A Neurochemical Basis for the Antipsychotic Activity of Loxapine: Interactions with Dopamine D_1, D_2, D_4 and Serotonin 5-HT_2 Receptor Subtypes

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Loxapine is a typical neuroleptic that shows great structural and functional homology to the atypical antipsychotic clozapine. Chronic loxapine treatment is usually associated with extrapyramidal symptoms (EPS), whereas clozapine treatment is not. Conversely, loxapine does not produce the agranulocytosis that often results from protracted clozapine treatment. Earlier studies of loxapine have usually implicated D_2 receptor blockade as the cause of the tardive dyskinesia that occurs with chronic treatment. More recently, loxapine's ability to potentiate serotonergic neurotransmission has also been implicated. In this study, the pharmacological affinities of loxapine for the dopamine D_1, D_2, D_4, as well as serotonin-2 (5-HT_2) and NMDA receptor subtypes, were investigated through direct radioreceptor assays. The findings indicate that loxapine displays an extremely strong binding affinity for dopamine D_4 and serotonin 5-HT_2 receptors, which suggests that both serotonergic and dopaminergic mechanisms contribute to the antipsychotic drug action and EPS associated with loxapine in the treatment of schizophrenia.

Key Words: loxapine, typical and atypical neuroleptic, dopamine receptor, serotonin receptor

INTRODUCTION

It has been well established through behavioral studies, dopamine turnover, and direct ligand binding assays (Baldessarini et al 1980; Seeman 1980, 1987, 1988, 1994) that all known antipsychotic drugs have the ability to block dopamine D_2 receptor activity. However, the discovery of many new receptor subtypes (Giros et al 1989; Van Tol et al 1991; Sunahara et al 1991; Seeman and Van Tol 1994) and the possibility of monoaminergic interactions have introduced elements of complexity and multiplicity to the mechanism of neuroleptic action.

Most antipsychotic drugs currently used for the treatment of schizophrenia can be classified as either typical (classical) or atypical neuroleptics. The therapeutic action of a typical antipsychotic drug is generally accompanied by adverse side effects such as neuroleptic-induced parkinsonism or tardive dyskinesia. In addition, atypical neuroleptics such as fluperlapine and clozapine show a striking dissociation between antipsychotic activity and the adverse side effects seen with classical neuroleptics (Burki et al 1977; Meltzer et al 1989).

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Loxapine is a mid-potency, typical neuroleptic belonging to the tricyclic dibenzoxazepine family. Several reports have shown that although loxapine treatment produces extrapyramidal side effects, the incidence of these are less frequent when compared to treatment with higher potency antipsychotics such as haloperidol or fluphenazine (Schwartz and Brotman 1992; Seeman 1981). A study (Carlyle et al 1993) comparing the use of loxapine versus haloperidol in the treatment of aggressive, demented patients showed that, even though both drugs proved to be equally efficacious, loxapine produced significantly fewer side effects than haloperidol, including extrapyramidal effects. Although this drug shares many structural and pharmacological properties with the atypical neuroleptic, clozapine, loxapine differs primarily in that it does not cause the agranulocytosis (depression of white blood cell counts) often associated with chronic clozapine treatment (De Paulo et al 1982).

Earlier studies of loxapine have suggested that the major pharmacological mode of action involves dopamine D_2 receptor antagonism, and to a lesser extent, blocking activity at D_1 receptors as well (Buckland et al 1992). Recent molecular cloning techniques have facilitated not only the identification of additional dopamine receptor subtypes, but also further classification of serotonin (5-HT) receptor isoforms (Bradley et al 1986; Saudou and Hen 1994; Hoyer et al 1994). Receptor binding studies have indicated that central serotonergic mechanisms may also be involved in the action of antipsychotic drugs. Interactions of dopamine and serotonin in the nigrostriatal system are well documented, (Chesselet 1984; Meltzer et al 1989) and loxapine has been shown not only to antagonize dopamine receptors, but also to potentiate serotonergic transmission by blocking 5-HT reuptake (Delini-Stula 1986).

In this study, direct radioreceptor assays of the D_1, D_2, D_4, and 5-HT_2 receptor subtypes were used in the presence of loxapine to determine the affinity of this drug for each of these receptor subtypes. Such an approach may provide pharmacological evidence for loxapine's apparent clinical efficacy. A better understanding of loxapine's mechanism of action may yield useful information for the rational design of new analogues of loxapine which may prove to be even more effective antipsychotics, devoid of extrapyramidal side effects.

