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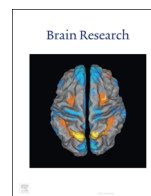
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Discriminative stimulus properties of 1.25 mg/kg clozapine in rats: Mediation by serotonin 5-HT₂ and dopamine D₄ receptors



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ABSTRACT

The atypical antipsychotic drug clozapine remains one of most effective treatments for schizophrenia, given a lack of extrapyramidal side effects, improvements in negative symptoms, cognitive impairment, and in symptoms in treatment-resistant schizophrenia. The adverse effects of clozapine, including agranulocytosis, make finding a safe clozapine-like a drug a goal for drug developers. The drug discrimination paradigm is a model of interoceptive stimulus that has been used in an effort to screen experimental drugs for clozapine-like atypical antipsychotic effects. The present study was conducted to elucidate the receptor-mediated stimulus properties that form this clozapine discriminative cue by testing selective receptor ligands in rats trained to discriminate a 1.25 mg/kg dose of clozapine from vehicle in a two choice drug discrimination task. Full substitution occurred with the 5-HT_{2A} inverse agonist M100907 and the two preferential D₄/5-HT₂/α₁ receptor antagonists Lu 37-114 ((S)-1-(3-(2-(4-(1H-indol-5-yl)piperazin-1-yl)ethyl)indolin-1-yl)ethan-1-one) and Lu 37-254 (1-(3-(4-(1H-indol-5-yl)piperazin-1-yl)propyl)-3,4-dihydroquinolin-2(1H)-one). Partial substitution occurred with the D₄ receptor antagonist Lu 38-012 and the α₁ adrenoceptor antagonist prazosin. Drugs selective for 5-HT_{2C}, 5-HT₆ muscarinic, histamine H₁, and benzodiazepine receptors did not substitute for clozapine. The present findings suggest that 5-HT_{2A} inverse agonism and D₄ receptor antagonism mediate the discriminative stimulus properties of 1.25 mg/kg clozapine in rats, and further confirm that clozapine produces a complex compound discriminative stimulus.

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1. Introduction

Clozapine (CLZ) is the prototype for atypical antipsychotic drugs (APDs) (also referred to as *second generation APDs*) based upon a negligible risk for extrapyramidal side effects (Matz et al. 1974), a lack of hyperprolactinemia (Meltzer and Fang, 1976; Meltzer et al. 1989a), an efficacy for negative symptoms (Molina et al., 2005), improvements in cognitive functioning (Meltzer and McGurk, 1999; Potkin et al. 2001), an ability to treat suicidality in schizophrenic patients (Meltzer, 1999), and an improvement in positive symptoms in treatment-resistant schizophrenia (Kane et al. 1988). Unfortunately, CLZ produces agranulocytosis in approximately 1% of patients (De Fazio et al. 2015; Idanpaan-Heikkilä et al. 1977), and while these effects are considered uncommon (De

Fazio et al. 2015), the severity of this condition has limited CLZ to an APD of last resort. Yet, due to the therapeutic efficacy and lack of extrapyramidal side effects by CLZ, drug development efforts continue with the goal of developing a safe CLZ-like atypical APD.

One method used to understand the behavioral stimulus properties of drugs is the drug discriminative paradigm. Drug discrimination allows researchers to identify the receptor-mediated stimulus properties of psychoactive drugs. The paradigm informs researchers about behaviorally relevant receptor actions and can be used as a screening tool for identifying compounds with similar neuro-behavioral pharmacological actions. The effects of a drug that subjects have been trained to discriminate from noticeably different effects, normally the drug's physiologically inert vehicle, serves as a discriminative stimulus, or cue, that can be evaluated by tests to determine if substitution for the cue occurs with other compounds.

The discriminative stimulus properties of CLZ have been established using this paradigm, with substitution for CLZ occurring

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with many other atypical APDs (Porter and Prus, 2009). Traditionally, drug discrimination studies with CLZ have used a training dose of 5.0 mg/kg. This dose produces in vivo D₂ receptor occupancy equivalent to that found by clinically-effective doses in humans, suggesting that this dose has clinical relevance (Kapur et al. 2003). In rats using a 5.0 mg/kg training dose of CLZ, full substitution (i.e., ≥ 80% CLZ-appropriate responding) has occurred with the atypical APDs olanzapine (Millan et al. 1999; Moore et al. 1992; Philibin et al. 2005; Prus et al. 2005a), quetiapine (Millan et al. 1999; Prus et al. 2005b) and melperone (Prus et al. 2004). Typical APDs (e.g., haloperidol, chlorpromazine; also known as first generation APDs) do not substitute for a 5.0 mg/kg CLZ training dose (Prus et al. 2004; Prus et al. 2005b). Full generalization does not occur from a 5.0 mg/kg CLZ training dose to all atypical APDs, however, including sertindole (Prus et al. 2005b), risperidone (Prus et al. 2005b), ziprasidone (Millan et al. 1999; although see Prus et al. 2005b), and zotepine (Goudie et al. 2004). Thus, less than half of atypical APDs tested in rats have produced full substitution for the traditional 5.0 mg/kg CLZ training dose in rats.

As is well known in the drug discrimination literature, the training dose of the training drug is an important variable and sensitivity to the discriminative stimulus properties of the training drug is usually increased as the dose of the training drug is reduced, which is indicated by leftward shifts in the generalization curve and a lower ED₅₀ value (Stolerman et al. 2011). Consistent with these general findings, studies using lower training doses of CLZ in rats have found that the discriminative cue generalizes to more atypical antipsychotic drugs than higher training doses. Porter et al. (2000) found full substitution for a 1.25 mg/kg training dose of CLZ with the atypical APDs risperidone and sertindole. Full substitution also occurred to olanzapine, although partial substitution (i.e., ≥ 60% CLZ-appropriate responding) occurred with quetiapine. In other low dose CLZ studies, full substitution also occurred with atypical APDs melperone (Prus et al. 2004) and zotepine (Goudie et al. 2004). To further study differences between these training doses in this paradigm, Prus et al. (2005a) trained rats to discriminate a 1.25 mg/kg dose versus a 5.0 mg/kg dose versus vehicle in a three choice drug discrimination task. In this study too, both quetiapine and sertindole induced full substitution for the 1.25 mg/kg CLZ discriminative stimulus, while risperidone partial substitution for this dose.

