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Brain receptors for antipsychotic drugs and dopamine: Direct binding assays

(schizophrenia/haloperidol/caudate nucleus/neuroleptics/butaclamol)

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ABSTRACT **In order to test the suggestion that antipsychotic drugs act by blocking dopamine receptors in the brain, the direct effects of such neuroleptic drugs were tested on the stereospecific binding of [^{3}H]dopamine and of [^{3}H]haloperidol to rat brain striata and their subfractions. The stereospecific component of binding was defined as that amount of [^{3}H]dopamine or [^{3}H]haloperidol bound in the presence of (−)-butaclamol (an inactive drug) minus that bound in the presence of (+)-butaclamol (a potent neuroleptic drug); 100 nM butaclamol was used for the [^{3}H]haloperidol assay, while 1 μM butaclamol was used for the [^{3}H]dopamine assay. Various antipsychotic drugs inhibited this stereospecific component in both the dopamine and haloperidol assays. These inhibitory potencies correlated with the clinical doses used for controlling schizophrenia.**

Antipsychotic or neuroleptic drugs are thought to act by blocking dopamine receptors in the nervous system (1–5). Hitherto, the evidence for this hypothesis has been indirect. For example, it is known that neuroleptic drugs inhibit dopamine-sensitive adenylate cyclase (5–7), increase the firing rate of dopamine neurons (8), decrease the stimulated release of dopamine from dopaminergic neurons (9), accelerate the turnover of dopamine (10, 11), and block the effects of such dopamine-mimetic drugs as amphetamine and apomorphine (11, 12). The potency of the drugs in these tests is not correlated 1:1 with their potencies in controlling schizophrenia (13, 5). For example, haloperidol and spiroperidol are 20–100 times more potent clinically than chlorpromazine, yet they are equal to or weaker than chlorpromazine in blocking the dopamine-sensitive adenylate cyclase (5–7). The presynaptic coupling-blockade actions of the neuroleptics (9) correlate slightly better with the clinical antipsychotic potencies, but even here the correlation is not 1:1.

Neuroleptics readily concentrate in cell membranes since the drugs are highly surface-active and fat-soluble (14, 15). This high nonspecific solubility in membranes has prevented the identification of specific binding sites for neuroleptics (16). The introduction of the new neuroleptic butaclamol, which has an active (+)-enantiomer and an inactive (−)-enantiomer (17–20), now permits the identification of stereospecific binding sites for neuroleptics along criteria already outlined for the opiate receptor (21, 22).

The present results describe stereospecific radioreceptor assays for [^{3}H]haloperidol and [^{3}H]dopamine, and provide the first direct evidence that neuroleptics compete with dopamine for stereospecific dopamine binding sites in direct proportion to their clinical potencies. Preliminary reports of these results may be found in refs. 4 and 23.

Abbreviations: Buffer A, 15 mM Tris·HCl, 5 mM Na_2EDTA, 1.1 mM ascorbate, 12.5 μM nialamide (pH 7.4); buffer B, buffer A minus nialamide; IC_{50}, inhibitory concentration for 50% reduction in binding.

MATERIALS AND METHODS

The majority of the experiments were done on homogenates or subcellular fractions of rat brain striatum, since this region contains abundant dopamine (24), and presumably abundant dopamine and neuroleptic receptors.

1. Preparation of Crude Homogenate of Rat Brain Striatum. Crude homogenates were prepared from rat striata using male albino Wister rats of between 150 and 250 g. The rats were sacrificed by cervical dislocation, and the brains immediately removed and rinsed in ice-cold 0.9% NaCl. The striata (each about 30 mg) were dissected out within 5 min, and 12–16 striata from 6 to 8 animals were pooled for homogenization in 12 ml of ice-cold 0.32 M sucrose for subcellular fractionation, or an ice-cold solution of 15 mM Tris·HCl, 5 mM Na_2EDTA, 1.1 mM ascorbate, and 12.5 μM nialamide, pH 7.4, (buffer A) for the P_2 fraction. Buffer A was degassed with N_2 for storage. Crude homogenates of the striata were made using a glass homogenizer with a Teflon piston with 0.13–0.18 mm clearance; the piston, rotating at 500 rpm, was passed up and down 20 times.

