Peripheral and central adrenoceptor modulation of the behavioural effects of clozapine in the paw test

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1 In rats, the atypical neuroleptic, clozapine, has been found to increase the hindlimb retraction time but not the forelimb retraction time, in the paw test. These parameters have predictive validity for the antipsychotic efficacy and extrapyramidal side-effects of drugs, respectively. The present study analysed to what extent drugs acting on adrenoceptors affect the behavioural effect of clozapine in the paw test.

2 The α_1 -adrenoceptor agonist, ST 587 but not the peripherally working α_1 -agonist, methoxamine, decreased the effect of clozapine on the hindlimb retraction time. The α_1 -antagonist phenoxybenzamine increased this effect of clozapine, and blocked the effect of ST 587 on clozapine at low doses. Only the combination of phenoxybenzamine with clozapine produced an increase in forelimb retraction time.

3 The α_2 -adrenoceptor agonist, clonidine, decreased the effect of clozapine on the hindlimb retraction time. This effect was neither antagonized by the α_2 -antagonist rauwolscine nor by the α_1 -antagonist phenoxybenzamine. Rauwolscine or the peripherally working α_2 -antagonist L-659,066 did not influence the effect of clozapine on the hindlimb retraction time. The forelimb retraction time was not affected by any of the drug combinations.

4 In contrast to the β_2 -adrenoceptor agonist, clenbuterol, which was ineffective, the peripherally acting β -agonist, (–)-isoprenaline, increased the effects of clozapine on the hindlimb retraction time. The β -antagonist, (–)-propranolol as well as the peripherally acting β -antagonist, nadolol decreased this effect of clozapine. Low doses of the peripherally acting β_1 -antagonist, atenolol, as well as low doses of the β_2 -antagonist, ICI-118,551, decreased the effect of clozapine. A low dose of nadolol blocked the effect of (–)-isoprenaline on clozapine. Only the combination of clenbuterol with clozapine produced an increase in forelimb retraction time.

5 It is concluded that blockade of central α_1 -adrenoceptors plays an important role in the effect of clozapine on the hindlimb retraction time. Furthermore, the effect of clozapine on the hindlimb retraction time is strongly modulated by peripheral β_1 - and/or β_2 -adrenoceptors. Given the predictive validity of the paw test, the presented data suggest that the α_1 -adrenoceptor antagonist properties of clozapine are important for its therapeutic effects, but not for its lack of extrapyramidal side-effects.

Keywords: Neuroleptics; peripheral adrenoceptors; central adrenoceptors; α_1 -adrenoceptors; α_2 -adrenoceptors; β_1 -adrenoceptors; β_2 -adrenoceptors; paw test

Introduction

Many studies point towards a role for the noradrenergic system in schizophrenia (Maas *et al.*, 1993; for review Van Kammen *et al.*, 1986). There are also many studies suggesting a role for α -adrenoceptors in the therapeutic effects of neuroleptics (Petersen, 1981; Marwaha & Aghajanian, 1982; Cohen & Lipinsky, 1986; Baldessarini *et al.*, 1992), although blockade of dopamine D_2 receptors is believed to be crucial (Creese *et al.*, 1976; Seeman *et al.*, 1976; Peroutka & Snyder, 1980; but see, Cohen & Lipinsky, 1986). However, clozapine is a much weaker antagonist of the dopamine D_2 receptor than most neuroleptics (Richelson & Nelson, 1984; Anderson, 1988; Farde & Nordström, 1992), while it is effective in patients who are unresponsive to other neuroleptics (Kane *et al.*, 1988). Moreover, clozapine is superior to most other neuroleptics with respect to lack of extrapyramidal side-effects (Angst *et al.*, 1971) and amelioration of negative symptoms (Honigfeld *et al.*, 1987). Together, this implies that clozapine must have a working mechanism that is different from that of other neuroleptics. One of the prominent features of clozapine is its potent α_1 - as well as α_2 -adrenoceptor antagonist properties (Hall *et al.*, 1986; Richelson & Nelson, 1984), which are even more pronounced since clozapine is administered in high doses (Wyatt, 1976). Therefore, the present study examined to what extent α_1 - and α_2 -adrenoceptors play a role in the effects of clozapine. Moreover, since α - and β -adrenoceptors can interact strongly

