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The Role of Muscarinic Cholinergic Receptors in the Discriminative Stimulus Properties of Clozapine in Rats

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KELLEY, B. M. AND J. H. PORTER. *The role of muscarinic cholinergic receptors in the discriminative stimulus properties of clozapine in rats.* PHARMACOL BIOCHEM BEHAV **57**(4) 707–719, 1997.—The present study examined the role of muscarinic receptors in the discriminative stimulus properties of clozapine. One group of rats was trained to discriminate the atypical antipsychotic clozapine (CLZ, 5.0 mg/kg, IP) from vehicle in a two-lever drug discrimination procedure, and a second group of rats was trained to discriminate the muscarinic cholinergic antagonist scopolamine (SCP, 0.125 mg/kg, IP) from saline. Complete cross-generalization was obtained for SCP in the CLZ-trained rats and for CLZ in the SCP-trained rats. The M1 muscarinic antagonist trihexyphenidyl substituted completely for both CLZ and SCP; however, the M2 antagonist BIBN 99 failed to substitute for either CLZ or SCP. In other substitution tests, the tricyclic antidepressant amitriptyline, the antihistamine promethazine, and cyproheptadine (5-hydroxytryptamine [5-HT]$_{2A}$/5-HT$_{2C}$, histamine, and muscarinic antagonist) substituted completely for CLZ and SCP. The tetracyclic antidepressant mianserin substituted completely in the CLZ-trained rats, but did not substitute for SCP. Compounds that produced partial substitution included the tricyclic antidepressant imipramine, the anxiolytic chlordiazepoxide, and the antipsychotic thioridazine. Other compounds tested only in the CLZ-trained rats that failed to produce reliable CLZ-appropriate responding included *N*-methyl-D-aspartic acid (NMDA, selective agonist for glutamate receptors), metergoline (5-HT$_{2A}$/5-HT$_{2C}$ antagonist), propranolol (beta noradrenergic antagonist), and phentolamine (alpha noradrenergic antagonist). All of the compounds that produced CLZ-appropriate responding (except for mianserin) display high binding affinities for muscarinic cholinergic receptors. The results of the present study demonstrated that muscarinic receptors (especially M1) play an important role in the mediation of the discriminative stimulus properties of CLZ in rats, and provide additional support for the importance of CLZ's anticholinergic properties as part of it's unique profile as an atypical antipsychotic. © 1997 Elsevier Science Inc.

Amitriptyline Antidepressants BIBN 99 Chlordiazepoxide Cholinergic Clozapine Cyproheptadine Discriminative stimulus effects-drug discrimination Fixed ratio schedule 5-hydroxytryptamine (serotonin) Imipramine Metergoline Mianserin Muscarinic receptors *N*-methyl-D-aspartic acid (NMDA) Neuroleptics Operant behavior Phentolamine Promethazine Propranolol Rats Scopolamine Thioridazine Trihexyphenidyl

THE atypical antipsychotic clozapine appears to be superior to conventional (typical) neuroleptics, such as haloperidol and chlorpromazine, in the treatment of schizophrenia. For example, symptom reduction has been reported as early as the first week of CLZ treatment and continued to be more effective than the typical neuroleptic chlorpromazine (11,35,49); CLZ produces fewer side effects (such as extrapyramidal motor effects) and is tolerated better than typical neuroleptics (11,35); and CLZ has been shown to be more proficient in reducing the severity of negative symptoms (35,14). Furthermore, it has been demonstrated that CLZ is effective in patients who are resistant to treatment with typical neuroleptics (26,35). Chronic treatment with CLZ does not appear to cause the development of tardive dyskinesia (9), and CLZ can actually reduce the severity of pre-existing tardive dyskinesia symptoms (41,45). The most serious side effect of CLZ is the development of agranulocytosis in 1–2% of patients, although close monitoring of white blood cells can greatly reduce the frequency of this complication (63).

CLZ, a dibenzodiazepine, is both structurally and pharmacologically different from typical neuroleptics such as haloperidol, a butyrophenone, and chlorpromazine, a phenothiazine.

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Unlike typical neuroleptics that potently antagonize dopamine D_2 receptors, CLZ binds with relatively low affinity to D_2 receptors (29). However, CLZ displays a high binding affinity for a number of other receptor systems and these differences have led to several hypotheses about the mechanisms underlying the unique clinical profile for atypical neuroleptics (18). One hypothesis (46) has argued that atypical antipsychotics such as CLZ have a ratio of 5-HT_{2A}/D_2 binding affinity favoring the 5-HT_{2A} receptors, whereas typical neuroleptics display a reverse binding profile (Following the recommendations of the Serotonin Club Receptor Nomenclature Committee [International Union of Pharmacology Committee for Receptor Nomenclature, see 28], we are using the new classification system for serotonin receptors. Thus, the "classical" 5-HT_2 receptors are now classified as 5-HT_{2A} receptors and 5-HT_{1C} receptors are now classified as 5-HT_{2C} receptors. This nomenclature is used throughout the article.). A second hypothesis has focused on the potent anticholinergic properties of atypical neuroleptics. Snyder et al. (59) proposed that the anticholinergic activity of atypical neuroleptics is responsible for their lack of extrapyramidal motor side effects. More recently, Tandon and Greden (64) have argued that cholinergic hyperactivity may be related to the development of negative symptoms in schizophrenia. Thus, CLZ's anticholinergic properties (i.e., antagonism of muscarinic receptors) may play an important role in its ability to alleviate negative symptoms.

A number of studies (7,21,25,50,68,72,73) have focused on the discriminative stimulus effects of the atypical antipsychotic CLZ in an attempt to determine the underlying neural mechanism(s) that mediates the discriminative stimulus properties of CLZ. Hoenicke et al. (25) have suggested that blockade of 5-HT_{2A} and 5-HT_{2C} receptors is the underlying mechanism responsible for the discriminative cue properties of CLZ based on results with pigeons trained to discriminate CLZ from vehicle. However, in drug discrimination studies using rats, Wiley and Porter (72,73) reported that the 5-HT_{2A}/5-HT_{2C} antagonist ritanserin failed to substitute for CLZ. Alternatively, Nielsen (50) has argued that muscarinic cholinergic antagonism mediates CLZ's discriminative stimulus properties, as evidenced by the ability of scopolamine and atropine to substitute for CLZ in rats trained to discriminate CLZ from vehicle. While there are a number of obvious differences between the Hoenicke et al. (25), the Wiley and Porter (72,73), and the Nielsen (50) studies (such as pigeons versus rats and cumulative dosing versus acute dosing procedures) many of the drugs (e.g., amitriptyline, cyproheptadine, fluperlapine, and promethazine) in the Hoenicke et al. study that substituted for CLZ demonstrate potent antagonism at a number of receptors including both cholinergic and serotonergic receptors. Thus, the receptor mechanism(s) involved in CLZ's discriminative stimulus effects remains unresolved.

In order to more precisely determine the role of muscarinic receptors in CLZ's discriminative stimulus properties in rats, the present study included two drug discrimination groups. One group of rats was trained to discriminate CLZ from vehicle and a second group of rats was trained to discriminative SCP from saline. If CLZ's discriminative stimulus properties are mediated by antagonism of muscarinic receptors in rats, then one would expect cross-generalization between CLZ and SCP (i.e., CLZ should substitute for SCP and SCP should substitute for CLZ). Furthermore, any drug that engenders CLZ-appropriate responding also should produce SCP-appropriate responding. The role of M1 and M2 muscarinic receptors in the discrminative stimulus properties of CLZ also was examined.

