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SPIPERONE: A LIGAND OF CHOICE FOR NEUROLEPTIC RECEPTORS

1. KINETICS AND CHARACTERISTICS OF *IN VITRO* BINDING

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Abstract—A binding assay for neuroleptic receptors has been developed with spiperone as the labelled ligand. As compared to haloperidol, spiperone showed a 2-times higher ratio of specific versus aspecific binding, a 10-fold greater association constant and a slower dissociation of the receptor ligand complex.

The receptor sites labelled by spiperone appeared to be for a great deal similar to those of haloperidol, however certain differences were apparent; the number of receptor sites per gram of tissue was found to be higher for the former; spiperone showed a biphasic receptor ligand dissociation curve which was not observed for haloperidol; also a slight difference in physical stability between spiperone and haloperidol binding sites was noted. Inhibition studies using antagonists and agonists in comparison with the pharmacological profile of the compounds showed that the receptor sites labelled by both ligands are mainly of dopaminergic nature, but also a serotonergic and to a minor extent a noradrenergic component should be involved. Within the striatum haloperidol binding sites seemed to be relatively more related to dopaminergic sites whilst the spiperone binding sites appeared to comprise a higher serotonergic component.

It is concluded that spiperone is a more suitable ligand than haloperidol for studying the neuroleptic receptors. The use of different labelled ligands provided evidence for the heterogeneity of the neuroleptic binding sites in the striatum.

The concept of the dopamine receptor blockade has been the main guideline for research on the mechanism of action of neuroleptics. The idea was originated from the observations that the behaviour provoked by dopamine-mimetic agents was readily antagonized by neuroleptic drugs (cf. ref. 1). An important contribution supporting the hypothesis was the finding that neuroleptics enhance considerably the dopamine turnover, which is in turn reversed by dopamine agonists [2]. Moreover, the behavioural and biochemical effects of neuroleptics appeared to be exclusively localized in the dopaminergic brain areas (cf. ref. 1).

Through recent *in vitro* binding studies more direct evidence was obtained of the existence of receptors in the brain which specifically bind dopamine and related agonist [3, 4] as well as neuroleptics [5, 6]. These binding sites mainly occurred in the striatum [5, 6] but were also found in other dopaminergic areas of the CNS [7]. [^{3}H]Haloperidol was used to label the neuroleptic receptors [3–9], and the dopamine receptor was more specifically labelled by [^{3}H]dopamine and [^{3}H]apomorphine [4]. The specificity of the binding was first assessed by the stereospecific displacement of the labelled ligand ((+)-butaclamol the active enantiomer, (−)-butaclamol inactive) and secondly by the highly significant correlation between the relative receptor affinity of large series of various neuroleptics and their pharmacological and clinical potency [4, 8, 9]. Subcellular distribution studies showed that the specific [^{3}H] haloperidol binding sites were mainly enriched in the microsomal cell fraction [7]. Thus in contrast to previous allegations, they are apparently not associated with synaptosomes [3, 6].

The heterogeneity of the neuroleptic receptor population has not yet been established. The pharmacological [10] and clinical [11] profile of neuroleptics include many aspects related to different brain functions. How heterogeneous is the receptor population in the striatum, the brain region used as prototype in most studies, in which way do the receptor sites in different brain regions differ and where are the receptors which are responsible for the antipsychotic action of the drug, localized? Regarding these questions we started *in vitro* binding study, using different [^{3}H]neuroleptics, to find out if the receptor sites can be differentiated.

In the present study the kinetics and binding characteristics of the new ligand [^{3}H]spiperone are compared to those of the classically used [^{3}H]haloperidol. The relative binding affinity of a large number of various compounds for [^{3}H]spiperone and [^{3}H]haloperidol binding sites is regarded in view of the differential pharmacological activity of the drugs.

MATERIALS AND METHODS

Tissue preparation. Female Wistar rats (150 g) were decapitated and the brains were immediately removed. The striatum was rapidly dissected and placed either in ice-cold sucrose (0.25 M) for the preparation of the microsomal cell fraction, or in Tris–HCl buffer (0.05 M, pH 7.7) for the preparation of a total particulate cell fraction. The microsomal cell fraction was obtained by homogenizing the tissue in 10 vol. of 0.25 M sucrose using a Duall homogenizer and subsequent centrifugations, at 1086 *g* for 10 min, at 30,894 *g* for 10 min and at 177,700 *g* for 60 min. All the particulate fractions were washed once. The total particulate cell fraction was prepared

according to Burt *et al.* [3]. The tissue was homogenized in 40 vol. of ice-cold Tris–HCl buffer with an Ultra Turrax. The homogenate was centrifuged at 4° at 35,000 *g* for 30 min, the pellets were twice washed with cold buffer and recentrifuged. Finally the pellets (microsomal or total particulate) were suspended in an appropriate vol. of salt-buffer according to Burt *et al.* [3], i.e. Tris–HCl, 0.05 M, pH 7.6 containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μM pargyline and 0.1% ascorbic acid. The tissue preparations were preincubated for 5 min at 37° and then kept in ice until they were used.

Incubation procedure. A standard assay was performed as follows: an incubation mixture of 2.2 ml of salt-buffer contained 0.025 g striatal tissue, 4.4 pmoles labelled ligand (final concentration 2×10^{-9} M) and 0.44 nmoles (−)-butaclamol (final concentration 2×10^{-7} M). Blanks were obtained by replacing the inactive (−)-butaclamol by the very potent (+)-enantiomer respectively in 1000-fold excess of [^{3}H]haloperidol or in 10,000-fold excess of [^{3}H]spiperone. The incubation was run for 10 min at 37° and stopped by rapid filtration under suction through Whatman GF/B glass fiber filters using a Millipore 3025 sampling manifold. Filters were washed twice with 5 ml of ice-cold Tris-buffer and transferred into counting vials. The time for filtration and washing did not exceed 8 sec. Instagel (10 ml) was added and vigorous shaking followed for 15 min. The radioactivity was measured in dis./min in a Berthold liquid scintillation spectrometer. The stereospecifically displaceable binding was calculated as the difference between the amount of radioactivity bound in the presence of (−)-butaclamol and (+)-butaclamol.

