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ORIGINAL INVESTIGATION

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NNC 19-1228 and NNC 22-0031, novel neuroleptics with a ''mesolimbic-selective'' behavioral profile

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Abstract NNC 19-1228 [1-(3(6-benzothiazolylcarbamoyloxy)propyl)-4-(6-flouro-1,2-benzisoxazol-3-yl)piperidine] and NNC 22-0031 [4-(6-flouro-1,2-benzisoxazol-3-yl)-1-(3-(3,4-methylenedioxyphenylcarbamoyloxy)propyl)piperidine] are newly developed compounds with an in vitro pharmacologic profile similar to that of clozapine, i.e., mixed dopamine (DA), 5-hydroxytryptamine (5-HT)$_2$ and α_1-adrenergic antagonist action. In pharmacological experiments in mice, the compounds inhibited DA D_2 receptor binding in vivo at doses that produced only moderate antagonism of methylphenidate (MPD)-induced stereotyped gnawing. However, the compounds were markedly more potent in blocking MPD-induced motility, a model which showed a high degree of sensitivity to α_1-adrenergic antagonism, but not 5-HT$_2$ antagonism. In rats, the NNC-compounds blocked conditioned avoidance responding and attenuated the discriminative stimulus effects of amphetamine, but failed to induce catalepsy. These results are discussed in terms of adrenergic, serotonergic and dopaminergic interactions which suggest that the NNC compounds may act as DA antagonists with mesolimbic selectivity, and thus may have efficacy as antipsychotics without coincident extrapyramidal side effects.

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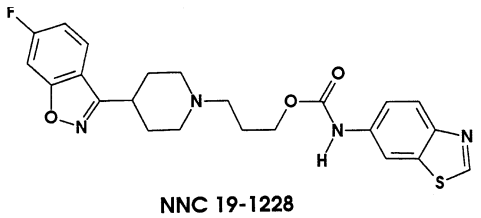
Introduction

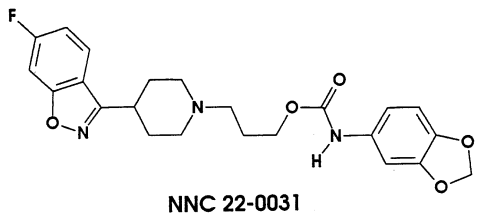
Neuroleptic drugs share the ability to block dopamine (DA) receptors in the brain (Seeman 1980). Most neuroleptics bind primarily to receptors of the D_2 type, although some neuroleptics also bind to DA D_1 receptors (e.g., chlorpromazine, clozapine, fluphenazine and flupenthixol; Hyttel et al. 1991). Dopamine D_1, D_2 are here used for pharmacologically defined DA receptors, while D1–D5 are used for the molecular DA receptor subtypes. In addition to blocking DA receptors, many neuroleptics also inhibit a variety of other neurotransmitter receptors (e.g., α-adrenergic, serotonergic, cholinergic and histaminergic; Richelson 1988). These additional actions may differentially give rise to various central and peripheral side effects (e.g., cardiovascular), and thus underlie the clinical profile of the neuroleptic in question. Neuroleptics further differ in their ability to counteract the ''negative'' and ''positive'' symptoms of psychosis (Meltzer et al. 1986) and induce sedation (Davis et al. 1983). Clozapine is generally considered as a compound with a very favorable profile, i.e., clozapine has low EPS (extrapyramidal side effect) potential and is also active in patients that are otherwise treatment resistant (Meltzer 1992a). Many efforts have been devoted recently towards the discovery of new compounds with a clozapine-like clinical profile, since agranulocytosis, which occurs in 1–2% of patients treated with clozapine, restricts the clinical utility of the drug (ibid). At a neurochemical level, these new ''atypical'' or ''limbic-selective'' neuroleptics incorporate some of the non-dopaminergic actions of clozapine; notably, these include 5-hydroxytryptamine (5-HT), α_1-adrenergic and to some extent muscarinic cholinergic antagonism.

In the present study, we have compared the neurochemical and pharmacological profiles of NNC 19-1228 [1-(3-(6-benzothiazolylcarbamoyloxy)propyl)-4-(6-flouro-1,2-benzisoxazol-3-yl)piperidine] and NNC 22-0031 [4-(6-flouro-1,2-benzisoxazol-3-yl)-1-(3-(3,4-methylenedioxyphenylcarbamoyloxy)-propyl)piperidine] with that of clozapine and several other reference neuroleptics (for structures of the NNC-compounds, see Fig. 1). The pharmacological models used to evaluate the action of these compounds included methylphenidate (MPD)-induced hypermotility and stereotyped gnawing behavior in the mouse. These two models were used to evaluate "striatal" and "limbic" DA antagonist action for a series of classical and newer putative "atypical" neuroleptics, respectively. The action of these compounds in the mouse models was then compared with their ability to inhibit the in vivo binding of [^{3}H]raclopride, a D_2 receptor antagonist, and [^{3}H]ketanserin, a 5-HT_2 antagonist. Furthermore, the MPD-induced gnawing and motility models were characterized with respect to the involvement of 5-HT_2 and α_1-adrenergic modulation. These neurotransmitter systems have been implicated in "atypical" neuroleptic action (e.g., in relation to the clinical profile of clozapine; Baldessarini et al. 1992; Meltzer 1992b) and were therefore of particular interest.

This in vivo profile of the NNC compounds was extended by studying amphetamine discrimination, conditioned avoidance behavior and catalepsy induction in the rat. Amphetamine discrimination has previously been shown to depend on the activation of mesolimbic DA systems (Nielsen and Jepsen 1985; Nielsen and Scheel-Krüger 1986), while conditioned avoidance behavior is used to detect the antipsychotic properties of compounds (e.g., clozapine) which are not readily active in other DA antagonist models (Arnt 1982). Finally, catalepsy was chosen as a classical rat model for EPS potential in the clinic (Sanberg 1980; Arnt et al. 1981, 1986; Morelli et al. 1981; Morelli and Di Chiara 1985). The in vivo results were compared with data obtained in a panel of radioligand binding assays selective for various families of receptors, and for molecular receptor subtypes within the DA family.



NNC 19-1228



NNC 22-0031

Fig. 1 Structures of NNC 19-1228 and NNC 22-0031

Materials and methods

Animals

Male NMRI mice (20 ± 2 g) and male Wistar rats (150 ± 10 g) were purchased from Moellegaard's Breeding Labs, LI. Skensved, Denmark. They were housed in group cages (mice: 20/cage; rats: 4/cage) placed in separate rooms at constant temperature (20 ± 1°C) and relative humidity (50 ± 10%). The animals had access to food (standard lab chow) and tap water ad libitum (except for the rats used in the drug discrimination experiments, below).