METHODS

Subjects

Thirty-two naive adult male Sprague–Dawley rats (290–340g) obtained from Harlan Sprague Dawley, Indianapolis, IN, served as subjects. Animals were housed individually in suspended wire cages in a temperature controlled (220°C) environment on a 12L:12D cycle (lights on at 0600). After one week of acclimation, the rats were reduced to 85% of their free-feeding weights by placing them on a food restricted diet (10–15g of Agway Prolab MHR 3000 rodent chow following experimental sessions). The animals' body weights were adjusted over the duration of the experiment to allow for normal growth. Water was available ad lib in the home cages.

Apparatus

All experimental sessions were conducted in four standard operant chambers (Lafayette Instruments, Lafayette, IN, Model 80001) housed in sound-attenuated chambers (Lafayette Instruments, Model 80015). Each chamber contained two identical response levers, mounted symmetrically on either side of the intelligence panel 6.5 cm above the grid floor. A pellet dispenser (Lafayette Instruments, Model 80200) delivered 45 mg food pellets (Formula P Purified Rodent Diet, P. J. Noyes, Lancaster, N.H.) into a food cup (2.5 cm above the grid floor) located between the two levers in the center of the intelligence panel. Fan motors provided ventilation as well as masking noise for each chamber. Two 7-w white house lights (one light centered over each lever) were located 18.5 cm above the grid floor. A WIN (486) computer using MED-PC software (Med Associates, Inc., St. Albans, VT) was used to control the operant schedule and record data.

Drugs

Clozapine (Sandoz Pharmaceuticals, Hanover, NJ) was prepared in a solution of 85% lactic acid (10–15 drops) and distilled water to a total volume of 50 ml (doses refer to the free base). Amitriptyline HCl (Merck Sharpe & Dohme, Rahway, NJ), trihexyphenidyl HCl, phentolamine mesylate (Research Biochemicals International, Natick, MA), imipramine HCl (CIBA-GIEGY, Summit, NJ), chlordiazepoxide HCl, cyproheptadine HCl, mianserin HCl, promethazine HCl, propranolol HCl, scopolamine HCl (Sigma Chemical Co., St. Louis, MO), metergoline HCl (Farmatalia, Milan, Italy), and thioridazine HCl (Sandoz) were dissolved in a solution of 0.9% saline (doses refer to the salt). *N*-methyl-D-aspartic acid (NMDA, Sigma), was dissolved in a solution of equimolar sodium hydroxide. BIBN 99 (supplied courtesy of Boehringer Ingelheim Pharmaceuticals, Biberach, Germany) was dissolved in a 0.1% HCl acid solution and sonicated for 10 min. The solution was then titrated with 0.1% NaOH (about 500 μl) until the solution obtained a pH of 5.0. Isotonic saline was used to bring the solution to volume. CLZ and thioridazine were administered 60 min prior to test sessions. SCP, amitriptyline, chlordiazepoxide, cyproheptadine, imipramine, metergoline, mianserin, phentolamine, promethazine, propranolol, and trihexyphenidyl were administered 30 min prior to test sessions. NMDA was administered 10 min prior to test sessions. All doses were administered intraperitoneally (IP) at a volume of 1 ml/kg of body weight, except for BIBN 99 which

was injected subcutaneously (also at a volume of 1 ml/kg) 60 min prior to test sessions.

Discrimination Training

All experimental sessions lasted 15 min and were conducted daily Monday through Friday. Experimental sessions usually were not conducted on the weekends, but the rats were maintained at 85% of their free-feeding body weights. At the beginning of the study the rats were randomly assigned to one of two conditions for Discrimination Training. Sixteen rats were trained to discriminate clozapine (5.0 mg/kg) from vehicle (CLZ-trained rats). The other 16 rats were trained to discriminate scopolamine (0.125 mg/kg) from vehicle (SCP-trained rats). In order to control for olfactory cues (see 19), the position of the drug-associated lever (right vs. left) was counterbalanced among the rats. Initially, the rats were trained to lever press with a single lever present in each operant box according to a fixed ratio 1 (FR 1) food reinforcement schedule. The ratio was gradually increased to a final schedule of FR 30 over 10 sessions. Prior to the first two training sessions, the rats were injected with vehicle; prior to training sessions three and four, the rats were injected with either CLZ (5.0 mg/kg) or SCP (0.125 mg/kg). The drug or vehicle injections for the remaining six sessions followed this double alternation sequence (vehicle, vehicle, drug, drug, vehicle, vehicle; VV,DD,VV) and only the correct lever was present in the operant box during these 10 sessions. Next, the rats received five sessions with drug injections with only the drug lever present; this was followed by five sessions with vehicle injections with only the vehicle lever present.

Beginning with session 21, both levers were installed for Discrimination Training and only responses on the correct lever resulted in the delivery of reinforcers with the completion of the FR 30 requirement during each experimental session. Responses on the incorrect lever reset the ratio requirement for the correct lever. Then the double alternation schedule (VV,DD,VV, counterbalanced among the rats) was resumed and continued throughout the experiment for training sessions. In order to complete Discrimination Training, a rat had to meet the following three evaluation criteria: (1) the first completed FR 30 must have been made on the appropriate lever; (2) percent of correct-lever responding during the 15 min test sessions must be equal to or greater than 85 %; and (3) response rate must equal or exceed 30 responses per minute (RPM). After completing Discrimination Training, Control Test sessions were conducted and consisted of a minimum of four test sessions with injections of either the training dose of the drug or the vehicle (counterbalanced order). Drug testing usually occurred on Tuesdays and Fridays, although occasionally testing occurred on other days (there was a minimum of two training sessions between test days). On test days responses on either lever delivered a reinforcer according to the FR 30 food reinforcement schedule. Discrimination training on the double alternation schedule continued on nontest days. Successful completion of the Control Tests required each rat to meet the three evaluation criteria on four of five consecutive test sessions. Rats that failed to meet the three evaluation criteria during Discrimination Training or during the Control Tests were removed from the study. A total of 13 rats in the CLZ-trained group and 10 rats in the SCP-trained group successfully completed Discrimination Training and met the three evaluation criteria.

Generalization and Substitution Testing

The 13 rats that successfully completed CLZ training were randomly assigned to two groups ($n = 7$ and $n = 6$). After testing scopolamine (0.0625–1.0 mg/kg), trihexyphenidyl (0.2–6.4 mg/kg), and amitriptyline (1.5–24.0 mg/kg) in the first group and NMDA (3.75–30.0 mg/kg; dose determination was repeated), mianserin (0.5–16.0 mg/kg), and metergoline (1.0–8.0 mg/kg) in the other, the 11 rats that continued to meet the three evaluation criteria were combined for the testing of imipramine (1.5–12.0 mg/kg). Then, a second CLZ generalization dose effect curve was obtained (0.156–10.0 mg/kg). Five rats successfully completed that testing and were tested with four additional drugs: promethazine (1.25–10.0 mg/kg); cyproheptadine (0.039– 2.5 mg/kg); chlordiazepoxide (2.5–10.0 mg/kg); and BIBN 99 (0.25–1.0 mg/kg). In the SCP-trained group, six drugs were tested: clozapine (2.5–20.0 mg/kg); amitriptyline (0.75–12.0 mg/kg); trihexyphenidyl (0.1–6.4 mg/kg); imipramine (1.5–12.0 mg/kg); mianserin (2.0–8.0 mg/kg); and thioridazine (2.5–20.0 mg/kg). Then, a second SCP generalization dose effect curve was obtained (0.03125–0.25 mg/kg). The five rats that successfully completed that testing were then tested with promethazine (1.25–10.0 mg/kg), cyproheptadine (1.25–10.0 mg/kg), chlordiazepoxide (2.5–10.0 mg/kg), and BIBN 99 (0.25–1.0 mg/kg). In order to be tested on a given test day, a rat had to meet the three evaluation criteria (described above) on the previous (training) day. For the first Generalization Tests with the training drugs, the doses for CLZ and SCP were administered according a randomized Latin Square design. During all other drug tests, the doses were given in ascending order. Between testing with each drug, Control Test sessions were conducted with the appropriate training drug and vehicle and the rats were required to meet the three evaluation criteria for one test session with vehicle and one test session with the training drug before the next drug could be tested. During the course of the study, rats that developed a preference for a specific lever position, whose responding or discrimination control deteriorated (as indicated by response rates consistently below 5 RPM or consistent failure to meet the evaluation criteria) or that became sick were removed from the study.

