Further characterization of the discriminative stimulus properties of the atypical antipsychotic drug clozapine in C57BL/6 mice: role of 5-HT_{2A} serotonergic and α_1 adrenergic antagonism

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Abstract

Rationale The discriminative stimulus properties of the atypical antipsychotic drug (APD) clozapine (CLZ) have recently been studied in C57BL/6 mice, a common background strain for genetic alterations. However, further evaluation is needed to fully characterize CLZ's discriminative cue in this strain of mice.

Objectives The objectives of the study were to confirm the previous findings using a shorter pretreatment time and to further characterize the receptor mechanisms mediating the discriminative stimulus properties of CLZ by testing APDs, selective ligands, and *N*-desmethylozapine (CLZ's major metabolite) in C57BL/6 mice.

Materials and methods C57BL/6 male mice were trained to discriminate 2.5 mg/kg CLZ (s.c.) from vehicle in a two-lever drug discrimination task.

Results Generalization testing with CLZ yielded an ED₅₀=1.19 mg/kg. Substitution testing with APDs showed that the atypical APDs quetiapine, sertindole, zotepine, iloperidone, and melperone fully substituted for CLZ ($\geq 80\%$ CLZ-appropriate responding), but aripiprazole did not. The typical APDs chlorpromazine and thioridazine substituted for CLZ (fluphenazine and perphenazine did not). The serotonin (5-HT)_{2A} antagonist M100907 and the α_1 -adrenoceptor antagonist prazosin fully substituted for CLZ. The H₁ histaminergic antagonist pyrilamine, dopamine agonist amphetamine, and the selective serotonin reuptake inhibitor fluoxetine did not substitute for CLZ. While *N*-desmethylozapine did not substitute for CLZ when tested alone, *N*-desmethylozapine plus a low dose of CLZ combined in an additive manner produced full substitution.

Conclusions CLZ's discriminative cue in C57BL/6 mice is a "compound" cue mediated in part by antagonism of 5-HT_{2A} and α_1 receptors.

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Introduction

Recently, we demonstrated that the atypical antipsychotic drug (APD) clozapine (CLZ) could serve as a discriminative stimulus in C57BL/6 mice trained to discriminate

2.5 mg/kg CLZ from vehicle (VEH) in a standard two-lever drug discrimination task (Philibin et al. 2005). Characterization of this wild-type strain in behavioral assays such as drug discrimination is important, as the C57BL/6 mouse has become a standard inbred mouse strain used for breeding and is used as the background strain for many genetically engineered transgenic and knockout mice. Holmes et al. (2002) have stated that the design and interpretation of studies on targeted gene mutations can be strengthened by fully characterizing the behavior of background mouse strains commonly used in such studies (see also, Banbury Conference on genetic background in mice 1997; Crawley 2003; Wolfer and Lipp 2000).

While the discriminative stimulus properties of CLZ have been studied primarily in rats (Goas and Boston 1978; Wiley and Porter 1992; Kelley and Porter 1997; Goudie et al. 1998; Porter et al. 2000; Prus et al. 2004, 2005a, b; 2006), other species, including pigeons (Hoenicke et al. 1992) and squirrel monkeys (Carey and Bergman 1997), have also been studied. The Philibin et al. (2005) study was the first to establish CLZ drug discrimination in mice, and results from that study suggested that there were differences between rats and mice. Specifically, Philibin et al. found that the 5-HT_{2A/2B/2C} antagonist ritanserin fully substituted for CLZ, while the muscarinic antagonist scopolamine did not. The CLZ discriminative cue was significantly attenuated by the serotonergic 5-HT_{2A/2C} agonist quipazine at doses that did not disrupt response rates. These data provided evidence that serotonergic mechanisms play an important role in mediating the discriminative stimulus properties of CLZ in C57BL/6 mice and were in direct contrast to studies that have used rats, which have found a strong muscarinic component for the clozapine cue but not serotonergic mechanisms. The only selective ligands that have consistently substituted for CLZ in rats are muscarinic cholinergic antagonists such as scopolamine and trihexyphenidyl (Nielsen 1988; Kelley and Porter 1997; Goudie et al. 1998; Millan et al. 1999; Prus et al. 2004; Porter et al. 2005), while serotonergic antagonists such as ritanserin (Wiley and Porter 1992, 1993) and M100907 (Goudie et al. 1998; Millan et al. 1999; Prus et al. 2004) fail to substitute for the CLZ cue in rats. However, it should be noted that the CLZ training dose influences this greatly. Porter et al. (2000) found that with a low (1.25 mg/kg) training dose of CLZ, more atypical APDs generalized to CLZ's discriminative cue and that muscarinic antagonism does not engender CLZ-appropriate responding (Prus et al. 2006). Goudie et al. (2004a) found that the atypical APD zotepine fully substituted for CLZ at a 2.0 mg/kg training dose, but not at a 5.0 mg/kg training dose. Serotonergic mechanisms for CLZ's discriminative cue also appear to be important in pigeons (see Hoenicke et al. 1992).