In vitro radioligand binding

Specific DA-D_1, DA-D_2 and 5-HT_2 binding was assessed as previously described by Andersen et al. (1992). Briefly, membranes prepared from rat striatal tissue were incubated with 0.1 nm ^{3}H-SCH 23390 [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine] (D_1), 0.3 nM ^{3}H-spiroperidol (D_2) or 0.4 nm ^{3}H-ketanserin (5-HT_2) for 60 min at 30°C. Free and bound ligand was separated by rapid filtration of samples through Whatman GF/B filters, and quantitated by liquid scintillation counting. Non-specific binding was assessed by measuring binding in the presence of an excess of *cis*-flupenthixol (D_1, D_2) or methysergide (5-HT_2). Specific α_1-adrenergic binding was similarly assessed by incubating rat forebrain membrane preparations with 0.5 nm ^{3}H-prazosin. Non-specific binding was determined in the presence of excess phentolamine. The results are shown as IC_{50} values of experiments performed in triplicate.

Binding to the following receptors and uptake sites were also assessed according to methods described by Suzdak et al. (1992): α- and β-adrenergic; muscarinic; 5-$HT1_A$; histamine H1, H2, H3; opiate μ and κ; strychnine-sensitive and insensitive glycine; glutamate; [AMPA, kainic and NMDA]; GABA-A; BZ (benzodiazepine); Cl-channels; GABA, DA, NE (norepinephrine), 5-HT and choline uptake sites.

Radioligand binding to the following sites was also assessed: batrachotoxinin (BTX, using ^{3}H-BTX according to Rock et al. 1991); 5-HT_{2c} [using ^{3}H-mesulergine according to Burris et al. (1991)]; adenosine A1 and A2 [using ^{3}H-PIA and ^{3}H-CGS 21680 according to Murphy and Snyder (1986) and Bruns et al. (1986), respectively]; calcium channels [using ^{3}H-nitrendipine according to Goldman and Pisano (1985)].

Finally, the ability of the NNC compounds to interact with DA receptor subtypes expressed in clonal cell lines were also assessed (below).

In vitro inhibition of radioligand binding to DA receptor subtypes

Clonal cell lines expressing the human cDNA of the dopamine-$D2_s$, -D3 and -D4.2 receptor subtypes were used as a source of receptor for radioligand binding assays. The human DA-$D2_s$ receptor stably expressed in Ltk^- cells (as described by Bunzow et al. 1988) was obtained from Dr. D.K. Grandy (Vollum Institute). The human D3 receptor was re-cloned by rt-PCR from HEK293 cells, introduced into the mammalian expression vector Zem219B and stably transfected into BHK-21tk^- cells using the lipofectin method (BRL). Cell membranes were prepared from both $D2_s$/Ltk^- and

D3/BHK-21tk$^-$ cells by hypotonic lysis (as described by Scheideler and Zukin 1990). Cell membranes prepared from CHO cells expressing the DA-D4.2 receptor were provided by Receptor Biology (Baltimore, Md., USA). The affinity of a test substance for a DA receptor subtype was determined by measuring its ability to compete in vitro for radioligand binding at receptor sites expressed on these cell membrane preparations. Radioligands were employed in each assay as follows: [^{3}H]spiperone (18.5 Ci/mmol), 0.3 nM final concentration ($D2_s$, D4.2); [^{3}H]R(+)7-OH-DPAT (146 Ci/mmol), 0.1 nM final concentration (D3). All assays were performed at 25°C in assay buffer consisting of 20 mM Hepes, pH 7.4, containing 2 mM $MgCl_2$. To initiate the assay, membranes, test substance and radioligand were mixed and incubated for 45 min. Samples are then passed through Whatman GF/B filters under vacuum and washed twice with 4 ml ice-cold assay buffer containing 0.1 M NaCl. Filters were placed in counting vials, 4 ml Ultima Gold (Packard) added and the radioactivity determined by liquid scintillation counting. Specific binding was defined as the total binding in controls minus the non-specific binding. Non-specific binding is assessed by determining the radioactivity present after including 3 µM *d*-butaclamol ($D2_s$, D4.2) or 5 µM quinpirole (D3) in place of test substance. The control specific binding was typically >90% of the total binding. The test value is given as K_i (the dissociation constant (nM) of the receptor-inhibitor complex) from experiments performed in triplicate. This value is calculated from the Cheng-Prusoff equation: $K_i = IC_{50}/(1+C/K_D)$, where C is the radioligand concentration and K_D the radioligand dissociation constant (calculated by Scatchard plot analysis of independent data from saturation binding experiments).

In vivo radioligand binding in mice

Groups of three mice were administered test substance PO and challenged 30 min later by IV administration of 8 µCi/mouse of ^{3}H-raclopride (DA-D_2), or 4 µCi/mouse of ^{3}H-ketanserin (5-HT_2). The mice were killed after 30 min, the striatum rapidly dissected and homogenized in ice-cold buffered saline (50 mM TRIS-HCl, pH 7.4, containing 120 mM NaCl and 4 mM $MgCl_2$). Aliquots of the homogenate were then passed through Whatman GF/C filters, washed in buffered saline and radioactivity present on the filters quantitated by liquid scintillation counting. In order to determine the amount of non-specific D_2 binding, 3 mg/kg *cis*-flupenthixol was administered IV 50 min prior to injecting ^{3}H-raclopride. In order to determine the amount of non-specific 5-HT_2 binding 100 mg/kg methysergide was administered SC 50 min prior to injecting ^{3}H-ketanserin. The ED_{50} for a test substance was defined as the dose which inhibits 50% of the specific radioligand binding.

MPD-induced stereotyped gnawing

The method previously described by Pederson and Christensen (1972) was used. Briefly, immediately upon injection of MPD (maximum dose: 60 mg/kg SC) mice were placed in pairs in chambers which rested on corrugated paper. The stereotyped gnawing of the paper was evaluated (absent or present) after 1 h in each case (five cages/dose of test compound). At least ten bite marks was required for each positive score in a test cage. Test drugs were injected at a predetermined time prior to MPD. Three to five doses of each test drug were then administered (ten mice in five pairs per dose of test drug).