Thioridazine (1.25–20.0 mg/kg), propranolol (2.5–30.0 mg/kg), and phentolamine (1.5–6.0 mg/kg) were tested in six CLZ-trained rats (5.0 mg/kg training dose) used in a previous drug discrimination study. The training and testing procedures in that study were almost identical to those in the present study. These six rats had previous testing with the following drugs: clozapine, haloperidol, ritanserin, MDL 72222 (5-HT_3 antagonist) and buspirone (see 72 for further details).

Data Analysis

The number of lever presses on each lever, the number of reinforcers earned, and the lever on which the first FR 30 was completed was recorded during each session. Also, during two-lever drug discrimination training and during all test sessions, percent of correct-lever responding (i.e., number of responses on the correct lever divided by the total number of responses $\times$ 100) and responses per minute were calculated. During Generalization and Substitution Testing, the percent of clozapine or scopolamine lever responding and response rates were calculated. Responding on the drug lever at 80% or greater was defined as complete substitution. ED_{50}'s (with 95% confidence intervals) were calculated with the least squares method of linear regression on the linear part of the dose-effect curve only for those drugs that produced complete substitution (see 23).

The %DLR for rats that had less than 5 responses per min were excluded from this analysis. Separate repeated measures analyses of variance (ANOVA) comparing response rates were performed for each drug. Significant ANOVA's were followed by Tukey post hoc tests (alpha = 0.05).

RESULTS

Cross-Generalization Testing

Thirteen rats in the CLZ-trained group successfully completed Discrimination Training and the Control Tests in an average of 52.5 sessions (range = 51–71 sessions). Ten rats in the SCP-trained group completed Discrimination Training and Control Tests in an average of 54.9 sessions (range = 51–61) from the very first day of lever-press training. Figure 1 shows mean percent drug lever responding (% DLR) and mean responses per minute (RPM) for the CLZ (upper left panel) and SCP (lower left panel) Generalization tests. The ED_{50} for the CLZ dose effect curve was 0.36 mg/kg (95% confidence interval [C.I.] = 0.026–5.026). Both the 5 and 10 mg/kg doses produced > 80% DLR. Analysis of response rates revealed that RPM for the 10 mg/kg dose of CLZ were significantly less ($F[5, 60] = 5.37$, $p < 0.001$) than for the other CLZ doses, but did not differ significantly from either the vehicle or CLZ control tests. In the SCP-trained rats both the 0.125 and 0.250 mg/kg doses of SCP produced > 80% DLR. The ED_{50} for the SCP dose effect curve was 0.04 mg/kg (95% C.I. = 0.017–0.102). There were no significant changes in response rates ($F[5, 40] = 0.64$, $p > 0.05$).

During cross-generalization testing, six rats tested from the CLZ-trained group (Fig. 1, upper right panel) displayed CLZ-appropriate responding at the three highest doses of SCP (0.25 mg/kg = 97% DLR, 0.50 mg/kg = 98.3% DLR, 1.0 mg/kg = 98% DLR). The other rat failed to substitute at any of the tested doses of SCP. The ED_{50} for the dose effect curve was 0.12 mg/kg (95% C.I. = 0.051–0.300). Response rates for the 1.00 mg/kg dose of SCP were significantly less ($F[7,42] = 3.24$, $p < 0.01$) than for the CLZ control point, the SCP vehicle, and the 0.0625 and 0.125 mg/kg doses. None of the SCP doses were significantly different from CLZ vehicle.

Seven of the SCP-trained rats (Fig. 1, lower right panel) produced SCP-appropriate responding at the three highest doses of CLZ (5.0 mg/kg = 89.3% DLR, 10.0 mg/kg = 98.6% DLR, 20.0 mg/kg = 100% DLR). One of the other two rats displayed 100% DLR at the 10.0 mg/kg dose, but failed to substitute at any other doses, while the other rat consistently chose the vehicle-lever. The ED_{50} for the dose effect curve was 3.09 mg/kg (95% C.I. = 1.237–7.738). The 20.0 mg/kg dose of CLZ significantly ($F[6, 48] = 4.297$, $p < 0.01$) reduced responding relative to all other doses and control tests.

Substitution Testing

Figure 2 presents the results of testing for the M1 antagonist trihexyphenidyl and the M2 antagonist BIBN 99. In the CLZ-trained rats (top left panel) trihexyphenidyl displayed complete substitution for CLZ in six rats at the 3.2 mg/kg dose (97.8% DLR) and five rats (data for one rat was excluded at this dose because his response rate was < 5 RPM) at the 6.4 mg/kg dose (100% DLR). The other rat demonstrated CLZ-appropriate responding only at the 0.80 mg/kg dose (99% DLR). The ED_{50} equaled 0.96 mg/kg (95% C.I. = 0.568–1.634). There were no significant changes in response rates ($F[7, 42] = 0.908$, $p > 0.05$). In the SCP-trained rats (top right panel) complete substitution was obtained with trihexyphenidyl at the 3.2 mg/kg dose for all eight rats and at the 6.4 mg/kg dose for seven rats (one rat was excluded because his response rate <5 RPM). The ED_{50} was 0.81 mg/kg (95% C.I. = 0.472–1.373). Again, there were no significant changes in response rates ($F[8, 56] = 1.505$, $p > 0.05$).

BIBN 99 did not produce CLZ-appropriate responding (Fig. 2, bottom left panel) or SCP-appropriate responding (bottom right panel) at the doses tested. There were no significant changes in response rates for the CLZ-trained rats ($F[4, 16] = 1.74$, $p > 0.05$). In the SCP-trained rats, the SCP control response rate was significantly lower than the VEH and BIBN 99 response rates.

Figure 3 displays the results of Substitution Testing for the three antidepressants amitriptyline, imipramine and mianserin. Amitriptyline (top left panel) produced complete substitution at the three highest doses in six CLZ-trained rats, reaching 98.3% DLR for the 12.0 mg/kg dose. The ED_{50} was 1.50 mg/kg (95% C.I. = 0.252–8.924). Analysis of response rates revealed no significant changes ($F[6, 30] = 1.247$, $p > 0.05$). For 9 SCP-trained rats (top right panel) 99.1% DLR was obtained at the 12.0 mg/kg dose of amitriptyline, and the ED_{50} equaled 1.78 mg/kg (95% C.I. = 0.874–3.625). There were no significant changes in response rates ($F[6, 48] = 1.045$, $p > 0.05$).

Imipramine produced full substitution (95.9% DLR) in seven CLZ-trained rats (Fig. 3, middle left panel) at the 12.0 mg/kg dose, and two rats at that dose showed partial substitution (68.5% DLR). The remaining two rats failed to produce appreciable drug-lever responding at any of the tested doses. The ED_{50} for the dose effect curve was 9.62 mg/kg (95% C.I. = 4.572–14.650). Response rates were significantly reduced ($F[5, 50] = 7.922$, $p < 0.0001$) at the 6.0 and 12.0 mg/kg doses relative to the vehicle and clozapine control points. In the SCP-trained group (middle right panel) three rats displayed complete substitution (93% DLR) and one rat partial substitution (68%) at the 12.0 mg/kg dose of imipramine. One rat's response rate was suppressed below 5 RPM at these two doses and not included. The ED_{50} equaled 8.08 mg/kg (95% C.I. = 4.306–11.854). Response rates were significantly lower ($F[6, 30] = 4.576$, $p < 0.01$) for the 12.0 mg/kg dose than for the vehicle control point.