Determination of IC_{50}-values. The concentration of the drug producing a 50 per cent inhibition of the stereospecifically displaceable binding of the labelled ligand under standard conditions was estimated graphically. Inhibition curves (log (dis./min) of bound ligand in ordinate versus −log (inhibitor concentration) in absciss) were composed of at least 5 points on the decline of the curve. The upper and lower limits of the curves were fixed respectively by the control values of the assays in the presence of (−)-butaclamol and the blanks in the presence of (+)-butaclamol. The intersection point between the inhibition curve and the line representing 50 per cent stereospecific binding gave the pIC_{50}-value (i.e. $-\log IC_{50}$). The mean $pIC_{50} \pm$ S.E.M. was calculated from three independent determinations using freshly prepared tissue and drug solutions, and performed in duplicate.

Drugs. Tetrahydroisoquinolins and *N,N*-dipropyldopamine were generously provided by Prof. Dr. J. Cannon. Tryptamine-HCl, serotonin-creatinine sulphate and dopamine-HCl were provided by Aldrich Europe, Belgium. Other drugs were obtained from various pharmaceutical companies. [^{3}H]Haloperidol (spec. act. 8.5 Ci/m-mole) and [^{3}H]spiperone (spec. act. 9 Ci/m-mole) were obtained from the Radiochemical department of Janssen Pharmaceutica. Stock solutions of the nonlabelled drugs were prepared at a concentration of 10^{-4} M in 0.1% cremophore (a surfactant obtained from B.A.S.F., Germany) [12].

RESULTS

In a standard binding experiment (see Methods) using a total particulate cell fraction the binding of

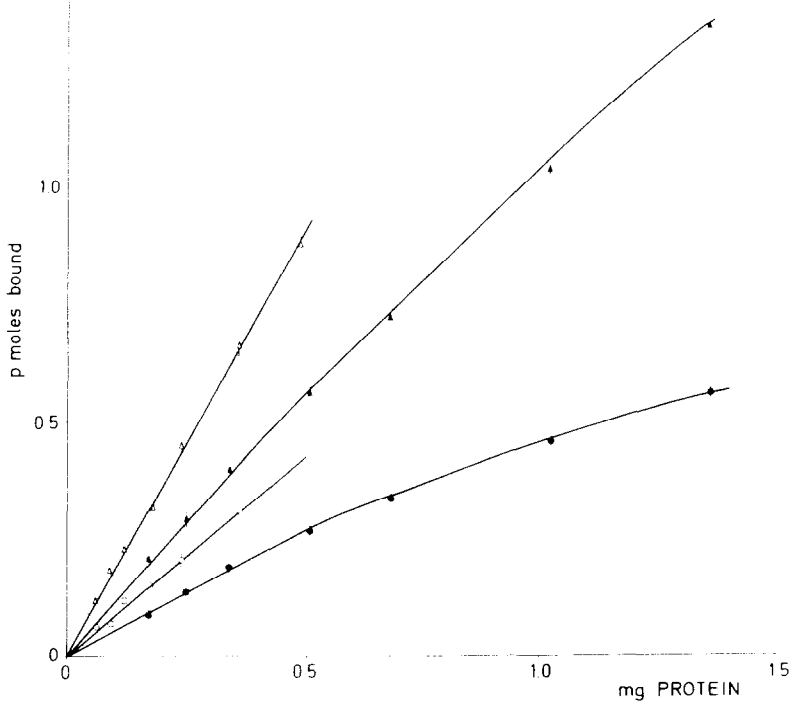

Fig. 1. Stereospecific displaceable binding (pmoles per 2.2 ml standard assay minus blank) of [^{3}H]spiperone (△ and ▲) and of [^{3}H]haloperidol (○ and ●) with increasing protein concentration using a microsomal cell fraction (open symbols) or a total particulate cell fraction (black symbols) from rat striatum.

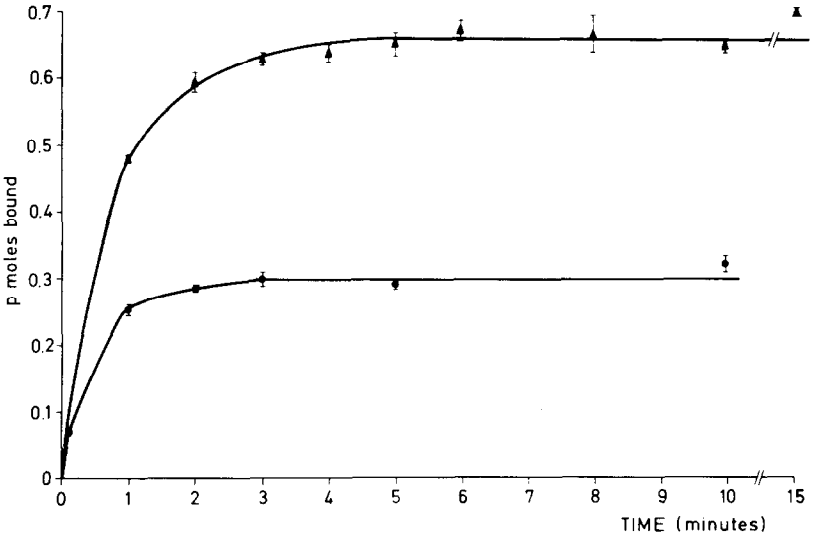

Fig. 2. Stereospecific binding (pmoles per 2.2 ml standard assay minus blank) using a rat striatal microsomal cell fraction of [^{3}H]spiperone (▲) and [^{3}H]haloperidol (●) along with incubation time.

labelled ligand (2×10^{-9} M) in the presence of (−)-butaclamol equalled the binding in the absence of non-labelled drug and amounted to 53 ± 3 pmoles/g tissue for spiperone and to 31 ± 1 pmoles/g tissue for haloperidol. Even at a concentration of 10^{-5} M (−)-butaclamol did not affect the binding of [^{3}H]spiperone. In contrast (+)-butaclamol appeared to be a potent inhibitor of the binding and displayed

