIZATION OF THE DISCRIMINATIVE STIMULUS PROPERTIES OF THE ATYPICAL ANTIPSYCHOTIC AMISULPRIDE IN C57BL/6 MICE

Timothy J. Donahue
donahuetj@vcu.edu

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By: TIMOTHY JOHN DONAHUE
Bachelor of Arts, St. Mary's Seminary University, Baltimore, Maryland 1973
Master of Education, Virginia Commonwealth University, 1976
Master of Humanities, University of Richmond, 1984
Master of Science, Virginia Commonwealth University, 2012

Director: Joseph H. Porter, Ph.D.
Professor of Psychology
Department of Psychology

Virginia Commonwealth University
Richmond, Virginia
November, 2014

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Abstract

CHARACTERIZATION OF THE DISCRIMINATIVE STIMULUS PROPERTIES OF THE ATYPICAL ANTIPSYCHOTIC AMISULPRIDE IN C57BL/6 MICE

By Timothy John Donahue, MS, MA, M.Ed.

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Major Director Joseph H. Porter, Ph.D.
Professor, Department of Psychology

Amisulpride, a benzamide derivative, is a second generation (atypical) antipsychotic drug used to treat both positive and negative symptoms of schizophrenia. Amisulpride is a relatively selective antagonist at dopamine D_2 and D_3 receptors and at serotonin 5-HT_{2B} and 5-HT_7 receptors. This is a unique binding profile as compared to both first generation (typical) and second generation antipsychotic drugs. It is approved in Europe and displays an atypical clinical profile with reduced extrapyramidal motor effects. The drug has a chiral center and is a mixture of two optical isomers: *(S)*-amisulpride and *(R)*-amisulpride. The therapeutic form of the drug is a mixture of these two enantiomers (*rac*-amisulpride). The present study used a two-lever drug discrimination assay to allow a direct comparison between amisulpride and its two isomers. Additionally, substitution testing was conducted with the typical antipsychotics haloperidol and chlorpromazine; the atypical antipsychotics olanzapine, clozapine, risperidone, quetiapine and aripiprazole; the antidepressants imipramine, fluoxetine, bupropion, mianserin; the anxiolytic

chlordiazepoxide; and the benzamide derivatives sulpiride, *(S)*-sulpiride , tiapride, nemonapride and zacopride; and selective ligands with receptor mechanisms relevant to amisulpride. C57BL/6 mice were trained to discriminate 10 mg/kg *rac*-amisulpride from vehicle in a two-lever drug discrimination task for food reinforcement in an average of 35.7 sessions (range 6-89). The amisulpride dose-response curve (0.078 – 10.0 mg/kg) yielded an ED_{50} = 0.64 mg/kg, 95% CI [.47, 0.84 mg/kg]. The *(S)*-amisulpride and *(R)*-amisulpride isomers fully substituted for amisulpride with a significant left-ward shift in the dose-response curve for *(S)*-amisulpride (ED_{50} = 0.33 mg/kg) as compared to *rac*-amisulpride and *(R)*-amisulpride. The benzamide derivatives sulpiride and the (*S*)-sulpiride isomer fully substituted for amisulpride; however, the benzamide derivative tiapride produced only partial substitution (76.4% DLR), and nemonapride (54.52% DLR) and zacopride (38.64% DLR) did not substitute for amisulpride. None of the other tested drugs (antipsychotics, antidepressants, anxiolytics, and selective ligands) substituted for rac-amisulpride's discriminative stimulus. These results showed that the *rac*-amisulpride stimulus was readily acquired in C57BL/6 mice, and that it has a unique and robust discriminative stimulus that is dose-dependent, time-dependent and stereoselective and is not shared with other antipsychotic or antidepressant drugs.

Characterization of the Discriminative Stimulus Properties of the Atypical Antipsychotic Amisulpride in C57BL/6 Mice

Synopsis of Antipsychotic Drugs in the Treatment of Schizophrenia

Research in the 1950s produced the first effective pharmacological treatments for the care of individuals diagnosed with schizophrenia. The first generation antipsychotics (also called typical antipsychotics), such as chlorpromazine and haloperidol, proved effective in treating some the positive symptoms of the disorder (e.g. hallucinations and delusions) but had serious drawbacks, including severe extrapyramidal motor side effects (EPS). In addition, the typical antipsychotics also were not effective in alleviating negative symptoms of the disorder (such as depression and anhedonia) and a significant proportion of individuals with the disorder proved to be treatment-resistant (J. M. Kane, Honigfeld, Singer, & Meltzer, 1988). Continued research led to more effective antipsychotic medications with less unwanted side effects ushering in a second generation of antipsychotic drugs known as atypical antipsychotics such as clozapine, risperidone, and olanzapine. Clozapine, first synthesized in 1958 by Wander AG, (compound HP-1854) is known as the prototypical atypical antipsychotic medication and proved effective in treating a range of symptoms of schizophrenia without producing EPS. However, clozapine was not without its problems as it was associated with a high incidence of agranulocytosis (a condition resulting in reduced production of white blood cells) in certain populations leading to it being withdrawn from the marketplace in 1975. It was reintroduced in 1989 in the USA with special guidelines and restrictions for use with treatment-resistant schizophrenic patients (Meltzer, 1997). This spurred additional research to develop other atypical antipsychotics and amisulpride was bought to the market in the mid-1990s in France by Sanofi-Aventis. Amisulpride is the focus of this dissertation. It is the intent of this dissertation that an

investigation of the discriminative stimulus properties of amisulpride will yield knowledge of the drug's underlying pharmacological mechanisms responsible for the drug's interoceptive (subjective) properties. Hopefully, this research also will yield valuable information as to the unique contribution amisulpride has made in treating schizophrenia as compared to typical antipsychotics.

Schizophrenia

Schizophrenia is a debilitating mental disorder involving major disruptions of perception, cognition, emotion, and behavior. Its cause remains a persistent and challenging mystery. The consequences for the individual and society are profound as most patients suffer from a lifetime of psychiatric disability, periodic hospitalizations, poor social adjustment, and disrupted family relationships. The overall U.S. cost of schizophrenia in 2002 was estimated at $62.7 billion (Wu et al., 2005). While there is some variation across countries and by race/ethnicity the worldwide prevalence rate for the disorder is approximately .07 % (Saha, Chant, Welham, & McGrath, 2005). Schizophrenia typically has an early onset appearing in late adolescence and the early 20s, and rarely does it affect individuals older than 45 years old (Mueser & McGurk, 2004). Compared to women, men tend to experience symptoms earlier, have a slightly higher incidence of the illness and respond more poorly to treatment (Cocchi et al., 2014; M. V. Seeman, 1982). It appears across all economic, social and cultural borders and it is a devastating disorder, with 4.9% of schizophrenics committing suicide during their lifetime, usually near illness onset (Palmer, Pankratz, & Bostwick, 2005). The etiology of schizophrenia is complicated and still unclear. Quite a few factors have been implicated. Genetic factors have been found to play a role as the illness appears to run in families (Sullivan, 2005); however, the role of genetics is complicated by the fact that monozygotic twins have a concordance rate of 50% for

schizophrenia implying that other environmental or organic factors play a significant role as well (Owen, Craddock, & O'Donovan, 2005). Research indicates that an interaction of genes and environmental factors such as exposure to viruses, prenatal malnutrition, complications during birth, and other unknown psychosocial variables play an important role in the development of schizophrenia (Fatemi & Folsom, 2009; Mirsky & Duncan, 1986). Other theories emphasize the role of brain pathology, neurodevelopmental factors, brain chemistry and the more recent plethora of studies surrounding epigenetic factors (Fatemi & Folsom, 2009; Gavin & Floreani, 2014; Hyde & Weinberger, 1990; Snyder, 1976; Trimble, 1991). Historically, psychiatry has progressed from merely observing symptoms to defining symptom clusters as part of an illness associated with a disorder and patterns of recovery. In 1896 the German psychiatrist Emil Kraepelin used the term "dementia praecox" (early dementia) to distinguish the dementia (and psychosis) that struck people in the late teens and early twenties from that seen in the elderly. Additionally he developed the first list of symptoms associated with the disorder emphasizing thought disorders. In 1911, the Swiss psychiatrist Eugen Bleuler coined the term "schizophrenia" from the Greek meaning split-mind (Tsuang, Faraone, & Green, 1999) in an effort to clearly differentiate the disorder from late onset dementia. Bleuler also detailed differences between the two in terms of onset, duration and possible remission/recovery (B. J. Cohen, 2003).

Symptoms. *The Diagnostic and Statistical Manual of Mental Disorders* (5th ed.; *DSM-5*) (American Psychiatric Association, 2013) is the most widely accepted reference used by clinicians and researchers for the classification and symptomatology of mental disorders. Table 1 shows the *DSM-5* diagnostic criteria for schizophrenia. Generally speaking, for a diagnosis of schizophrenia, an individual is required to exhibit two (or more) of the following symptoms for a

Table 1.

Diagnostic criteria for schizophrenia

Diagnostic Criteria for Schizophrenia Disorder
A. Two (or more) of the following, each present for a significant portion of time during a 1-month period (or less if successfully treated). At least one of these must be (1), (2), or (3): 1. Delusions. 2. Hallucinations. 3. Disorganized speech (e.g., frequent derailment or incoherence). 4. Grossly disorganized or catatonic behavior. 5. Negative symptoms (i.e., diminished emotional expression or avolition). **B**. For a significant portion of the time since the onset of the disturbance, level of functioning in one or more major areas, such as work, interpersonal relations, or self-care, is markedly below the level achieved prior to the onset (or when the onset is in childhood or adolescence, there is failure to achieve expected level of interpersonal, academic, or occupational functioning). **C.** Continuous signs of the disturbance persist for at least 6 months. This 6-month period must include at least 1 month of symptoms (or less if successfully treated) that meet Criterion A (i.e., active-phase symptoms) and may include periods of prodromal or residual symptoms. During these prodromal or residual periods, the signs of the disturbance may be manifested by only negative symptoms or by two or more symptoms listed in Criterion A present in an attenuated form (e.g., odd beliefs, unusual perceptual experiences). **D**. Schizoaffective disorder and depressive or bipolar disorder with psychotic features have been ruled out because either 1) no major depressive or manic episodes have occurred concurrently with the active-phase symptoms, or 2) if mood episodes have occurred during active-phase symptoms, they have been present for a minority of the total duration of the active and residual periods of the illness. **E**. The disturbance is not attributable to the physiological effects of a substance (e.g., a drug of abuse, a medication) or another medical condition. **F**. If there is a history of autism spectrum disorder or a communication disorder of childhood onset, the additional diagnosis of schizophrenia is made only if prominent delusions or hallucinations, in addition to the other required symptoms of schizophrenia, are also present for at least 1 month (or less if successfully treated).

Adapted from *Diagnostic and statistical manual of mental disorders* (5th ed). (American Psychiatric Association, 2013)

significant portion of time during a 1-month period: delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior and the presence of negative symptoms, e.g. depression (Criterion A). Additionally, for a significant portion of time since the onset of disturbance, the individual should exhibit social/occupational dysfunction in areas such as work, school, and interpersonal relations or markedly below normal self-care (Criterion B). These symptoms should persist for at least 6 months (Criteria C) and the clinician should rule out both Schizoaffective Disorder and Mood Disorder (Criteria D). The diagnosis is precluded if the disturbance is due to the direct physiological effects of medication or substance abuse (Criteria E). Finally, if the individual has a history of Autistic Disorder or another Pervasive Developmental Disorder, the additional diagnosis of schizophrenia should be applied only if prominent delusions or hallucinations are present.

British psychiatrist and researcher Timothy Crow proposed that the symptoms of schizophrenia be further refined into two main categories, positive and negative symptoms (Crow, 1980). Positive symptoms are produced by the individual that are outside the usual behavioral repertoire of human beings. That is, they are behaviors that exist, which should not be present, such as auditory hallucinations (hearing voices), delusional thoughts (being persecuted), or rambling and incoherent speech (word salad). The individual may also exhibit bizarre motor behaviors such as purposeless and unstimulated motor activity (catatonic excitement).

Disorganized thinking. The most pronounced positive symptom of schizophrenia is disorganized thinking. Individuals with the disorder often express thoughts that are loosely connected, appear in random order, and bear little association to relevant situations. Further, thoughts are not expressed in coherent, meaningful language. Disorganized thinking is mainly seen in the form of many varied delusions and distinguishing among them can be difficult

(Spitzer, 1990). Simply, delusions are false beliefs not subject to change by reason or experience (Tsuang et al., 1999). Individuals may express delusions of grandeur, such as thinking one is a famous movie star, one is omnipotent, or is all-knowing. Further, delusions can be expressed in the form of control; for example, believing that others are controlling one's thoughts or that one is controlling the thoughts of others.

Cognitive impairments. Closely related to disorganized thinking are other cognitive impairments. Previously, the loss of normal cognitive abilities has been framed as a negative symptom; however, due to its unique characteristics, *DSM-5* delineates cognitive impairments into a separate category of symptoms. Most schizophrenics have some degree of cognitive deficiency (Meltzer, Thompson, Lee, & Ranjan, 1996). These include: disorganized thoughts, difficulty concentrating/and or following instructions, difficulty completing tasks, memory problems, impairments in delayed recall, visuomotor skills, distractibility, impairments in delayed recognition, perceptual skills, and IQ (Keefe, 2007). The degree of cognitive impairment is important as it is a major predictor of the individual's functional outcome (Green, Kern, Braff, & Mintz, 2000). The more severe the cognitive deficit, the more difficult it is to treat the patient and the less favorable the outcome. The National Institute of Mental Health established the MATRICS™ initiative (Measurement and Treatment Research to Improve Cognition in Schizophrenia) to clarify for researchers how the issue of cognitive deficits should be approached (Green et al., 2004). Initiatives such as these will hopefully stimulate research toward the development of novel therapeutic agents tailored specifically for cognitive impairments associated with schizophrenia. Until such cognitive deficits are clearly defined from other symptoms of the disorder, the burden falls on pharmaceutical companies to empirically demonstrate the efficacy of a drug promoted for the treatment of schizophrenia or, failing that,

delineate which symptoms a promised treatment will and will not provide therapeutic relief (Laughren & Levin, 2006).

Disorganized behavior. Disorganized behavior is another salient symptom of schizophrenia. Disorganized behavior is defined as behaviors that are not in accord with the usual, customary socially acceptable repertoire of behaviors and does not express clear intent and purpose. A common example of such behavior is a motor disturbance known as catatonic behavior. This may consist of episodes of uncontrolled, agitated, and disorganized behavior, such as pacing around a ward aimlessly. Behavior can appear repetitive, hyperactive, destructive and even violent. The schizophrenic patient may exhibit mannerisms, habitual movements that usually involve a single body part such as, grimaces, tics, moving lips soundlessly, fidgeting with fingers, or hand wringing. At the opposite extreme, catatonic behavior may be expressed as a complete absence of motor actions, such as sitting rigid and motionless in a chair for hours on end, unresponsive to external stimuli.

Negative symptoms. While positive symptoms are the most pronounced symptoms of schizophrenia, negative symptoms are no less troublesome. Negative symptoms are behaviors that should normally be present, but are absent (Andreasen, Flaum, Swayze, Tyrrell, & Arndt, 1990). Negative symptoms may be expressed in the form of a marked decrease or almost absence of cognitive, emotional, and motor behaviors. Typical examples would include: avolition (lack of initiative), blunt, flat or restricted affect (emotionally void), anhedonia (lack of pleasure), alogia (absence or poverty of speech), poor eye contact, decreased spontaneous movements, and diminished emotional responsiveness as seen in the muted ability to feel intimacy or closeness to others. The catatonic stupor, a total lack of movement and verbal behavior, is an extreme example of a negative symptom. A person may appear poorly groomed, unable to persist at a

task, and withdrawn from social activities sitting motionless in a chair for hours on end, completely unresponsive to environmental stimuli (Will, 1972). Traditionally, negative symptoms have proven to be more difficult to treat than positive symptoms (Möller, 1998). Such symptoms clearly underscore the fact that schizophrenia results in a marked loss of the basic behavioral components necessary for effective social interaction (Curran & Monti, 1982).

No two individuals with the disorder present with identical symptoms; each patient has a unique combination of behavioral and cognitive difficulties. Indeed, only a few of the symptoms need be present for a diagnosis of schizophrenia to be made. *DSM-5* cautions that there is not "one type" of schizophrenia and its authors saw fit to drop all previous schizophrenic subtypes that appeared in earlier versions of the manual such as paranoid, disorganized, catatonic type, etc. Current thinking emphasizes the presence of specific symptoms within a continuum and a combination of positive or negative symptoms unique to each individual.

Pharmacological Treatments for Schizophrenia

Throughout history, the treatment of individuals with schizophrenia and related disorders has been nothing if not misguided, ineffective and, in many cases, inhumane. Those afflicted with the disorder were subjected to a wide range of treatments such as beatings, isolation, bloodletting, crude medical procedures, exorcism, and generally restricted to asylums or imprisoned in jails under notorious and dehumanizing conditions (Alexander & Selesnick, 1966). The French physician Philippe Pinel (1745-1826) was the first advocate for the development of more humane treatment of mental patients. Pinel promoted a medical model of mental illness based on the belief in organic causes for mental illness (Pinel, 1804). Pinel was one of the early founders of psychiatry through his work at the Bicêtre Hospital in Paris, and he is remembered as the "father of psychiatry." Yet, even with care for the mentally ill generally improving

throughout the late 1800s and through most of the 1900s, most individuals with schizophrenia were still confined to institutional care with little in the way of hope for treating the disorder. By 1955, more than half a million psychotic patients in the United States were confined to mental institutions (Julien, Advokat, & Comaty, 2010). From today's perspective, it is difficult to fathom how the treatments pursued at that time could have ever been seen as therapeutic and as advanced medical practices. Treatments included carbon dioxide (CO2) inhalation (Lovenhart, Lorenz, & R.M., 1929), injections of apomorphine or the barbiturate sodium amytal (Thorner, 1935), comas induced by insulin (Sakel, 1937), convulsive treatment induced by injections of camphor and metrazol (von Meduna, 1935), and electroconvulsive shock (Cerletti, 1956; Shorter & Healy, 2007). Hindsight bias aside, one might suggest that these treatments could be considered the prelude to the first pharmacological treatments for schizophrenia.

First generation typical antipsychotic medications. The development of pharmacological treatments for schizophrenia began in the 1940s with the French surgeon Henri Laborit (Hamilton & Timmons, 1994). Convinced that a patient's own fears of surgery were a major attributing factor to many of the deaths associated with surgery, Laborit experimented with various drugs to reduce presurgical anxiety. In 1950, S. Courvoisier and her associates tested Paul Charpentier's new compound chlorpromazine (4560 RP) discovered while Charpentier was working for the French pharmaceutical company Laboratoires Rhône-Poulenc (Charpentier P & Jacob R, 1952). Courvoisier found that it prolonged sleep induced by barbiturates in rodents and inhibited conditioned avoidance-escape responding in mice (Pichot, 1996). The conventional sedatives at that time merely blocked autonomic responses but had no antianxiety (anxiolytic) effect. Laborit experimented with chlorpromazine combining it with promethazine and an analgesic to produce a presurgical "lytic cocktail" which indeed lessened presurgical fears

(Laborit, Huguenard, & Alluame, 1952). When administered prior to surgery, patients became calm, mildly sedated, and the post-surgical complications and death rates were greatly reduced. This conscious but nonchalant state (being indifferent to what occurs around them) would come to be known as a "neuroleptic" state from Greek world *lepsis* (seizure), thus to "seize" the neuron. Laborit's cocktail-induced state bore a striking resemblance to the detached state and behaviors exhibited by individuals with schizophrenia such as emotional flatness, apathy, and a loss of initiative. Thus, drugs treating this detached schizophrenic state came to be known as "neuroleptics" and represent the first-generation (typical) of antipsychotic drugs (Julien et al., 2010). These typical antipsychotics were derived from a class of drugs known as phenothiazines and were the first drugs to successfully treat the symptoms of schizophrenia. Laborit correctly predicted that the main ingredient in his "lytic cocktail", chlorpromazine, may have application for psychiatric disorders (Stip, 2002). As the history of pharmacological agents demonstrates, drugs effective for the treatment of one medical condition often lead to the drug being used for the treatment of other medical conditions (W. W. Shen, 1999). Excitement grew over the use of chlorpromazine when Parisian psychiatrists Hamon, Delay and Deniker used it in psychiatric hospitals with astounding results (Deniker, 1990). It quieted down many frenetic positive symptoms of individuals with schizophrenia and related disorders, although it did little to treat depression. In a short period of time, it was marketed in France for the treatment of schizophrenia. In 1953 it was brought to the European market under the trade name Largactil®. The drug's powerful effect in managing some of the symptoms of schizophrenia led to its expanded use throughout Europe and North America. It was approved for use in the United States in 1955 and marketed under the trade name Thorazine®. The discovery of the therapeutic effects of chlorpromazine sparked what has come to be known as the drug revolution in

psychiatry (Lopez-Munoz et al., 2005). Chlorpromazine's impact cannot be overstated. This was the beginning of psychopharmacology and ushered in a new era in the treatment of mental disorders (Thompson, 1997).

Following the introduction of chlorpromazine, the late 1950s and 1960s would see the development of other neuroleptics, such as haloperidol, benperidol, droperidol, loxapine and molindone, all from a class of drugs known as butyrophenones (Julien et al., 2010). These typical neuroleptics shared a similar mechanism of action: reducing dopamine activity in the brain, chiefly as antagonists at dopamine D_2 or D_2-like receptors (Meltzer, 1991).

As clinical practice has shown, dopamine antagonism proved to be rather important, if not indispensable, for the therapeutic effects of antipsychotic medications. However, dopamine antagonism is also responsible for the undesirable extrapyramidal motor side effects. Additionally, dopamine antagonism (alone) has been found to be ineffective for treating the negative symptoms of schizophrenia. The extrapyramidal system is a neural network found in the central nervous system that is part of the motor system responsible for involuntary reflexes and modulation of movement (i.e. coordination). It is located primarily in the reticular formation of the pons and medulla, and target neurons in the spinal cord involved in reflexes, locomotion, complex movements, and postural control. These tracts include the nigrostriatal (mesostriatal) pathway, basal ganglia, cerebellum, the vestibular nuclei, and different sensory areas of the cerebral cortex and serve regulatory functions by moderating motor activity without directly innervating motor neurons (Purves et al., 2001). Extrapyramidal motor side effects are often devastating and in some cases, permanent. They may include: Parkinsonian-like tremors, rigidity, involuntary tics, involuntary movements and body restlessness known as akathesia (Jeste & Caliguiri, 1993). Another side-effect noticed by clinicians of early neuroleptic drugs

was tardive dyskinesia, a devastating neurological syndrome characterized by repetitive, involuntary movements such as grimacing, lip smacking, pursing of the lips, and or excessive eye blinking. Sadly, tardive dyskinesia tended to appear late in the course of treatment and often after discontinuation of the drug with symptoms continuing for years and was untreatable (Crane, 1968). Although such side effects were regrettable, many in the psychiatric field came to view such effects as an indication and expectation of whether or not a drug was a true neuroleptic, and therefore considered to be a necessary and unavoidable part of therapeutic treatment (van Rossum, 1966). The risk of extrapyramidal motor effects, the presence of tardive dyskinesia, the failure of the early neuroleptics to alleviate the negative symptoms, was further complicated by the fact that many patients were treatment-resistant to the early antipsychotics. This scenario was the catalyst for researchers to develop a "second-generation" of antipsychotics. These improved medications would be known as "atypical" antipsychotics and provided psychiatrists with alternative therapeutic medications that could treat a wider range of schizophrenic symptoms with less adverse side effects (Julien et al., 2010).

Second generation atypical antipsychotic medications. The second-generation antipsychotics were introduced into the United States with clozapine in 1989 followed by: risperidone (1994), olanzapine (1996), sertindole (withdrawn from U.S. markets in 1998, but available in certain European countries), quetiapine (1997), ziprasidone (2001), aripiprazole (2002), paliperidone (2006), iloperidone (2009) and asenapine (2009). Amisulpride (Solian®) was available in the mid-1990s in Europe and Australia but not in the United States (Julien et al., 2010).

Clozapine (Clozaril®) was the first atypical antipsychotic with demonstrated superiority over first-generation antipsychotics. It was developed by the European pharmaceutical company

Wander Pharmaceutical Company in 1958, later acquired by Sandoz in 1967. It was the prototypical second-generation antipsychotic medication and remains, today, as the "gold standard" medication for treatment-resistant patients, as well as being the first drug to prove effective in treating both the positive and negative symptoms of schizophrenia. (Hippius, 1999; Meltzer, 1994). Clozapine was the first antipsychotic with greatly reduced extrapyramidal motor side effects, the major concern with the first-generation drugs (Arnt & Skarsfeldt, 1998a; Ellenbroek, 1993a; J. M. Kane et al., 1988). Additionally, clozapine was not linked to tardive dyskinesia, which was a significant problem for typical antipsychotic medications such as haloperidol (Meltzer & Luchins, 1984). However, clozapine's initial success suffered a major setback in 1975 when it was linked to agranulocytosis during a clinical trial in Finland that resulted in the deaths of several patients (Anderman & Griffith, 1977; Idnpn-Heikkil, Alhava, & Olkinuora, 1975; Lahdelma & Appleberg, 2012). Subsequently the drug was voluntarily withdrawn from the market. Despite thorough investigations, the exact reason for those deaths remains a mystery. However, some clinicians in Europe and the United States continued to use clozapine, and the positive results they witnessed led to a groundswell of support for the drug's reintroduction to the general marketplace. This ground swell of support for the drug prompted the FDA in 1989 to allow the drug to be used with the restrictions that it carry a "Black Box Warning", combined with the restriction that the drug be used only for treatment-resistant patients with required mandatory and regular white blood cell tests (Volavka et al., 2002). Patients are classified as treatment-resistant if their condition shows little or no improvement from the administration of two other antipsychotic medications (Chakos, Lieberman, Hoffman, Bradford, & Sheitman, 2001; J. Kane, Honigfeld, Singer, Meltzer, & 1988). Due to the medical risks associated with agranulocytosis, fear of litigation, and expenses related to blood cell testing

clozapine remains closely monitored and limited to a smaller segment of the patients suffering from schizophrenia.

Researchers continued in the development of atypical antipsychotics hoping to prevent the agranulocytosis associated with clozapine as well as other unwanted side effects such as metabolic syndromes associated with many antipsychotics. These metabolic syndromes included significant weight gain, a propensity to produce glucose intolerance (leading to diabetes), elevation in blood lipids, and cardiac electrographic abnormalities (Gupta, Dadheech, Yadav, Sharma, & Gautam, 2014). As the second-generation antipsychotics grew in popularity, so did their "off label" use for conditions such as depression, bipolar disorder, dysthymia, dementia, autism spectrum disorders, anxiety disorders, borderline personality disorder, anger, aggression, and various behavioral control disorders. This off label use spurred the coining of new terms for these drugs specifying their use in a wider variety of psychiatric conditions. Terms such as *mood stabilizers* and *neuromodulators* entered the psychiatric lexicon (Crystal, Olfson, Huang, Pincus, & Gerhard, 2009). The section below will now profile a rather unique atypical antipsychotic, amisulpride, which is the primary focus of this dissertation.