Mianserin produced complete substitution in five CLZ-trained rats (Fig. 3, bottom left panel) at the 4.0 mg/kg dose (100% DLR) and at the 8.0 mg/kg dose (98.6% DLR). Four rats displayed complete substitution at the 16.0 mg/kg dose (99.3% DLR), three rats substituted at the 2.0 mg/kg dose (100% DLR), and two rats substituted at the 1.0 mg/kg dose (99.0%). The ED_{50} was calculated to be 1.50 mg/kg (95% C.I. = 0.446–5.278). There were no significant changes in response rates across doses ($F[7, 35] = 1.018$, $p > 0.05$).

Mianserin did not produce any reliable SCP-like responding in the SCP-trained rats (Fig. 3, bottom right panel). One rat did display SCP-appropriate responding at all three doses (3.0 mg/kg = 94% DLR, 4.0 mg/kg = 100% DLR, 8.0 mg/kg = 100% DLR), and one other rat displayed SCP-like responding at the 2.0 mg/kg (99% DLR). No ED_{50} was calculated for this dose effect curve. The 8.0 mg/kg dose produced a significant reduction ($F[4, 28] = 3.385$, $p < 0.05$) in responding relative to the vehicle control test.

Figure 4 shows the results of Substitution Testing for cyproheptadine and promethazine. Cyproheptadine produced CLZ-appropriate responding in all five of the CLZ-trained rats at the 1.25 and 2.5 mg/kg doses (top left panel). At the lower doses complete substitution was seen with three rats at the 0.156 mg/kg dose (98.4% DLR), four rats at the 0.3125 mg/

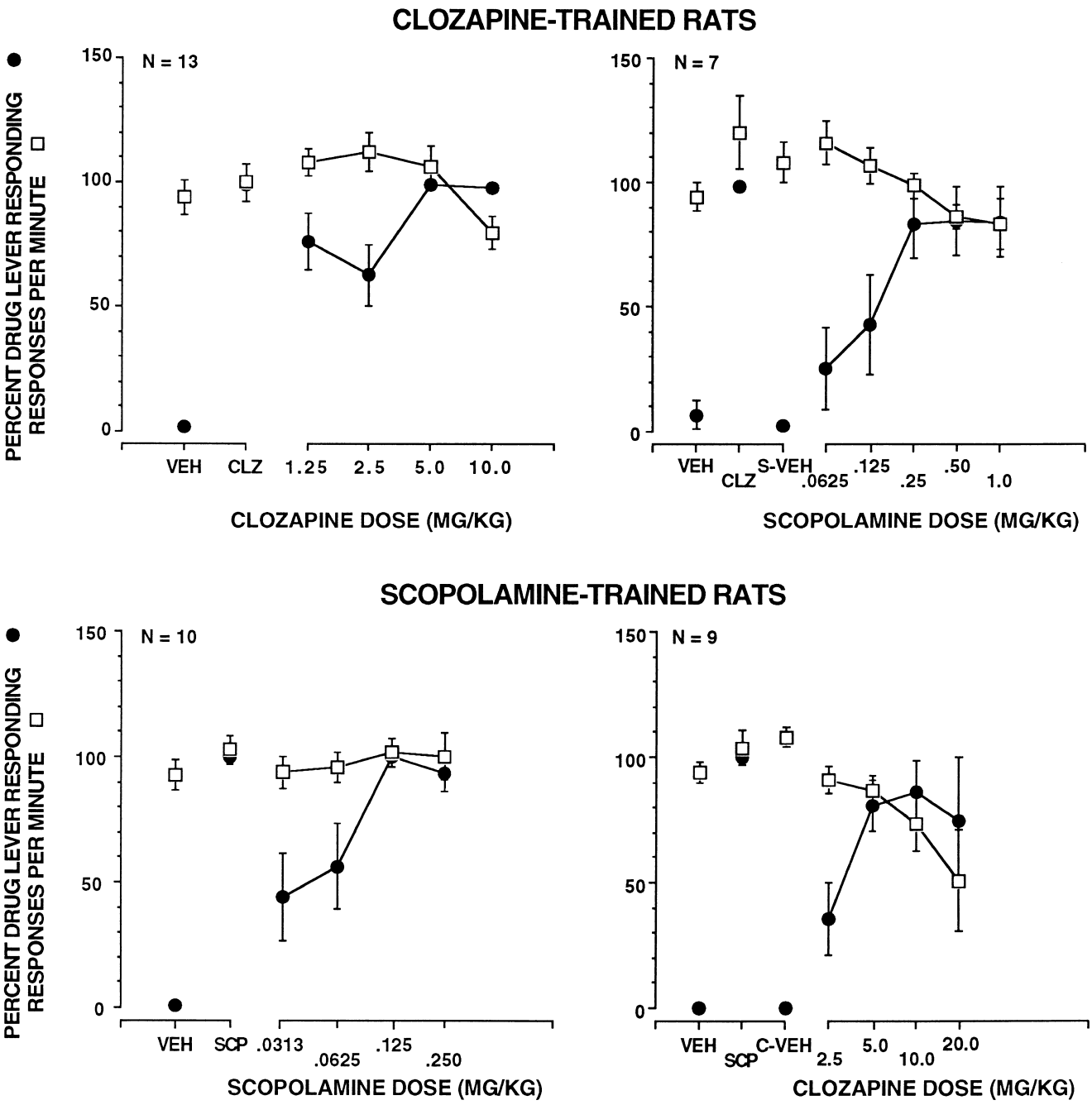

FIG. 1. Generalization testing with the training drugs is shown for the CLZ-trained rats (upper left panel) and the SCP-trained rats (lower left panel). Substitution testing with SCP in the CLZ-trained rats (upper right panel) and with CLZ in the SCP-trained rats (lower right panel) also is shown. The vehicle (VEH) and drug Control Tests, mean % DLR (± SEM), mean responses per min (±SEM), and the number (n) of rats tested are shown for each testing condition. The CLZ vehicle (C-VEH) and SCP vehicle (SCP-VEH) also were tested prior to substitution testing.

kg dose (97.1% DLR), and 3 rats at the 0.625 mg/kg dose. The ED_{50} for this dose effect curve equaled 0.12 mg/kg (95% C.I. = 0.033–0.448). A significant increase ($F[7, 28] = 4.453$, $p < 0.01$) in response rates was observed for the four highest doses (0.3125 to 2.5 mg/kg). In the SCP-trained rats (top right panel) four rats displayed full substitution at the 10.0 mg/kg dose (the fifth rat had 77.7% DLR). Three rats displayed full substitution at the 5.0 mg/kg dose (100% DLR), and one rat substituted (80.7% DLR) at the 2.5 mg/kg dose. The ED_{50} was 3.49 mg/kg (95% C.I. = 2.644–4.602). Response rates for the 10.0 mg/kg dose of cyproheptadine and the SCP control test were significantly less than for all other doses ($F[5, 20] = 9.18$, $p < 0.001$).