The pharmacological mechanisms that differentially mediate 1.25 mg/kg and 5.0 mg/kg CLZ training doses in rats are poorly understood. Most generalization testing with selective receptor ligands have been primarily conducted in 5.0 mg/kg CLZ-trained rats. The results from these investigations suggest that the 5.0 mg/kg CLZ training dose is mediated primarily by muscarinic receptor antagonism, based on full stimulus generalization occurring to muscarinic receptor antagonists (Goudie et al. 1998; Kelley and Porter, 1997). Thus, muscarinic receptor antagonism may explain the full stimulus generalization that has occurred from the 5.0 mg/kg CLZ training dose to atypical APDs with moderate to high affinities for muscarinic receptors, such as olanzapine and quetiapine (Schotte et al. 1996), while full stimulus generalization has not occurred to atypical APDs with a weak affinity for muscarinic receptors, such as melperone and ziprasidone (Bolden et al. 1992; Schotte et al. 1996).

The present study was conducted to characterize the receptor-mediated stimulus properties of a 1.25 mg/kg CLZ discriminative stimulus in rats. Ligands selective for dopamine, 5-HT, muscarinic, noradrenergic, and histaminergic receptors were tested for stimulus generalization in these animals given that CLZ binds with an appreciable affinity for these receptors (Arnt and Skarsfeldt, 1998; Schotte et al. 1996). In addition, three putative new antipsychotics were included in the study and two of those

compounds were multitarget compounds interacting with D₄/5-HT₂/α₁ receptors that have been speculated to support the clinical efficacy of CLZ (Brunello et al. 1995; Meltzer, 2007).

2. Results

2.1. Binding affinities

The binding affinities for Lu 37-254, Lu 37-114, and Lu 35-138 for selected receptors with potential relevance to clozapine's mechanism of action are listed in Table 1 and are expressed as K_i or IC₅₀. In general, these compounds had relatively similar binding affinities at the receptors investigated, although there are some notable differences. Each compound had low nanomolar affinities at the dopamine D₄ receptor, and somewhat lower affinities at the dopamine D₂ receptor, ranging from 75 (Lu-35-138) to 228 nM (Lu 37-254). Lu 37-254 and Lu 37-114 had low nanomolar affinity for the 5-HT₂ receptor, while Lu 35-138 had lower affinity for this target in the range of 260 nM. Lu 35-138 and Lu 37-254 had low affinity for the 5-HT_{2C} receptor at 520, and 1200 nM respectively, while Lu 37-114's affinity for this target was approximately 91 nM. Each of these three compounds has moderately strong affinities at the α₁ adrenergic receptor, ranging from 6.3 nM in the case of Lu 37-114 to 45 nM for Lu 35-138. Finally, Lu 37-254 and Lu 37-114 have low (1900 nM) to moderate (75 nM) affinities for the 5-HT transporter, respectively, while Lu 35-138 has low nanomolar affinity for this target. In summary, the rank order (from highest affinity to lowest affinity) for these compounds is as follows: Lu 37-254, D₄ > 5-HT₂ > α₁ > D₂ >> 5-HT_{2C}; Lu 37-114, D₄ > 5-HT₂ > α₁ > 5-HT_{2C} > D₂; Lu 35-138, D₄ > α₁ > D₂ > 5-HT₂ > 5-HT_{2C}. For the 5-HT transporter, Lu-35-138 had the highest affinity followed by Lu-37-114, which had a moderate affinity; Lu-37-254 had a low affinity for the transporter.

2.2. Drugs that produced full substitution for clozapine

2.2.1. Clozapine

The results of substitution testing with the atypical APD CLZ are shown in Fig. 1 (left panels). CLZ produced fully generalized for itself at the training dose (99.0% ± SEM=0.37; ED₅₀=0.20 mg/kg, 95% confidence interval [C.I.]=0.16–0.26 mg/kg), 2.5 mg/kg (98.3% ± SEM=0.62) and at a 5.0 mg/kg dose (93.0% ± 2.98). A significant decrease in response rates was observed at the 5.0 mg/kg dose (F(6, 186)=19.93, P < 0.0001).

2.2.2. Clozapine time course

Substitution testing and response rate results for the CLZ training dose (1.25 mg/kg) across different time points are shown in Fig. 1 (right panels). Again the pretreatment time used for CLZ training sessions was 60 min. The 1.25 mg/kg CLZ training dose

Table 1

Pharmacological profile of Lu 37-254, Lu 37-114, and Lu 35-138 at selected dopaminergic, serotonergic, and adrenergic receptor targets. Affinity data are presented as K_i, except where noted otherwise.

Compound	Target (K _i ; nM)					IC ₅₀ (nM)
	D ₂	D _{4,2}	5-HT ₂	5-HT _{2C}	α ₁	
Lu 37-254	228 ^a	2.6 ^a	13 ^a	1200 ^a	29 ^a	1900
Lu 37-114	120	2.5	4.0	91	6.3	75
Lu 35-138	753 ^b	5.0 ^c	260 ^c	520 ^c	45 ^c	3.2 ^c

^a Data presented in this cell is an IC₅₀ value.

^b Data originally published in Bang-Andersen et al. (2002).

^c Data originally published in Hertel et al. (2007).

