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The Discriminative Stimulus Properties of the Atypical Antipsychotic Clozapine in C57BL/6 Mice

Scott D. Philibin
Virginia Commonwealth University

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By
Scott D. Philibin
Department of Psychology

Master of Science, Virginia Commonwealth University, 2003
Bachelor of Science, Virginia Commonwealth University, 2000

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

Virginia Commonwealth University
Richmond, Virginia

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Table of Contents

List of Tables

List of Figures

ABSTRACT

Serotonin and α1 adrenergic receptor antagonism may contribute to atypical antipsychotic drug effects. Clozapine (2.5 mg/kg) drug discrimination in C57BL/6 mice may selectively screen atypical antipsychotic drugs. Previous data show that the atypical antipsychotics olanzapine, risperidone, ziprasidone but not the typical antipsychotic haloperidol fully substitutes for clozapine. The present study demonstrated that the atypical antipsychotics quetiapine, sertindole, zotepine, iloperidone, melperone fully substituted for clozapine but aripiprazole did not. The typical antipsychotics fluphenazine and perphenazine failed to fully substitute for clozapine but chlorpromazine and thioridazine fully substituted for clozapine. This model does not differentiate between atypical and typical antipsychotic drugs but it may be useful in the detection of antipsychotics with potent serotonin and α1 adrenergic receptor antagonist actions.

Introduction

Etiology and phenomenology of schizophrenia

Schizophrenia is a severe and complex neurological disorder. Approximately 1% of the population worldwide is afflicted with schizophrenia. The disease commonly manifests in late adolescence or early adulthood with roughly equal prevalence among young men and women. A genetic predisposition (endophenotype) linked to an environmental "insult" may be required for expression of the disorder. Schizophrenia is associated with a high degree of mortality as approximately 5% of patients commit suicide.

The observed syndrome was initially termed *dementia praecox* based on the early onset of the disorder, progressive deterioration in cognition and generally poor prognosis (Kraeplin, 1896). It was later termed *schizophrenia*, emphasizing the fragmentation of volition, behavior, emotion as well as cognition (not split or multiple personality disorder) (Bleuler, 1930). The current diagnostic criteria for schizophrenia is defined in the *Diagnostic and Statistical Manual – IV (DSM-IV)* (American Psychiatric Association [APA] Task Force on DSM-IV, 1994)

Schizophrenic symptoms are generally characterized into positive, negative, and cognitive symptoms. Positive symptoms, such as delusions and/or hallucinations, are conceptualized as abnormalities in normal function (e.g. reality testing); whereas, negative symptoms of schizophrenia have been categorized as deficits in normal functioning. Delusions may consist of irrational or false beliefs of grandeur or persecution even in the face of direct contradictive evidence. Those suffering from schizophrenia may experience positive symptoms like hallucinations (e.g., false auditory stimuli, such as hearing inner voices giving them

instructions). Negative symptoms can consist of affective flattening, social withdrawal, anhedonia, alogia and avolition.

A striking feature of schizophrenia is the early onset, persistence and progressive worsening of cognition in patients with schizophrenia. Cognitive impairments in schizophrenia consist of deficits in executive functioning, verbal learning and memory, vigilance, working memory and fine motor performance. The cognitive aspects of schizophrenia are receiving increased attention as targets in the development of novel antipsychotic drugs (Laughren & Levin, 2005; Meltzer & McGurk, 1999). This new focus is also evident in the development of current programs such as the Measurement and Treatment Research to Improve Cognition in Schizophrenia (NIMH-MATRICS).

There are also schizophrenic subtypes according to the DSM-IV. The patient experiencing systematic delusions of persecution represents *paranoid* schizophrenia. *Disorganized* or hebephrenic schizophrenia is particularly severe and characterized by an early onset, a wide range of symptoms and an extensive deterioration of personality. *Catatonic* schizophrenia can result in the exhibition of abnormal postures by the patients for extended periods of time.

Psychopharmacology of Schizophrenia

The introduction of antipsychotic drugs in the 1950s transformed the treatment of schizophrenia. Use of antipsychotic drugs to effectively treat psychosis contributed greatly to the community mental health movement and increased the drive for deinstitutionalization of patients. The first widely accepted hypothesis for a neurochemical dysfunction in schizophrenia was the "dopamine (DA) hypothesis". This notion postulates schizophrenia is linked to a hyperfunctional

DA system or an excess of DA neuronal activity in the mesolimbic system. This theory is based largely on pharmacological evidence; antipsychotic drugs (e.g., phenothiazines) may treat psychosis by decreasing the level of dopaminergic activity and some drugs (e.g., amphetamine) may be psychotomimetic by increasing dopaminergic activity.

The approval of clozapine in the United States in the 1990s initiated a vast improvement in the pharmacotherapy of schizophrenia. Clozapine is the prototype for a novel class of antipsychotic drugs superior to conventional neuroleptic agents, such as haloperidol. Clozapine is not a neuroleptic, i.e. it does not produce catalepsy in rodents. Clozapine is not associated with extrapyramidal symptoms (EPS) and refutes the notion that therapeutic efficacy and EPS are inextricably linked. Clozapine has a complex receptor binding profile and its mechanism of action in the treatment of schizophrenia has not been determined.

Antipsychotic drugs used for the treatment of schizophrenia vary in their ability to selectively treat this wide array of symptoms (Kane, 1999). Encouragingly, investigations of antipsychotic drug actions are furthering our understanding of how receptor specific effects exert their therapeutic effects. The selective targeting of receptor subtypes in the pharmacological treatment of schizophrenia will ultimately lead to antipsychotic drugs with increased therapeutic efficacy and a reduced side effect liability.

The complex actions of the atypical antipsychotic clozapine on various neurotransmitter receptor subtypes (see Table 1) have focused drug development on multiple receptor targets. Clozapine is atypical because it does not demonstrate the EPS profile of typical antipsychotic drugs like haloperidol and chlorpromazine (Arnt & Skarsfeldt, 1998; Ellenbroek, 1993). Clozapine has increased therapeutic efficacy for both the positive and negative symptoms of

DISSOCIATION RATE CONSTANTS (K_i) FOR ANTIPSYCHOTIC DRUGS AT SELECTED NEUROTRANSMITTER RECEPTOR SUBTYPES (Schotte et al. 1996)

Species *(except where indicated):*	RAT	RAT	RAT	RAT
Source *(except where indicated:*	Frontal Cortex	Striatum	Striatum	Total Cortex
ATYPICALS	**5-HT2A**	**D2**	**M**	**α 1**
Clozapine	3.3	150	34	23
Olanzapine	1.9	17	26	60
Risperidone	0.16	3.3	>5,000	2.3
Quetiapine	120	310	1,020	58
Sertindole	0.85	7.4	>5,000	1.8
Ziprasidone	0.31	9.7	>5,000	12
Zotepine	0.91	13	550	3.4
Iloperidone	0.2[5]	3.3[5]	6000[5]	0.31[5]
Melperone	102.0[5]	180[5]	10,000[5]	180[5]
Aripiprazole	8.7[7]	3.3[7]	6,780 (M_1)[7]	25.7[7]
CLOZAPINE METABOLITE				
N-desmethylclozapine	10.9[8]	115.2[8]	67.6 (M_1)[8]	104.8[8]
TYPICALS				
Haloperidol	25	1.4	4,670	19
Fluphenazine	19[6]	0.8[4]	1,900[4]	9.0[4]
Perphenazine	5.6[6]	1.4[4]	1,500[4]	10.0[4]
Chlorpromazine	3.3[2]	1.2[1]	378[3]	14[3]
Thioridazine	6.3[1]	7.9[1]	18[4]	5[4]

1. Roth et al. 1995 (rat brain) 2. Leysen et al.. 1982 (rat cortex) 3. Hals et al. 1986 (rat brain) 4. Richelson 1984 (human brain, K_d) 5. Richelson and Souder 2000 (human brain, K_d) 6.Wander et al. 1987 (human cortex, K_d) 7. Shapiro et al. 2003 (human cloned) 8. Roth et al. 2000 (human cloned)

schizophrenia, and it does not increase serum prolactin levels (Meltzer, 1992). Clozapine is not associated with tardive dyskinesia, and can actually attenuate pre-existing tardive dyskinesia (Meltzer & Luchins, 1984). Clozapine has a high efficacy in a substantial number of schizophrenic patients that are resistant to traditional antipsychotic drug treatment (Kane, Honigfeld, Singer, & Meltzer, 1988; Meltzer, 1997) and also shows reduced risk of suicide relative to typical antipsychotic drugs (Meltzer, 2001).

Clozapine is a dibenzodiazepine that binds with a relatively low affinity for D_2 receptors and has a relative higher binding affinity for the D_1 receptor (Schotte, Janssen, Gommeren, Luyten, Van Gompel, Lesage, De Loore, & Leysen, 1996). It also has a slightly more potent affinity for the D_4 than the D_2 receptor. Typical or conventional neuroleptic antipsychotics such as haloperidol, a butyropherone, and chlorpromazine, a phenothiazine, have the opposite relative dopamine receptor potencies ($D_2 >> D_1$); these agents are potent dopamine D_2 antagonists. Clozapine also has high affinity for several other neurotransmitter receptor subtypes including serotonergic, muscarinic, noradrenergic and histaminergic systems (Schotte et al., 1996). The diverse receptor binding profile of clozapine is believed to contribute to its unique atypical antipsychotic effects and many novel antipsychotic drugs also display complex receptor binding profiles (see Table 1). Spatial distributions or the regional selectivity of dopamine receptor tracts is another distinction made between atypical and typical antipsychotic drugs.

Antipsychotic drugs target different subcortical structures including the brain stem, hypothalamus, basal ganglia, and mesolimbic system (Bradley, 1986). Typical antipsychotics antagonize dopamine receptors in both the mesolimbic pathway and the striatal pathway (Bunney & Grace, 1978; Chiodo & Bunney ,1983; 1985). Atypical antipsychotics, such as clozapine, bind

to dopamine receptor subtypes that are expressed primarily in the mesolimbic system and cortex. These receptors are only weakly expressed in the basal ganglia and may provide one explanation for the lack of EPS associated with atypical antipsychotic drugs (Bradley, 1986).

The Dopamine Hypothesis of Antipsychotic Effects

There are five major subtypes of dopamine receptors (D_1-D_5) in the human brain (Seeman, 1992; Seeman, Corbett, Nam, & Van Tol, 1996). Types 1 and 5 have shared structural and signaling properties that comprise the D_1-receptor class (Sunahara, Guan, O'Dowd, Seeman, Laurier, L.G., Ng, G., George, Torchia, Van Tol, & Niznik,1990; Sunahara, Niznik, Weiner, Stormann, Brann, Kennedy, Gelernter, Rozmahel, Yang, & Israel, 1991). Types 2-4 are also somewhat similar in structure and comprise the D_2-receptor class. Dopamine D_2 receptors appear to possess more clinical relevance to antipsychotic drug effects.

The D_1 and D_5 (also called D_{1b}) are G protein (G_s) coupled receptors. The heterotrimeric G proteins cause sequential activation of adenylate cyclase, cyclic AMP-dependent protein kinase, and mediate D_1-like receptor signaling. The increased phosphorylation that results from the combined effects of activating cyclic AMP-dependent protein kinase and inhibiting protein phosphatase 1 regulates the activity of many receptors, enzymes, ion channels, and transcription factors. The D_1-like receptor also signals via phospholipase C-dependent and cyclic AMP-independent mobilization of intracellular calcium. D_1 and D_5 receptors are primarily found in the cerebral cortex and hippocampus (D_1 is also expressed in the caudate nucleus) (Neve, Seamans, & Trantham-Davidson, 2004).

Dopamine D_1 receptor antagonists do not demonstrate therapeutic effects in the treatment of psychotic symptoms (Karlson, Smith, Farde, Harnryd, Sedvall, & Wiesel, 1995; Den Boer,

van Megen, Fleischhacker, Louwerens, Slaap, Westenberg, Burrows, & Srivastava, 1995; de Beaurepaire, Labelle, Naber, Jones, & Barnes, 1995). Also, therapeutic dose ranges of various antipsychotic drugs occupy low or negligible levels of D_1 receptors in the brains of psychotic patients (Farde & Nordstrom, 1992). Although therapeutic dose levels of clozapine occupy approximately 36-59% of brain dopamine D_1 receptors, there is little evidence to support the importance of the D_1 receptor in the unique therapeutic effects of clozapine.

The heterotrimeric G proteins mediate D_2-like receptor signaling. These pertussis toxin-sensitive G proteins regulate some effectors, such as to decrease adenylate cyclase, via their G-alpha subunits, but regulate many more effectors such as ion channels, phospholipases, protein kinases, and receptor tyrosine kinases as a result of the receptor-induced liberation of G-betagamma subunits. In addition to interactions between dopamine receptors and G proteins, other protein-protein interactions also occur that are critical for regulation of dopamine receptor signaling (Neve et al., 2004).

Typical antipsychotic drugs block post-synaptic dopamine D_2 receptors (Carlsson & Lindqvist 1963). The clinical potencies of antipsychotic drugs, as well as to induce EPS, directly correlate with their ability to antagonize dopamine D_2 receptors (Seeman, Staiman, Lee, & Chau-Wong,1974; Seeman, Chau-Wong, Tedesco, & Wong, 1975; Seeman, Lee, Chau-Wong, & Wong, 1976). This is not the case with D_3 or D_4 receptor subtypes. The dopamine D_2 receptors are 60%-80% consistently occupied by therapeutic doses of antipsychotic drugs when directly measured using positron emission tomography (PET) or single photon emission tomography (SPET) (Farde, Nordstrom, Halldin, Wiesel, & Sedvall., 1992a; Farde, Nordstrom, Wiesel, Pauli, Halldin, & Sedvall, 1992b; Nyberg, Nordstrom, Halldin, & Farde, 1995; Kapur, Remington,

Zipursky, Wilson, & Houle,1995; Kapur, Remington, Jones, Wilson, DaSilva, Houle, & Zipursky, 1996; Kapur, Zipursky, Remington, Jones, DaSilva, Wilson, & Houle, 1998; Kapur, Cho, Jones, McKay, & Zipursky, 1999; Heinz, Knable, & Weinberger, 1996) in the human striatum (i.e., the caudate nucleus and/or the putamen). Clozapine and another atypical antipsychotic drug, quetiapine, however, had low D_2 receptor occupancy.

Dopamine D_2 receptors are primarily expressed in the caudate nucleus, putamen, nucleus accumbens, amygdala, hippocampus, and cerebral cortex. Dopamine D_2 receptors expressed in the caudate nucleus and putamen are suggested to contribute to the EPS effects of these drugs and D_2 receptors expressed in regions such as the amygdala, hippocampus, and cortical areas may be the sites important for antipsychotic efficacy (Stevens, 1973) . However, if the D_2 receptor is not a common target for all antipsychotic drugs then novel mechanisms of action can be postulated.

Clozapine is a consistent exception to the "70%" rule of D_2 receptor occupancy. Therapeutically effective doses of clozapine occupy only up to approximately 50% of striatal brain dopamine receptors, as measured by various radioligands using PET (Farde et al. 1992; 1992b; Kapur et al. 1996; 1998; 1999; Karbe et al. 1991; Louwerens, Buddingh, Zijlstra, Pruim, Paans, Vaalburg, & Sloof, 1993; Conley, Medoff, Wong, & Tamminga, 1995; Conley, Zhao, Wong, & Tamminga, 1996) or SPET (Busatto, Pilowsky, Costa, Ell, Verhoeff, & Kerwin.1995; Klemm, Grunwald, Kasper, Menzel, Broich, Danos, Reichmann, Krappel, Rieker, Briele, Hotze, Moller, & Biersack,1996; Scherer, Tatsch, Schwarz, Oertel, Konjarczyk, & Albus, 1994; Pilowsky, Busatto, Taylor, Costa, Sharma, Sigmundsson, Ell, Nohria, & Kerwin, 1996; Su, Breier, Copolla, Hadd, Elman, Adler, Malhotra, Watsky, Gorey, Weinberger, & Pickar, 1996a;

Su, Breier, Copolla, Hadd, Elman, Adler, Malhotra, Watsky, Gorey, Hough, Weinberger, & Pickar, 1996b; Pickar, Su, Weinberger, Coppola, Malhotra, Knable, Lee, Gorey, Bartko, Breier, & Hsiao, 1996). The conclusion that D_2 receptors are not the main antipsychotic target for clozapine (Farde et al. 1992b; Brunello et al. 1995) is premature because it does not take into consideration extrastriatal D2 receptors which are just beginning to be studied with appropriate ligands such as 11C- epidipride. It is beyond the scope of this dissertation to consider the possible importance of the ability of clozapine and other atypical antipsychotic drugs to differentially occupy cortical and ventral tegmental D2 receptors compared to typical antipsychotic drugs. This is an emerging story of great importance (Meltzer, HY, personal communication, 5/10/2006).

The dopamine hypothesis of schizophrenia may be oversimplified. Administration of the dopamine precursor L-DOPA actually alleviates psychotic symptoms in some chronic refractory schizophrenic patients (Alpert & Friedhoff 1980). Clinical reduction of psychotic symptoms is also not an immediate consequence of antipsychotic drug treatment. Attenuation of psychotic symptoms does not generally appear until after several weeks of chronic administration, presumably following the stabilization of the dopaminergic receptor system in response to chronic treatment with potent D_2 antagonists. Amphetamine-induced psychosis in normal human controls, in contrast, shows immediate improvement after acute antipsychotic drug administration (Zeidman, Oscherwitz, & Addario, 1975).

These discrepancies suggest important differences between the dopamine hypothesis of the disorder and the pathology of the disease. The dopamine hypothesis, however, remains critical to our understanding of the neuropathology of schizophrenia. Investigating and testing

the dopamine hypothesis have allowed many scientists doing basic and clinical research to increase our knowledge of the function of dopamine and how it relates to the behavioral pharmacology of schizophrenia. Novel antipsychotic agents that are relatively weaker dopamine antagonists also target other neurotransmitter receptor systems. There is a growing body of evidence from the heterogeneous group of atypical antipsychotic drugs with their diverse receptor binding profiles that dopamine is not the only important neurotransmitter receptor target for an effective antipsychotic drug.

Serotonergic actions are crucial for atypical antipsychotic effects

The indoleamine lysergic acid diethylamide (LSD) is a serotonergic agonist that has hallucinogenic properties. Knowledge of these psychotomimetic pharmacological effects helped initiate a search for a link between schizophrenia and the serotonin receptor system (Wooley & Shaw 1954). Serotonin (5-HT) receptor based mechanisms may be an important component for the actions of atypical antipsychotic drugs.

A principle characteristic among atypical antipsychotic drugs that differentiate them from typical antipsychotic drugs is more potent 5-HT_{2A} receptor antagonism relative to weaker dopamine D_2 receptor antagonism (Meltzer, Matsubara, & Lee, 1989). This pharmacological profile is consistent with the atypical antipsychotics clozapine, olanzapine, risperidone, quetiapine and ziprasidone (Creese, Burt, & Snyder, 1976; Meltzer, 1999; Meltzer & Stahl 1976; Kapur & Seeman 2000; Reynolds, 1996). The 5-HT_{2A}/D_2 model has generated many novel antipsychotic drugs with efficacy for psychosis, cognition and low EPS, e.g. asenapine, laurisdone, iloperidone and perospirone. Subsequent *in vivo* studies have also demonstrated that

atypical antipsychotic drugs have a higher receptor binding affinity for 5-HT_{2A} versus D_2 receptors (Stockmeier, DiCarlo, Zhang, Thompson, & Meltzer, 1993; Matsubara, 1993).

Clozapine has a high affinity for several other 5-HT neurotransmitter receptor subtypes implicated in its unique effects, including 5-HT_{2A}, 5-HT_{1A}, 5-HT_{2C}, 5-HT_3, 5-HT_6 and 5-HT_7 (Meltzer & Nash 1991). 5-HT_{1A} receptor agonist activity may also be an important factor that can contribute to an atypical antipsychotic drug profile (Vander-Maelen, 1990; Ichikawa, Ishii, Bonaccorso, Fowler, O'Laughlin, & Meltzer, 2001; Wadenberg & Ahlenius 1991). Atypical antipsychotics clozapine, quetiapine, ziprasidone and aripiprazole are direct 5-HT_{1A} agonists, but risperidone and olanzapine are not. However, all of these agents increase dopamine release in the medial prefrontal cortex (PFC) of rats; this effect is blocked by the 5-HT_{1A} antagonist WAY100635 (Ichikawa et al., 2001). The increase in dopamine release in the medial PFC by these atypical agents demonstrates another common component of the 5-HT_{2A}/D_2 antagonists, including aripiprazole. The 5-HT_{2A} and 5-HT_{1A} subtypes may be the two most important 5-HT receptors for unique antipsychotic action.

5-HT_{2A} receptors are widely distributed in brain areas, especially in the cortex (Hoyer, Pazos, Probst, & Palacios, 1986); they are found in a number of regions, including the olfactory tubercle, frontal, parietal, cingulate, and entorhinal cortices, midbrain, thalamus, dentate gyrus, caudate-putamen, nucleus accumbens and septum (Leysen, Gommeren, Heylen, Luyten, Van de Weyer, Vanhoenacker, Haegeman, Schotte, Van Gompel, Wouters, & Lesage, 1996). 5-HT_{2A} and 5-HT_{1A} receptors are co-localized on the cortical and hippocampal pyramidal glutamatergic neurons (Hirose, Sasa, Akaike, & Takaori,.1990; Jakab & Goldman-Rakic, 1998) and on GABAergic interneurons (Willins, Deutch, & Roth, 1997). Both receptor subtypes are implicated

in schizophrenia. 5-HT_{2A} receptors located on GABAergic interneurons have modulatory control of neuronal inhibition (Cozzi & Nichols 1996; Abi-Saab, Bubser, Roth, & Deutch, 1999). 5-HT_{2A} receptors are also found in the substantia nigra and ventral tegmentum, important dopamine tracts in the nigrostriatum and mesocorticolimbic areas. This suggests a potential modulatory role in the effects of various aspects of antipsychotic effects.