(e.g. Stone *et al.*, 1987), the possible modulation of the effects of clozapine by β -adrenoceptors was also studied. The animal model used was the so-called paw test which models both the antipsychotic efficacy and the extrapyramidal side-effects of drugs (Ellenbroek *et al.*, 1987). With regard to a large number of criteria (such as false positives and false negatives, response to chronic treatment, interactions between neuroleptics and anticholinergics, etc.), this model has been shown to have predictive validity for both the antipsychotic efficacy and the extrapyramidal side-effects of drugs (Ellenbroek *et al.*, 1987; Ellenbroek & Cools, 1988; Ellenbroek, 1993).

Methods

The procedure followed was similar to that described by Prinssen *et al.* (1993). Male Wistar rats (weighing between 220 and 250 g) were housed individually 24 h before the experiment. Experiments were performed between 10 h 00 min and 16 h 00 min, and rats were used only once. On the day of the experiment, the rats received one or two intraperitoneal injections. In the case of single injections, each rat was injected at $t = 0$ min. In the case of two injections, the first injection (an adrenoceptor antagonist) was given at $t = -10$ min and the second (clozapine) at $t = 0$ min. When a (putative) adrenoceptor antagonist was combined with its agonist, the two drugs were administered as a cocktail at $t = -10$. The injection volume was always 1 ml kg⁻¹, except for phenoxybenzamine dissolved in propylene glycol

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(0.5 ml kg⁻¹). All drugs were dissolved in distilled water, except for clozapine to which a drop of HCl was added (1 N), phenoxybenzamine which was dissolved in propylene glycol, and the combination of clonidine or ST 587 and phenoxybenzamine which was dissolved in 50% alcohol. Control injections consisted of distilled water, since combinations of propylene glycol or 50% alcohol with clozapine did not differ from the combination of distilled water with clozapine (data not shown).

Thirty minutes after the last injection the paw test was performed and repeated at 40 and 50 min. In the paw test a rat was placed on a Perspex platform which has four holes. The rat was placed on the platform by positioning first the hind- and then the forelimbs in the holes. Two variables were measured in this test, namely the time it takes the animal to retract its first hindlimb (hindlimb retraction time; HRT) and the time it takes the animal to retract its first forelimb (forelimb retraction time; FRT) with a minimum time of 1 s and a maximum time of 60 s. Since only robust effects are considered to be important, the window (1 to 60 s) was chosen so that both significant decreases and significant increases could be easily detected. The individual score was calculated for each animal as the mean of the three trials. The data are presented as the median value of these means per group ($n = 6-15$ rats) together with the corresponding 25 and 75 percentiles. Group differences were calculated with a two-tailed Mann-Whitney U-test.

The following drugs were used (for the sake of clarity, peripherally working drugs are defined as drugs that do not readily pass the blood-brain barrier): the atypical neuroleptic clozapine (Sandoz, The Netherlands), the α_1 -agonist ST 587 (2-(2-chloro-5-trifluoromethyl-phenyl imino)imidazolidine; Boehringer Ingelheim, Germany), the peripherally working α_1 -agonist, methoxamine HCl (Wellcome, U.S.A.), the α_1 -antagonist, phenoxybenzamine HCl (SK&F, U.S.A.), the α_2 -agonist, clonidine HCl (Boehringer Ingelheim, The Netherlands), the α_2 -antagonist rauwolscline HCl (Carl Roth, Germany), the peripherally working α_2 -antagonist L 659,066 ((2R-trans)-N-(2-(1,3,4,6,7,12b-hexahydro-2'-oxospiro(2H-benzo-furo(2,3-a)quinolizine-2,3-imidazolin)-3'-yl)ethyl)methane-sulphonamide monohydrochloride; Merck, U.S.A.), the β_2 -agonist clenbuterol HCl (Sigma, The Netherlands), the peripherally working β -agonist, (-)-isoprenaline HCl (Sigma, U.S.A.), the β -antagonist, (-)-propranolol HCl (RBI, U.S.A.), the peripherally working β -antagonist, nadolol (Sigma, St. Louis, U.S.A.), the peripherally working β_1 -antagonist ($\pm$)-atenolol (RBI, U.S.A.) and the β_2 -antagonist, ICI-118,551 (erythro-DL-1-(7-methylindan-4-yloxy)-3-(isopropylaminobutan-2-ol) hydrochloride; Zeneca, U.S.A.).