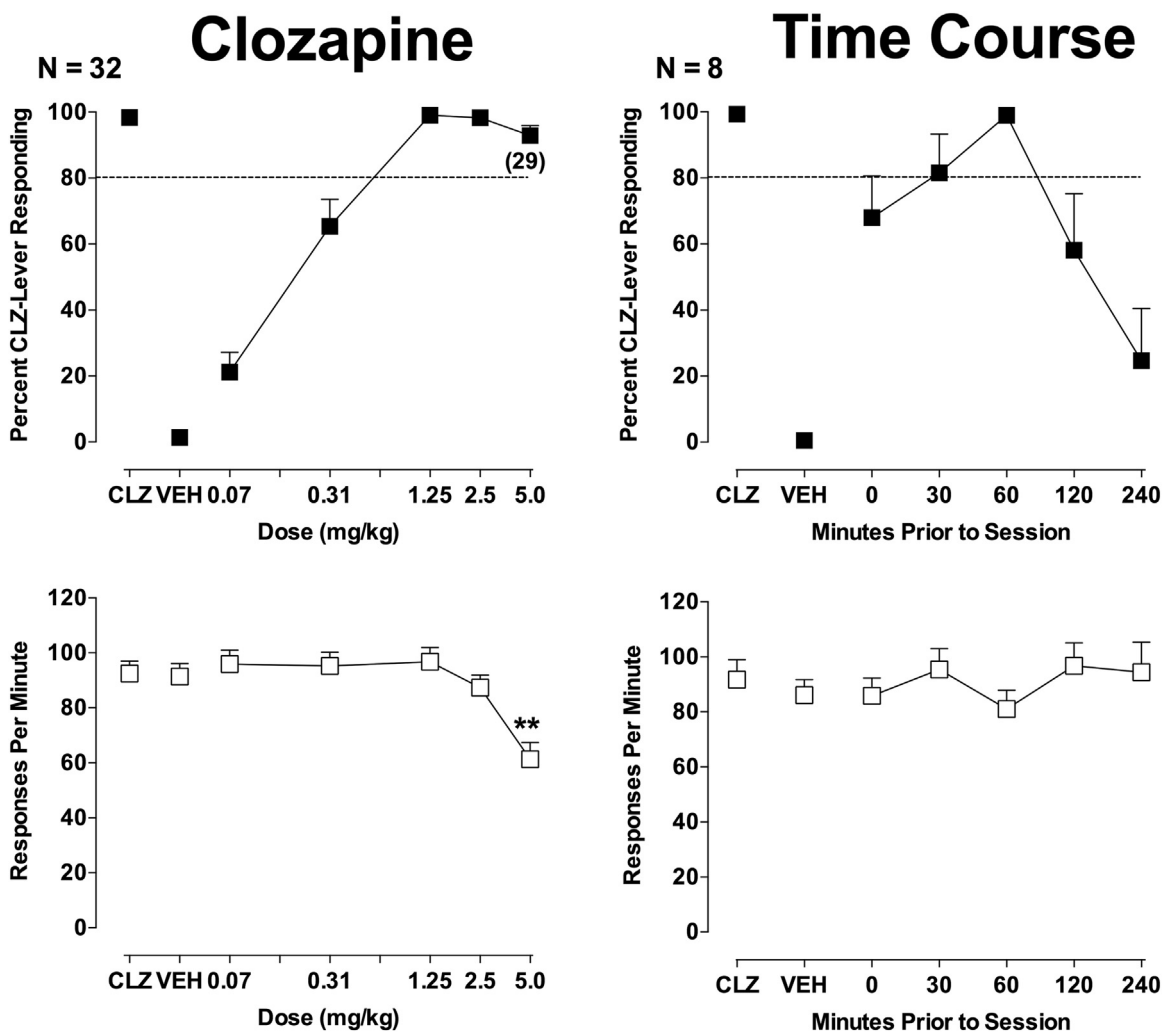


Fig. 1. Left: Substitution testing with the atypical APD CLZ. The top panel shows percent CLZ-lever responding and the bottom panel shows response per minute for each dose tested in male Sprague Dawley rats (N=32) trained to discriminate a 1.25 mg/kg dose of CLZ (60 min prior to session) from vehicle in a two-choice drug discrimination task. Right: The training dose of CLZ was tested at different pretreatment times prior to a test session and assessed for percent CLZ-lever responding (top panel) and responses per minute (bottom panel) (N=8). The figures include a test with the CLZ training dose (noted as CLZ on the left of the abscissa) and vehicle (noted as VEH on the abscissa). Data are displayed as means (\pm SEM). Rats not meeting the response rate minimum were excluded from calculation for percent drug lever responding but were included in the response rate calculation. N refers to the number of rats tested and included in the analysis unless noted otherwise in parentheses. ** $P < 0.01$ compared to VEH.

administered 30 min ($81.6\% \pm \text{SEM} = 11.8$) and 60 min ($99.0\% \pm \text{SEM} = 0.3$) prior to testing produced full generalization from the CLZ training dose. However, the 0 min, 120 min, and 240 min pre-session response rates did not differ significantly across the different time points ($P > 0.05$).

2.2.3. M100907

The 5-HT_{2A} receptor inverse agonist M100907 (Fig. 2, left panels) produced full substitution for CLZ at the 1.0 mg/kg dose ($85.6\% \pm \text{SEM} = 10.95$; $\text{ED}_{50} = 0.04$ mg/kg, 95% C.I. = 0.01–0.14 mg/kg). Response rates did not differ significantly across the doses tested.

2.2.4. Lu-37-114

The D₄/5-HT₂ α_1 receptor antagonist Lu-37-114 (Fig. 2, middle panels) also produced full substitution, for the 10.0 mg/kg dose ($98.1\% (\pm \text{SEM} = 0.95)$; $\text{ED}_{50} = 0.24$ mg/kg, 95% C.I. = 0.004–13.031). A small, but significant decrease in response rates was observed ($F(7,70) = 3.60$, $P < 0.01$) at the 5.0 mg/kg dose but not at the 10.0 mg/kg dose.