Drug discrimination procedures provide an *in vivo* behavioral measure of drug effects that correlates well with

the *in vitro* pharmacological mechanisms of the drug (Colpaert and Balster 1988; Holtzman 1990). Two-lever drug discrimination allows for the classification of drugs in terms of their subjective effects and determination of the pharmacological mechanisms that underlie the discriminative stimulus properties of the training drug. This process is more difficult with APDs as they represent a rather heterogeneous drug class displaying diverse and varying receptor binding profiles (see Table 1). Also, it should be noted that *in vitro* activity of drugs does not always correlate perfectly with *in vivo* activity (see Arnt and Skarsfeldt 1998). However, there has been a great deal of drug discrimination research with APDs, especially with the atypical APD CLZ that remains the prototype or gold standard for atypical APDs. Drug discrimination has proven to be useful in the screening of a number of putative atypical antipsychotic drugs (e.g., Moore et al. 1992; Bruhwiler et al. 1997; Tang et al. 1997; Millan et al. 1998a, b; 1999; 2000); however, there have been atypical APDs such as risperidone (Hoenicke et al. 1992; Fiorella et al. 1997; Goudie and Taylor 1998; but cf. Porter et al. 2000), sertindole (Goudie and Taylor 1998; but cf. Porter et al. 2000), and ziprasidone (Millan et al. 1999) that fail to reliably substitute for CLZ in rat studies. In contrast, both risperidone and ziprasidone fully substituted for CLZ's discriminative cue in C57BL/6 mice, suggesting that the CLZ drug discrimination assay with C57BL/6 mice may provide a useful preclinical model for screening clozapine-like APDs (Philibin et al. 2005; sertindole was not tested).

CLZ displays a diverse receptor binding profile (Arnt and Skarsfeldt 1998; Schotte et al. 1996), and as shown in Table 1, CLZ has a strong affinity for serotonin_{2A} (5-HT_{2A}), histamine H₁, cholinergic muscarinic, and α_1 -adrenoceptor receptors, but a relatively weak affinity for dopamine (DA) D₂ receptors. CLZ's potent 5-HT_{2A} receptor antagonism relative to its weak DA D₂ receptor antagonism has been suggested as the principal pharmacological characteristic that differentiates CLZ and other atypical APDs from typical APDs that are potent DA D₂ antagonists (Meltzer et al. 1989). However, there is great diversity both between and within these two classes of APDs with regard to their receptor binding affinities. Thus, it is important to develop preclinical assays that can help in the identification and development of potential antipsychotic agents. The pharmacological specificity of the drug discrimination assay allows for the *in vivo* mechanism(s) of action of antipsychotic agents to be characterized and may provide important information for the development of more selective drug treatments for schizophrenia.

One of clozapine's major metabolites, *N*-desmethylozapine (NDMC), is found in many species, including rats, mice, guinea-pigs, dogs, monkeys, and humans (Bun et al. 1999). Recently, there has been increasing interest in the

Table 1 Dissociation rate constants (K_i , nanomolar) are shown for antipsychotic drugs at selected neurotransmitter receptor subtypes (from Schotte et al. 1996, except where indicated)

Species (except where indicated) Tissue (except where indicated)	Rat Frontal cortex 5-HT _{2A}	Rat Striatum D ₂	Rat Striatum M	Rat Total cortex α_1	Guinea pig Cerebellum H ₁
Atypical APDs					
Clozapine	3.3	150.0	34	23.0	2.1
Olanzapine	1.9	17.0	26	60.0	3.5
Risperidone	0.16	3.3	>5,000	2.3	2.6
Quetiapine	120.0	310.0	1,020	58.0	19.0
Sertindole	0.85	7.4	>5,000	1.8	570.0
Ziprasidone	0.31	9.7	>5,000	12.0	110.0
Zotepine	0.91	13.0	550	3.4	3.4
Iloperidone ^a	0.2	3.3	6,000	0.31	12.3
Melperone ^a	102.0	180.0	>10,000	180.0	580.0
Aripiprazole ^b	8.7	3.3	6,780 (M ₁)	25.7 (α_{1A})	25.1
Typical APDs					
Haloperidol	25.0	1.4	4,670	19.0	730.0
Fluphenazine	19.0 ^c	0.8 ^d	1,900 ^d	9.0 ^d	21.0 ^d
Perphenazine	5.6 ^c	1.4 ^c	1,500 ^d	10.0 ^d	8.0 ^d
Chlorpromazine	3.3 ^e	1.2 ^f	378 ^g	14.0 ^g	9.0 ^d
Thioridazine	6.3 ^f	7.9 ^f	18 ^d	5.0 ^d	16.0 ^d

These values should be used for general comparisons only since the species, conditions, tissues, and assays varied among the studies 5-HT_{2A} serotonin 5-HT_{2A} receptors, D₂ dopamine D₂ receptors, M cholinergic muscarinic receptors, α_1 α_1 adrenoceptors, H₁ histamine H₁ receptors, K_i equilibrium dissociation constant of the competitive inhibitor, K_D dissociation equilibrium constant

^a Richelson and Souder 2000 (human brain, K_D)

^b Shapiro et al. 2003 (human cloned)

^c Wander et al. 1987 (human cortex, K_D)

^d Richelson and Nelson 1984 (human brain, K_D)