Concomitant blockade of 5-HT_{2A} receptors and D_2 receptors causes marked increases in extracellular dopamine concentrations in the mesocortical projection areas relative to the nigrostriatal and mesolimbic pathways (Volonte, Monferini, Cerutti, Fodritto, & Borsini, 1997; Kuroki, Meltzer, & Ichikawa, 1999; Rollema, Lu, Schmidt, Sprouse, & Zorn, 2000; Rowley, Needham, Kilpatrick, & Heal, 2000; Stephenson et al. 2000; Westerink, Kawahara, De Boer, Geels, De Vries, Wikstrom, Van Kalkeren, Van Vliet, Kruse, & Long, 2001; Ichikawa, Li, Dai, & Meltzer. 2002). These cortical effects on dopamine release of clozapine are not reproduced by the typical antipsychotic drug haloperidol (Marcus, Malmerfelt, Nyberg, & Svensson. 2002). These neurochemical data are consistent with behavioral data showing the potentiation of antipsychotic effects of D_2 receptor antagonists by 5-HT_{2A} antagonists in animal models of antipsychotic effects.

For example, ritanserin increases the effect of raclopride to block conditioned avoidance responding in rats (Wadenberg, Salmi, Jimenez, Svensson, & Ahlenius, 1996). Low-dose haloperidol was augmented with ritanserin to improve negative symptoms (Duinkerke, Botter, Jansen, van Dongen, van Haaften, Boom, van Laarhoven, & Busard, 1993) and monotherapy with ritanserin has been shown to improve both positive and negative symptoms in the absence of any dopamine D_2 antagonistic effects (Wiesel, Nordstrom, Farde, & Eriksson.1994).

Ritanserin, unfortunately, is associated with adverse cardiovascular side effects. Other 5-HT_{2A} antagonists, such as M100907, SR46349-B and ACP-103 could possibly be used as adjunctive antipsychotic treatments or as a monotherapeutic approach. It may be possible to generate a clozapine-like profile with haloperidol by augmentation with a 5-HT_{2A} receptor antagonist such as M100907 (Liegeois, Ichikawa, & Meltzer, 2002).

While numerous atypical antipsychotic drugs bind with high affinity to 5-HT_{2A} receptors (Meltzer et al. 1989), this is also true of some typical antipsychotic drugs, such as chlorpromazine and spiroperidol. Further, there are various atypical antipsychotic drugs that do not bind with high affinity to 5-HT_{2A} receptors, such as the substituted benzamide agents sulpiride, sultopride, amisulpiride, raclopride, remoxipride and tiapride (Chivers, Gommeren, Leysen, Jenner, & Marsden, 1988). These substituted benzamides are highly specific D_2/D_3 antagonists (Burt, Enna, Creese, & Snyder, 1975; Seeman et al. 1975; Sokoloff, Giros, Martres, Bouthenet, & Schwartz, 1990; Gessa, Canu, Del Zompo, Burrai, & Serra, 1991). This class is differentiated from the 5-HT_{2A}/D_2 receptor antagonist class; D_3 antagonist actions may be crucial for the effects of these atypical agents.

However, the 5-HT_{2A}/D_2 receptor antagonist model has had a great impact on the development of many compounds that fit this receptor binding profile (see review by Meltzer, 1999). There are increasing data obtained from microdialysis and electrophysiological studies suggesting how 5-HT_{2A} receptor antagonists modulate dopaminergic activity differentially in the nigrostriatal, mesolimbic, and mesocortical systems (Kuroki et al. 1999; Liegeois et al. 2002; Moghaddam & Bunney 1990). The $5HT_{2A/2B/2C}$ antagonist ritanserin had little effect on the dopamine system in any brain region by itself, but dopamine release in the prefrontal cortex was

observed when combined with the $D_{2/3}$ antagonist raclopride while the striatum remained unaffected (Andersson et al. 1995).

5-HT_{2A} receptor antagonism may also be involved in the lowered risk of suicide in schizophrenic patients by its effects on dopamine and norepinephrine release (Meltzer et al. 2003). More potent 5-HT_{2A} receptor blockade may allow for therapeutic doses at relatively low D_2 occupancy rates: 55% 5-HT_{2A} versus 30%-50% D_2 (Leysen et al. 1996) versus 80%-100% occupancy rates for haloperidol. 5-HT_{2A} receptor antagonist actions may also be a crucial effect for the treatment of refractory patients (Meltzer 1997). 5-HT_{2A} receptor antagonist actions that modulate dopamine appear to be important for the unique therapeutic effects of atypical antipsychotic drugs on cognition, negative symptoms and antipsychotic actions.

5-HT_{2A} receptor antagonism concurrent with weaker D_2 blockade may allow atypical antipsychotics to increase dopamine release in the medial prefrontal cortex while having a smaller effect on dopamine mesolimbic release. This may be crucial for the atypical antipsychotic advantages for cognition, negative symptoms and antipsychotic effects. 5-HT_{2A} receptors have been implicated in psychosis, negative symptoms, EPS and mood disorders (Leysen et al. 1996; Meltzer & Fatemi 1996). This notion is supported with results from a range of mixed 5-HT_{2A}/D_2 receptor antagonists, including the atypical antipsychotics risperidone, olanzapine, ziprasidone, zotepine and quetiapine.

Muscarinic Cholinergic Receptor effects on EPS and Psychosis

The highest affinity for a muscarinic receptor subtype for clozapine or olanzapine is for the M_1 receptor (Bymaster, Hemrick-Luecke, Perry, & Fuller 1996). There are currently five identified muscarinic receptor subtypes termed M_1-M_5 (Peralta et al. 1987; Bonner et al. 1987;

Buckley et al. 1988). Clozapine and olanzapine have similar high receptor binding affinities for the muscarinic cholinergic receptor subtypes, particularly the M_1 receptor subtype (Bymaster et al., 1996). The major difference between clozapine and olanzapine is that olanzapine is nonselective for dopamine and muscarinic receptors. Clozapine, in contrast, is much more selective (>10 fold) for muscarinic than dopamine receptors. Clozapine was originally thought to be a full muscarinic antagonist and olanzapine was developed as an agent with potent antimuscarinic properties.

Oxotremorine-induced tremors in rodents are blocked by both clozapine and olanzapine (Moore, Tye, Axton, & Risius, 1992). Clozapine blocks oxotremorine and arecoline-induced accumulation of acetylcholine in the rat striatum (Racagni, Cheney, Trabucchi, & Costa, 1976). The muscarinic antagonists atropine and scopolamine, however, attenuate the effect of clozapine to increase extracellular dopamine levels and dopamine metabolites in the striatum; these muscarinic antagonists are ineffective against similar effects of haloperidol and thioridazine (Rivest & Marsden, 1991; Meltzer, Chai, Thompson, & Yamamoto. 1994). These results may indicate that central muscarinic receptors modulate dopamine in the actions of clozapine but not in the mechanism of typical antipsychotic drugs.

There are muscarinic subtypes located in limbic structures of the brain associated with schizophrenia, such as the nucleus accumbens and the prefrontal cortex (Levey, Kitt, Simonds, Price, & Brann, 1991). Colocalization of muscarinic and dopamine receptors are found (Weiner, Levey, & Brann, 1990; Bernard, Normand, & Bloch, 1992) and muscarinic receptors on dopamine nerve terminals potentiate dopamine release (Anden & Stock 1973; Bymaster, Reid, Nichols, Kornfeld, & Wong, 1986; Bymaster, Wong, Mitch, Ward, Calligaro, Schoepp,

Shannon, Sheardown, Olesen, & Suzdak, 1994). These interactive effects with dopamine have properties most similar to the M_1 receptor subtype but a lack of selective muscarinic antagonists does not permit definitive conclusions.

In vivo effects of clozapine and olanzapine are weaker than would be predicted by in vitro radioligand binding studies (Moore et al., 1997; Beasley et al., 1997; Arnt & Skarsfeldt, 1998). Clozapine and olanzapine have dissimilar characteristics compared to prototypical muscarinic antagonist ligands with reference to muscarinic receptor binding affinities across binding assays conducted in different ionic strength preparations. Whether the muscarinic actions of clozapine and olanzapine are important for their therapeutic effects has not been determined.

Anticholinergic effects may contribute to the lack of EPS seen with clozapine and olanzapine (Bymaster et al., 2003). Concurrent administration of anticholinergic drugs with typical antipsychotic drug treatment reduces EPS without attenuating the therapeutic effects, supporting a link between the cholinergic and dopaminergic systems. Treatment with anticholinergic agents to treat neuroleptic-induced EPS remains a mainstay of treatment. The alleviation of severe motor side effects by antimuscarinic drugs is also supported in preclinical research as they reverse cataleptic effects of typical antipsychotic drugs (Erzin-Waters, Muller, & Seeman, 1976; Sayers, Burki, Ruch, & Asper, 1976; Ahlenius & Hillegaart 1986).

Coadministration of antimuscarinic agents to alleviate the movement disorders induced by chronic typical antipsychotic drug treatment does not, however, result in a clozapine-like clinical profile (Sayers, Burki, Ruch, & Asper, 1975; Lowe, Seeger, & Vinick, 1988). This would support the notion that antimuscarinic drugs only abolish the induced EPS side effects, leaving antipsychotic potency relatively unaffected. The role of cholinergic neurotransmission in

the antipsychotic effects of clozapine and olanzapine may be a unique effect. There is recent research that focuses on muscarinic receptor subtypes as potential therapeutic targets for atypical antipsychotic drugs (Bymaster, Felder, Tzavara, Nomikos, Calligaro, & McKinzie, (2003).

Early evidence suggested that stimulation of the central cholinergic receptor system with the use of cholinesterase inhibitors was beneficial in some schizophrenic patients (Pfeiffer & Jenney ,1957; Rosenthal & Bigelow, 1973). 50% of patients suffering from Alzheimer's disease have psychotic symptoms (White & Cummings, 1996). Delusions appearing in Alzheimer's patients are reduced by the cholinesterase inhibitor physostigmine (White & Cummings, 1996). Xanolemine is a relatively selective muscarinic partial agonist at M_1 and M_4 receptors that reduced psychotic symptoms in Alzheimer's patients (Bodick, Offen, Levey, Cutler, Gauthier, Satlin, Shannon, Tollefson, Rasmussen, Bymaster, Hurley, Potter, & Paul, 1997). Preclinical research may also indicate potential antipsychotic effects involved with anticholinergic neurotransmission.

In animal models of psychotomimetic effects known to be mediated by dopamine, the xanomeline analog, 6-(3-propylthio-1,2,5-thiadiazol-4yl)-1-azabicyclo[3.2.1]-octane (PTAC), and oxotremorine RS86 and pilocarpine antagonized these effects (Bymaster et al., 1998; Fink-Jensen, Kristensen, Shannon, Calligaro, Delapp, Whitesitt, Ward, Thomsen, Rasmusseen, Sheardown, Jeppesen, Sauerberg, & Bymaster, 1998). Dopamine D_1 and D_2 receptor agonist induced contralateral rotation in unilaterally 6-OHDA-lesioned rats was attenuated by these cholinergic agents. PTAC also inhibits conditioned avoidance responding in rats, apomorphine-induced climbing in mice, and spontaneous locomotor activity in rats without the induction of

catalepsy (Bymaster et al., 1998). These *in vivo* effects were blocked by the muscarinic antagonist scopolamine.

These data suggest that anticholinergic effects influencing dopaminergic neurotransmission mediate the effect of PTAC on the animal models of psychosis. While this provides compelling evidence for a role of cholinergic neurotransmission in the symptomology of psychosis, this may not be the only means by which an atypical antipsychotic profile can be achieved. It is important to note that the atypical antipsychotic risperidone has a pharmacological profile similar to that of olanzapine except that it possesses negligible muscarinic affinity.

Noradrenergic Receptor Mediated Effects

Clozapine and olanzapine have high affinity for the α-adrenergic receptor but no affinity for the β–adrenergic receptor at concentrations up to 10 μM (Moore et al. 1993). Clozapine has roughly equal affinities for both α_1 and α_2 adrenergic receptor subtypes (K_i = 7 and 8 nM respectively). Olanzapine has a 10-fold higher selective affinity for the α_1 adrenergic receptor (K_i = 19 nM) than for the α_2 adrenergic receptor (Moore et al., 1992). Many atypical antipsychotic drugs, such as sertindole, risperidone, quetiapine, zotepine and ziprasidone have a high α adrenergic receptor binding affinity (Schotte et al., 1996).

It has been suggested that α_1 adrenergic receptor blockade contributes to the diminished side effects of clozapine (Baldessarini, Huston-Lyons, Campbell, Marsh, & Cohen, 1992). Chronic administration of haloperidol coadministered with the α_1 adrenergic antagonist prazosin produces the characteristic pattern of A10 depolarization with no effect on A9, but this was not effective with the α_2 adrenergic antagonist idazoxan (Chiodo & Bunney 1985). The α_1 adrenergic receptor blockade of clozapine may differentially affect nigrostriatal and mesolimbic

dopamine release and contribute to the lack of EPS. This is not easy to support, however, using neuroleptic-induced catalepsy as a model of EPS. A potentiating effect of α_1 adrenergic receptor blockade with prazosin or WB4101 has been found on haloperidol-induced catalepsy but the α_2 antagonists yohimbine, RX 82002 and MK-912 attenuated this effect dose dependently (Kalkman, Neumann, Hoyer, & Tricklebank, 1998). Clozapine treatment causes upregulation of α_1 adrenergic receptors but not dopamine receptors in the forebrain. Typical antipsychotic drugs only cause upregulation of dopamine receptors.

It is difficult to paint a clear picture of the relevance of noradrenergic transmission with respect to antipsychotic activity. A behavioral assay attempting to produce the pharmacological profile of clozapine in an animal model of antipsychotic efficacy demonstrated that α_1 adrenergic, D_1, and 5-HT_2 antagonist in combination was required; no two combinations alone had any effect (Prinssen, Ellenbroek, & Cools, 1994b). There are now an increasing number of new antipsychotic drugs that target α adrenergic receptor subtypes.

In vivo receptor mechanisms of action

The mechanism(s) of action for clozapine and other atypical antipsychotic drugs in the treatment of schizophrenia may be concurrent actions on several neurotransmitter receptor subtypes. Much evidence suggests there may be multiple target sites important for the therapeutic effects and reduced side effects of atypical antipsychotic drugs. The neurotransmitter receptor systems dopamine, serotonin, acetylcholine, glutamate, and norepinephrine are all candidate targets for the selective treatment of symptoms. It is therefore important to characterize the *in vivo* and *in vitro* receptor binding profiles of atypical antipsychotic drugs to help elucidate the pharmacology of schizophrenia.

The receptor mechanisms of action also can be examined using classical behavioral pharmacological techniques. Preclinical researchers have used a variety of animal models to characterize the receptor-mediated effects of clozapine *in vivo*. This research approach allows the study of the functional effects of drugs in the living organism. One method by which atypical antipsychotic drugs can be differentiated from typical antipsychotic drugs is to evaluate the correspondence between receptor profiles. While *in vitro* results cannot always be transferred to studies *in vivo* where receptors are present in their natural environment, pharmacological properties produce many behavioral specific actions that reflect receptor activity. Thus, both *in vitro* and *in vivo* models of mechanism of pharmacological action are necessary.

An animal model that has predictive validity is crucial for the development of antipsychotic drugs and elucidating their mechanism of action. There is currently a lack of adequate preclinical models that help elucidate the mechanisms underlying crucial differences between the actions of typical vs. atypical antipsychotic drugs. Further, many of these models are capable of differentiating between antipsychotic and other psychotropic drugs but only a few seem to be able to differentiate between typical and atypical antipsychotics. Examples of models that do strive to distinguish the two major classes of antipsychotic drugs are the paw test and prepulse inhibition (PPI) in rats (Geyer & Ellenbroek 2003). A model with predictive validity should be useful in the detection of putative atypical antipsychotic drugs.

Face validity refers to the phenomenological similarity between the model and the disease. Construct validity refers to theoretical constructs of schizophrenia based on the pathophysiological processes underlying the disease. However, in practical application of these

criteria, the elements of predictive validity related to therapeutic response are most commonly used to establish criteria for model validity.

Assessment of Preclinical Model Validity

1. Antipsychotic drugs of different chemical classes. Antipsychotic drugs belong to a number of structurally unrelated chemical classes and therefore, various chemical classes of antipsychotic drugs should also be effective in an animal model: Phenothiazines (chlorpromazine, fluphenazine, perphenazine and thioridazine), thioxanthenes (thiothixene and flupenthixol), butyrophenones (haloperidol, benperidol, iloperidone and melperone), dibenzazepines (clozapine, olanzapine and loxapine), benzamides (sulpiride and remoxipride), piperazinyl-dibenzothiazepines (quetiapine). The model is validated by its sensitivity to various antipsychotic drugs.

2. No false positives. Nonantipsychotic drugs should not have any effect. The model should be insensitive to nonantipsychotic drugs. Severe motor effects should be differentiated from the primary measure (e.g., anhedonia vs. motor disruption). For example, antidepressants from a number of pharmacological classes, including tricyclic antidepressants, selective serotonin or norepinephrine reuptake inhibitors, monoamine oxidase inhibitors may reduce response rate and increase differential rates of reinforcement (DRL) schedules in rats that may resemble antipsychotic effects and produce false positive results. Based on criterion one, all antipsychotic drugs should be effective in the model. However, the atypical antipsychotic drugs retain a special position. Many traditional models of the behavioral actions of neuroleptic drugs are based on EPS-like motor effects. The atypical antipsychotic drugs do not cause EPS motor

side effects so they have often gone undetected in traditional animal models (Moore & Gershon 1989).

3. No false negatives. A valid assay for atypical antipsychotic drug effects should selectively differentiate atypical antipsychotic drugs from all other pharmacological classes, including typical antipsychotics. Further, the pharmacological effect in the behavioral model should be subthreshold to EPS-like motor effects. It should also be noted that newer atypical antipsychotic drugs with purported novel mechanisms of action may not be detected in a model established based on receptor-mediated effects of an older therapeutic agent.

Preclinical Models with Predictive Validity

The incomplete understanding of the causes and mechanisms of schizophrenia restrict preclinical model development. An integration of findings across animal models and further refinement of the criteria used to assess model validity will help further our understanding of the complex pharmacology of schizophrenia. The evidence of predictive validity will facilitate the development of batteries of tasks to characterize different behavioral aspects of antipsychotic drug effects. The current impetus of research focuses on the ability of animal behavioral models to selectively differentiate atypical from typical antipsychotic drugs, while still being able to distinguish antipsychotic from non-antipsychotic agents. The effects of typical and atypical antipsychotic drugs have been characterized in various animal models. Conditioned avoidance response (CAR) and catalepsy (CAT) are two standard preclinical tests extensively used to predict antipsychotic activity and motor side-effect liability, respectively.

Conditioned Avoidance Response. The selective ability of antipsychotic drugs to inhibit conditioned avoidance response (CAR) has been utilized for over 50 years. Reinforcement is

used to control the CAR behavior experimentally. Aversive electric shocks can be administered to the subject and any response that permits the organism to escape from or prevent the occurrence of the electric shock will be reinforced. Behavior that terminates the aversive stimulus is called *escape* behavior. Behavior that delays the occurrence of the aversive stimulus is called *avoidance* behavior. In most CAR paradigms, animals are conditioned to make an active response (e.g., locomotion in a shuttle box or pole climbing) to avoid or escape foot shock.

Early models based on the inhibition of the CAR by antipsychotic drugs have been characterized in several animal species (Barrett, 1983; Cook & Catania, 1964; Janssen, Niemeggeers, & Schellekens, 1966; Spealman & Katz, 1980; Wenger, 1979; Worms, Broekkamp, & Lloyd, 1983). The general finding is that non-cataleptic doses of antipsychotic drugs disrupt avoidance without disrupting escape. CAR does not, however, detect the atypical antipsychotic sulpiride (Arnt, 1982; Kuribara & Tadokoro 1981; Van Der Heyden, 1989). The atypical antipsychotics clozapine and thioridazine inhibit the CAR, whereas the typical antipsychotic prothipendyl has no effect (Janssen et al., 1966). High doses of thioridazine are necessary in order to block CAR (Blackburn & Phillips 1989). Thus, the two classes of antipsychotic drugs have inconsistent effects in CAR studies (Moore et al., 1992; Sanger, 1985).

CAR procedures are not specific to antipsychotic drugs. CAR has a number of false positives probably due to the motor involvement of the task. Arnt (1982) examined the effects of 22 non-antipsychotic drugs on the CAR. 11 of the drugs tested inhibited CAR. Among a wide range of non-antipsychotic compounds, α_1-adrenergic antagonists, benzodiazepines, a barbiturate, GABA agonists, morphine and a serotonin agonist inhibited the CAR at doses inducing other motor disturbances. Adenosine agonists also reduce CAR in the rat (Martin,

Rossi, & Jarvis, 1993). The α-adrenergic activity of some antipsychotic drugs (e.g. clozapine and chlorprothixene) may contribute to CAR inhibition. Sanger (1985) found similar results with clozapine and chlordiazepoxide in the shuttle box avoidance procedure using rats.