MATERIALS AND METHODS

Membrane preparation — human striatal

Human striata were homogenized in 10 vol of homogenization buffer (0.25 M sucrose, 50 mM Tris-HCL, 1 mM EDTA, 0.1 mM PMSF, pH 7.4) in a Wheaton glass homogenizer and centrifuged at 1,000 × g for 10 min. The resulting supernatant was saved and the pellet resuspended in homogenization buffer as before. The supernatants were pooled and centrifuged at 40,000 × g for 30 min. The pellet was washed again in 10 vol resuspension buffer (50 mM Tris-HCL, 1 mM EDTA, 0.1 mM PMSF, pH 7.4) and centrifuged at 40,000 × g for 30 min. The final pellet was resuspended in 2 vol resuspension buffer and stored at -80°C in small aliquots. On the day of use, membranes were thawed on ice and resuspended in assay buffer for D_1 and D_2 receptor assays: (50 mM Tris-HCL, 1 mM EDTA, 5 mM MgC_2 and 0.1% ascorbic acid, pH 7.4).

Membrane preparation — human cortical

Human cortex samples were processed and membranes were prepared in the same manner as the striatal membranes. On the day of the assay, the cortical membranes were thawed on ice and diluted in assay buffer for the 5-HT_2 assay (50 mM Tris, pH 7.4).

Membrane preparation — bovine cortical

Bovine cortex was homogenized in 20 vol of homogenization buffer (10 mM HEPES, 5 mM EDTA, pH 7.4) and centrifuged at 1,000 × g for 10 min and the supernatant saved. This step was repeated and the supernatants were centrifuged at 40,000 × g for 30 min. The resulting pellet was resuspended in 20 vol of resuspension buffer (10 mM HEPES, 1 mM EDTA, pH 7.4); the mixture incubated at 37°C for 30 min and centrifuged at 40,000 × g for 30 min. The previous step was repeated and then the pellet was washed (resuspended and centrifuged) 2 more times. The final pellet was resuspended in 2 vol of resuspension buffer (which also serves as the assay buffer for NMDA receptor binding) and frozen at -80°C until the day of the assay.

Membrane preparation — COS cells

COS cells were maintained in α-Minimum Essential Media (α-MEM) supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin in a humid, 37°C environment containing 5% CO_2. COS cells were transfected with the human dopamine D_4 receptor cDNA using the technique of electroporation (Spencer 1991). Seventy-two hours after transfection, confluent cell cultures were harvested using PBS with 1mM EDTA and centifuged at 1000 × g for 10 min. Resulting pellets were frozen at -80°C until the day of the binding assay, at which time the cell pellets were thawed on ice and resuspended in 10 vol of cell binding buffer (50 mM Tris-HCL, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, 120 mM NaCl, pH 7.4) using a polytron at half maximal setting for 30 sec. The homogenate was centrifuged at 40,000 × g for 30 min and the final pellet resuspended in cell binding buffer and used for dopamine D_4 receptor binding assays.

Table 1

Competition of loxapine and various radiolabelled ligands for binding to cell surface receptors

Ligand	Receptor Subtype	Brain Region	Human K_i (nM)	Bovine K_i (nM)
[^{3}H] SCH23390	D_1	Caudate	26 ± 3.0	62 ± 7.0
[^{3}H] spiroperidol	D_2	Striatum	24 ± 1.9	26 ± 2.2
[^{3}H] spiroperidol	D_4	COS cells transfected with D_4	7.5 ± 1.4	-
[^{3}H] ketanserin	5-HT_2	Frontal cortex	6.2 ± 0.95	6.6 ± 0.92
[^{3}H] MK801	NMDA	Cortex	-	no effect

Each value is an average of 4 to 6 separated experiments ± SEM. The K_i values were calculated using the Cheng-Prusoff equation (1973):