^e Leysen et al. 1982 (rat cortex)

^f Roth et al. 1995 (rat brain)

^g Hals et al. 1986 (rat brain)

possibility that NDMC may be a novel APD because of its interesting in vitro receptor binding profile. Like CLZ, it is a potent 5-HT_{2A} antagonist, yet in contrast to CLZ, it is a partial agonist at DA D₂ (Lameh et al. 2007; Natesan et al. 2007) and at DA D₃ receptors (Burstein et al. 2005; Novi et al. 2007). It has been suggested that NDMC's unique muscarinic agonist properties may provide one molecular explanation for the superior clinical effects of CLZ (Weiner et al. 2004) and may more effectively treat the cognitive deficits that are observed in schizophrenic patients (Li et al. 2005). The initial clinical trials with ACP-104 (i.e., NDMC) focused on its safety, tolerability, and pharmacokinetics (Tamminga et al. 2006); however, a recent press release (Reuters, June 16, 2008) reported that ACP-104 did not display antipsychotic efficacy as compared to placebo in a Phase IIb clinical trial.

Because of the cross-species differences in the underlying mechanisms for CLZ's discriminative stimulus properties and the purported relevance of serotonergic actions in the therapeutic effects of clozapine (Meltzer et al. 1989), one goal of the present study was to extend the findings of the Philibin et al. (2005) study and to further characterize

the discriminative stimulus properties of the atypical APD clozapine in C57BL/6 mice by testing additional atypical and typical APDs and several selective ligands to better understand the pharmacological basis of the discriminative stimulus properties of CLZ in C57BL/6 mice. These results will serve as a foundation for future use of CLZ drug discrimination in genetically modified mice. A second goal was to compare the discriminative stimulus properties of CLZ to its major metabolite NDMC.

Materials and methods

Materials

Thirty adult male C57BL/6 wild-type mice weighing 20–25 g (Harlan Laboratories, Indianapolis, IN, USA) were used as subjects. All research was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2003), and the Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all procedures. The mice were

food-restricted to 90–95% of their free-feeding body weights. Water was available ad libitum in the home cages, and the mice were housed individually in a temperature-controlled vivarium (22–24°C) under a 12-h light/dark cycle (0600/1800 hours).

Apparatus

Drug discrimination experiments were conducted in four standard computer-interfaced mouse operant conditioning chambers (Model ENV-307A; Med Associates, St. Albans, VT, USA), with two retractable levers positioned equidistantly from a liquid dipper. Experimental events and data collection during these experiments were controlled by Med-PC for Windows software (Med Associates).

Drugs

Table 2 lists the drugs and drug classes tested in the present study. Clozapine (gift from Novartis, Hanover, NJ, USA), sertindole, aripiprazole, and M100907 (gift from Lundbeck, Copenhagen, Denmark), quetiapine (gift from Zeneca Pharmaceuticals, Wilmington, DE, USA), iloperidone and zotepine (HY Meltzer), fluphenazine (E.R. Squibb and Sons, New Brunswick, NJ, USA), and perphenazine, prazosin, and pyrilamine (Sigma Chemical Company, St. Louis, MO, USA) were dissolved in distilled water with two to three drops of lactic acid (pH values were approximately 4.0). Chlorpromazine HCL (Sigma), thioridazine HCL (Novartis), melperone, and fluoxetine (HY Meltzer) were dissolved in distilled water. Drugs were administered subcutaneously (s.c.) at a volume of 10 ml/kg body weight with a 30-min pre-session injection time. All doses refer to the salt form of the drugs.

Procedures

Training Mice were initially trained to lever press (only the vehicle-appropriate lever was present, and the mice received a VEH injection 30-min prior to the training sessions) under a fixed ratio 1 (FR 1) schedule for liquid food reinforcers (0.02 ml of sweetened condensed milk, by volume: one part condensed milk, one part sugar, and three parts water) during daily 15-min sessions. The FR requirement was gradually increased until stable responding occurred under an FR 10 reinforcement schedule (ten to 15 sessions). The training dose of 2.5 mg/kg CLZ was selected in order to provide a comparison to the results from the Philibin et al. (2005) study, in which 2.5 mg/kg CLZ was used. Also, based on the results of the time-course data from that study, a 30-min injection time was selected. Single-lever training was continued with only the CLZ-appropriate lever present for five sessions with mice

Table 2 Results of generalization testing in C57BL/6 mice trained to discriminate clozapine from vehicle in two-lever drug discrimination from the present study (indicated in **BOLD**) and from Philibin et al (2005)