2. Preparation of P_2 Fraction. The crude homogenate was centrifuged at 1000 × *g* for 15 min at 4° in a Sorvall RC2B refrigerated centrifuge. The supernatant was saved and the pellet was resuspended in 10 ml of 0.32 M sucrose and recentrifuged at 1000 × *g*. The pellet (P_1) was discarded, and the two supernatants were pooled and centrifuged at 20,000 × *g* for 30 min at 4°. The supernatant was discarded, and the pellet (P_2) was saved for further use.

3. Preparation of Crude Synaptosome Fraction. The P_2 fraction was in 6 aliquots layered over a discontinuous sucrose density gradient composed of 4.5 ml of 1.2 M sucrose and 4 ml of 0.8 M sucrose in polycarbonate centrifuge tubes. The tubes were centrifuged at 50,000 × *g* for 2 hr at 4° in a Beckman L265B Spinco centrifuge, using a swinging bucket rotor.

4. Dopamine Binding Assays. The binding of [^{3}H]dopamine and the effects of various neuroleptics on this binding was studied by two methods:

(*a*) *"Inulin + Filter" Method for Measuring [^{3}H]Dopamine Binding.* For the binding assay, the following aliquots were added to a 12 × 75 mm glass test tube, using Eppendorf pipettes with polypropylene tips (delivery reproducibility of ±2%; Brinkmann Instrument Co.): 100 μl of a 0.025% solution of [*carboxyl*-^{14}C]inulin (2.03 Ci/g, Mallinckrodt nuclear), 200 μl of 3.5 nM [^{3}H]dopamine (3,4-dihydroxyphenyl [*ethyl*-1-^{3}H(N)]ethylamine, specific activity, 8.4 Ci/mmol; New England Nuclear Corp., Boston), 200 μl of various solutions of neuroleptics or nonradioactive dopamine, and finally, 200 μl of the crude synaptosome suspension. All stock solutions were prepared in buffer A and kept

ice-cold. The mixture was incubated on ice for 30 min and then quickly filtered, using a vacuum pump, through 0.45 μm Millipore filters prewetted with buffer. The underside of each filter was then blotted briefly and its radioactivity ^{14}C and ^{3}H radioactivity was determined in a liquid scintillation vial containing 10 ml of Aquasol (New England Nuclear Corp.) (25). The radioactive inulin served to correct for the residual supernatant containing the trapped, but unbound, [^{3}H]dopamine left on and in the filter paper as well as between and within the disrupted membranes (26).

(*b*) *Centrifugation Method for Measuring* [^{3}H]*Dopamine Binding.* The majority of the [^{3}H]dopamine assays were done with the P_2 fraction and using a centrifugation method (27). The P_2 was resuspended in a buffer consisting of 15 mM Tris·HCl, 5 mM Na_2EDTA, and 1.1 mM ascorbate (pH 7.4) (buffer B), and the suspension was incubated at 37° for 1 hr. The fraction was then washed twice by centrifugation at 20,000 × *g* for 30 min at 4° and resuspension in buffer B. The final suspension, rehomogenized with 20 strokes as before, contained 0.7 mg of protein per ml.

The binding of [^{3}H]dopamine was done in 10 × 75 mm glass tubes containing 0.5 ml of 3 nM [^{3}H]dopamine (see *Section 4a*, above), 0.5 ml of buffer with various concentrations of neuroleptics or butaclamol, and 0.5 ml of the washed P_2 suspension. The stock solutions and suspension were kept on ice. The mixture was incubated at 22° for 15 min with Vortex mixing every 5 min. If the incubation was at 4°, the absolute amount of binding was reduced by only about 20%; this small temperature effect compares with about a 20-fold difference when one studies dopamine uptake (28, 29) in the higher and more usual dopamine concentration range of 50–800 nM with appropriate cofactors (30–32). After incubation, the tubes were centrifuged at 20,000 × *g* at 4° for 20 min. The supernatant was decanted, and the top of the pellet was rinsed twice with 1 ml of buffer A. The tube was inverted and drained, and the inside walls were wiped with cotton-tipped sticks. One ml of Aquasol was added, and the pellet was dislodged by vigorous mixing. The pellet dissolved within 3 hr and the tube contents were transferred to a liquid scintillation tube with two 1-ml washes of Aquasol. The vials were monitored for ^{3}H. All determinations were made in triplicate at least.