$$K_i = \frac{IC_{50}}{1 + L/K_D}$$

Receptor binding assays — dopamine, 5-HT_2, NMDA receptors

To perform the receptor binding assays, 0.8 nM of [^{3}H] SCH23390 (D_1 receptor antagonist), 0.5 nM [^{3}H] spiroperidol (D_2 and D_4 receptor antagonist), 0.5 nM of [^{3}H] ketanserin (5-HT_2 receptor antagonist), and 2.0 nM [^{3}H] MK801 (NMDA receptor antagonist) were incubated with 150 µg of membrane proteins in a final volume of 1 ml. Nonspecific binding was determined in parallel assays in the presence of 1 µM (+) butaclamol (D_2 and D_4 assays), 10 µM cis-flupenthixol (D_1 assays), 2 µM methysergide (5-HT_2 assays) and 50 µM MK801 (NMDA assays). Assays using [^{3}H] spiroperidol also included 50 nM ketanserin to occlude the presence of serotonergic sites. For the competition experiments, varying concentrations of loxapine were included in the assay tubes. Incubations for the D_1, D_2, 5-HT_2 and NMDA receptors were performed at 25°C for 90 min, 25°C for 60 min, 37°C for 15 min and 25°C for 120 min, respectively. D_4 receptor binding assays with COS cells were incubated at 22°C for 120 min using the cell binding buffer described in the membrane preparation section. At the end of the incubation, the bound and free ligands were separated by rapid filtration on Whatman GF/B filters, which were washed 3 times with 5 ml of cold filtration buffer: (50 mM Tris-HCL, 1.0 mM EDTA, pH 7.4) for the [^{3}H] spiroperidol and [^{3}H] SCH23390 assays, (50 mM Tris-HCL, pH 7.4) for [^{3}H] ketanserin assays, and (10 mM HEPES, 1 mM EDTA, pH 7.4) for [^{3}H] MK801 assays. Bound radioactivity was measured using a Beckman Scintillation Counter (model LS 5000TA).

Data analysis

The binding data were analyzed as previously described (Kazmi and Mishra 1987). In brief, curves were analyzed using weighted nonlinear curve-fitting programs obtained from GraphPad Prism software (San Diego CA, USA), and IC_{50} values were obtained from these curves. Data were analyzed for either 1 site or multiple binding sites including statistical analysis comparing "goodness of fit" between 1 or 2 affinity state models.

RESULTS

The results of competition experiments carried out with loxapine in human and bovine D_1, D_2, D_4, 5-HT_2 and NMDA receptors are summarized in Table 1. All competition curves exhibited a single class of binding sites, in agreement with several previously published reports (see Seeman 1980). [3H] SCH23390 binding in the presence of loxapine gave K_i values of 26 ± 3.0 nM and 62 ± 7.0 nM in the human and bovine membranes, respectively (see Figure 1). A K_i value of 24 ± 1.9 nM was obtained with [^{3}H] spiroperidol and loxapine assays using human caudal membranes. Assays involving bovine membranes yielded an almost an equal K_i of 26 ± 2.2 nM, as shown in Figure 2. Figure 3 illustrates the binding of [^{3}H] spiroperidol and loxapine, producing an K_i of 7.5 ± 1.4 nM in membranes made from COS cells, which were transfected with human D_4 cDNA. It must be noted that, because of the unavailability of a specific ligand to measure D_4 affinity, the best alternative was to use these transfected COS cells. As such, the results using transfected cells must be interpreted with caution until new ligands become available to make a direct comparison with results from human caudal membranes. Furthermore, the competition between loxapine and the 5-HT_2 antagonist [^{3}H] ketanserin in both the human and bovine frontal cortex yielded an almost equal and surprisingly high affinity of loxapine for the 5-HT_2 receptors (6.2 ± 0.95 nM and 6.6 ± 0.92 nM, respectively) (see Figure 4). Lastly, [^{3}H] MK801 and loxapine binding was not displaced by any concentration of loxapine in bovine cortical membranes (see Figure 5).