Anticholinergic agents do not attenuate therapeutic effects in patients suffering from schizophrenia. Anticholinergic drugs attenuate inhibition of CAR by typical antipsychotic drugs. It has been shown that the muscarinic antagonist scopolamine reduces the effectiveness of haloperidol or fluphenthixol inhibition of CAR (Arnt, 1982; Fibiger et al., 1975; Setler et al., 1976). The effects of scopolamine on haloperidol were much more potent than effects on fluphenthixol-induced suppression of CAR, possibly due to greater selectivity of haloperidol for the D_2 receptor (Arnt, Christensen, & Hytell, 1981). Additional antimuscarinic activity of typical antipsychotics may moderately attenuate CAR inhibition (Arnt, 1982).

Antagonism at 5-HT_{2A} receptors potentiates the effect of haloperidol (Wadenberg, Browning, Young, & Hicks, 2001a) and raclopride (Wadenberg, Hicks, Richter, & Young, 1998) in a CAR task in rats. Phenylpiperazines, such as the serotonin agonist meta-chlorophenylpiperazine (MCPP), have also been reported to block CAR in the Fischer-344 rat (Martin, Elgin, Mathiasen, Davis, Kesslick, Baldy, Shank, DiStefano, Fedde, & Scott, 1989). The largest body of CAR data however implicates a dopamine mechanism. The antipsychotics haloperidol, risperidone, olanzapine and quetiapine are effective in the CAR model at a lower level of D_2 occupancy than required for catalepsy (Wadenberg et al. 2001a; Wadenberg, Soliman, VanderSpek, & Kapur, 2001b).

Catalepsy. Another classic model used as a drug screen for antipsychotics is the catalepsy test. In the catalepsy test the laboratory animal is placed in an abnormal position and latency to

correct the imposed body posture is measured. Animals are generally placed with their forepaws elevated approximately 5-10 cm. The most popular variations of the catalepsy are the bar or the wood block test. Wadenberg and colleagues (2001b) observed that dopamine plays a pivotal role in catalepsy as it was observed in animals receiving the typical antipsychotic haloperidol and the atypical antipsychotics risperidone and olanzapine, but only at doses that produced a D_2 receptor occupancy $\geq$ 85%.

However, there are false positives in catalepsy also. The D_2 occupancy of quetiapine did not cross the 85% threshold (up to 100 mg/kg) and it did not show catalepsy (Wadenberg et al., 2001b). The atypical antipsychotics clozapine, thioridazine and prothipendyl do not cause catalepsy at high doses (Arnt, 1982; Ellenbroek, 1992). Further, there are several false positives (i.e., non-antipsychotic drugs that do cause catalepsy). The opiates (Ellenbroek, Peeters, Honig, & Cools, 1987; Kuschinsky & Hornykiewicz ,1972), acetylcholine agonists (Costall & Naylor 1973) produce catalepsy. The cannabinoids (acting at CB_1 receptors) cause CAT in mice (Varvel, Bridgen, Tao, Thomas, Martin, & Lichtman, 2005).

In relevance to catalepsy as a predictor of EPS, anticholinergic antagonists attenuate neuroleptic-induced catalepsy. Haloperidol-induced catalepsy in mice is reversed by the muscarinic antagonist atropine (Klemm, 1985). Similar results with haloperidol and the muscarinic antagonist scopolamine were also obtained (Ellenbroek & Cools 1988). Catalepsy rapidly and reliably detects the potential of pharmacological agents to produce EPS. Catalepsy is increasingly used for the screening of drugs with therapeutic potential in the treatment of Parkinson's disease, such as the $\alpha 2$ adrenoreceptor antagonists (Pinna, Volpini, Cristalli, & Morelli, 2005) and the serotonin 5-HT_{1A} agonists (Bantick, De Vries, & Grasby, 2005). CAR

and catalepsy models may have some predictive accuracy for antipsychotic drugs because they share a common underlying mechanism: dopamine D_2 occupancy (Wadenberg et al., 2001b).

Intracranial Self-Stimulation. Antipsychotic drugs suppress levels of operant lever responding for intracranial self-stimulation (ICSS) (Worms et al., 1983). ICSS models are largely based on dopamine receptor mechanisms and neural pathways related to reward and neuroleptic-induced anhedonia (Ettenberg et al., 1981). The catecholamines dopamine and noradrenaline have been implicated in ICSS (Crow, 1972; 1976). However, antagonism of α adrenergic receptor subtypes is not effective in other ICSS studies (Montgomery, Grottick, & Herberg, 2003; Zarevics, Weidley, & Setler, 1977). The typical antipsychotic drug chlorpromazine has been compared to lithium chloride in ICSS procedures (Takigawa, Fukuzako, Ueyama, & Tominaga, 1994). More recently, it has been described that atypical as well as typical antipsychotic drugs induce characteristic within-session response decrements in operant behaviors, including ICSS (Montes, Chaatoufel, & Ferrer, 2005; Takigawa, Fukuzako, Ueyama, & Tominaga, 1994).

In ICSS procedures, animals are implanted with an electrode inserted through the skull into specific brain regions. The electrode is connected to a lever that delivers a small electrical current (100-250 μA) when pressed. The electrical current serves as a positive reinforcer and rats rapidly learn to lever press for brain electrical stimulation (Olds, 1976). (Wauquier, 1976; 1979) implanted electrodes in the medial forebrain bundle in the lateral hypothalamus and tested a large series of antipsychotic drugs. All antipsychotic drugs tested, including clozapine and thioridazine, attenuated ICSS. While ICSS is less sensitive to the atypical antipsychotic drugs

(especially thioridazine) than the typical antipsychotic drugs, various chemical classes of antipsychotic drugs have been shown to be effective in the model.

Unfortunately, a number of false positives occur in ICSS procedures with nonantipsychotic agents. Cholinergic agonists produce inhibition of ICSS (Pradhan, 1976). Noradrenergic antagonists decrease ICSS behavior (Bailey & Pradhan 1975). The antidepressants desipramine and amitriptyline reduce ICSS (Wauquier, 1976). Benzodiazepines potentiate ICSS (Wauquier, 1976). The opiates morphine, piritramide and fentanyl increase ICSS at lower doses and reduce ICSS at higher doses (Wauquier, 1976). Treatment with anticholinergic drugs counters the ICSS rate suppression produced by a large number of antipsychotic drugs.

ICSS procedures allow for the testing of a large range of doses. ICSS paradigms also possess few false negatives with typical antipsychotic drugs. However, ICSS procedures are less sensitive to the effects of atypical antipsychotic drugs. Antipsychotic drug selectivity and the development of tolerance are limitations of the ICSS model.

Blockade of Drug-induced Hyperlocomotion. Antipsychotic drugs block the effects of dopamine agonists to increase locomotor activity at doses below those that produce stereotypy. The nucleus accumbens has been implicated in these stimulant effects on behavior (Costall & Naylor 1975; Pijnenberg, Honig, & van Rossum, 1975; Pijnenberg & van Rossum 1973) as well as, the olfactory tubercle (Cools, 1986). Direct infusion of dopamine into the nucleus accumbens produces increases in locomotion (Pijnenberg, 1977). Most studies have focused on the attenuation of the stimulant effects of dopamine agonists in this procedure.

Hyperactivity induced by dopamine was antagonized with several antipsychotics including haloperidol, fluphenazine and pimozide dose dependently (Costall & Naylor, 1976). Many antipsychotics were tested on dopamine-induced hyperactivity caused by direct injections of the dopamine agonist 6,7 ADTN into the nucleus accumbens (Arnt, 1983). All antipsychotic drugs except sulpiride blocked hyperlocomotion. Sulpiride was inactive after peripheral injection, whereas intra-accumbens sulpiride antagonized 6,7-ADTN-induced hyperactivity. Thioridazine and sulpiride showed a much more pronounced effect in the Costall and Naylor (1976) study as compared to the Arnt (1983) investigation. Notably, clozapine was found to be potently effective across both studies.

Arnt (1983) tested several other compounds for effects on 6,7 ADTN-induced locomotion. The α_1 adrenoreceptor antagonist prazosin but not aceperone attenuated the dopamine related hyperactivity. 6,7 ADTN-induced locomotion was potentiated after application of the serotonin antagonist methsergide and direct injections of serotonin into the nucleus accumbens blocked amphetamine-induced locomotion (Costall, Hui, & Naylor, 1979b; Jones, Mogenson, & Wu, 1981), suggesting serotonin agonists may be a false positive. Opiates are also able to reverse dopamine stimulated locomotor activity (Costall, Fortune, & Naylor, 1978).

The α_2-adrenergic receptors modulate the effects of amphetamine on locomotion in mice (Luttinger & Durivage 1986) and adrenoreceptor α_2 agonists dose dependently attenuate amphetamine induced locomotion in rats (Poleszak & Malec 2000) but may also be potential antipsychotic agents (Kalkman & Loetscher 2003). Anticholinergic agents were ineffective at reversing the effects of haloperidol on locomotion induced by intra-accumbens injections of

dopamine in one study (Costall et al., 1979a), however, Arnt et al. (1981) reversed the haloperidol blockade of 6,7-ADTN-induced hyperactivity with the antimuscarinic scopolamine.

Several other drugs without apparent direct effects on the dopamine system also increase activity when injected directly into the nucleus accumbens, like picrotoxin (Jones et al., 1981) and the glutamate agonist N-methyl-D-aspartate (NMDA) (Hamilton, De Belleroche, Gardiner, & Herberg, 1986). More recent data shows an effect of stimulation of dopamine $D_{1/5}$ receptors, possibly in the medial prefrontal cortex that is associated with inhibitory actions on locomotor activity and d-amphetamine-induced hyperactivity (Isacson, Kull, Wahlestedt, & Salmi, 2004). There are apparently multiple receptor mechanisms that are effective in this model.

More recent hyperlocomotion models have focused on the effects of (+/-) 3,4-methylenedioxymethamphetamine (MDMA), which releases dopamine and serotonin *in vivo* and increase locomotor activity (Kehne, Ketteler, McCloskey, Sullivan, Dudley, & Schmidt, 1996). MDMA-stimulated dopamine release is reduced by the selective 5-HT_{2A} receptor antagonist [R-(+)-a- (2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinem ethanol] (MDL 100,907 or M100907), as well as with other agents with potent 5-HT_{2A} antagonist activity (ritanserin, clozapine, MDL 28,133A, or methiothepin). Agents that block 5-HT_{1A} (propranolol), D_2 (haloperidol), or D_1 receptors (SCH 23390) also reduced MDMA-stimulated locomotion. The contribution of different receptors to MDMA-stimulated locomotion suggests the potential utility of this model for characterizing putative atypical antipsychotic compounds with multiple receptor targets.

Studies have compared the effects of various antipsychotic drugs on locomotor hyperactivity induced by the two different psychotomimetics phencyclidine (PCP) and

amphetamine (Sams-Dodd, 1998). The PCP model may be more sensitive to potent 5-HT_{2A} antagonist effects (Maurel-Remy, Bervoets, & Millan, 1995). This model may be better suited to detect atypical antipsychotic drug effects than the amphetamine model.

The dopamine-induced locomotor paradigm has the ability to detect most antipsychotics but is not selective for this pharmacological class. The amphetamine dose used to stimulate locomotion is also a critical factor for which receptor-mediated effects are important in this model (Arnt, 1995). The development of tolerance is a weakness in the amphetamine-induced locomotion paradigm (Sams-Dodd, 1998).

The Paw Test. Although many of these models are capable of differentiating between antipsychotic and other pharmacological classes, only a few seem to be able to differentiate between typical and atypical antipsychotics. The paw test is an assay established in rats that has been shown to screen the unique effects of antipsychotic drugs and to distinguish atypical from typical antipsychotic drugs (Ellenbroek et al., 1987). The paw test differentiates between typical antipsychotic drugs which prolong both the forelimb (FRT) and hindlimb retraction time (HRT) at equipotent doses and atypical neuroleptics which are much more potent in prolonging HRT than in prolonging FRT. Thus, the FRT is believed to be indicative of EPS and the HRT of antipsychotic effects. The underlying mechanism may be based on results showing that the neostriatum and the nucleus accumbens play different roles in modulating forelimb and hindlimb rigidity (Ellenbroek, Schwartz, Sontag, Jaspers, & Cools, 1985; Ellenbroek, Van den Hoven, & Cools, 1988).

Atypical antipsychotic drugs selectively increase HRT at doses lower than those that increase FRT. Typical antipsychotic drugs produce HRT and FRT latencies at equipotent doses.

Correspondingly, the muscarinic antagonist scopolamine blocked the FRT, but not the HRT; chronic neuroleptic treatment reduced the FRT, but not the HRT. The nonantipsychotic drugs desipramine, diazepam and morphine do not influence the variables measured in the paw test, although morphine does produce catalepsy (Ellenbroek et al., 1987). Approximately 25 antipsychotic drugs have been tested in the paw test and all increase HRT (Geyer & Ellenbroek 2003).

Over 20 non-antipsychotic drugs have been tested and were without effect in the paw test (Geyer & Ellenbroek 2003). These include the antihistamine phenothiazine promethazine, the opiate morphine, the benzodiazepine diazepam, the tricyclic antidepressant desipramine (Ellenbroek et al., 1987; Ellenbroek & Cools 1988). Serotonergic agents ritanserin, ketanserin, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OHDPAT), and 1-(2,5dimethoxy-4-iodophenyl)-2-aminopropane (DOI) had no effect in the paw test (Ellenbroek, Prinssen, & Cools, 1994).

The role of serotonin 5-HT_{1A} and 5-HT_2 in the effects of atypical and typical antipsychotic drugs has been characterized in the paw test (Ellenbroek et al., 1994). The differential effects of 8-OHDPAT and DOI on the various antipsychotics (e.g., fluphenazine, thioridazine and risperidone) illustrate the important differences in the mechanism(s) of antipsychotic drugs. Adrenoreceptor drugs are also able to modulate the behavioral effects of clozapine using the paw test in rats (Prinssen et al., 1994a). Combinations of noradrenergic, serotonergic and dopaminergic agents mimic the profile of clozapine in the paw test (Prinssen et al., 1994b).

The paw test possesses a large degree of predictive validity. Unfortunately, only a few laboratories have attempted to replicate this procedure (Grimm & See 1998; Guan, Dai, & Zhu,

2000), so the paw test has not been as extensively evaluated as some other models across laboratories. It importantly attempts to differentiate atypical from typical antipsychotic drugs.

Drug Discrimination. Drug discrimination is a procedure in which animals are trained to detect the interoceptive stimulus effects of an administered dose of a training drug versus saline/vehicle (i.e., a nondrug condition). Different doses of the same training drug or a different training drug may also be established in this model. Drug discrimination studies are most commonly two-lever operant procedures. Several species have been utilized in the drug discrimination paradigm, primarily rats, pigeons and monkeys. Humans have also been used in this procedure.

The drug discrimination procedure is used to investigate various pharmacological aspects of the stimulus properties of the drug. These effects include mechanism of action, onset and duration of activity, structure-activity relations, activity of metabolites, tolerance and withdrawal, identification and development of potential antagonists and similarity of effect to other agents. Drug discrimination is an important, useful and versatile tool in central nervous system pharmacology for investigating receptor-mediated effects on behavior.

Drugs are able to function effectively as discriminative stimuli in the control of behavior (Schuster & Balster 1977). For example, it was demonstrated early on that atropine and pentobarbital produce two qualitatively different states (discriminative stimulus effects) (Overton, 1966). There is a high level of pharmacological specificity in the classification of drugs using drug discrimination procedures and this led to the "one cue per pharmacological class" idea.

Drug discrimination procedures can be used to determine stimulus gradients as well as generalization curves with novel compounds tested for their ability to substitute for the training drug. Attenuating or potentiating effects of receptor subtypes important for the discriminative cue can be investigated using selective ligands. The discriminative stimulus properties of drugs provides an *in vivo* measure that correlates well with *in vitro* results in the study of the neural basis of drug effects on behavior. Characterizing the effects of clozapine using drug discrimination procedures can increase our understanding of the mechanism(s) of action of atypical antipsychotic drugs *in vivo* by identifying receptor subtypes that may be important for atypical antipsychotic effects.

Antipsychotic drugs were first used in a drug discrimination procedure in the 1960s (Stewart, 1962). Rats were trained to discriminate 4.0 mg/kg chlorpromazine vs. saline using shock avoidance in a three-compartment test chamber (similar to a T-maze). The phenothiazines acepromazine, perphenazine, and prothipendyl fully substituted, but the phenothiazine prochlorperazine and the tricyclic antidepressant imipramine failed to substitute for chlorpromazine.

Chlorpromazine (5.0 mg/kg) drug discrimination using a T-maze (shock avoidance) could not be established (Overton, 1966) and initial attempts to establish discriminative stimulus control with chlorpromazine (1.0 mg/kg) could not be obtained in rats in a two-lever operant procedure (Harris & Balster 1971). However, some subsequent attempts have been successful. A chlorpromazine (1.0 mg/kg) versus saline drug discrimination in a two-lever operant procedure was successfully established with food reinforcement on one lever and shock punishment on the other lever (Barry, Steenberg, Manian, & Buckley, 1974). The chlorpromazine metabolites were

tested and only one (7-OH-CPZ) generalized to the chlorpromazine discriminative stimulus cue. Importantly, it was shown that quaternary chlorpromazine (which does not cross the blood-brain barrier) failed to substitute for chlorpromazine indicating that the discriminative stimulus effects were centrally mediated. Chlorpromazine has since been established as a training drug in two other drug discrimination studies (Goas & Boston, 1978; Porter, Covington, III, Varvel, Vann, & Warren, 1998). Drug discrimination using haloperidol vs. saline has also been established using a two-lever operant task for food reward (Colpaert et al. 1976), then subsequently by (McElroy, Stimmel, & O'Donnell, 1989), who demonstrated that chlorpromazine substituted for haloperidol (0.05 mg/kg).

While the typical antipsychotic drugs are somewhat difficult to train using drug discrimination procedures, the atypical antipsychotic drug clozapine possesses robust discriminative stimulus properties. Clozapine (6.9 mg/kg) was first established as a training drug in a two-lever operant procedure (Goas & Boston, 1978). They also trained another group of rats to discriminate chlorpromazine (2.0 mg/kg) vs. vehicle. Haloperidol and clozapine substituted in chlorpromazine-trained rats but the non-antipsychotic drug chlordiazepoxide did not. In clozapine-trained rats, haloperidol, chlordiazepoxide, and atropine all failed to substitute for clozapine (i.e., the generalization between clozapine and chlorpromazine was asymmetrical). Goas and Boston (1978) subsequently established a drug-drug discrimination using 8.8 mg/kg clozapine vs. 4.24 mg/kg chlorpromazine and showed that haloperidol substituted for chlorpromazine. Clozapine (20 mg/kg) and haloperidol (2.5 mg/kg) were subsequently established as training drugs in a T-maze discrimination procedure (Overton, 1982).

Gauvin, Goulden, & Holloway (1994) established a three-choice paradigm in rats trained to discriminate haloperidol vs. saline vs. amphetamine as a single pharmacological continuum (agonist-antagonist) that was hypothesized to represent a parallel subjective or interoceptive stimulus continuum associated with the drug injections. A three-choice drug discrimination study with clozapine vs. chlorpromazine vs. vehicle distinguished the atypical antipsychotics clozapine and olanzapine from the typical antipsychotics chlorpromazine and haloperidol; however, the stimulus properties of the atypical APD risperidone were similar to chlorpromazine, but not to clozapine (Porter, Prus, Vann, & Varvel, 2005).

Drug discrimination studies demonstrate that chlorpromazine generalizes to both olanzapine (Porter et al., 1999) and to haloperidol (McElroy et al., 1989). Clozapine generalizes to both olanzapine (Porter & Strong, 1996; Porter, J.H., Varvel, Vann, Philibin, & Wise, 2000) and chlorpromazine (Goas & Boston, 1978; Porter et al., 1998). Olanzapine produces full substitution for both clozapine (Goudie & Taylor, 1998; Moore et al., 1993; Moore et al., 1992; Porter et al., 2000) and chlorpromazine (Porter et al., 1998). There is evidently some degree of similarity between the discriminative stimulus properties of clozapine, olanzapine, and chlorpromazine and between chlorpromazine and haloperidol.

A recent drug discrimination study from our lab conducted in rats (Prus, Philibin, Pehrson, Stephens, Cooper, Wise, & Porter, 2005a) has shown that the atypical antipsychotics olanzapine, quetiapine, and ziprasidone produced full substitution for 5.0 mg/kg clozapine, whereas the atypical antipsychotics risperidone and sertindole produced partial substitution (60-79 %DLR). While the typical antipsychotic, thioridazine, produced full substitution for the 5.0 mg/kg clozapine training dose, but the typical antipsychotics chlorpromazine, fluphenazine, and

haloperidol failed to substitute for clozapine. In a subgroup of 1.25 mg/kg clozapine-trained rats, ziprasidone produced strong partial substitution (73.0 %DLR) for the 1.25 mg/kg clozapine dose.

Based on these findings, some atypical antipsychotic drugs (i.e., quetiapine and ziprasidone) produce full substitution ($\geq$ 80% DLR) only for the 5.0 mg/kg clozapine dose, whereas other atypical antipsychotic drugs (i.e., sertindole and risperidone) produce full substitution only for the 1.25 mg/kg clozapine dose. This shows that both the low and high training doses may be important for the screening of putative atypical antipsychotic drugs. Therefore, a three-lever drug discrimination procedure in rats using high (5.0 mg/kg) versus low (1.25 mg/kg) dose-clozapine versus vehicle was established to test if this procedure more selectively differentiates between atypical and typical antipsychotic drugs (Prus, Philibin, Pehrson, & Porter, 2005b). These data further suggest that the lower 1.25 mg/kg clozapine training dose engenders partial to full generalization to more atypical antipsychotic drugs than does the higher 5.0 mg/kg training dose.