Results

Effects of drugs acting on adrenoceptors

The purpose of this study was to analyse the interaction of drugs acting on adrenoceptors with clozapine. To avoid the use of doses of drugs that were effective on their own, all drugs used in interaction with clozapine, were also tested alone in high doses. In case of strong behavioural effects of such a drug, a sub-threshold dose was used in the interaction study with clozapine. ST 587 (1 mg kg⁻¹), phenoxybenzamine (10 mg kg⁻¹), rauwolscline (5 mg kg⁻¹), L 659,066 (5 mg kg⁻¹), (-)-isoprenaline (5 mg kg⁻¹), (-)-propranolol (10 mg kg⁻¹), nadolol (10 mg kg⁻¹), atenolol (1 mg kg⁻¹) and ICI-118,551 (1 mg kg⁻¹) did not significantly influence HRT or FRT compared with controls (distilled water, median values: HRT 1.1 s; FRT: 1.0 s). Methoxamine (10 mg kg⁻¹) and clenbuterol (10 mg kg⁻¹) induced small, but significant increases in HRT (median value of HRT: 2.9 and 4.3 s, respectively). Since higher doses of clonidine induced strong effects on the HRT [(clonidine (0.3 mg kg⁻¹) induced a HRT of 22.0 s (median value)], the highest dose used in this study was

limited to 0.1 mg kg⁻¹, a dose that was without significant effects.

Effects of clozapine alone

In line with earlier findings, clozapine (15–20 mg kg⁻¹) significantly increased the HRT ($P < 0.01$; Figure 1), but not the FRT (data not shown) compared with controls (distilled water; see above).

Effects of α_1 -selective drugs on clozapine

ST 587 (0.25–1 mg kg⁻¹) decreased the effect of clozapine (20 mg kg⁻¹) on the HRT (Figure 1): higher doses produced stronger effects. On the other hand, the peripherally acting α_1 -agonist, methoxamine (10 mg kg⁻¹) combined with clozapine (20 mg kg⁻¹), did not influence the HRT (median value: 56.6 s) compared with clozapine alone (median value: 60.0 s; Figure 1). Phenoxybenzamine increased the effect of clozapine (15 mg kg⁻¹) on the HRT (Figure 1): the lowest dose which was significantly effective, already produced a maximal increase (60.0 s). The effect of ST 587 on clozapine could be antagonized by phenoxybenzamine (1 mg kg⁻¹; Figure 3), in a dose that itself did not affect clozapine (15 mg kg⁻¹; Figure 1).

Figure 1 The effect of ST 587 and phenoxybenzamine on the hindlimb retraction time (HRT) induced by clozapine. The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the HRT are shown. * $P < 0.05$; ** $P < 0.01$.

Figure 2 The effect of clonidine and rauwolscline on the hindlimb retraction time (HRT) induced by clozapine. The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the HRT are shown. ** $P < 0.01$.

The FRT was not affected by any of the combinations, apart from the combination of phenoxybenzamine (10 mg kg⁻¹) and clozapine (15 mg kg⁻¹) that produced a small, but significant effect ($P < 0.05$) on the FRT (median value: 6.2 s) compared with clozapine alone (median value: 1.0 s).

Effects of α_2 -selective drugs on clozapine

Clonidine (0.03–0.1 mg kg⁻¹) significantly decreased the effect of clozapine (20 mg kg⁻¹) on the HRT (Figure 2): higher doses could not be tested since they produced strong effects when given alone (see above). Rauwolscline (1–5 mg kg⁻¹; Figure 2), as well as L-659,066 (5 mg kg⁻¹; HRT, median value: 35.8), did not influence the effect of clozapine (15 mg kg⁻¹) on the HRT. The inhibitory effect of clonidine (0.1 mg kg⁻¹) on clozapine (20 mg kg⁻¹) could not be antagonized by relatively high doses (see Discussion) of rauwolscline (1 mg kg⁻¹) or phenoxybenzamine (1 mg kg⁻¹; Figure 3).