Amisulpride

History. Amisulpride was introduced by the French pharmaceutical company Sanofi-Aventis in the mid-90s, and marketed as Solian®, Sulpitac®, Amitrex® or Soltus®. Merger acquisitions of Sanofi-Aventis delayed amisulpride's introduction and marketing in the United States. Subsequently, Sanofi-Aventis decided not to pursue the U.S. market where numerous atypical medications where already present. Thus, amisulpride is not approved by the Food and Drug Administration (FDA) for use in the United States, but it is used in Europe (France, Germany, Italy, Switzerland, Russia, United Kingdom, etc.) and in Australia to treat psychoses,

schizophrenia, and depression (Abbas et al., 2009). It shows clinical efficacy for both positive and negative symptoms of schizophrenia with a low incidence of extrapyramidal motor side effects (Delcker, Schoon, Oczkowski, & Gaertner, 1990).

Amisulpride originated from the development of benzamides, second-generation atypical antipsychotics designed to alleviate the positive and negative symptoms of schizophrenia without producing unwanted side effects. It became evident that clinically effective drugs for schizophrenia share D_2 dopamine receptor antagonist properties (Seeman, P, 1992). Dopamine receptor occupancy of 50% - 60% in the central nervous system, specifically D_2 receptors, appears to be necessary to elicit antipsychotic activity. However, increased receptor occupancy amounts of 70% - 80% are believed to be responsible for the extrapyramidal motor side effects (Farde et al., 1992). The current interpretation of the dopamine hypothesis holds that antipsychotic effects of these drugs are linked to activity at limbic dopamine receptors, whereas, antagonism of dopamine receptors in the striatum is responsible for extrapyramidal motor side effects. The mesolimbic pathway is a key area for memory and motivational behaviors. When antipsychotics block the dopamine receptors in this pathway, intense emotions associated with schizophrenia are often reduced. Additionally, a blockade of dopamine receptors in the mesocortical dopamine pathway produces a reduction of positive symptoms such as hallucinations, disordered thinking, and delirium. Antipsychotics that antagonize the nigrostriatal dopamine pathway are linked to extrapyramidal motor side effects. Thus, compounds possessing selectivity for limbic and mesocortical structures while exerting minimal antagonism on nigrostriatal receptors would function as ideal antipsychotic drugs: producing few unwanted motor side effects and treating both the positive and negative symptoms of schizophrenia (Perrault, Depoortere, Morel, Sanger, & Scatton, 1997). The benzamides met the challenge of a

compounds that were clinically effective through their action of preferentially blocking limbic versus striatal dopamine receptors (Zivkovic, Guidotti, Revuelta, & Costa, 1975).

Benzamides. In 1958, the French company Delagrange produced a range of medicinal compounds from the benzamides. This class includes, for example, metoclopramide which was used to treat gut disturbances. Metoclopramide is a D_2 receptor antagonist and a mixed 5-HT_3 receptor antagonist/ 5-HT_4 receptor partial agonist (Donnerer, 2003). In 1962, at St. Anne's Hospital in Paris, Psychiatrist Pierre Deniker observed that some patients in the clinic who were taking metoclopramide exhibited neuroleptic-like extrapyramidal side effects. Although these effects were rare, Deniker hypothesized (correctly) that metoclopramide might be a neuroleptic. Delagrange synthesized a wide variety of benzamides and chose sulpiride for antipsychotic testing. Animal testing revealed that sulpiride had a lower risk of causing catalepsy, and the human studies found it was less likely to be associated with both extrapyramidal side effects and tardive dyskinesia (Borenstein et al., 1969). Fortunately, the compound also was effective in treating depression and anxiety. The first clinical data on sulpiride's effectiveness for treating psychotic and dysthymia symptoms were presented in Paris in 1968 at the Académie française. It was used throughout the 1970s in both France and Japan (Healy, 2002). Healy notes that had the dopamine hypothesis existed at the time of the introduction of sulpiride, it would have created a more mysterious puzzle because neuroleptic effects were to become associated with D_2 receptor antagonism. Sulpiride is a very potent D_2 receptor antagonist, K_i = 8.20 (Kessler et al., 1993), yet it was associated with fewer extrapyramidal side effects than typical antipsychotics. However it was not without its problems including hyperprolactinemia and the necessity of increased use of additional drugs for managing adverse effects, including stomatological, dermatological, and musculoskeletal or joint side effects, constipation, and pneumonia (Lai, Hsieh, Yang, & Lin,

2014; Wang & Sampson, 2014). Research continued with the benzamides producing several derivatives, including amisulpride. Amisulpride would be found to be an effective medication for treating the positive symptoms of schizophrenia, as well as treating depression and dysthymia with few extrapyramidal side effects; valued attributes of an "atypical" antipsychotic.

Receptor binding profile. Amisulpride [(± amino-4-N-(1-ethyl-2 pyrrolidinyl) methylsulphonyl-5-methoxy-2-benzamide)] is a substituted benzamide derivative that has a relatively narrow range of effects on dopaminergic and serotonergic transmission. Figure 1 illustrates the chemical structure and molecular weight of amisulpride, including the racemic form of the drug (*rac*-amisulpride) and its two isomers. The racemic form of amisulpride constitutes the actual therapeutic drug. It is used as an atypical antipsychotic and has a unique

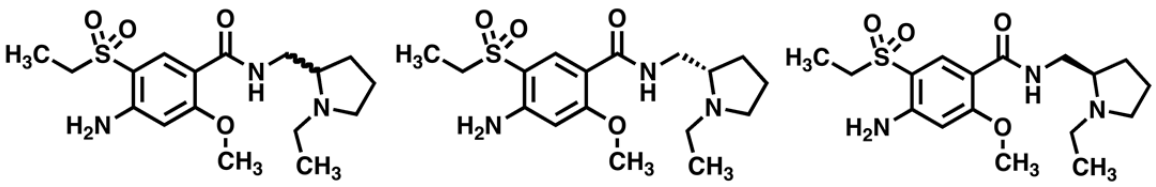

Figure 1. Chemical Structure of *rac*-amisulpride and its two isomers.

binding profile. Amisulpride has an affinity for dopamine D_2 (K_i = 1.3) and D_3 (K_i = 2.4) (P. Sokoloff et al., 1992) receptors where it has been shown *in vitro* and *in vivo* to display antagonistic effects at both of these dopamine receptors (Chivers, Gommeren, Leysen, Jenner, & Marsden, 1988; Perrault et al., 1997; Scatton et al., 1994; P Sokoloff, Giros, Martres, Bouthenet,

& Schwartz, 1990). Amisulpride binds to serotonin 5-HT_7 (K_i = 11.50) receptors (Abbas et al., 2009) where it displays potent antagonistic activity (Abbas et al., 2009; P Sokoloff et al., 1990). Amisulpride also binds to serotonin 5-HT_{2B} (K_i = 13.0) (Abbas et al., 2009). A recent unpublished binding assay found that at the serotonin 5-HT_{2B} receptor, *rac*-amisulpride displays weak antagonistic activity, about 500 fold lower potency, compared to its antagonistic activity at serotonin 5-$HT7_A$ (Bryan L. Roth, personal communication, November 11, 2013). The drug has no other relevant pharmacological interactions having negligible affinity for other receptors. Table 2 presents the known binding profile of amisulpride. It has been well established that the effectiveness of many antipsychotic drugs depends on their blockade of postsynaptic dopaminergic sites. What is of particular interest with amisulpride is its activity at dopamine D_3 presynaptic autoreceptors. Also, numerous studies confirm that amisulpride has a greater affinity for dopamine D_3 versus D_2 receptors (de Bartolomeis et al., 2013; Scatton et al., 1994; Stone, Bressan, Erlandsson, Ell, & Pilowsky, 2005). *Ex vivo* binding studies in the rat brain show that amisulpride is twice as selective for dopamine D_3 as for D_2 receptors (Scatton et al., 1977). It has particular selectivity for dopamine D_3 autoreceptors which are located mainly on cells in the limbic system (Möller, 2003; Perrault et al., 1997; Scatton et al., 1977; Scatton et al., 1994; Schoemaker et al., 1997). Rodent studies reveal that at high doses (40-80 mg/kg), it exhibits dopaminergic blocking activity on postsynaptic sites similar to that of typical antipsychotic medications; however, at low doses (<10 mg/kg) it increases dopaminergic transmission by blocking autoreceptors on the presynaptic terminals (Möller, 2003). This presynaptic blockade , in turn, leads to increased dopaminergic transmission (Sanger, Perrault, Schoemaker, & Scatton, 1999), a finding supported by laboratory techniques designed to measure extracellular dopamine levels which found amisulpride at dose of 1, 3 and 10 mg/kg did indeed increase dopamine

Table 2.

Dissociation rate constants for rac-amisulpride.

Drug Name	Receptor								
	5-HT$_{1B}$	**5-HT$_{2A}$**	**5-HT$_{2B}$**	**5-HT$_{6}$**	**5-HT$_{7}$**	**D$_{2}$**	**D$_{3}$**	**D$_{4}$**	**α$_{2A}$**
Rac-amisulpride	1,744.0[c]	2,000.0[a]	13.0[c]	4,154.0[c]	11.5[c]	1.3[b]	2.4[b]	2,369.0[c]	1,114.0[c]

Constants *(K_i, nM)* for 5-HT, serotonin receptors; α, adrenergic alpha receptors; D, dopamine receptors
Amisulpride has been tested and has no significant binding (Ki > 10,000 nM) at the following receptors: 5-HT$_{1A}$, D$_1$, D$_5$, α$_{1A}$, α$_{1B}$, H$_1$, M$_1$, M$_5$

[a]Schoemaker et al., 1997; rat cerebral cortex
[b]Sokoloff et al., 1992; human cloned cDNA cells
[c]Abbas et al., 2009; human cloned cDNA cell

release from presynaptic D_3 neurons (Schoemaker et al., 1997). It is suspected that the increased dopamine release via amisulpride's antagonism of presynaptic autoreceptors is also the underlying mechanism responsible for the drug's therapeutic effect in alleviating depression, a topic that will be addressed further in this paper. Dopamine D_3 antagonism is of much interest as a target for therapeutic neuroleptic agents primarily due to the location of D_3 receptors in neural circuits, primarily the nucleus accumbens and cerebral cortex, areas believed to be dysfunctional in schizophrenia (Schwartz, Diaz, Pilon, & Sokoloff, 2000; Schwartz, Levesque, Martres, & Sokoloff, 1993).

Theories of atypicality. There appear to be two schools of thought as to why atypical antipsychotics, given their binding profile at dopamine D_2 and D_3 receptors, show greatly reduced risks of extrapyramidal side effects. In one of the first attempts to define atypicality on the basis of receptor mechanisms, Meltzer stated that one criteria is that a drug would have a higher binding affinity to 5-HT_2 serotonin receptors relative to D_2 dopamine receptors (Meltzer, 1989). In a study of 38 typical and atypical antipsychotic medications that have affinities to binding sites in rat brains, Meltzer found the distinguishing characteristic of atypical antipsychotics was that these drugs exhibit a higher binding affinity for serotonin as compared to dopamine receptors. From that study he concluded that a medication characterized as an atypical should yield a pKi ratio of 5-HT_2/D_2 value ≥ 1.2. Interestingly, amisulpride (which was not included in Meltzer's study) does not meet that criteria; its pKi ratio of 5-HT_{2B}/D_2 is 0.976 which is < 1.2. This illustrates difficulty of defining atypicality solely on the basis of receptor binding. Abbas concludes, "Thus, despite having a pharmacological profile reminiscent of a typical

antipsychotic in that it exhibits high D_2 affinity and low 5-HT_{2A} affinity, amisulpride *therapeutically* resembles atypical antipsychotics" (Abbas, 2010, p. 2).

Kapur and Seeman argue an alternative theory known as the "fast-off" theory (Kapur & Seeman, 2001). This theory holds that atypical antipsychotics have low affinities for the dopamine D_2 receptor and are loosely bound to and rapidly released from this receptor. Accordingly, atypical antipsychotics bind more loosely to D_2 receptors than dopamine itself, while typical antipsychotics bind more tightly than dopamine. In a dissociation-time course investigation of 31 antipsychotics, Seeman et al. found that atypical antipsychotics such as amisulpride dissociate in less than 60 seconds from dopamine receptors; whereas, atypical antipsychotics such as haloperidol dissociate more slowly over a 30-minute time span (P. Seeman, 2002). These findings would tend to support the "fast off" theory as a distinguishing characteristic separating atypical from typical antipsychotics and possibly be a reason accounting for the therapeutic effects of atypical antipsychotics and their reduced incidence of extrapyramidal side-effects. Figure 2 illustrates Seeman's findings.

However, a recent radioligand binding study challenges the notion that the rate of reversibility of dopamine antagonism is the salient characteristic of atypicality (Sahlholm et al., 2014) . Sahlholm et al. used human GIRK1 and GIRK4 cDNA cells transfected into *Xenopus laevis* oocytes, then, with an electrophysiology-based assay that provides greater temporal resolution than in previous studies, they compared the dopamine D_2 dissociation rates of the typical antipsychotics chlorpromazine and haloperidol to 10 atypical antipsychotics (including clozapine, amisulpride, and sulpiride) and 5 experimental compounds, all possessing high affinities for dopamine D_2 receptors. The results showed that while there was wide variability in dissociation rates among the antipsychotics tested, the small and nonsignificant differences

observed between chlorpromazine on the one hand and amisulpride, clozapine and quetiapine on the other hand do not support the argument that the rate of reversibility of dopamine D2 antagonism is the distinguishing feature delineating atypical vs. typical antipsychotics. The authors concluded that "other factors, such as engagement of serotonin receptors, functional selectivity for D_2R signaling pathways, or subpopulation – or brain region-selective D_2R occupancy, may be the critical determinants of antipsychotic atypicality" (Sahlholm et al., 2014, p. 154). Thus, the current trend emerging in the field suggests that the debate is larger than dissociation rates or ratios of receptor binding. The debate is widening and calls a more comprehensive explanation of what exactly delineates atypical from typical antipsychotic medications. The reason why atypical antipsychotics are more effective than typical

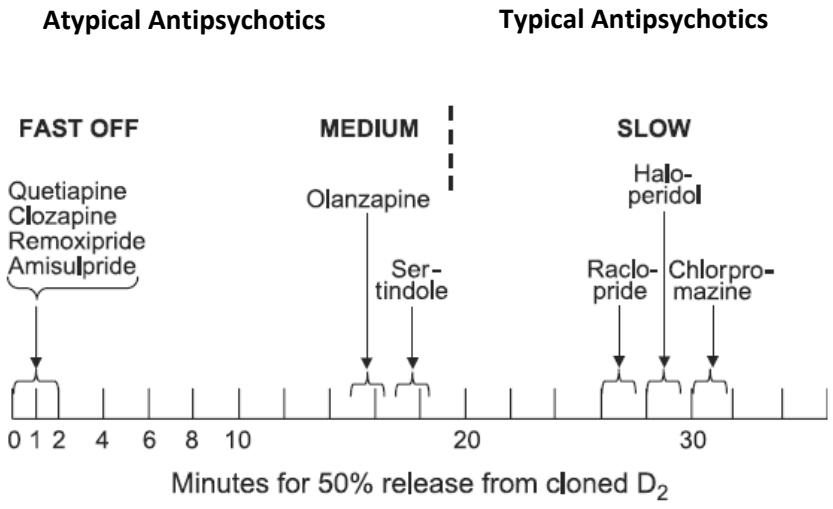

Figure 2. Dissociation rates of *rac*-amisulpride and relevant antipsychotics medications. Using human cloned D2 receptors were equilibrated in with various tritium-labelled antipsychotic drugs followed by a high concentration of raclopride or dopamine to displace the antipsychotic drug from the receptor. The atypical antipsychotics displayed rapid dissociation (< 60 seconds), olanzapine and sertindole showed a medium dissociation rates, and the typical antipsychotics exhibited much slower dissociation rates (from Seeman, P., 2002, p. 30).

antipsychotics in treating schizophrenia would have to incorporate a whole host of factors such as the role of genetic variations, interactions among other neurotransmitters involved in schizophrenia, and other intracellular processes initiated by antipsychotics (da Silva Alves, Figee, Amelsvoort, Veltman, & de Haan, 2008). It may be that the label of "atypical antipsychotic" is a therapeutic clinical label and not one derived solely from receptor mechanisms. Until these issues are resolved, clinicians will most likely rely on the rule of thumb that if a medication treats the major symptoms of schizophrenia (positive and negative) and produces less extrapyramidal motor side effects (compared to typical antipsychotics) then said medication is referred to as an atypical antipsychotic.

The answer to the question of what exactly accounts for the difference in receptor mechanisms between typical and atypical antipsychotics also may provide a clue in explaining any potential differences found in the discriminative cue properties of atypical antipsychotics (e.g. amisulpride) versus typical antipsychotics (e.g. haloperidol) as well as the discriminative cue differences among atypical antipsychotics (e.g. amisulpride vs. clozapine, olanzapine, etc.). This research hopes to shed more light on this subject.

Pharmacokinetic properties. The pharmacokinetic properties of a drug address how the drug is handled by the body relative to factors such as: amount of drug absorption, distribution, time course, elimination, and half-life. Until recently, pharmacokinetic analyses of amisulpride in humans have been conducted with assays such as: high performance liquid chromatography coupled with UV/visible detection (Péhourcq, Ouariki, & Bégaud, 2003), fluorescence detection (Malavasi, Locatelli, Ripamonti, & Ascalone, 1996), single mass spectrometry (Kratzsch, Peters, Kraemer, Maurer, & Maurer, 2003), and tandem mass spectrometry (Mogili, Kanala, Challa, Chandu, & Bannoth, 2011). Table 3 presents the general pharmacokinetic properties of

amisulpride in human subjects receiving 50 mg/day for the treatment of dysthymia. Interestingly, amisulpride is relatively slow to cross the blood brain barrier (BBB), a factor offset clinically by administering the drug in higher doses than other antipsychotics. This slow penetration is also responsible for elevated prolactin levels; an adverse side effect discussed later in this paper. Amisulpride has been shown to be well tolerated (Widlöcher, Allilaire, Guérard des Lauriers, & Lecrubier, 1990). A recommended starting dose for acute schizophrenia is ~800 mg/day (Y. Lecrubier et al., 2001). Using this dose, it has been found that amisulpride is rapidly absorbed having an oral bioavailability of ≈ 50% with peak plasma concentrations occurring at 1 and 3 hours after oral administration, the second peak larger than the first. The drug's absorption rate is significantly reduced by ingestion of a meal with high in carbohydrates, but is not affected by a meal high in fat. Protein binding of amisulpride appears minimal and the volume of distribution is large (Noble & Benfield, 1999). The total body clearance is 32.8 hours with renal clearance at 18.7 hours. Terminal elimination half-life of a single radiolabelled amisulpride dose (200mg orally) is 12 hours with 51 – 71% eliminated in feces and 24 – 47% in urine (Bianchetti, Canal, & Rosenzweig, 1995; Dufour & Desanti, 1988; Noble & Benfield, 1999). Amisulpride is absorbed via the gastrointestinal tract and evenly distributed to all body systems with minimal (≤ 17%) binding to plasma proteins (Rosenzweig et al., 2002). Whether administered intravenously or orally, elimination occurs mainly via the kidneys. A minor amount is metabolized hepatically, and produces two inactive metabolites (Bergemann, Kopitz, Kress, & Frick, 2004; Lambert & Naber, 1999; Malavasi et al., 1996). A study utilizing liquid chromatography-tandem mass spectrometry of amisulpride in human plasma found that the metabolites of amisulpride to be of minor relevance as less than 5% of the drug undergoes

Table 3.

Pharmacokinetic properties of rac-amisulpride.

Amisulpride	**Route**	**Dose**	**$t_{1/2}$ (h)**	**CL (L/h)**	**F (%)**
Caukell et.al. (1996)[a]	PO	50 mg	12.1	--	47
Nobel & Benfield (1999)[b]	IV	50 mg	--	32.8	--
	PO	50 mg	12	--	≈50
Rosenzweig et.al. (2002)[c]	PO	50 mg	1.3±0.1	31.2-41.6	48-51
Sparshatt et.al. (2009)[d]	PO	50 mg	12	--	48

In human studies:
a = mean weight 70.0 ± 7.0 kg
IV, intravenous; PO, oral; $t_{1/2}$, elimination half-life; CL, clearance;
F, bioavailability; --, not reported

[a]Coukell, et al., 1996
[b]Noble et al., 1999
[c]Rosenzweig et al., 2002
[d]Sparshatt et al., 2009

metabolism (Gschwend, Ring, & Martin, 2006). The pharmacokinetics of the enantiomers of racemic amisulpride reveal that the plasma concentration profiles of *(S)*-amisulpride and *(R)*-amisulpride are closely parallel with *(S)*-amisulpride showing higher concentrations in a ratio of ~1.3 for C_{max} and AUC (Rosenzweig et al., 2002).

The pharmacokinetic properties of amisulpride in rodents are pertinent to this research. In a study just published (Noh et al., 2014), researchers used a rapid and simple chromatographic assay to determine amisulpride time course bioavailability in rat plasma using tandem mass spectrometry. Its findings are presented in Figure 3. The assay characterized the time course of the plasma concentration of amisulpride at a dose of 10 mg/kg following oral administration in three rats. Two peaks are noticeable at about 0.5 and 2.5 h, and the plasma concentrations decayed mono-exponentially thereafter. The investigators presented the following pharmacokinetic findings: the maximum concentration was 80 ± 18 ng/ml; the elimination rate constant was 0.24 ± 0.08 h^{-1}; the half-life was 2.9 ± 1.0 h; the AUC_{24th} was 450 ± 120 ng·h/ml; and the total clearance was 22.2 ± 5.8 l/h/kg (Noh et al., 2014). These findings combined with that of Perrault et al. (1997) support the one hour pre-session injection time used in this study.

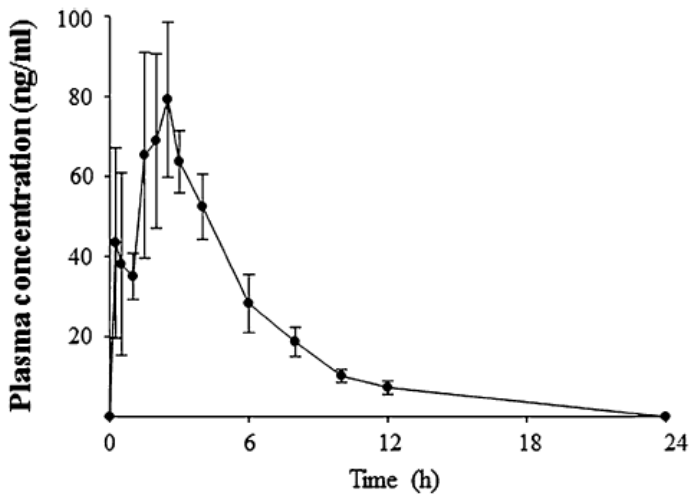

Figure 3. Time course of plasma concentrations of amisulpride in rats after a single oral administration of 10 mg/kg amisulpride (n = 3) (Noh et al., 2014)

Pharmacodynamic properties. The pharmacodynamic properties of a drug account for the biochemical and physiological effects of the drug, particularly at receptor sites. A basic underlying principle of pharmacology is that any behavioral and psychological effects induced by a drug are derived from the drug's interaction with receptors (Julien et al., 2010). While the complete mechanism of action of amisulpride has yet to be determined, it is known that it has a high affinity for dopamine D_2 and D_3 receptors (Cudennec, Fage, Benavides, & Scatton, 1997; Schoemaker et al., 1997) in the limbic system and at serotonin 5-HT_{2B} and 5-HT_{7A} also in limbic areas (Abbas et al., 2009). It does not bind to dopamine D_1, D_4, or D_5 receptors to any appreciate extent. The drug is unique in that at low doses (≤10 mg/kg) *in vivo* (rodents) it preferentially blocks *presynaptic* D_2 and D_3 dopamine autoreceptors, facilitating both dopamine release and dopaminergic neurotransmission for limbic rather than striatal receptors. Higher doses of amisulpride (while still antagonizing presynaptic autoreceptors) block *postsynaptic* receptors, thereby inhibiting dopaminergic hyperactivity. (Coukell, Spencer, & Benfield, 1996; R. H.

Roth, 1984; Schoemaker et al., 1997). Amisulpride also is significantly more efficacious in reducing the negative symptoms of schizophrenia as compared to typical antipsychotic drugs such as haloperidol (S. Leucht, 2004; S Leucht, Pitschel-Walz, Engel, & Kissling, 2001; Möller, 2000). *In vivo* preclinical studies (Abbas et al., 2009) verify that amisulpride has potent 5-HT_7 antagonistic effects, making it useful in the treatment of the negative as well as the positive symptoms of schizophrenia. Table 4 summarizes the relevant pharmacodynamic properties of amisulpride.

Selectivity. A brief clarification is in order to discuss what is meant in the characterization of amisulpride as a *selective* dopamine D_2, D_3 receptor antagonist. Much research has been done unraveling the neurotransmitter dopamine (3-hydroxytyramine, which is synthesized from the amino acid tyrosine) since its discovery almost sixty years ago (Carlsson, Lindqvist, & Magnusson, 1957). While dopamine is an integral player in a wide variety of normal physiological functions and behaviors, it also is implicated in a host of abnormal behaviors. Changes in brain dopaminergic function underlie the dopamine hypothesis of schizophrenia (Carlsson, 2001; Creese, Burt, & Snyder, 1976; Snyder, 1976; Snyder, Taylor, Coyle, & Meyerhoff, 1970).

Dopamine receptors belong to a large class of proteins known as G protein-coupled receptors, also referred to as metabotropic receptors. They are a seven-transmembrane domain structure coupled to three G-proteins sub units: alpha (α), beta (β) and gamma (γ). Upon activation by a ligand in the extracellular space, the seven-transmembrane structure undergoes a conformational change and releases its subunits, each of which can open or close an adjacent ion channel and/or initiate a wide variety of intracellular activities. Many of these intracellular activities involve second- messenger mechanisms affecting a whole host of functions within the cell such as

Table 4.

Pharmacodynamic properties of rac-amisulpride.