The three-lever procedure has also shown that many selective ligands fail to fully substitute for clozapine at either training dose but that the muscarinic cholinergic receptor plays a stronger role in the 5.0 mg/kg training dose (Prus, Philibin, Pehrson, & Porter, 2006). Full substitution engendered by mianserin showed additional evidence that some antidepressant agents may produce clozapine-like discriminative stimulus properties. These data further support the notion that clozapine may have a compound discriminative stimulus cue.

The Discriminative Stimulus Properties of Clozapine

Typical antipsychotic drugs have failed to fully substitute for clozapine in drug discrimination procedures. Thus, there are no false positives with typical antipsychotics tested in

clozapine-trained animals. However, there are atypical antipsychotics (e.g., risperidone and sertindole) that have failed to substitute for clozapine, indicating some false negatives in this model.

The clozapine discriminative cue may be a complex cue involving concurrent blockade of multiple receptor subtypes (Carey & Bergman, 1997; Goudie & Taylor, 1998; Goudie & Smith, 1999; Porter et al., 2000; Prus et al., 2005a; 2006). The specific complex of receptor subtypes generating the discriminative stimulus of clozapine remains undetermined. Clozapine drug discrimination studies have compared the discriminative stimulus properties of typical and atypical antipsychotics and selective receptor ligands with the discriminative stimulus properties of clozapine.

Selective blockade of dopamine receptors does not engender clozapine-appropriate responding in drug discrimination procedures. The dopamine D_1 antagonist SCH 23390 fails to substitute for clozapine in rats (Franklin & Tang, 1994; Goudie et al., 1998; Porter et al., 1999) and in pigeons (Hoenicke, Vanecek, & Woods, 1992). D_2 antagonists do not substitute for clozapine in rats (Browne & Koe, 1982; Franklin & Tang, 1994; Goas & Boston, 1978; Goudie et al., 1998; Porter et al., 1999; Tang, Franklin, Himes, Smith, & Tenbrink, 1997; Villanueva, Arezo, & Rosecrans, 1992; Wiley & Porter, 1993) or in squirrel monkeys (Carey & Bergman 1997) Sulpiride (another D_2 antagonist) does not substitute for clozapine in rats (Ortmann, Meisberger, Bischoff, Hauser, Bittiger, & Waldmeier, 1986) or in pigeons (Hoenicke et al., 1992). D_4 and D_3 antagonists similarly fail to substitute for clozapine in rats (Goudie, Baker, Smith, Prus, Svensson, Cortes-Burgos, Wong, & Haadsma-Svensson, 2001; Goudie, Smith, Taylor, Taylor, & Tricklebank, 1998).

Clozapine had only partial effect in blocking the reinforcing and discriminative stimulus effects of cocaine (van Campenhout, De Haes, & Meert, 1999; Vanover, Piercey, & Woolverton, 1993). In fact, rats trained to discriminate the D_2/D_3 agonist, 7-OH-DPAT fully generalized to clozapine but not to haloperidol (Dekeyne, Rivet, Gobert, & Millan (2001). Clozapine has D_2/D_3 antagonist activity but these effects were attributed to 5-HT_{1A} agonist actions at autoreceptors. Clozapine did not substitute for the D_2/D_3 antagonist tiapride in rats (Cohen, Sanger, & Perrault, 1997). The ability to antagonize the discriminative stimulus of the dopamine agonist d-amphetamine does not correlate with other atypical antipsychotic drugs similar to clozapine (Arnt, 1996).

Blockade of α and β adrenergic receptors generally fail to engender clozapine appropriate responding using rats or pigeons. Phentolamine and propranolol, α and β adrenergic antagonists respectively, do not substitute for clozapine in rats (Kelley & Porter 1997). The selective α_1 antagonist, prazosin, does not substitute for the clozapine discriminative stimulus in rats (Nielsen, 1988) or in pigeons (Hoenicke et al., 1992) nor does the selective α_2 antagonist yohimbine substitute for clozapine in rats (Goudie et al., 1998; Franklin & Tang, 1994). However, S18327, a putative atypical antipsychotic with α_1 and α_2 adrenergic antagonist properties, fully substituted for clozapine (Millan, Brocco, Rivet, Audinot, Newman-Tancredi, Maiofiss, Queriaux, Despaux, Peglion, & Dekeyne, 2000). The H_1 histaminergic antagonist mepyramine (pyrilamine) does not substitute in clozapine-trained rats (Goudie et al., 1998) nor does pyrilamine in pigeons (Hoenicke et al., 1992). Full generalization to clozapine has been demonstrated by the histamine H_1 receptor antagonists promethazine and cyproheptadine,

although these compounds serve as antagonists at multiple 5-HT and muscarinic receptors as well (Kelley & Porter, 1997).

NMDA failed to substitute for clozapine in rats (Kelley & Porter, 1997), but (Schmidt & Volz, 1992) reported that NMDA substituted for clozapine in rats trained to discriminate clozapine vs. saline in a T-maze. Clozapine but not haloperidol has been shown to antagonize the discriminative stimulus of MK-801 (Corbett, 1995) but this was not supported in another laboratory (Smith, Boyer-Millar, & Goudie, 1999). Further, clozapine failed to alter the behavioral effects of PCP in two-lever drug discrimination and mixed signaled-unsignaled differential reinforcement of low rates of responding (DRL) procedures (Compton, Slemmer, Drew, Hyman, Golden, Balster, & Wiley, 2001).

The theory that concurrent antagonism of 5-HT_{2A} and 5-HT_{2C} serotonin receptors mediates the clozapine discriminative stimulus (Hoenicke et al., 1992) is based on findings with pigeons trained to discriminate 1.0 mg/kg (IM) clozapine vs. vehicle. However, in other drug discrimination studies, ritanserin (5-$HT_{2A/2B/2C}$ antagonist) does not substitute for clozapine in rats trained to discriminate clozapine vs. vehicle (Wiley & Porter 1992) or in rats trained to discriminate clozapine vs. haloperidol (Wiley & Porter 1993). Antagonism of 5-$HT_{2A/2C}$ receptors resulted in clozapine appropriate responding in pigeons (Hoenicke et al., 1992), but this has not been demonstrated in rats. Ketanserin (another 5-$HT_{2A/2C}$ antagonist) does not substitute for clozapine in rats (Franklin & Tang, 1994; Goudie et al., 1998; Nielsen, 1988; Tang et al., 1997) or in pigeons (Hoenicke et al., 1992).

The selective 5-HT_{2A} antagonist MDL 100,907 does not substitute for clozapine in rats (Goudie et al., 1998) but clozapine does generalize to MDL100,907 (Dekeyne et al., 2003). 5-

HT_{2C} selective antagonists do not substitute for clozapine (Goudie et al., 1998; Wiley & Porter, 1992). The 5-HT_3 receptor antagonists MDL 72222 (Wiley & Porter, 1992; 1993) and ondansetron (GR38032F) also fail to generate clozapine responding in rats (Goudie et al., 1998) or in pigeons (Hoenicke et al., 1992). 5-$HT_{2B/2C}$ receptor antagonists SB 200646 or SDZ SER 082 do not generalize to clozapine in rats (Goudie et al., 1998). 5-HT_{1A} agonists S-14506 in rats (Goudie et al., 1998) and 8-OH-DPAT in pigeons (Hoenicke et al., 1992) and buspirone in rats (Franklin & Tang, 1994; Wiley & Porter, 1992; 1993) also fail to substitute for clozapine. 5-HT_1 antagonists do not attenuate the clozapine discriminative stimulus (Goudie et al., 1998); this is despite evidence of 5-HT_{1A} agonist actions of clozapine (Newman-Tancredi et al., 1996).

5-HT_{1A} agonists demonstrated cross-generalization with S-16924 and 5.0 mg/kg clozapine (Millan et al., 1999). The discriminative stimulus effects of the 5-HT_{2C} agonist mCPP were not attenuated by clozapine; however, the mCPP-stimulated phosphoinositide turnover was fully antagonized by clozapine (Fiorella, Rabin, & Winter, 1996). Clozapine blocks the discriminative stimulus effects of the 5-$HT_{2A/2C}$ agonist DOI (Schreiber, Brocco, & Millan, 1994). Clozapine was shown to partially block the discriminative stimulus effects of the 5-HT_{2A} agonists 2,5-dimethoxy-4-methylamphetamine (DOM) or lysergic acid (LSD) and neither DOM nor LSD generalized to clozapine (Palumbo & Winter, 1994). The blockade of (-) DOM-induced stimulus control has been used to identify antipsychotics with potential 5-HT_{2A} antagonist properties (Fiorella, Helsley, Rabin, & Winter, 1997).

Nicotinic cholinergic receptors also do not appear to be involved in the clozapine discriminative stimulus (Villanueva et al., 1992). Brioni, Kim, O'Neil, Williams, & Decker (1994) reported that clozapine failed to substitute in nicotine drug discrimination but did partially

attenuate the nicotine cue. The muscarinic cholinergic receptor system does appear to play a role in the discriminative stimulus properties of clozapine.

Muscarinic antagonists reliably substitute in clozapine drug discrimination studies (Goudie et al., 1998; Kelley & Porter, 1997; Nielsen, 1978; Millan et al., 1999). Antagonism of the M_1, but not M_2, muscarinic receptor appears to be an important component of the clozapine discriminative stimulus (Kelley & Porter 1997; Prus et al., 2004). Cross-generalization that was established between clozapine-trained rats and scopolamine-trained rats by Kelley and Porter (1997) demonstrates a shared mechanism for the discriminative stimulus effects of these two training drugs.

Generalization observed between clozapine and muscarinic antagonists may be due to the similar muscarinic antagonist actions of these drugs (Franklin & Tang, 1994). The clozapine cue appeared to be blocked with the muscarinic agonist oxotremorine (1.0 mg/kg) by Nielsen (1988), although, this effect could not be evaluated statistically due to strong rate suppression. There are two significant exceptions to the theory that the clozapine discriminative stimulus is mediated solely by anticholinergic mechanisms.

The benzodiazepine chlordiazepoxide showed partial substitution in rats for both clozapine and scopolamine, yet has no affinity for muscarinic receptors (Kelly & Porter, 1997). Also, the tricyclic antidepressant mianserin substituted for clozapine, but failed to substitute for scopolamine in rats (Kelly & Porter, 1997). Antimuscarinic actions may be sufficient, but are not an essential component for drugs that fully substitute for clozapine.

Training dose is an important factor in clozapine drug discrimination procedures that affects which drugs substitute for the training drug, rates of responding, and the ability of drugs

to attenuate the stimulus cue of the training drug (Colpaert et al., 1976). Training dose can also affect the involvement of different receptor mechanisms mediating the discriminative stimulus properties of clozapine. Previous drug discrimination studies using antipsychotic training drugs do not always distinguish atypical from typical antipsychotic drugs.

However, more recent studies have shown that the atypical antipsychotics olanzapine, sertindole, risperidone, and zotepine fully generalize to the clozapine discriminative stimulus when a lower training dose (< 5.0 mg/kg) was used (Porter et al., 2000; Goudie & Taylor, 1998; Smith et al., 1998). These data suggest that a lower training dose may be a more sensitive assay for differentiating atypical from typical antipsychotic drugs. Therefore, investigating the effects of antipsychotic drugs in low dose clozapine drug discrimination procedures may be more relevant to the identification of receptor targets important for the clinical effects of atypical antipsychotic drugs.

As previously noted, strong muscarinic cholinergic antagonism is sufficient to engender full generalization in rats trained to discriminate 5.0 clozapine vs. vehicle (Nielson, 1988; Kelley & Porter, 1997); however, the low affinity of sertindole and risperidone for muscarinic cholinergic receptors suggests this is not the case at a low (1.25 mg/kg) training dose (Porter et al., 2000). Further, the muscarinic antagonist scopolamine failed to substitute in rats trained to discriminate 1.25 mg/kg clozapine vs. vehicle (Wise, Vann, Philibin, Carter, Varvel, Pehrson, Silver, & Porter, 2001). Prus et al. (2005), however, demonstrated that the M_1 preferring muscarinic antagonist trihexyphenidyl fully substitutes for 1.25 mg/kg clozapine but not 5.0 mg/kg clozapine. Full substitution was seen for clozapine with the α_1 adrenergic receptor

antagonist prazosin and a single dose of the 5-HT_{2A} receptor antagonist M100907 (Wise et al., 2001).

Many atypical antipsychotic drugs with multiple receptor binding sites of action have been detected in clozapine drug discrimination procedures. Clozapine drug discrimination with a low training dose appears to selectively differentiate atypical from typical antipsychotic agents in rats (Porter et al., 2000). Further refinements in the drug discrimination model, such as training dose, training drug, and animal species, may prove useful in the discovery of novel antipsychotic agents with superior therapeutic efficacy and reduced side effect vulnerability.

Rationale

Drug discrimination is used as an *in vivo* mechanistic study of the differences and similarities between antipsychotic drugs. The recent development of transgenic and gene-targeted knockout animals provides a new and powerful tool to investigate the molecular basis of drug effects that complements behavioral pharmacology (Gold, 1996). Unfortunately, these molecular techniques are currently only readily available in mice - the vast majority of behavioral models used to study antipsychotic drugs have been conducted in rats. While the majority of clozapine drug discrimination studies have been conducted in rats, standard drug discrimination procedures can also be used with mice (Philibin, Prus, Pehrson, & Porter, 2005).

Establishing the use of wild type mice in a clozapine drug discrimination procedure that has been extensively characterized in rats will allow for the future use of genetic mutant mouse models to be compared in highly reliable assay for receptor specific effects. This technique is particularly useful when a receptor knockout mouse exists for a recently identified receptor subtype for which no existing selective ligand is available. Advances in molecular neuroscience have greatly facilitated behavioral pharmacologists in the investigation of the genetic basis underlying the behavioral effects of drugs. Deletion of a target gene that controls the production of a neurotransmitter is somewhat analogous to administering a pharmacological neurotransmitter receptor antagonist. Molecular approaches such as gene targeted knockout mutations, expression of an exogenous transgene and the disruption of cellular expression of genes with antisense oligonucleotides are now being successfully used. The use of these

molecular approaches in antipsychotic drug research will undoubtedly help lead to the discovery of new and improved agents for the treatment of schizophrenia.

Philibin et al. (2005) established the atypical antipsychotic clozapine as a training drug using a two-lever drug discrimination operant procedure in C57BL/6 mice. The atypical APDs olanzapine, risperidone and ziprasidone fully substituted for the discriminative cue of clozapine, while the typical APD haloperidol failed to substitute for clozapine. Generalization testing with selective ligands showed that the serotonin 5-$HT_{2A/2B/2C}$ antagonist ritanserin fully substituted for clozapine and that the 5-HT receptor agonist quipazine significantly blocked the clozapine discriminative cue without disrupting response rates. The muscarinic receptor antagonist scopolamine, the dopamine agonist amphetamine, and the serotonin agonist quipazine failed to substitute for clozapine. These results demonstrated that antagonism of 5-HT receptors plays a crucial role in the discriminative stimulus of clozapine in C57BL/6 mice (Philibin et al., 2005).

If serotonin mechanisms are critical in this assay then agents with potent serotonergic antagonist actions should resemble clozapine in this procedure. Results obtained from this mechanistic model can be used concomitantly among a battery of behavioral tests predictive of antipsychotic or EPS side effects to facilitate the discovery of novel antipsychotic agents. The present study was designed to further explore the clozapine drug discrimination model with C57BL/6 mice as established in the Philibin et al. (2005) study. Additional typical and atypical antipsychotic drugs and selective ligands were tested in order to further validate the model and to establish the underlying pharmacological mechanisms that mediate the discriminative stimulus properties of clozapine in C57BL/6 mice.

Hypothesis.

Atypical APDs that are potent 5-HT2A receptor antagonists and relatively weaker D2 antagonists will fully substitute for clozapine. Typical APDs that are more potent D2 antagonists will fail to substitute for clozapine. Non-antipsychotic agents will fail to substitute for clozapine.

Methods

Subjects

Thirty experimentally naïve, male C57BL/6 wild type mice (20-25g) obtained from Harlan Laboratories were housed individually in clear plastic cages (18 X 29 X 13 cm) with steel wire fitted tops and wood chip bedding. Mice were transported daily (Monday-Friday) from an animal colony room (12 hour light-dark cycle, lights on at 7 a.m., 22-24° C) to the laboratory where experimental training and testing sessions occurred. After one week of acclimation, the mice were slightly food deprived. To initiate the lever press response, mice were maintained at 90-95% of their free feeding body weights (20-25g) by restricting their daily ration of standard rodent chow (water available ad libitum). When rates of responding were established and stabilized on both levers, the mice were allowed to gradually gain weight to free feed status as drug discrimination training progressed as long as the mouse maintained 80% accuracy on the appropriate levers. The Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) were followed and the Institutional Animal Care and Use Committee at Virginia Commonwealth University (VCU) approved the procedures used in the present study (IACUC Protocol 0301-3155). Drug discrimination training started with 30 mice to ensure that at least 20 mice successfully acquired the task. In this within-subjects design, each mouse served as its own control and eight to ten mice per group were sufficient to detect treatment effects (power = 0.8, alpha = 0.05).

Drugs

Clozapine (atypical antipsychotic drug; gift from Novartis, Hanover, NJ), N-desmethylclozapine (clozapine metabolite; gift from Sepracor, Inc., Marlborough, MA)

sertindole (atypical antipsychotic drug; gift from Lundbeck, Copenhagen, Denmark), quetiapine (atypical antipsychotic drug; gift from Zeneca Pharmaceuticals, Wilmington, Del.), iloperidone (atypical antipsychotic drug; gift from HY Meltzer, Vanderbilt University, Nashville, TN), zotepine (atypical antipsychotic drug; gift from HY Meltzer), aripiprazole (atypical antipsychotic drug; gift from Lundbeck), fluphenazine (typical antipsychotic drug; E.R. Squibb and Sons, New Brunswick, N.J.), perphenazine (typical antipsychotic drug; Sigma Chemical Company, St. Louis, MO), M100907 (5-HT_{2A} antagonist; gift from Lundbeck), prazosin (adrenergic α_1 antagonist; Sigma Chemical Company) and pyrilamine (histaminergic H_1 antagonist; Sigma Chemical Company) were dissolved in distilled water with a few drops of lactic acid. Chlorpromazine HCL (typical antipsychotic drug; Sigma Chemical Company), thioridazine HCL (typical antipsychotic drug; gift from Novartis, Hanover, NJ) melperone (atypical antipsychotic drug; gift from HY Meltzer) and fluoxetine (SSRI; gift from HY Meltzer) were dissolved in distilled water. All drugs were administered subcutaneously (S.C.) at a volume of 10 ml/kg body weight with a 30 min presession injection time. Doses for all compounds refer to the salt form.

Apparatus

Testing was conducted in five standard computer-interfaced operant conditioning chambers (Model ENV-307A, Med Associates Inc., East Fairfield VT) with two retractable response levers in the left and right positions (8 cm apart) on the intelligence panel. The levers extend 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 the recessed food trough into which a liquid dipper delivers 0.02 ml of sweetened-condensed milk (by volume: one part condensed milk, one part sugar, and two parts water). The inner test chambers consist of a 15 cm L X 11.5

cm D X 17.5 cm H area surrounded by an aluminum chassis box with a single Plexiglas side door. Test chambers are housed in sound attenuated cubicles. Standard MED-PC software (Med Associates Inc.) controlled the operant schedule and recorded data.

Training procedures

Magazine Training. The lever was not available to the mouse during the fifteen-minute session. Liquid reinforcer was delivered noncontingently on a fixed-time 5 second (FT 5 sec) intermittent delivery schedule (i.e., a single presentation of sweetened milk was delivered by raising the dipper cup every 5 seconds automatically for 5 seconds).

Lever Press Training. Lever press training began upon completion of magazine training with a single lever extended inside the chamber. Each mouse was placed in the operant chamber and trained to press the levers for 0.02 ml of sweetened condensed milk on a fixed ratio one (FR1) schedule of reinforcement, in which the milk reinforcer (dipper was available for 3 sec.) was delivered after every lever press. Mice were trained to lever press on a single lever (the vehicle lever). The position of the drug-associated lever (left vs. right) was counterbalanced between the mice to control for olfactory cues (Extance & Goudie, 1981). The value of the FR was gradually increased over several sessions until FR10 was obtained. Mice were injected daily with vehicle 30 min prior to each test session. The mice then began drug lever training on the alternate lever on the FR10 schedule (only drug lever present) and received clozapine injections 30 min prior to each test session. Once responding on the FR10 schedule was stable, the mice received the training drug or vehicle injections according to a double alternation sequence (i.e., DDVVDDVV) with only the appropriate lever presented inside the operant chamber. Once

baseline rates of responding stabilized, both levers were extended in the operant chambers for the remaining sessions. Clozapine and its vehicle were administered 30 min. prior to testing.

Drug Discrimination Training. Mice were trained to discriminate 2.5 mg/kg clozapine on drug days. On days when 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. During the first ten sessions of two-lever discrimination training five consecutive sessions of drug lever training was followed by five consecutive sessions of vehicle lever training. The double alternation schedule subsequently resumed and was used throughout the remainder of the study. Mice received two-lever training until the training criteria were passed during 5 of 6 consecutive sessions.

Drug Discrimination Criteria. Successful discrimination training was evaluated and assessed according to three criteria: (1) the first completed FR10 was on the appropriate lever, (2) 80% or greater of the total responding occurred on the appropriate lever and (3) response rate equaled or exceeded 10 responses per minute. Control vehicle and clozapine (2.5 mg/kg) tests were administered and passed prior to generalization testing with all new test drugs. During control and test sessions, responses on both levers were reinforced according to the FR10 schedule and the FR reset when switching occurred. The three training criteria also had to be met during the training session immediately prior to all test sessions.