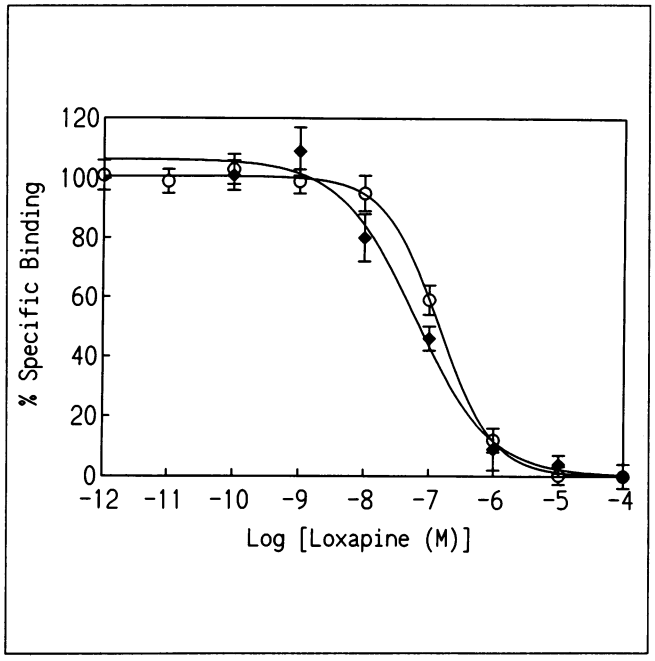

Figure 1. Computer-fitted competition curve of [^{3}H] SCH23390 (D_1 receptor antagonist) binding in the presence of loxapine in human caudal membranes (♦) and bovine striatal membranes (○). Each value for each curve is the average of 4 separate experiments ± SEM.

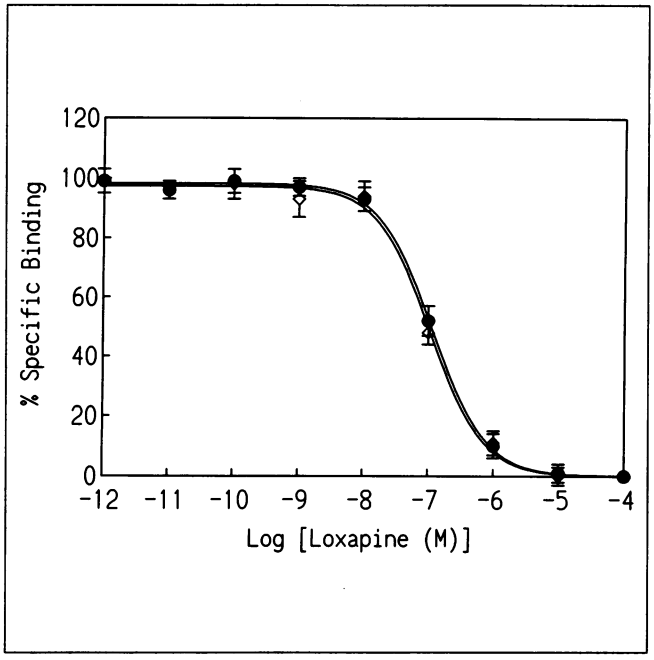

Figure 2. Computer-fitted competition curve of [^{3}H] spiroperidol (D_2 receptor antagonist) binding in the presence of loxapine in human caudal membranes (◊) and bovine striatal membranes (●). Each value for each curve is the average of 4 separate experiments ± SEM.

In comparing competition experiments involving the human membranes only, the rank order of potency of loxapine for the various receptors appears to be as follows: 5-$HT_2 \geq D_4 >>> D_1 > D_2$. In a very similar fashion, the rank order of potency for loxapine to the bovine tissues is as follows: 5-$HT_2 >>> D_2 \geq D_1$. Although the order of affinity for D_1 and D_2 are reversed in the 2 species, it is clear that, in both cases, the affinity of loxapine for 5-HT_2 and for D_4 is much greater than any of the other affinities.

DISCUSSION

Over the past 2 decades, loxapine has been prescribed in Canada as an effective antipsychotic drug for the treatment of schizophrenia. Unfortunately, the greatest drawback to the therapeutic potential of this drug is the presence of extrapyramidal signs and symptoms (EPS) such as tardive dyskinesia. Nevertheless, it must be mentioned that, of the neuroleptics used in clinical practice, loxapine appears to have a relatively lower incidence of extrapyramidal side effects than the high potency neuroleptics (Schwartz and Brotman 1992; Seeman 1981; Carlyle et al 1993). In order to better characterize the pharmacological properties of loxapine and, further, to provide a clear understanding of its clinical efficacy and undesirable side effects, the effects of loxapine on the various receptor subtypes in 2 mammalian species were investigated. The data clearly illustrate that loxapine causes a significant inhibition of ligand binding to both serotonergic and dopaminergic receptors, whereas its interactions with the NMDA receptor are negligible.

Although the therapeutic effects of typical neuroleptics have been attributed to D_2 receptor blockade, so have the extrapyramidal syndromes such as tardive or acute dyskinesias (Burt et al 1977; Seeman 1980, 1987). The atypical neuroleptic clozapine shows a distinct dissociation between antipsychotic activity and extrapyramidal effects, and rarely displays the tardive dyskinesia and cataleptogenesis associated with chronic typical neuroleptic treatment. A possible explanation for this dissociation may be that clozapine has a higher affinity for the D_4 receptor than for the D_2 receptor (Van Tol 1994). Therefore, the clinical benefit of clozapine may lie in its D_4 receptor antagonism. The lack of EPS may be due to its low affinity for the D_2 receptor. Neuroleptic-induced EPS is thought to involve D_2 receptors in the basal ganglia. D_4 receptors have been shown to have a predominantly cortical and mesolimbic distribution in the brain. Thus, the lack of EPS associated with clozapine may also be due to its low affinity for striatal D_2 receptors (Seeman 1995).