The FRT was not affected by any of the drug combinations.

Figure 3 All groups received 20 mg kg⁻¹ clozapine. The left part of the figure shows that the inhibition of clozapine by ST 587 is antagonized by phenoxybenzamine. The right part of the figure shows that the inhibition of clozapine by clonidine is neither antagonized by rauwolscline (Rauwol) nor by phenoxybenzamine (PBZ). The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the hindlimb retraction time (HRT) are shown. * $P < 0.05$.

Figure 4 The effect of clenbuterol and (-)-propranolol on the hindlimb retraction time (HRT) induced by clozapine. The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the HRT are shown. * $P < 0.05$; ** $P < 0.01$.

Effects of β -selective drugs on clozapine

Clenbuterol (1–10 mg kg⁻¹) did not influence the effect of clozapine on the HRT (15 mg kg⁻¹; Figure 4). In contrast, the peripherally acting β -agonist, (-)-isoprenaline (1–5 mg kg⁻¹) increased the effects of clozapine (15 mg kg⁻¹) on the HRT (Figure 5): the lowest dose that was significantly effective, already produced maximal effects (60.0 s). (-)-Propranolol (0.1–10 mg kg⁻¹), nadolol (1–10 mg kg⁻¹), atenolol (0.1–1 mg kg⁻¹) and ICI-118,551 (0.1–1 mg kg⁻¹) decreased the effect of clozapine (20 mg kg⁻¹) on the HRT (Figures 4–6). The effect of (-)-isoprenaline (5 mg kg⁻¹) on clozapine (15 mg kg⁻¹) was blocked by a dose of nadolol (1 mg kg⁻¹) that itself had no effect (Figure 5).

The FRT was not affected, apart from the combination of clenbuterol (10 mg kg⁻¹) and clozapine (15 mg kg⁻¹), which produced a significant increase in the FRT (median value: 15.7 s) compared with clozapine alone (median value: 1.0 s).

Discussion

The role of α_1 -adrenoceptors in the effects of clozapine

In line with earlier findings, clozapine (15–20 mg kg⁻¹) enhanced the hindlimb retraction time (HRT) in the paw test. The α_1 -agonist, ST 587, decreased this effect of clozapine. The effect of ST 587 appeared to be α_1 -adrenoceptor-specific: (1) ST 587 is highly selective for α_1 - over α_2 -adrenoceptor stimulation (De Jonge *et al.*, 1981); (2) the effect of ST 587 could be attenuated by a dose of the α_1 -antagonist, phenoxybenzamine, that itself did not affect clozapine; (3) when combined with clozapine, higher doses of phenoxybenzamine produced effects opposite to those of ST 587.

A high dose (cf. Liebau *et al.*, 1989) of the peripherally acting α_1 -agonist, methoxamine, did not alter the effect of clozapine on the HRT. Even when a possible effect of methoxamine was obscured by the ceiling effect of clozapine, the contribution of peripheral α_1 -adrenoceptors to the effects of ST 587 can be excluded since the effective dose of ST 587 (0.5 mg kg⁻¹) was 20 times smaller than the ineffective dose of methoxamine (10 mg kg⁻¹). Thus, the attenuation of the effects of clozapine by α_1 -adrenoceptor stimulation did not seem to involve peripheral α_1 -adrenoceptors. Therefore, it is concluded that central α_1 -adrenoceptor stimulation decreased the effects of clozapine on the HRT in the paw test. These

Figure 5 The effect of (-)-isoprenaline and/or nadolol on the hindlimb retraction time (HRT) induced by clozapine. The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the HRT are shown. ** $P < 0.01$; † $P < 0.05$, compared with the combination of (-)-isoprenaline (5 mg kg⁻¹) and clozapine.