Selectivity for dopamine D_2 and D_3 receptors[a]
In vitro: high affinity for and blockade of human dopamine D_2 and D_3 receptors (K_i <3nmol/L)
No appreciable affinity for D_1 and D_4 or D_5 receptors
Affinity for and blockade of 5-$HT_{2B,}$ and 5-HT_7 but no significant affinity for other serotonin receptor types and none for histamine H_1, muscarinic, or α-adrenergic receptors
Ex vivo: higher affinity for D_3 than for D_2 receptors (selectivity ratio = 2)
Selectivity for limbic structures[b]
Preferential blockade of dopamine agonist-induced hypermotility *vs* stereotypies, lack of induction of extrapyramidal motor side effects
Selectivity for presynaptic D_2 and D_3 autoreceptors at low doses[b]
Preferential blockade of apomorphine-induced yawning and hypomotility; potentiation of the incentive value of food in a place preference paradigm
Endocrine effects in humans[c]
Mean prolactin level increased from 7.89 (predose baseline) to 36.96 mg/L 5 hours after administration of a single 50 mg dose of amisulpride in 21 healthy volunteers; after a further 3 days of amisulpride administration (50 mg twice daily), predose and postdose (5 hours) prolactin levels on day 5 were 41.77 and 47.23 mg/L, respectively
Endocrine adverse events during amisulpride treatment for dysthymia suggest at least some dopamine receptor antagonism at low dosages
K_i (nM) = binding constant

K_i (nM) = binding constant

Note. Adapted from (Noble & Benfield, 1999)
[a]Scatton et al., 1997; rat brain
[b]Schoemaker et al., 1997; rat brain
[c]Noble et al., 1999; human cloned cDNA cells

metabolism, enzyme activity, protein synthesis, and gene expression. These receptors are divided into two major classes on the basis of their structural, pharmacological, and biochemical properties (Beaulieu & Gainetdinov, 2011). The classes are D_1 subtype receptors (D_1 and D_5) and D_2 subtype receptors (D_2, D_3, D_4). Dopamine D_1 receptors are found exclusively on postsynaptic neurons. Dopamine D_2 and D_3 receptors are more complex as they are found on both presynaptic and postsynaptic sites. The presynaptic sites act as autoreceptors critical for negative feedback mechanisms that adjust neuronal firing rate, synthesis, and release of dopamine in response to dopamine's presence in the extracellular milieu (Wolfe & Roth, 1990). G-protein receptors represent a dynamic system and amisulpride, through its antagonistic action, disrupts the normal dynamic processes of these receptors.

While dopamine receptors are distributed throughout the central nervous system, there appear to be three main pathways: the nigrostriatal, the mesolimbic and the tuberoinfundibular (Jaber, Robinson, Missale, & Caron, 1996). The regional distribution of dopamine receptors and their location on the neuron (presynaptic versus postsynaptic sites) is directly related to the function of ligands with dopaminergic affinities. D_2 receptors are found mainly in the striatum, olfactory tubercle, nucleus accumbens, substantia nigra and pituitary. While amisulpride has an affinity for D_2 receptors, it has a greater affinity for with D_3 receptors that possess a more limited distribution, primarily in the limbic areas such as the shell of the nucleus accumbens, olfactory tubercle and islands of Calleja (Jaber et al., 1996; Missale, Nash, Robinson, Jaber, & Caron, 1998; P. Sokoloff et al., 1992). Electrophysiological assays designed specifically to measure the effects of amisulpride at D_2 and D_3 in specific regions of the rat brain (10 mg/kg) confirmed that amisulpride does indeed have "limbic selectivity" primarily in the ventral tegmental area (Di

Giovanni, Di Mascio, Di Matteo, & Esposito, 1998) confirming earlier research that amisulpride increases dopaminergic neuronal activity in the mesolimbic versus the mesostriatal region (Schoemaker et al., 1997). Another measure of selectivity of amisulpride has to do with the drug's functional effect on receptor intracellular signaling transduction pathways. Dopamine D_1 receptors are generally coupled to $G\alpha_s$ and *stimulate* the production of the second messenger cAMP and the activation of protein kinase A (PKA). Amisulpride has an affinity for dopamine D_2 class (D_2 and D_3 receptors) which are coupled to $G\alpha_{i/o}$ and *negatively* regulate cyclic adenosine monophosphate/protein kinase A resulting in a decrease of protein kinase PKA in intracellular activity (Beaulieu & Gainetdinov, 2011; Missale et al., 1998) of which the net effect is the inhibition of dopamine levels in extracellular space in postsynaptic sites, and conversely, an increase in dopamine on presynaptic sites through a blockade of autoreceptors responsible for negative feedback messages, thereby having a disinhibition effect. In a very relevant study on the specific signaling pathway of amisulpride, Park et al., used immunostaining of SH-SY5Y human cells to determined that amisulpride (compared to haloperidol) increased the levels of Akt and GSK-3^{β} phosphorylation effectively increasing the levels of phosphor-CREB, BDNF, and Bcl-2 regulation of the $^{\beta}$-arrestin 2-dependent pathway via blockade of the D_2 receptors. This signaling pathway, they believe provides a critical clue as to the mechanism underlying the functional effect of amisulpride (Park et al., 2011). Natesan et al. utilized fluorescent spectrometry to demonstrate another signaling pathway of amisulpride. With rats as subjects, they demonstrated that amisulpride induces *c-fos*, a protein that is a transcription factor instrumental as a mediator in multiple signaling cascade pathways which, in turn, bind to DNA in the nucleus and activate genes, particularly in the limbic regions (specifically the nucleus accumbens) when D_2/D_3 receptor occupancies exceed 60% (Natesan, Reckless, Barlow,

Nobrega, & Kapur, 2008). The link between *c-fos* induction in the nucleus accumbens and the therapeutic effects of antipsychotics has been well established, although deeper understanding of exactly how this improves the symptoms of schizophrenia remains unanswered (A. Y. Deutch & Duman, 1996; A.Y Deutch, Lee, & Iadarola, 1992; Kontkanen, Lakso, Wong, & Castren, 2002). Another interesting intracellular action of amisulpride is its region-specific action on gene expression (induction). Utilizing an autoradiographic signal assay on sectioned rat brain tissue, de Bartolomeis et al. investigated the effects of postsynaptic signaling differences, specifically gene expression, between amisulpride and haloperidol. Specifically, they were investigating the different levels of key gene expression between the two drugs and whether those genes are region-specific in the brain. The genes of interest were *Arc, c-fos, Zif-268, Norbin and Homer* ; all key regulators of synaptic plasticity and linked to schizophrenia and the pharmacodynamic effects of antipsychotic drugs. Results showed that amisulpride (compared to haloperidol) has a very unique signaling profile. It impacts preferentially on receptors in limbic forebrain areas and initiates a unique pattern of postsynaptic genes in these regions, principally *c-fos* and *Zif-268*. As well, this gene activity is dose-dependent with a dose range of 10 – 35 mg/kg (de Bartolomeis et al., 2013)

In summary, regarding amisulpride, the term "selective" refers to four components: first, the relative selective affinity of amisulpride for dopamine D_2/D_3 receptors; second, its brain region specificity for limbic versus striatal structures; third, its preference at low doses for presynaptic dopamine receptors, while at high doses for postsynaptic receptors, and fourth; its intracellular activation of Akt/GSK-3$^{\beta}$-arrestin 2-dependent signaling system and *c-fos* and *Zif-268* induction. These factors contribute to the use of the descriptive term of amisulpride being a *selective* benzamine derivative.

Role of dopamine autoreceptors. An autoreceptor is a G-protein receptor situated on the membrane of a presynaptic neuron. Its main function is to serve as a negative feedback mechanism, regulating the synthesis and release of neurotransmitters secreted by the neuron that the autoreceptor is located on. Once dopamine is released by a presynaptic neuron, it travels across the synapse to bind to postsynaptic dopamine receptors and that released dopamine is simultaneously detected by the autoreceptor (on the presynaptic site), which triggers a cascade of events inside the presynaptic neuron such as regulating the synthesis and further release of dopamine; typically this action is a negative feedback response inhibiting the release of additional dopamine into the synaptic cleft. As mentioned earlier, *rac*-amisulpride has been demonstrated (particularly at low doses) to have an affinity for and possesses an antagonistic effect on dopamine D_3 autoreceptors. In effect, *rac*-amisulpride inhibits the negative feedback activity of D_3 autoreceptors resulting in an increase of dopamine in the synaptic cleft. This action (in addition to antagonism of dopamine D_2 post synaptic receptors) appears to be one of the important characteristics of amisulpride in improving cognitive functions in patients with schizophrenia, as well as alleviating the negative symptoms of the disorder (Horacek et al., 2006; McKeage & Plosker, 2004).

Adverse effects. As with many medications for the treatment of schizophrenia amisulpride is not without unwanted adverse effects. Clinical studies in which amisulpride is used at low does (50-100 mg/day) for the treatment of depression or dysthymia report that the most common adverse effects are weight gain, headache, dry mouth, somnolence and constipation(Noble & Benfield, 1999; Ravizza & Investigators., 1999). Endocrine effects are another category of unwanted side effects, including: galactorrhoea, amenorrhoea, breast pain, decreased libido, high prolactin risk and menstrual dysfunction (Boyer, Lecrubier, Puech,

Dewailly, & Aubin, 1995; Smeraldi, 1998). Patients receiving higher doses (400-800) for the treatment of positive symptoms of schizophrenia report many of the same adverse effects seen with lower dose treatment: weight gain and elevated serum prolactin levels (Peuskens, Pani, Detraux, & DeHert, 2014). The elevated prolactin levels are caused by the drug's poor ability to cross the BBB, a factor necessitating administering high doses of the drug relative to dosage levels for other antipsychotics. As the drug is slow in penetrating the BBB, this causes a saturation of dopamine receptors in the periphery, such as the pituitary (which is outside the BBB) contributing to elevated blood prolactin levels (Natesan et al., 2008). Regarding extrapyramidal effects, the European First episode Schizophrenia Trial (EUFEST) collected data from 50 sites in 13 European countries (including Israel) specifically assessing rates of extrapyramidal effects including parkinsonism, akathesia, dystonia and dyskinesia. The study (n=490) compared amisulpride (200-800 mg/day) to three other second-generation antipsychotics: olanzapine (5-20 mg/day), quetiapine (200-750 mg/day) and ziprasidone (40-160 mg/day). Results showed that the amisulpride group reported 2.1% of patients showing mild parkinsonism, 0% showing akathesia, dyskinesia or dystonia (Janusz K. Rybakowski et al., 2014). Another multicentered study comparing one group of patients taking amisulpride 400 mg/day and a second group taking 800 mg/day found only 5% of all patients complaining of minor adverse effects such as headache, constipation, abdominal discomfort and somnolence and no reports of extrapyramidal adverse effects (Lee et al., 2012). While many studies report few extrapyramidal effects with amisulpride, the European Schizophrenia Outpatient Health Outcomes Study conducted a massive 3-year research project (N=7728) on the incidence of extrapyramidal symptoms and tardive dyskinesia in patients taking olanzapine, risperidone, quetiapine, amisulpride and clozapine compared to haloperidol (Novic, Haro, Bertsch, &

Haddad, 2010). The study sought to examine the clinical correlations of the incidence of extrapyramidal effects and treatment medication. While the results showed much lower incidents of adverse motor effects of those taking atypical antipsychotics versus typical antipsychotics (e.g. haloperidol), the incidence of extrapyramidal effects among the atypical antipsychotic group were quite interesting. Figure 4 illustrates the results of the European Schizophrenia Outpatient Health Outcomes Study.

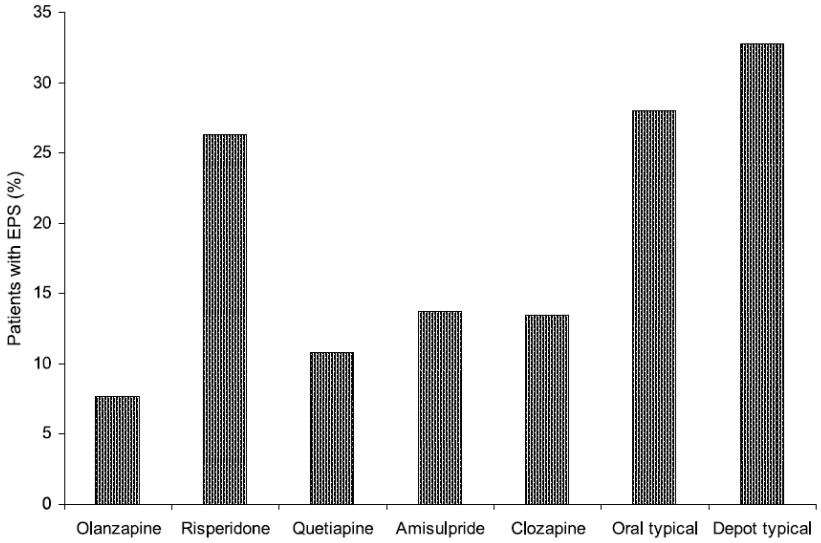

Figure 4. Incidence of extrapyramidal effects by treatment cohort in the European Schizophrenia Outpatient Health Outcomes Study. A total of 7728 patients began treatment with a single antipsychotic drug at baseline. At the beginning of the study, 4893 patients (63%) reported no EPS and 6921(89.6%) reported no tardive dyskinesia. After 3 years of treatment, there were significant differences between treatment cohorts in the incidence of EPS, which ranged from 7.7% (Olanzapine) to 32.8% (depot injection/typical). With olanzapine as the reference drug, patients treated with risperidone, amisulpride, clozapine, and both oral and depot typical antipsychotics were more likely to develop EPS compared with patients treated with olanzapine.

Note: *Adapted from* (Novic et al., 2010)

Dosage for treatment of depression. Before proceeding, it is necessary to briefly discuss the dysthymia and how is it different from depression (discussed earlier in this paper). Both dysthymia and depression are separate mood disorders with many overlapping symptoms (depressed mood, disturbed sleep, low energy, poor concentration, suicidal ideation). Dysthymia generally has fewer or less symptoms of depression but it last longer, perhaps as long as two years. It is incorrect to view dysthymia as a mile form of depression as it lacks a number of signature symptoms of depression, most notably: anhedonia, psychomotor symptoms (e.g. lethargy or agitation), physical symptoms and the fact that an episode of depression need last only two weeks versus two years (American Psychiatric Association, 2013). Treatment of dysthymia is similar to that for depression: psychotherapy and medication.

There are clinical studies validating the effectiveness of amisulpride in the treatment of dysthymia (Racagni, Canonico, Ravissa, Pani, & Amore, 2004; Rocca et al., 2002; Zanardi & Smeraldi, 2006). There are studies where amisulpride is used in the treatment of both dysthymia and depression (Komossa, Depping, Gaudchau, Kissling, & Leucht, 2010). And there are clinical studies validating the effectiveness of amisulpride for depression *and* the negative symptoms of schizophrenia (Danion, Rein, & Fleurot, 1999; Kim et al., 2007; Y. Lecrubier, Quintin, Bouhassira, Perrin, & Lancrenon, 2006; Peuskens, Moller, & Puech, 2002; J.K. Rybakowski et al., 2012). It is a challenge attempting to cleanly delineate among the studies using amisulpride for dysthymia, depression or the negative symptoms of schizophrenia as many studies use different diagnostic criteria for each of the disorders and there is a great deal of overlap among the symptoms and terminology.

Studies indicate that with patients with a diagnosis of only dysthymia, amisulpride 50 mg/day is as effective as amitriptyline 25 to 75 mg/day or fluoxetine 20 mg/day (Y. Lecrubier, Boyer,

Turjanski, & Rein, 1997; Noble & Benfield, 1999). Table 5 provides data compiled from four randomized double-blind comparative studies of amisulpride and traditional antidepressants in patients with dysthymia, depression in remission, or mild to moderate depression. Pre and post-tests were given on the Montgomery-Asberg Depression Rating Scale (MADRS). The overall conclusion of the studies in Table 5 provides evidence that amisulpride is as effective or equal other antidepressants (amineptine, amitriptyline, imipramine, fluoxetine) used in the treatment of these mood disorders. It also exhibits good tolerability and minimal side effects. What is of particular interest is that the dose used to treat dysthymia is lower than the dose used to treat the positive symptoms of schizophrenia (Noble & Benfield, 1999).

Additionally, the literature regarding the use of amisulpride in treating the negative symptoms of schizophrenia indicates that low doses of amisulpride have proven to be effective in regards to alleviating the negative symptoms of schizophrenia with the optimum dose range of 50-150 mg/day (Boyer et al., 1995). Anhedonia, a hallmark negative symptom of schizophrenia, is believed to be related to dopamine D_2/D_3 receptors in the limbic brain areas, and the therapeutic effects of antidepressant medications increase in the sensitivity of D_2/D_3 dopamine receptors in these brain areas (Willner, 1997). In similar research, Speller et al. conducted a one-year, low-dose neuroleptic study of patients with schizophrenia characterized by persistent negative symptoms comparing amisulpride and the typical antipsychotic haloperidol (Speller, Barnes, Curson, Pantelis, & Alberts, 1997). That study found that a regimen of low dose of amisulpride 100-150 mg per day was more effective in treating flattened affect and avolition-apathy in patients with chronic schizophrenia than comparable low doses of 3.0-4.5 mg per day haloperidol. Another placebo-controlled clinical study conducted by Danion et al. evaluated the efficacy and safety of low doses of amisulpride in schizophrenic patients with predominantly

Table 5.

Efficacy of amisulpride (AMIS) in patients with dysthymia: summary of data from randomized double-blind comparative studies.

Reference	Diagnosis (% of pts) [mean MADRS score]	Study Duration (mo)	Drug and dosage (mg/day) [no. of pts]	Response rate (% of pts)		Reduction from Baseline in rating Scores (%)		Overall Efficacy[c]
				MADRS[a]	CGI[b]	MADRS	CGI	
				Severity		Severity		
Comparison with tricyclic antidepressants								
Boyer et al.[i]	PDYS (NR) or PDYS and Single episode of mild major depression (NR) [18]	3	AMIS 50 [101[d]] AMIN 200[107[d]] PL [105[d]]		63* 64* 33	48* 46* 21		AMIS≡AMIS >PL
Ravizza et al.[j]	PDYS (98%) or single Episode of major depression in partial remission (2%), PL responders excluded[f] [21 (AMIS) or 22 (AMIT)] [e]	6	AMIS 50 [165[d]] AMIT 25-75[g][85]	60 63	67 68	51 53		AMIS≡AMIT
Lecrubier et al.[k]	PDYS (41%), PDYS and mild or moderate major depression (41%) or isolated chronic major depression in partial remission (18%) [25]	6	AMIS 50 (1 wk) then 100 [54[h]] IMIP 100 [51[h]] PL [51[h]]	72* 69* 33	53* 48* 31		36* 31* 16	AMIS≡IMIP >PL
Comparison with fluoxetine (FLUX)								
Smeraldi[l]	PDYS (94%) or single episode of major depression in partial remission (6%), PL responders excluded[e] [21AMIS) or 22 (FLUX)]	3	AMIS 50 [139] FLUX 20 [129]	74 67	78 66	62 56		AMIS≡FLUX

[a] MADRS score reduced by ≥50%
[b] Pts considered 'much improved' or 'very much improved' for CGI 2 item.
[c] Based on primary efficacy end-points [reduction in MADRS total score and CGI response rate (Lecrubier et al.), CGI response rate (Boyer et al.) and MADRS response rate (Smeraldi)] except in Ravizza et al. (efficacy was a second end point).
[d] Intention-to-treat analysis.
[e] Trial designed primarily to assess tolerability; efficacy variables were secondary end-points.
[f] Pts received PL during a 1-wk run-in period prior to active treatment and those with a ≥20% decrease in MADRS score and MADRS score ≤13 were excluded.
[g] Patients received 50 mg/day for 2wk and then 25, 50 or 75 mg/day thereafter depending on clinical response and tolerability.
[h] Per-protocol analysis.

AMIN = amineptine; **AMIT** = amitriptyline; **FLUX** = fluoxetine; **CGI** = Clinical Global Impression; **IMIP** = imipramine; **MADRS** = Montgomery-Asberg Depression Rating Scale; **NR** = not reported; **PDYS** = primary dysthymia; **PL** = placebo; pts = patients; > indicates efficacy significantly ($p < 0.05$) better than that of comparator; ≡ indicates similar efficacy; * $p < 0.05$ *vs* PL.

[i] (Boyer, Lecrubier, Stalla-Bourdillon, & Fleurot, 1999)
[j] (Ravizza & Investigators., 1999)
[k] (Y. Lecrubier et al., 1997)
[l] (Smeraldi, 1998)

Note. Adapted from (Noble & Benfield, 1999)

primary negative symptoms with 242 patients (64% men and 36% women) in 35 treatment centers in four countries (Danion et al., 1999). That study found that lowed doses of 50 - 100 mg/day amisulpride consistently showed a clear superiority over placebo in improving primary negative symptoms in patients with schizophrenia.

In comparison to other atypical antipsychotic drugs, amisulpride possesses a unique receptor binding profile which appears to be dose-dependent. *In vivo* studies with rodents demonstrate that low-dose (≤ 10 mg/kg) amisulpride selectively antagonizes presynaptic dopamine autoreceptors controlling dopamine synthesis and release in limbic structures; whereas with higher doses (40-80 mg/kg) postsynaptic dopamine D_2 receptor occupancy and blockade is evident (Schoemaker et al., 1997). Perhaps, this dose-effect is responsible for the drug's clinical effectiveness in treating both the positive and negative symptoms of schizophrenia.

Interestingly, the benzamide sulpiride has also been demonstrated to exhibit a clinically dose-dependent profile. In an animal model of depression, rats were subjected chronically (12 weeks) to a variety of mild, unpredictable stressors and within 2 weeks exhibited depressive like behaviors. Treatment with various antidepressants (sulpiride, tricyclic antidepressants desmethylimipramine or amitriptyline) found that sulpiride attenuated (or reversed) depression-like behaviors in the least amount of time (2 weeks) compared to the other medications (7 weeks) (Sampson, Willner, & Muscat, 1991). Clinical human trials have shown that sulpiride at low doses (100-500 mg/day) is effective for treating chronic depression and, at higher doses (300-1,200 mg/day), it is effective for treating the positive symptoms of the disorder (Alfredsson, Harnryd, & Wiesel, 1984; Benkert & Holsboer, 1984).

Dosage for treatment of schizophrenia. Apparently, among clinicians, there are a few unsettled questions surrounding the appropriate starting dose of amisulpride in the treatment of

schizophrenia. For example, is dose titration or the use of a loading dose necessary or could one give 800 mg/day beginning with the first day of treatment? To compare the efficacy, tolerability and subjective experience between a group receiving an initial dose of 800 mg/day and a group titrating up from an initial dose of 400 mg/day Lee et al. (2012) conducted an extensive 6-week, randomized, multicentered, open-label study to examine this question. Their results found that slow titration from 400 mg/day only delayed maximal efficacy by weeks relative to starting with an initial dose of 800 mg/day. With regard to safety, more adverse effects were seen in the group beginning with 800 mg/day, with the more serious effects being minimal weight gain, and elevated serum prolactin levels. Regardless of the beginning dose, the study found no statistically significant difference between the two groups in overall incidence of adverse events, including extrapyramidal effects. Another result was that subjects reported an improvement in subjective measure of quality of life and attitudes towards amisulpride regardless of dose group (Lee et al., 2012). A similar study conducted by Möller et al. found that 62% of patients receiving a fixed dose of 800 mg/day reported higher ratings on Clinical Global Impression scores than those receiving titrated doses (Möller, Boyer, & Fleurot, 1997). Another dose-range study compared fixed doses of amisulpride (400, 800 and 1200 mg/day) to haloperidol (16 mg/day). In that study, Puech et al. found an interesting bell-shaped dose-response curve on scores on the Simpson-Angus Scale (a measure of extrapyramidal syndromes) among groups receiving amisulpride 100 mg/day (52%), 400 mg/day (66%), 800 mg/day (78%) and 1200 mg/day (66%). Interestingly, the 800 mg/day group represented the peak of the curve with the highest incidence of EPS. Overall, subject data reported on the Brief Psychiatric Rating Scale (BPRS) indicated that the dose range of 400 to 800 mg/day are optimal for amisulpride and that these doses were superior to haloperidol (16 mg) in the treatment of acute episodes of schizophrenia (Puech,

Fleurot, & Rein, 1998). In summary, the research is consistent in finding that doses ranging from 400-800 mg/day provide the best relief for the positive symptoms of schizophrenia.

Drug Discrimination as a Behavioral Assay

Drug discrimination is an important behavioral assay for studying the *in vivo* pharmacology of drugs. Its methodology consists of a laboratory investigation of the interoceptive effects (i.e. subjective effects) of a training drug as a stimulus cue for performing a specific behavioral response (Solinas, Panlilio, Justinova, Yasar, & Goldberg, 2006). Basically, drug discrimination is a behavioral procedure whereby an organism must recognize a particular drug state, choose a correct response, and receive reinforcement (Young, 2009). It has been used to study a wide assortment of drugs, test compounds, therapeutic agents, and drugs of abuse. In a standard study, an animal such as a mouse or rat is trained via Pavlovian and operant (Skinnerian) principles of learning to associate an interoceptive, subjective state with a particular behavioral response such as lever pressing. The subject learns to discriminate the internal stimuli associated with a particular drug (called a training drug) from those stimuli of a vehicle state (non-drug agent such as saline). After training in a drug discrimination paradigm, the subject can recognize the specific interoceptive cues of different drugs. As the drugs themselves serve as the discriminative stimuli, this procedure proves quite useful in studying the pharmacological profile of the drug (Harris & Balster, 1971; Overton, 1966). After the subjects learn to discriminate the training drug from vehicle, testing usually proceeds with different doses of the training drug (generalization testing) followed by the introduction of novel agents for the purpose of substitution testing. Substitution testing can provide critical information as to the receptor binding profile of the drug that contributes to the discriminative stimulus properties of that drug.

Also, drugs belonging to the same pharmacological class tend to substitute for each other. For example, animals trained to discriminate dihydroetorphine will provide similar behavioral responses to the stimulus cue of heroin and morphine (Beardsley & Harris, 1997). If a novel drug does not substitute for a training drug, this suggests that the underlying pharmacological mechanisms of the two drugs are different. Drug discrimination also is an important technique for assessing a wide variety of factors related to drugs such as: sex, genetic strains, pharmacological history, genetic manipulations (knockout subjects), and other neurobiological factors that may influence the interoceptive property of a chemical agent. Research has shown high reliability between the discriminative effects of drugs in animals and that of humans (Kamien, Bickel, Hughes, Higgins, & Smith, 1993).

There are a number of procedural variables that are important components in the drug discrimination paradigm. Procedures may vary depending on the design of the study, such as: the route of injection (e.g., subcutaneous, intraperitoneal), the pre-injection time (typically 30 to 60 minutes), the reinforcement schedule (often fixed ratio 30 or 10 reinforcement schedules), and the reinforcers, (most likely food pellets or liquid, such as water or sweetened milk). Despite these differences in procedure, results from drug discrimination studies tend to be quite consistent within the same drug class (Porter & Prus, 2009). If differences are found across studies, these differences are usually related to the dose of the training drug and the species utilized.

Brief History of Drug Discrimination with Antipsychotic Drugs. Drug discrimination has its roots in early theories of state dependent learning dating from the early 1800s. While psychology's enthusiasm in state dependent learning waxed and waned throughout the 19th century into the 20th century a milestone was reached with the publication of Conger's research

on the stimulus effects of alcohol on approach and avoidance behavior (Conger, 1951). Many hold that this was the first drug discrimination study. This was followed by other studies such as in 1962 when Stewart trained rats to discriminate 4.0 mg/kg (i.p.) of the typical antipsychotic chlorpromazine from saline in a shock-avoidance task using a three-compartment test chamber (Stewart, 1962). She found that the phenothiazines acepromazine, perphenazine, and prothipendyl fully substituted for chlorpromazine, but that the phenothiazines prochlorperazine and the tricyclic antidepressant imipramine did not substitute. In 1966 Overton attempted to establish discrimination with a 5.0 mg/kg (i.p.) dose of chlorpromazine in a T-maze (shock avoidance) procedure; no discrimination could be established (Overton, 1966). The first drug discrimination study on chlorpromazine in a two-lever operant task with rats as subjects was conducted by Barry et al. (1974). They successfully trained rats to discriminate 1.0 mg/kg chlorpromazine from saline. This was the first study that also included testing of the discriminative stimulus properties of metabolites, in this case metabolites of chlorpromazine.