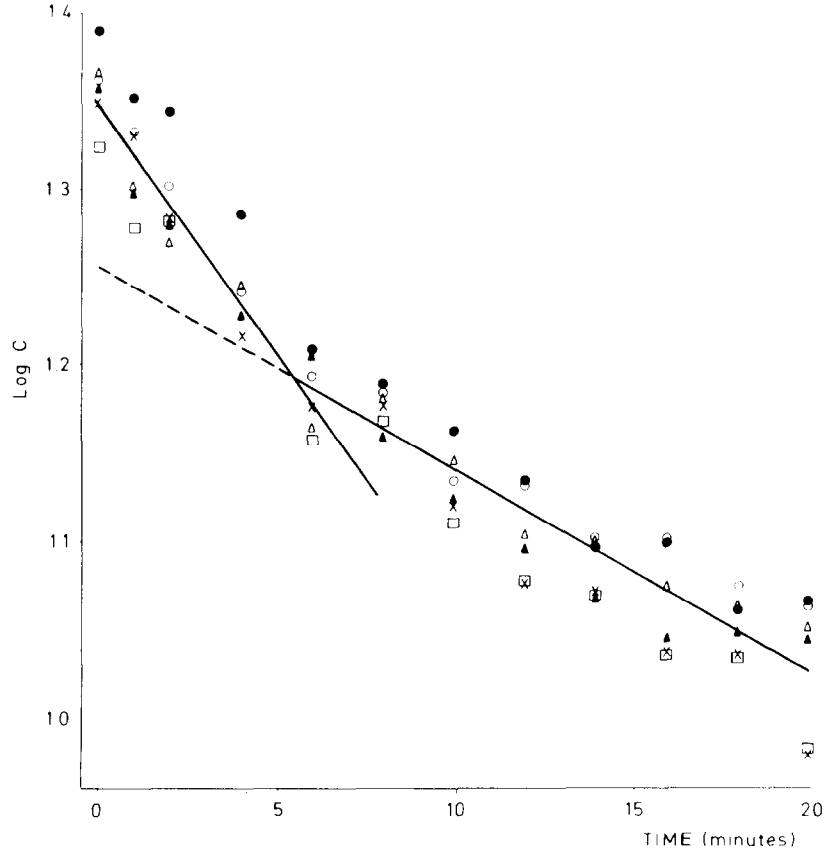

Fig. 3. Dissociation of [^{3}H]spiperone, bound in the presence of (−)-butaclamol to rat striatal microsomal fraction under standard conditions, represented in a first-order reaction plot, i.e. log C versus time (C = pmoles bound per gram tissue). The lines are determined by points obtained from six different experiments each represented by a different symbol.

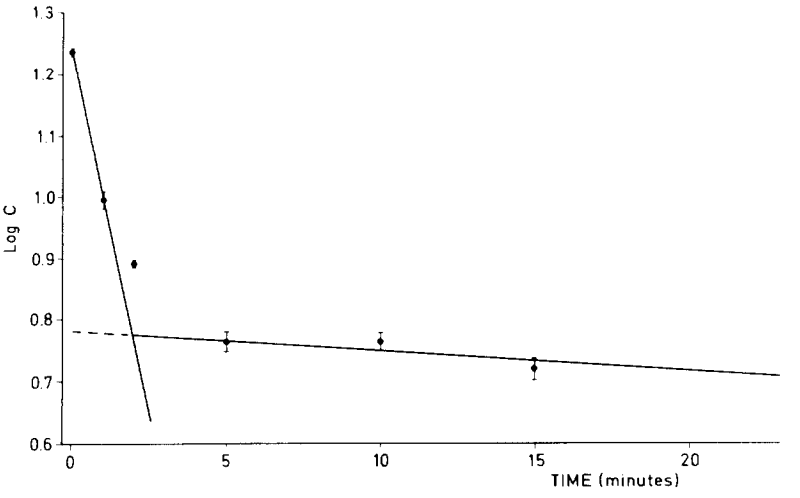

Fig. 4. First-order reaction plot of the dissociation of [^{3}H]haloperidol bound to rat striatal microsomal fraction in the presence of (−)-butaclamol under standard conditions. Each point represents the mean of six different experiments.

an IC_{50}-value of spiperone binding of 3.98×10^{-8} M. The residual labelled ligand retained on the filter in the presence of excess of (+)-butaclamol was taken as the blank value. Using a total particulate cell fraction blanks were 10.8 ± 0.8 pmoles/g tissue for spiperone and 12.1 ± 0.5 pmoles/g tissue for haloperidol. Thus, the stereospecifically displaceable part of the binding of spiperone (43 ± 3 pmoles/g tissue) constituted at least 80 per cent of the total binding and in a standard assay it was 2.26 times greater than the "stereospecific" binding of haloperidol (19.0 ± 0.9 pmoles/g tissue), which represented 61 per cent of the total binding.

Figure 1 shows that for both ligands the stereospecifically displaceable binding was linear with protein concentration up to 0.5 mg per 2.2 ml incubation mixture. When a total particulate tissue preparation was used the average binding per mg protein was 1.14 pmoles for spiperone and 0.51 pmole for haloperidol. Using the receptor enriched microsomal fraction [7] the binding was respectively 1.9 pmoles/mg protein for spiperone and 0.88 pmole/mg for haloperidol.

In all further experiments the tissue preparations were diluted to 0.025 g original tissue per incubation mixture of 2.2 ml, corresponding to 0.36 mg protein for a microsomal fraction (used in all kinetic investigations) and to 1.02 mg protein for a particulate fraction (used for the determination of IC_{50}-values of drugs).

Association. The association between the ligand and the stereospecific receptor sites occurred very rapidly. For both haloperidol and spiperone, equilibrium of binding was achieved within 2 min (Fig. 2a, b). Only two points were taken in the course of the reaction (time 0 and 1) and this was insufficient to look for a linear transformation of the association curve.