5. Neuroleptic Binding Assays. (*a*) *Centrifugation Method for Measuring* [^{3}H]*Haloperidol Binding.* The P_2 pellet was resuspended in buffer A, centrifuged, resuspended in the buffer, and incubated at 37° for 60 min with constant shaking; this preincubation step greatly improved the amount of [^{3}H]haloperidol finally bound. The material was then washed twice in buffer A (with 20,000 × *g* centrifugations) and finally suspended at a protein concentration of 1 mg/ml in buffer A.

The [^{3}H]haloperidol binding was done in 10 × 75 mm Pyrex glass test tubes containing 0.5 ml of 0.9–3 nM [^{3}H]haloperidol, 0.5 ml of buffer A containing various concentrations of neuroleptics, butaclamol, or dopamine-mimetic drugs, and 0.5 ml of the washed and preincubated P_2 suspension. All samples were in quintuplicate.

After incubating for 30 min at 22°, the tubes were centrifuged (20,000 × *g* for 20 min at 4°). Two 0.5-ml aliquots of each supernatant were pipetted into liquid scintillation vials; Aquasol was added and the vials were monitored for ^{3}H. The method for calculating the number of mol of drug adsorbed per mg of protein has been published (33).

Radioactive haloperidol was obtained by catalytic exchange labeling (using 25 Ci of $^{3}H_2O$; procedure done by New England Nuclear Corp., Boston) and then purifying by silica gel thin-layer radiochromatography, using 9:1 chloroform/methanol; final specific activity was 0.1 Ci/mmol. Dr. P. A. J. Janssen, Dr. J. Heykants, and Dr. J. Brugmans generously donated a sample of [^{3}H]haloperidol specifically labeled in the F-containing ring (0.09 Ci/mmol). [^{3}H]Haloperidol was also obtained from CIS radioactive products (IRE Belgique). The relatively insoluble compounds, such as haloperidol and spiroperidol (spiperone) or apomorphine, were first dissolved at high concentration in ethanol; dilutions were made such that the final ethanol concentration was less than 0.01%.

(*b*) *Dialysis Method for Measuring* [^{3}H]*Haloperidol Binding.* The binding of [^{3}H]haloperidol was also done by dialysis. Sextuplicate samples of 1 ml each of the washed and pre-incubated P_2 fraction (*Section 5a*, above) were placed in boiled and rinsed dialysis bags (7 cm long and, when flat, 1 cm wide). The bags were placed in 16 × 125 mm culture tubes (15 ml, screw cap with rubber liner) containing 10 ml of 1 nM [^{3}H]haloperidol and 100 nM of either (+)-butaclamol or (−)-butaclamol, and also a third nonradioactive neuroleptic. The tubes were rotated in the dark at 4° for 2.5 days in the presence of penicillin or streptomycin. Duplicate aliquots (0.4 ml) were than taken from inside and outside the bag, placed in 10 ml of Aquasol, and monitored for ^{3}H.

(*c*) *Filter Method for Measuring* [^{3}H]*Haloperidol Binding.* The majority of the [^{3}H]haloperidol binding assays were done using the crude homogenate and a filtration method. The freshly prepared crude homogenate in buffer A was centrifuged at 40,000 × *g* for 15 min at 4°, the supernatant was discarded, and the pellet was rehomogenized in buffer A and preincubated for 1 hr at 37°. At this point the suspension was often subdivided and stored at −20°. After thawing, the material was rehomogenized and washed once in buffer A.

The binding of [^{3}H]haloperidol was done in triplicate in 22 × 150 mm Pyrex glass boiling tubes, containing aliquots of 100 μl of 100 nM (+)- or (−)-butaclamol, 100 μl of another neuroleptic or dopamine-mimetic drug, 200 μl of 3 nM [^{3}H]haloperidol, and 200 μl of the preincubated crude homogenate. The mixture was incubated at 22° with Vortex mixing every 5 min. After 30 min, 12 ml of ice-cold buffer A were injected into the boiling tube with a syringe within 1.5 sec, and the entire contents were poured immediately onto a prewetted glass fiber filter (GF/C, Whatman); filtration, using a vacuum pump, was completed in 1 sec. The filter papers were removed from the filtering manifold (Millipore no. 3025) and placed in 10 ml of Aquasol in a liquid scintillation vial for ^{3}H monitoring. The results were calculated in terms of femtomoles of haloperidol bound per mg of homogenate protein.