Colpaert et al., in 1976, were the first to test the typical antipsychotic haloperidol as the training drug in a two-lever operant discrimination, training rats to discriminate 0.02 mg/kg (s.c.) haloperidol from saline. This was an onerous task requiring over 80 training sessions (Colpaert F, Niemegeers, & Janssen, 1976). Colpaert also was the first to introduce the procedure of using a fixed-ratio (FR 10) schedule of reinforcement as a component in the methodology (Glennon, Torbjorn, & Frankenheim, 1991). The first drug discrimination study on an atypical antipsychotic utilizing clozapine in addition to the typical antipsychotic chlorpromazine as training drugs was conducted by Goas and Boston in 1978 with rats as subjects. While haloperidol, clozapine, and the muscarinic antagonist benztropine mesylate produced full substitution for chlorpromazine, none of the tested drugs (chlorpromazine, haloperidol,

chlordiazepoxide, or atropine) substituted for clozapine (Goas & Boston, 1978). In 1982 Overton utilized a T-maze drug discrimination procedure demonstrating that clozapine and haloperidol could be established as training drugs; however, no drug discrimination could be established with chlorpromazine, fluphenazine, haloperidol, or thioridazine as the training drugs (Overton, 1982).

Drug discrimination plays a unique role in the investigation of the biochemical, neurological, and pharmacological properties of antipsychotic medications. As a tool it is useful as a behavioral assay in the preclinical development of medications (A. J. Goudie & Smith, 1999; Porter & Prus, 2009). It also contributes to our understanding of the pharmacological mechanisms that mediate the discriminative stimulus properties between and within antipsychotics and provides a method of analysis for charting the stimulus effects of various doses of a particular drug. Furthermore, it provides researchers with an assay to measure the extent to which a drug generalizes to various doses of itself and the degree to which a novel drug may substitute for a training drug. There are many preclinical behavioral tests used in screening drugs in the development of antipsychotic medications (Arnt & Skarsfeldt, 1998a; Ellenbroek, 1993b; Geyer & Ellenbroek, 2003); however, what makes drug discrimination unique is that it measures the interoceptive effects of a training drug as a stimulus cue for performing a specific behavioral response. Thus, as a *behavioral* assay and paradigm, it has been used over many years to aid in classifying drugs, identifying the underlying pharmacological mechanisms mediating the stimulus properties of a drug, and providing information on the role of genetics in drug response (Arnt & Skarsfeldt, 1998a; A. J. Goudie & Smith, 1999; Porter & Prus, 2009).

Colpaert, an enthusiastic advocate for drug discrimination, uses almost poetic language in speaking of the drug discrimination paradigm to "reveal exquisite molecular specificity" of

drugs (Colpaert, 1999), and to provide valuable information about drug activity including: "kinetic and temporal features, reversibility of receptor ligand binding, stereospecificity, structure-activity relationships, agonist-antagonist interactions, pA_2 characteristics, receptor supersensitivity, central vs. peripheral sites of drug's actions, and neurotoxicological effects of therapeutic agents" (Colpaert, 1999, p. 338). Not only does the drug discrimination paradigm allow us to study the physiological mechanisms underlying the subjective and perceptual effects of a drug, it also is a valuable model for pathology, and provides a credible, empirical and scientific way to investigate subjective states. In so doing, Colpaert holds that drug discrimination "makes contributions to neurobiology that are unique, realizing that it does the astonishing feat of bridging "hard" molecular processes to the "soft" realm of subjectivity that at one point seemed forever beyond the realm of science (Colpaert, 1999). This author of this dissertation has long shared the belief that everything psychological has its roots in the biological. Drug discrimination is one of the tools to investigate that relationship.

Drug Discrimination with atypical antipsychotics. After the early drug discrimination studies investigating typical antipsychotics, much of the recent focus has centered on the atypical antipsychotic drug clozapine, a dibenzodiazepine. Clozapine continues to be the "gold standard" and "prototypical" atypical antipsychotic medication against which all antipsychotic drugs are compared. The binding profile of clozapine shows that it has a high affinity for dopaminergic D_4 receptors, serotonergic 5-$HT_{2A/2B/2C}$, 5-HT_6, and 5-HT_7 receptors; cholinergic M_1 M_2, receptors; adrenergic α_1 and α_2 receptors; and histaminergic H_1 receptors and moderate affinity for dopamine D_1, D_2, D_3, and D_5 receptors (Arnt & Skarsfeldt, 1998a; Bymaster et al., 1996; Davies, Compton-Toth, Hufeisen, Meltzer, & Roth, 2004; E. Richelson, 1999; Schotte et al., 1996). The receptor activity of clozapine shows that it has mixed agonist-antagonist properties. Generally, it

exhibits antagonist action at the aforementioned receptors (Meltzer, 1994). However, there is evidence to suggest that clozapine acts as an agonist or partial agonist at peripheral and central muscarinic subtype receptors and that the drug's activity may also be dependent on tissue, metabolic process and site (e.g. central vs. peripheral) (Olianas, Maullu, & Onali, 1999; Smith GC et al., 2014). For example, Zorne et al. (1994) provided evidence that clozapine behaves as a full agonist at the cloned human M_4 receptor expressed in Chinese hamster ovary CHO cells, while acting as an antagonist at muscarinic M_1,M_2, M_3, and M_5 receptors (Zeng, Le, & Richelson, 1997). Agonistic properties of clozapine may explain some of its unique therapeutic properties (Porter & Prus, 2009); specifically, that agonistic activity at 5-HT_{1A} receptors contributes to the treatment of negative and cognitive symptoms, mood enhancement, and, the reduction of extrapyramidal motor side effects (although what accounts for this reduction of motor effects is unknown) (M.J. Millan, 2000). Nielsen contends that muscarinic cholinergic antagonism (in rats) is key to clozapine's discriminative stimulus properties (Nielsen, 1988), a finding reinforced by Kelley and Porter (B.M. Kelley & J.H. Porter, 1997) and others (A. J. Goudie, Smith, Taylor, Taylor, & Tricklebank, 1998). Nicotinic cholinergic receptors have not been shown to be important in the discriminative stimulus properties of clozapine (Prus, Philibin, Pehrson, & Porter, 2006; Villanueva, Arezo, & Rosecrans, 1992). Clozapine's lower affinity for dopamine receptors suggests that the drug's antagonistic effect at these receptors does not seem to be significant as a mediating factor for clozapine's discriminative stimulus properties. In contrast, antagonism of D_2 receptors is thought to inhibit the ability of some antipsychotic drugs to substitute for clozapine (Carey & Bergman, 1997; Cole, Field, Sumnall, & Goudie, 2007). The preponderance of the evidence indicates that clozapine has a diverse and multifaceted binding

profile and a compound (complex) discriminative stimulus that is not fully understood (A. J. Goudie & Smith, 1999; Porter & Prus, 2009).

Beyond Clozapine. To date, only four atypical antipsychotics have been utilized as the training drug in drug discrimination studies. They are: clozapine (A. Goudie & Taylor, 1998; Brian M. Kelley & Joseph H. Porter, 1997; Nielsen, 1988), olanzapine (Porter & Strong, 1996), quetiapine (A. J. Goudie, Smith, & Millan, 2004), and ziprasidone (Wood et al., 2007). Each of these compounds exhibit a greater affinity for 5-HT_{2A} receptors over D_2 receptors; however, like clozapine, they have diverse binding profiles for other receptors as well (Schotte et al., 1996). Olanzapine has a receptor-binding profile resembling clozapine, but has a much higher affinity for D_1 and D_2 dopamine receptors. Ziprasidone has a higher affinity for 5-HT_{1A} and 5-HT_7 receptors. Olanzapine, quetiapine, and ziprasidone all have strong affinities for α_1-adrenoceptors, while only olanzapine exhibits a strong affinity for muscarinic receptors (M.J. Millan, 2000; E. Richelson, 1999; Schotte et al., 1996).

The only study in which a stereoisomer of an antipsychotic has been used as a training drug utilized the enantiomer *(S)*-amisulpride as a discriminative cue in C57BL/6 mice (Donahue et al., 2014). Interestingly, the enantiomer *(S)*-amisulpride has a very different profile than the four atypical antipsychotics previously used in drug discrimination studies. *(S)*-amisulpride exhibits a higher affinity at dopamine D_2 and D_3 receptors where it is twice as potent as *rac*-amisulpride and 20-50 times more potent than *(R)*-amisulpride (Castelli, Mocci, Sanna, Gessa, & Pani, 2001; Marchese, Ruiu, et al., 2002). In a study to determine the effects of the amisulpride isomers on rat catalepsy and the radioligand binding affinities for the isomers, Marchese et al. determined that *(S)*-amisulpride bound to the dopamine D_2 receptor with higher affinity than the racemic form of the drug and *(R)*-amisulpride with none of the isomers showing significant

affinity for 5-HT_2 serotonin receptors (Marchese, Bartholini, et al., 2002). Table 6 presents the binding profile of *rac*-amisulpride and its isomers.

Preliminary data. Donahue et al. investigated the discriminative stimulus cue properties of *(S)*-amisulpride, the enantiomer thought to underlie the actions of *rac*-amisulpride, the therapeutic form of the drug (Donahue et al., 2014). Male C57BL/6 mice were trained to discriminate *(S)*-amisulpride (10 mg/kg, s.c.) form vehicle in a standard two-lever drug discrimination paradigm. The *(S)*-amisulpride stimulus was rapidly acquired and was shown to be dose-related, time dependent (effective between 30 and 120 min) and stereoselective: *(S)*-amisulpride (ED_{50}=1.77 mg/kg) was approximately three times more potent than *rac*-amisulpride (ED_{50}=4.94 mg/kg) and ten times more potent than *(R)*-amisulpride (ED_{50} = 15.84 mg/kg). Generalization testing showed the *(S)*-amisulpride stimulus generalized completely to *rac*-amisulpride (ED_{50}-=4.78), to *(R)*-amisulpride (ED_{50}=22.36), to the benzamide analog sulpiride (ED_{50} = 12.67 mg/kg), but did not fully generalize (≥ 80% drug lever responding) to the typical antipsychotic drug haloperidol nor to the atypical antipsychotic drugs clozapine (partial substitution of 65%), nor to aripiprazole. These results with *(S)*-amisulpride were encouraging and lead to the current investigation into the discriminative stimulus properties of the racemic form of the drug, *rac*-amisulpride used for treatment.

Amisulpride in drug discrimination studies. To date, *rac*-amisulpride has not been used as a training drug in drug discrimination studies with rodents, but it has been tested in several drug discrimination in which other drugs were trained as the discriminative stimulus. Cohen et al. tested numerous drugs for their ability to substitute to the discriminative stimulus of tiapride including: amisulpride, sulpiride, sultopride, clebopride, raclopride, metoclopramide, remoxipride and numerous others non-benzamide drugs. They demonstrated

Table 6.

Comparison of K_i binding profiles of amisulpride and its isomers compared to antipsychotics on [^{3}H]YM-09151- nemonapride (D_2), [^{3}H]ketanserin (5-HT_{2A}) and [^{3}H]clonidine (α_2) binding.

Drug	(Rat Striatum), D_2, K_i (nM) ± S.E.M.	(Rat Cortex), 5-HT_{2A}, K_i (nM) ± S.E.M.	(Rat Cortex), α_2, K_i (nM) ± S.E.M.
(RS)-amisulpride	9.8 ± 0.4	> 5000	783 ± 27
(S-) amisulpride	5.2 ± 01[a]	> 5000	1528 ± 45[a]
(R+)-amisulpride	244 ± 12[b]	> 5000	375 ± 14[a]
Haloperidol	1.11 ± 0.04[b]	42.2 ± 0.81	> 5000
Risperidone	3.5 ± 0.6[a]	0.6 ± 0.01	16 ± 3

Ki of the different forms of amisulpride and haloperidol, and risperidone for different [^{3}H] ligands. Data represent mean (± S.E.M.) of four independent experiments. Statistical Differences vs *(RS)*-amisulpride were calculated using one-way ANOVA followed by Neumann-Keuls test for multiple comparisons ([a]P<0.05 or [b]P<0.01 vs/ *(RS)*-amisulpride).

Note. Adapted from (Marchese et al., 2002).

that in rats trained to discriminate tiapride (2.2 mg/kg) from vehicle amisulpride fully substituted for tiapride (ED_{50} = 4.0 mg/kg) with significant rate suppression as compared to vehicle. They also found that the benzamide sulpiride (ED_{50} = 18.0 mg/kg) completely substituted for tiapride (2.2 mg/kg) and also produced significant reduction in rate as compared to vehicle (C. Cohen, Sanger, & Perrault, 1997). Two studies have tested amisulpride in rats trained to discriminate the atypical antipsychotic drug quetiapine. Smith et al. found that amisulpride did not substitute for quetiapine (10 mg/kg training dose) at any dose tested (range 3-80 mg/kg) and produced significant rate suppression at 80 mg/kg compared to vehicle (Judith A. Smith & Andrew J. Goudie, 2002). In a second study, Goudie et al. found that amisulpride did not substitute for the discriminative stimulus properties of the antipsychotic quetiapine (10 mg/kg) nor to clozapine (5 mg/kg) trained rats (A. J. Goudie et al., 2004).

With the limited drug discrimination research utilizing atypical antipsychotics, combined with the fact that *rac*-amisulpride has yet to be investigated as a training drug, there remains more to be discovered about the subjective effects of these medications and the neural receptor mechanisms underlying their discriminative stimulus properties. To add to the body of knowledge on this subject, this author believes that there is much to be gained by an investigation of the discriminative stimulus properties of the rather unique and effective atypical antipsychotic amisulpride. An examination of the drug's interoceptive properties promises to yield relevant information regarding the pharmacological mechanisms underlying amisulpride's discriminative stimulus and may shed light on the medication's possible therapeutic effect.

Rationale

Amisulpride is an atypical antipsychotic medication developed in the 1990s. It has a unique binding and clinical profile, possessing a high affinity for dopamine D_2 and D_3 receptors and for serotonin 5-HT_{2B} and 5-HT_7 receptors with a preferential activity in the limbic region of the brain. Its activity appears to be dose-dependent. At high doses it blocks postsynaptic D_2 / D_3 receptors and at low doses it selectively blocks dopamine D_2 and D_3 presynaptic autoreceptors amplifying dopaminergic transmission (Coukell et al., 1996; Cudennec et al., 1997; Schoemaker et al., 1997). This low-dose effect appears to be responsible for the drug's efficacy as an antidepressant. Amisulpride is chiral in nature possessing two optical isomers: *(S)*- amisulpride and *(R)*-amisulpride. The racemic form, *rac*-amisulpride, is a 50/50 mixture of the two enantiomers. The more active enantiomer is *(S)*-amisulpride insofar as its ability to bind to dopamine D_2 (K_i =1.3 nM*)* and D_3 receptors (K_i =2.4 nM*)*, where it is twice as potent as the racemic form and 20 to 50 times more potent than *(R)*- amisulpride in displacing radioligands from dopamine D_2 and D_3 receptors (Castelli et al., 2001). The racemic form of the drug has proven its efficacy in treating both positive and negative symptoms and the value of its clinical use in the treatment of schizophrenia (Möller, 2000). It has shown to be well tolerated with the incidence of extrapyramidal motor symptoms (especially for low doses) similar to that of placebo (Noble & Benfield, 1999). Amisulpride's binding affinity and antagonist action at dopamine D_2 and D_3 receptors is intriguing because classic typical antipsychotics antagonize these same receptors and it is precisely this mechanism that is believed to produce extrapyramidal motor side effects (Strange, 2001). Yet, compared to typical antipsychotics, amisulpride's propensity to produce unwanted extrapyramidal effects is almost negligible (Geddes, Freemantle, Harrison, & Bebbington, 2000). For example, the typical antipsychotic

haloperidol has antagonistic action with high affinity for the dopamine D_2 (K_i = 0.50 nM*)* and D_3 (K_i =12 nM) receptors (B. L. Roth, 2014), while amisulpride also has high affinity at dopamine D_2 (K_i =1.3 nM) and D_3 receptors (K_i =2.4 nM*)* (P Sokoloff et al., 1990). High affinity and antagonism at these receptor sites are known to play an important role in producing extrapyramidal motor side effects; yet, this is not seen with amisulpride. What accounts for this discrepancy? Is it that amisulpride is more selective at D_2 and D_3 receptors? Is it that haloperidol, unlike amisulpride, also has antagonism and moderate affinity for D_1 receptors (K_i = 83 nM)? Is it the speed with which amisulpride releases from dopamine receptors as compared to typical antipsychotics such as haloperidol (Kapur & Seeman, 2001)? It is the ratio of serotonin-dopamine receptor activity (Meltzer, 1989)? Perhaps it is the action of amisulpride at autoreceptors or the particular dopamine pathway (limbic versus striatal) in the brain that is affected? Perhaps it is amisulpride's activity at serotonin 5-HT_{2B} and 5-HT_{7A} receptors. This is a puzzle that this dissertation seeks to examine and, hopefully, suggest some answers.

With the knowledge that drug discrimination is an impressive, dynamic and cogent *in vivo* assay for determining the subjective effects of drugs and for studying the *in vivo* receptor mechanisms that mediate a drug's discriminative stimulus and perhaps therapeutic effects, utilizing this paradigm will allow for a direct comparison between the therapeutic form of the atypical antipsychotic amisulpride (*rac*-amisulpride) and other typical and atypical antipsychotics, and also representative compounds from the major classes of antidepressants. This comparison will be followed by testing selective ligands to better parse out underlying neural receptor mechanisms responsible for the discriminative stimulus cue of *rac*-amisulpride.

The overall goal of this study is to use the drug discrimination paradigm as a behavioral assay to examine the ability of male C57BL/6 mice to discriminate the atypical antipsychotic

drug *rac*-amisulpride from vehicle. To date, there are no published drug discrimination studies of *rac*-amisulpride as the training drug with mice or rats. C57BL/6 mice are chosen for this study as they have been demonstrated to be an excellent model for preclinical studies of medications used for schizophrenia (Laurent & Podhorna, 2004; Powell, Zhou, & Geyer, 2009; Xu, Yang, McConomy, Browning, & Li, 2009). As such, this research is an original study in the effort to investigate the discriminative stimulus properties of *rac*-amisulpride.

There were two major aims of this study. The first aim was to establish *rac*-amisulpride as a discriminative stimulus in a standard two-lever drug discrimination procedure in C57BL/6 mice. The purpose of this aim was to determine if *rac*-amisulpride has a discriminative stimulus that can be detected by C57BL/6 mice. This aim began with generalization testing of *rac*-amisulpride to develop a dose-response curve. Next, we proceeded to substitution testing with the drug's two isomers, and continued substitution testing with a wide variety of typical and atypical antipsychotics, other benzamide derivatives, and other medications known for their antidepressant and anxiolytic effects. The rationale employed was that if *rac*-amisulpride does have a discriminative stimulus cue, and knowing the drug's receptor mechanisms at dopamine D_2 and D_3 and serotonin 5-HT_{2B} and 5-HT_{7A}, then, by substitution testing with other known antipsychotics, we could begin the process of delineating, through comparison and contrast, those receptor mechanisms most likely responsible for the discriminative cue properties of the *rac*-amisulpride. Table 7 shows for the binding profile of the antipsychotics tested in the present study. The inclusion of substitution testing with antidepressant and anxiolytic compounds was

Table 7.

Receptor binding affinity Ki values (nM) for tested antipsychotic drugs at relevant receptor targets

	Receptor												
Drug Name	**5-HT$_{1A}$**	**5-HT$_{1B}$**	**5-HT$_{1D}$**	**5-HT$_{2A}$**	**5-HT$_{2B}$**	**5-HT$_{7}$**	**D$_1$**	**D$_2$**	**D$_3$**	**D$_4$**	**α$_{1A}$**	**α$_{1B}$**	**M$_1$**
Rac-amisulpride	NSB[a]	1,744.0[c]	--	2,000.0[a]	13.0[c]	11.5[c]	--	1.3[c]	2.4[l]	2,369.0[c]	NSB[a]	NSB[a]	NSB[a]
Haloperidol	7,930.0[b]	165.0[d]	--	78.0[b]	165.0[g]	--	25.0[b]	0.5[d]	12.0[d]	2.3[d]	12.0[d]	8.0[d]	NSB[d]
Chlorpromazine	3,115.0[d]	1,489.0[f]	452.0[d]	3.2[d]	6.0[d]	21.0[k]	25.0[n]	2.8[l]	5.0[d]	12.3[s]	0.28[d]	0.81[d]	47.0[d]
Clozapine	770.0[b]	390.0[d]	--	13.0[d]	31.30[i]	6.3[k]	53.0[e]	69.0[o]	83.0[e]	39.0[d]	1.6[d]	7.0[d]	14.0[d]
Aripiprazole	5.6[d]	833.0[d]	63.0[d]	4.6[d]	0.36[d]	10.0[d]	387.0[d]	0.95[d]	5.35[d]	514.0[d]	25.0[d]	34.0[d]	6776.0[d]
Risperidone	427.0[d]	53.6[d]	22.3[d]	0.19[d]	41.58[d]	6.6[d]	60.6[d]	4.9[d]	12.2[d]	18.6[d]	5.0[d]	9.0[d]	NSB[d]
Olanzapine	--	509.0[d]	150.0[h]	3.0[d]	12.0[j]	100.0[e]	10.0[e]	3.7[p]	2.0[e]	9.6[s]	7.3[e]	--	1.9[t]
Quetiapine	830.0[e]	5,000.0[g]	560.0[h]	366.0[d]	--	290.0[m]	390.0[e]	69.0[q]	9.2[r]	1164.0[s]	7.0[b]	--	120.0[b]

5-HT, serotonin receptors; D, dopamine receptors; α, adrenergic alpha receptors; M, muscarinic receptors; --, not tested; NSB, no significant binding (Ki > 10,000 nM)

[a] (Schoemaker et al., 1997); rat cerebral cortex
[b] (Bymaster et al., 1996); rat cortex
[c] (Abbas et al., 2009); cloned human cDNA cells
[d] PDSP Certified data; human cloned 5-HT cells
[e] (Arnt & Skarsfeldt, 1998b); rat cloned D_1 cells
[f] (Titeler, Lyon, Bigorna, & Schneider, 1987); rat striatum
[g] (Schotte et al., 1996); rat striatum
[h] (Elliott Richelson & Souder, 2000); human brain tissue
[i] (Wainscott, Lucaites, Kursar, Baez, & Nelson, 1996)
[j] (Bymaster et al., 1999); CHO-K1 cells transfected with human muscarinic receptors
[k] (B. L. Roth, Ciaranello, & Meltzer, 1992); rat cloned receptors
[l] (P Sokoloff et al., 1990); rat cloned D_2 cells
[m] (Schotte et al., 1996); rat brain
[n] (Anderson, 1989); rat frontal cortex
[o] (P. Sokoloff et al., 1992); Cloned D_3 receptor from human mammillary bodies
[p] (Philip Seeman, Roy Corbett, & Van Tol, 1997); human cloned D_2 receptor
[q] (Arnt & Skarsfeldt, 1998b); rat striatum
[r] (Burstein et al., 2005); human cloned NIH-3T3 cells
[s] (B. L. Roth, Tandra, Burgess, Sibley, & Meltzer, 1995); cells transfected with rat D_4 cDNA cells subcloned into pcDNA3 cells
[t] (Bymaster et al., 1996); rat cortex

warranted as *rac*-amisulpride is quite effective in treating the negative symptoms of schizophrenia (e.g. depression) and may further elucidate the role of serotonin 5-HT_{2B} and 5-HT_{7A} in amisulpride's discriminative stimulus cue. Recall that *rac*-amisulpride is also prescribed for treating dysthymia and depression.

The second aim was to conduct substitution testing with selective ligands that are either agonists or antagonists at specific receptor sites responsible for the effects of *rac*-amisulpride. Specifically, selective agonists and antagonists for dopamine D_2 and D_3 and for serotonin 5-HT_{2B} and 5-HT_7 receptors were tested. Thus, building upon knowledge gained in our general substitution testing, this specific ligand testing enabled us to more precisely investigate the underlying neural receptors responsible for the discriminative stimulus effects of *rac*-amisulpride.

Methods

Subjects

Thirty-five experimentally naïve, adult male C57BL/6 inbred mice (20-25 g) obtained from Harlan Laboratories (Indianapolis, IN) were housed individually in clear plastic cages (18 X 29 X 13 cm) secured on a ventilated rack with wood chip bedding (sanichips, Teklad, Madison, WI). Mice were transported daily (6-7 days per week) from the vivarium (12 hour light-dark cycle, lights on at 6 a.m.) to the laboratory where experimental training and testing sessions occurred. The vivarium temperature remained between 22 and 24 degrees Celsius. After one week of acclimation to the vivarium and handling, the subjects were food deprived to 85-90% of their free feeding body weights and were maintained on a food restricted diet of standard rodent chow (Harlan Teklad Lab Diets, Teklad LM-485). Water was available *ad libitum* in the home cages. The mice were randomly assigned to two cohort groups. Cohort 1 (n = 16) were used for substitution testing to *rac*-amisulpride 10 mg/kg. Cohort 2 (n = 19) were used for selected ligand substitution testing to 10 mg/kg *rac*-amisulpride. The *Guide for Care and Use of Laboratory Animals (National Research Council, 2011)* was followed and the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU) approved the procedures that were used in the present study (IACUC Protocol AM10284).

Drugs

Rac-amisulpride, *(S)*-amisulpride and *(R)*-amisulpride (gift from Drug Discovery Program, Georgetown University, Washington, D.C.), clozapine (gift from Novartis, East Hanover, N.J.), haloperidol, sulpiride, chlorpromazine, tiapride, risperidone, chlordiazepoxide HCl, quinpirole HCl, bupropion HCl and apomorphine (Sigma-Aldrich Chemical Co., St. Louis, MO),

olanzapine (gift from Eli Lily, Indianapolis, IN), fluoxetine HCl (National Institute of Mental Health Chemical Synthesis and Drug Supply Program, Bethesda, MD), imipramine and zacopride (Gift from A.H. Robbins Pharmaceuticals, Richmond, VA), aripiprazole, nemonapride and LP-44 HCl (National Institute of Mental Health Chemical Synthesis and Drug Supply Program), mianserin (Research Biochemical International, Natick, MA), quetiapine (Gift from Zeneca Pharmaceuticals, Wilmington, DE), SB-269970 HCl, BW 723C86 HCL, SB-204741, racolopride and *(S)*-sulpiride (Tocris, Minneapolis, MN). Fluoxetine HCl, chlordiazepoxide HCl, raclopride HCl, bupropion HCL, SB-269970 HCl, BW 723C86 HCL, LP-44 HCL and quinpirole HCl were the salt form of the drug and dissolved in saline. All other drugs were the free base form and were dissolved in distilled water with a small quantity (approximately two drops) of 85% lactic acid, with sodium hydroxide used as a buffer to insure a pH balance of approximately 7.0. Doses and pretreatment times were based on preliminary studies and previous studies in the literature (C. Cohen et al., 1997; Collins, Jackson, Koek, & France, 2014; DiPilato et al., 2014; Donahue et al., 2014; Furmidge, Exner, & Clark, 1991; Galici, Boggs, Miller, Bonaventure, & Atack, 2008; McElroy, Stimmel, & O'Donnell, 1989; Morita et al., 2005; Perrault et al., 1997; Philibin, Prus, Pehrson, & Porter, 2005; Philibin et al., 2009; Prus et al., 2006; Schechter, 1983; Shelton & Nicholson, 2013; Ukai, Mori, & Kameyama, 1993; Upton, Stean, Middlemiss, Blackburn, & Kennett, 1998; Young & Glennon, 2002; Young & Johnson, 1991). All drugs were administered subcutaneously (s.c.) in an injection volume of 10.0 ml/kg body weight.