Testing procedures

Generalization testing. Generalization or substitution testing normally occurred on Tuesdays and Fridays with a minimum of 2 days between tests including both a passed drug and

a vehicle training session. After successful completion of vehicle and clozapine control tests, a generalization dose effect curve was determined for clozapine (0.3125 - 5.0 mg/kg). Substitution tests were conducted with various atypical and typical antipsychotic drugs, an antidepressant drug and selective ligands for serotonergic, noradrenergic or histaminergic receptors. New control tests were performed between each new test drug with the training drug and vehicle to assess clozapine discriminative stimulus control.

Data Analysis

The percentage of animals in which the correct first FR (FFR) was obtained provided one index of stimulus control. The number of responses on each lever was recorded and converted into percent drug lever responding (%DLR) by dividing the number of responses on the drug lever by total responses on both levers and multiplying by 100. Responses per minute (RPM) for each session were calculated. ED_{50} values [with 95% confidence intervals [C.I.] were calculated for %DLR data using the least squares method of linear regression with the linear portion of the dose effect curve. ED_{50} values were calculated for test drugs that fully substituted for the training dose of clozapine (full substitution $\geq$ 80% DLR; partial substitution was $\geq$ 60 to < 80% DLR). A repeated-measures analysis of variance (ANOVA) comparing responses per minute was performed for each drug (GB-STAT software; Dynamic Microsystems, Inc., Silver Spring, MD). Significant ANOVAS were followed by Newman-Keuls post-hoc tests ($p < 0.05$). To record %DLR, animals had to earn at least one reinforcer (FR10) or have responses per minute $\geq$ 2.0 (30 lever press responses per session).

Results

Acquisition of two-lever discrimination

Twenty six out of thirty mice reached training criteria in an average of 14.8 (SEM $\pm$ 1.6) sessions with a range of 6-34 sessions. Three mice were removed from the study because they were unable to complete the clozapine dose response curve.

Clozapine generalization

The mean %DLR ($\pm$SEM) and the mean responses per minute ($\pm$SEM) for the clozapine generalization curve for the 2.5 mg/kg training dose is shown in Fig. 1. Full generalization to the clozapine cue was obtained at 2.5 mg/kg and 5.0 mg/kg. Generalization testing yielded an ED_{50} = 1.19 (95% C.I. 1.09 - 1.30 mg/kg). Response rates were significantly suppressed by 5.0 mg/kg ($F_{6, 132} = 115.3$, $P<0.0001$) with only 4 of 23 animals meeting the rate criterion at that dose (i.e. earned a reinforcer or had $\geq$ 2.0 RPM).

Replication of clozapine generalization dose effect

Generalization curves for the 2.5 mg/kg clozapine training dose in the current study (2) are superimposed over previously obtained data (1) in C57BL/6 mice (Philibin et al. 2005) in Fig.2.

Quetiapine generalization

Quetiapine (Fig. 3) fully substituted (98.0% DLR) for clozapine at 10.0 mg/kg. Partial substitution (60.7% DLR) for clozapine was seen at 5.0 mg/kg. Generalization testing yielded an ED_{50} = 1.92 (95% C.I. 1.07 - 3.47 mg/kg). Response rates were significantly suppressed at 5.0 mg/kg ($F_{6, 36} = 10.7$, $P<0.0001$).

Sertindole generalization

Sertindole (Fig. 4) fully substituted (82.9% DLR) for clozapine at 40.0 mg/kg. Generalization testing yielded an ED_{50} = 9.64 (95% C.I. 4.97- 18.71 mg/kg). Response rates were not significantly different from vehicle at any of the doses tested (2.5 – 40.0 mg/kg).

Zotepine generalization

Zotepine (Fig. 5) fully substituted (88.5% DLR) for clozapine at 5.0 mg/kg. Partial substitution (66.8% DLR) for clozapine was seen at 2.5 mg/kg. Generalization testing yielded an ED_{50} = 2.12 (95% C.I. 1.76 - 2.56 mg/kg). Response rates were significantly suppressed at 5.0 mg/kg ($F_{5, 35}$ = 34.6, P<0.0001).

Iloperidone generalization

Iloperidone (Fig. 6) fully substituted (89.8% DLR) for clozapine at 0.4 mg/kg. Generalization testing yielded an ED_{50} = 0.19 (95% C.I. 0.14 - 0.25 mg/kg). Response rates were significantly suppressed at 0.4 mg/kg ($F_{6, 42}$ = 20.6, P<0.0001).

Melperone generalization

Melperone (Fig. 7) fully substituted (94.8% DLR) for clozapine at 2.0 mg/kg. Generalization testing yielded an ED_{50} = 2.22 (95% C.I. 1.56- 3.16 mg/kg). Response rates were significantly suppressed at 2.0 mg/kg ($F_{6, 42}$ = 20.7, P<0.0001).

Aripiprazole generalization

Aripiprazole (Fig. 8) (1.25 – 10.0 mg/kg) failed to substitute for clozapine. Response rates were significantly suppressed at 5.0 and 10 mg/kg ($F_{5, 30}$ = 20.9, P<0.0001).

Chlorpromazine generalization

Chlorpromazine (Fig. 9) fully substituted (94.5% DLR) for clozapine at 4.0 mg/kg. Partial substitution (67.2% DLR) for clozapine was seen at 2.0 mg/kg. Generalization testing

yielded an ED_{50} = 1.37 (95% C.I. 1.12 - 1.69 mg/kg). Response rates were significantly suppressed at 4.0 mg/kg ($F_{5, 35}$ = 22.8, P<0.0001).

Thioridazine generalization

Thioridazine (Fig. 12) fully substituted (97.5% DLR) for clozapine at 20.0 mg/kg. Generalization testing yielded an ED_{50} = 5.85 (95% C.I. 4.20 - 8.14 mg/kg). Response rates were not significantly different from vehicle at any of the doses tested (2.5 – 20.0 mg/kg).

Fluphenazine generalization

Fluphenazine (Fig. 10) (0.125 – 2.0 mg/kg) failed to substitute for clozapine. Fluphenazine never generated above vehicle (>20% DLR) appropriate responding. Response rates were significantly suppressed at 1.0 and 2.0 mg/kg ($F_{6, 36}$ = 10.65, P<0.0001).

Perphenazine generalization

Perphenazine (Fig. 11) (0.125 – 2.0 mg/kg) failed to substitute for clozapine. Fluphenazine never generated above vehicle (>20% DLR) appropriate responding. Response rates were significantly suppressed at 1.0 and 2.0 mg/kg ($F_{6, 36}$ = 10.65, P<0.0001).

M100907 generalization

The 5-HT_{2A} antagonist M100907 (Fig. 13) fully substituted (87.55% DLR) for clozapine at 5.6 mg/kg. Generalization testing yielded an ED_{50} = 1.95 (95% C.I. 1.34805- 2.817627 mg/kg). Response rates were significantly suppressed at 0.3, 1.0, 3.0 and 5.6 mg/kg ($F_{7, 49}$ = 10.02, P<0.0001).

Prazosin generalization

The α_1 antagonist prazosin (Fig. 14) fully substituted (81.5% DLR) for clozapine at 2.8 mg/kg. Generalization testing yielded an ED_{50} = 1.68 (95% C.I. 1.04812- 2.70317 mg/kg). Response rates were significantly suppressed at 2.0 and 2.82 mg/kg ($F_{6, 42} = 5.7$, $P<0.0005$).

Pyrilamine generalization

The H_1 histamine antagonist pyrilamine (Fig. 15) (5.0 – 40.0 mg/kg) failed to substitute for clozapine. Maximum clozapine appropriate responding was 38.9%. Response rates were significantly suppressed at 40.0 mg/kg ($F_{5, 35} = 22.3$, $P<0.0001$).

Fluoxetine generalization

The selective serotonin reuptake inhibitor (SSRI) fluoxetine (Fig. 16) (3.75 – 15.0 mg/kg) failed to substitute for clozapine. Maximum clozapine appropriate responding was 13.1%. Response rates were significantly suppressed at 15.0 mg/kg ($F_{4, 28} = 3.9$, $P<0.05$).

Clozapine generalization

A second generalization curve with clozapine for the 2.5 mg/kg training dose was obtained prior to testing N-desmethylclozapine (Fig. 17). Maximum clozapine appropriate responding was 99.6% at the 2.5 mg/kg training dose. Generalization testing yielded an ED_{50} = 1.065 (95% C.I. 0.92- 1.23 mg/kg).

N-desmethylclozapine generalization

The clozapine metabolite n-desmethylclozapine (Fig. 18) (2.5 – 10.0 mg/kg) failed to substitute for clozapine. Maximum clozapine appropriate responding was 39.2%. Response rates were significantly suppressed at 5.0 mg/kg and 10.0 mg/kg ($F_{4, 32} = 30.75$, $P<0.0001$).

Figure 1. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for generalization curves for the 2.5 mg/kg clozapine training dose. The dashed line at 80% drug-lever responding indicates full generalization to the training dose of clozapine. Control tests were conducted with either clozapine (2.5 mg/kg) or vehicle prior to testing. Mice had to have a rate of at least 2.0 responses per minute or have earned a reinforcer (FR10) to be included in percentage drug-lever responding data. Significant differences in rates of responding are indicated by *asterisks* (*$P<0.05$, **$P<0.01$).

Figure1. Clozapine Generalization

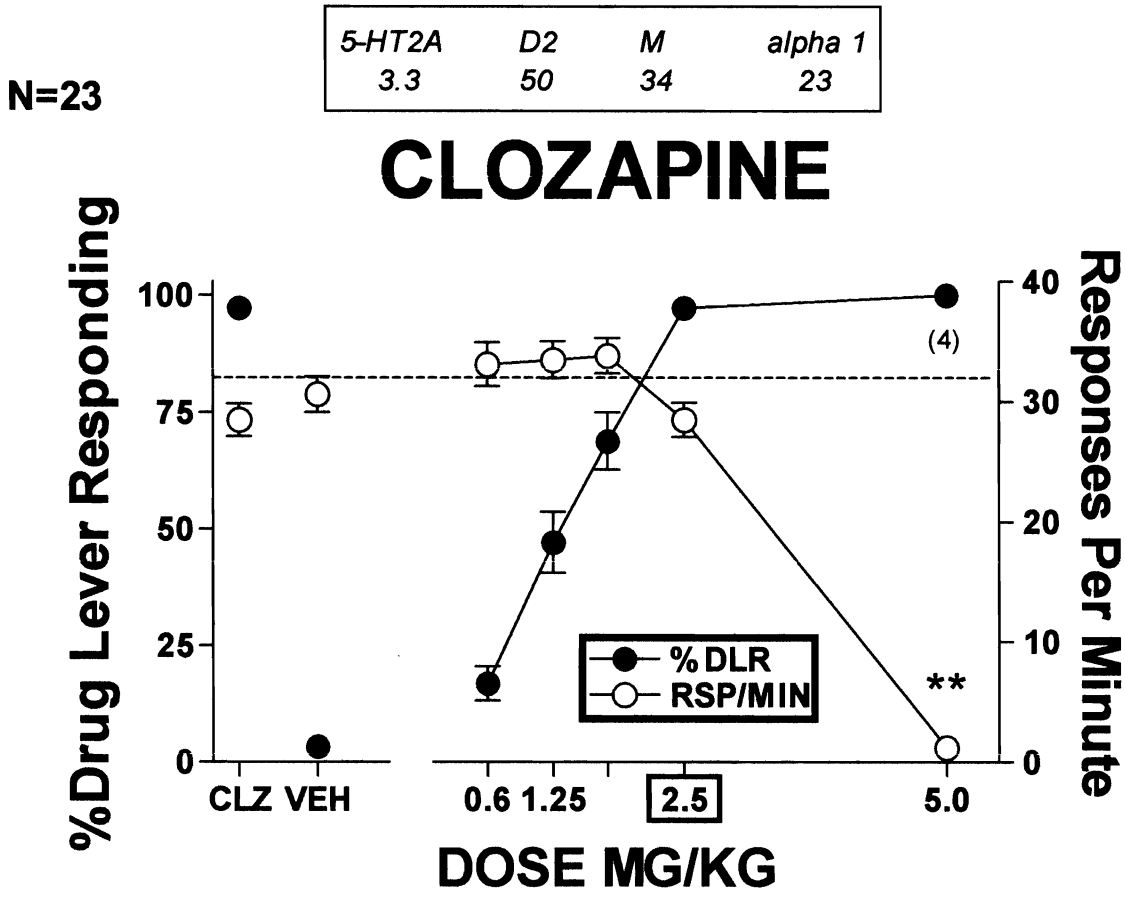


Figure 2. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) for generalization curves for the 2.5 mg/kg clozapine training dose in the current study (2) are superimposed over previously obtained data (1) in C57BL/6 mice (Philibin et al. 2005). Indicators of significant differences in rates of responding have been omitted. All other details are the same as Fig. 1.

Figure 2.

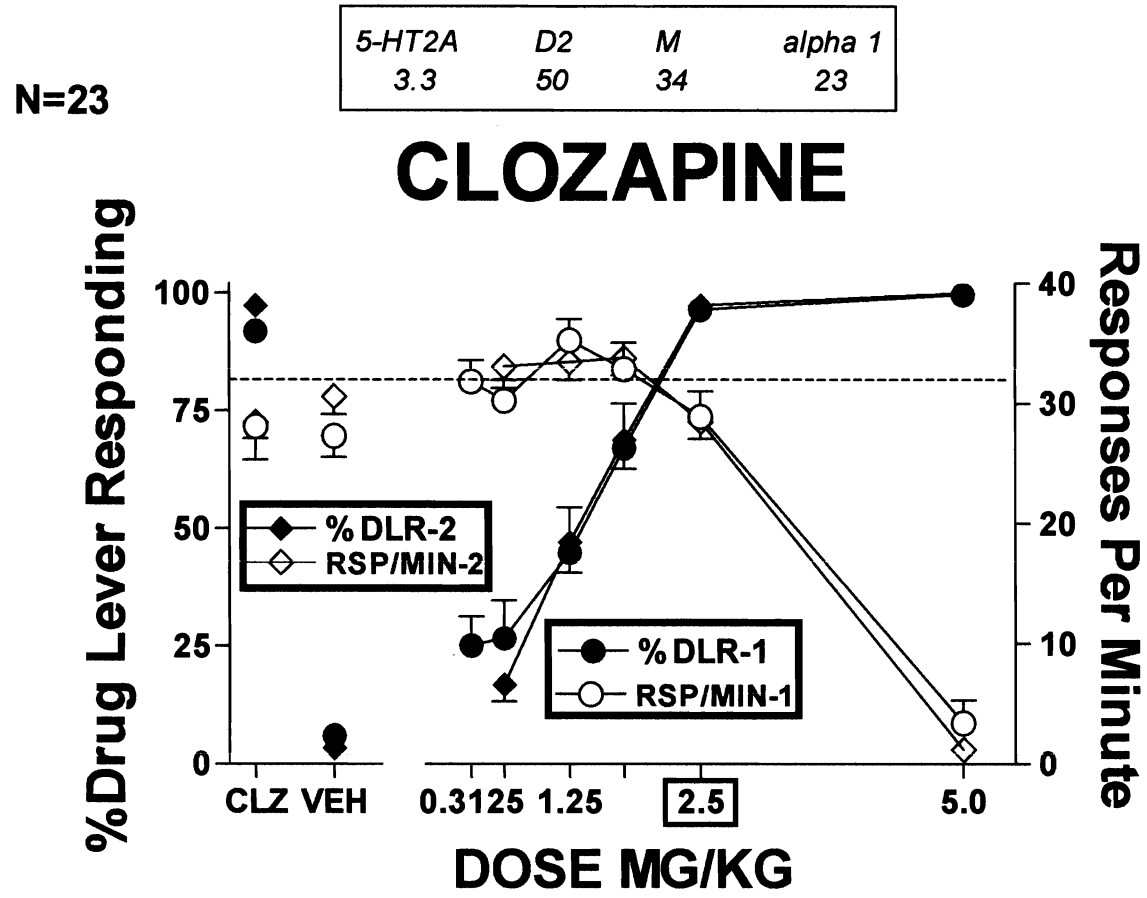

5-HT2A
3.3
D2
50
M
34
alpha 1
23
N=23
CLOZAPINE
%Drug Lever Responding
Responses Per Minute
100
75
50
25
0
40
30
20
10
0
% DLR-2
RSP/MIN-2
% DLR-1
RSP/MIN-1
CLZ
VEH
0.3125
1.25
2.5
5.0
DOSE MG/KG

Figure 3. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for quetiapine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 3.

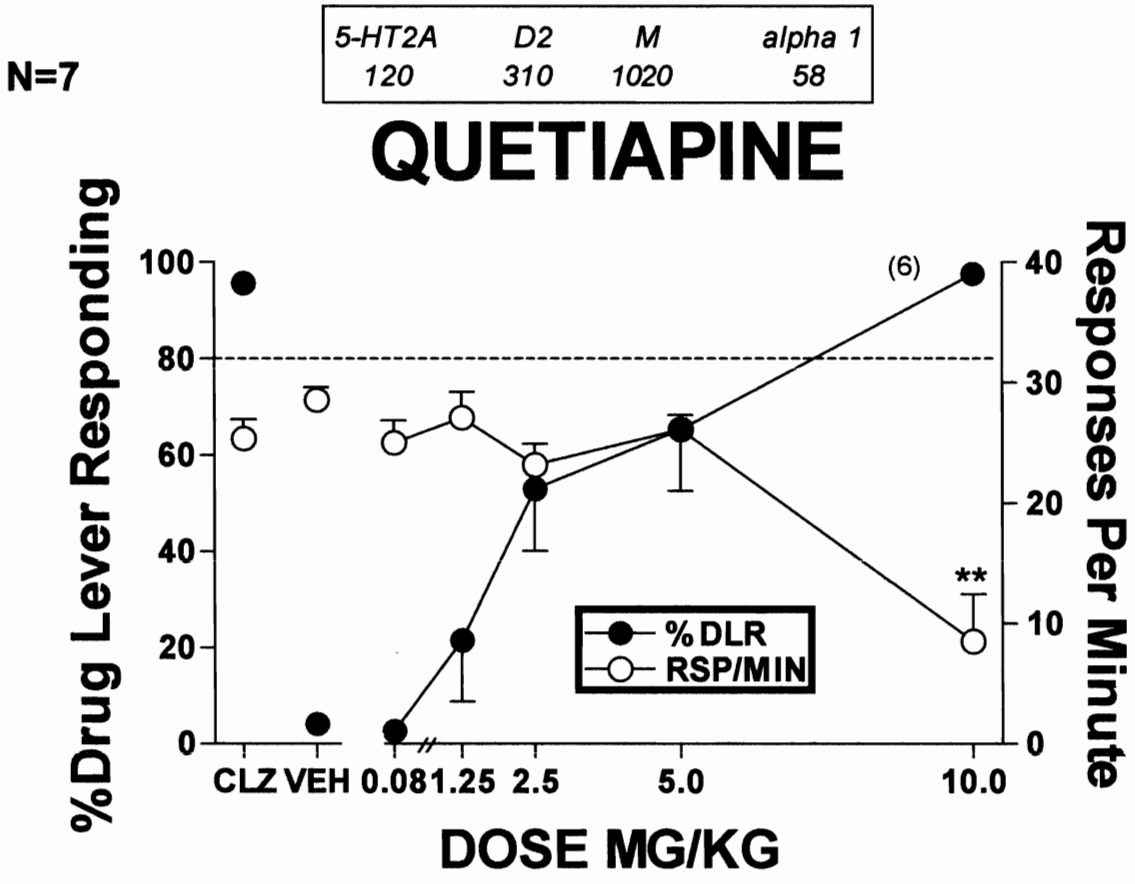

N=7
5-HT2A 120
D2 310
M 1020
alpha 1 58
QUETIAPINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
(6)
**
% DLR
RSP/MIN
CLZ VEH 0.08 1.25 2.5 5.0 10.0
DOSE MG/KG

Figure 4. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for sertindole generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 4.