MPD-induced motility

Mice were habituated for 120 min to an acrylic glass chamber (width, length, height: 29 × 29 × 38 cm); this was placed within a frame of photocells (4 × 4) located 1 cm above the floor. A computer located in an adjacent room recorded each photocell interruption. The photocell chambers were placed in a dimly lit sound-isolated and fan-ventilated chest (four cages/chest) which also provided masking noise. Following the habituation period (above), MPD (30 mg/kg) was injected SC and the locomotor behavior recorded for the following 2 h. Test drugs were injected at a predetermined interval prior to MPD. Three to five doses of test drug were administered. Each dose was administered to four mice; these four mice were placed in the same chamber (above). The data are expressed as percent inhibition of control MPD values (saline pretreatment, 30 mg/kg MPD). In some (interaction) experiments (below), shifts in the dose response of MPD were used.

Amphetamine discrimination

Subjects

Sixteen male Wistar rats (Møllegård, Ry, Denmark) weighing approximately 250 g at the beginning of the experiment were used. The rats were housed in pairs in a colony room with water available at all times. Lighting was provided between 0600 and 1800 hours. The animals were kept at approximately 80% of free feeding weight by restricting their access to food.

Apparatus

Eight operant chambers equipped with two response levers, cue lights, a house light and a food magazine were used (Coulbourn Instruments, Lehigh Valley, Pa.). The food pellets used were 45 mg Dustless Precision Pellets (Bio-Serv, Frenchtown, N.J., USA). Experiments were run and data recorded by a PC by procedures programmed using MedPC software (Med Associates, East Fairfield, Vt., USA).

Discrimination training

A Drug Lever and a No Drug Lever was assigned to each rat in a balanced fashion. Prior to trials, animals received an injection of *d*-amphetamine (drug) or no injection (no drug). In order to produce a food pellet, the animals were required to press the lever appropriate to the pretreatment (drug or no drug). Animals were run on a single alternation schedule with an increase in the fixed ratio (FR) response requirement every other day until an FR10 was reached. Presses on the incorrect lever had no other programmed consequences than to reset the FR value on the correct lever. When reaching an FR10, animals were switched to a double alternation schedule in which pairs of two consecutive drug sessions and two consecutive no drug sessions alternated. A session lasted until 50 food pellets had been earned, or until 15 min had elapsed, whichever occurred first. Before entering the test phase, rats were required to meet a set of criteria (Swedberg et al. 1988). Briefly, each rat had to reach a level of 90% correct responding for eight consecutive sessions with no more than nine responses on the incorrect lever prior to the first reinforcement. The two sessions following immediately after the eighth criterion session were acquisition test sessions in which the training conditions (Drug or No Drug) were tested.

Drug discrimination testing

In the testing phase, animals were run according to a single alternation schedule and test sessions were interspersed between the training sessions. Test sessions were identical to training sessions,

with the single exception that both levers were now ''correct'' such that ten consecutive presses on any of the levers would produce a food pellet.

Test sessions were typically run on Tuesdays and Fridays, provided that the animals performed according to the criterion on the training days. If training day performance fell below criterion for any rat on a single training day, the upcoming test was postponed for that rat and it was tested again only after completing two consecutive training sessions during which criterion was met. Test drugs were injected SC 30 min (haloperidol, clozapine) or IP 120 min (NNC compounds).

Data analysis

Discrimination results are expressed as the mean of the individual percentages of correct responding during drug and no drug sessions, respectively. Rates of responding is a measure independent of the discriminative effects and reflects the rate at which the rats responds on a lever irrespective of whether the drug or no drug lever was chosen. Rates of responding are expressed as the mean number of responses per second.

Conditioned Avoidance Responding (CAR)

Rats were trained to perform a shuttle response in Coulbourn Instruments two-way shuttle boxes (model E99-36) in order to avoid an electric shock (unconditioned stimulus; UCS) (1 mA) through the grid floor. The shock was signalled by an 82 dB tone (conditioned stimulus; CS) from a ''Sonalert'' tone generator/speaker mounted in the wall of the experimental chamber. The specific experimental conditions were as follows: intertrial interval: 25 s; CS-UCS interval 10 s; maximum UCS duration: 1 min; sessions were terminated after 45 min or the completion of 40 trials, whichever come first. Avoidance responses were reinforced with 10 s of shock-free time added to the next intertrial interval. The control of experimental events as well as the collection of response data were accomplished by a PC located in an adjacent room.

Induction of catalepsy

The method was similar to that described by Morelli and Di Chiara (1985). Briefly, rats were injected with the test compound and placed individually on an inclined (70°) wire-mesh screen (0.8 mm steel wire, 7 mm mesh). The extremities of the animals were gently abducted. The latency to move any extremity was used to define the intensity of catalepsy according to the following scale from 0 to 3: (0) latency <15 s; (1) 15–29 s; (2) 30–59 s; (3) >60 s.

Following injection of the test compound, the animals underwent the catalepsy test following 5, 15, 30, 90 and 120 min. This procedure of multiple testing was used, since it increases the likelihood of observing neuroleptic catalepsy (Klemm 1985).

Drugs

The following drugs were dissolved in water and injected in a volume of 10 ml/kg (mice) or 1 ml/kg (rats) with exceptions as noted (below).

d-Amphetamine sulphate (Bie & Berntsen, Copenhagen, Denmark); chlorpromazine hydrochloride (Sigma, St Louis, Mo., USA); cinanserin hydrochloride (Bristol-Myers Squibb, Princeton, N.J. USA) clebopride maleinate (Allmiral Laboratories, Barcelona, Spain); clozapine (free base) (Sandoz Pharma A/G, Basel, Switzerland), in dilute HCl, made up to volume with water or suspended in 5% duphasol-x (drug discrimination studies); flupenthixol, *cis*(Z), dihydrochloride (H. Lundbeck A/S, Copenhagen, Denmark); haloperidol (free base) (Janssen Pharmaceutica, Beerse, Belgium), in propylene glycol and tartaric acid (1 mol) 1:1, made up to volume with water or suspended in 5% duphasol-x (drug discrimination studies); ketanserin tartrate (Janssen Pharmaceutica); MPD hydrochloride (Ciba-Geigy, Basel, Switzerland); NNC 19-1228 [1-(3-(6-benzothiazolylcarbamoyloxy)-propyl)-4-(6-fluoro-1,2-benzisoxazol-3-yl)piperidine, oxalate; synthesized at Novo Nordisk A/S]; NNC 22-0031 [4-(6-fluoro-1,2-benzisoxazol-3-yl)-1-(3-(3,4-methylenedioxy-phenylcarbamoyloxy)-propyl)piperidine, oxalate; synthesized at Novo Nordisk A/S]; suspended in 5% duphasol-x; prazosin hydrochloride (Pfizer, Brussels, Belgium); risperidone (free base) (Janssen Pharmaceutica), dissolved as per haloperidol (above); ritanserin (free base) (Janssen Pharmaceutica); sertindole (free base, kindly synthesized by Dr. Rolf Hohlweg, Department of Medicinal Chemistry, Novo Nordisk A/S); setoperone (free base) (Janssen Pharmaceutica), dissolved as per haloperidol (above); spiperone (free base) (Janssen Pharmaceutica), dissolved as per haloperidol; *l*-sulpiride (Sigma), in acetic acid, made up to volume with water; tefludazine dihydrochloride (H. Lundbeck A/S); thioridazine hydrochloride (Sandoz Pharma A/G); tiaspirone hydrochloride (Bristol-Myers Squibb, Wallingford, Conn. USA); trifluoperazine dihydrochloride (Rhône-Poulenc Pharma Norden A/S, Denmark). All doses refer to the forms indicated above.