Loxapine is regarded as a typical neuroleptic since it induces catalepsy and tardive dyskinesia, and is thought to

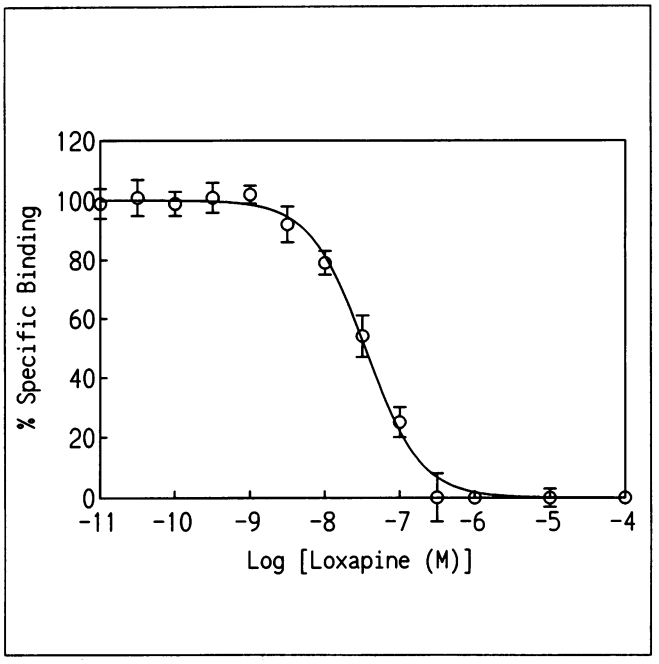

Figure 3. Computer-fitted competition curve of [^{3}H] spiroperidol binding in the presence of loxapine in COS cell membranes transfected with human D_4 receptor cDNA. Each value is an average of 6 experiments ± SEM.

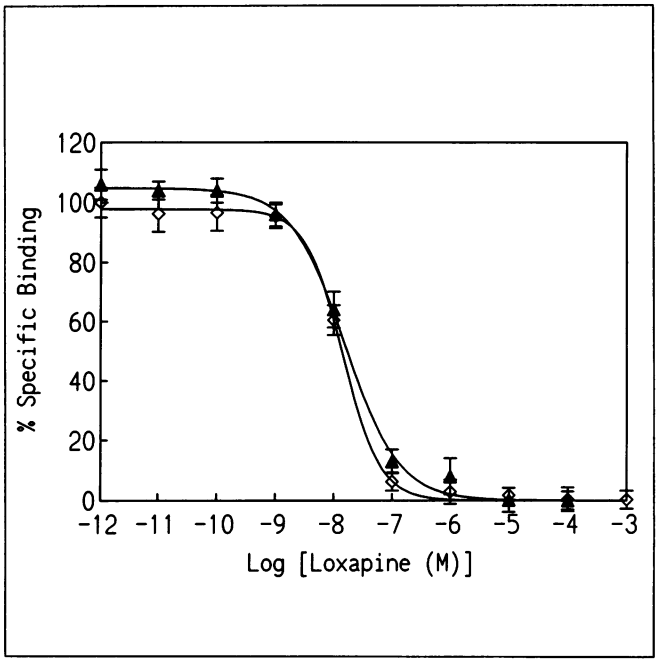

Figure 4. Computer-fitted competition curve of [^{3}H] ketanserin (5-HT_2 antagonist) binding in the presence of loxapine in human cortical membranes (◊) and bovine cortical membranes (▲). Each value is an average of 4 experiments ± SEM.