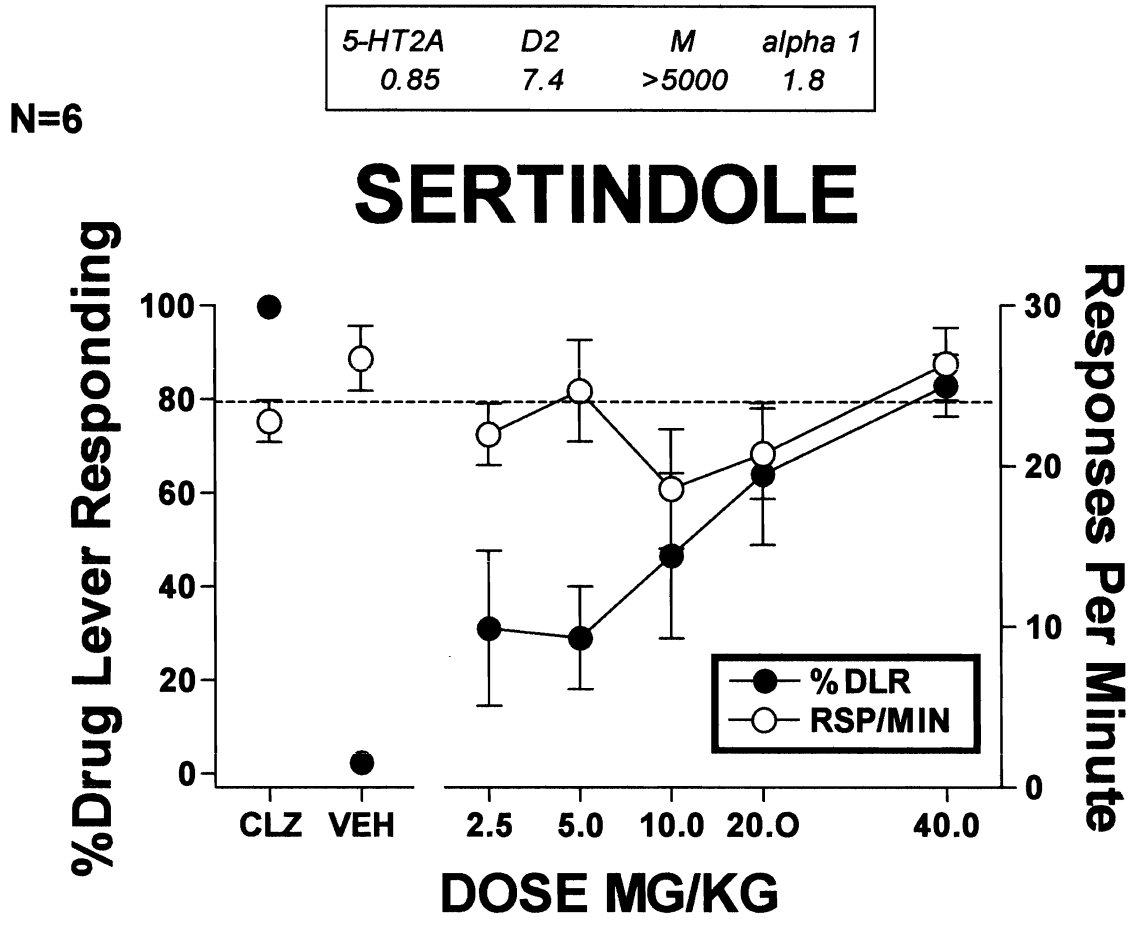

5-HT2A
0.85
D2
7.4
M
>5000
alpha 1
1.8
N=6
SERTINDOLE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
30
20
10
0
CLZ
VEH
2.5
5.0
10.0
20.O
40.0
%DLR
RSP/MIN
DOSE MG/KG

Figure 5. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for zotepine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 5.

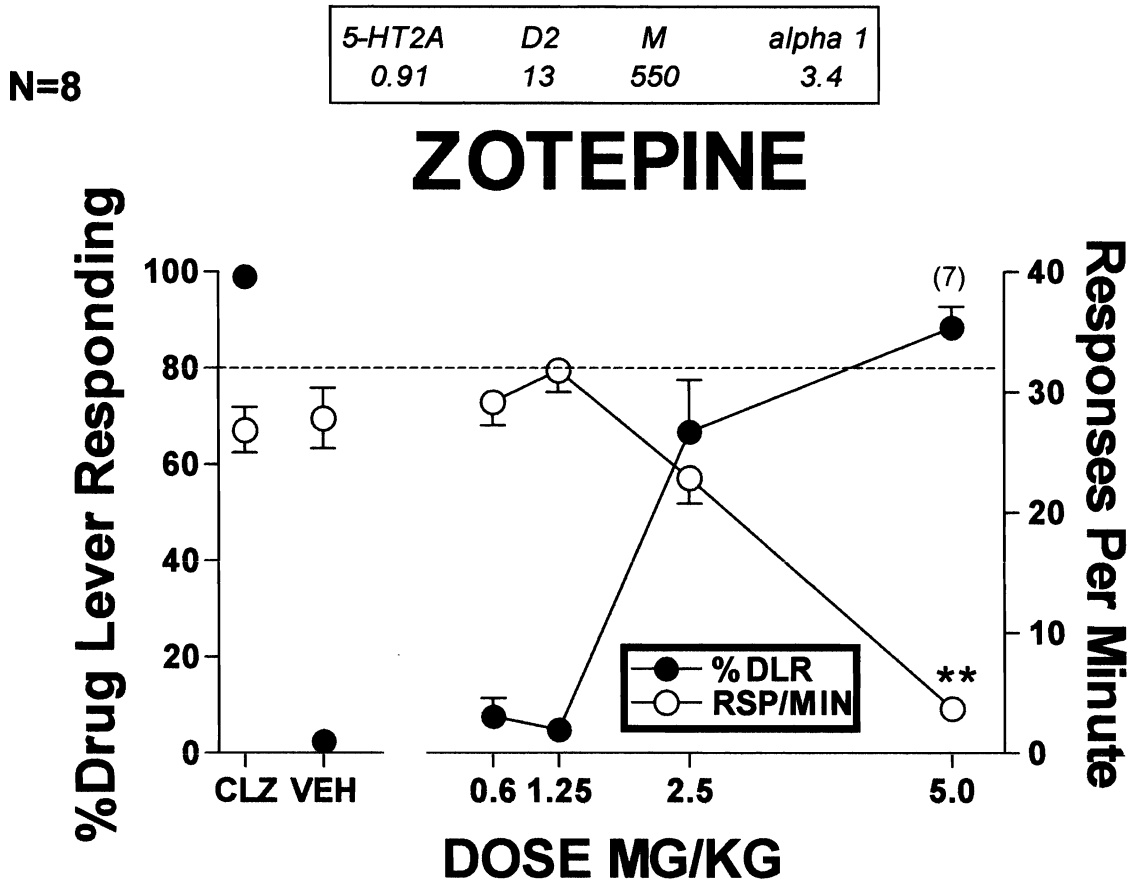

5-HT2A
0.91
D2
13
M
550
alpha 1
3.4
N=8
ZOTEPINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
(7)
**
% DLR
RSP/MIN
CLZ VEH
0.6 1.25
2.5
5.0
DOSE MG/KG

Figure 6. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for iloperidone generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 6.

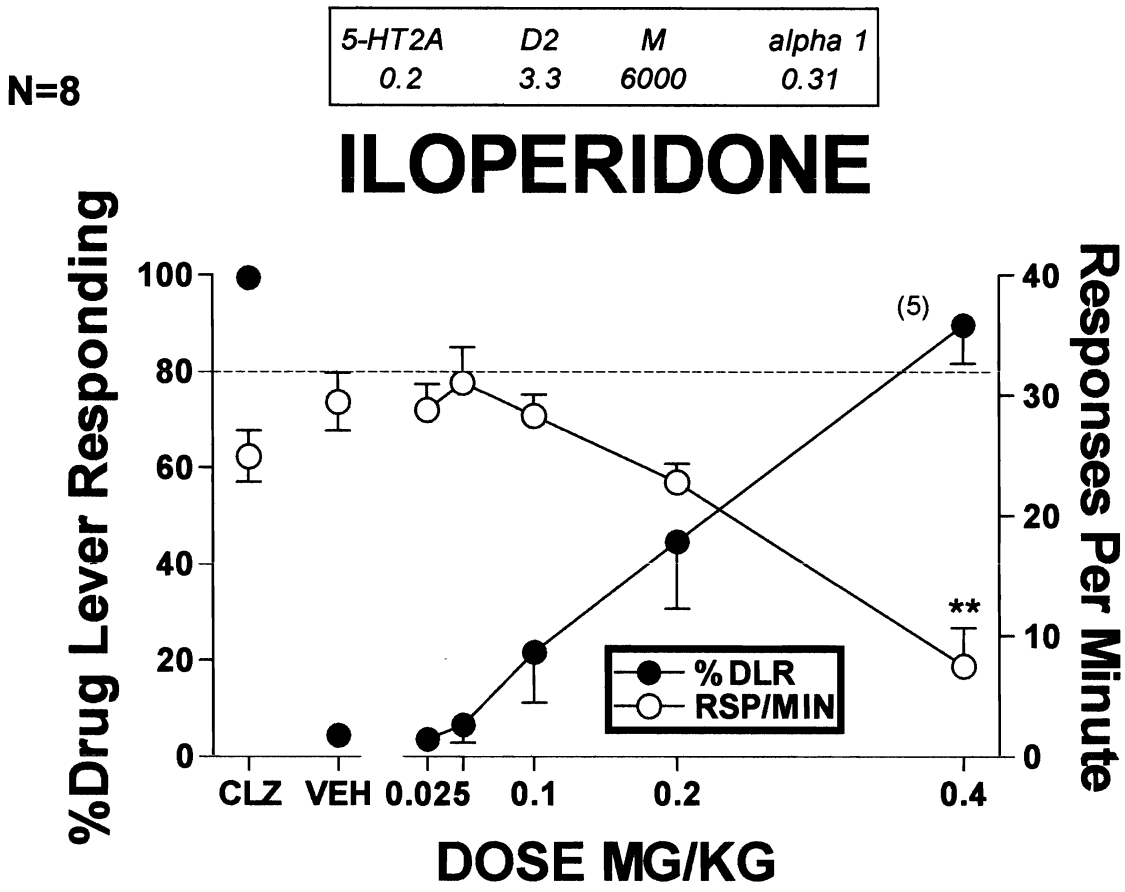

5-HT2A
0.2
D2
3.3
M
6000
alpha 1
0.31
N=8
ILOPERIDONE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
(5)
**
% DLR
RSP/MIN
CLZ
VEH
0.025
0.1
0.2
0.4
DOSE MG/KG

Figure 7. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for melperone generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 7.

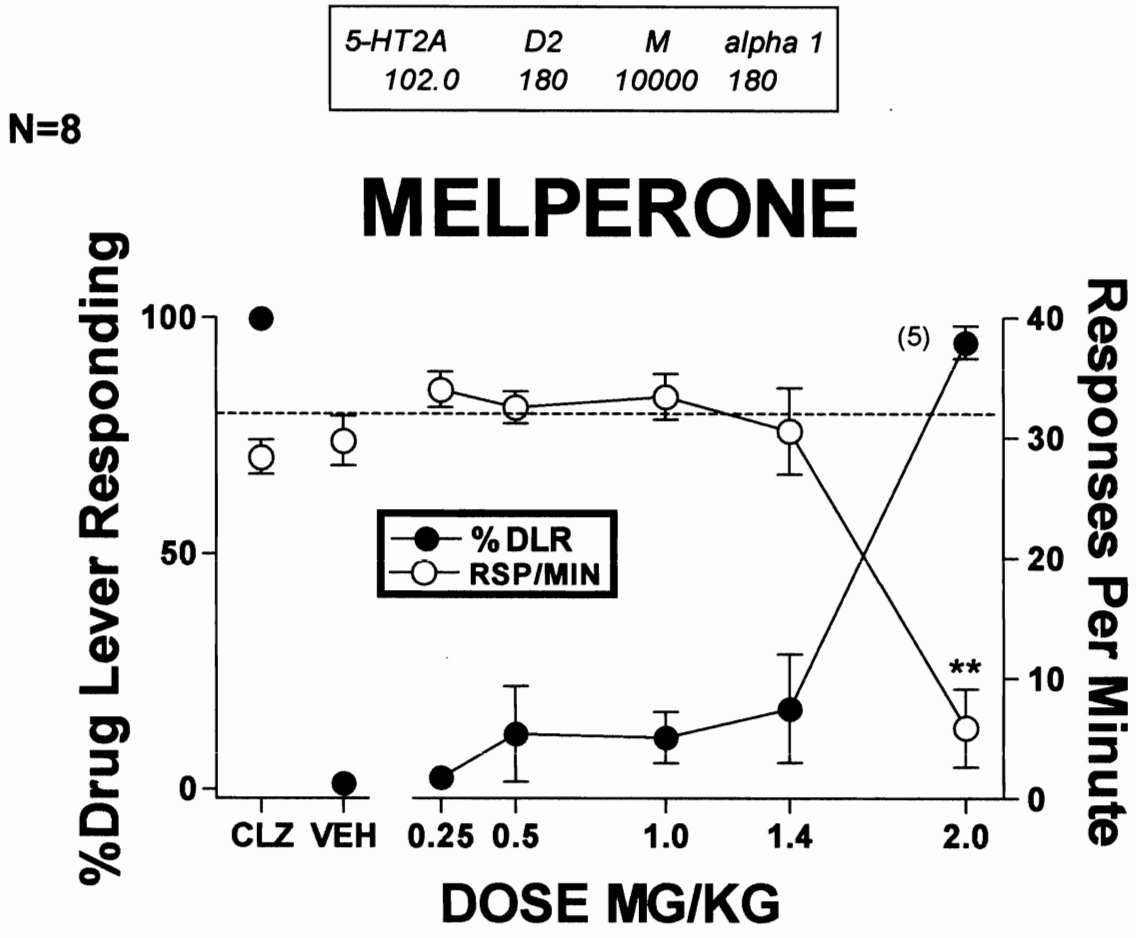

5-HT2A
102.0
D2
180
M
10000
alpha 1
180
N=8
MELPERONE
%Drug Lever Responding
Responses Per Minute
100
50
0
40
30
20
10
0
(5)
**
% DLR
RSP/MIN
CLZ
VEH
0.25
0.5
1.0
1.4
2.0
DOSE MG/KG

Figure 8. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for aripiprazole generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 8.

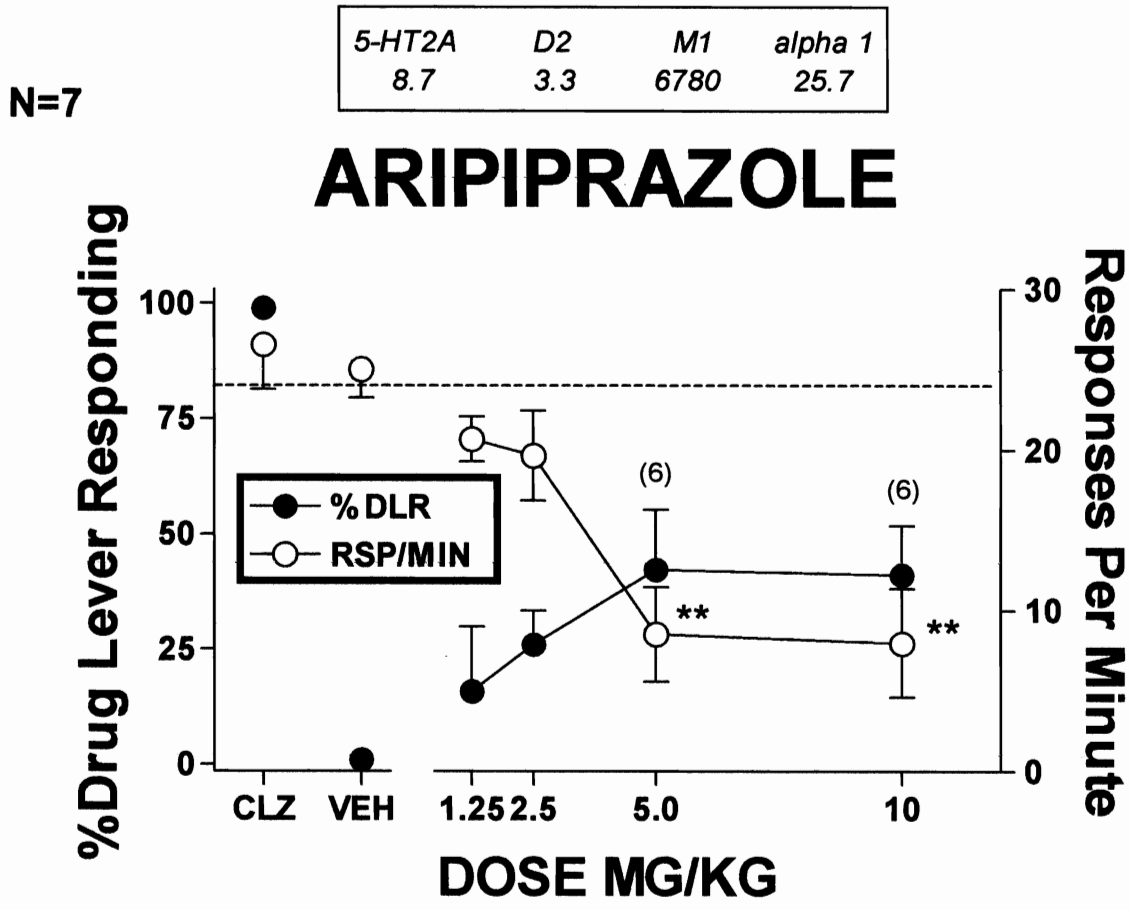

5-HT2A
8.7
D2
3.3
M1
6780
alpha 1
25.7
N=7
ARIPIPRAZOLE
%Drug Lever Responding
Responses Per Minute
100
75
50
25
0
30
20
10
0
% DLR
RSP/MIN
(6)
(6)
**
**
CLZ
VEH
1.25
2.5
5.0
10
DOSE MG/KG

Figure 9. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for chlorpromazine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 9.

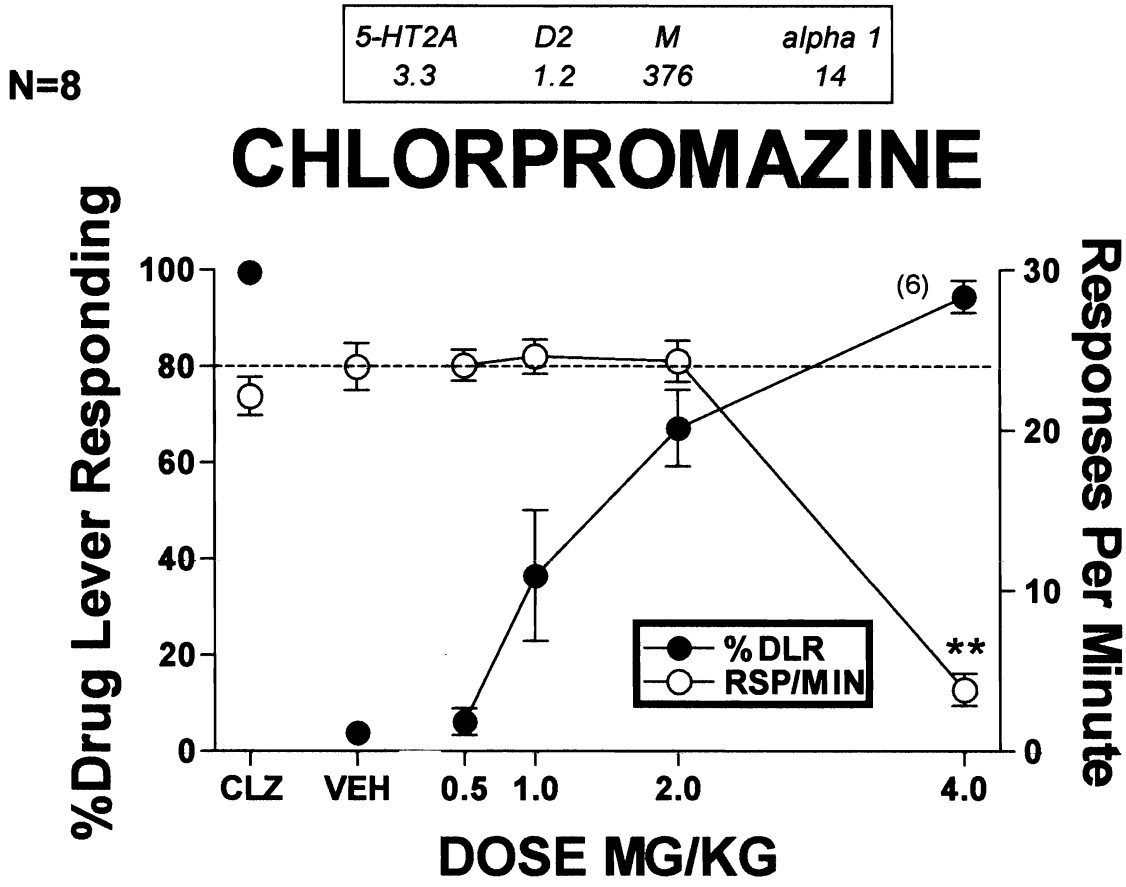

5-HT2A
3.3
D2
1.2
M
376
alpha 1
14
N=8
CHLORPROMAZINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
30
20
10
0
(6)
**
%DLR
RSP/MIN
CLZ
VEH
0.5
1.0
2.0
4.0
DOSE MG/KG

Figure 10. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for thioridazine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 10.