Statistical analysis

The behavioral data were subjected to computer programmed log-probit analysis which yielded ED_{50} values. The gnawing behavior was analyzed in terms of inhibition of the level of gnawing induced by 60 mg/kg MPD (100% gnawing) using as minimum 0% gnawing. The hyperactivity data was analyzed in terms of inhibition of the level of photocell interruptions induced by 30 mg/kg MPD using as minimum the level induced by saline (alone). In the interaction experiments (above), ED_{50} values for MPD were generated using as minimum 0% gnawing and saline-locomotor activity levels, respectively.

The data from the interaction experiments were subjected to covariance analysis using the SAS GLM procedure. Post-hoc analysis was conducted by constructing least square means for the drug versus saline treatments. This analysis yielded a *P*-value for the two treatment means (drug versus saline) being equal.

The drug discrimination accuracy data were subjected to log-probit analysis yielding an ED_{50} value for amphetamine-cue inhibition based on the maximum being 100% and the minimum being 0% amphetamine-lever responses. The accuracy data were further analyzed using a repeated measures analysis of variance. Post-hoc least significant difference intervals were constructed in order to compare treatment effects with control.

The CAR data were also analyzed in terms of inhibitory potency (ED_{50}) of the test drugs in blocking CAR using as maximum 100% effective avoidance behavior (the animals avoiding the shock in all trials) and as minimum 0 (the animals performing only escape behavior). Animals that were affected by the test drug to the extent that they were showing consecutive escape failures were not allowed to complete such sessions but were taken out of the test chamber and the experiment was interrupted on such days. As a measure of non-specific disruption of the conditioned behavior, the ratio of total duration of shock-time divided with the number of escape responses (average escape latency) was calculated. The escape latency in untreated animals was usually below 2 s. The dose increasing the escape latency above 2 s was said (arbitrarily) to define a minimum effective dose (MED) for escape-latency effects. This term, rather than an ED_{50} was used because the calculation of an ED_{50} would require doses of drugs leading to relatively long exposure of the animals to electric shock; this was considered unnecessary for ethical reasons. The 2-s criterion level was chosen since well-trained animals rarely had average escape latencies above this level.

Results

In vitro radioligand binding profile

Similar to haloperidol, NNC 19-1228 and NNC 22-0031 had high affinity and moderate selectivity for D2/D3 receptor subtypes within the DA receptor family (Table 1). Further, both NNC-compounds had high affinities towards 5-HT_2 and α_1-adrenergic receptors (Table 2). This latter profile was also found for a number of reference neuroleptics (ibid). NNC 19-1228 at higher concentrations displaced binding from ^{3}H-DHA-labelled beta-receptors (IC_{50} = 488 nM), ^{3}H-batrachotoxinin-labelled sodium channels (IC_{50} = 786 nM), DA-uptake sites (IC_{50} = 817 nM) and noradrenaline uptake sites (IC_{50} = 682 nM). NNC 22-0031 at higher concentrations displaced binding to ^{3}H-pyrilamine-labelled histamine receptors (IC_{50} = 330 nM) and ^{3}H-mesulergine-labelled 5-HT_{2c} receptors (IC_{50} = 108 nM). Both NNC-compounds failed to bind significantly to a number of other neurotransmitter receptor or uptake sites (IC_{50} > 1000 nM).

Table 1 Dopamine receptor profile NNC 19-1228 and NNC 22-0031. Binding affinities (Ki: nM) to DA receptor subtypes expressed in clonal cell lines. See Methods for other details

Compound	Ki (nM)				
	D1	D2	D3	D4	D5
NNC 19-1228	46	3.4	4	32	49
NNC 22-0031	35	3.9	1.2	66	–
Haloperidol	34	2	4.7	1.4	18

Table 2 Receptor binding profiles of NNC 19-1228 and NNC 22-0031. Binding affinities of NNC 19-1228, NNC 22-0031 and reference neuroleptics to DA, 5-HT_2 and α_1-adrenergic receptors in the rat brain using ^{3}H-SCH23390, ^{3}H-Spiroperidol, ^{3}H-ketanserin and ^{3}H-prazosin, respectively, as radioligands. See Methods for other details

Compound	IC_{50} (nM)			
	D_1	D_2	5-HT_2	α_1
Clozapine	173	134	25	32
Flupenthixol	1.7	0.6	6	2
Haloperidol	127	4	128	50
NNC 19-1228	26	5	0.6	3
NNC 22-0031	15	5	4	4
Sertindole	47	2	0.9	10
Spiperone	741	0.2	3	20
Sulpiride (−)	>3000	73	3000	>3000
Tefludazine	64	6	4.5	9
Thioridazine	34	16	41	4
Tiaspirone	128	1.4	3	6
Trifluperazine	13	2	43	35

Displacement of in vivo radioligand binding

Table 3 summarizes the ability of clozapine, haloperidol, NNC 19-1228 and NNC 22-0031 to displace radioligand binding from DA D_2 and 5-HT_2 receptors in the mouse brain in vivo. Both NNC-compounds and clozapine were remarkably more effective in inhibiting 5-HT_2 receptor as compared to D_2 receptors. In contrast, haloperidol was equally effective at both sites. In general, the compounds differed only marginally in their ability to displace 5-HT_2 receptor binding, but exhibited large differences in their ability to displace D_2 receptor binding.