Dissociation. Release of the labelled drug bound in the presence of either (−)-butaclamol or (+)-butaclamol was investigated. The experiments were performed by adding to the incubation mixture a 1,000-fold excess of cold ligand after equilibrium of the binding of the labelled ligand was achieved. For [^{3}H]spiperone the blanks (incubated in the presence of (+)-butaclamol) were initially 5.6 pmoles/g tissue. After addition of the cold drug, the blank values diminished very rapidly to 3.4 pmoles/g, and after approximately 6 min they remained constant at this level for at least 1 hr. The release of spiperone in the blanks appeared to be a very complex process and could not be transformed into a linear reaction-time plot. The release of [^{3}H]spiperone bound in the presence of (−)-butaclamol showed first-order reaction kinetics (see Fig. 3). The first-order dissociation curve (log C versus time) was constituted of two straight lines with the intersection point at 6 min, which appeared reproducible in 6 independently performed experiments. The regression line [13] through the points between 0 and 6 min had a slope $b = -0.028\ min^{-1}$, with a standard error of b: $s_b = 0.002\ min^{-1}$. The apparent half-life of dissociation calculated from the slope in the initial phase was 11 min. The regression line through the second part of the dissociation curve (from 8 to 18 min) had a slope $b = -0.012\ min^{-1}$ with $s_b = 0.001\ min^{-1}$ yielding $k'_{-1} = 0.028 \pm 0.002\ min^{-1}$ and $t'_{1/2} = 25$ min. To calculate the first order reaction constant and the half-life of dissociation of the rapid phase, the values of this phase were corrected for the contribution of the slow phase in the initial part of the curve. The resulting straight line had a slope $b = 0.1269\ min^{-1}$ yielding $k_{-1} = 0.292\ min^{-1}$ and $t_{1/2} = 2.4$ min. Release of [^{3}H]haloperidol bound in the presence of (−)-butaclamol occurred much faster, and after 2 min the initial blank values were obtained. A log C versus time plot (Fig. 4) showed two parts (Fig. 4), after the first 2 min, a very slow dissociation was observed, which ran parallel for assays and blanks. The slope of the regression line through the points between 5 and 30 min was $b = -0.003$, with $s_b = 0.0005$ yielding k_{-1} 0.007 ± 0.001 and $t_{1/2} = \sim 99$ min. After correcting the values in the rapid phase for the contribution of the slow phase the line in the log ΔC versus

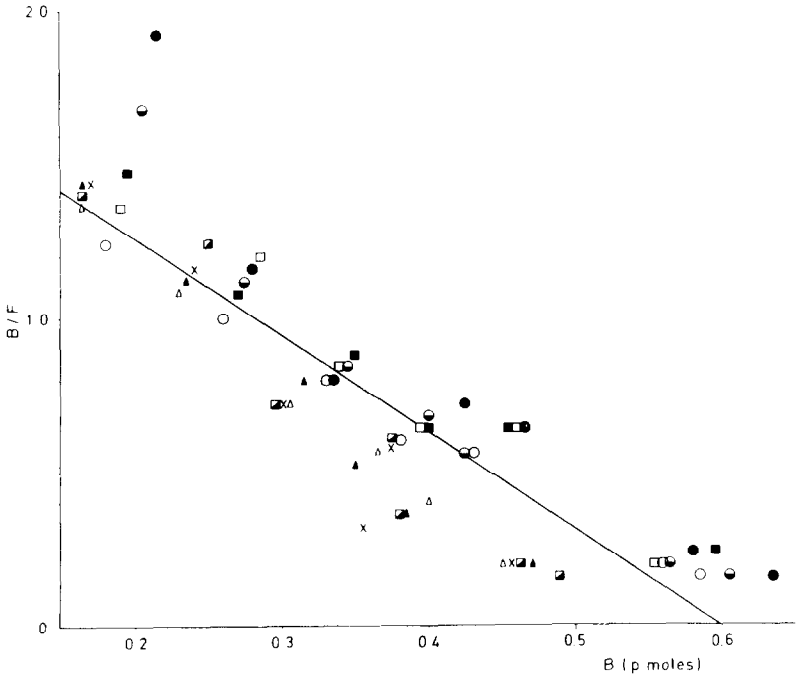

Fig. 5. Scatchard plot of stereospecific [^{3}H]spiperone binding. B = pmoles stereospecifically bound ligand per assay using a microsomal fraction (incubation mixture of 2.2 ml contains 0.36 mg protein). F = total pmoles of ligand present in the incubation mixture minus B. The ligand concentration ranged from 0.2 to 5 nM; nine experiments were performed independently, each represented by a different symbol. The regression line [13] through 58 points revealed: slope $b = -3.2$ (pmoles/2.2 ml)$^{-1}$, $s_b = 0.2$(pmoles/2.2 ml)$^{-1}$; abscissa intercept: $-a/b = 0.60$ (pmoles/2.2 ml), $s_{a/b} = 0.04$ (pmoles/2.2 ml).

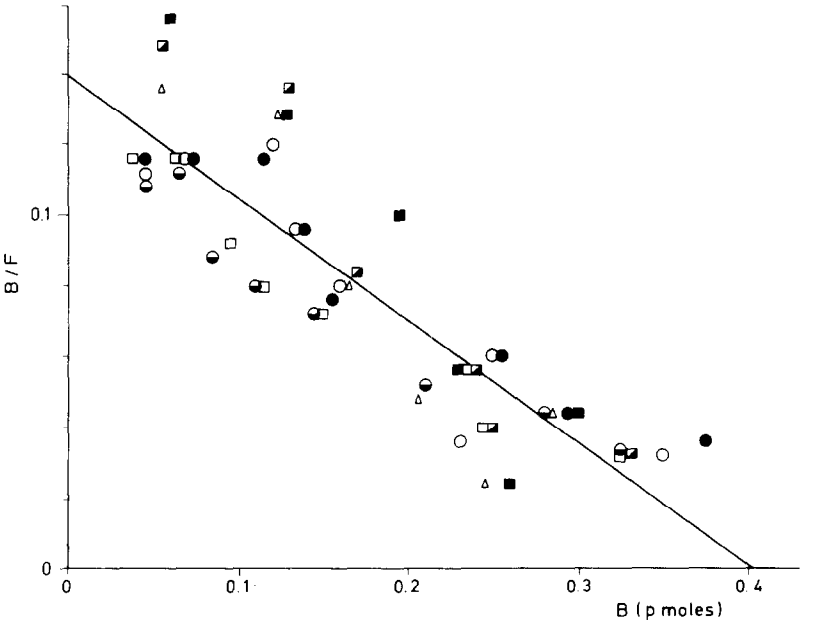

Fig. 6. Scatchard plot of stereospecific [^{3}H]haloperidol binding (cf. legend to Fig. 5). Seven experiments were performed independently, each represented by a different symbol. The regression line [13] through 50 points revealed: slope $b = -0.35$ (pmoles/2.2 ml)$^{-1}$ $s_b = 0.03$ (pmoles/2.2 ml)$^{-1}$: abscissa intercept: $-a/b = 0.40$ (pmoles/2.2 ml), $s_{a/b} = 0.03$ (pmoles/2.2 ml).

time plot revealed a slope $b = 0.551$, yielding $k_{-1} = 1.27\,min^{-1}$ and $t_{1/2} = 0.55\,min$ (not shown in the figure).