Apparatus

Testing was conducted in six standard computer-interfaced operant conditioning chambers (Model ENV-307A, Med Associates Inc., St. Albans, VT) each containing two retractable levers in the left and right positions (8 cm apart) on the front panel of the operant

chamber. The levers extended 0.8 cm into the chamber and were positioned 2.5 cm above a grid floor constructed of parallel stainless steel rods. Centered between them was a recessed food trough into which a liquid dipper delivered 0.02 ml of sweetened-milk (by volume: 150 ml powdered milk, 150 ml sugar, and 500 ml water). The inner test chambers consisted of a 15 cm L X 11.5 cm D X 17.5 cm H area surrounded by an aluminum framed box with a single Plexiglas side door. Test chambers were housed in sound attenuating chambers equipped with ventilation fans. MED-PC software (Version 4.2, Med Associates Inc.) was used to control the operant sessions and record data.

Training Procedures

Phase I: Lever-press training. The mice were trained to lever press using a modified autoshaping procedure to respond on a single extended lever (J. E. Barrett & Vanover, 2003). Mice were randomly assigned to an operant box placed in which a single lever (the vehicle-paired lever) was extended inside the chamber. Each subject was placed in the operant chamber for a 15 minute session and trained to press the levers for 0.02 ml of sweetened milk on a fixed ratio one (FR 1) schedule of reinforcement, in which the reinforcer was delivered after every lever press (dipper was available for 3 sec.). Subjects were trained to lever press on a single lever (i.e. the vehicle-paired lever) until drug administration began. The position of the drug-associated lever (left vs. right) was counterbalanced for each subject to control for olfactory cues (Extance & Goudie, 1981). In between each session, the interior of the operant boxes and the levers were wiped down with a solution of water and 10% ethyl alcohol to further control for olfactory cues. The value of the FR was gradually increased over the next 7-8 sessions until FR 10 was obtained. After response rates were consistently higher than 10 responses per minute, two-lever drug discrimination training began.

Phase II: Drug discrimination acquisition training. Following initial lever-press training, the mice then began single-lever training (errorless training). Subjects were injected daily with vehicle 60 minutes prior to each training session. Only the vehicle-associated lever was extended in the test chamber and responding was reinforced according to the FR 10 schedule. This vehicle training continued for 5 sessions (days) until rates stabilized. The mice then began errorless training on the drug appropriate lever. The mice were administered 10 mg/kg *rac*- amisulpride injections 60 minutes prior to training sessions and were only presented with the *rac*-amisulpride-associated lever (opposite of the vehicle-associated lever). The training dose of 10 mg/kg *rac*-amisulpride and the pre-session injection time of 60 minutes was chosen based on previous drug discrimination studies in the literature (C. Cohen et al., 1997; Perrault et al., 1997) and from a study done in our lab study on the discriminative stimulus properties of *(S)*-amisulpride (Donahue et al., 2014). Our training dose and pre-session injection time also was recently supported in a blood plasma assay utilizing a sensitive LC-MS/MS method (tandem mass spectrometry) for determining amisulpride concentrations in rat plasma, and a preclinical pharmacokinetic study in the rat (Noh et al., 2014). Once response rates stabilized at over 10 responses per minute (5 sessions/days for vehicle, ~10 sessions/days for *rac*-amisulpride), two-lever drug discrimination training began. During two-lever training sessions both levers were extended into the operant chamber. The subjects were administered *rac*-amisulpride and vehicle injections according to a double alternation sequence (i.e., DDVVDDVV). On days when the drug was administered, only responding on the drug-associated lever was reinforced. On days when vehicle was administered, only responding on the vehicle associated lever was reinforced. Responses on the incorrect lever reset the ratio requirement on the correct lever to 10. Subjects

received two-lever drug discrimination training until the training criteria were passed during 5 of 6 consecutive sessions.

Drug discrimination training criteria. Successful discrimination training was evaluated and assessed according to three criteria: (1) the first completed fixed ratio (FFR) of the FR 10 schedule was executed on the appropriate lever, (2) 80% or greater of total responses made during the session occurred on the appropriate lever, and (3) response rate for the session was equal to or exceeded 10 responses per minute (RPM). Control tests with vehicle and amisulpride were administered and had to be passed prior to generalization testing with all drugs. During control test sessions, responses on both levers were reinforced according to the FR 10 schedule and the FR requirement was reset when switching between levers occurred. The three training criteria also had to be met for two consecutive training sessions immediately prior to all drug test sessions.

Phase III: Generalization and Substitution testing and time course. After successful completion of vehicle and *rac*-amisulpride control tests, a *rac*-amisulpride generalization dose effect curve was determined. Next, the substitution testing was conducted with the two isomers of *rac*-amisulpride, antipsychotics drugs, antidepressant drugs and selective ligands. Six subjects were randomly assigned to the testing of a drug and each animal received one injection of each dose tested. Table 8 shows drugs tested for substitution to *rac*-amisulpride, doses, injection times and literature references. Table 9 shows antidepressants used for substitution testing and their binding affinities at receptors relevant to *rac*-amisulpride. Table 10 shows the selected ligands used for substitution testing and their binding affinities at receptors relevant to *rac*-amisulpride.

Table 8. *Drugs tested for substitution to rac-amisulpride: doses and injection times*

	Drug	Doses Tested (mg/kg)								Inj. time	Citation: Inj. time & doses
1	*rac*-amisulpride	0.078	0.3125	0.625	2.5	10.0	20.0			60 mins	Perrault et al. 1997
2	*(S)*-amisulpride	0.0078	0.156	0.3125	0.625	1.25	2.5	5.0		60 mins	Perrault et al. 1997
3	*(R)*- amisulpride	0.156	0.3125	0.625	1.25	2.5	5.0	10.0	20.0	60 mins	Perrault et al. 1997
4	Clozapine	0.625	1.25	1.78	2.5	3.54				30 mins	Philibin et al. 2009
5	Haloperidol	0.00625	0.025	0.05	0.10	0.20	.40			30 mins	McElroy et al. 1989
6	Sulpiride	0.78	3.125	6.25	12.50	25.0	50.0			60 mins	Cohen et al. 1997
7	Chlorpromazine	0.125	0.25	0.50	1.0	2.0	4.0			30 mins	Philibin et al. 2009
8	Tiapride	2.5	10.00	20.0	40.0	56.6	80.0			30 mins	Cohen et al. 1997
9	Olanzapine	0.0625	0.125	0.25	0.5	1.0				30 mins	Philibin 2005
10	Risperidone	0.0625	0.125	0.25	0.50					60 mins	Philibin 2005
11	Fluoxetine	5.0	10.0	20.00	40.00					30 mins	Philibin et al. 2009
12	Imipramine	5.0	10.0	20.00						30 mins	Schechter 1983
13	Aripiprazole	0.15625	0.31250	0.625	1.25					30 mins	Philibin et al. 2009
14	Chlordiazepoxide	2.5	5.0	10.00	20.00	40.0	56.60			30 mins	Shelton & Nicholson ,2013
15	Mianserin	0.5	1.0	2.0						30 mins	Prus, et al. 2006
16	Quetiapine	2.5	5.0	7.10	10.0					30 mins	Philibin et al. 2009
17	(S)-sulpiride	2.5	5.0	10.0	20.0	40.0				60 mins	Ukai et al. 1993
18	Nemonapride	0.01	0.032	0.056	0.10					60 mins	Furmidge et al. 1991
19	Bupropion	1.0	10.0	32.0	56.0					15 mins	Young & Glennon, 2002
20	Zacopride	1.0	10.0	32.0	56.0					60 mins	Young & Johnson, 1991
21	Apomorphine	0.032	0.10	0.32	1.0					15 mins	Collins et al. 2014

Table 9

Receptor binding affinity Ki values (nM) for antidepressant drugs at relevant receptor targets to rac-amisulpride

	Receptor												
Drug Name	**SERT**	**5-HT$_{1A}$**	**5-HT$_{1B}$**	**5-HT$_{2B}$**	**5-HT$_{2C}$**	**5-HT$_{6}$**	**5-HT$_{7}$**	**M$_1$**	**M$_5$**	**D$_2$**	**D$_3$**	**DAT**	**NET**
Amisulpride	--	NSB[a]	1,744.0[c]	13.0[c]	--	4,154.0[c]	11.5[c]	NSB	NSB	1.3[c]	2.4[l]	--	--
Fluoxetine	2.0[b]	8313.0[b]	6165.0[e]	5,030.0[g]	23.98[i]	1,770.0[k]	--	702.0[b]	2,700.0[q]	--	--	784.0[n]	119.0[v]
Imipramine	8.7[b]	--	--	--	94.0[j]	209.0[k]	1,000.0[m]	42.0[q]	83.0[q]	726.0[r]	387.0[t]	8,500.0[o]	11.0[b]
Mianserin	1,000.0[j]	398.0[d]	2,801.0[f]	50.11[h]	1.99[i]	19.9[p]	111.0[m]	--	--	674.0[s]	2,841.0[u]	9,400.0[o]	22.0[w]
Bupropion	1000[x]	NSB[y]	--	--	NSB[y]	--	--	--	--	NSB[x]	--	570[y]	940[x]

SERT, serotonin transporter; 5-HT, serotonin receptors; α, adrenergic alpha receptors; D, dopamine receptors; DAT, dopamine transporter; NET, norepinephrine transporter; --, not tested; NSB, no significant binding (Ki > 10,000 nM)

[a] (Schoemaker et al., 1997); rat cerebral cortex
[b] (Owens, Morgan, Plott, & Nemeroff, 1997); rat cortex
[c] (Abbas et al., 2009); human cloned cDNA cells
[d] (Boess & Martin, 1994); human cloned [^{3}H]8-OH-DPAT cells
[e] (Wong, Threlkeld, & Robertson, 1991); rat cerebral cortex
[f] (Matsumoto, Combs, & Jones, 1992); rat spinal cord
[g] (Rothman et al., 2000); human brain cloned 5-HT$_{2B}$ cells
[h] (Glusa & Pertz, 2000); rat cloned 5-HT$_{2B}$ receptors in AV-12 cells
[i] (Sanders-Bush & Breeding, 1988); rat choroid plexus
[j] (Pälvimäki et al., 1996); rat brain
[k] (Stebben, Ansanay, Brockaert, & Dumuis, 1994); rat striatal cDNA cloned cells
[l] (P. Sokoloff et al., 1992); human cloned D$_3$ receptor, human mammillary bodies
[m] (Y. Shen et al., 1993); rat cloned kidney G protein-coupled receptors
[n] (Letchworth et al., 2000); rat striatum
[o] (Tatsumi, Groshan, Blakely, & Richelson, 1997); human cloned dopamine cells
[p] (Unsworth & Molinoff, 1994); 5-HT receptor in mouse neuroblastoma N18TG2 cells
[q] (Stanton, Bolden-Watson, Cusack, & Richelson, 1993); human cloned M$_5$ cells
[r] (Runyon et al., 2001); rat cloned 5-HT$_{2A}$ cells (NIH3T3)
[s] (Kessler et al., 1993); rat hippocampus
[t] (Toll et al., 1998); human cloned D$_3$ receptors
[u] (Fernandez et al., 2005); human cloned D$_3$ receptors
[v] (Béïque, Lavoie, de Montigny, & Debonnel, 1998); rat brain
[w] (Wikström, Mensonides-Harsema, Cremers, Moltzen, & Arnt, 2002); rat brain
[x] (Anderson, 1989); rat brain
[y] (Sanchez & Hyttel, 1999); rat brain

Table 10.

Selective ligands for substitution test: Receptor Binding affinity Ki values (nM) and activity at relevant receptor targets.

Drug	D_2	D_3	5-HT_{2B}	5-HT_{7A}
Amisulpride	Antagonist Ki = 1.3[a]	Antagonist Ki = 2.4[a]	Antagonist Ki = 13[a]	Antagonist Ki = 11.5[a]
Raclopride	Antagonist Ki = 4.8[b]	Antagonist Ki = 1.8[c]	--	--
Quinpirole	Agonist Ki = 8.0[d]	Agonist Ki = 5.1[e]	Agonist Ki = 302[f]	--
SB-269970	--	--	--	Antagonist Ki = 1.26[g]
LP-44 HCl	--	--	--	Agonist Ki = 0.22[h]
SB-204741	--	--	Antagonist pKi = 7.8[i]	--
BW-723C86	--	--	Agonist Ki = 12.58[j]	--

5-HT, serotonin receptors; D, dopamine receptors; --, not tested; NSB, no significant binding (Ki > 10,000 nM); pKi, the negative logarithm to base 10 of the equilibrium dissociation constant

[a] (Abbas et al., 2009); human cloned cDNA cells
[b] (Andersen, 1988); mouse brain
[c] (Strange, 2001); rat cloned CHO cells
[d] (Levant, Grigoriadis, & DeSouza, 1992); rat brain
[e] (P Sokoloff et al., 1990); rat cloned CHO cells
[f] (Knight et al., 2004); human cloned high expressing CHO-K1 cells
[g] (Lovell et al., 2000); human cloned receptors in HEK 293 cells
[h] (Leopoldo et al., 2004); RNA from cloned rat kidney cells
[i] (Forbes, Jones, Murphy, Holland, & Baxter, 1995); rat stomach fundus
[j] (Kennett, Bright, Trail, Baxter, & Blackburn, 1996); rat stomach cells

A brief explanation of our order of dosing is relevant to address the question of order effects as a possible confound. For the order of doses used on each drug tested we first conducted a research of the literature to determine the range of doses for pertinent behavioral effects such as substitution to a relevant test drug, or a significant decrease in rate of responding. We then utilized an ascending order approach in the administration of different doses of each drug. First, we administered low doses of each drug that we suspected would not produce any behavioral effects and proceeded in ascending order to higher doses based on a logarithmic scale. This incremental ascending order approach is a conservative method to detect and therefore avoid any toxic effects of higher doses of certain drugs. If it became evident that a dose of a drug produced severe rate suppression, testing at that dose was stopped and no further animals tested at that dose. Also, since we used the ascending order method in the substitution phase of our study, we used this same method in subsequent combination testing to preserve consistency in methodology.

A time-course study with the 10 mg/kg training dose of *rac*-amisulpride also was conducted to confirm that our pre-session injection time was the optimal period for the bioavailability of 10 mg/kg *rac*-amisulpride. Six mice were selected at random and required to pass control points for both *rac*-amisulpride and vehicle. The mice were randomly assigned to one of two groups, an ascending or descending pre-session injection time period. The ascending group was administered 10 mg/kg *rac*-amisulpride with increasing pre-session time periods in the following order: 0, 15, 30, 60, 120, 240, and 480 minutes prior to testing. The descending time period group received the training dose with a reversed pre-session time period: 480, 240, 60, 50, 15, and 0 minutes. As in generalization and substitution testing, each subject had to successfully meet the three training criteria for two consecutive training sessions immediately

prior to all test sessions.

Operational definitions of dependent variables. One measure of stimulus control is the first fixed ratio (FFR). This is defined as the subject's first set of 10 continuous and uninterrupted responses on either of the two levers. If a subject begins responding on one lever, and then switches to the opposite lever without completing 10 consecutive responses on the initial lever, the counter is reset to 0 and does not record a first fixed ratio until 10 uninterrupted responses were completed on one lever. Another measure of behavior is the percent of drug lever responding (%DLR). This is calculated by counting the number of responses on the appropriate drug lever in a 15 minute session and dividing the quotient by the total number of responses made on both levers, then multiplying that number by 100 to convert the decimal to a percentage. Test drugs that achieve response percentages at 80% or higher are considered full substitution. Response rate was calculated as responses per minute (RPM) for each 15 minute session.

Data analysis. For all sessions, the Med-PC software was programmed to calculate the percent drug-lever responding (%DLR) on the condition-appropriate lever, dividing the number of responses on that lever by the total number of responses on both levers and then multiplying the result by 100. Responses per minute were calculated by taking the total number of responses on both levers and dividing by 15 min (i.e. the session length). A subjects % DLR data were excluded from data analysis if it did not complete a fixed ratio (i.e. did not receive a reinforcer) or if responses per minute were < 2.0. However, all response rate data were included in the calculation of responses per minute, even it was 0 (i.e. no responses during the session). Full substitution to the drug stimulus cue was defined as ≥ 80% DLR. Partial substitution to the drug cue was defined as ≥60% DLR and <80%DLR. No substitution to the drug stimulus cue was defined as <60% DLR (Porter, Walentiny, Philibin, Vunck, & Crabbe, 2008). For all drugs that

produced full substitution for *rac*-amisulpride an effective dose 50% (ED_{50}) value with 95% confidence intervals were calculated for %DLR data using the least squares method of linear regression with the linear portion of the dose effect curve (Bliss, 1967; Goldstein, 1964). The ED_{50} represents the calculated drug dose at which animals would be expected to make 50% of their responses on the drug designated lever. A one-way repeated-measures analysis of variance (ANOVA) comparing responses per minute were calculated for each drug (GraphPad Prism version 6.0 for Windows; GraphPad Software, San Diego, CA, USA). Significant ANOVAS were followed by Dunnett's post hoc tests comparing the doses to vehicle when appropriate ($p < 0.05$). For the time-course experiment, a one-way repeated measures ANOVA comparing %DLR at the different time points was conducted. Significant ANOVAs were followed by Newman-Keuls post hoc tests ($P<0.05$).

Results

rac-Amisulpride Acquisition Discrimination and Generalization Dose Effect Curves

The results of the acquisition training for both cohort groups of mice that successfully trained to discriminate 10 mg/kg *rac*-amisulpride from vehicle are shown in Figure 5. In cohort 1, fourteen of the 16 mice met acquisition criteria in a mean (± SEM) of 35.71 ± 6.18 training sessions (range of 6-89 sessions). Two mice failed to meet acquisition criteria in 121 training sessions; therefore, they were removed from the study. For cohort 2, all nineteen mice met acquisition criteria in a mean (± SEM) of 41.58 ± 4.47 training sessions (range of 7-75 sessions).

Generalization testing (Figure 5, lower panels) for cohort 1 yielded an ED_{50} = 0.73 mg/kg 95% CI [0.47, 1.13 mg/kg]. Partial generalization to the *rac*-amisulpride discriminative cue was attained at 2.5 mg/kg (66.41% DLR) and full generalization was attained at the training dose of 10.0 mg/kg (94.65 DLR), and 20 mg/kg (94.99% DLR). A one-way repeated measures ANOVA revealed that there was a significant effect of doses on response rate, $F(6,78) = 4.81$, $p = 0.0003$. A Dunnett's post hoc test revealed a significant increase in response rates at doses 2.5, 10 and 20 mg/kg as compared to vehicle.

Generalization testing for cohort 2 yielded an ED_{50} = 0.56 mg/kg 95% CI [0.42, 0.76 mg/kg]. Two mice in this group failed to establish consistent stimulus control and were removed from the study. Partial generalization to the *rac*-amisulpride discriminative cue was attained at 0.625 (67.10%DLR) and 2.5 mg/kg (76.73% DLR). Full generalization was attained at 10.0 mg/kg (96.31% DLR). A one-way repeated measures ANOVA revealed that there was a significant effect on response rates $F(4,25) = 7.50$, $p = 0.0004$. However, a Dunnett's post hoc test failed to reveal any significant differences between drug and vehicle.

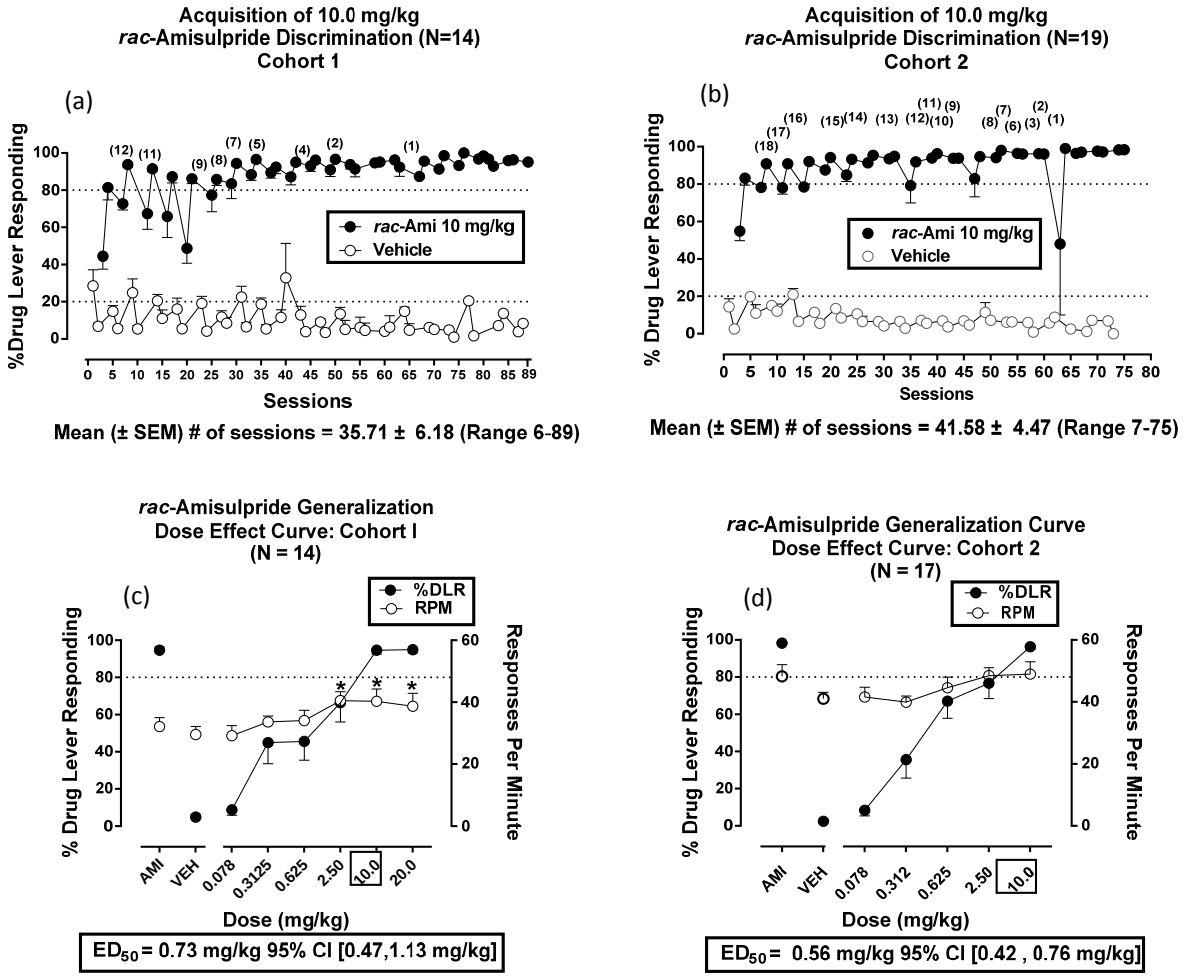

Figure 5. rac-amisulpride acquisition discrimination and *rac*-amisulpride generalization dose effect curves. Panel (a) shows acquisition of two-lever discrimination is shown for the 10 mg/kg *rac*-amisulpride (AMI) training dose for cohort 1 and panel (b) shows acquisition data for cohort 2. Mean percentage drug lever responses (± SEM) are presented separately for drug injections *(closed circles)* and vehicle (VEH) injections *(open circles).*The *dashed line* at 80% indicates drug-appropriate responding and the *dashed line* at 20% indicates 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 who had not yet met acquisition

criteria. Panel (c) shows *rac*-amisulpride generalization dose effect curve in C57BL6 mice trained to discriminate 10mg/kg *rac*-amisulpride from vehicle in cohort 1, and panel (d) shows *rac*-amisulpride generalization does effect curve for cohort 2. The dashed line at 80% indicates drug-appropriate responding indicating full generalization to the training drug *rac*-amisulpride 10 mg/kg. Prior to generalization testing, control test sessions were conducted with both *rac*-amisulpride (10 mg/kg) and vehicle (VEH). Left *ordinate*: Percentage of drug lever responses (%DLR) on the *rac*-amisulpride designated lever after s.c. administration of *rac*-amisulpride. Right *ordinate*: Animals' response rates (responses per minute [RPM]) are shown (significantly different from vehicle, * $p < 0.05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$). AMI and VEH are the control tests showing % DLR and RPM after the standard pre-session injection interval of 60 min. *Abscissa*: drug doses.

***rac*-Amisulpride Time Course**

Time course data shown in Figure 6 demonstrated that the 10 mg/kg training dose of *rac*-amisulpride produced full substitution at the 30 minute s.c. pre-session injection time point (average drug lever responding = 86.5%), and at the training 60 minute time point (average drug lever responding = 91.8%). Partial substitution was seen at the 15 minute (average drug lever responding = 67.5%) and at the 120 minute (average drug lever responding = 77.6%) injection time points. A one-way repeated measures ANOVA for percent drug-lever responding (%DRL) was significant, $F(7,35) = 13.03$, $p < 0.0001$, and a Dunnett's multiple comparison post hoc test was used to determine which injection times were significantly different from the training injection time of 60 minutes. Compared to the 60 minute injection time, 0 minutes and 480 minutes produced significantly lower %DLR ($p = 0.001$). The 15 minute, 30 minute, and the 120 minute injection times were not significantly different ($p = 0.05$) from the 60 minute injection time. A one-way repeated measures ANOVA for responses per minute showed there was no significant effect of injection time, $F(7,35) = 1.16$, $p = 0.35$.

Isomer Substitution and *rac*-amisulpride Generalization For Both Cohort Groups.

Generalization testing for combined cohort groups (N=31) yielded an $ED_{50} = 0.64$ mg/kg 95% CI [0.47, 0.84 mg/kg] as shown in Figure 7 panel (a). Partial generalization to the *rac*-amisulpride discriminative cue was attained at 2.5 mg/kg (72.07% DLR) and full generalization was attained at 10.0 mg/kg (95.56 DLR). A one-way repeated measures ANOVA revealed that there was a significant effect of drug on response rates, $F(5,50) = 4.81$, $p = 0.0001$. A Dunnett's post hoc test revealed a significant increase in response rates at doses 2.5, and 10 mg/kg, as compared to vehicle.