Behavioral experiments

MPD-induced gnawing behavior

In antagonism experiments, most neuroleptics blocked the gnawing behavior (Table 4), although (−)-sulpiride was inactive in oral doses up to 300 mg/kg. Clozapine had no MPD-antagonistic effect in doses up to 100 mg/kg. Doses of 150 mg/kg and higher produced mortality and severe ataxia although the gnawing behavior was inhibited at these higher doses, yielding an apparent ED_{50} of 122 mg/kg. Both NNC compounds were relatively devoid of MPD-antagonistic effect in this model, although doses of 100 mg/kg incompletely attenuated the MPD-induced gnawing to 50–60% (see Fig. 2). For haloperidol, complete D_2 receptor antagonism in vivo coincided with full blockade of MPD-induced gnawing, whereas, for the NNC-compounds, full antagonism of the binding corresponded only to a partial blockade of the MPD-induced gnawing (ibid).

In the interaction experiments, 1 mg/kg prazosin slightly but non-significantly shifted the dose-responses of MPD to the right (see Fig. 3). ED_{50} of MPD was 19 mg/kg, versus 30 mg/kg for prazosin (t = 1.55, P < 0.18). A 0.2 mg/kg dose of the serotonergic antagonist ritanserin also slightly but non-significantly shifted the dose-response of MPD (ibid); from an ED_{50} of 32 mg/kg in saline-pretreated animals to 50 mg/kg in the ritanserin-pretreated mice (t = 1.69, P < 0.15) (ibid). A higher dose of ritanserin (2 mg/kg) significantly shifted the MPD dose-response [saline-pretreated animals: ED_{50} = 20 mg/kg; ritanserin pretreated mice, ED_{50} = 55 mg/kg (t = 6.62, P < 0.0012) (ibid)]. The 5-HT_2 antagonist cinanserin had no effect at 20 mg/kg on the MPD dose-response curve (ibid) while a higher dose (40 mg/kg) produced a marginal shift to the right [ibid; ED_{50}s in saline and cinanserin-pretreated animals were 25 and 35 mg/kg, respectively (t = 1.11, P < 0.3)]. Finally, the ability of ketanserin (10 mg/kg IP) to affect the inhibitory potency of haloperidol against MPD was investigated. Ketanserin did not produce a consistent change in the anti-MPD effect of haloperidol (Fig. 4).

Table 3 In vivo receptor binding experiments. Results from in vivo receptor binding experiments. The results are shown as ED_{50} values in mg/kg following oral administration 60 min prior to decapitation

Ligand	ED_{50} (mg/kg)				
	Receptor	Clozapine	Haloperidol	NNC 19-1228	NNC 22-0031
[^{3}H]Raclopride	D_2	145	0.12	16	7
[^{3}H]Ketanserin	5-HT_2	2.8	0.7	0.9	0.6

Table 4 DA-antagonists: behavioral profile in the mouse. The table shows the ability of NNC 19-1228, NNC 22-0031 and reference neuroleptics to inhibit MPD-induced hyperactivity and MPD-induced gnawing following oral (60 min) pretreatment

Compound PO 60 min	ED_{50} (mg/kg)		Ratio, ED_{50}
	MPD activity	MPD gnawing	Gnawing/activity
Clozapine	40	(122)[a]	(3)
Flupenthixol	0.5	0.9	1.8
Haloperidol	0.3	0.9	3
NNC 19-1228	1.7	94	55
NNC 22-0031	1.7	100[b]	59
Sertindole	3.3	29	8.8
Spiperone	0.3	0.5	1.7
Sulpiride (–)	117	>300	>2.6
Tefludazine	0.39	5.5	14
Thioridazine	13	9.3	0.7
Tiaspirone	40	136	3.4
Trifluperazine	2.9	0.8	0.3

[a] Antagonism associated with toxicity
[b] Incomplete dose-response; see Fig. 2

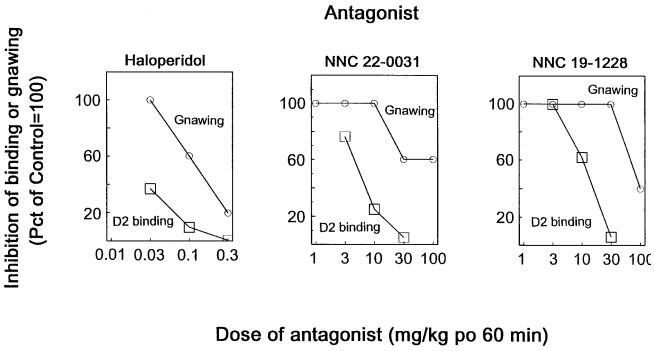


Fig. 2 Inhibition of D_2 receptor binding in vivo and blockade of MPD-induced gnawing. Ability of haloperidol, NNC 22-0031 and NNC 19-1228 to inhibit the in vivo binding of [^{3}H]raclopride to mouse striatal dopamine receptors as related to inhibitory potency of the compounds against MPD-induced gnawing. The compounds were given PO 60 min prior MPD or to death (in vivo binding experiments)

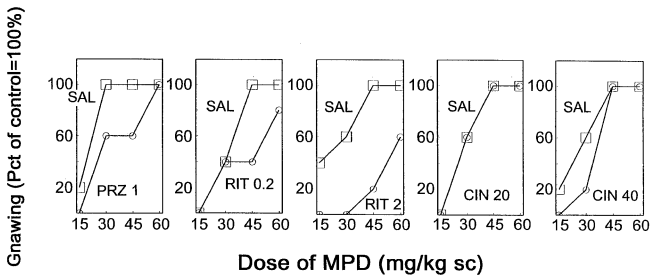


Fig. 3 Modulation of MPD-induced gnawing behavior. The influence of various pretreatments on the dose-response of MPD in the gnawing test. Saline (*SAL*) or prazosin 1 mg/kg (*PRZ*); ritanserin 0.2 and 2 mg/kg (*RIT*); or cinanserin 20 and 40 mg/kg (*CIN*)

MPD-induced hyperactivity

MPD dose-dependently induced hyperactivity with a maximum effect of 45 mg/kg. Higher doses decreased locomotor behavior (data not shown). The dose of 30 mg/kg MPD was used as the standard dose for induction of hyperactivity. In antagonism experiments, NNC 19-1228 and NNC 22-0031 were potent inhibitors of the 30 mg/kg MPD-induced hyperactivity with ED_{50} values of 1.7 mg/kg (Table 3). All other neuroleptics tested also antagonized MPD in this model; however, with varying potencies from 0.3 mg/kg