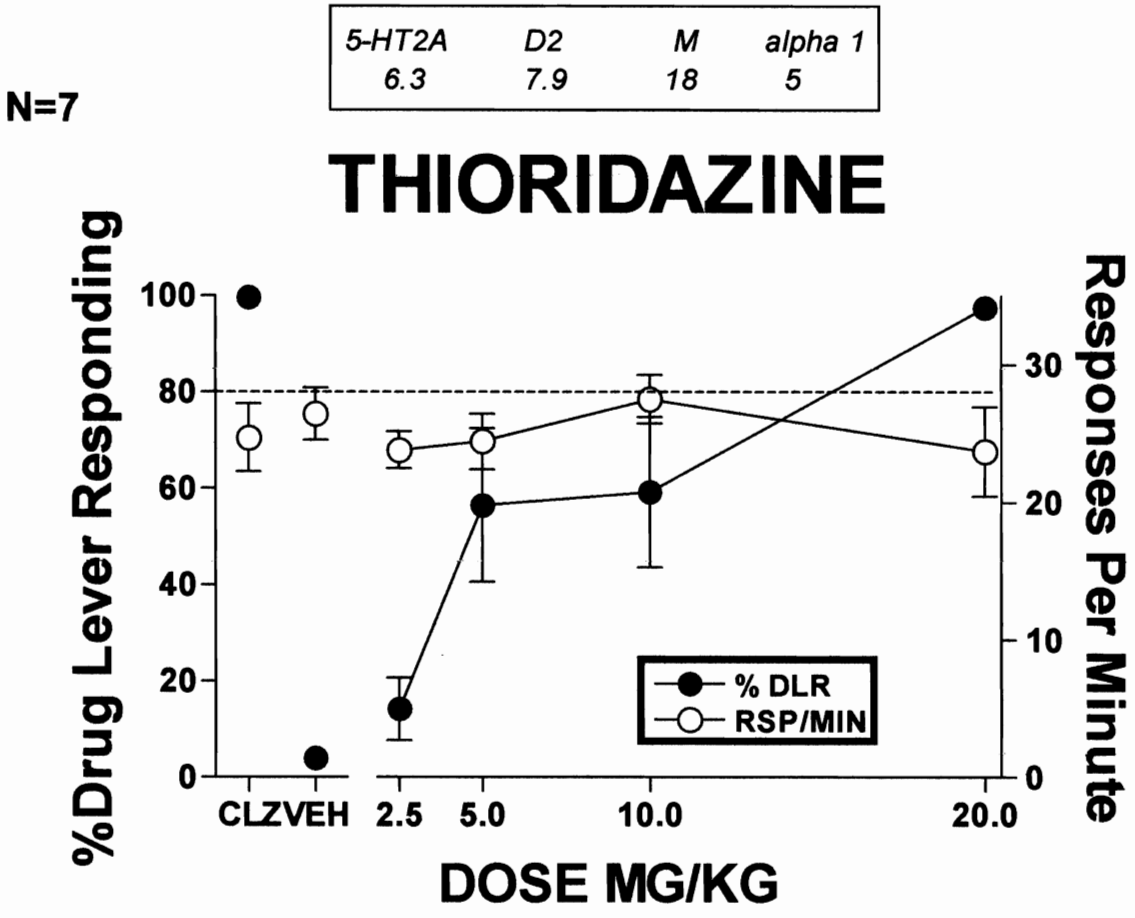

5-HT2A
6.3
D2
7.9
M
18
alpha 1
5
N=7
THIORIDAZINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
30
20
10
0
% DLR
RSP/MIN
CLZVEH
2.5
5.0
10.0
20.0
DOSE MG/KG

Figure 11. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for fluphenazine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 11.

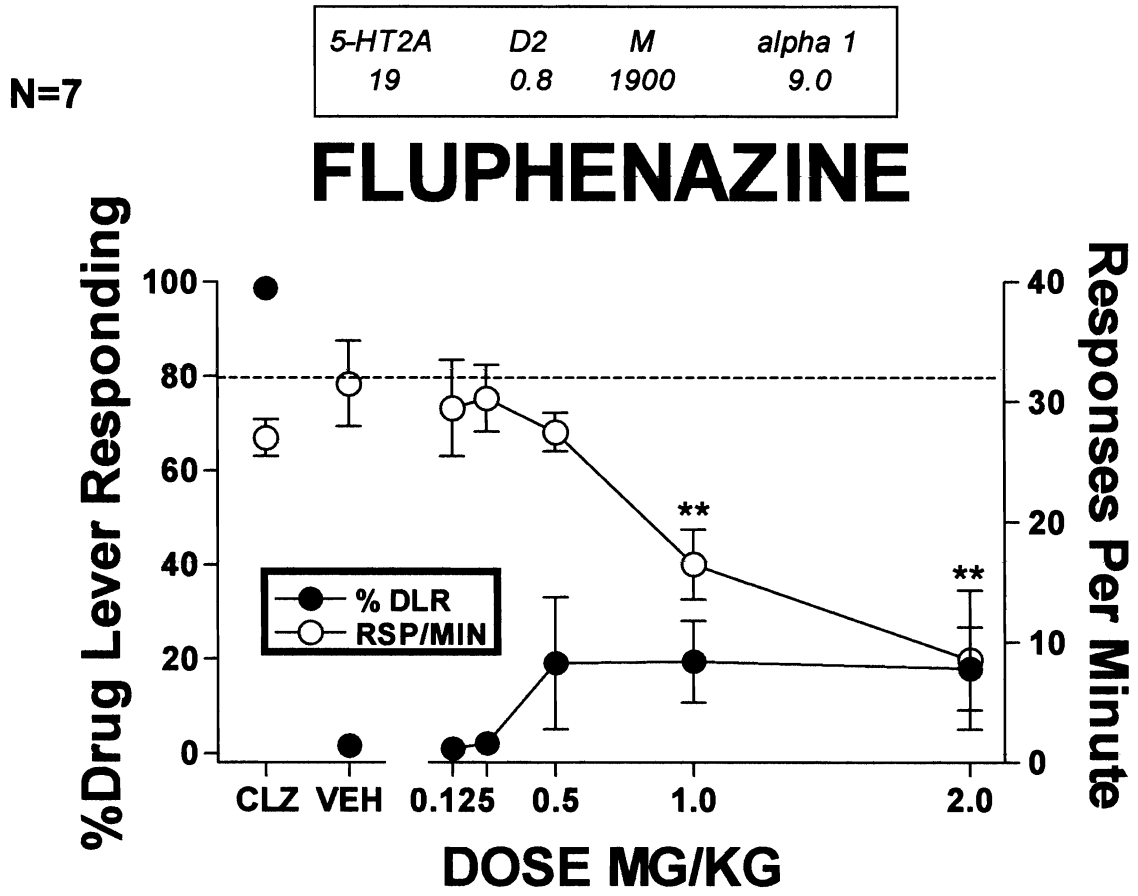

N=7
5-HT2A 19
D2 0.8
M 1900
alpha 1 9.0
FLUPHENAZINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
% DLR
RSP/MIN
**
**
CLZ
VEH
0.125
0.5
1.0
2.0
DOSE MG/KG

Figure 12. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for perphenazine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 12.

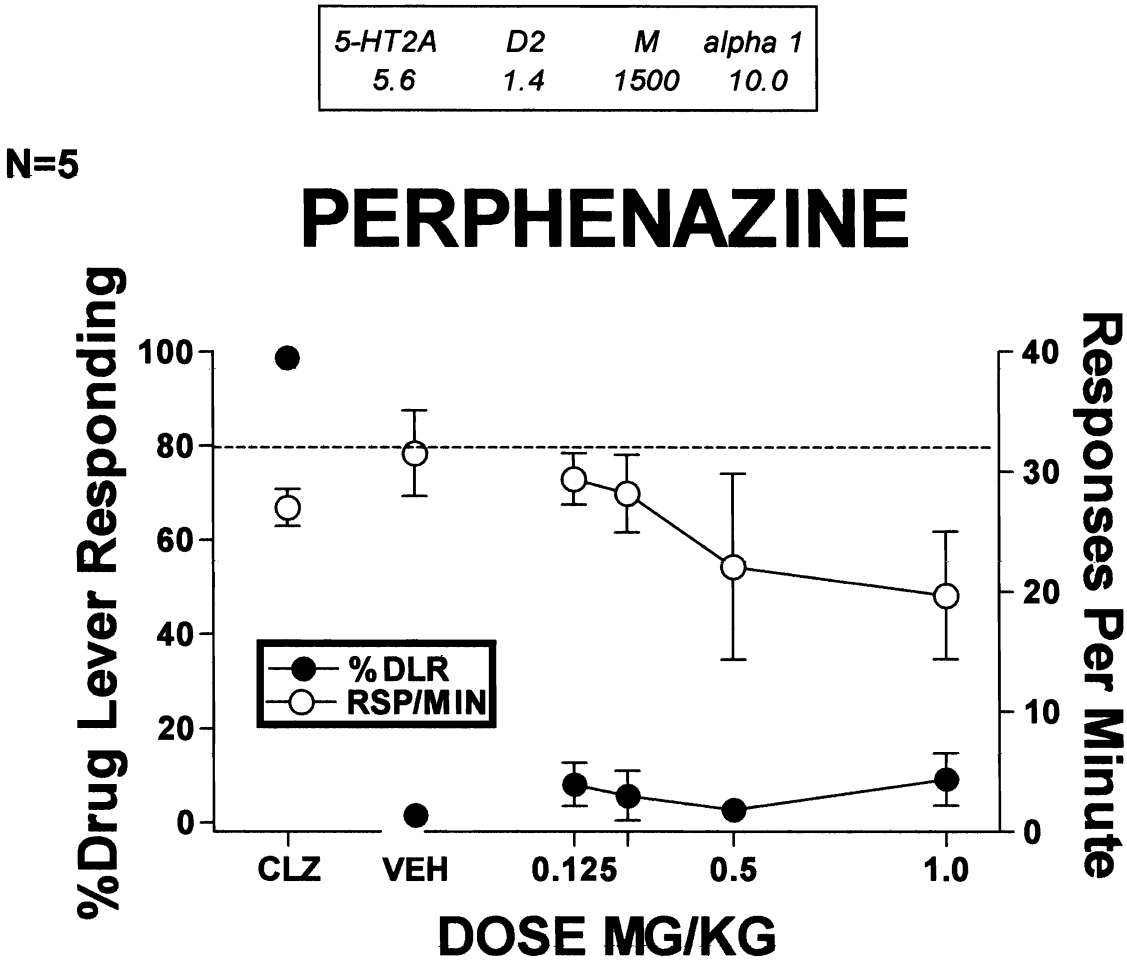

5-HT2A
5.6
D2
1.4
M
1500
alpha 1
10.0
N=5
PERPHENAZINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
% DLR
RSP/MIN
CLZ
VEH
0.125
0.5
1.0
DOSE MG/KG

Figure 13. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for M100907 generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 13.

5-HT2A	D2	M	alpha 1
1.92	>10000	>10000	128.0

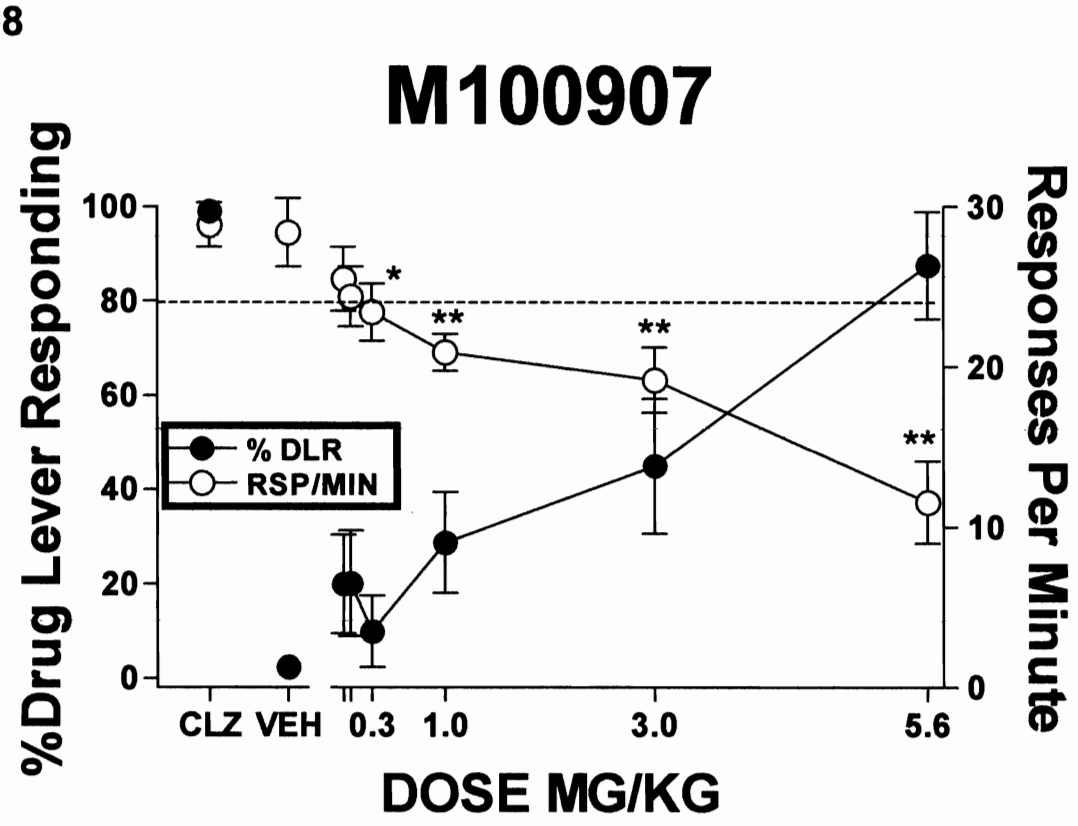


Figure 14. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for prazosin generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 14.

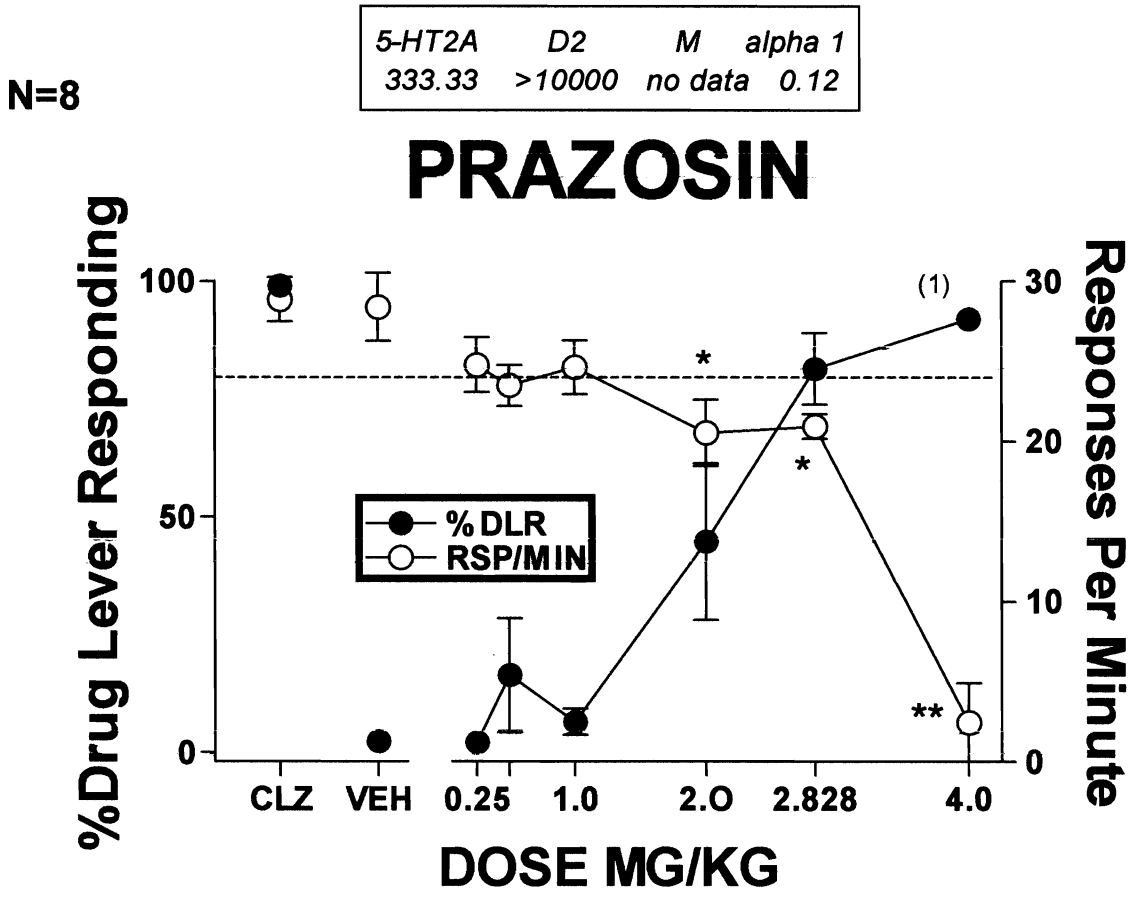

5-HT2A D2 M alpha 1
333.33 >10000 no data 0.12
N=8
PRAZOSIN
%Drug Lever Responding
Responses Per Minute
100
50
0
30
20
10
0
(1)
*
*
**
% DLR
RSP/MIN
CLZ
VEH
0.25
1.0
2.O
2.828
4.0
DOSE MG/KG

Figure 15. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for pyrilamine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 15.

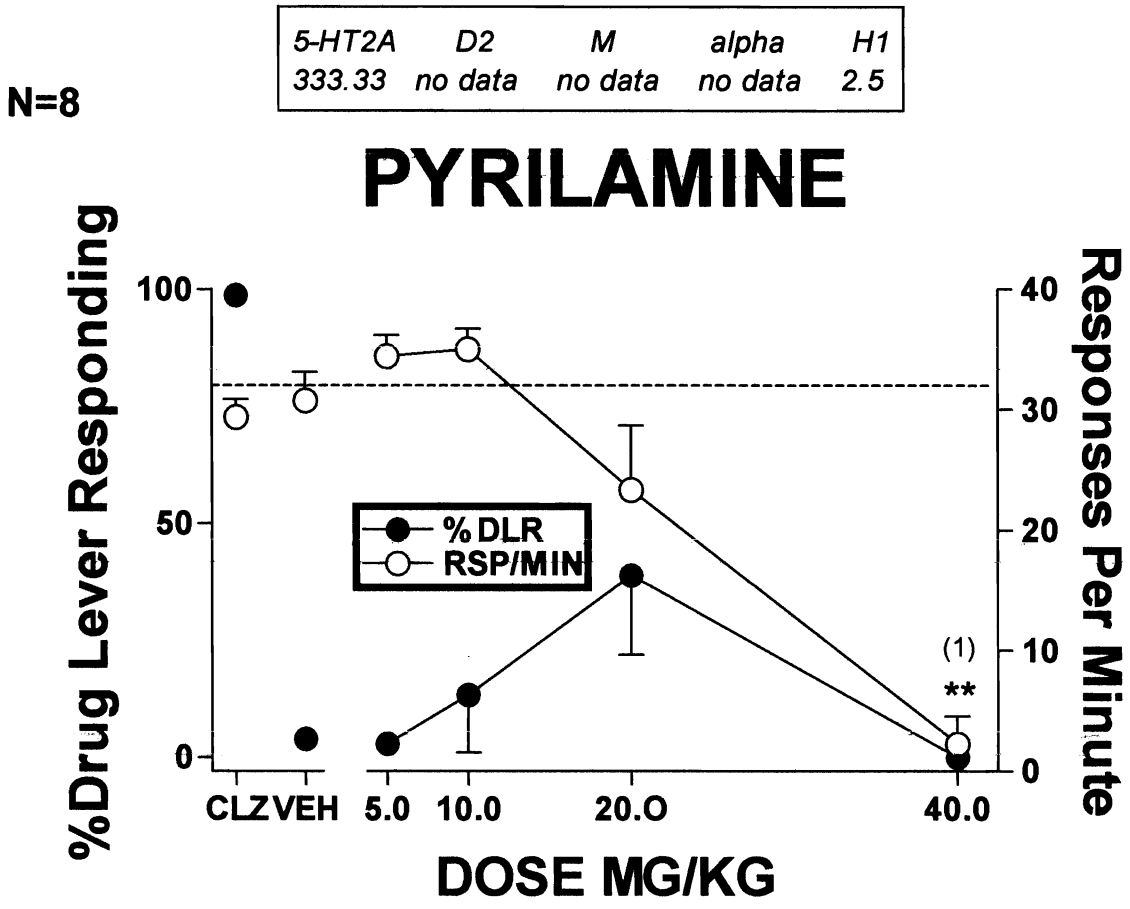

5-HT2A
333.33
D2
no data
M
no data
alpha
no data
H1
2.5
N=8
PYRILAMINE
%Drug Lever Responding
Responses Per Minute
100
50
0
40
30
20
10
0
%DLR
RSP/MIN
(1)
**
CLZVEH
5.0
10.0
20.O
40.0
DOSE MG/KG

Figure 16. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for fluoxetine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 16.

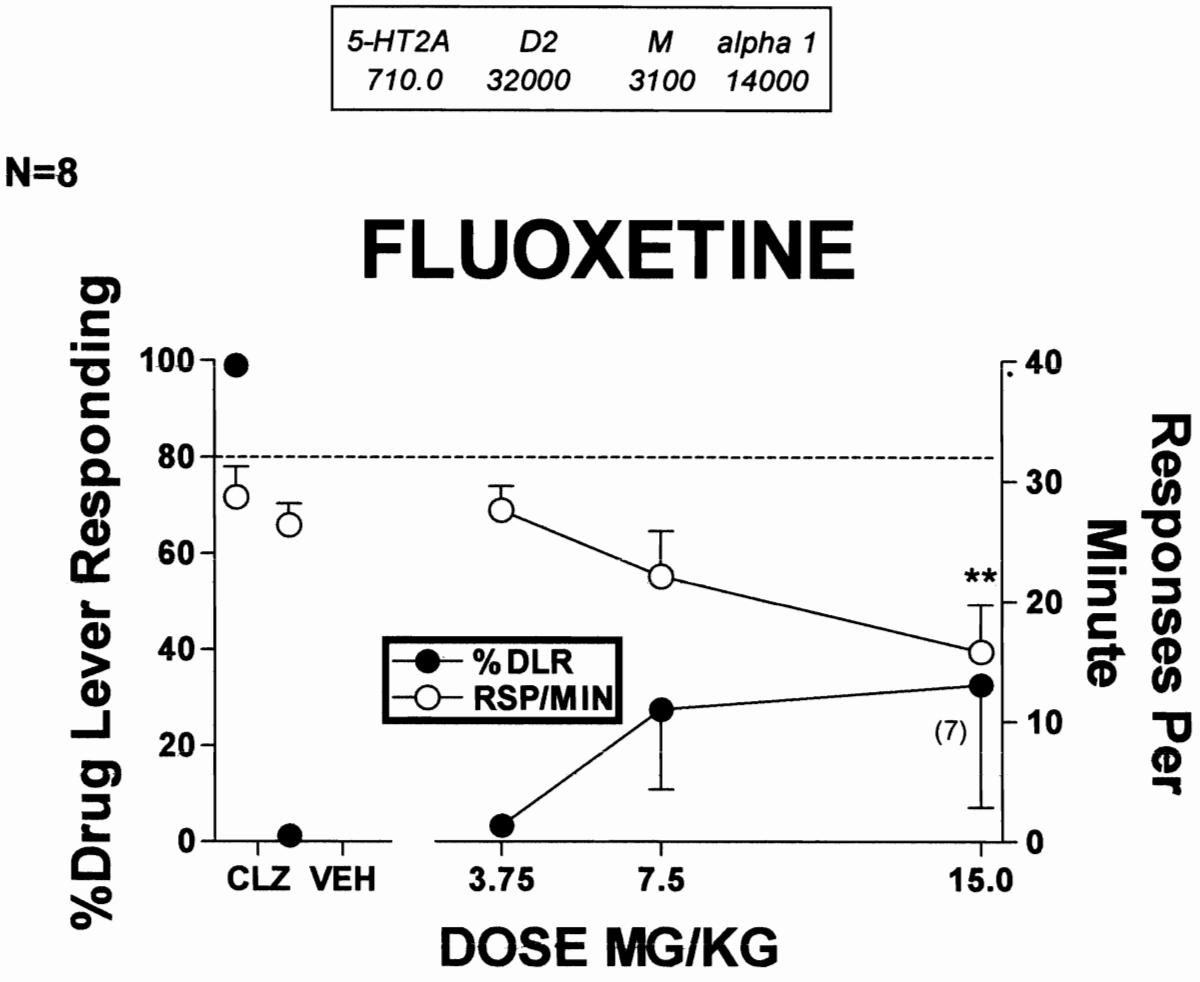

5-HT2A
710.0
D2
32000
M
3100
alpha 1
14000
N=8
FLUOXETINE
%Drug Lever Responding
Responses Per Minute
100
80
60
40
20
0
40
30
20
10
0
%DLR
RSP/MIN
**
(7)
CLZ
VEH
3.75
7.5
15.0
DOSE MG/KG

Figure 17. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for clozapine generalization curves for the 2.5 mg/kg clozapine training dose prior to testing N-desmethyl clozapine. All other details are the same as Fig. 1.