Figure 6 All groups received 20 mg kg⁻¹ clozapine. The effect of atenolol and ICI-118,551 on the hindlimb retraction time (HRT) induced by clozapine is shown. The doses used (in mg kg⁻¹) are shown in the figure. Median values and percentiles (25 and 75%) of the HRT are shown. **P* < 0.05; ***P* < 0.01.

data together with the fact that clozapine potently antagonizes α_1 -adrenoceptors (Hall *et al.*, 1986; Mennon *et al.*, 1988) strongly suggest that blockade of central α_1 -adrenoceptors contribute significantly to the HRT elicited by clozapine. Since the HRT is a parameter with predictive validity for the antipsychotic efficacy of drugs (Ellenbroek *et al.*, 1987; Ellenbroek & Cools, 1988; Ellenbroek, 1993), the present study implies that inhibition of central α_1 -adrenoceptors is important for the antipsychotic effects of clozapine. On the other hand, a high dose of phenoxybenzamine alone (10 mg kg⁻¹) did not increase the HRT, suggesting that blockade of α_1 -adrenoceptors alone is not enough to produce such an effect. Indeed, recent studies have shown that the 5-hydroxytryptamine 5-HT₂ receptor antagonist properties (Ellenbroek *et al.*, 1994) as well as the dopamine D₁ receptor antagonist properties, but not the dopamine D₂ receptor antagonist properties of clozapine (Ellenbroek *et al.*, 1991) also play a role in the effects of clozapine on the HRT. We are now studying to what extent combined antagonism of 5-HT₂ receptors, dopamine D₁ receptors and α_1 -adrenoceptors may produce a clozapine-like profile in the paw test.

As previously reported (Ellenbroek *et al.*, 1987), unlike classical neuroleptics, clozapine did not induce an increase in the forelimb retraction time (FRT). When combined with clozapine, ST 587 did not induce an increase in FRT, indicating that the α_1 -antagonist properties of clozapine do not play a role in its lack of FRT. Since the FRT is a parameter with predictive validity for extrapyramidal side-effects (Ellenbroek *et al.*, 1987; Ellenbroek & Cools, 1988), the present data indicate that the α_1 -antagonist properties of clozapine do not play a role in its lack of extrapyramidal side-effects. Moreover, phenoxybenzamine in combination with clozapine, did induce an increase in FRT, suggesting that additional blockade of α_1 -adrenoceptors together with clozapine, may increase, but not decrease, extrapyramidal side-effects (cf. Chiodo & Bunney, 1985).

The role of α_2 -adrenoceptors in the effects of clozapine

As mentioned, clozapine enhanced the HRT in the paw test. The higher dose of clonidine (0.1 mg kg⁻¹) decreased this effect of clozapine. This effect of clonidine could not be blocked by a relatively high dose of rauwolscine (cf. Timmermans *et al.*, 1981), suggesting that α_2 -adrenoceptor stimulation does not affect clozapine with respect to the HRT. This is underlined by the finding that both rauwolscine and L 659,066 did not modulate the effect of clozapine on the HRT. Since the HRT is a parameter with predictive validity

for the antipsychotic efficacy of drugs (see above), the present study implies that inhibition of α_2 -adrenoceptors does not contribute to the antipsychotic effects of clozapine.

Since clonidine also has α_1 -adrenoceptor agonist properties (Liebau *et al.*, 1989), the role of these adrenoceptors was evaluated as well. However, a dose of phenoxybenzamine (1 mg kg⁻¹) that strongly attenuated the effect of ST 587, did not attenuate the effect of clonidine on clozapine. This suggests that the effects of clonidine on clozapine are not due to its α_1 -adrenoceptor properties. However, until more is known about the pharmacology of the many different α_1 - and α_2 -adrenoceptor subtypes that have recently been identified (Johnson & Minneman, 1986; Morrow & Creese, 1986; Bylund *et al.*, 1988) a role for these receptor subtypes cannot be fully excluded. Moreover, the effect of clonidine may involve an interaction between α_1 - and α_2 -adrenoceptor (sub)types. Alternatively, other receptors for which clonidine has a high affinity may be involved, e.g. imidazoline receptors (Molderings *et al.*, 1993).

As mentioned clozapine did not induce an effect on the FRT; nor did the combination of clonidine with clozapine induce an increase in FRT, indicating that the α_2 -antagonist properties of clozapine do not play a role in its lack of effect on FRT. Since the FRT is a parameter with predictive validity for extrapyramidal side-effects (see above), the present data indicate that the α_2 -antagonist properties of clozapine do not play a role in its lack of extrapyramidal side-effects.