RESULTS

The binding of [^{3}H]dopamine to sonicated synaptosomes as well as to the P_2 fraction is shown in Fig. 1. The dissociation constant for binding was in the nM range. Unlabeled dopamine (6 nM) displaced the maximum amount of [^{3}H]dopamine, approximately 40 fmol/mg for the P_2 fraction. Haloperidol also competed with [^{3}H]dopamine for binding, and the inhibitory concentration for 50% reduction in binding (IC_{50}) was 10 nM; phenylephrine, up to 0.1 mM, had no effect on [^{3}H]dopamine binding (not shown). Apomorphine and clozapine also competed with [^{3}H]dopamine (Table 1, column 1).

Table 1. IC_{50} values (concentrations for 50% reduction in binding of [^{3}H]dopamine or [^{3}H]haloperidol)

	[^{3}H]Haloperidol binding			[^{3}H]Dopamine binding
Method	Centrifugation	Dialysis SSB	Filter SSB	Centrifugation SSB
Concentration of isotope	0.3 nM	1 nM	1 nM	1 nM
Type of fraction	P_2	P_2	C.H.	P_2
IC_{50} values				
Haloperidol	3 nM	4 nM	20 nM	10 nM
Chlorpromazine	—	—	120 nM	450 nM
Promazine	—	—	5 μM	1.7 μM
Molindone	—	60 nM	—	—
Spiroperidol	—	—	0.4 nM	—
Clozapine	150 nM	180 nM	—	—
Apomorphine	600 nM	—	700 nM	50 nM
Nialamide	—	>1 nM	—	—
Serotonin	0.1 mM			
Norepinephrine	>0.1 mM			
Phenylephrine	>0.1 mM			
Diazepam	0.1 mM			
Diphenylhydantoin	>0.1 mM			

SSB refers to stereospecific binding, which is the amount of [^{3}H]dopamine or [^{3}H]haloperidol bound in the presence of (−)-butaclamol minus the amount bound in the presence of (+)-butaclamol; 100 nM butaclamol was used for the [^{3}H]haloperidol assay, while 1 μM butaclamol was used for the [^{3}H]dopamine assay. C.H. indicates crude homogenate (*Materials and Methods, Section 1*). Glycine, glutamate, and phenobarbital had no effect at 0.1 mM.

Fig. 2 illustrates the competition between the enantiomers of butaclamol and [^{3}H]dopamine as well as between butaclamol and [^{3}H]haloperidol in the P_2 fraction. The (+)-enantiomer of butaclamol was 200 times more effective than the (−)-enantiomer in reducing the binding of [^{3}H]dopamine or [^{3}H]haloperidol. The maximum difference between the displacements produced by the two enantiomers occurred at butaclamol concentrations of 10^{-6} M in the [^{3}H]dopamine assay, and 10^{-8} M in the [^{3}H]haloperidol assay; this maximum difference will be referred to as the stereospecific difference and it amounted to 40 fmol/mg for [^{3}H]dopamine and 55 fmol/mg for [^{3}H]haloperidol. Identical results were obtained with the crude homogenate in the case of [^{3}H]haloperidol, and consequently further detailed work on [^{3}H]haloperidol binding was done with the crude homogenate, and using the glass fiber filter technique.

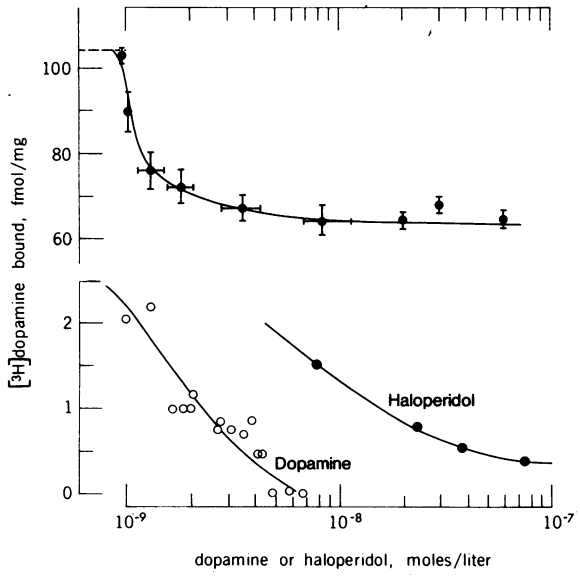

FIG. 1. (*Top*) Binding of [^{3}H]dopamine to the P_2 fraction of rat striatal homogenate, using the centrifugation assay. An average of ten separate assays were used for each point. Concentration of [^{3}H]dopamine was 1 nM. (*Bottom*) Binding of [^{3}H]dopamine to sonicated synaptosomes (rat striatum), using [^{14}C]inulin as a marker for inaccessible water, and filtration through Millipore filters. An average of 30 separate assays were used for each point. The vertical bars are standard deviations, while the horizontal lines represent the concentration range encompassed.