Figure 17.

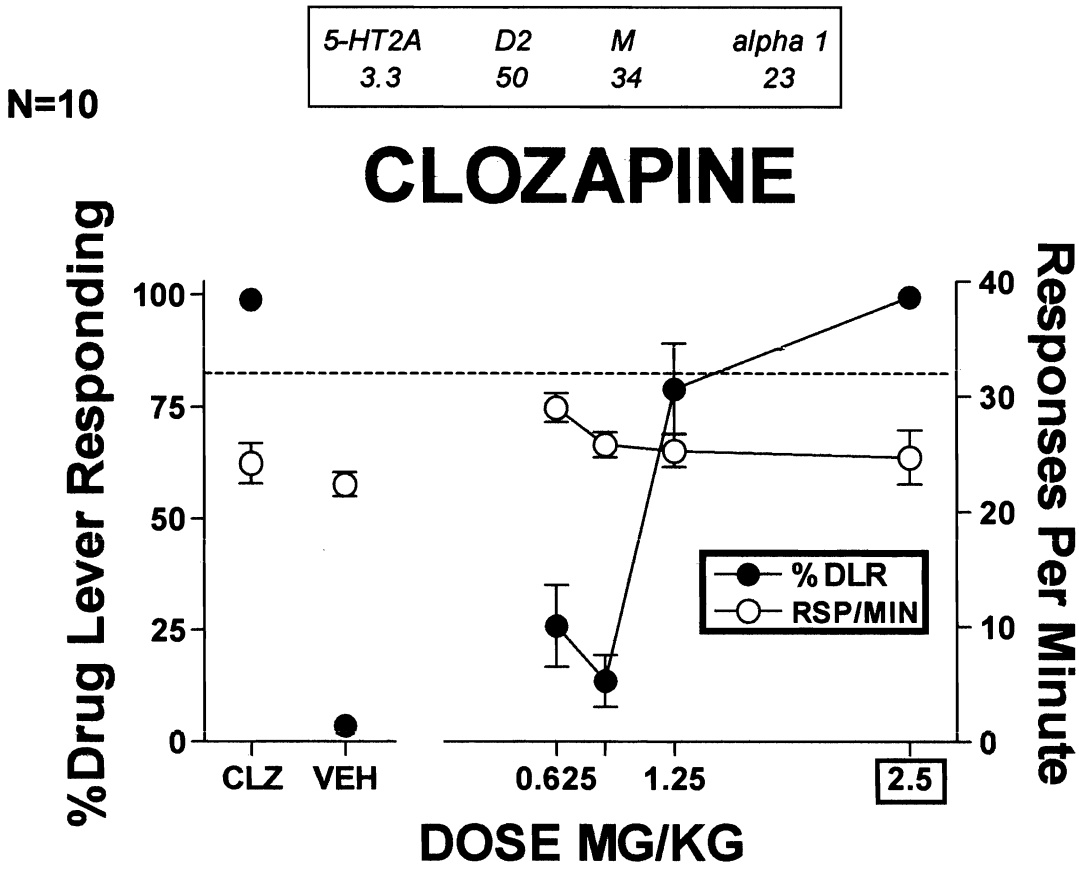
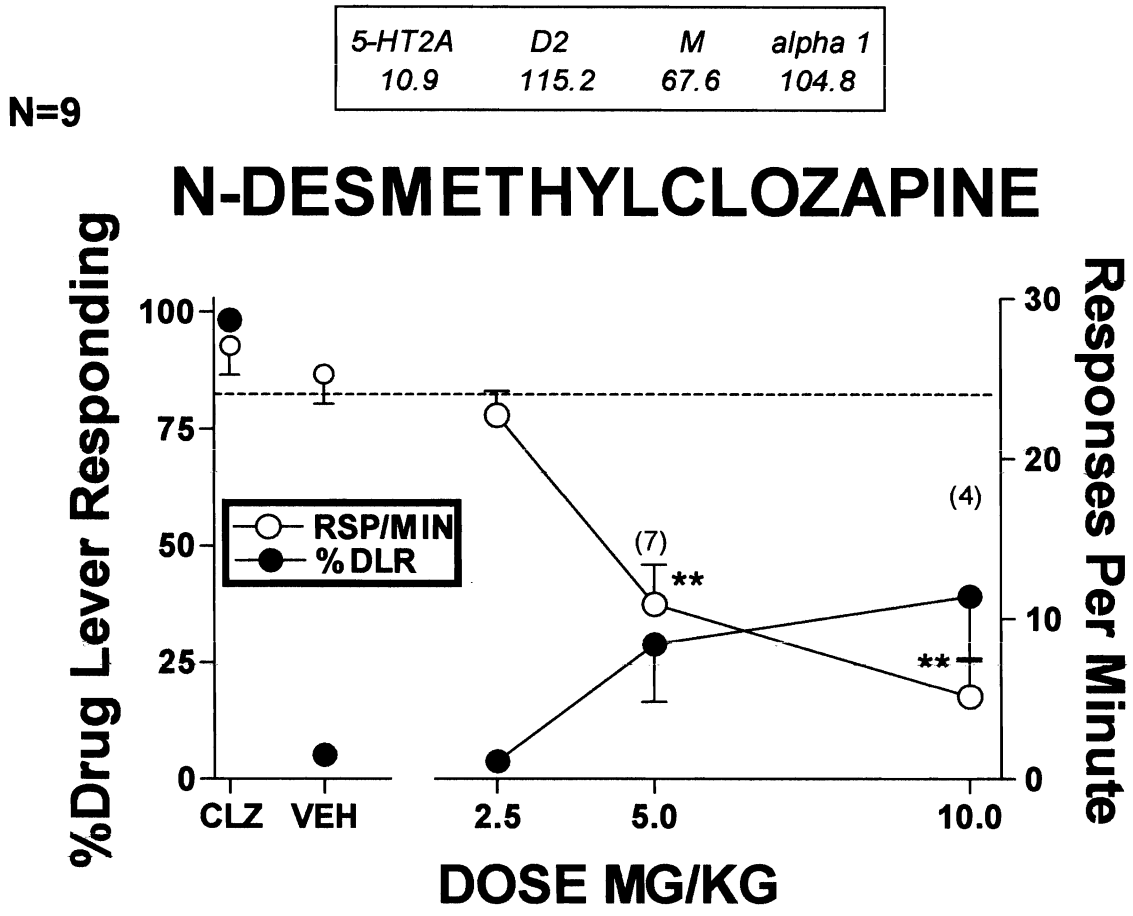

5-HT2A
3.3
D2
50
M
34
alpha 1
23
N=10
CLOZAPINE
%Drug Lever Responding
Responses Per Minute
100
75
50
25
0
40
30
20
10
0
% DLR
RSP/MIN
CLZ
VEH
0.625
1.25
2.5
DOSE MG/KG

Figure 18. Mean percentage drug-lever responding (±SEM) and mean responses per minute (±SEM) are shown for N-desmethylclozapine generalization curves for the 2.5 mg/kg clozapine training dose. All other details are the same as Fig. 1.

Figure 18.


5-HT2A
10.9
D2
115.2
M
67.6
alpha 1
104.8
N=9
N-DESMETHYLCLOZAPINE
%Drug Lever Responding
Responses Per Minute
100
75
50
25
0
30
20
10
0
RSP/MIN
%DLR
(7)
**
(4)
**
CLZ
VEH
2.5
5.0
10.0
DOSE MG/KG

Discussion

Clozapine discrimination and generalization

The present study obtained remarkably similar data to that previously reported by Philibin et al. (2005). Generalization testing with clozapine yielded an ED_{50} = 1.19 in the present study as compared to an ED_{50} = 1.14 in the Philibin et al. (2005) study. However, the acquisition of the drug discrimination task was more rapid in the current study. Mice in the present study met the training criteria in an average of 14.8 (SEM $\pm$ 1.6) sessions with a range of 6-34 sessions; whereas, in the Philibin et al. (2005) study the mice required an average of 35.6 ($\pm$2.84, range 15-52) training sessions. This may be due to the increased experience of the experimenter with the drug discrimination procedure with mice. Another possible factor was that the 60 minute presession injection time used in the Philibin et al. (2005) study was changed to 30 minutes (based on time course data from that study that showed no significant difference between the 60 and 30 minute time points).

Classification of antipsychotic drugs

It may be an oversimplification to assign the antipsychotic agents to two homogeneous classes; the typical and atypical antipsychotic drugs (see review by Arnt & Skarsfeldt, 1997). Rather, each novel antipsychotic drug has its own unique pharmacological profile that is determined by its neurotransmitter receptor binding profile. The atypical antipsychotic drugs are, themselves, a heterogeneous group of agents but there are some similarities in their receptor binding affinities (see Table 1.). It is therefore, essential to characterize the receptor mediated effects of various antipsychotic agents in order to increase our understanding of the complex pharmacology of schizophrenia.

In the clozapine drug discrimination procedure, the 5-HT_{2A} receptor antagonist M100907 engendered clozapine appropriate responding in C57BL/6 mice. This extends previous findings that the 5-$HT_{2A/2B/2C}$ receptor antagonist ritanserin fully substitutes for clozapine in C57BL/6 mice (Philibin et al., 2005). The atypical antipsychotics olanzapine, risperidone, and ziprasidone (from the Philibin et al., 2005 study) and sertindole, quetiapine, iloperidone, melperone, and zotepine (from the present study) fully substituted for clozapine in C57BL/6 mice. All of these atypical APDs bind potently to 5-HT receptors and these data suggest that clozapine (at least in C57BL/6 mice) produces an interoceptive cue that is mediated via 5-HT receptor antagonism. These findings also demonstrate that the discriminative stimulus properties of clozapine are similar to those of these atypical antipsychotic drugs.

While both clozapine and olanzapine are potent antimuscarinic agents, the selective muscarinic antagonist scopolamine failed to fully substitute for clozapine (Philibin et al., 2005). This suggests that antimuscarinic effects are not necessary or sufficient to engender clozapine-appropriate responding in C57BL/6 mice. This notion is further supported by the ability of some atypical antipsychotic agents with negligible muscarinic affinities (e.g., risperidone and sertindole) to fully substitute in clozapine-trained C57BL/6 mice. The failure of N-desmethylclozapine to generalize may be due to its being a partial dopamine agonist rather than a D_2 antagonist (Burstein, Ma, Wong, Gao, Pham, Knapp, Nash, Olsson, Davis, Hacksell, Weiner, & Brann, 2005). This hypothesis is supported by the finding that aripiprazole, another partial DA agonist, also did not generalize. Thus, although both NDMC and aripiprazole are potent 5-HT2A antagonists, and selective 5-HT2a antagonists such as M100907 generalize to clozapine, 5-HT2A partial agonism may prevent the discrimination cue from being detected. The partial DA

agonism would lead to a diminished release of DA in subcortical, but not cortical regions. This would suggest further research should consider the role of mesolimbic DA release in the discrimination cue. A way to test this would be to combine amphetamine with clozapine or to block the effect of dopamine release by pretreatment with low dose haloperidol.

While this procedure detects the vast majority of atypical antipsychotic drugs tested, several typical antipsychotic drugs also fully substituted for clozapine in this assay. Typical antipsychotic drugs that tend to have higher receptor binding affinities for 5-HT_{2A} receptors relative to haloperidol, such as chlorpromazine and thioridazine, fully substituted for clozapine. Chlorpromazine, unlike clozapine, is associated with EPS, tardive dyskinesia and neuroleptic malignant syndrome, but similar to clozapine, has serotonergic, muscarinic and dopaminergic antagonist effects. Full substitution for clozapine with chlorpromazine may be due to its higher affinity for 5-HT receptors or other concurrent receptor effects - i.e., a compound cue similar to that of clozapine. Chlorpromazine represents a false positive in this model as a selective assay for antipsychotic agents that have reduced EPS liability. Thioridazine is a low-potency phenothiazine that is slightly less potent than chlorpromazine. While, thioridazine has a lower propensity to cause EPS than most conventional neuroleptic agents, it is classified as a typical antipsychotic drug. Thioridazine has slightly higher potency at 5-HT_{2A} receptors relative to D_2 but this is not a ratio greater than 2:1. In this regard, thioridazine is more similar to clozapine, but unlike clozapine, thioridazine is associated with EPS.

The only atypical antipsychotic drug tested that failed to fully substitute for clozapine in C57BL/6 mice was aripiprazole. Aripiprazole, 7-{4-[4-(2,3-dichlorophenyl)-1-piperazinyl]-butyloxy}-3,4-dihydro-2(1*H*)-quinolinone, is a novel antipsychotic with a somewhat different

mechanism of action from other atypical antipsychotic drugs. Aripiprazole has 5-HT_{2A} blocking properties and suppresses DA activity by the partial DA agonist mechanism. Similar to many antipsychotic agents, aripiprazole binds with high affinity to dopamine D_2 receptors (Kikuchi, Tottori, Uwahodo, Hirose, Miwa, Oshiro, & Morita, 1995; Lawler, Prioleau, Lewis, Mak, Jiang, Schetz, Gonzalez, Sibley, & Mailman, 1999); however, whereas older antipsychotics are believed to exert their effects through antagonism of D_2 (and 5-HT_2) receptors, aripiprazole may exert its therapeutic effects through partial agonism of the D_2-family of receptors (Inoue, Domae, Yamada, & Furukawa, 1996).

Aripiprazole displays dopamine D_2 receptor antagonist activity *in vivo* (e.g., blockade of apomorphine-induced stereotypy) and D_2 receptor agonist activity in an *in vitro* model of dopaminergic hypoactivity (blockade of increased dopamine synthesis in reserpine-treated rats) (Kikuchi et al., 1995). There is also evidence that aripiprazole has antagonist actions at several 5-HT receptor subtypes relevant to schizophrenia, such as 5-HT_{1A} and 5-HT2A antagonism (Bruins Slot, De Vries, Newman-Tancredi, Cussac, 2002; Jordan, Koprivica, Dunn, Tottori, Kikuchi, Altar, 2004).

It is unclear why aripiprazole failed to substitute for clozapine in the current study. Aripiprazole demonstrates dopamine D_2 receptor antagonist actions *in vivo* (e.g., blockade of apomorphine-induced stereotypy) and that could potentially prevent generalizable doses from being tested before rate suppression occurs in this operant task, similar to the effects seen with olanzapine in clozapine-trained nonhuman primates (Carey & Bergman 1997). In that study, olanzapine fully substituted for clozapine in squirrel monkeys, but only when a D_2 agonist was co-administered with olanzapine and blocked the rate-suppressant effects of olanzapine.

The failure of the clozapine metabolite N-desmethylclozapine to substitute for the clozapine discriminative stimulus may be due to M1 agonism, partial dopamine agonism, or strong mu opiate activity. These different receptor effects from clozapine would require additional studies to characterize in relation to the clozapine.

Rate suppressant effects

Clozapine drug discrimination in C57BL/6 mice successfully detected many atypical antipsychotic drugs. However, many of the atypical agents (as well as the typical antipsychotic chlorpromazine) substituted for clozapine only at rate suppressant doses. Control test points obtained with the training dose of clozapine have rates of responding that are no different from vehicle control test points. Also, several antipsychotic drugs that fully substitute for clozapine have done so at doses that do not decrease rates of responding (e.g., olanzapine, sertindole and thioridazine). Finally, all drugs that failed to fully substitute for clozapine were tested up to rate suppressant doses. Therefore, rate suppression is neither a requirement nor is it sufficient to engender clozapine-appropriate responding in C57BL/6 mice.

Conclusions

The potent blockade of 5-HT_{2A} receptors appears to be an integral component of the discriminative stimulus properties of clozapine in C57BL/6 mice. The differentiation of antipsychotic drugs in this model appears to be based, at least in part, on 5-HT_{2A} receptor antagonism. However, clozapine drug discrimination in C57BL/6 mice does not completely differentiate atypical from typical antipsychotic drugs (as noted above in the discussion). Clozapine drug discrimination procedures used in C57BL/6 mice may be useful for the study of receptor specific mechanisms of action in the development of putative antipsychotic agents with potent 5-HT_{2A} receptor antagonist actions. This may provide a receptor-specific *in vivo* assay useful in the development of novel pharmacotherapies for schizophrenia.

The α_1 adrenoreceptor antagonist prazosin also fully substituted for clozapine in the current study. Many antipsychotic drugs bind to the α_1 adrenoreceptor subtype with relatively high affinity. Comparison with dopamine D_2 receptor affinities suggests that antipsychotic blockade of α_{1A} and/or α_{1B} adrenoceptors may contribute to the antipsychotic activity of many atypical and several typical antipsychotic drugs (Cahir & King 2005). Full substitution obtained with the α_1 adrenergic antagonist prazosin for clozapine suggests that this paradigm may be useful for the detection of antipsychotic agents with potent α_1 adrenergic receptor antagonist actions.

Future directions

Much evidence indicates that atypical antipsychotic drugs similar to clozapine can be differentiated from the prototypical typical antipsychotic haloperidol in the paw test (a model of antipsychotic and EPS effects) and pre-pulse inhibition (see review by Geyer & Ellenbroek,

2003) and the discriminative stimulus of clozapine (Goudie & Taylor, 1998; Porter et al., 2000; Philibin et al., 2005). However, there is an imperative need to target schizophrenic symptoms with more selective pharmacological treatment.

Atypical antipsychotic drugs such as clozapine, olanzapine, risperidone and quetiapine have substantially reduced the EPS liability of antipsychotic drug treatment but these agents are only moderately effective in the treatment of negative and cognitive executive symptoms. Thus, there is a continued focus on the need for superior antipsychotic drugs. Correlating the differences and similarities between atypical and typical antipsychotic drugs on a behavioral and neurochemical level will help increase our understanding of the complex pharmacology of schizophrenia and help lead to improved agents with greater therapeutic efficacy and reduced side effect liability.

Results from these two clozapine drug discrimination studies in mice demonstrate that this procedure can be successfully established in C57BL/6. The C57BL/6 is a standard inbred mouse strain used for breeding and as the background strain for genetically engineered mutations. The use of inbred strains of mice (e.g., C57BL/6 versus DBA/2) offers strong advantages to investigations of the role of specific neurotransmitter receptor systems in the effects of pharmacological agents (e.g., dopamine). Further, establishing this procedure in mice opens the door for the future behavioral phenotyping of transgenic and knockout mice in this model. Targeted mutation of genes expressed in the mouse brain is now allowing for the increased integration of molecular genetics and behavioral neuroscience.

Rapid advances in biomedical research will be facilitated in the future by emerging technologies such as conditional transgenics and knockouts. Conditional knockouts are

engineered to restrict the effects of the mutation either spatially or temporally. This technique can be used to induce the mutation only in a specific period of adulthood, avoiding the compensatory mechanisms during development or to a certain cell type or region that allows for anatomical specificity. Transgenic and knockout techniques are now being used to develop gene therapy strategies for human genetic disorders. Animal models that permit the dissection of the genetic basis of behavior will no doubt aid the development of new treatment strategies for genetic disorders such as schizophrenia.

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VITA

Scott D. Philibin was born on July 26, 1971 in Frankfurt, West Germany at a U.S. military hospital. His family subsequently moved to Newport News, VA when Scott was two years old. He completed high school and, after a brief stint at a community college, he transferred to Virginia Commonwealth University (VCU). He received his B.S. in the year 2000 in general psychology. He received his M.S. in 2003 and Ph.D. in 2006 in the experimental biopsychology program mentored by Dr. Joseph H. Porter. He is now preparing to head out west to start a postdoctoral position in the behavioral genetics laboratory of Dr. John C. Crabbe at Oregon Health and Science University (OHSU) in Portland. Happy trails...