Scatchard analysis. Plotting B/F versus B (B = pmoles bound, F = total amount of ligand in pmoles per incubation mixture minus B) for stereospecifically displaceable [^{3}H]spiperone and [^{3}H]haloperidol binding revealed for both ligands a straight line in the concentration range between 0.2 and 5 nM. From 5 nM on, the aspecific binding started to increase rapidly and a bending of the curve was observed. Figure 5 shows the Scatchard plot for "stereospecific" [^{3}H]spiperone binding. From the slope the association equilibrium constant $K_a = (7.0 \pm 0.5)\ 10^9\ M^{-1}$ was obtained, the concentration of stereospecific receptor sites given by the abscissa intercept amounted to (0.27 ± 0.02) pmoles/ml incubation mixture containing 0.36 mg protein of a striatal microsomal fraction. Therefrom the maximum stereospecific binding appeared to be 1.7 pmoles/mg protein. The B/F versus B plot for [^{3}H]haloperidol binding is shown in Fig. 6. The association equilibrium constant derived from the slope of the plot was $K_a = (7.7 \pm 0.7)\ 10^8\ M^{-1}$, and the concentration of receptor sites calculated from the abscissa intercept was (0.18 ± 0.01) pmoles/ml. Thus the maximum stereospecific binding per mg protein for haloperidol is 1.1 ± 0.1 pmoles.

Thermal inactivation. Thermal inactivation accomplished by preincubation of the tissue preparation at different temperatures (37°, 50°, 80°) was investigated for stereospecific and aspecific binding in salt-buffer as well as in Tris-buffer without salts. In the presence of salts, the binding was initially lower than in Tris-buffer. Also blank values, measured in the presence of (+)-butaclamol, were lower in a medium containing salts. However, the presence of salts appeared to protect the receptor against thermal inactivation. Preincubation at 37° up to 1 hr caused only a very slight decrease of binding activity (see Fig. 7), however, in Tris-buffer the decrease for both ligands was twice as great as in the presence of salts. When the preincubation temperature was raised to 50° the loss of binding in the presence of salts was for haloperidol 2.2 and for spiperone 5 times faster than at 37°, whereas in Tris-buffer the inactivation was enhanced 10 and 17 times. Preincubation at 80° for both ligands resulted in both conditions in a complete loss of binding within 5 min. The aspecific binding was not affected by thermal inactivation. The aspecific binding only diminished from 10.9 pmoles/g tissue at 37° to 7.3 pmoles/g tissue at 50° in the case of spiperone and preincubation in Tris-buffer.

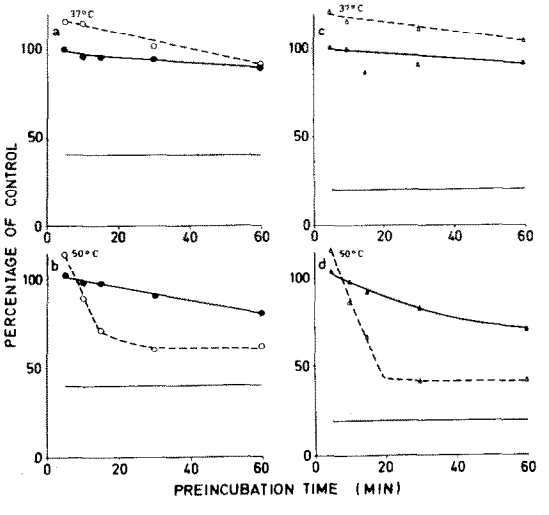

Fig. 7. Thermal inactivation of binding sites in a striatal microsomal fraction for [^{3}H]haloperidol (a, b) and for [^{3}H]spiperone (c, d). Preincubation and incubation in Tris-buffer (– – –) or in salt buffer (— — —). Binding is expressed as the percentage of control binding under standard conditions, which amounted to 18 pmoles/g for [^{3}H]haloperidol and to 30 pmoles/g for [^{3}H]spiperone. The two upper lines in each figure represent the respective binding in the presence of (–)-butaclamol, the two lower lines the binding in the presence of (+)-butaclamol.

Inhibition studies. Two different series of compounds were investigated for inhibition of [^{3}H]spiperone and [^{3}H]haloperidol binding. The first series comprised various antagonists for dopaminergic, serotonergic and noradrenergic action. The pharmacological activity of the compounds was assessed in the ATN-test described by Niemegeers [15], showing in the same experimental session the anti-apomorphine, anti-tryptamine and anti-norepinephrine activity of the drugs. The pIC_{50}-values for [^{3}H]haloperidol and [^{3}H]spiperone binding together with the pharmacological data are given in Table 1. Plotting pIC_{50}-values of spiperone versus those of haloperidol showed a linear correlation (Fig. 8). Calculation of the regression line yielded slope $b = 0.87$ with $s_b = 0.07$, ordinate intercept $a = 0.49$ with the standard error of the intercept $s_a = 0.09$ and correlation coefficient $r = 0.92$. The slope of the line did not appear to be significantly different from 1. The Spearman rank correlation coefficient [14] was highly significant $r_s = 0.904$. The most potent inhibitors in the series were the butyrophenones fluspiperone, spiperone and benperidol, whereas the sedative drugs (e.g. azaperone, promazine, thioridazine, chlorpromazine, pipamperone) were among the weakest inhibitors. Surprising was the extremely high affinity of metopimazine, a noradrenaline antagonist, which reached the activity of the most potent neuroleptics. Aceperone, an α-adrenergic receptor blocker and pizotifen, a serotonin antagonist were only slightly active but did not deviate from the correlation line. On an average, the IC_{50}-values for spiperone binding were 3 times greater than the IC_{50}-values for haloperidol binding (the ratio is given by the antilog of the ordinate intercept of the correlation line). Exceptions were the aminoethyl benzamides, sulpiride and metoclopramide which were 20-fold and 30-fold weaker inhibitors for spiperone than for haloperidol binding. The second series of compounds, shown in Table 2, included a variety of neurotransmitters and neurotransmitter agonists. Again the correlation between the pIC_{50}-values for both labelled ligands appeared to be highly significant (Fig. 9). The regression line was characterized by a slope $b = 0.88$ with $s_b = 0.04$, an intercept $a = 0.05$ and a correlation coefficient $r = 0.98$. The Spearman rank correlation coefficient was $r_s = 0.95$. In the second series, the ratio of IC_{50}-values of spiperone versus haloperidol binding amounted to 10 for ergotamine, the most active compound, and to 2 for the