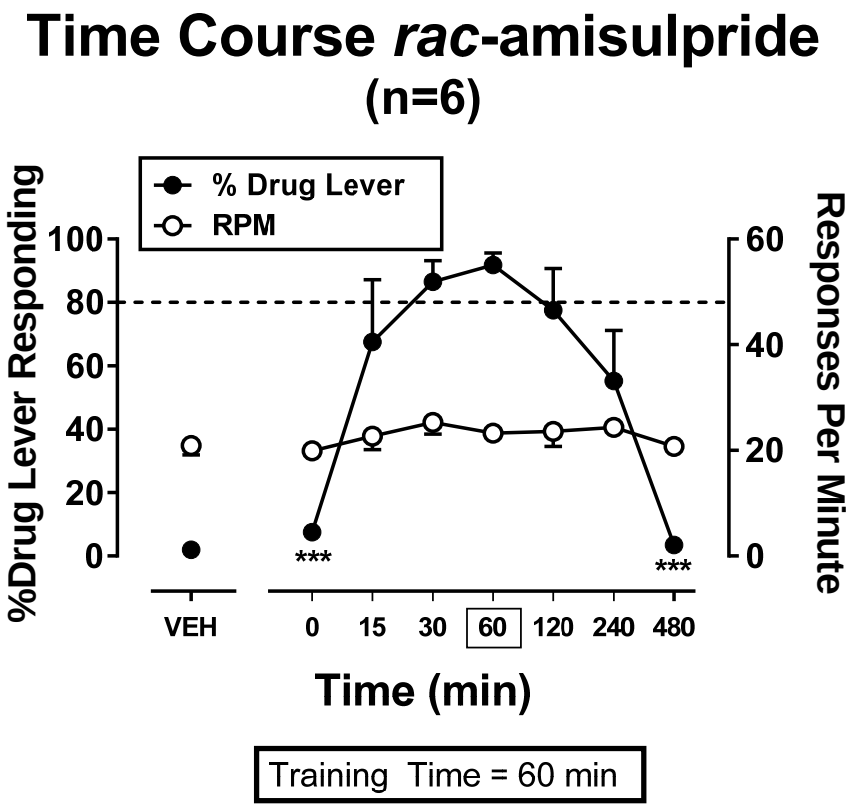

Figure 6. Time course of 10 mg/kg *rac*-amisulpride. Time course data are shown for 0, 15, 30, 60, 120, 240 and 480 minute pre-session s.c. injection times for the 10 mg/kg training dose of *rac*-amisulpride. For percent drug lever responding, significant differences from the pre-session injection time (60 min) are indicated by asterisks (*** $p = .001$). For responses per minute, there were no significant differences as compared to the vehicle (VEH) control.

Substitution testing for the combined *rac*-amisulpride cohorts and for the two isomers *(S)*-amisulpride and *(R)*-amisulpride is shown in Figure 7. (*S*)-amisulpride (panel b) produced full substitution for *rac*-amisulpride at 1.25 (94.29% DLR), 2.5 (97.94% DLR), and 5.0 mg/kg (84.92 % DLR). Substitution testing revealed an ED_{50} 0.33 mg/kg 95% CI [0.25, 0.45 mg/kg]. A one-way repeated measures ANOVA found (*S*)-amisulpride produced a significant effect on response rates, $F(6, 36) = 3.03$, $p = 0.02$. A Dunnett's post hoc test revealed significant increases at doses 1.25, 2.5 and 5.0 mg/kg as compared to vehicle.

The isomer (*R*)-amisulpride produced full substitution for *rac*-amisulpride at 1.25 mg/kg (80.78% DLR), 5 mg/kg (90.38%DLR), and 10 mg/kg (90.39% DLR) as shown in Figure 7 panel (c). Substitution testing revealed an ED_{50} 0.68 mg/kg 95% CI [0.43, 1.11 mg/kg]. There was no significant change in response rates at any dose tested $F(7,42) = 0.88$, $p = 0.53$.

Benzamide derivatives substitution testing for sulpiride and (*S*)-sulpiride isomer.

The substitution testing of the benzamide derivatives sulpiride and its (*S)*-sulpiride isomer are shown in Figure 8. The atypical antipsychotic sulpiride substituted for *rac*-amisulpride at 25.00 mg/kg (81.61% DLR), and 50.00 mg/kg (82.65% DLR) as shown in panel (a). Substitution testing revealed an $ED_{50} = 7.29$ mg/kg 95% CI [3.73, 14.28 mg/kg]. A one-way repeated measures ANOVA found sulpiride produced a significant effect on response rates, $F(6, 30) = 2.71$, $p = 0.03$. A Dunnett's post hoc test revealed that 3.125 mg/kg sulpiride significantly suppressed response rate as compared to vehicle.

The *(S)*-sulpiride isomer substituted for *rac*-amisulpride at 40.00 mg/kg (82.18% DLR). Substitution testing revealed an $ED_{50} = 9.12$ mg/kg 95% CI [4.60, 18.08 mg/kg] as shown in Figure 8 panel (b). A one-way repeated measures ANOVA found that sulpiride produced no significant effects on response rates, $F(5,25) = 1.64$, $p = 0.186$.

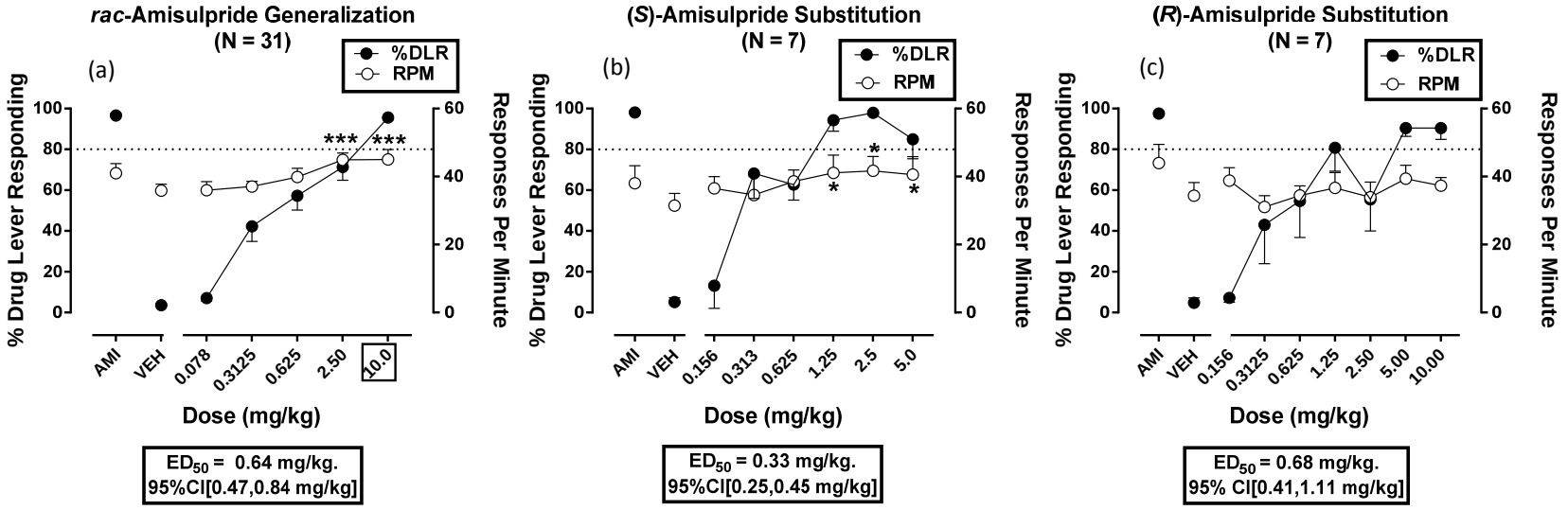

Figure 7. *Rac*-amisulpride generalization curve for combined groups, *(S)*-amisulpride, and *(R)*-amisulpride substitution. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for *rac*-amisulpride substitution curve for combined groups. Panel (b) shows generalization testing for the isomer *(S)*-amisulpride, and panel (c) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the isomer (*R*)-amisulpride, * $p < 0.05$, *** $p < .001$. All other details are the same as Figure 5.

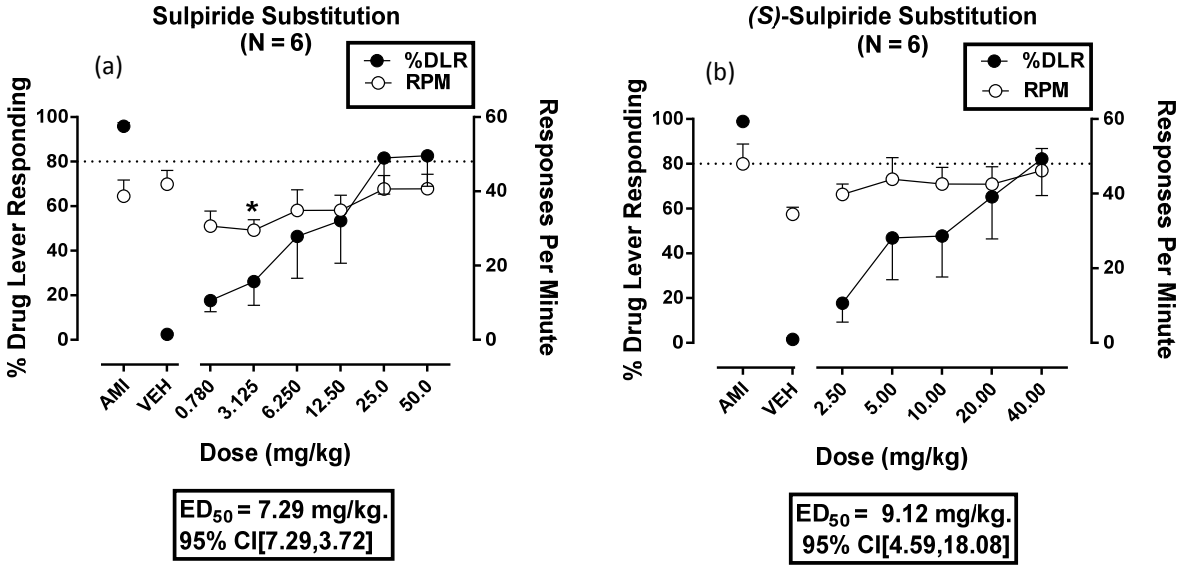

Figure 8. Substitution testing of benzamide derivatives sulpiride and (*S)*-sulpiride. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the benzamide derivative sulpiride substitution curve, and panel (b) for the benzamide derivative (*S)*-sulpiride isomer, * $p < .05$. All other details are the same as Figure 5.

Substitution testing of benzamide derivatives tiapride, nemonapride and zacopride

The results for substitution testing of the benzamide derivatives tiapride, nemonapride and zacopride are shown in Figure 9. Panel (a) shows the atypical antipsychotic tiapride produced a very high partial substitution for *rac*-amisulpride at 40 mg/kg (76.41%DLR). No other doses of tiapride substituted for *rac*-amisulpride. There were no significant changes in response rates $F(6,30) = 0.45$, $p = 0.45$.

The benzamide atypical antipsychotic nemonapride did not substitute for *rac*-amisulpride at any of the tested doses (0.01 – 0.10 mg/kg) as shown in Figure 9 panel (b). Maximum %DLR was seen at the 0.032 mg/kg dose (54.52% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(3, 20) = 16.82$, $p < 0.0001$. A Dunnett's post hoc test revealed significant rate suppression at the 0.10 mg/kg dose as compared to vehicle.

Zacopride, a selective 5-HT_3 antagonist and 5-HT_4 agonist, did not substitute for *rac*-amisulpride at any of the doses tested (1.0 – 56.0 mg/kg) as shown in Figure 9 panel (c). Maximum %DLR was seen at 0.032 mg/kg dose (38.64% DLR). A one-way repeated measures ANOVA found that zacopride produced no significant effects on response rates, $F(4, 25) = 1.10$, $p = 0.38$.

Substitution Testing of Typical Antipsychotic Medications: Haloperidol, Chlorpromazine and Apomorphine

The typical antipsychotic haloperidol did not substitute for rac-amisulpride at any of the tested doses (0.00625 – 0.20 mg/kg) as shown in Figure 10 panel (a). Maximum %DLR was seen at 0.10 mg/kg (41.61 %DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(5,25) = 2.63$, $p = 0.05$. However a Dunnett's post hoc test failed to reveal any significant differences between drug and vehicle response rates.

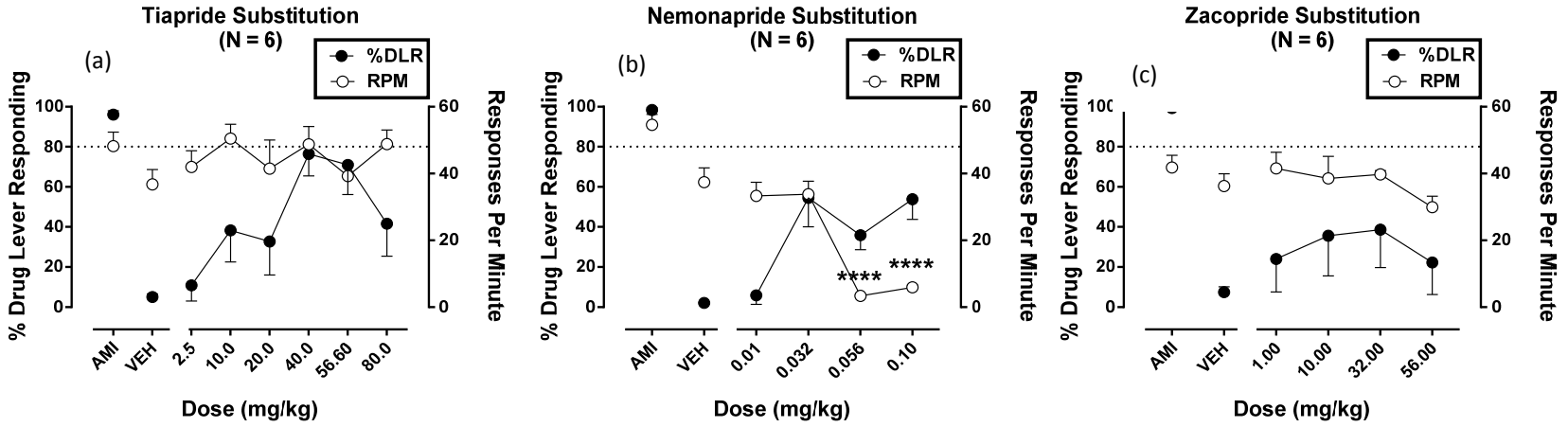

Figure 9. Substitution testing of benzamide derivatives tiapride, nemonapride and zacopride. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the benzamide derivative tiapride substitution curve, panel (b) for the benzamide derivative nemonapride, and panel (c) for the benzamide derivative zacopride, ****$p < .0001$. All other details are the same as Figure 5

The typical antipsychotic chloropromazine did not substitute for *rac*-amisulpride at any of the tested doses (0.125 – 2.0 mg/kg) as shown in Figure 10 panel (b). Maximum % DLR was seen at 0.50 mg/kg (57.14 % DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(5, 25) = 11.44$, $p < .0001$. A Dunnett's post hoc test revealed that 2.0 mg/kg chlorpromazine significantly suppressed response rates as compared to vehicle. Two animals were tested at 4.0 mg/kg chlorpromazine; however, that dose produced complete suppression of responding and no further animals were tested at this dose.

Apomorphine, a non-selective D_1, D_2, D_3, D_4 and D_5 agonist, did not substitute for rac-amisulpride at any of the tested doses (0.032 mg/kg) as shown in Figure 10 panel (c). Maximum %DLR was seen at 0.032 mg/kg dose (12.91% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(3,20) = 7.84$, $p = 0.0012$. A Dunnett's post hoc test revealed significant rate suppression at 0.032, and 0.32 mg/kg apomorphine as compared to vehicle. Two animals were tested at 1.0 mg/kg apomorphine; however, that dose produced complete suppression of responding and no further animals were tested at this dose.

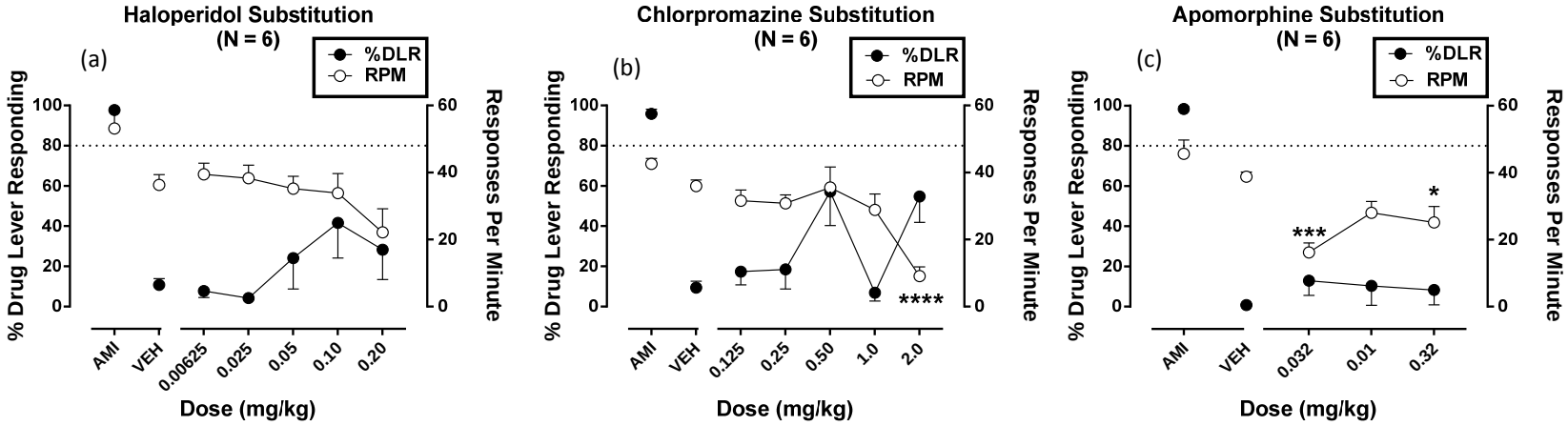

Figure 10. Substitution testing of typical antipsychotics: haloperidol, chlorpromazine, and the dopamine agonist apomorphine. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the typical antipsychotic haloperidol substitution curve, panel (b) for the typical antipsychotic chlorpromazine, and panel (c) for the dopamine agonist apomorphine, * $p < .05$, *** $p < 0.001$, **** $p < 0.0001$. All other details are the same as Figure 5.

Substitution testing of atypical antipsychotics: olanzapine, clozapine, risperidone, quetiapine, and aripiprazole.

The atypical antipsychotic olanzapine did not substitute for *rac*-amisulpride at any of the tested doses (0.0625 – 1.0 mg/kg) as shown in Figure 11 panel (a). Maximum %DLR was seen at 0.5 mg/kg dose (20.03% DRL). There were no significant changes in response rates as compared to vehicle at any dose tested $F(4,20) = 0.79$, $p = 0.55$.

The atypical antipsychotic clozapine did not substitute for *rac*-amisulpride at any of the tested doses (0.625 – 2.5 mg/kg) as shown in Figure 11 panel (b). Maximum %DLR was seen at 1.78 mg/kg dose (29.25% DLR). A one-way repeated measures ANOVA revealed clozapine produced a significant effect on response rates, $F(4, 20) = 19.44$, $p < 0.0001$. A Dunnett's post hoc test revealed that 1.78 and 2.5 mg/kg clozapine significantly suppressed response rates as compared to vehicle.

The atypical antipsychotic risperidone did not substitute for *rac*-amisulpride at any of the tested doses (0.625 – 2.5 mg/kg) as shown in Figure 11 panel (c). Maximum %DLR was seen at 0.25 mg/kg dose (36.39% DLR). A one-way repeated measures ANOVA revealed that risperidone produced a significant effect on response rates, $F(3, 15) = 32.80$, $p < 0.0001$. A Dunnett's post hoc test revealed that 0.125 and 0.25 mg/kg risperidone significantly suppressed response rates as compared to vehicle.

The atypical antipsychotic quetiapine did not substitute for *rac*-amisulpride at any of the tested doses (2.5 – 7.10 mg/kg). Maximum %DLR was seen at 7.10 mg/kg dose (44.58% DLR) as shown in Figure 11 panel (d). A one-way repeated measures ANOVA found a significant difference in response rates compared to vehicle, $F(3,20) = 8.80$, $p = 0.0006$. A Dunnett's post hoc test revealed significant response rate suppression at 7.10 mg/kg quetiapine as compared to

vehicle. Two animals were tested at 10.0 mg/kg quetiapine; however, that dose produced complete suppression of responding and no further animals were tested at this dose.

The atypical antipsychotic aripiprazole did not substitute for *rac*-amisulpride at any of the tested doses (0.15625 – 1.250.0 mg/kg) as shown in Figure 11 panel (e). Maximum %DLR was seen at 1.5625 mg/kg (38.04% DLR). A one-way repeated measures ANOVA revealed aripiprazole produced a significant effect on response rates, $F(4, 25) = 7.49$, $p = 0.0004$. A Dunnett's post hoc test revealed that 0.625 and 1.25 mg/kg aripiprazole significantly suppressed response rates as compared to vehicle.

Substitution testing of antidepressants fluoxetine and imipramine

The selective serotonin reuptake inhibitor antidepressant fluoxetine did not substitute for *rac*-amisulpride at any of the tested doses (5.0 – 40.0 mg/kg) as shown in Figure 12 panel (a). Maximum %DLR was seen at 20.0 mg/kg dose (5.28% DLR). A one-way repeated measures ANOVA revealed that fluoxetine produced a significant effect on response rates, $F(4, 20) = 11.88$, $p < 0.0001$. A Dunnett's post hoc test revealed that 20.0 and 40.0 mg/kg fluoxetine significantly suppressed response rates compared to vehicle.

The tricyclic antidepressant imipramine did not substitute for *rac*-amisulpride at any of the tested doses (5.0 – 20.0 mg/kg) as shown in Figure 12 panel (b). Maximum %DLR was seen at 5.0 mg/kg dose (2.60% DLR). A one-way repeated measures ANOVA revealed that imipramine produced a significant effect on response rates, $F(3, 15) = 8.65$, $p = 0.0014$. A Dunnett's post hoc test revealed that 20.0 mg/kg imipramine significantly suppressed response rates compared to vehicle.

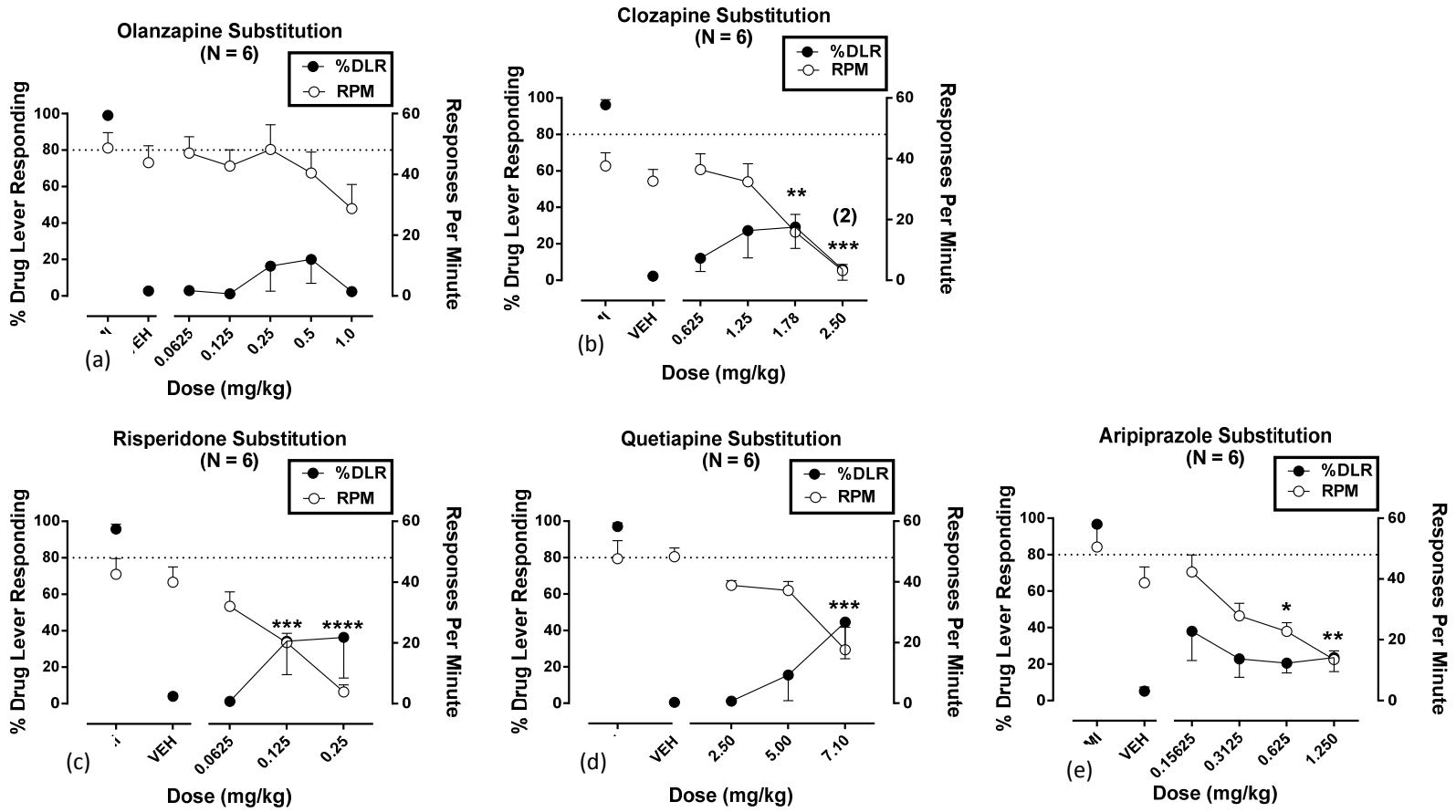

Figure 11. Substitution testing of atypical antipsychotics: olanzapine, clozapine, risperidone, quetiapine, and aripiprazole. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the atypical antipsychotic olanzapine substitution curve, panel (b) for clozapine, panel (c) for risperidone, panel (d) for quetiapine and panel (e) for aripiprazole, * $p < .05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All other details are the same as Figure 5.

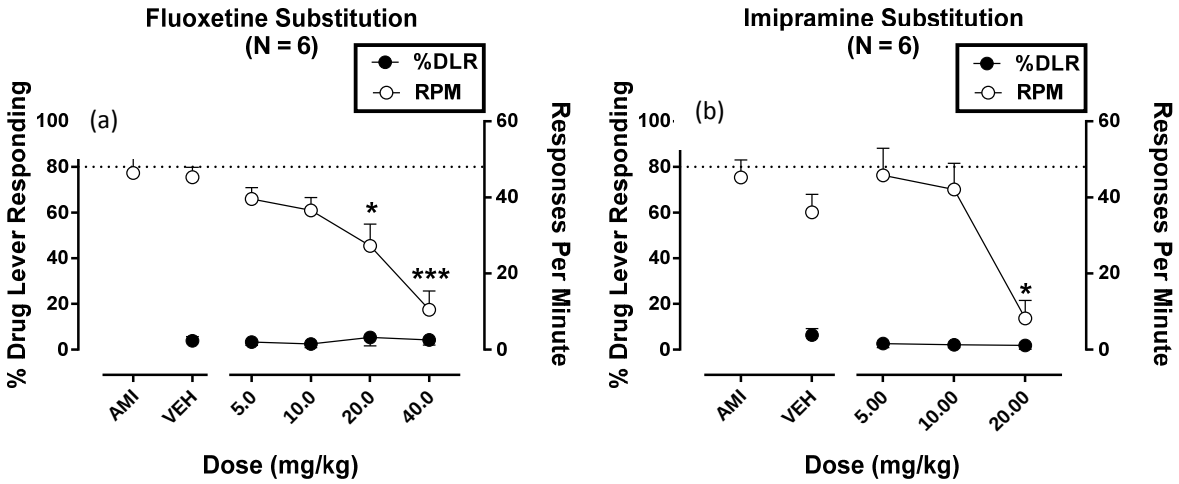

Figure 12. Substitution testing of antidepressants fluoxetine and imipramine. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the selective serotonin reuptake inhibitor fluoxetine substitution curve, and right panel (b) for the tricyclic antidepressant imipramine, * $p < .05$, *** $p < 0.001$. All other details are the same as Figure 5.