2.2.5. Lu 37-254

The D₄/5-HT₂ α_1 receptor antagonist Lu 37-254 (Fig. 2, right panels) produced full substitution at the 2.5 mg/kg dose ($81.3\% \pm \text{SEM} = 11.56$; $\text{ED}_{50} = 0.92$ mg/kg, 95% C.I. = 0.37–2.27 mg/kg) and partial substitution at the 1.25 mg/kg ($61.6\% \pm \text{SEM} = 18.01$) and 5.0 mg/kg dose ($69.3\% \pm \text{SEM} = 15.15$). The 5.0 mg/kg dose also produced a significant decrease in response rates ($F(5,35) = 5.11$, $P < 0.01$).

2.3. Drugs that did not produce full stimulus generalization

The results of substitution testing with all other compounds are shown in Table 2. All compounds were tested up to doses that produced a significant decrease in response rates relative to vehicle control ($P < 0.05$), except for ORG 38,457, chlordiazepoxide, Lu 38-012, Lu 35-138, SB-271,046, and RO 8554. Doses that produced significant differences in response rates relative to vehicle also are indicated in Table 2. The α_1 adrenoceptor antagonist prazosin produced partial substitution for CLZ at the 2.0 mg/kg dose ($68.8\% \pm \text{SEM} = 17.3$), and the D₄ receptor antagonist Lu 38-012 also produced partial substitution at the 5.0 mg/kg dose

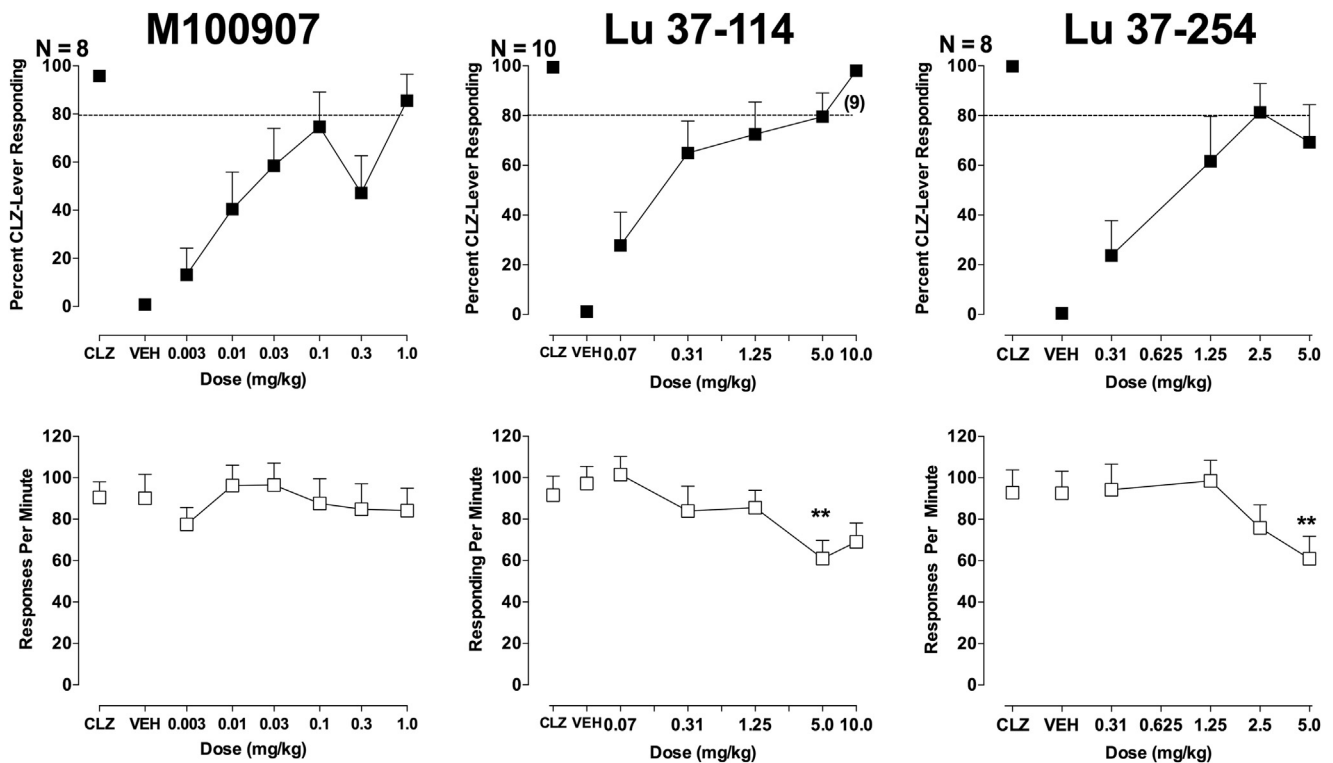


Fig. 2. Substitution testing with the 5-HT_{2A} receptor inverse agonist M100907 (left), the D₄/5-HT₂/α₁ receptor antagonist Lu 37-114 (middle), and the D₄/5-HT₂/α₁ receptor antagonist Lu 37-254. See Fig. 1 for other details. **P < 0.01 compared to VEH.

(60.9% ± SEM=12.9). No other compounds listed in this table produced partial substitution for clozapine.

3. Discussion

The present study evaluated a series of selective receptor ligands as well as ligands with multiple actions for the purpose of elucidating the discriminative stimulus properties of a 1.25 mg/kg CLZ training dose in rats. As noted in the introduction, the 1.25 mg/kg training dose screens atypical and from typical APDs more effectively. CLZ produced full stimulus generalization to itself up to a 5.0 mg/kg training dose, and the discriminative stimulus effects of the training dose are evident from 30 to 60 min post injection. Full stimulus generalization occurred from CLZ to only a limited number of ligands, including the selective 5-HT_{2A} receptor inverse agonist M100907 and the D₄/5-HT₂/α₁ preferring receptor antagonists Lu 37-114 and Lu 37-254. Beyond this, partial stimulus generalization occurred to the D₄ receptor antagonist Lu 38-012 and α₁ adrenoceptor antagonist prazosin.