The effects of various neuroleptics were tested on the butaclamol stereospecific difference. For example, Fig. 3 shows that the IC_{50} for chlorpromazine on the stereospecific difference in [^{3}H]dopamine binding was 450 nM where the concentration of either (+)- or (−)-butaclamol was 1 μM. Further IC_{50} values for other neuroleptics, as well as apomorphine, competing for [^{3}H]dopamine binding, are listed in Table 1 (last column).

Sample data for the competition between various neuroleptics and [^{3}H]haloperidol are shown in Fig. 4. In this figure the ordinate is expressed in terms of the % of the maximum stereospecific difference [in the presence of 100 nM (+)- or (−)-butaclamol]. Examples of two different methods, the dialysis method and the filtration method, are shown in Fig. 4. In the filtration method, 12 ml of ice-cold buffer A were flooded into the incubation mixture immediately before filtering. This step served to lower the amount of nonspecific adsorption to the glass fiber filters; this amount of nonspecific adsorption was determined in triplicate for every drug concentration tested, and the amount was subtracted from the total binding in the presence of the membranes. This flooding step, however, lowered the free concentration of all drugs, and desorption from the stereospecific binding sites occurred. For example, waiting 15 sec between the addition of buffer A and the filtration completely abolished the stereospecific difference.

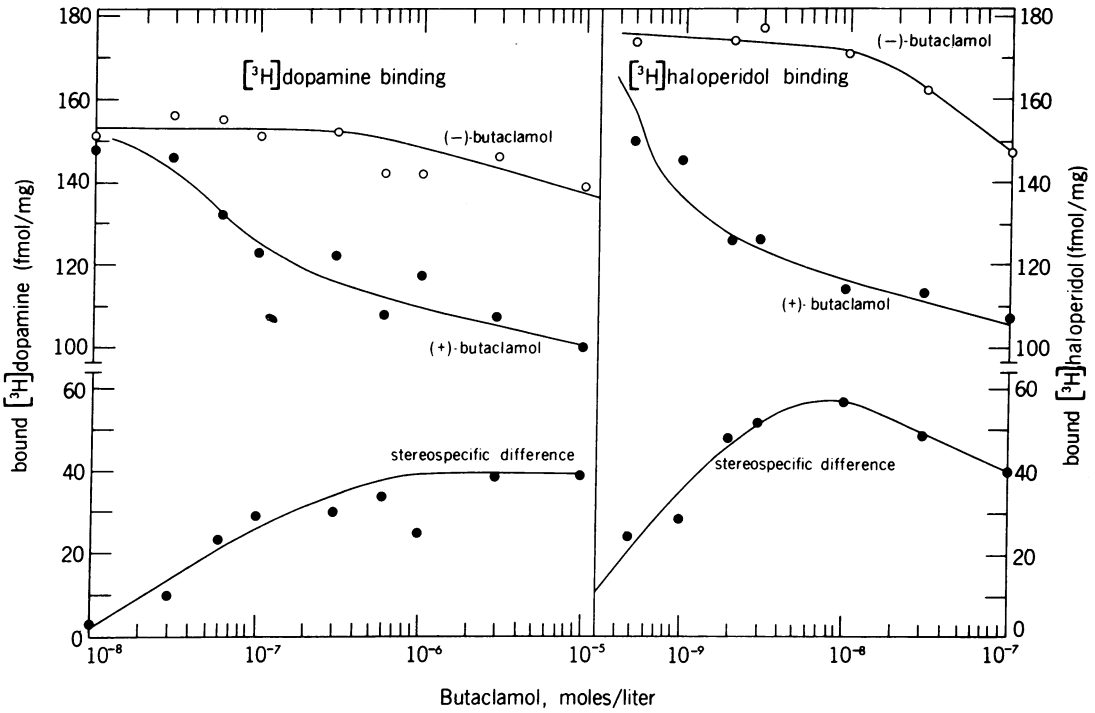

FIG. 2. Competition between butaclamol and [^{3}H]dopamine (*left*), and between butaclamol and [^{3}H]haloperidol (*right*) for binding to rat striatal P_2 fraction and using the centrifugation assay for both. Three to six determinations per point. "Stereospecific difference" refers to the difference in the amount of ligand bound in the presence of (−)-butaclamol minus that in the presence of (+)-butaclamol. The maximum stereospecific difference is approximately the same for [^{3}H]dopamine and [^{3}H]haloperidol.