Substitution testing of bupropion, mianserin, and chlordiazepoxide.

The antidepressant bupropion (aminoketone class) did not substitute for *rac*-amisulpride at any of the tested doses (1.0 – 56.0 mg/kg) as shown in Figure 13 panel (a). Maximum %DLR was seen at 56.0 mg/kg dose (14.25% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(4, 25) = 18.65$, $p < 0.0001$. A Dunnett's post hoc test revealed significant response rate suppression at 56.00 mg/kg bupropion as compared to vehicle.

The tetracyclic antidepressant mianserin did not substitute for *rac*-amisulpride at any of the tested doses (0.50 – 2.00 mg/kg) as shown in Figure 13 panel (b). Maximum %DLR was seen at 0.50 mg/kg dose (40.26% DLR). A one-way repeated measures ANOVA revealed that mianserin produced a significant effect on response rates, $F(3, 20) = 65.11$, $p < 0.0001$. A Dunnett's post hoc test revealed that 2.00 mg/kg mianserin HCl significantly suppressed response rates compared to vehicle.

The anxiolytic chlordiazepoxide did not substitute for *rac*-amisulpride at any of the tested doses (2.5 – 56.6 mg/kg) as shown in Figure 13 panel (c). Maximum %DLR was seen at the 56.6 mg/kg dose (17.10% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(6, 35) = 3.50$, $p = 0.0084$. However, a Dunnett's post hoc test failed to reveal any significant difference between chlordiazepoxide as compared to vehicle.

Substitution testing of selected dopaminergic ligands raclopride and quinpirole

The results for the selected dopaminergic ligands raclopride and quinpirole are shown in Figure 14. Panel (a) shows the substituted benzamide raclopride (D_2, D_3 antagonist) did not fully substitute for *rac*-amisulpride at any of the tested doses (0.025-0.40 mg/kg). However, there was partial substitution at 0.10 mg/kg (62.68 %DLR). A one-way repeated measures ANOVA found no significant differences in response rates, $F(5, 30) = 1.48$, $p = 0.23$.

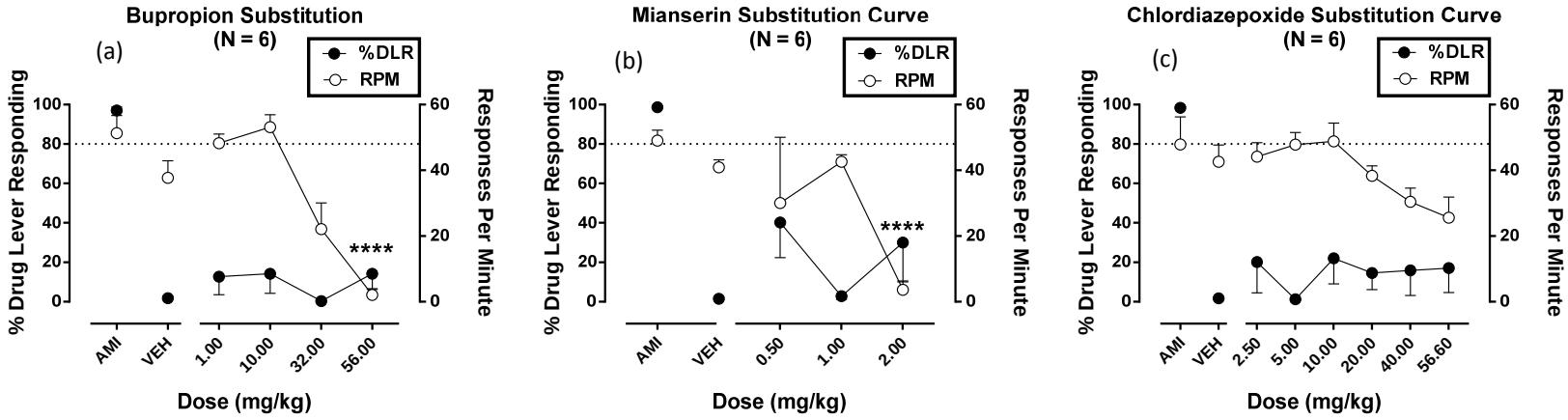

Figure 13. Substitution testing of bupropion, mianserin, and chlordiazepoxide. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the antidepressant bupropion substitution curve, panel (b) for the tetracyclic antidepressant mianserin, and left panel (c) the anxiolytic chlordiazepoxide, **** $p < 0.0001$. All other details are the same as Figure 5.

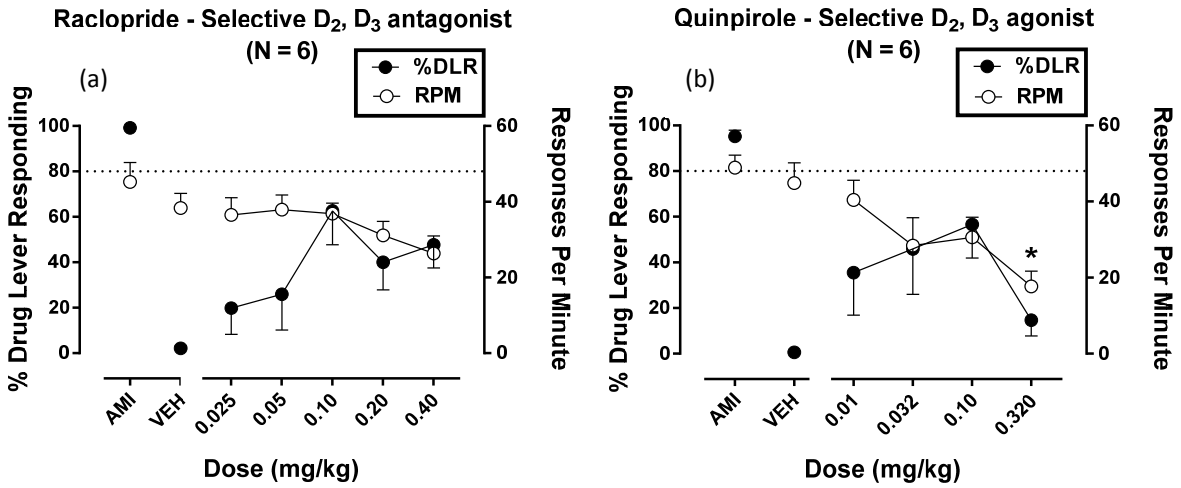

Figure 14. Substitution testing of selective dopaminergic ligands raclopride and quinpirole. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the dopamine antagonist raclopride substitution curve, and panel (b) for the dopamine agonist quinpirole, * $p < .05$. All other details are the same as Figure 5.

The compound quinpirole (selective D_2 and D_3 agonist) did not fully substitute for *rac*-amisulpride at any of the tested doses (0.01-0.32 mg/kg) as shown in Figure 14 panel (b). Maximum %DLR was seen at the 0.10 mg/kg dose (56.64 %DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(4, 25) = 3.73$, $p = 0.0164$. A Dunnett's post hoc test revealed significant rate suppression at 0.32 mg/kg quinpirole as compared to vehicle.

Substitution testing of selective serotonin ligands BW-723C86 and SB-204741

The compound BW 723C86 (selective 5-$HT2_B$ agonist) did not substitute for *rac*-amisulpride at any of the tested doses (1.0 – 32.00 mg/kg) as shown in Figure 15 panel (a). Maximum %DLR was seen at 10.00 mg/kg (17.91% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(3, 20) = 15.19$, $p < 0.0001$. A Dunnett's post hoc test revealed significant rate suppression at a dose 32.00 mg/kg as compared to vehicle.

The compound SB-204741 (5-HT_{2B} antagonist) did not fully substitute for *rac*-amisulpride at any of the tested doses (1.0 – 4.00 mg/kg) as shown in Figure 15 panel (b). Maximum %DLR was seen at 2.00 mg/kg (17.64 %DLR). A one-way repeated measures ANOVA found no significant differences in response rates, $F(3, 20) = 0.182$, $p = 0.91$.

Substitution testing of selective serotonin ligands LP-44 and SB-269970

The compound LP-44 (selective 5-HT_7 agonist) did not substitute for *rac*-amisulpride at any of the tested doses (1.0 – 32.00 mg/kg) as shown in Figure 16 panel (a). Maximum %DLR was seen at 10.00 mg/kg (50.11% DLR). A one-way repeated measures ANOVA found a significant difference in response rates, $F(4, 25) = 4.80$, $p < 0.0054$. A Dunnett's post hoc test revealed significant rate suppression at 32.00 mg/kg as compared to vehicle.

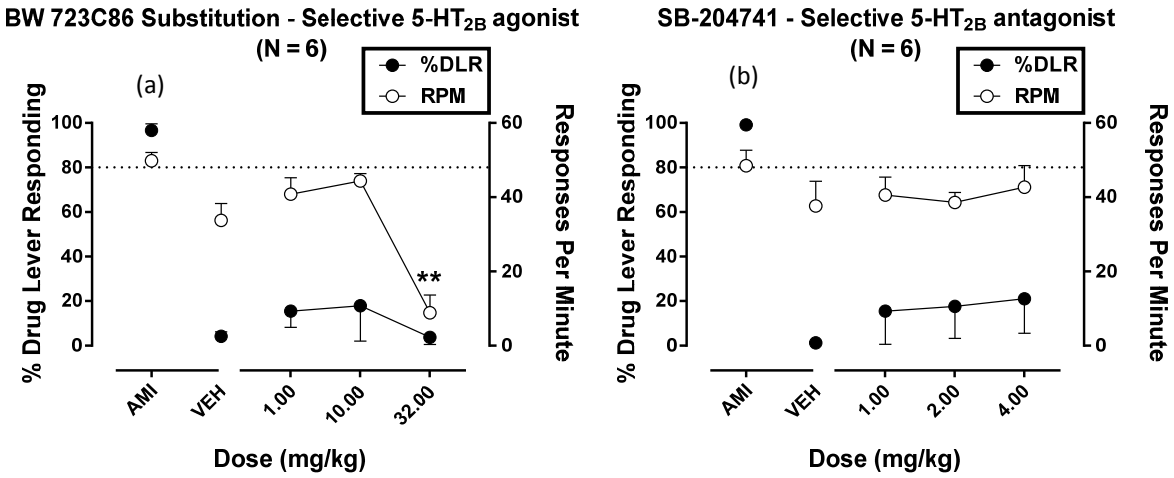

Figure 15. Substitution testing of the selective serotonin ligands BW 723C86 and SB-204741. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the 5-HT_{2B} agonist BW 723CC86, and panel (b) for the 5-HT_{2B} antagonist SB-204741, ** $p < .01$. All other details are the same as Figure 5.

The compound SB-269970 (selective 5-HT_7 antagonist) did not fully substitute for *rac*-amisulpride at any of the tested doses (0.32 – 56.0 mg/kg) as shown in Figure 16 panel (b). Maximum %DLR was seen at 32.00 mg/kg dose (36.74 %DLR). A one-way repeated measures ANOVA found no significant differences in response rates, $F(6, 35) = 0.97$, $p = 0.463$.

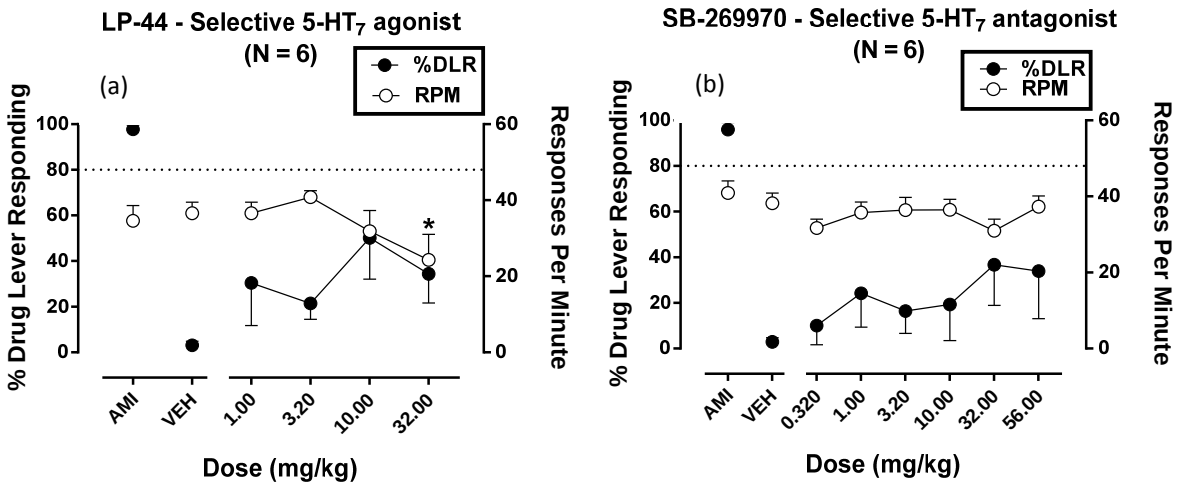

Figure 16. Substitution testing of selective serotonin ligands LP-44 and SB-269970. Panel (a) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the selective serotonin ligand LP-44 (5-HT_7 agonist). Panel (b) shows mean percent drug lever responding (± SEM) and mean responses per minute (± SEM) for the selective serotonin ligand SB-269970 (5-HT_7 antagonist), * $p < .05$. All other details are the same as Figure 5.

Discussion

***rac*-Amisulpride as a discriminative stimulus.** The results of the present study demonstrated that the atypical antipsychotic *rac*-amisulpride can exert reliable discriminative stimulus control in male C57BL/6 mice at doses that do not significantly suppress rates of responding. A previous study conducted by this author (Donahue et al., 2014) demonstrated that the isomer *(S)*-amisulpride (10 mg/kg training dose) also exerts a robust discriminative stimulus in C57BL/6 mice, and that the *(R)*-amisulpride isomer and *rac*-amisulpride produced full substitution for *(S)*-amisulpride. Results from that study will aid, through comparison, in the analysis of the findings of this dissertation project. This study is original as, to date; there are no published studies on the discriminative stimulus properties of *rac*-amisulpride, the therapeutic form of the drug, with any species. The general goal of this study was to build upon the data demonstrated in Donahue et al. (2014) by utilizing the drug discrimination paradigm as a behavioral assay to investigate the discriminative stimulus properties of *rac*-amisulpride. The first aim was to establish *rac*-amisulpride as a discriminative stimulus and compare, through substitution testing, the discriminative stimulus properties of *rac*-amisulpride to the enantiomers *(S)*- amisulpride and *(R)*-amisulpride as well as a wide variety of typical and atypical antipsychotics, other benzamide derivatives, and other medications known for their antidepressant and anxiolytic effects. The second aim was to conduct substitution testing with selective ligands that are either agonists or antagonists at specific receptor sites responsible for the effects of *rac*-amisulpride. Specifically, selective agonists and antagonists for dopamine D_2 and D_3 and for serotonin 5-HT_{2B} and 5-HT_7 receptors were tested. This specific ligand testing enabled us to more precisely investigate the underlying neural receptors responsible for the discriminative stimulus effects of *rac*-amisulpride.

The present study utilized *rac*-amisulpride (10 mg/kg) as the training drug. The acquisition differences among the two groups of animals reveals that Cohort 1 acquired the discriminative cue in fewer sessions (35.7) compared to Cohort 2 (41.58) and their respective ranges overlap. These differences in acquisition between the two groups are minimal and may be accounted for by the differences in sample size in the three groups. The training dose (10 mg/kg) and pre-injection time (60 min) in the present study were based upon published drug discrimination research from our lab as well as the pharmacological profile of amisulpride in mice and rats in other behavioral studies (Donahue et al., 2014; Perrault et al., 1997).

Comparison of dose-effect curves. A comparison of the generalization curves for the racemic and two isomeric forms of amisulpride yielded interesting information regarding %DLR and respective ED_{50} values. As seen in Table 11 and Figure 17, the ED_{50} values for *rac*-amisulpride and the (*R*)-amisulpride isomer are very similar to each other; however, there was a significant leftward shift for the (*S*)-amisulpride isomer. This leftward shift may be due to the differences in potency for *(S)*-amisulpride relative to *(R)*-amisulpride and the racemic form. It has been demonstrated that *(S)*-amisulpride is twice as potent as *rac*-amisulpride and 20 to 40 times more potent than *(R)*-amisulpride in displacing radioligands from dopamine $D_{2/3}$ receptors (Castelli et al., 2001). It is also interesting that in our study (*R*)-amisulpride (ED_{50} = 0.68 mg/kg. 95% CI[0.41, 1.11 mg/kg.]) and *rac*-amisulpride (ED_{50}=0.46 mg/kg. 95% CI[0.47, 0.84]) show overlapping intervals indicating that the respective ED_{50} values were not statistically different. This suggests that the *R*-isomer (while not as potent as the *S*-isomer) is equipotent with *rac*-amisulpride and shared discriminative stimulus properties with *rac*-amisulpride and, in fact, may contribute to the discriminative stimulus properties of *rac*-amisulpride. This finding is intriguing and warrants further investigation as to whether or not (*R)*-amisulpride possesses a

Table 11.

Comparison of ED_{50} values for the three forms of amisulpride.

Drug Form	**ED_{50} Value**	**95% Confidence Interval**
rac-amisulpride (N=31)	ED_{50} = 0.64 mg/kg	0.47 – 0.84 mg/kg
(S)-amisulpride (N=7)	ED_{50} = 0.33 mg/kg	0.25 – 0.45 mg/kg
(R)-amisulpride (N-7)	ED_{50} = 0.68 mg/kg	0.41 – 1.11 mg/kg

Values based on free base weights of the drugs.

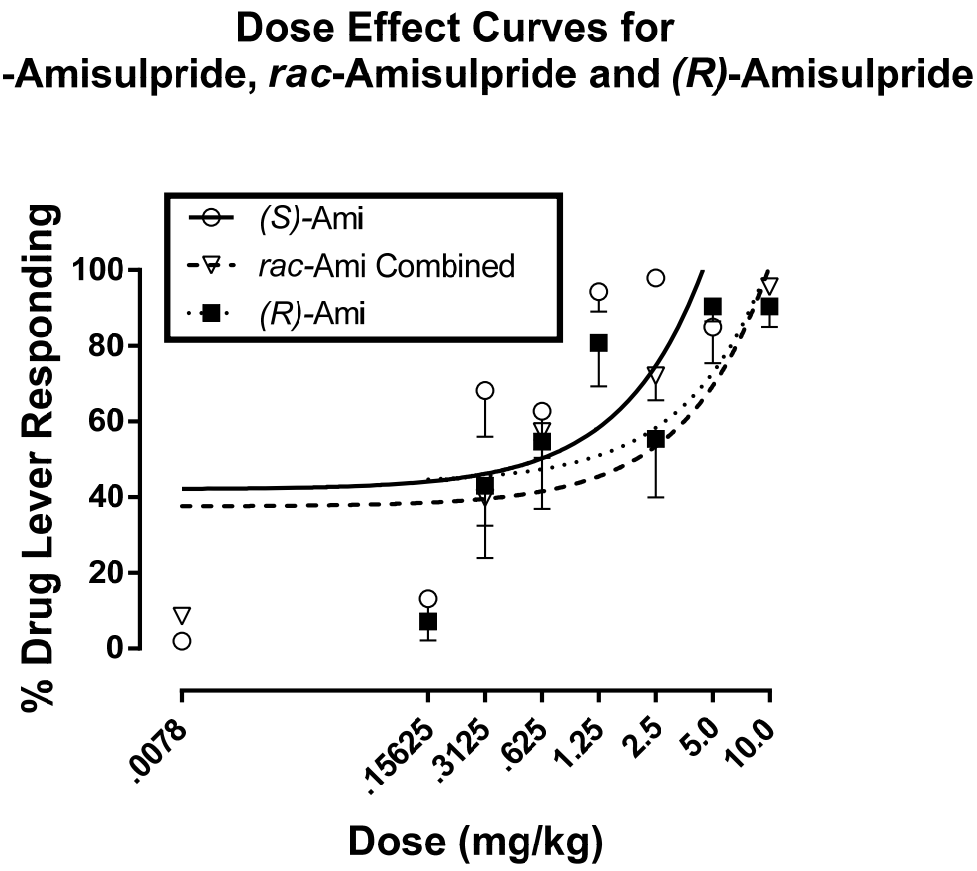

Figure 17. Dose-effect curves for *(S)*-amisulpride, *rac*-amisulpride and *(R)*-amisulpride. The % drug lever responding data for *rac*-amisulpride and its isomers are redrawn on a log base 10 scale with least squares regression lines to illustrate the significant ($p<0.05$) leftward shift of *(S)*-amisulpride dose-effect curve relative to *rac*-amisulpride and *(R)*-amisulpride.

discriminative stimulus that can be trained in C57BL/6 mice and, if so, at what dose? Furthermore, this suggests that (*R*)-amisulpride may contribute to the therapeutic properties of *rac*-amisulpride and may in fact have unique therapeutic properties of its own for the treatment of schizophrenia and/or depression.

***rac*-Amisulpride time course.** Time course data shown in Figure 3 revealed a rather symmetrical inverted U shaped curve for %DLR responding across various time points with no significant changes in response rates. The data demonstrated that with a 60 minute s.c. pre-injection time, 10 mg/kg training dose of *rac*-amisulpride produced partial substitution at 15 minutes (67.5% DLR) and 120 minutes (77.55% DLR). Full substitution was produced at 30 minutes (86.54% DLR) and 60 minutes (91.78% DLR) with a significant decline at 0 minutes (7.53% DLR), 240 minutes (55.26% DLR), and 480 minutes (3.50% DLR). The finding that full substitution was achieved at 60 minute time point is consistent with existing research using the same post injection time period to achieve maximum behavioral effects in mice and rats (Donahue et al., 2014; Manzaneque & Navarro, 1999; Perrault et al., 1997; Scatton et al., 1994). The elimination rate appears consistent with expected normal half-life elimination. Interestingly, this author's previous study of *(S)*-amisulpride (10 mg/kg training dose) (Donahue et al., 2014) showed a 30 minute time point produced only partial substitution (70.28% DLR) compared to the full substitution at 30 minutes (86.54% DLR) for *rac*-amisulpride at that same dose. This difference may be due to absorption rates, and/or slower elimination rate or the difference in potency at certain receptors between the racemic form of the drug and its isomers.

Benzamide derivatives substitution testing. Of particular interest to this study was the testing of the benzamide derivatives, the class of drugs to which *rac*-amisulpride belongs. Tested benzamides included: the atypical antipsychotic sulpiride, *(S)*-sulpiride (Figure 10), tiapride, nemonapride and zacopride (Figure 13). Binding affinities for the benzamides are shown in Table 12. This study showed that both sulpiride and *(S)*-sulpiride fully substituted for *rac*-amisulpride. Sulpiride substituted for *rac*-amisulpride at 25.00 mg/kg (81.61% DLR), and 50.00 mg/kg (82.65% DLR) revealing an ED_{50} = 7.29 mg/kg. *(S)*-sulpiride substituted for *rac*-amisulpride at 40.00 mg/kg (82.18% DLR) revealing an ED_{50} = 9.12. While sulpiride showed a significant suppression of response rates as compared to vehicle, *(S)*-sulpiride show no significant effects on response rates. This is particularly interesting as both sulpiride and *(S)*-sulpiride both display a high binding affinity and antagonistic action at dopamine D_2 and D_3 receptors, an affinity and antagonism shared by *rac*-amisulpride. However, unlike *rac*-amisulpride, sulpiride and *(S)*-sulpiride show no affinity for any serotonin receptors. This provides us with a clue that perhaps the discriminative stimulus properties of *rac*-amisulpride are particularly related to its affinity for and antagonism of dopamine D_2 and D_3 receptors. Also interesting was that tiapride showed very high partial substitution for rac-amisulpride at 40 mg/kg (76.41% DLR), again with no significant effects on response rates. Tiapride also has no known affinity for serotonin receptors (unlike *rac*-amisulpride), but does have affinity for dopamine D_2 receptors (K_i = 31.0) as does *rac*-amisulpride (K_i = 1.3). However, tiapride has an affinity for dopamine D_4 (K_i =14.0) receptors while *rac*-amisulpride has no affinity for D_4 receptors. Perhaps the shared affinity at D_2 receptors provides a clue as to why tiapride showed high partial substitution for *rac*-amisulpride. Recall in our introduction that Cohen et al. (1997) trained rats to discriminate tiapride and amisulpride produced tiapride-

Table 12

Receptor binding affinity K_i values (nM) of tested benzamide drugs at relevant receptor targets

	Receptor										
Drug Name	**5-HT$_{1A}$**	**5-HT$_{1B}$**	**5-HT$_{2A}$**	**5-HT$_{2B}$**	**5-HT$_3$**	**5-HT$_6$**	**5-HT$_7$**	**α_{2A}**	**D$_2$**	**D$_3$**	**D$_4$**
Rac-amisulpride	NSB[a]	1,744.0[c]	2,000.0[a]	13.0[c]	--	4,154.0[c]	11.5[c]	1,114.0[c]	1.3[c]	3.8[l]	2,369.0[c]
Sulpiride	589.0[b]	--	NSB[d]	--	--	5,000.0[f]	3,000.0[g]	682.0[h]	8.2[i]	0.71[m]	54.0[n]
(S)-sulpiride	--	--	--	--	--	--	--	--	9.9[n]	6.4[o]	
Tiapride	--	--	--	--	--	--	--	--	31.0[j]	--	14.0[j]
Nemonapride	58.0[p]	--	--	--	--	--	--	--	0.04[k]	0.06[q]	--
Zacopride	--	--	--	--	8.97[e]	--	--	--	--	--	--

5-HT, serotonin receptors; α, adrenergic alpha receptors; D, dopamine receptors; --, not tested; NSB, no significant binding (Ki > 10,000 nM)

[a] (Schoemaker et al., 1997); rat cerebral cortex
[b] (Raymond et al., 1989) ; COS-7 human cloned cells
[c] (Abbas et al., 2009) ; human cloned cDNA cells
[d] (B. L. Roth et al., 1992) ; rat brain
[e] (Kilpatrick, Bunce, & Tyers, 1990); rat cortex
[f] (B. L. Roth et al., 1994) ; rat cloned HEK-293 receptor cells
[g] (Ruat et al., 1993); rat cloned hypothalamus cDNA cells
[h] (Boyajian & Leslie, 1987); rat brain
[i] (Kessler et al., 1993); rat cortex
[j] (Burstein et al., 2005); human monoamine G protein-coupled receptors
[k] (Philip Seeman & Van Tol, 1995); pig anterior pituitary
[l] (P Sokoloff et al., 1990); rat brain cloned G protein-coupled receptors
[m] (Lawler et al., 1999); rat cloned C-6 glioma cells
[n] (Zahniser & Dubocovich, 1983); rat striatum
[o] (Kapur & Seeman, 2001); human cloned cDNA cells
[p] (Toll et al., 1998); D3-receptor-containing CHOp- cells
[q] (Tang, Todd, Heller, & O'Malley, 1994); rat cloned D_3 cells transfected into mouse Ltk- fibroblasts

appropriate responding; however, the present study showed that tiapride produced only partial substitution for amisulpride. This is an interesting asymmetrical cross-generalization between tiapride and *rac*-amisulpride. One possible explanation is that tiapride's binding profile only shares one receptor affinity with *rac*-amisulpride (dopamine D_2) and that this (alone) is insufficient to fully produce amisulpride-like drug lever responding. As to why amisulpride fully substituted for tiapride in Cohen et al. (1997) remains a curious question.