As noted earlier, CLZ binds to a multitude of receptors, each of which has at one time or another been investigated as a potential mediator of atypical antipsychotic actions (Meltzer, 2002). Among these receptor actions, antagonism of 5-HT₂ receptors appears to be an important component that is shared by nearly every atypical antipsychotic drug on the market. A receptor binding profile that includes preferential antagonism of 5-HT_{2A} receptors over D₂ receptor remains the most consistent and reliable profile for developing an atypical antipsychotic drug (Meltzer and Massey, 2011; Meltzer et al. 1989b; Schotte et al. 1996). Amisulpride remains one of the only atypical APDs lacking an affinity for 5-HT_{2A} receptors (Abbas et al. 2009).

The present study found full substitution by a 1.0 mg/kg dose of M100907, which is supportive of 5-HT_{2A} receptor inverse agonism mediating these stimulus effects. The current study did not find,

however, partial or full substitution by the 5-HT_{2A/2B/2C} receptor antagonist ritanserin. In other studies, the M100907 was found to produce full substitution for a 1.25 mg/kg CLZ dose in individual rats (Prus et al. 2004), and full substitution for a M100907 discriminative stimulus occurred with CLZ in rats (Dekeyne et al. 2003). The dose of M100907 that produced full substitution for CLZ in the present study was higher than a dose of M100907 (0.01 mg/kg) found sufficient to completely block the discriminative stimulus effects of the 5-HT_{2A/2C} receptor agonist (2,5-dimethoxy-4-iodophenyl)-2-aminopropan (DOI) (Schreiber et al. 1994). These past findings suggest that the receptor mechanisms mediating the stimulus effects of M100907 for the doses used in the present study may involve more than 5-HT_{2A} receptors, since M100907 exhibits a moderate affinity for 5-HT_{2C} receptors and α₁ adrenoceptors (Pehok et al. 2006). Coinciding with this, Philibin et al. (2009) reported that both M100907 and α₁ adrenoceptor antagonist prazosin substituted for a CLZ discriminative cue in male C57BL/6 mice.

Full substitution occurred with the D₄/5-HT₂/α₁ receptor antagonists Lu 37-114 and Lu 37-254 in the present study. As noted earlier partial substitution occurred with the selective D₄ receptor antagonist Lu 38-012 and the α₁ adrenoceptor antagonist prazosin. Substitution did not occur to the 5-HT_{2C} receptor antagonist ORG38457. Taking these findings together, D₄ receptor antagonism may represent part of the clozapine discriminative cue, which has long been considered a compound stimulus (Goudie et al. 1998). Given that Lu 37-114 and Lu 37-254 also bind to 5-HT₂ and α₁ receptors, the additional antagonism of these receptors may have established stimulus properties more similar to those produced by CLZ than established by either action alone. No substitution occurred for Lu 35-138, which also is an antagonist for D₄ receptors and α₁ adrenoceptors, but this compound also inhibits 5-HT reuptake (Hertel et al. 2007). Enhanced 5-HT concentrations produced by Lu 35-138 may run counter to clozapine's pharmacological profile by activating, rather than blocking, 5-HT₂ receptors.

Table 2
Drugs that did not produce full substitution for CLZ.