Drug	ED ₅₀ (mg/kg)	Maximum % clozapine-lever responding
Atypical antipsychotic drugs		
Clozapine (2.5 mg/kg training dose)	1.19	97.4
Clozapine (2.5 mg/kg training dose)	1.14	99.7
Clozapine (2nd curve)	1.05	99.8
Aripiprazole	–	41.2
Iloperidone	0.19	89.8
Melperone	1.56	94.8
Olanzapine	0.24	87.3
Quetiapine	1.92	98.0
Risperidone	0.07	95.0
Sertindole	9.64	82.9
Zotepine	2.12	88.5
Ziprasidone	0.27	93.6
N-Desmethylozapine	–	49.9
N-Desmethylozapine+ 0.625 mg/kg Clozapine	3.53	83.6
Typical antipsychotic drugs		
Chlorpromazine	1.37	94.5
Fluphenazine	–	19.4
Haloperidol	–	51.6
Perphenazine	–	57.1
Thioridazine	5.85	97.5
Selective ligands		
Amphetamine (DA agonist)	–	8.1
M100907 (5-HT _{2A} antagonist)	1.95	87.6
Prazosin (α ₁ -adrenoceptor antagonist)	1.68	92.0
Pyrilamine (H ₁ histaminergic antagonist)	–	38.9
Quipazine (5-HT agonist)	–	5.3
Ritanserin (5-HT _{2A/2B/2C} antagonist)	2.08	94.5
Scopolamine (muscarinic antagonist)	–	62.3
Fluoxetine (SSRI)	–	13.1

ED₅₀ values are shown for those drugs that fully substituted for clozapine (i.e., >80% clozapine-lever responding; dashes indicate that the drug did not fully substitute for clozapine). The maximum % clozapine-lever responding is shown for all drugs tested. All drugs were administered s.c. Full substitution for clozapine=>80% clozapine-lever responding. Partial substitution for clozapine=>60% to <80% clozapine-lever responding. No substitution for clozapine=<60% clozapine-lever responding

receiving an injection of 2.5 mg/kg CLZ each day. All remaining training and test sessions were conducted with both levers available to the mice. Prior to these training sessions, mice received either a 2.5 mg/kg CLZ or VEH

injection according to a double alternation sequence (i.e., CLZ, CLZ, VEH, VEH, CLZ, CLZ, etc.), and only responses on the condition-appropriate lever were reinforced. A response on the incorrect lever reset the FR counter to 10. The position of the CLZ-appropriate lever (left versus right) was counterbalanced between groups to control for olfactory cues (Extance and Goudie 1981). The discrimination training criteria for all subjects consisted of (1) completing the first FR on the correct lever, (2) at least ten responses per minute (RPM), and (3) at least 80% condition-appropriate responding for five out of six consecutive training sessions.

Testing After the subjects met the three training criteria, substitution testing began and normally was conducted two times each week with a minimum of two training sessions between each drug test. Also, the training criteria had to be met for at least two training sessions between each drug test session. Control tests with the training drug CLZ (2.5 mg/kg) and VEH were completed prior to testing each drug in order to determine that CLZ was still maintaining good discriminative control. Test sessions differed from training sessions in that FR 10 responding on either lever resulted in delivery of reinforcers (responses on one lever reset the FR 10 requirement on the other lever).

After substitution testing, with atypical and typical APDs and with selective ligands, was completed (see Table 2 for list of the tested drugs), the CLZ generalization curve was re-established in seven mice. Then, substitution testing with NDMC was conducted (2.5, 5.0, and 10.0 mg/kg doses). Because NDMC did not fully substitute for CLZ, it was decided to repeat the NDMC dose–response determination, but in combination with a low dose of CLZ that did not engender full CLZ-appropriate responding in order to determine if NDMC could potentiate the discriminate cue of CLZ. The 0.625 mg/kg dose of CLZ was selected as it produced only 32.1% DLR responding when tested alone.

Data analysis

The number of lever presses on each lever during test sessions was recorded for each mouse and converted into percent drug-lever responding (% DLR) and response rate. Response rate was calculated as the mean RPM for each session. Percent drug-lever responding was not included in the dose–response curves for mice that failed to obtain a reinforcer or if response rate fell below two RPM. ED_{50} values (using the least-squares method of linear regression on the linear portion of the curves) and 95% confidence intervals were calculated for the % DLR substitution curves, in which at least one drug dose generated 80% or more CLZ-appropriate responding. Full substitution for the

CLZ cue was defined as 80% or greater CLZ-appropriate responding, while partial substitution was defined as CLZ-appropriate responding $\geq 60\%$ and $<80\%$. A repeated-measures analysis of variance (ANOVA) was conducted to assess significant effects of drug doses on response rates and to analyze differences in % DLR for the CLZ time course (GB-STAT software, V10.0; Dynamic Microsystems, Silver Spring, MD, USA). Significant ANOVAs were followed by Newman–Keuls post hoc tests ($p < 0.05$).

Results

Clozapine generalization testing

Twenty-six out of 30 mice reached training criteria in an average of 14.8 (SEM $\pm$ 1.6) sessions with a range of six to 34 sessions. Three mice did not complete the CLZ generalization curve because of unreliable performance and were removed from the study. The mean % DLR ($\pm$ SEM) and the mean RPM ($\pm$ SEM) for the CLZ generalization curve for the 2.5 mg/kg training dose are shown in Fig. 1. Full generalization to the CLZ cue was obtained at 2.5 and 5.0 mg/kg. Generalization testing yielded an ED_{50} =1.19 (95% CI=1.09–1.30 mg/kg). Response rates were significantly reduced by 5.0 mg/kg CLZ ($F[6,132]=115.3$, $p < 0.001$) with only four of 23 animals meeting the response rate criterion at that dose (i.e., earned a reinforcer or had >2.0 RPM); however, these four mice displayed 100% CLZ-appropriate responding.