Table 1. pIC_{50}-values of *in vitro* binding and *in vivo* pharmacological activity of various antagonists

No.	Drug	pIC_{50} (M) ± S.E.M. of ligand binding to rat striatal tissue		Activity in ATN-test in rats*			
		[^{3}H]-Haloperidol binding	[^{3}H]-Spiperone binding	APO	TRYP	NE	Relative potency apomorphine antagonism
1	Fluspiperone base	9.4 ± 0.1	8.74 ± 0.04	----	---	0	1.6
2	Spiperone base	9.4 ± 0.1	8.90 ± 0.03	----	---	0	1.6
3	Benperidol HCl H_2O	9.05 ± 0.04	8.35 ± 0.06	----	-	0	1.0
4	Metopimazine base	8.8 ± 0.2	8.0 ± 0.1	0	0	----	-
5	Octoclothepine maleate	8.5 ± 0.2	8.7 ± 0.2	----	----	---	4.2
6	Methithepine maleate	8.0 ± 0.1	8.0 ± 0.2	----	----	----	6.3
7	Trifluperidol HCl	8.8 ± 0.1	7.85 ± 0.06	----	--	-	1.6
8	Droperidol base H_2O	8.7 ± 0.1	8.1 ± 0.1	----	-	-	1.2
9	Pimozide base	8.5 ± 0.1	7.80 ± 0.05	----	0	0	4.8
10	Butaclamol (+) HCl	8.0 ± 0.2	7.7 ± 0.1	----	---	0	9.6
11	Haloperidol base	8.5 ± 0.1	7.69 ± 0.06	----	-	0	1.6
12	Ethomoxane	8.3 ± 0.1	7.80 ± 0.1	----	--	--	7.2
13	Bromperidol base	7.8 ± 0.2	7.72 ± 0.05	----	-	0	2.8
14	Fluphenazine 2 HCl	7.8 ± 0.2	7.41 ± 0.07	----	--	-	5.5
15	Moperone HCl	8.0 ± 0.2	7.45 ± 0.05	----	-	0	1.8
16	Trifluoperazine 2 HCl	8.0 ± 0.1	7.55 ± 0.09	----	-	0	3.6
17	Flupenthixol 2 HCl	7.7 ± 0.2	7.23 ± 0.05	----	---	---	9.6
18	Thiothixene base	8.2 ± 0.3	7.55 ± 0.05	----	-	--	22
19	Oxiperomide base	7.93 ± 0.08	7.7 ± 0.4	----	-	0	3.2
20	Chlorprothixene HCl	7.55 ± 0.05	7.25 ± 0.05	----	---	---	17
21	Clothiapine base	7.4 ± 0.2	7.1 ± 0.1	----	---	--	9.6
22	Clopimozide base	8.1 ± 0.3	7.03 ± 0.06	----	0	0	15
23	Halopemide base	8.0 ± 0.1	6.9 ± 0.1				-
24	Penfluridol base	7.6 ± 0.1	7.2 ± 0.2	----	0	0	22
25	Pipamperone base	6.5 ± 0.2	5.9 ± 0.1	---	----	--	307
26	Chlorpromazine HCl	6.9 ± 0.1	6.85 ± 0.07	----	---	---	25
27	Thioridazine HCl	7.4 ± 0.2	6.65 ± 0.05	---	--	----	406
28	Pizotifen maleate	6.6 ± 0.1	6.0 ± 0.2	0	----	0	-
29	Azaperone base	6.7 ± 0.2	6.50 ± 0.05	----	----	----	93
30	Aceperone base	6.5 ± 0.1	6.3 ± 0.1	-	-	----	14900
31	Promazine HCl	6.6 ± 0.2	6.0 ± 0.1	----	---	----	307
32	Sulpiride base	7.1 ± 0.1	6.06 ± 0.06	----	--	0	2140
33	Clozapine base	6.4 ± 0.2	5.9 ± 0.2	---	----	---	615
34	Metoclopramide 2 HCl 2 H_2O	6.95 ± 0.05	5.8 ± 0.1	----	--	0	66

* Activity score: ---- very strong antagonist; --- strong antagonist; -- moderate antagonist; - weak antagonist; 0 no antagonistic effect.

weakest inhibitor fitting the correlation line, (3,4-dihydroxy phenylalanine) 2-imidazoline. Noradrenaline was a 15-times weaker inhibitor of spiperone than of haloperidol binding whereas serotonin showed only a 2-fold difference in IC_{50}-value for spiperone and haloperidol.

DISCUSSION

From the kinetic parameters of the binding, summarized in Table 3, it was obvious that [^{3}H]spiperone is a far more suitable ligand than [^{3}H]haloperidol to label the neuroleptic receptors in rat striatum. Indeed, in a standard assay (see Methods) the total amount of bound ligand was 70 per cent higher for spiperone than for haloperidol and, moreover, spiperone exerted a lower aspecific binding. The rate of ligand-receptor association was very fast for both compounds and binding equilibrium was achieved within 2 min. Of importance for the labelling experiments, was the relatively slow rate of ligand-receptor complex dissociation observed with spiperone. The investigations of the spiperone-receptor binding were much easier to perform than those of haloperidol, which dissociated from the receptor with a half-life of 0.55 minutes at 37°. Although the filtration technique was very rapid and filtration was completely accomplished within 8 sec, the low tissue labelling observed with haloperidol might in fact partly be due to its rapid dissociation from the receptor. It is indeed conceivable that during the washing procedure, which is inherent in the technique, the probability to flush away specifically-bound ligand was higher for haloperidol than for spiperone. In that respect a certain amount of information on the [^{3}H]haloperidol binding might be lost. The dissociation curves showed a different picture for both ligands: biphasic for [^{3}H]spiperone, monophasic for [^{3}H]haloperidol (the second part of the curve completely paralleled the blank line). That indicates that spiperone labels a heterogeneous population of receptors with different ligand-receptor dissociation rates. However, the question remained whether the initial dissociation of the