accelerate dopamine turnover in the striatum (Sayers et al 1975, 1977). Structurally, clozapine and loxapine belong to the class of dibenzoxazepines and are highly similar, only diverging in the spatial location of a chlorine atom, and the replacement of the diazepine group in clozapine with an oxazepine structure in loxapine (Coupet et al 1979; Matsubara and Meltzer 1989). The presence of EPS may result from the relatively strong affinity of loxapine for dopamine D_2 receptors. The results support the hypothesis that D_2 receptor antagonism could be the cause of neuroleptic-induced EPS, since loxapine has a strong affinity for D_2 in both the human and bovine species. Yet, like clozapine, loxapine shows a preferential and higher binding affinity for dopamine D_4 (see Figure 3). It should be noted, however, that these experiments were carried out in transfected COS cells. Thus, the major pharmacological difference between loxapine and clozapine appears to be the higher affinity of loxapine, as compared to clozapine, for the D_2 receptor. And so, although loxapine is a typical neuroleptic, and causes EPS possibly because of its affinity for D_2 receptors, it may produce fewer EPS than other typical neuroleptics since, like clozapine, it has an affinity for the D_4 receptor (Seeman 1995).

The dopaminergic mechanism of neuroleptic activity is now generally accepted and involves the increase of dopamine D_2 receptor density in the brain (Buckland et al 1992). Recently, the role of serotonin in the mediation of antipsychotic drug action has also been investigated by several groups (Lee and Tang 1984; Matsubara and Meltzer 1989; Wei and Niu 1990). Matsubara and Meltzer (1984) found that, in the rat frontal cortex, both clozapine and loxapine were able to down-regulate 5-HT_2 receptors after acute treatment. Chronic drug treatment with loxapine and clozapine in the rat brain also significantly decreased 5-HT_2 receptors by approximately 50%, but the treatment had no effect on D_2 receptor density (Lee and Tang 1984). In the study outlined in this paper, loxapine displayed a very strong affinity for the 5-HT_2 receptor in bovine and human tissues, almost equal to its affinity for the D_4 receptor in transfected COS cells, which suggests that acute loxapine treatment could be effective in the down-regulation of serotonin receptors.

In conclusion, the results indicate that clozapine and loxapine share similar affinities for 5-HT_2 receptors in the human and bovine brains, and for D_4 dopamine receptors in transfected cells. This fact is in agreement with previous investigations that have been performed in the rat brain (Coupet et al 1979) and in COS-7 transfected cells (Van Tol et al 1991). The fact that these 2 drugs differ only in terms of loxapine's affinity for the D_2 receptor, unlike clozapine, may

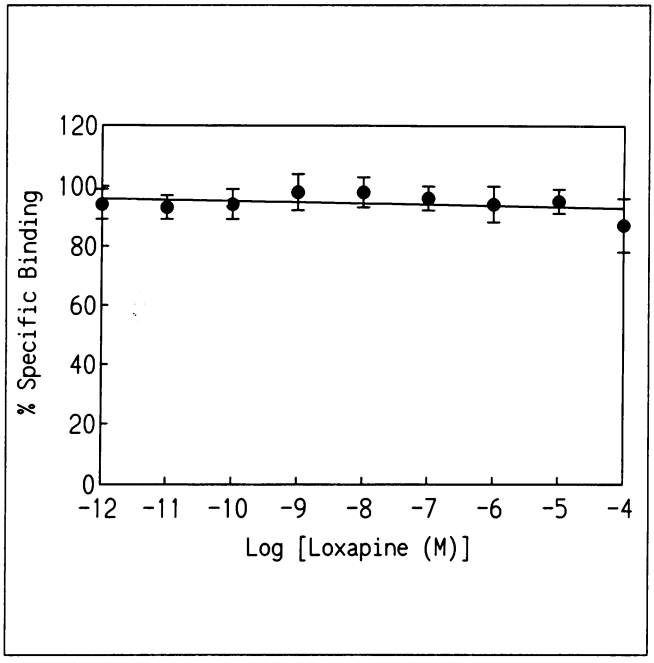

Figure 5. Computer-fitted competition curve of [^{3}H] MK801 (NMDA receptor antagonist) binding in the presence of loxapine in bovine cortical membranes. Each value is the average of 4 separate experiments ± SEM.

explain the EPS that is associated with loxapine but not with clozapine. The drug-receptor interactions defined by this study contribute to the understanding of the manner in which the typical neuroleptic loxapine may exert its therapeutic effects as well as associated EPS. Further work may probe the interactions of loxapine with dopamine and serotonin receptors at the molecular level of mRNA expression and at the functional level (e.g., activation and inhibition of second messengers) in order to characterize the occurrence of tardive dyskinesia further or the clinical mode of action of loxapine. The design of loxapine analogues addressing both positive and negative symptoms of schizophrenia could provide a greater therapeutic potential for schizoaffective disorders in the future.

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