Substitution testing with atypical antipsychotic drugs

The results of substitution testing for the atypical APDs quetiapine (upper panel), sertindole (middle panel), and zotepine (lower panel) are presented in Fig. 2. Quetiapine fully substituted (98.0% DLR) for CLZ at 10.0 mg/kg. Partial substitution (60.7% DLR) for CLZ was seen at 5.0 mg/kg with an ED_{50} =1.92 (95% CI=1.07–3.47 mg/kg). Response rates were significantly reduced at 10.0 mg/kg ($F[6,36]=10.7$, $p < 0.0001$). Sertindole fully substituted (82.9% DLR) for CLZ at 40.0 mg/kg with an ED_{50} =9.64 (95% CI=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 fully substituted (88.5% DLR) for CLZ at 5.0 mg/kg and partial substitution (66.8% DLR) for clozapine was seen at 2.5 mg/kg with an ED_{50} =2.12 (95% CI=1.76–2.56 mg/kg). Response rates were significantly reduced at 5.0 mg/kg ($F[5,35]=34.6$, $p < 0.001$).

The results of substitution testing for the atypical APDs iloperidone (upper panel), melperone (middle panel), and aripiprazole (lower panel) are presented in Fig. 3. Iloper-

Fig. 1 The mean percent drug-lever responding (% DLR) and mean responses per minute (RPM) for the clozapine generalization curve in C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine (CLZ) from vehicle (VEH) are shown. The dashed line at 80% DLR indicates full generalization to CLZ. Prior to generalization testing, control tests were conducted with the CLZ training dose and VEH. Mice that failed to earn a reinforcer or with response rates below two RPM were not included in the % DLR data (number of mice included is shown in parentheses). For the response rate data, significant differences from VEH are indicated by asterisks (** $p < 0.01$)

idone fully substituted (89.8% DLR) for CLZ at 0.4 mg/kg with an $ED_{50} = 0.19$ (95% CI = 0.14–0.25 mg/kg). Response rates were significantly reduced at 0.4 mg/kg ($F[6,42] = 20.6$, $p < 0.001$). Melperone also fully substituted (94.8% DLR) for CLZ at 2.0 mg/kg, producing an $ED_{50} = 1.56$ (95% CI = 1.39–1.76 mg/kg). Response rates were significantly reduced at 2.0 mg/kg ($F[6,42] = 20.7$, $p < 0.001$). Aripiprazole (1.25–10.0 mg/kg) failed to substitute for CLZ (maximum of 42.4% DLR at the 5.0 mg/kg dose). Response rates were significantly reduced at 5.0 and 10 mg/kg ($F[5,30] = 20.9$, $p < 0.001$).

Substitution testing with typical antipsychotic drugs

The results of substitution testing for the typical APDs chlorpromazine (upper left panel), thioridazine (lower left panel), fluphenazine (upper right panel), and perphenazine (lower right panel) are presented in Fig. 4. Chlorpromazine fully substituted (94.5% DLR) for CLZ at 4.0 mg/kg. Partial substitution (67.2% DLR) for CLZ was seen at 2.0 mg/kg with an $ED_{50} = 1.37$ (95% CI = 1.12–1.69 mg/kg), and response rates were significantly reduced at 4.0 mg/kg ($F[5,35] = 22.8$, $p < 0.001$). Thioridazine also fully substituted (97.5% DLR) for CLZ at 20.0 mg/kg with an $ED_{50} = 5.85$ (95% CI = 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 (0.125–2.0 mg/kg) failed to substitute for CLZ. Fluphenazine never generated above VEH-appropriate responding (i.e., >20% DLR). Response rates were significantly reduced at 1.0 and 2.0 mg/kg ($F[6,36] = 10.65$, $p < 0.001$). Perphenazine (0.125–2.0 mg/kg) also failed to substitute for CLZ. Response rates were significantly reduced at 2.0 mg/kg ($F[4,24] = 8.66$, $p < 0.001$), with only

ATYPICAL ANTIPSYCHOTIC DRUGS

Fig. 2 Substitution testing with the atypical antipsychotic drugs quetiapine (upper panel), sertindole (middle panel), and zotepine (bottom panel) are shown for C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine from vehicle. See Fig. 1 for other details

two mice meeting the response rate criterion (they displayed 57.1% DLR at that dose).