Promethazine also produced complete substitution in both

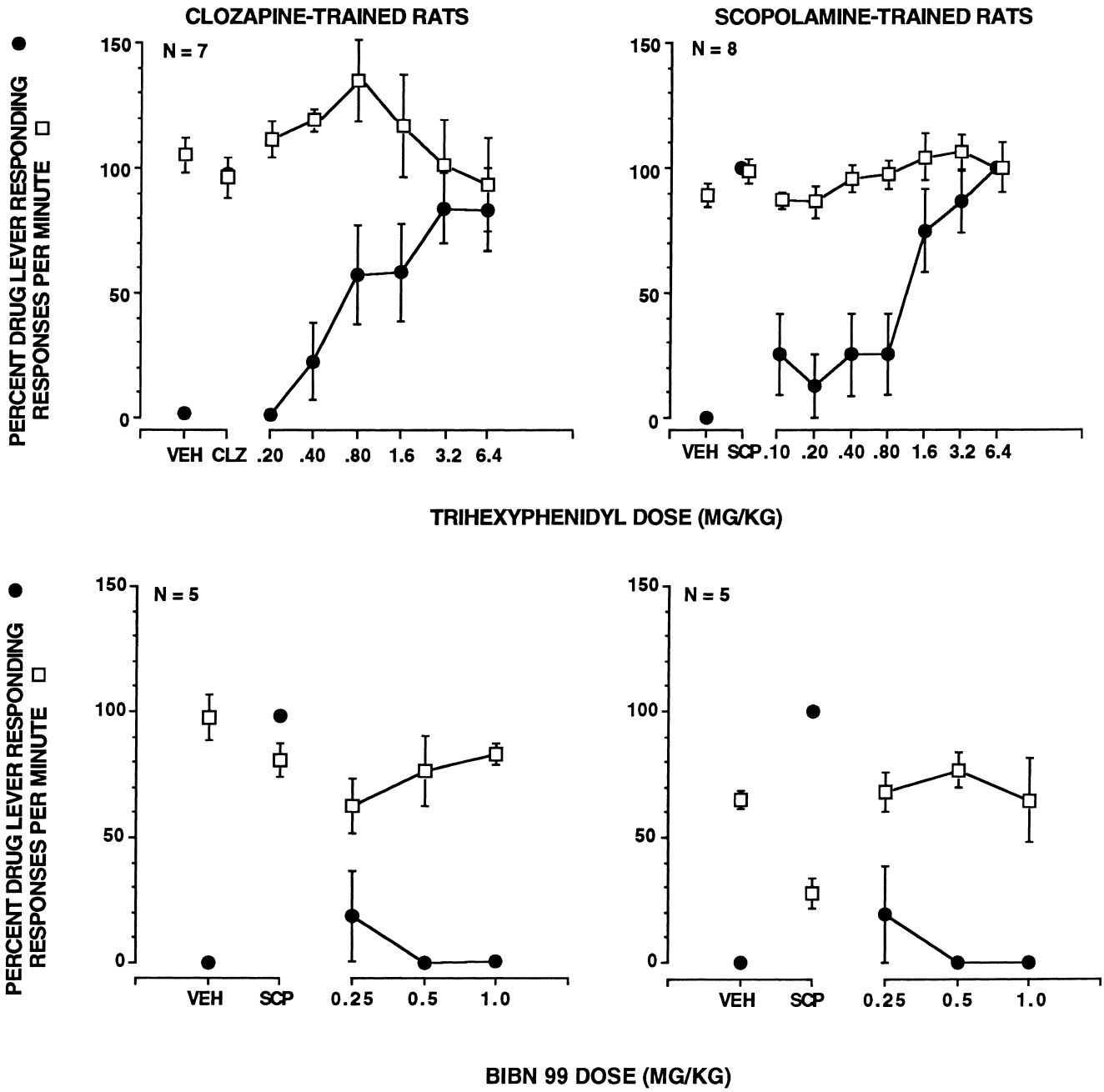

FIG. 2. Results of substitution testing with trihexyphenidyl (top two panels) and with BIBN 99 (bottom two panels) are shown for the CLZ-trained rats (left panels) and for the SCP-trained rats (right panels). Other details are the same as in Fig. 1.

the CLZ-trained and SCP-trained rats. All five rats displayed CLZ-like responding (Fig. 4, bottom left panel) at the three highest doses of promethazine, and two rats showed full substitution at the lowest dose (2.5 mg/kg = 97.8%). The ED_{50} for this dose effect curve was 1.11 mg/kg (95% C.I. = 0.165–7.506). There were no significant differences in response rates ($F[5, 20] = 2.41$, $p > 0.05$). In the SCP-trained rats (bottom fight panel) five rats showed complete substitution at the 10.0 mg/kg dose, and three rats substituted at the 5.0 mg/kg (99.9% DLR) and 2.5 mg/kg (98.9% DLR) doses. The ED_{50} equaled 2.10 mg/kg (95% C.I. = 0.689–6.381) Again, there were no significant differences in response rates ($F[5, 20] = 0.874$, $p > 0.05$).

Other Tested Compounds

The highest mean %DLR for the other compounds that did not engender 80% or greater drug-appropriate responding in either the CLZ-trained rats or the SCP-trained rats are listed in Table 1 (the compounds that did produce full substitution are shown for comparison). The binding affinities for all of the compounds tested are also shown for muscarinic