Drug (type)	Dose	Number of Subjects tested (no. responding)	Percent clozapine lever responding (SEM)	Responses per minute (SEM)
Dopamine Compounds				
Haloperidol (typical anti-psychotic drug and D ₂ -receptor preferring antagonist)	Vehicle	14 (12)	0.7 (0.2)	93.6 (8.4)
	0.05	14	4.1 (1.1)	86.7 (10.3)
	0.1	14	16.6 (5.0)	61.5 (7.4)**
	0.2	14	24.4 (5.8)	7.9 (1.2)**
	Vehicle	9	9.1 (8.5)	77.6 (11.4)
	0.3125	12	20.6 (11.0)	89.9 (7.4)
	0.625	12	23.9 (12.5)	86.5 (11.7)
	1.25	12	20.1 (10.9)	77.5 (10.0)
	2.5	12	24.8 (12.5)	74.2 (7.9)
	5.0	12	60.9 (12.9)	75.4 (8.7)
10.0	11	23.4 (12.0)	77.3 (10.6)	
Lu 35-138 (D ₄ /α ₁ antagonist and 5-HT reuptake inhibitor)	Vehicle	5	0.5 (0.2)	65.4 (9.3)
	5.0	5	5.0 (3.9)	59.1 (14.6)
	10.0	5	6.2 (4.5)	53.9 (6.0)
	20.0	4	5.3 (4.4)	49.9 (12.7)
d-Amphetamine (D ₂ agonist)	Vehicle	17 (14)	0.7 (0.4)	71.6 (6.6)
	0.25	17 (9)	3.0 (2.7)	68.1 (7.1)
	0.5	16 (1)	14.5 (8.7)	61.6 (10.1)
	1.0	14	11.1 (8.1)	38.0 (11.6)
	2.0	9	1.0	1.9 (1.1)**
Serotonin compounds				
Ritanserin (5-HT _{2A/2B/2C} antagonist)	Vehicle	20	0.6 (0.7)	97.0 (10.7)
	0.5	7	27.6 (12.1)	62.1 (10.8)
	1.0	20	8.8 (12.6)	88.8 (10.5)
	2.0	20	29.8 (11.1)	93.2 (10.1)
	4.0	20	44.1 (16.4)	88.7 (11.4)
	8.0	6	49.9 (17.2)	66.5 (14.4)*
ORG 38,457 (5-HT _{2C} antagonist)	Vehicle	7	0.0 (0.0)	18.2 (8.9)**
	1.25	7	0.5 (0.5)	85.4 (10.9)
	2.5	7	28.1 (18.1)	78.8 (15.9)
	5.0	6	0.1 (0.0)	93.9 (9.5)
	10.0	6	16.4 (15.1)	80.2 (14.5)
SB-271,046 (5-HT ₆ antagonist)	Vehicle	12	8.5 (7.1)	85.6 (11.8)
	1.25	12	18.1 (10.9)	90.2 (9.2)
	2.5	12	30.3 (12.5)	82.2 (10.5)
	5	12	9.1 (6.5)	74.8 (10.7)
RO 8554 (5-HT ₆ antagonist)	Vehicle	7	0.3 (0.1)	65.7 (11.4)
	1.0	7	28.8 (18.3)	67.9 (7.3)
	3.0	7	1.6 (0.7)	60.4 (9.6)
	10.0	7	6.3 (6.1)	53.2 (9.0)
SB-258,741 (5-HT ₇ partial inverse agonist)	Vehicle	10	0.6 (0.3)	83.7 (12.3)
	1.25	10	18.8 (11.9)	94.3 (11.7)
	2.5	10	10.4 (9.8)	90.0 (11.6)
	5.0	10	9.2 (8.9)	89.5 (10.5)
10.0	9	12.4 (10.4)	60.6 (13.4)*	
Noradrenergic compounds				
Prazosin (α ₁ antagonist)	Vehicle	9	2.1 (2.0)	102.9 (12.1)
	0.125	10	11.3 (9.7)	79.9 (9.7)
	0.25	5	18.5 (9.9)	52.9 (13.9)
	0.5	10	54.6 (12.6)	76.2 (11.7)
	1.0	5	56.8 (22.1)	48.2 (10.5)*
	2.0	7	68.8 (17.3)	53.6 (13.3)**
Muscarinic compounds				
Scopolamine (muscarinic antagonist)	Vehicle	21	4.9 (2.6)	82.9 (7.8)
	0.03125	22	28.6 (8.9)	87.2 (6.3)
	0.125	21	33.3 (9.3)	76.1 (7.2)**
	0.05	20	39.2 (8.4)	45.9 (6.7)**
Trihexyphenidyl (M ₁ muscarinic antagonist)	Vehicle	13	1.0 (0.5)	100.7 (10.3)
	0.75	14	23.0 (8.7)	110.5 (8.7)
	3.0	14	47.5 (12.1)	114.6 (9.4)
	6.0	14	47.9 (10.7)	91.0 (9.3)
12.0	11	48.6 (10.0)	53.3 (8.1)**	
Histamine compounds				
Pyrilamine (H ₁ antagonist)	Vehicle	10	1.0 (0.5)	72.1 (7.8)
	5.0	10	18.3 (9.8)	68.9 (8.9)

Table 2 (continued)

Drug (type)	Dose	Number of Subjects tested (no. responding)	Percent clozapine lever responding (SEM)	Responses per minute (SEM)
	10.0	10	32.8 (14.5)	76.3 (7.7)
	20.0	10	10.4 (9.9)	72.6 (8.9)
	40.0	5	32.1 (20.0)	23.8 (14.9)**
Anxiolytic compounds				
Chlordiazepoxide (anxiolytic)	Vehicle	9	0.5 (0.3)	81.1 (9.6)
	1.25	10	21.3 (12.3)	102.0 (13.4)
	2.5	9	24.3 (13.3)	91.5 (17.9)
	5.0	10	34.1 (14.7)	100.0 (15.1)
	10.0	10	21.9 (13.0)	88.7 (14.0)
	20.0	8	40.9 (16.4)	83.2 (10.5)

** P < 0.01.

* P < 0.05 compared to vehicle.

Clozapine exhibits a high affinity for D₄ receptors, as do many other APDs including olanzapine, risperidone and haloperidol (Bymaster et al. 1996; Roth et al. 1995). As noted previously, the 1.25 mg/kg dose of clozapine fully generalizes to all atypical APDs tested so far, with the exception of quetiapine (Porter et al. 2000). Quetiapine lacks an appreciable affinity for D₄ receptors, perhaps accounting for partial substitution for clozapine in this previous study. Yet, many typical APDs exhibit a high affinity for D₄ receptors, but do not include full or partial substitution. A potential difference between atypical and typical APDs regarding D₄ receptor binding is that many atypical APDs exhibit a greater affinity for D₄ receptors over D₂ receptors, whereas the vast majority of typical APDs have a greater affinity for D₂ receptors than D₄ receptors (Roth et al. 1995). This might explain a lack of full stimulus generalization from a 1.25 mg/kg dose of clozapine to quetiapine, but not account for full substitution for risperidone, which engenders a greater affinity for D₂ receptors than for D₄ receptors (Porter et al. 2000). Neither does a high affinity for D₄ and 5-HT_{2A} receptors appear to only explain substitution for clozapine by atypical, but not typical, APDs (Porter et al. 2000), as many typical APDs also exhibit a high to moderate affinity for 5-HT_{2A} receptors (although typical APDs have a stronger affinity for D₂ receptors compared to 5-HT_{2A} receptors) (Roth et al. 1995). It may instead be the case that antagonism of both D₄ and 5-HT_{2A} receptors produces clozapine-like discriminative stimulus effects, but that additional antagonism of D₂ receptors with an affinity greater than 5-HT_{2A} receptors, makes these stimulus effects unlike clozapine. Thus, drugs that also have a strong affinity for D₂ receptors do not produce full stimulus generalization from clozapine.

Stimulus effects of higher training doses of CLZ in rats are clearly mediated by muscarinic receptor antagonism, based on substitution by muscarinic receptor antagonists atropine (Nielsen, 1988), scopolamine (Goudie et al. 1998; Kelley and Porter, 1997; Nielsen 1988) and trihexyphenidyl (Kelley and Porter, 1997; Prus et al. 2004). The present study did not find partial or full substitution by either scopolamine or trihexyphenidyl. Prus et al. (2006) also did not find substitution for a 1.25 mg/kg training dose of CLZ by scopolamine, while Prus et al. (2004) did find full substitution for this training dose by trihexyphenidyl. Overall, a key distinction between the discriminative stimulus effects of these two training doses appears to be the prominence of muscarinic antagonism with the higher training dose.