Substitution testing with selective ligands

The results of substitution testing for M100907 (upper left panel), prazosin (upper right panel), pyrilamine (lower left panel), and fluoxetine (lower right panel) are presented in Fig. 5. The 5-HT_{2A} antagonist M100907 fully substituted (87.55% DLR) for CLZ at 5.6 mg/kg, yielding an $ED_{50} = 1.95$ (95% CI = 1.35–2.82 mg/kg). Response rates were significantly reduced at 0.3, 1.0, 3.0, and 5.6 mg/kg ($F[7,49] = 10.02$, $p < 0.001$). The α_1 antagonist prazosin fully substituted (81.5% DLR) for CLZ at 2.8 mg/kg, producing an $ED_{50} = 1.68$ (95% CI = 1.04812–2.70317 mg/kg). Response rates were significantly reduced at 2.0, 2.82, and

Fig. 3 Substitution testing with the atypical antipsychotic drugs iloperidone (*upper panel*), melperone (*middle panel*), and aripiprazole (*bottom panel*) are shown for C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine from vehicle. See Fig. 1 for other details

4.0 mg/kg ($F[6,42]=5.7$, $p<0.001$). The one mouse that met the response rate criterion at the 4.0 mg/kg did display full substitution for CLZ (92.0% DLR). The H_1 histamine antagonist pyrilamine (5.0–40.0 mg/kg) failed to substitute for CLZ, with a maximum CLZ-appropriate responding of 38.9%. Response rates were significantly reduced at 40.0 mg/kg ($F[5,35]=22.3$, $p<0.001$). Finally, the selective serotonin reuptake inhibitor (SSRI) fluoxetine (3.75–15.0 mg/kg) also failed to substitute for CLZ. Maximum CLZ-appropriate responding was 13.1% at the 15.0 mg/kg dose. Response rates were significantly reduced at that dose ($F[4,28]=3.9$, $p<0.05$).

Figure 6 (top panel) shows the re-determination of the CLZ generalization curve for the seven mice used to study NDMC. The ED_{50} value for this curve (1.05 mg/kg; 95%

CI=0.86–1.29 mg/kg) was very similar to that for the first CLZ generalization curve (see Fig. 1; $ED_{50}=1.19$ mg/kg). Substitution testing with NDMC (Fig. 6, middle panel) did not yield any CLZ-appropriate responding (maximum of 49.9% DLR at the 10.0 mg/kg dose of NDMC for the three mice that met response rate criterion). There was a significant reduction in response rates at both the 5.0 and 10.0 mg/kg doses of NDMC. However, when the NDMC doses were combined with the low, 0.625 mg/kg dose of CLZ, full substitution for the CLZ cue was obtained at the 10.0 mg/kg dose of NDMC and partial substitution (62.5% DLR) at the 5.0 mg/kg dose, yielding an $ED_{50}=3.53$ mg/kg (95% CI=2.06–6.07 mg/kg). Of the six mice that met the response rate criterion for inclusion at the 10.0 mg/kg dose, five of them had 100% CLZ-appropriate responding. NDMC produced a significant reduction in response rates at the 10.0 mg/kg dose.

Discussion

A summary of the results from the present study and from the Philibin et al. (2005) study is shown in Table 2. ED_{50} values are shown for the antipsychotic drugs and selective ligands that fully substituted for CLZ ($\geq 80\%$ DLR), and maximum % CLZ-lever responding is shown for all tested drugs. The mice in the present study readily acquired the CLZ discrimination and, in fact, met the training criteria faster than in the Philibin et al. (2005). The only significant methodological difference between the two studies was that a 60-min pre-session injection time was used in the Philibin et al. (2005) study, and a 30-min pre-session time was used in the present study, but this did not affect the generalization curves for CLZ, as the ED_{50} values in both studies were virtually identical (see Table 2).

In the present study, the atypical APDs iloperidone, melperone, quetiapine, sertindole, and zotepine and the typical APDs chlorpromazine and thioridazine fully substituted for CLZ in C57BL/6 mice in the present study. All of these APDs, except melperone, bind potently to 5-HT_{2A} receptors, suggesting that CLZ (in C57BL/6 mice) produces an interoceptive cue that is mediated (at least in part) by 5-HT_{2A} receptor antagonism. Interestingly, the selective serotonin reuptake inhibitor fluoxetine failed to substitute for CLZ, indicating that it was not sufficient to simply increase serotonin levels to produce CLZ-appropriate responding. Fluoxetine does not display any significant occupancy of 5-HT_{2A} receptors, although it does display up to 43% occupancy of 5-HT_{2C} receptors at a 20 mg/kg dose (Palvimäki et al. 1999). Also, it should be noted that Dekeyne et al. (2003) trained rats to discriminate the 5-HT_{2A} antagonist M100907 and reported that the atypical APDs CLZ, quetiapine, risperidone, and ziprasidone all

Fig. 4 Substitution testing with the typical antipsychotic drugs chlorpromazine (*upper left panel*), thioridazine (*lower left panel*), fluphenazine (*upper right panel*), and perphenazine (*lower right panel*) are shown for C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine from vehicle. See Fig. 1 for other details

fully substituted for M100907, although the typical APD, haloperidol, which has only modest 5-HT_{2A} activity, also substituted for M100907, possibly through blockade of α_1 adrenoceptors.