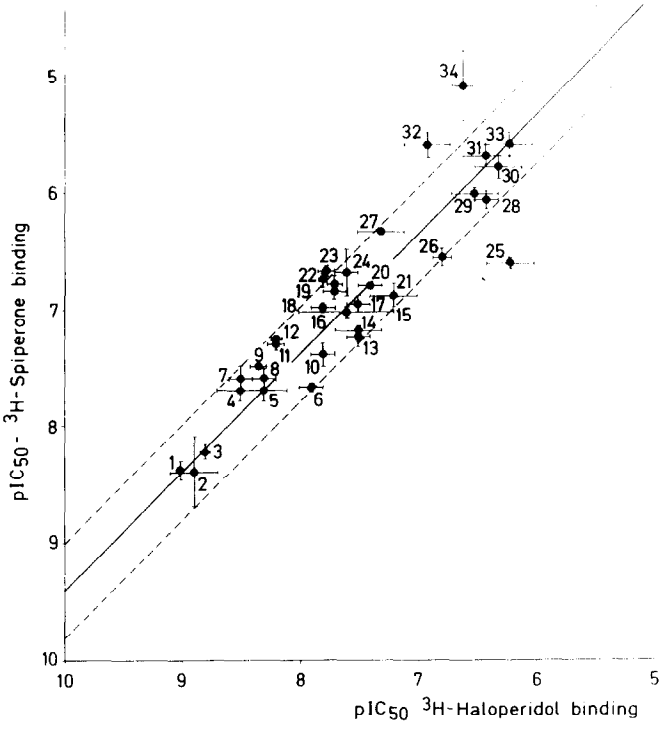

Fig. 8. Correlation between pIC_{50}-values for stereospecific [^{3}H]spiperone binding and [^{3}H]haloperidol binding of various antagonists listed in Table 1.

haloperidol-receptor complex was also composed of two (or more) parts. It could not be answered due to methodological limitations. The equilibrium association constant, deduced from the slope of the Scatchard plot, was 10 times greater for spiperone than for haloperidol. That indicated indeed that spiperone had a higher overall affinity for the receptor sites than haloperidol. The concentration of receptor sites per mg protein was 1.5 times higher for spiperone than for haloperidol. Maybe additional sites, dif-

Table 2. pIC_{50}-values of neurotransmitters and neurotransmitter agonists for [^{3}H]haloperidol and [^{3}H]spiperone binding in rat striatal preparation

No.	Drug	pIC_{50} (M) ± S.E.M. [^{3}H]Haloperidol binding	[^{3}H]Spiperone binding
a	Ergotamine	8.4 ± 0.1	7.4 ± 0.2
b	2-(*N*,*N*-Dipropyl)amino 5,6-dihydroxy tetralin	7.83 ± 0.07	6.9 ± 0.2
c	Apomorphine	7.3 ± 0.1	6.3 ± 0.1
d	2-(*N*,*N*-Dipropyl)amino 6,7-dihydroxy tetralin	7.2 ± 0.1	6.50 ± 0.03
e	2-Amino 6,7-dihydroxy tetralin	6.8 ± 0.1	6.0 ± 0.2
f	Piribedil	6.06 ± 0.06	5.6 ± 0.1
g	*N*,*N*-dipropyl dopamine	6.3 ± 0.2	5.60 ± 0.05
h	2-Amino 5,6-dihydroxy tetralin	5.85 ± 0.04	4.95 ± 0.09
i	Dopamine	6.0 ± 0.1	5.2 ± 0.1
j	Bufotenin	4.7 ± 0.1	4.4 ± 0.05
k	Tryptamine	4.6 ± 0.1	4.05 ± 0.05
l	Serotonin	4.5 ± 0.1	4.10 ± 0.05
m	β-Phenethylamine	4.4 ± 0.1	3.8 ± 0.1
n	(3,4-Dihydroxy phenylamine) 2-imidazoline	3.86 ± 0.05	3.55 ± 0.07
o	Noradrenaline	4.93 ± 0.05	3.75 ± 0.15
	Glycine	Not active at 1 mM	Not active at 1 mM
	γ-Aminobutyric acid	Not active at 1 mM	Not active at 1 mM
	Tryptophan	Not active at 1 mM	Not active at 1 mM
	l-Dopa	25 per cent inhibition at 0.1 mM	Not active at 1 mM
	Acetylcholine	Not active at 1 mM	Not active at 1 mM
	Histamine	25 per cent inhibition at 1 mM	Not active at 1 mM

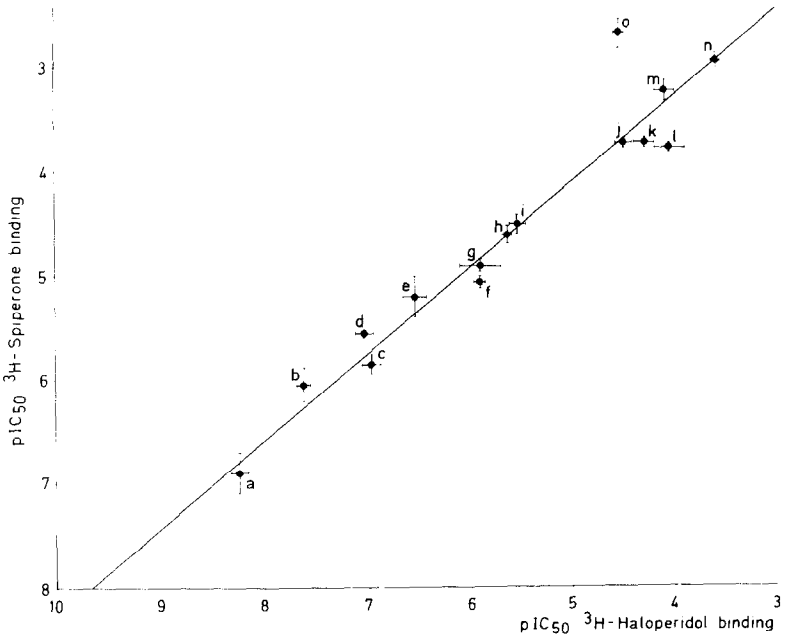

Fig. 9. Correlation between pIC_{50} values for stereospecific [^{3}H]spiperone binding and [^{3}H]haloperidol binding of the agonists listed in Table 2.

ferent from those common for both ligands, are labelled by spiperone. When taking into account both, the biphasic dissociation curve and the higher density of spiperone receptor sites it can be assumed that in the conditions of the present experiments spiperone labelled a more heterogenous receptor population than haloperidol.