The role of β -adrenoceptors in the effects of clozapine

The peripherally acting β -agonist, (–)-isoprenaline, increased the effect of clozapine on the HRT, which could be attenuated by an ineffective dose of the peripherally acting β -antagonist, nadolol (1 mg kg⁻¹). Moreover, when given alone, higher doses of nadolol decreased the effects of clozapine. Together, these data imply that peripheral β -adrenoceptors modulate the effect of clozapine on the HRT. The decrease in the effect of clozapine on the HRT by β -adrenoceptor blockade seems to be mediated via β_1 - as well as β_2 -adrenoceptors, since atenolol and ICI-118,551 were both effective in doses that are receptor-specific (Bilsky *et al.*, 1983). The significant effect of a low dose of atenolol (0.1 mg kg⁻¹) furthermore underlines the involvement of peripheral receptors. The β_2 -agonist, clenbuterol, had no effect on clozapine, in contrast with (–)-isoprenaline. This was especially surprising since selective β_2 -adrenoceptor blockade did affect clozapine (see above). These data may indicate that stimulation of β_2 -adrenoceptors, as opposed to non-selective β -adrenoceptor stimulation, is not sufficient to enhance the effect of clozapine on the HRT. However, one has to be careful since clenbuterol also seems to possess β_1 -antagonist properties (Ordway *et al.*, 1987). Since a β_1 -antagonist (atenolol) decreased the clozapine-induced HRT, the possibility cannot be excluded that the lack of effect of clenbuterol is due to its ability to interact with both β_1 - and β_2 -adrenoceptors in an opposite manner.

As mentioned, clozapine did not induce an increase on the FRT. Neither did the combination of any of the peripherally acting drugs with clozapine produce an effect on the FRT. Interestingly, clenbuterol, which readily passes the blood-brain barrier, did induce an increase in FRT, when combined with clozapine, suggesting that central β_2 -adrenoceptors may be involved in this increase in FRT.

Since clozapine has a very low affinity for β -adrenoceptors (Hall *et al.*, 1986), it becomes difficult to ascribe the effects of peripheral β -adrenoceptor agents on the clozapine-induced increase of HRT to clozapine's ability to interact with β -adrenoceptors. While the precise mechanisms of action by which peripheral β -adrenoceptors might modulate the effects of clozapine are unknown, we can exclude the following ones. First, the effects of the β -ligands do not seem to be mediated by a change in the pharmacokinetics of clozapine.

Propranolol has been shown to increase, and not to decrease, the plasma concentration of neuroleptics (the classical neuroleptic, chlorpromazine and the atypical neuroleptic, thioridazine; clozapine was not studied), thereby increasing their therapeutic efficacy (Peet *et al.*, 1980; Silver *et al.*, 1986). Indeed, (–)-propranolol has been found to increase, but not to decrease, the effects of haloperidol on the HRT (unpublished observations). Second, the effect of the β -ligands could not be ascribed to a direct influence on the extrafusal fibres in the hindlimb muscles, thereby influencing the contractability of these muscles, despite the fact that these muscle fibres contain β_2 -adrenoceptors (Buckenmeyer *et al.*, 1990): for, both β_1 - and β_2 -adrenoceptors were found to be involved in the effects studied. Moreover, (–)-propranolol increased the effects of haloperidol on the HRT (see above), while it decreased the effects of clozapine (Figure 4): such effects seem incompatible with a direct action on muscle fibres. Further research on the mechanisms of action by which peripheral β -adrenoceptors modulate the effects of clozapine is necessary.

Summary

The present study shows that central α_1 -adrenoceptor stimulation decreases the effects of clozapine on the hindlimb

retraction time (HRT), a parameter with predictive validity for the antipsychotic efficacy of drugs. As discussed, these data imply that blockade of central α_1 -adrenoceptors by clozapine is important for the antipsychotic effects of clozapine. The present study also shows that blockade of β_1 - and/or β_2 -adrenoceptors decreased the effect of clozapine on the HRT, an effect that seemed to be mediated by peripheral receptors. The forelimb retraction time (FRT), a parameter with predictive validity for extrapyramidal side-effects, on which clozapine has no effect, was not increased when clozapine was combined with α_1 -agonists. Therefore, these data imply that α_1 -adrenoceptors do not play a role in the lack of extrapyramidal side-effects of clozapine.

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