The atypical APD melperone substituted for CLZ, but has a lower affinity for 5-HT_{2A} receptors than do the other APDs discussed above. It does display a preferential ratio for 5-HT_{2A} receptors relative to D₂ receptors, as do all of

the atypical APDs that fully substituted for CLZ in the present study (and in the Philbin et al. 2005 study). However, this cannot explain the ability of the typical APDs, chlorpromazine and thioridazine, that do not display a preferential ratio for 5-HT_{2A} receptors (thioridazine has sometimes been considered to be an atypical APD—see Meltzer et al. 1989 for discussion) to fully substitute for CLZ. Both APDs do display potent antagonist activity at

Fig. 5 Substitution testing with the selective ligands M100907 (5-HT_{2A} antagonist; *upper left panel*), prazosin (α_1 antagonist; *lower left panel*), pyrilamine (H₁ histamine antagonist; *upper right panel*), and the selective serotonin reuptake inhibitor fluoxetine (*lower right panel*) are shown for C57BL/6 mice trained to discriminate 2.5 mg/kg clozapine from vehicle. See Fig. 1 for other details

Fig. 6 Substitution testing with the clozapine (CLZ) metabolite *N*-desmethylozapine (NDMC) is shown for C57BL/6 mice trained to discriminate 2.5 mg/kg CLZ from vehicle (VEH). The CLZ dose response re-determination is shown in the upper panel. NDMC substitution testing is shown in the middle panel. Combination testing of NDMC with a low, 0.625 mg/kg dose of CLZ is shown in the bottom panel (* $p < 0.05$). See Fig. 1 for other details

α_1 -adrenoceptors, and it was found in the present study that the α_1 -adrenoceptor antagonist prazosin fully substituted for CLZ. Thus, one possible explanation for the ability of chlorpromazine and thioridazine to produce CLZ-appropriate responding may be α_1 antagonism. Many APDs bind to the α_1 -adrenoceptor subtype with relatively high affinity. Comparison with dopamine D_2 receptor affinities suggests that antipsychotic blockade of α_{1A} - and/or α_{1B} -adrenoceptor receptors may contribute to the antipsychotic activity of many atypical and several typical APDs (Cahir and King 2005; see also Dekeyne et al. 2003). For example, Goudie et al. (2004a, b) suggested that α_1 -adrenoceptors play a role

in the discriminative stimulus properties of the atypical APDs zotepine and quetiapine. Full substitution with the α_1 antagonist prazosin in the present study suggests that CLZ drug discrimination in C57BL/6 mice may be useful for the detection of antipsychotic agents with potent α_1 -adrenoceptor receptor antagonist actions.

The 5-HT_{2A} receptor antagonist M100907 engendered CLZ-appropriate responding in the present study, extending the previous finding that the 5-HT_{2A/2B/2C} receptor antagonist ritanserin fully substituted for CLZ in C57BL/6 mice (Philibin et al. 2005). However, a very high dose of M100907 was needed to produce CLZ-appropriate responding in the present study (ED₅₀=1.95 mg/kg). Dekeyne et al. (2003) trained rats to discriminate M100907 and found an ED₅₀ of 0.002 mg/kg—approximately a 1,000-fold difference in potency. This suggests the possibility that the 5-HT_{2A} antagonist properties of M100907 may not have been responsible for the CLZ-appropriate responding in the present study. While not as potent at other receptors, M100907 does bind to 5HT_{2C}, D₂, and α_1 receptors (Dekeyne et al. 2003). Also, ritanserin is an antagonist at both 5-HT_{2A} and 5-HT_{2C} receptors. Thus, it is possible that the ability of M100907 to substitute for CLZ in C57BL/6 mice may be related to activity at 5-HT_{2C} receptors. Interestingly, this would agree somewhat with findings from the Hoenicke et al. (1992) study with CLZ discrimination in pigeons. They concluded that antagonism of both 5-HT_{2A} AND 5-HT_{2C} receptors was important for CLZ's discriminative cue.

While both CLZ and olanzapine are potent antimuscarinic agents, the selective muscarinic antagonist scopolamine failed to fully substitute for CLZ in C57BL/6 mice (see Philibin et al. 2005); however, scopolamine did partially substitute (62.3% DLR) for CLZ. This suggests that antimuscarinic effects alone are not sufficient to engender CLZ-appropriate responding in C57BL/6 mice, although it is possible that muscarinic antagonism might be more relevant in C57BL/6 mice with a different training dose (see Goudie et al. 2004a). This notion is further supported by the ability of some atypical APDs with negligible muscarinic affinities (e.g., iloperidone, melperone, quetiapine, risperidone, sertindole, and ziprasidone) to fully substitute for CLZ in C57BL/6 mice. Thus, it appears that CLZ's discriminative cue in C57BL/6 mice can best be described as a "compound" cue in which activity at one or more receptors (5-HT_{2A}, α_1 , muscarinic, and perhaps D₂) may be sufficient to produce CLZ-appropriate responding. Thus, the ability of either typical or atypical APDs to substitute for CLZ may reflect a combination of activity at two or more receptors (i.e., a compound cue; see Goudie et al. 1998, Porter et al. 2000).