More information about the heterogeneity of the binding sites could be obtained from differences in the physical stability of the receptors on one hand and from a differential susceptibility to various inhibitors on the other. In the recent thermal inactivation studies the influence of a physiological salt concentration on the binding appeared to be quite similar for both ligands. At 37° the binding sites were quite stable and the curves were identical with both ligands. At a higher temperature, especially in Tris-buffer, the binding sites of spiperone appeared to be more rapidly degraded than those for haloperidol, thus pointing to a slightly different thermal inactivation of both kinds of receptors.

The inhibition study of the two series of compounds revealed that the receptor sites labelled by spiperone as well as by haloperidol are for a great part involved in the dopaminergic system, however also a serotonergic component seemed to be involved and perhaps, a noradrenergic one. Secondly it appeared that a higher concentration of a non-labelled drug is required for inhibition of spiperone binding than for inhibition of haloperidol binding. This is inherent in the higher affinity of spiperone for the receptor sites but may also be related to the heterogeneity of the receptor population.

From Fig. 8 and Table 1 it further appeared that nearly all the antagonists apparently fit the same correlation line. Those are compounds which in the ATN-test appeared to be either strong apomorphine antagonists devoid of anti-norepinephrine activity, or pure norepinephrine antagonists, or pure 5-HT antagonists, as well as compounds which display a mixed antagonism against two or three of these neurotransmitters. A clear distinction between the different

Table 3. Parameters of spiperone and haloperidol binding

	Spiperone	Haloperidol
Bound ligand (2×10^{-9} M) to particulate fraction		
+ (−)-butaclamol	53 ± 3 pmole.g^{-1} tissue	31 ± 1 pmole.g^{-1} tissue
+ (+)-butaclamol	10.8 ± 0.8 pmole.g^{-1} tissue	12.1 ± 0.5 pmole.g^{-1} tissue
percentage stereospecific	79.6%	61%
Association constant	$K_a = (7.0 \pm 0.5)\ 10^9\ M^{-1}$	$K_a = (7.7 \pm 0.7)\ 10^8\ M^{-1}$
Receptor density per mg protein in a microsomal fraction	$N = 1.7 \pm 0.1$ pmole/mg protein	$N = 1.1 \pm 0.1$ pmole/mg
First order dissociation rate constant	$k_{-1} = 0.292 \pm 0.005\ min^{-1}$ $k'_{-1} = 0.028 \pm 0.002\ min^{-1}$	$k_{-1} = 1.27\ min^{-1}$ $k'_{-1} = 0.007 \pm 0.001\ min^{-1}$
Half-life of dissociation	$t\ 1/2 = 2.4$ min $t'\ 1/2 = 25$ min	$t\ 1/2 = 0.55$ min $t'\ 1/2 = 99$ min

receptor sites could at this stage not yet be made. However, careful inspection of the position of the points relative to the regression line revealed that all of the strong tryptamine antagonists lay to the right hand side of the correlation line, thus indicating that spiperone binding was more than averagely inhibited by these compounds. Although there was a similar tendency in the relative receptor affinity tested *in vitro* and the relative potency of the compounds for apomorphine antagonism (Table 1), the correlation did not exist throughout. Discordances between *in vitro* and *in vivo* observations may always be ascribed to the inability of the compound to reach the appropriate receptor sites *in vivo* (blood brain barrier, aspecific adsorption; unpublished results) in that respect the antiemetic metopimazine is envisaged. However, the less striking variations between the potency ranks provided a good indication that not just one aspect of the neuroleptic action is reflected in the *in vitro* binding assay, but that indeed various components are involved. Multiple regression analysis between the pIC_{50}-values for binding and the various ED_{50}-values in the ATN-test (C. J. E. Niemegeers, to be published) may reveal the relative contribution of the various components in the receptor binding (in preparation). The assumption that the spiperone, more than haloperidol, should label also neuroleptic receptor sites which are related to serotonergic mechanisms, is further substantiated by very recent results obtained from *in vitro* binding studies in the frontal cortex. In this brain area neuroleptic receptor binding was only detectable with spiperone as the labelled ligand, and the most potent inhibitors appeared to be the compounds which in the ATN-test displayed a high anti-tryptaminergic activity, whilst the pure dopamine antagonists were much less active (in preparation). The conclusions which can be drawn from the inhibitory action of the agonists belonging to the second series are very similar to the foregoing conclusions concerning the antagonist. The greater part of the agonists were dopamine-mimetic, so that the decline of the correlation line was defined by the dopaminergic agents. Here also spiperone binding seemed to be relatively more influenced by indoleamines than by catecholamines. The IC_{50} ratios for spiperone versus haloperidol binding were for the serotonin-like compounds 2 to 3 while for the dopaminergic compounds the ratios were between 5 and 10.

Other neurotransmitters (as e.g. gaba, acetylcholine etc.) seemed not to interfere with the neuroleptic receptor binding.

The main conclusions from the present investigations are:

(1) Using a striatal microsomal fraction, the kinetic picture of spiperone binding was far more favourable than that of haloperidol for labelling the receptor sites. Due to its high affinity constant, the slow dissociation rate and the low aspecific binding, spiperone is a very suitable ligand for specific *in vivo* labelling of receptor sites (see papers II and III of this series).

(2) Assuming that the kinetic data were not influenced by certain methodological restrictions, indications were obtained that even within the striatum, the binding sites labelled by spiperone were somewhat different from those labelled by haloperidol.

(3) Spiperone is of considerable interest in view of the heterogeneity of the receptor population related to neuroleptic activity.

(4) The correlation between the IC_{50}-values of spiperone and haloperidol binding for various compounds and comparison of results obtained *in vitro* with data from the *in vivo* ATN-test showed that the neuroleptic binding sites on the microsomal fraction of the striatum are heterogeneous and not only related to the action of dopamine but also to that of 5-hydroxytryptamine and noradrenaline. In pharmacological experiments, spiperone appeared to be a better tryptamine antagonist than haloperidol, the higher involvement of spiperone in serotonergic mechanisms was also to be seen in the *in vitro* study using the striatal tissue.

It can be postulated that when studying different brain regions which are more or less specifically related to the action of a certain neurotransmitter, a more clearcut differentiation can be made between sites labelled either by haloperidol or by spiperone. The study of the binding kinetics in different brain regions may provide further information concerning the mechanism and duration of action of the neuroleptic agents. Finally, it seems no longer utopian that by perseverance and careful investigation the specific receptor sites responsible for a specific pharmacological or clinical effect may be identified.

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