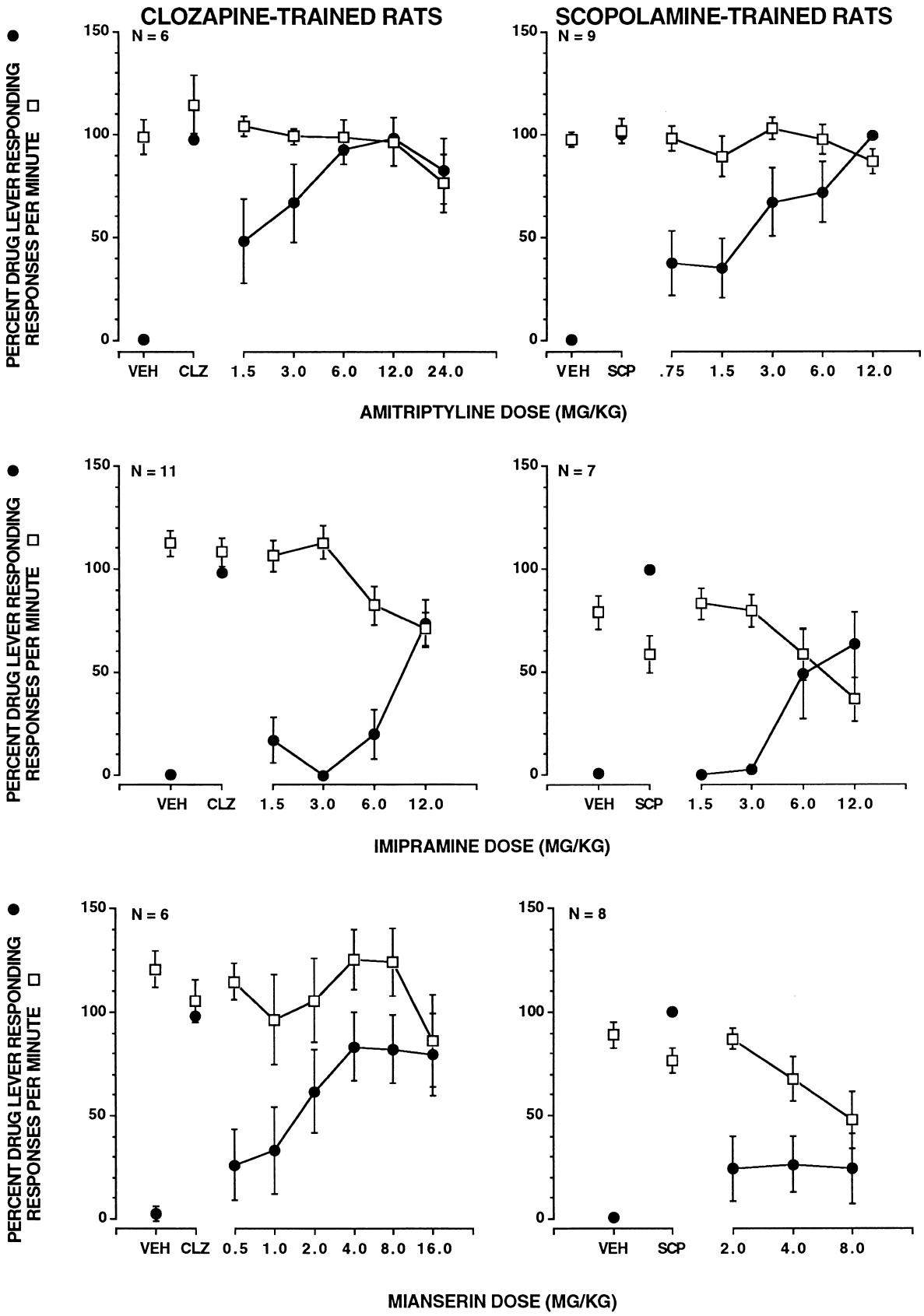

FIG. 3. Results of substitution testing with amitriptyline (top two panels), imipramine (middle two panels), and mianserin (bottom two panels) are shown for the CLZ-trained rats (left panels) and for the SCP-trained rats (right panels). Other details are the same as in Fig. 1.

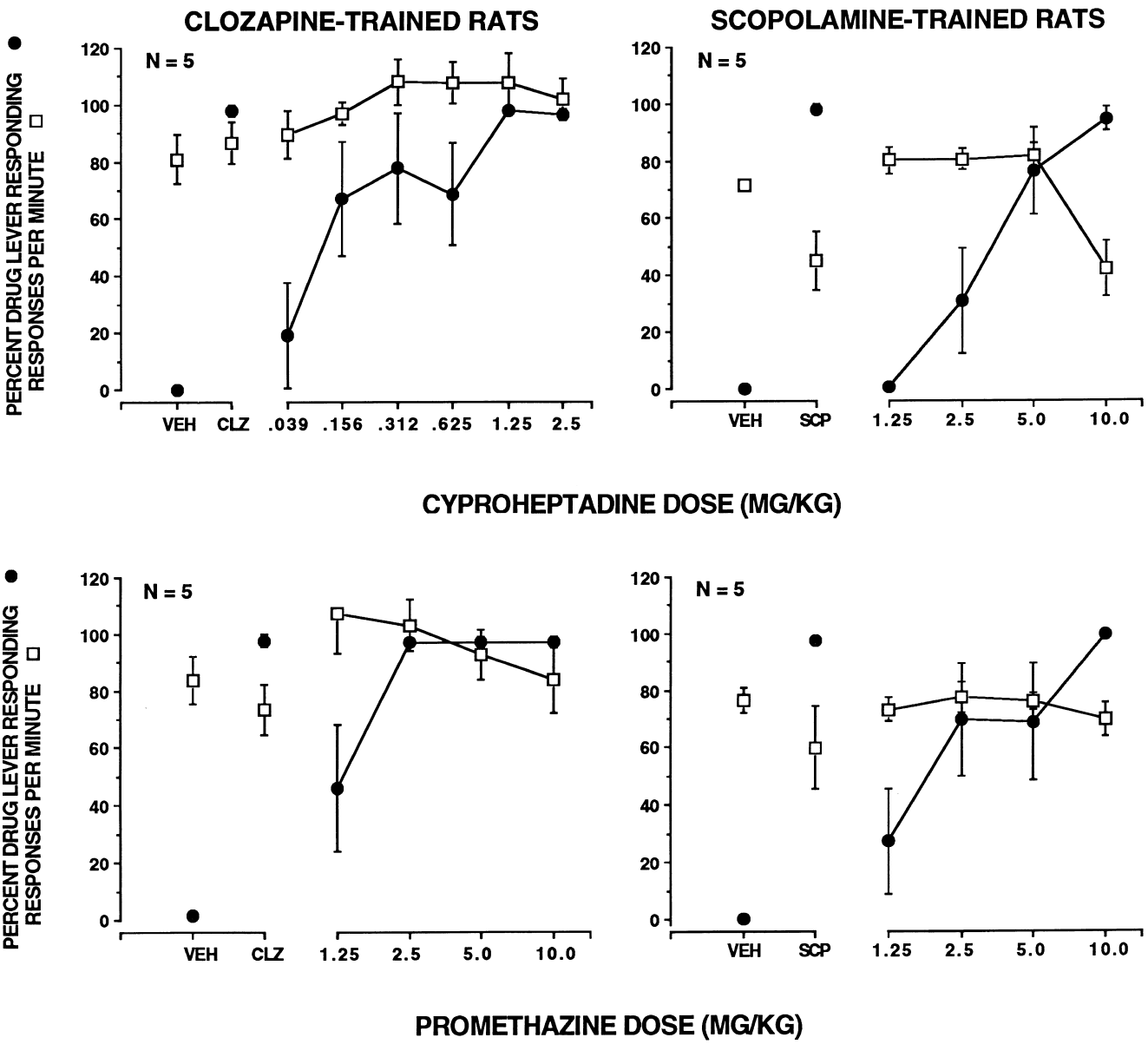

FIG. 4. Results of substitution testing with cyproheptadine (top two panels) and promethazine (bottom two panels) are shown for the CLZ-trained rats (left panels) and for the SCP-trained rats (right panels). Other details are the same as in Fig. 1.

cholinergic (ACh-M) receptors and for the serotonin 5-HT_{2A} and 5-HT_{2C} receptors.

Chlordiazepoxide produced partial substitution for SCP at the 10.0 mg/kg dose with three rats displaying greater than 80% DLR (93.2% DLR); however response rates were significantly suppressed to 45.2% of vehicle response rates at that dose ($F[4, 16] = 3.73, p < 0.05$). Three rats also displayed greater than 80% DLR (93.1% DLR) for CLZ (only one of these also substituted for SCP). There were no significant differences in response rates for the CLZ-trained rats ($F[4, 16] = 1.078, p > 0.05$).

Thioridazine produced greater than 80% DLR (97.3% DLR) in three of the CLZ-trained rats at the 20 mg/kg dose; however, response rates were significantly suppressed from VEH response rates ($F[6, 30] = 7.389, p < 0.0001$) at both the 10.0 and 20.0 mg/kg doses. In the SCP-trained rats four rats displayed complete substitution (97% DLR) at the 5.0 mg/kg dose, and one rat displayed partial substitution (73% DLR). One other rat displayed complete substitution at the three lower doses (99.6% DLR), but not at the 20.0 mg/kg dose (0% DLR). Analysis of response rates revealed a significant reduction ($F[5, 35] = 4.143, p < 0.005$) for the 10.0 and 20.0 mg/kg doses as compared to the vehicle control point.