The only atypical APD that failed to fully substitute for CLZ in C57BL/6 mice is aripiprazole, which has a

mechanism of action different from other atypical APDs. Like many antipsychotic agents, aripiprazole binds with high affinity to dopamine D_2 receptors (Kikuchi et al. 1995; Lawler et al. 1999); however, unlike other APDs, aripiprazole is believed to exert its therapeutic effects through partial agonism of the D_2 -family of receptors (Inoue et al. 1996; Lawler et al. 1999). Aripiprazole also displays 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). It has also been shown that aripiprazole dose dependently attenuated the discriminative stimulus properties of S32504, which is a highly selective agonist at dopamine D_3 receptors but is a less potent agonist at D_2 receptors (Millan et al. 2007). There is evidence that aripiprazole has antagonist actions at several 5-HT receptor subtypes relevant to schizophrenia, such as 5-HT $_{1A}$ and 5-HT $_{2A}$ receptors (Bruins Slot et al. 2006). However, it should be noted that aripiprazole fully substituted for the 5-HT $_{1A}$ agonist LY293284, demonstrating in vivo agonist activity at this receptor (Marona-Lewicka and Nichols 2004), and aripiprazole has been characterized as a potent, partial agonist at human 5-HT $_{1A}$ receptors (Jordan et al. 2002). Aripiprazole's in vivo dopamine D_2 receptor antagonist activity may have prevented generalizable doses (i.e., higher doses) from being tested before response rates were suppressed, as has been seen with olanzapine in CLZ-trained nonhuman primates (Carey and Bergman 1997) and rats (Cole et al. 2007).

The results of the present study and Philibin et al. (2005) demonstrate that there are significant differences in the discriminative stimulus properties of CLZ in rats and mice. While CLZ appears to have a compound cue in both species, the relative importance of receptor subtypes seems to differ. In rats, only muscarinic cholinergic antagonists consistently substitute for CLZ (Nielsen 1988; Kelley and Porter 1997; Goudie et al. 1998; Millan et al. 1999; Prus et al. 2004; Porter et al. 2005), while 5-HT $_{2A}$ and α_1 -adrenoceptor antagonists fail to substitute for CLZ (Wiley and Porter 1992, 1993; Goudie et al. 1998; Millan et al. 1999; Prus et al. 2004). In C57BL/6 mice, both 5-HT $_{2A}$ and α_1 -adrenoceptor antagonists reliably produce full substitution for CLZ, whereas cholinergic antagonists do not (Philibin et al. 2005; the present study).

Finally, the present study investigated CLZ's major metabolite NDMC to determine if it shares discriminative stimulus properties with CLZ. NDMC failed to substitute for CLZ, producing a maximum of 49.9% CLZ-lever responding at a dose of 10.0 mg/kg for three of the seven mice (response rates were too suppressed to include the % DLR data for the other four mice; see Fig. 6). This finding is similar to that reported by Prus et al. (2006) in a three-

lever CLZ discrimination task with rats. In the present study, a very low dose of CLZ (0.625 mg/kg) was tested in combination with NDMC. As the dose of NDMC was increased, the percent of CLZ-appropriate responding was increased and reached full substitution at the combination of 10 mg/kg NDMC+0.625 mg/kg CLZ. These results suggest that NDMC does share some similarities with CLZ in terms of its discriminative stimulus properties, as it was able to potentiate the discriminative cue properties of a low dose of CLZ (see bottom panel in Fig. 6). In addition, it appears that the co-administration of CLZ with NDMC was able to attenuate the rate-suppressing effects of the 10 mg/kg dose of NDMC, as response rates were markedly higher than when NDMC was administered alone (see middle panel in Fig. 6). NDMC displays strong partial agonist activity at M_1 receptors (Davies et al. 2005; Lameh et al. 2007; Natesan et al. 2007), and while CLZ has traditionally been considered to be a muscarinic antagonist, more recent work has shown that CLZ is a partial agonist at M_1 , M_2 , and M_3 receptors and is a full agonist at M_4 receptors (see Raedler et al. 2007). Thus, CLZ and NDMC also appear to share similarities in terms of their activity at muscarinic receptors (which may or may not be related to their discriminative cue properties).

In summary, CLZ drug discrimination in C57BL/6 mice successfully detected all but one of the atypical APDs in the present study and in the Philibin et al. (2005) study. Atypical APDs such as clozapine, olanzapine, risperidone, and quetiapine have substantially reduced the EPS liability of APD treatment, but these agents are only moderately effective in the treatment of negative and cognitive executive symptoms (Bilder et al. 2002). Thus, there is a continued need for the development of APDs that are more effective for the treatment of these symptoms of schizophrenia. While the discriminative stimulus properties of APDs may not be related to their antipsychotic efficacy, understanding APDs on both a behavioral and neurochemical level will help increase our understanding of the complex pharmacology of schizophrenia and lead to improved agents with greater therapeutic efficacy and reduced side effect liability. Also, the use of inbred strains of mice (e.g., C57BL/6 versus DBA/2, Porter et al. 2008) offers strong advantages to investigations of the role of specific neurotransmitter receptor systems in the effects of pharmacological agents (see Holmes et al. 2002; Crawley 2003; Wolfer and Lipp 2000). Establishing this model in C57BL/6 mice opens the door for the future behavioral phenotyping of transgenic and knockout mice in CLZ drug discrimination. Targeted mutation of genes expressed in the mouse brain is now allowing for the increased integration of molecular genetics and behavioral neuroscience. Animal models that permit the dissection of the genetic basis of behavior will no doubt aid the develop-

ment of new treatment strategies for genetic disorders such as schizophrenia.

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