Stimulus properties elicited by muscarinic receptor antagonism for the higher training dose of CLZ in rats, in turn, may overshadow the stimulus properties elicited by 5-HT_{2A} or D₄ receptors found in the 1.25 mg/kg CLZ training dose. In rats trained to discriminate a 1.25 mg/kg dose of CLZ versus a 5.0 mg/kg dose of CLZ versus vehicle, the primary difference between these stimuli

consisted of partial substitution for a 5.0 mg/kg dose, but not a 1.25 mg/kg dose, with scopolamine (Prus et al. 2006). Further, in this same study, partial substitution occurred for the 1.25 mg/kg CLZ dose, but not the 5.0 mg/kg dose, with ritanserin. Comparatively, 5-HT_{2A} receptor inverse agonism may elicit weaker stimulus effects than muscarinic receptor antagonism. For example, Dekeyne et al. (2002) reported that training M100907 (0.16 mg/kg) as a discriminative stimulus required approximately 70 sessions, whereas Kelley and Porter (1997) reported that training scopolamine (0.125 mg/kg) as a discriminative stimulus required approximately 50 sessions.

While full stimulus generalization did not occur from CLZ to the α_1 adrenoceptor antagonist prazosin, the level of CLZ-appropriate responding was over 60% (i.e., partial substitution), beyond “chance level choice” in a two lever task. Goudie et al. (1998) also reported a maximum of 67% substitution for a 5.0 mg/kg CLZ training dose with prazosin in rats. α_1 adrenoceptor antagonism is another receptor mechanism shared by many, but not all, atypical APDs as well as typical APDs (Schotte et al. 1996). In a study that trained rats to discriminate the typical APD chlorpromazine from 5.0 mg/kg CLZ from vehicle in a three-choice drug discrimination procedure in rats, prazosin produced full substitution for chlorpromazine, but not CLZ (Porter et al. 2005). Given these findings, it does not appear that α_1 adrenoceptor antagonism is unique to the stimulus properties of either dose of CLZ in rats, nor does it appear to generate stimulus effects unique to atypical APDs.

Beyond the data discussed so far, the remaining receptor ligands explored do not appear relevant to the stimulus properties of a 1.25 mg/kg CLZ training dose in rats. Based on the present findings, receptors lacking a role in this cue include D₂ receptors, H₁ histamine receptors, benzodiazepine sites on GABA_A receptors (i.e., chlordiazepoxide), and 5-HT₆ receptors. It is worth noting that not all compounds failing to produce at least partial substitution were tested up to rate-suppressant doses, although a wide range of doses was tested for each compound. Differences in the mediation of the discriminative stimulus properties of CLZ do occur between species, however. In male C57/BL mice, full substitution for CLZ with ritanserin (Philibin et al. 2005) and M100907 (Philibin et al. 2009) has been reported, and the discriminative stimulus effects of CLZ have been blocked by pretreatment with the 5-HT agonist quipazine (Philibin et al. 2005). These data suggest that 5-HT_{2A} receptor antagonism mediates the discriminative stimulus properties of CLZ in mice. In pigeons, 5-HT₂ receptors also exhibit CLZ-like stimulus effects (Hoenicke et al. 1992). Despite the apparent greater prominence of 5-HT mediated stimulus effects for CLZ in mice or pigeons, which would be more representative of what is thought to be highly important for atypicality, the CLZ discriminative stimulus in mice does not adequately screen atypical from typical APDs (Philibin et al. 2009) and most atypical APDs have yet to be tested in pigeons (Hoenicke et al. 1992).

The present study explored the discriminative stimulus properties of the prototypical atypical APD CLZ in rats, using a 1.25 mg/kg training dose of clozapine with has effectively screened typical from atypical APDs in past studies. Thus, this training dose in the drug discrimination paradigm appears to have utility as a screening model in APD development. There appears to be a basis for 5-HT_{2A} receptors, which would fit with currently established models for atypicality. Moreover, D₄ receptor antagonism also appears to mediate the discriminative stimulus properties of CLZ, especially when antagonism of both 5-HT_{2A} and D₄ receptors occur. Such models would be important as CLZ, despite being discovered well over half a century ago, remains one of the most, if not the most, effective atypical APDs available for clinical use.

4. Experimental procedure

4.1. Subjects

Experiments were conducted in 56 male Sprague Dawley rats (Harlan, Indianapolis, IN) with new cohorts of rats added over time. All rats were individually housed under constant temperature and humidity conditions and a 12 h light/dark cycle. Rats all weighed over 300 g prior to any experimental procedures taking place. The rats were food restricted to maintain 85% of free-feeding weights, but free-access to water was provided in the home cages. All procedures were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and followed the Guide for the Care and Use of Laboratory Animals (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research, National Academies Press, 2011).

4.2. Apparatus

All drug discrimination sessions were conducted in four standard computer-operated two lever (retractable) rat operant chambers equipped with food pellet delivery and housed in sound-attenuating cubicles with fans installed for ventilation and masking noise (Med Associates, St. Albans, VT). Experimental events were controlled by and data were collected using Med-PC version 3.0 (Med-Associates). A light near the top of each chamber provided illumination during all experimental sessions. Food reinforcers consisted of 45 mg powderless food pellets (Noyes Precision Pellets, Formula P, Research Diets, Inc., New Brunswick, NJ).