The other compounds that failed to reliably substitute for clozapine included metergoline, NMDA, phentolamine, and propranolol. Propranolol produced 54.0% DLR at the 25 mg/

TABLE 1

THE HIGHEST MEAN %DLR PRODUCED BY EACH DRUG AND ED_{50} VALUES (FOR DRUGS PRODUCING >80% DLR) ARE SHOWN FOR THE CLOZAPINE-TRAINED RATS AND THE SCOPOLAMINE-TRAINED RATS. THE BINDING AFFINITIES FOR ACh-M RECEPTORS ARE EXPRESSED AS K_D OR K_i VALUES (nM), AND BINDING AFFINITIES FOR 5-HT_{2A} AND 5-HT_{2C} RECEPTORS ARE EXPRESSED AS pK_i OR pK_D VALUES

	CLZ Group		SCP Group		Binding Affinities		
Drug	%DLR	ED_{50} (mg/kg)	%DLR	ED_{50} (mg/kg)	ACh-M (K_D or K_i)	5-HT_{2A} (pK_D or pK_i)	5-HT_{2C} (pK_D or pK_i)
Drugs producing >80% drug-appropriate responding							
Amitriptyline	98.3	1.50	99.1	1.78	6.9[A] 34.3[B] 12.0[D]	8.2[C]	7.7[C]
Clozapine (1)	99.9	0.36	86.3	0.12	3.1 (m1)[E]	7.6[F]	8.1[F]
Clozapine (2)	97.0	0.13	NT	—	48.0 (m2)[E]		
Cyproheptadine	98.2	0.11	94.7	3.13	19.0[G]	8.5[C]	7.9[C]
Mianserin	83.3	1.50	26.0	ND	Inactive[G] 469.0[B]	8.4[C]	8.9[C]
Promethazine	97.0	1.11	99.3	2.10	13.0[H]	ND[H]	ND[H]
Scopolamine (1)	84.4	3.09	99.8	0.04	1.1 (m1)[E] 2.0 (m2)[E]	Inactive	Inactive
Scopolamine (2)	NT	—	99.3	0.03			
Trihexyphenidyl	83.9	0.96	100.0	0.81	1.6 (m1)[E] 7.0 (m2)[E]	Inactive	Inactive
Drugs producing >60% and <80% drug-appropriate responding							
Chloriazepoxide	56.6	ND	69.9	ND	Inactive[I]	Inactive	Inactive
Imipramine	73.5	ND	63.0	ND	182.0[B]	7.2[C]	7.0[C]
Drugs producing <60% drug-appropriate responding							
BIBN 99	18.5	ND	19.3	ND	0.32 (m2)[J] 9.4 (m1)[J]	Inactive	Inactive
Metergoline	43.6	ND	NT	—	Inactive[G]	7.6[C]	8.6[C]
NMDA (1)	49.8	ND	NT	—	Inactive[K]	Inactive	Inactive
NMDA (2)	16.8	ND	NT	—			
Phentolamine	24.3	ND	NT	—	Inactive[L]	6.1[M]	6.1[M]
Propranolol	54.0	ND	NT	—	Inactive[L]	5.7[M]	6.8[M]
Thioridazine	58.4	ND	58.3	ND	190.0[H] 18.0[D] 2.7 (m1)[E] 14.0 (m2)[E]		

These values should be used for general comparisons only since the conditions, tissues and assays varied among the studies.

ND = Not Determined; NT = Not Tested; %DLR = Percent Drug Lever Responding; K_D = dissociation equilibrium constant; K_i = equilibrium dissociation constant of the competitive inhibitor; $pK_i = -\log K_i$; $pK_D = -\log$ mol/L. There were 2 dose determinations for clozapine, scopolamine and NMDA.

[A]42, K_D (nM), rat cortex; [B]22, K_D (nM), rat brain; [C]32, pK_i, rat frontal cortex (5-HT_{2A}), pig choroid plexus (5-HT_{2C}); [D]54, K_D (nM), human brain caudate; [E]5, K_D (nM), cloned human receptors; [F]8, pK_i, rat frontal cortex (5-HT_{2A}), pig choroid plexus (5-HT_{2C}); [G]39, K_i (nM), rat striatum; [H]62, K_i (nM), rat cortex; [I]24, 12, selective for benzodiazepine receptors on the $GABA_A$ receptor complex; [J]16, pK_i, rabbit vas deferens; [K]70, selective for NMDA glutamate receptors; [L]15, does not inhibit [^{3}H] QNB binding; [M]27, pK_D, pig cortex (5-HT_{2A}), pig choroid plexus (5-HT_{2C}).

kg dose, but that represented only two rats producing greater than 80% DLR. Response rates were suppressed to 25.0% of vehicle response rates at that dose. During the first dose determination with NMDA, three rats produced greater than 80% DLR at the 30.0 mg/kg dose, but when a second dose determination was conducted none of the rats substituted at that dose. There was only one rat in the phentolamine dose determination that produced greater than 80% DLR (at the 3.0 mg/kg dose). None of these compounds were tested in the SCP-trained rats.

DISCUSSION

The present study confirms previous reports (7,21,25,50, 68,72,73) that the atypical antipsychotic CLZ exerts strong discriminative stimulus effects in a two-lever operant task.

One of the difficulties in determining the neurochemical mechanisms responsible for CLZ's discriminative stimulus properties (and its antipsychotic effects) is that CLZ interacts with a large number of different neurotransmitter systems, including dopamine D_1 and D_2 (13,46,48), D_3 (60), D_4 (67), and D_5 (61), cholinergic muscarinic (54), including m1, m2, m3, m4, and m5 subtypes (4), serotonin 5-HT_{2A} (8,46,55), 5-HT_{2C} (8,55), 5-HT_3 (71), and 5-HT_6 and 5-HT_7 (56), adrenergic alpha 1 and alpha 2 (29,54), and histamine H1 (10,54) receptors.

The results of the present study clearly demonstrated that blockade of cholinergic receptors plays an important role in CLZ's discriminative stimulus effects in rats. In particular, antagonism of muscarinic M1 receptors appears to be sufficient for eliciting CLZ-appropriate responding. Cross-generalization between CLZ and SCP was shown in that SCP elicited CLZ-appropriate responding in CLZ-trained rats and CLZ elicited SCP-appropriate responding in SCP-trained rats. Such cross-generalization is generally considered to be unique to drugs that share a common mechanism for their discriminative stimulus effects (see 58). Also, the discriminative stimulus properties of SCP are less complicated than CLZ's and are mediated by central muscarinic receptors (33,34,52,66). Although SCP displays strong affinities for each of the muscarinic receptor subtypes (5), antagonism of the M1 receptor appears to be the mechanism mediating SCP's discriminative stimulus properties as both Jung et al. (34) and the present study (see Fig. 2) demonstrated that trihexyphenidyl generates SCP-appropriate responding in rats trained to discriminate SCP from saline in two-lever discrimination tests. Neither SCP or trihexyphenidyl display any binding affinity to 5-HT_{2A} or 5-HT_{2C} receptors (see Table 1). The highly selective (30 fold difference in M2/M1 binding affinity) M2 antagonist BIBN 99 (16) did not substitute for either SCP or for CLZ at the tested doses. Trihexyphenidyl has four times greater affinity for m1 receptors relative to m2 (5) and SCP has a higher affinity for M1 receptors than for M2 receptors (20) and a higher affinity for m1 than for m2, m3, m4, and m5 receptor subtypes (5). In addition, CLZ's strongest affinity for muscarinic receptors is at the m1 receptor site (5), and trihexyphenidyl substituted completely for CLZ in the present study. Finally, nicotinic cholinergic receptors do not appear to play a role in CLZ's discriminative stimulus properties as Villanueva et al. (69) reported that nicotine does not substitute for CLZ in CLZ-trained rats and that CLZ does not substitute for nicotine in nicotine-trained rats. Also, they found no interaction between CLZ and nicotine when various doses of CLZ and nicotine were combined, although Brioni et al. (6) recently reported that CLZ produced a significant attenuation of the nicotine cue.

Mianserin was the only drug tested in the present study that substituted for CLZ but failed to produce reliable SCP-appropriate responding. Mianserin's asymmetrical substitution with the two training drugs in the present study is interesting, since there was complete cross-generalization between CLZ and SCP. While miaserin failed to generate SCP-appropriate responding in the present study, we (36) recently reported that SCP does produce mianserin-appropriate responding in rats trained to discriminate mianserin (4.0 mg/kg) from saline (again, mianserin did not produce SCP-appropriate responding; see Barry and Krimmer [2] for discussion of asymmetrical generalization between drugs).