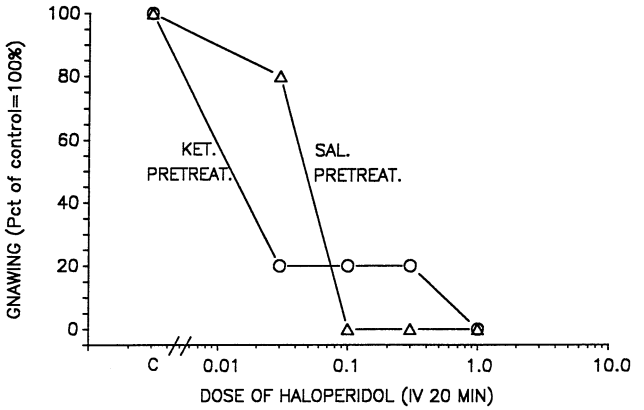


Fig. 4 Haloperidol antagonism of MPD induced gnawing: effect of ketanserin 10 mg/kg IP. Ability of ketanserin (KET, 10 mg/kg) to influence the inhibitory effect of haloperidol on MPD-induced gnawing

(haloperidol, risperidone and spiroperidol) to 117 mg/kg (sulpiride). When comparing ratios between antagonistic potencies in the MPD-gnawing and hyperactivity models, respectively, the NNC compounds had the highest ratios (55–59). In contrast, many conventional neuroleptics were equipotent in the two models (e.g., haloperidol and spiroperidol) while some compounds had moderate selectivity for antagonizing MPD-hyperactivity (ratios from 8 to 35, e.g., sertindole, tefludazine and risperidone).

In the interaction experiments, prazosin (1 mg/kg) prevented MPD from increasing motility in a dose-dependent manner (as post-hoc analysis of the effect of dose was non-significant, $t = 1.99$, $P < 0.14$). The ED_{50} (8 mg/kg) of MPD in saline-pretreated mice was increased to a level of greater than 30 mg/kg after prazosin treatment. Ritanserin-pretreatment at a dose of 2 mg/kg did not significantly change the ED_{50} for MPD (ED_{50} of MPD was 5 mg/kg and 8 mg/kg (ibid) in saline and ritanserin-pretreated animals, respectively). Finally, cinanserin in doses of 20 or 40 mg/kg did not significantly alter the dose-response of MPD (ibid).

Conditioned avoidance responding

NNC 19-1228 and NNC 22-0031 were moderately potent antagonists of conditioned avoidance responding (Table 5) with ED_{50}s of 15 and 5.9 mg/kg, respectively. These potencies were similar to that of clozapine (11 mg/kg) but considerably lower than those of haloperidol (0.2 mg/kg) and spiroperidol (0.09 mg/kg). In general, all of the tested neuroleptics blocked the conditioned avoidance responding with potencies ranging from that of haloperidol (above) to sulpiride (30 mg/kg). The antagonism of the conditioned avoidance responding by the NNC compounds occurred without effect on escape responding. With some neuroleptics (e.g., sertindole and tefludazine), effects on escape responding were seen with doses at or close to the ED_{50} for blocking the conditioned avoidance responding.

Catalepsy testing

NNC 19-1228 and NNC 22-0031 failed to induce catalepsy in doses of up to 100 mg/kg (Table 5). In this respect they resembled clozapine and sertindole, which were also devoid of any ability to produce catalepsy, even in very high doses (64 and 100 mg/kg, respectively). The potencies of the various neuroleptics for inducing catalepsy ranged from 0.25 mg/kg (spiroperidol) and up to 300 mg/kg (sulpiride). When comparing the ratios between blockade of conditioned avoidance responding and induction of catalepsy, NNC 19-1228 and NNC 22-0031 had ratios greater than 7 and 17, respectively, whereas the ratio for conventional neuroleptics (e.g., haloperidol and spiroperidol) were 4 and 2.8, respectively. Sertindole and tefludazine also had high ratios (>33 and 32, respectively), whereas that of clozapine was >5.8.

Amphetamine cue

The drug treatments significantly affected both the discriminative stimulus properties of amphetamine [$F(21,136) = 3.94$, $P < 0.006$] and the rate of responding [$F(21,164) = 12.49$, $P < 0.0001$]. Post-hoc analysis showed that the cueing effects of amphetamine were markedly attenuated by haloperidol in doses

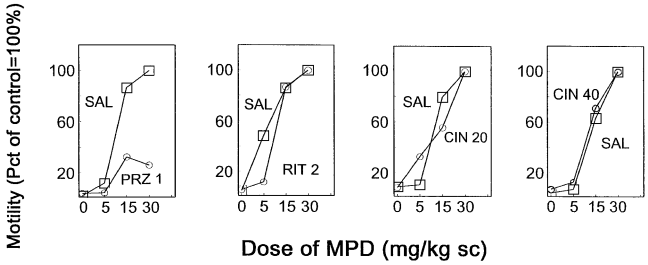


Fig. 5 Modulation of MPD-induced motility. Ability of various pretreatments to inhibit the dose-response of MPD in the motility test (see legend to Fig. 3 for other details)

Table 5 DA-antagonists: behavioral profile in the rat. The table shows the ability of NNC 19-1228, NNC 22-0031 and reference neuroleptics to inhibit CAR and catalepsy in the rat. Also shown is the inhibitory effect on escape responding and the ratio between catalepsy induction and CAR inhibition. The data are expressed as ED_{50} (or MED; escape-response inhibition) in mg/kg following IP 120-min pretreatment interval

Compound IP 120 min	ED_{50} (mg/kg)		Escape, MED	Ratio Catalepsy/CAR
	CAR	Catalepsy		
Clozapine	11	>64	>30	>5.8
Flupenthixol	0.38	1	0.8	2.6
Haloperidol	0.2	0.8	>0.3	4
NNC 19-1228	15	>100	>20	>7
NNC 22-0031	5.9	>100	>10	>17
Sertindole	3	>100	3	>33
Spiperone	0.09	0.25	>0.1	2.8
Sulpiride (–)	30	300	>30	10
Tefludazine	0.15	4.8	0.3	32
Thioridazine	28	64	>30	2.3
Tiaspirone	2.8	9.2	>3	3.3
Trißuperazine	0.7	1.8	>1	2.6