4.3. Drugs

The following drugs were administered: the atypical APD CLZ (gift from Novartis Pharmaceutical Corporation, East Hanover, NJ), the typical APD haloperidol (Sigma Chemical Company, St. Louis, MO), the psychostimulant D-amphetamine (Sigma-Aldrich, St. Louis, MO), the anxiolytic chlordiazepoxide (Sigma-Aldrich), the dopamine D₄ receptor antagonist Lu 38-012 (Hertel et al. 2007) (Lundbeck, Copenhagen-Valby, Denmark), the muscarinic receptor antagonist scopolamine (Sigma-Aldrich), the M₁ receptor preferring antagonist trihexyphenidyl (Sigma-Aldrich), the serotonin (5-HT)_{2A/2B/2C} receptor antagonist ritanserin (Research Biochemical International, Natick, MA), the 5-HT_{2A} receptor inverse agonist M100907 (Sigma-Aldrich), the 5-HT_{2C} receptor antagonist ORG 38,457 (Tocris), the 5-HT₆ receptor antagonist RO-8554 (gift from Roche Pharmaceuticals, Palo Alto, CA), the 5-HT₆ receptor antagonist SB 271,046 (Tocris), the 5-HT₇ receptor partial inverse agonist SB 258,741 (Tocris), the α_1 adrenoceptor antagonist prazosin (Research Biochemical International), the histamine H₁ receptor antagonist pyrilamine (Research Biochemical International), the D₄ receptor antagonist and 5-HT reuptake inhibitor Lu 35-138 (Bang-Andersen et al. 2007; Hertel et al. 2007) (Lundbeck), the preferential D₄/5-HT₂/ α_1 receptor antagonists 1-(3-(4-(1H-indol-5-yl)piperazin-1-yl)propyl)-3,4-dihydroquinolin-2(1H)-one (Bang-Andersen et al. 2002) (Lu 37-254) (Lundbeck) and (S)-1-(3-(2-(4-(1H-indol-5-yl)piperazin-1-yl)ethyl)indolin-1-yl)ethan-1-one (Lu 37-114 (Bang-Andersen et al. 2007) (Lundbeck). All drugs were dissolved in CLZ vehicle (de-ionized H₂O with 1-2 drops of lactic acid), except for the Lundbeck compounds, which were dissolved in a 10% 2-hydroxypropyl- β -cyclodextrin solution. All drugs were administered intraperitoneally at a volume 1 ml/kg body weight. Doses of scopolamine (HCl), SB 271,046 (HCl), prazosin (HCl), pyrilamine (maleate), Lu 37-114 (HCl), Lu 35-138 (HCl) and Lu 37-254 (HCl) doses were in the salt form, and doses for all other compounds refer to the base form. CLZ and RO-8554 were

administered one hour prior to session and all other drugs were administered 30 min prior to session. Injection routes, pre-injection times, and doses for these drugs were based on previous studies in this laboratory and at Lundbeck.

4.4. Binding and functional assays

Binding assays at the dopamine D₂ and D_{4.2} receptors, the 5-HT₂ and 5-HT_{2C} receptors, and α_1 adrenergic receptors for Lu 37-254 and Lu 37-114 were performed as described previously (Balle et al. 2003). Additionally, the 5-HT uptake functional assay was performed as described in Hertel et al. (2007).

4.5. Behavioral procedures

Drug discrimination training and generalization testing procedures were identical to those reported previously (Porter et al. 2000). Briefly, a fixed ratio 30 schedule was used for pellet delivery. Rats were injected with either vehicle or a 1.25 mg/kg dose of CLZ 60 min prior to a 15 min training session. CLZ and vehicle training sessions were administered according to a double-alternation sequence (i.e., DDVDD, etc). Every incorrect response reset the fixed ratio 30 counter. Drug discrimination training criteria consisted 5 out of 6 consecutive sessions with the following: 1) first fixed ratio 30 emitted on the condition-appropriate lever, 2) at least 80% or greater condition-appropriate responding, and 3) response rates of at least 30 responses per minute. Rats were required to meet these criteria prior to substitution testing. Before a substitution test, a rat had to have both a CLZ and a vehicle training session since the previous test and have the session immediately prior to a test meet the three training criteria listed above. A test session was identical to a training sessions except that a fixed ratio 30 completed on either lever produced a reinforcer. Doses for each drug tested were administered in ascending order, and only one dose was administered per day. Three cohorts of rats were used for testing the compounds in this study. All rats were tested with CLZ first (although only 32 rats were rested across a range of doses to provide a dose response curve), but to minimize the influence of drug history, different subsets of animals from each new group of rats were tested with different drugs and the sequence of drug testing varied randomly for each animal. Further, some drugs were tested in rats from multiple groups. Rats from first cohort were included in the CLZ time course and in tests for ritanserin, M100907, ORG 38,457, scopolamine, trihexyphenidyl, prazosin, pyrilamine, amphetamine, RO-8554, and Lu 35-138. Rats from the second cohort were included in tests for Lu 37-114, Lu 38-012, Lu 37-254, SB 258,741, chlordiazepoxide, and SB 271,046. Rats from the third cohort of rats were included in tests for haloperidol, ziprasidone, ritanserin, scopolamine, pyrilamine, and D-amphetamine. The number of subjects tested with each drug are indicated in the figures and in Table 2.

4.6. Data analysis

Percent CLZ-appropriate responding and responses per minute were reported as means (\pm the standard error of the mean [SEM]) in dose-effect curves. Full substitution for the CLZ discriminative cue was defined as 80% or greater CLZ-appropriate responding, and partial substitution was defined as 60% or greater and less than 80% CLZ-appropriate responding. For drugs that produced full substitution for CLZ, ED₅₀ values were obtained for the dose-effect curves (with 95% confidence levels) using a least squares linear regression analysis (Goldstein, 1964). If an animal's response rates fell below 5 responses per minute, the percent lever responding data for that particular dose were not included in the dose-effect curve or the ED₅₀ calculations. A one factor repeated measures

analysis of variance (ANOVA) was conducted to assess differences in response rates for a drug across doses, and for statistically significant F values, Newman-Keuls post hoc multiple comparison tests were conducted to identify rate-suppressant doses relative to vehicle control.

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