All of the compounds that produced full substitution for CLZ (amitriptyline, CLZ, cyproheptadine, promethazine, SCP, and trihexyphenidyl) display nanomolar affinity for muscarinic receptors (see Table 1), with the exception of mianserin which has a K_D of 469.0 nM (22). Because of the tremendous variability between studies in terms of the species, tissues, and assays reported in Table 1, a correlational analysis of muscarinic and/or serotonergic binding affinities with ED_{50}'s and %DLR for the drugs tested in the present study really is not appropriate; however, a visual inspection of trends can be made. For example, imipramine and thioridazine displayed partial (but symmetrical) substitution for both CLZ and SCP, and have more moderate binding affinities for muscarinic receptors than the drugs that fully substituted for CLZ (with the exception of mianserin). The interaction of many antidepressants with muscarinic receptors as antagonists also has been shown in functional assays. For example, both amitriptyline and imipramine block oxotremorine (muscarinic agonist) induced hypothermia and tremors (53). Also, it has been shown that the rate-suppressing effects of oxotremorine on operant responding (both fixed ratio and fixed interval schedules) are reversed by scopolamine, atropine, and amitriptyline (38) and by thioridazine and clozapine (38). The compounds in the present study that failed to produce reliable CLZ-appropriate responding (see Table 1) either have no affinity for muscarinic receptors (i.e., chlordiazepoxide, metergoline, NMDA, phentolamine, propranolol) or display only minimal affinity for muscarinic receptors (i.e., imipramine).

Hoenicke et al. (25) have suggested that blockade of both 5-HT_{2A} and 5-HT_{2C} receptors mediates the discriminative stimulus effects of CLZ in pigeons. They argued that the drugs which produced full substitution for CLZ (cyproheptadine, metergoline, mianserin, pizotifen, and fluperlapine) block both 5-HT_{2A} and 5-HT_{2C} receptors (27,46,55); whereas, the drugs that failed to generate CLZ-appropriate responding in their study either have minimal or no serotonergic antagonism or they are selective for 5-HT_{2A} vs. 5-HT_{2C} receptors (see 25). If blockade of both 5-HT_{2A} and 5-HT_{2C} receptors is the necessary pharmacological mechanism responsible for CLZ's discriminative stimulus effects, then both mianserin and metergoline should have substituted for CLZ in the present study; however, only mianserin produced CLZ-appropriate responding in rats. Interestingly, metergoline demonstrates no significant affinity for muscarinic receptors; whereas, mianserin does display some (albeit weak) affinity for muscarinic receptors (see Table 1). This suggests the possibility that blockade of 5-HT_{2A} and 5-HT_{2C} receptors alone is not sufficient in rats to generate CLZ-appropriate responding, but that some blockade of muscarinic receptors must also occur. This suggestion is also supported by the finding that ritanserin, a selective 5-HT_{2A} and 5-HT_{2C} antagonist (27), does not reliably substitute for CLZ in rats (72,73). Hoenicke et al. (25) suggested that promethazine may have a putative 5-HT_{2C} antagonist action in pigeons because it produced CLZ-appropriate responding; however, promethazine's cholinergic binding properties may be important, as promethazine displays a strong affinity (K_i = 13 nM) for muscarinic receptors (62). Also, the lack of clozapine-appropriate responding produced by the selective serotonin antagonist ritanserin (72,73) or by metergoline (the present study) cannot be attributed to a lack of tolerance to CLZ's anticholinergic effects because the rats in the CLZ discrimination group received two to three injections of CLZ every week over a period of many months. Examination of the rates of responding for the control tests with vehicle and CLZ (see figures) reveals no significant differences. This indicates that the rats had developed tolerance to any rate suppressant effects of CLZ that may have been present initially.

The finding that the anxiolytic chlordiazepoxide produced partial substitution for both SCP and CLZ in the present study

is interesting since chlordiazepoxide is a selective agonist for the benzodiazepine site on the $GABA_A$ receptor complex and is inactive at both muscarinic and serotonergic receptors (12,24). However, it has been shown that benzodiazepines produce reductions of 5-HT turnover in cortical areas, although the exact mechanism for these effects is not clear (40). Also, it has been shown that the benzodiazepine antagonist flumazenil increases levels of acetylcholine in the hippocampus; whereas, the benzodiazepine agonist diazepam decreases acetylcholine levels (30).

In addition to muscarinic and serotonergic receptors, a number of other neurotransmitter receptors have been studied, but none appear to be viable candidates as pharmacological mediators of CLZ's discrminative stimulus effects. Antagonism of 5-HT_3 receptors with MDL 72222 in rats (72,73) and ondansetron (GR38032F) in pigeons (25) does not generate CLZ-appropriate responding. Also, stimulation of serotonergic receptors does not appear to produce CLZ-appropriate responding in either rats or pigeons. The 5-HT_{1A} agonist 8-OH-DPAT does not substitute for CLZ in pigeons (25) and buspirone (also a 5-HT_{1A} agonist) does not substitute for CLZ in rats (72,73).

Blockade of dopamine receptors also does not appear to be sufficient to produce CLZ-like responding in either rats or pigeons. The dopamine D_1 antagonist SCH 23390 does not substitute for CLZ in rats (50,68) or pigeons (25). Likewise, the D_2 antagonist haloperidol fails to produce CLZ-appropriate responding in rats (7,68,72,73), and the D_2 antagonist sulpiride does not substitute for CLZ in either rats (51) or pigeons (25). The precise role of dopamine D_4 receptors remains to be determined since CLZ is the only neuroleptic that displays a high affinity for these receptors (67), and there are no selective D_4 antagonists available for testing. A similar problem exists for dopamine D_3 (60) and D_5 (61) receptors.

The present study found that phentolamine (alpha noradrenergic antagonist) and propranolol (beta noradrenergic antagonist) did not substitute for CLZ in rats, and previous studies have reported that prazosin, a selective alpha-1 antagonist (43), does not substitute for CLZ in rats (50) or pigeons (25). Thus, blockade of adrenergic alpha and beta receptors does not appear to produce CLZ-appropriate responding in either rats or pigeons. Likewise, NMDA failed to substitute for CLZ in the present study suggesting that stimulation of glutamate NMDA receptors is not sufficient to produce CLZ-appropriate responding in rats. This result is in contrast to a report by Schmidt (57) that NMDA (7.5, 15.0, and 30.0 mg/kg, SC) substituted in rats trained to discriminate CLZ from saline in a T-maze. While there are obvious differences between the T-maze and two-lever discrimination procedures, it is not clear why NMDA produced CLZ-appropriate responding in the Schmidt study and not in the present study, especially since there is no evidence to indicate that CLZ interacts directly with NMDA receptors.

The precise pharmacological or neurochemical mechanism(s) responsible for CLZ's antipsychotic effects remain unclear, and current theories about schizophrenia and the clinical effects of neuroleptic drugs have suggested that serotonergic (3,31,44,46) or cholinergic (59,64) systems may play an important role in the neuropathology of schizophrenia and may prove to be the underlying neural mechanisms responsible for CLZ's unique profile as an atypical neuroleptic (1,18). Some clinical evidence for the importance of cholinergic and serotonergic antagonist properties of antipsychotic drugs has already been shown. For example, the M1 antagonist trihexyphenidyl demonstrates efficacy in alleviating negative symptoms in certain populations of schizophrenics (64,65). Similarly, the selective 5-HT_{2A}/5-HT_{2C} antagonist ritanserin has been shown to produce a decrease in the negative symptoms of schizophrenics who were being treated with typical neuroleptics (17). If either or both of these theories prove to be correct, having established animal models (like drug discrimination procedures) based on these pharmacological mechanisms (i.e. cholinergic and serotonergic) will prove to be very important for the identification of other putative atypical antipsychotic drugs (e.g., olanzapine; see 47).

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