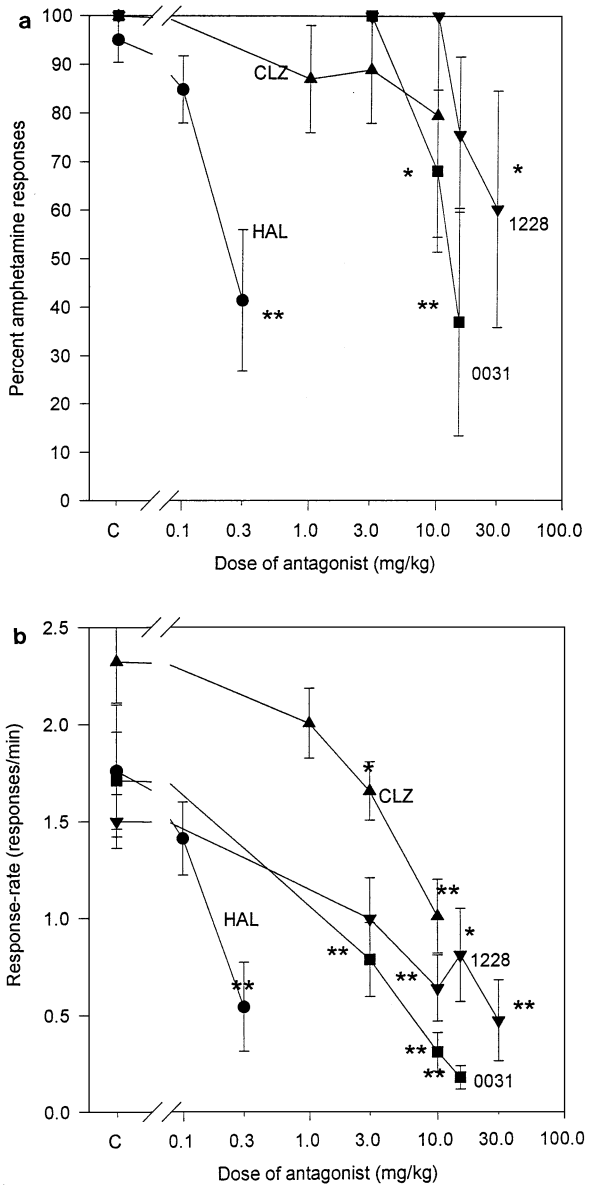


Fig. 6a, b Amphetamine cue. Potency of haloperidol (*HAL*), clozapine (*CLZ*), NNC 22-0031 and NNC 19-1228 to inhibit the cueing effect of 1 mg/kg amphetamine sulphate. Also shown is the ability of the drugs to affect the response rate during the test session. *C* denotes the effect of vehicle control session. **a** Amphetamine discrimination accuracy. **b** Amphetamine discrimination response rates. *Indicates statistically significant difference from amphetamine + vehicle levels ($P < 0.05$; ANOVA). **$P < 0.01$

(0.3 mg/kg) which also decreased the rate of responding (Fig. 6a,b). NNC 22-0031 had a similar profile, although the effects on amphetamine cueing were seen at higher doses (10–20 mg/kg) (ibid). Neither haloperidol nor NNC 22-0031 produced a complete inhibition of the amphetamine cue at any dose tested. NNC 19-1228 attenuated amphetamine induced cueing partially (maximal 40% inhibition at 30 mg/kg). Clozapine failed to block amphetamine discrimination in doses (5–10 mg/kg) which significantly decreased response rates (ibid).

Discussion

NNC 19-1228 and NNC 22-0031 are newly identified compounds with a neurochemical profile similar to clozapine. That is, the NNC compounds are mixed DA/5-HT_2/α_1 receptor antagonists. In contrast to clozapine, however, the NNC-compounds are relatively devoid of muscarinic antagonist action and in this respect resemble sertindole, a recently described compound with a high degree of mesolimbic selectivity in rat electrophysiological experiments (Sanchez et al. 1991; Hyttel et al. 1992). With respect to DA receptor subtype interactions, the NNC compounds had a profile which resembled haloperidol, i.e., high D2 and D3 affinity as compared to the affinity for other DA receptor subtypes.

In pharmacological experiments in mice, MPD was selected as an indirect DA agonist with special relevance for psychosis, since it has been shown dramatically to worsen schizophrenic symptoms (Segal and Janowsky 1978). MPD-induced motility is considered a useful model, since the hyperactivity of DA agonists involves the mesolimbic DA pathways (ibid) thought to be involved in the antipsychotic action of neuroleptic drugs (Creese and Iversen 1975; Kelly and Iversen 1976; Joyce et al. 1983). MPD-induced stereotyped gnawing behavior is considered a "classical" central stimulant-induced behavior induced by indirect DA mimetics. Its anatomical origin is believed selectively to involve the nigrostriatal DA pathway (Costall et al. 1977; Koob et al. 1977; Iversen and Alpert 1982). All neuroleptics tested blocked MPD-induced hyperactivity; however, many compounds were less active in inhibiting MPD-induced gnawing behavior. In fact, when comparing the relative ability of the selected compounds to block MPD-induced gnawing behavior (expressed as ratios, Table 4), interesting differences were noted. Many compounds with a high inhibition of MPD-gnawing versus inhibition of MPD-induced hypermotility ratio have previously been identified as "mesolimbic-selective" in various pharmacological models (e.g., tefludazine, tiaspirone; Svendsen et al. 1986) or in the clinic [clozapine (see, however, below) risperidone, sulpiride, sertindole; Castelao et al. 1989; Borison et al. 1992; Kane 1992; Lader 1992; Meltzer 1992a,b; McEvoy et al. 1993; McKenna and Bailey 1993). The profile of clozapine was difficult to ascertain, since the apparent antagonism of the MPD-induced gnawing only occurred at high doses which produced toxicological effects.

The NNC compounds, in particular, show a marked degree of separation in the above MPD tests suggestive of mesolimbic DA antagonistic selectivity. It has previously been demonstrated that the ability of drugs to block MPD-induced gnawing parallels the inhibition of D_2 receptor blockade in vivo (Nielsen and Andersen 1992). Thus, it can be asked if the relative inability of the NNC compounds to inhibit

MPD-induced gnawing is due to a situation where the compounds are unable fully to block the D_2 receptors. This possibility is unlikely, since the in vivo binding experiments showed that the NNC compounds fully occupied the D_2 receptor at doses which produced only a partial block of MPD-induced gnawing. Under the same conditions, haloperidol produced MPD-gnawing antagonism in parallell with its ability to block D_2 receptor binding in vivo, as has previously been described (ibid). Thus, the possibility exists that the non-DAergic effects of the NNC compounds may have modulated its functional DA antagonistic effect (below). An alternative explanation is that the NNC compounds exerted their particular profile because of a selective DA receptor subtype interaction. However, this is also unlikely due to the similarity of the profile of NNC compounds to that of haloperidol.