Nemonapride did not substituted for *rac*-amisulpride and showed significant rate suppression compared to vehicle. The failure for nemonapride to fully substitute for rac-amisulpride may be because nemonapride has an affinity only for dopamine D_2 receptors, and perhaps, as we saw with sulpiride, affinity for D_3 is required for substitution to *rac*-amisulpride. Zacopride, a diagnostic compound displaying antagonism at and a highly potent and selective binding affinity for serotonin 5-HT_3 receptor was also included in the benzamide testing. It did not substitute for *rac*-amisulpride and produced no significant effects on response rates compared to vehicle. Again, that zacopride has affinity for only serotonin 5-HT_3 receptor probably accounts for its inability to substitute for *rac*-amisulpride

Typical antipsychotic drugs substitution. The present study demonstrated that the typical antipsychotic medications haloperidol, chlorpromazine or the dopamine non-selective agonist apomorphine did not substitute for *rac*-amisulpride at any of the doses tested (Figure 8) and all three drugs showed significant rate suppression as compared to vehicle. Haloperidol has proved to be a difficult drug to establish as a discriminative stimulus (Colpaert F et al., 1976; McElroy et al., 1989) although it has been used in drug discrimination studies with drugs such as amphetamine(Haenlein, Caul, & Barrett, 1985) and nicotine (R. J. Barrett, Caul, & Smith, 2004). There are no studies showing that haloperidol has substituted for any atypical antipsychotic

medications; thus, it is not surprising that it did not substitute for *rac*-amisulpride. Donahue et al. (2014) found that *(S)*-amisulpride did not generalize to haloperidol, which supports the finding of this study regarding *rac*-amisulpride. The failure of haloperidol to substitute for *(S)*-amisulpride and *rac*-amisulpride suggests the difference in binding profiles between *rac*-amisulpride and haloperidol most likely accounts for this. See Table 7 for binding affinities at relevant receptors. Structurally, haloperidol is a butyrophenone that has strong binding affinity with antagonistic effects at dopaminergic $D_{1\text{-}5}$ and adrenergic α_{1A} and α_{1B} receptors and sigma$_{1\text{-}2}$ receptors. Chlorpromazine has a similar binding profile with strong affinity to and antagonistic action at dopaminergic $D_{1\text{-}5}$ and adrenergic α_{1A} and α_{1B} receptors and muscarinic M_1 receptors. Goas and Boston (1978) were the first to show that haloperidol substituted for chlorpromazine in a drug discrimination study with rats (Goas & Boston, 1978) a finding later supported by McElroy et al. (1989), which showed that chlorpromazine substituted for haloperidol in rats trained to discriminate 0.05 mg/kg (i.p.) haloperidol from vehicle (McElroy et al., 1989). Thus, there is cross-generalization between the two drugs. In contrast, *rac*-amisulpride binds selectively to dopaminergic $D_{2/3}$ and to serotonin 5-HT_{2B} and 5-HT_7 receptors and it is most likely that the difference in binding profiles between *rac*-amisulpride and haloperidol and chlorpromazine accounts for the fact that neither of the later drugs substituted for *rac*-amisulpride. It also may be that *rac*-amisulpride more quickly dissociates from dopamine receptors than either haloperidol or chlorpromazine (P. Seeman, 2002) (see Figure 2) and that this may be a factor for the failure of either typical antipsychotic to substitute for *rac*-amisulpride.

The non-selective dopamine agonist apomorphine, a compound sometimes used in the treatment of Parkinson's disease, did not substitute for *rac*-amisulpride and produced severe rate suppression compared to vehicle at higher doses. Apomorphine is not an antipsychotic drug and

was used as a negative control because of its agonistic activity and strong binding affinity to dopamine D_1 (K_i = 7.2)(Burt, Creese, & Snyder, 1976), dopamine D_2 (K_i = 2.3) and dopamine D_3 (K_i = 2.2) (Sautel et al., 1995), and dopamine D_4 (K_i = 4.3)(M. J. Millan et al., 2002). Considering that apomorphine and *rac*-amisulpride have opposite effects at dopamine $D_{2/3}$ receptors and that apomorphine does not bind, as *rac*-amisulpride does, to dopamine serotonin 5-HT_{2B}, or to 5-HT_{7A}, it is not surprising that it does not substitute for *rac*-amisulpride.

Atypical antipsychotic drug substitution. This present study found that the atypical antipsychotics olanzapine, clozapine, risperidone, quetiapine and aripiprazole did not substitute for *rac*-amisulpride at any of the tested doses (see Figure 9). Additionally, all of the drugs except olanzapine produced significant suppression of response rates compared to vehicle. See Table 7 for the binding affinities of these antipsychotics at receptors relevant to *rac*-amisulpride. There are studies to show that many of these atypical antipsychotics (with the exception of *rac*-amisulpride) substitute for each other in drug discrimination assays. For example, olanzapine binds with high affinity to dopamine $D_{1\text{-}4}$ receptors, and serotonin 5-HT_{2A} and 5-HT_{2B}, and adrenergic α_{1A}, and muscarinic M_1 where it displays antagonistic action at all of these receptor sites. Moore et al. (1992) showed that in rats trained to discriminate 5.0 mg/kg (i.p) clozapine from vehicle olanzapine produced full substitution (Moore, Tye, Axton, & Risius, 1992). Porter and Strong (1996) were successful in training rats to discriminate 0.5 mg/kg (i.p.) olanzapine from vehicle in a drug discrimination assay and that clozapine fully substituted for olanzapine in a dose-dependent manner (Porter & Strong, 1996). Olanzapine also was shown to show partial substitution to risperidone (Porter, McCallum, Varvel, & Vann, 2000). While olanzapine appears to share similar binding affinities and antagonist effects with other atypical

antipsychotics, this study concluded that the difference in binding profiles between olanzapine and *rac*-amisulpride appear to prohibit one from substituting for each other.

Clozapine, a dibenzodiazepine, binds to many receptors displaying a lower affinity to dopamine D_2 receptors but higher affinity for dopamine D_1, D_4, and serotonergic 5-$HT_{2A/2C}$, 5-HT_6, 5-HT_7, cholinergic $M_{1\text{-}4}$, adrenergic $\alpha_{1\text{-}2}$, and histaminic H_1 receptors (Arnt & Skarsfeldt, 1998a; Bymaster et al., 1996; E. Richelson, 1999; Schotte et al., 1996). Generally, clozapine is characterized as an antagonist at these receptors, but it has been shown to act as a week partial agonist at M_1 receptors, and as an agonist at M_4 and 5-HT_{1A} receptors (Davies et al., 2004; Weiner et al., 2004). It has been shown in drug discrimination studies with rats/mice that clozapine fully substitutes for olanzapine (Porter & Strong, 1996; Porter, Varvel, Vann, Philibin, & Wise, 2000), quetiapine (J. A. Smith & A. J. Goudie, 2002) and chlorpromazine (Goas & Boston, 1978; Porter, Villanueva, & Rosecrans, 1999). While clozapine may share similar stimulus properties with the aforementioned drugs it failed to substitute for *rac*-amisulpride and produced significant rate suppression compared to vehicle. Our preliminary investigation demonstrated that clozapine failed to fully substitute for *(S)*-amisulpride (Donahue et al., 2014) which makes it not surprising that clozapine did not substitute for *rac*-amisulpride. The selective binding profile of *rac*-amisulpride versus the rather extensive binding profile of clozapine combined with the latter drug's mixed antagonistic/agonistic effects most likely accounts for clozapine's failure to substitute for *rac*-amisulpride.

The atypical antipsychotic risperidone did not substitute for *rac*-amisulpride at any of the tested doses and produced significant rate suppression as compared to vehicle (Figure 9). Interestingly, risperidone was developed in an attempt to replicate clozapine's effectiveness without clozapine's side effects (agranulocytosis), so it is understandable that its mechanism of

action would be similar to that of clozapine (Schatzberg & Nemeroff, 2009). It has a high affinity for serotonin 5-HT_{1D}, 5-HT_{2A}, 5-HT_7, dopamine $D_{2\text{-}4}$, adrenergic $\alpha_{1A\text{-}1B}$ and moderate affinity at histamine H1 and dopamine D_1, where it acts as an antagonist at all these sites. Unlike *rac*-amisulpride, risperidone has higher affinity at serotonin 5-HT_{2A} receptors than for D_2 receptors. Risperidone has been shown to substitute fully for clozapine (Philibin et al., 2005; Porter, Varvel, et al., 2000; Porter et al., 2008), partially substitute for olanzapine (Porter, McCallum, et al., 2000), and fully substitute for quetiapine (J. A. Smith & A. J. Goudie, 2002). It is most likely that the dissimilarity in binding profiles between rac-amisulpride and risperidone account for why risperidone did not substitute for *rac*-amisulpride in this study.

Quetiapine, an atypical antipsychotic, likewise did not fully substitute for *rac*-amisulpride and produced severe rate suppression compared to vehicle. Table 7 shows that quetiapine has moderate affinity for and antagonizes dopamine D_2 receptors, a strong affinity for and acts as a partial agonist at histamine H_{1A} receptors, a strong affinity for and antagonizes adrenergic α_1 receptors, and a weak affinity and antagonistic action at serotonin 5-HT_{1A}, 5-HT_{2A} receptors. In drug discrimination studies where it was used as a test drug it showed full substitution to clozapine (Carey & Bergman, 1997; A. J. Goudie et al., 1998). The difference in binding profiles between *rac*-amisulpride and quetiapine are most likely the reason quetiapine did not fully substitute for *rac*-amisulpride.

The novel atypical antipsychotic aripiprazole, a benzisoxazole, is unique among the atypical antipsychotics, as it reduces dopaminergic neurotransmission acting as a partial agonist (versus antagonist) at dopamine $D_{2/3}$ receptors where it shows high affinity (see Table 7). Aripiprazole also has high affinity for and partial agonistic effects at serotonin 5-$HT_{1A,}$ 5-$HT_{2A,}$ 5-HT_{2B} , and shows a high affinity for and antagonistic effects at serotonin, and 5-HT_7 , dopamine D_3 and

adrenergic α_{1A}, α_{1B}, α_{2C}, receptors . The present study showed that aripiprazole did not substitute for *rac*-amisulpride at any of the doses tested and produced significant rate suppression compared to vehicle (Figure 9). As well, the preliminary study in our lab showed that aripiprazole also did not fully substitute for *(S)*-amisulpride and produced significant rate suppression compared to vehicle (Donahue et al., 2014). Our results suggest that the difference in binding profiles of *rac*-amisulpride as compared to aripiprazole helps explain why aripiprazole did not substitute for *rac*-amisulpride.

Antidepressant substitution. A number of antidepressant medications representing a wide class of drugs were tested in this study (Figures 11 and 12) and the binding affinities are presented in Table 9. The selective serotonin reuptake inhibitor fluoxetine did not fully substitute for rac-amisulpride and produced significant suppression of rates compared to vehicle. Fluoxetine has a high affinity for serotonin 5-HT_{2c} receptors where it inhibits the reuptake of serotonin into the presynaptic neuron, principally at serotonin 5-HT_{2c} receptor with no binding affinity for dopamine receptors. Additionally, serotonin acts a potent inhibitor of serotonin transporter proteins (SERT). This profile is quite different than *rac*-amisulpride and most likely contributes to the inability of fluoxetine to substitute for *rac*-amisulpride at any of the doses tested.

Bupropion is categorized as an "atypical" antidepressant as it acts via dual inhibition of norepinephrine and dopamine reuptake with negligent serotonergic effect or effects on post synaptic receptors (Stahl et al., 2004). It did not substitute for rac-amisulpride and produced significant rate suppression as compared to vehicle. As bupropion displays no binding affinity to any receptors relevant to *rac*-amisulpride it is understandable why it would fail to substitute.

The tricyclic antidepressant imipramine also did not substitute for *rac*-amisulpride at any of the tested doses and produced significant rate suppression. Imipramine has high binding affinity to SERT, serotonin 5-$HT_{2c,}$ norepinephrine transporter (NET) and moderate affinity for muscarinic M_1 and M_2 receptors. Its principal mechanism of action is in inhibiting neuronal uptake of serotonin and norepinephrine. This dissimilarity to *rac*-amisulpride in receptor binding affinity and mechanism of action is most likely a factor for why it failed to substitute for *rac*-amisulpride.

The tetracyclic antidepressant mianserin did not substitute for *rac*-amisulpride at any of the tested doses and produced significant rate suppression. Mianserin displays a high affinity for serotonin 5-HT_{2C}, 5-HT_6 and NET receptors. Mianserin acts as a weak inhibitor of NET and an antagonist at serotonin 5-HT_{2C}, 5-$HT_{6.}$ Of interest to this study, is that mianserin also displays a moderate affinity for serotonin 5-HT_{2B} receptors (K_i = 50.11). *Rac*-amisulpride also has an antagonistic action at 5-HT_{2B} receptors although its binding at that receptor displays a higher affinity at that receptor (K_i = 13.0) than that of mianserin. Apparently, this lone similarity was not sufficient to enable mianserin to fully substitute for *rac*-amisulpride.

The benzodiazepine anxiolytic chlordiazepoxide did not substitute for *rac*-amisulpride at any of the tested doses and produced a significant suppression of response rates. Chlordiazepoxide has an affinity for benzodiazepine (BZD) sites at $GABA_A$ receptors where it exerts an agonistic effect increasing the binding of GABA to $GABA_A$ receptors. This binding profile and action is quite different from that of *rac*-amisulpride and most likely accounts for the failure of chlordiazepoxide to substitute for *rac*-amisulpride. Thus, it is apparent from the evidence in this study that the discriminative stimulus of *rac*-amisulpride at the training dose of 10 mg/kg is not shared with the antidepressant drugs tested.

Selective ligand testing. Selective ligand testing revealed interesting information in our investigation into the neural receptors responsible for the discriminative stimulus effects of *rac*-amisulpride. Compounds were selected (see Table 10) that display a high affinity for receptors relevant to *rac*-amisulpride and exerted either agonistic or antagonistic effects at those receptors.

Rac-amisulpride displays a high affinity for dopamine D_2 and D_3 receptors where it exerts antagonistic effects. The compound raclopride is selective ligand displaying a high affinity for dopamine $D_{2/3}$ where it exerts antagonistic effects, quite similar to *rac*-amisulpride at those receptors. Raclopride has no affinity for serotonin receptors. Our study revealed that raclopride failed to substitute for rac-amisulpride at any of the tested doses and had no significant effect on rate. The compound quinpirole is a selective ligand displaying a high affinity for $D_{2/3}$ receptors where it exerts an agonistic effect at those receptors. Our study revealed that quinpirole failed to substitute for *rac*-amisulpride at any of the doses tested and produced a significant suppression of rates compared to vehicle. This suggests that the discriminative stimulus effects of *rac*-amisulpride are not solely dependent upon activity at dopamine $D_{2/3}$ receptors.

To investigate the contribution of serotonin 5-HT_{2B} receptors to the discriminative stimulus effects of *rac*-amisulpride, the present study tested the compound SB-204741 which has a high affinity for 5-HT_{2B} receptors and exerts an antagonist effect at that receptor (a property shared with *rac*-amisulpride). It was found that SB-204741 did not substitute for *rac*-amisulpride at any of the doses tested with no significant effects on response rates. We tested the selective compound BW 723C86, which has a high affinity for 5-HT_{2B} receptors, but exerts an agonist effect at that receptor, unlike *rac*-amisulpride. Our results showed that BW 723C86 did not substitute for rac-amisulpride at any of the doses tested and produced significant rate suppressive

effects. These results indicate that activity at serotonin 5-HT_{2B} is not sufficient, in and of itself, to account for the discriminative stimulus properties of *rac*-amisulpride.

To investigate the contribution of the serotonin receptor 5-HT_7 to the discriminative stimulus property of *rac*-amisulpride this study tested the selective compound SB-269970 which has a high affinity for serotonin 5-HT_7 receptors and exerts an antagonistic effect at that receptor similar to *rac*-amisulpride. We found that SB-269970 did not fully substitute for *rac*-amisulpride at any of the doses tested and had no effect on rate compared to vehicle. We tested the selective compound LP-44 which has a high affinity for serotonin 5-HT_7 receptors but exerts an agonist action at that receptor. Our results showed that it failed to substitute for rac-amisulpride at any of the doses tested with no significant effect on rate. This testing demonstrated that the discriminative stimulus property of *rac*-amisulpride is not solely dependent upon activity at the serotonin 5-HT_7 receptor.

That none of the selected ligands substituted for *rac*-amisulpride provides evidence that the discriminative stimulus property of *rac*-amisulpride, most likely, cannot be attributed to the action at any one specific receptor relevant to the drug. This assessment is tempered by our results that showed sulpiride and *(S)*-sulpiride both fully substituted for *rac*-amisulpride. This is interesting as both sulpiride and *(S)*-sulpiride have rather specific and limited receptor affinities to only dopamine D_2 and D_3 receptors. A curious question is why would sulpiride and (*S*)-sulpiride substitute for *rac*-amisulpride when the similar selective ligand raclopride (having identical binding affinity and antagonistic action) did not substitute fully for *rac*-amisulpride? One could surmise that the discriminative stimulus property of *rac*-amisulpride does not completely reside in its receptor affinities but may be related to more complicated and undetermined intracellular processes pertinent to dopamine and serotonin G-protein events. Or,

that the molecular structures of sulpiride and (*S*)-sulpiride and *rac*-amisulpride are similar enough to account for why sulpiride and (*S*) sulpiride substituted fully for *rac*-amisulpride; a similarity not shared by raclopride.

Autoreceptors. The literature is replete with studies indicating that medications having a high affinity for and antagonistic action at dopamine D_2 receptors produce a decrease in motor responses in animal models; and, in human studies, trigger a wide range of behavioral deficits one would usually associate with negative symptoms in schizophrenia such as: dysphoria, anhedonia, depression, akathesia, low libido, sedation or narcolepsy. The prevailing thought is that a blockade of the dopamine D_2 receptor (and other dopamine receptors) is responsible for these behavioral deficits. *Rac*-amisulpride, despite exhibiting a strong affinity for and antagonistic action at dopamine D_2 receptors did not produce any rate suppression effects as compared to vehicle in our study, nor does the clinical literature report that it has significant behavioral deficits in humans as compared to other medications with a similar profile at dopamine D_2 receptors (e.g. haloperidol). It is plausible to suggest that *rac*-amisulpride's affinity for and antagonistic action at dopamine D_2 receptors is possibly offset by its inhibition of autoreceptors on presynaptic dopamine D_3 sites, which actually *increase* dopamine availability in the synaptic cleft. Perhaps this increase in dopamine via D_3 autoreceptor inhibition is sufficient to offset the decrease of dopamine availability produced by the blockade of dopamine at D_2 receptors. A mentioned earlier, it is suggested that *rac*-amisulpride's ability to increase dopamine via D_3 antagonism is widely believed to account for the drug's efficacy in treating depression-like symptoms, and this may account for why we found no rate suppression effects of our tested doses of rac-amisulpride on C57BL/6 mice.

Future studies. Future drug discrimination studies in our lab will test the role played by the enantiomer *(R)*-amisulpride. Our investigations to date have demonstrated that *(R)*-amisulpride does fully substitute for both *(S)*-amisulpride and *rac*-amisulpride. An investigation of *(R)*-amisulpride as the training drug and substitution testing with many of the same compounds tested in this study will provide a more complete picture of the racemic form of amisulpride and its two isomers.

Our lab is also currently conducting combination testing of the selected ligands used in the present study to examine the ability of these selected ligands to attenuate or potentiate the discriminative stimulus effects of *rac*-amisulpride (10 mg/kg training dose). Specifically, we are investigating whether the discriminative stimulus effects of *rac*-amisulpride will be potentiated when combined with raclopride which has a similar high affinity for and antagonistic effect on dopamine $D_{2/3}$ receptors; and, whether the dopamine $D_{2/3}$ agonist quinpirole combined with *rac*-amisulpride will attenuate *rac*-amisulpride's discriminative effect. We are investigating whether the combination of *rac*-amisulpride and the compound SB-269970 (which has strong affinity for and antagonistic action at serotonin 5-HT_{7A} receptors) will potentiate *rac*-amisulpride's discriminative effect; and whether the compound LP-44 (with a high affinity for and agonistic action at serotonin 5-HT_{7A} receptors) combined with *rac*-amisulpride will attenuate *rac*-amisulpride's discriminative effect. We are also investigating whether *rac*-amisulpride's discriminative effect will be potentiated by the compound SB-204741 (with a high affinity for and antagonistic action at serotonin 5-HT_{2B}); and whether the compound BW-723C86 (with a high affinity for and agonist action at serotonin 5-HT_{2B}) will attenuate *rac*-amisulpride's discriminative effect. We hope the results of this combination testing will reveal additional clues regarding the underlying receptor mechanisms involved in *rac*-amisulpride's discriminative

effect with C57BL/6 mice. Recall that none of these selective ligands when given alone substituted for *rac*-amisulpride. If *rac*-amisulpride's discriminative effect is not affected by this selective ligand combination testing, then this is more evidence to suggest that the discriminative stimulus effect of *rac*-amisulpride is not to be found at activity at any one receptor site. Instead, it would suggest it is a compound stimulus residing in *rac*-amisulpride's unique binding profile and antagonistic action at dopamine D_2, D_3 *and* serotonin 5-HT_{2B}, and 5-HT_{7A}. Or, perhaps that activity of *rac*-amisulpride at autoreceptors mediates its unique discriminative stimulus cue.

Also, future tests could investigate the utilization of transgenic or knockout mice (KO) to discern genetic influences on various receptors that are perhaps responsible for the discriminative stimulus property of *rac*-amisulpride. With the results of our study on *(S)*-amisulpride and this current study on *rac*-amisulpride it would be very interesting to conduct a study with knock out mice with inactivated genes for specific receptors (dopamine D_2, D_3 or serotonin 5-HT_{2B}, 5-HT_7) to further delineate those receptors responsible for the discriminative stimulus properties of rac-amisulpride in C57BL/6 mice.

Conclusion: The use of *rac*-amisulpride, the therapeutic form of the drug, in this drug discrimination study extended the results of our previous study with *(S)*-amisulpride. Through our testing of numerous typical and atypical antipsychotics, antidepressants, anxiolytics, and selective ligands we investigated a wide range of receptor mechanism thought to be relevant to the discriminative stimulus properties of *rac*-amisulpride in a drug discrimination assay. While this investigation ruled out a number of receptor mechanisms thought to be important in the discriminative stimulus property of *rac*-amisulpride, we reached a similar conclusion as in our preliminary investigation with *(S)*-amisulpride. The exact pharmacological properties that are the basis for the discriminative stimulus properties of *rac*-amisulpride remain an open question. That

being said, we are confident that our results have narrowed the field and established a number of important findings. Of all the drugs tested for substitution to 10 mg/kg *rac*-amisulpride, only certain benzamide derivatives substituted fully (sulpiride and *S*-sulpiride) or partially (raclopride, tiapride) for *rac*-amisulpride. This indicates that the molecular structures of these compounds are similar enough to allow them to substitute for *rac*-amisulpride; a similarity not shared among all benzamide derivatives tested. We found that the *S*-isomer and *R*-isomer substituted fully supporting the conclusion that the discriminative stimulus of *rac*-amisulpride is stereoselective. We demonstrated that none of the antipsychotics tested (typical or atypical) nor the antidepressants (regardless of class of drug) substituted for *rac*-amisulpride; nor did the anxiolytic chlordiazepoxide. Through our testing of selected dopamine and serotonin ligands (antagonists and agonists) we demonstrated that the discriminative stimulus of *rac*-amisulpride appears not to be mediated solely via affinity for and functional activity at dopamine $D_{2/3}$ or serotonin 5-HT_{2B} and 5-HT_7 receptors. These findings led us to conclude that the benzamide atypical antipsychotic *rac*-amisulpride can serve as a discriminative stimulus in C57BL/6 mice and that its discriminative stimulus is dose-dependent, time-dependent and stereoselective.

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Vita

Timothy J. Donahue was born December 30, 1950 on Guam (U.S. Territory) in the Mariana Islands and is an American citizen. His father was a career officer with the U.S. Army and raised his family in many locations in the U.S.A. and abroad before retiring in Northern Virginia. Tim graduated from Annandale High School in 1969, and went to St. Mary's Seminary & University in Baltimore, Maryland, where he received his Bachelor of Arts, *cum laude*, in Humanities: Behavioral Sciences Concentration (1973). He received a Master of Education from Virginia Commonwealth University (1976), and a Master of Humanities from the University of Richmond (1984). He began a career in teaching in 1973 at Our Lady of Lourdes Elementary School in Richmond Virginia where he taught for three years, and then at Hermitage High School (Henrico County Public Schools) for thirty-four years where he taught AP Psychology. He retired from public school teaching in 2010 to pursue graduate studies in biopsychology at V.C.U. Tim is an adjunct faculty member for the V.C.U. Department of Psychology and also teaches a college dual-enrollment course in biological psychology at the Maggie L. Walker Governor's School in Richmond.

He is a recipient of the *R.E.B. Award for Teaching Excellence*, sponsored by the R.E.B. Foundation of Richmond which awarded him a generous grant to travel and interview acclaimed neuroscientists. He was awarded a *Neuroscientist-Teacher Travel Award* by the Society of Neuroscience given to teachers who have established effective relationships with SfN neuroscientists to help them teach neuroscience in the classroom. He also received *The Hollins University Teaching Award*, supported by an endowment established by Mary Bernhardt Decker '58 and James DeWitt Becker, honoring secondary school teachers who have devoted their lives to preparing students to achieve and excel in a higher education setting.

Tim is married to Marilee R. Donahue of Youngwood, Pennsylvania and has three children, Kevin, Caitlin and Eric.

Publications

Peer-Review Journal Publications

Donahue TJ, Hillhouse TM, Webster KA, Young R, De Oliveira EO, Porter JH (2014) (S)-amisulpride as a discriminative stimulus in C57BL/6 mice and its comparison to the stimulus effects of typical and atypical antipsychotics. *European Journal of Pharmacology*. 734:15-11. doi: 10.1016/j.ejphar.2014.03.047