The present data indicate that neither MPD-induced gnawing nor MPD-induced motility was influenced by 5-HT_2 antagonism. Only a relatively high dose of the 5-HT_2 antagonist ritanserin affected MPD-induced gnawing. However, at this high dose a DA receptor interaction is likely, based on its in vitro selectivity for DA/5-HT_2 receptors and also on its in vivo DA receptor binding affinity (Leysen et al. 1988; Schotte et al. 1989; Saller et al. 1990). This apparent lack of effect of 5-HT_2 blockade on MPD-induced gnawing behavior was further substantiated by the inability of the selective 5-HT_2 antagonist cinanserin (Leysen et al. 1981) in 20 and 40 mg/kg to affect MPD. Further, a high dose of the selective 5-HT_2 antagonist ketanserin was also unable significantly to affect the antagonist action of haloperidol against MPD-induced gnawing. Previous work in rodents has shown that 5-HT_2 antagonists potentiate certain DA functions (see discussion in Saller et al. 1990). However, opposite or weak interactions have also been described (Schmidt et al. 1992; Wadenberg 1992). Further, interactions between the DA and 5-HT_2 receptor systems may occur only under some circumstances; for example, when DA receptors are blocked (Sorensen et al. 1992) or stimulated (Schmidt et al. 1992) in certain anatomical areas (i.e., Devaud and Hollingsworth 1991). In the primate, most results support the view that 5-HT is unimportant for neuroleptic-induced dyskinesias (Liebman et al. 1989); see also discussion in Saller et al. (1990).

The results from the MPD-gnawing and motility interaction studies showed clearly that MPD-induced motility was strongly affected by doses of the α_1 antagonist prazosin but that MPD's dose-response for gnawing was not affected. In contrast, neither ritanserin nor cinanserin affected the motility. Thus, the pronounced α_1-blocking potency of many of the presently tested mesolimbic-selective neuroleptics, and in particular the NNC compounds, may underlie the preferential ability of these compounds to block MPD-induced hypermotility. This, however, does not explain why the NNC compounds and sertindole were relatively devoid of an antagonistic effect against MPD-induced gnawing, since the NNC compounds clearly occupied the D_2 receptors in vivo at doses which would otherwise have been expected to block MPD-induced gnawing. Further research into this issue is needed.

The role of α_1-adrenergic antagonism as a means to modulate or synergise mesolimbic DA antagonist activity is supported by electrophysiological data from Svensson and Ahlenius (1982), Grenhoff and Svensson (1993) and Andersson et al. (1993). Further, combining prazosin and haloperidol during chronic dosing appears to convey A10 DA-selective depolarization inactivation (Chiodo and Bunney 1985), a chronic model for mesolimbic neuroleptic specificity (White and Wang 1983). Additional evidence of synergism and interaction between α-adrenergic receptors and DA systems have been noted previously (Liebman et al. 1981; Waldmeier et al. 1982; Cohen and Lipinski 1986; Eshel et al. 1990). These interactions appear to be most prominent for mesolimbic DA systems (ibid). Similar to clozapine, NNC 22-0031 was shown by Fink-Jensen and Kristensen (1994) to exert mesolimbic selectivity in its ability to activate c-fos protein immunoreactivity. Interestingly, this effect of clozapine may not depend on α-adrenergic mechanisms (Fink-Jensen et al. 1995).

It is of interest to note that sertindole, an agent with pronounced electro-physiological A10 DA selectivity, possesses marked α-adrenergic antagonist action and preferentially blocks stimulant-induced hypermotility (Arnt and Sánchez 1993). The profile of chlorpromazine also deserves comment, since the profile of this conventional neuroleptic would suggest some mesolimbic selectivity. However, it can be speculated that the extensive metabolism of chlorpromazine with many active metabolites (Morselli 1977) may overshadow the effect of the native compound.

In the rat, all the tested neuroleptics blocked conditioned avoidance responding whereas some compounds were remarkably weak in inducing catalepsy. Thus, classical neuroleptics (e.g., haloperidol, trifluoperazine, spiperone) had relatively low CAR/catalepsy ratios, whereas compounds with a high degree of purported mesolimbic selectivity (e.g., sertindole, tefludazine, clozapine) had high ratios. Interestingly, risperidone did not exhibit a high degree of selectivity in the rat (although it did in the mouse). Both NNC compounds exhibited a high degree of apparent mesolimbic selectivity, as they inhibited CAR at low doses and failed to induce catalepsy even in very high doses. Previous characterization of the conditioned avoidance response (Arnt 1982) has revealed that in addition to dopaminergic blockade, α-adrenergic antagonism may play a role. This may explain the relatively potent ability of the NNC compounds to inhibit the conditioned avoidance responding as these compounds, as well as clozapine, sertindole and tefludazine, possess potent α-adrenergic antagonist activity.

It was interesting that NNC 22-0031 attenuated amphetamine's cueing effect, since this model has previously been shown to depend on activation of mesolimbic DA systems (Nielsen and Jepsen 1985; Nielsen and Scheel-Krüger 1986). Interestingly, α-adrenoceptor activation appears not to play a role in amphetamine discrimination, as prazosin is devoid of any amphetamine-antagonistic effects under conditions very similar if not identical to the presently used (Arnt 1992). Under the present circumstances, clozapine was unable to block the cueing effect of amphetamine, since a high level of response suppression precluded further testing. Although clozapine has previously been found to block amphetamine discrimination (ibid), this was only achieved with considerable response suppression. Thus, the differences in experimental procedures used here in comparison with Nielsen and Jepsen (1985; e.g., water versus food deprivation) may have given rise to differential sensitivities in the response-suppressive effects of clozapine.

In summary, the present results indicate that NNC 19-1228 and NNC 22-0031 are interesting new neuroleptic compounds to explore. Neurochemically, the compounds share mixed DA, 5-HT and α-antagonism with clozapine. The pharmacological data in both mice and rats indicate high mesolimbic DA antagonistic specificity coupled with low potential for extrapyramidal side effects. This profile may be due to interactions with non-DAergic receptors, in particular α_1-adrenergic receptors.

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