DISCUSSION

In this series of experiments it has been essential to rule out artifacts (34–36) concerning the stereospecific action of butaclamol; no stereospecific differences were observed for [^{3}H]dopamine or [^{3}H]haloperidol binding to filter papers, or to erythrocyte membranes, albumin, or myelin. It is possible that the stereospecific inhibition of binding of [^{3}H]dopamine by the enantiomers of butaclamol actually represents inhibition of active or facilitated dopamine transport or uptake into the cytoplasmic space of the synaptosome. There are several points that argue against this possibility. First, the dissociation constant for [^{3}H]dopamine binding in the present experiments is of the order of 1 nM, about 200-fold less than that measured for the K_m for [^{3}H]dopamine uptake (29, 30–32). Second, the preparations were greatly disrupted, as indicated by the large inulin space and as seen on

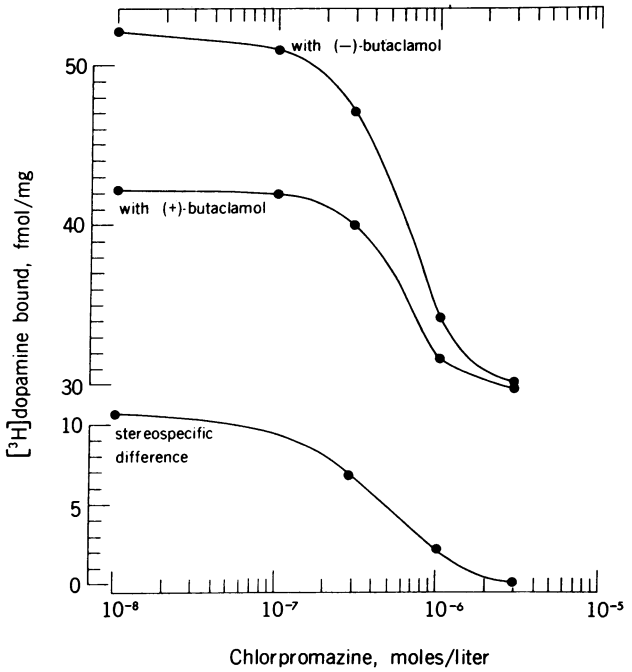

FIG. 3. The effect of chlorpromazine on the binding of [^{3}H]dopamine to rat striatal P_2 fraction (centrifugation method). In the absence of chlorpromazine, the amount of [^{3}H]dopamine binding is defined as the amount bound in the presence of 1 μM (−)-butaclamol minus that in the presence of 1 μM (+)-butaclamol.

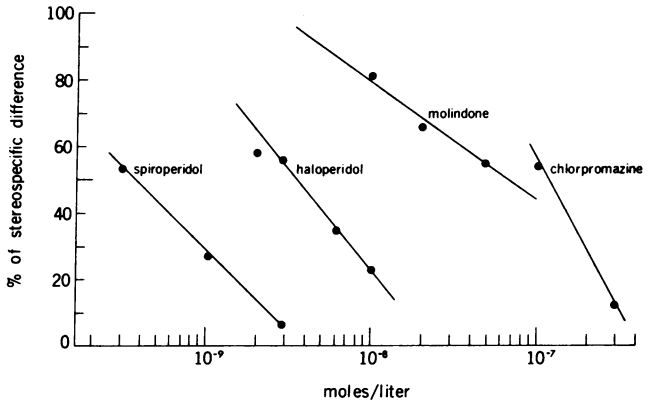

FIG. 4. The effects of various neuroleptics on the binding of [^{3}H]haloperidol to rat striatal fractions. One hundred percent represents the difference in [^{3}H]haloperidol binding in the presence of 100 nM (−)-butaclamol minus that in the presence of 100 nM (+)-butaclamol. The results for chlorpromazine and spiroperidol were obtained on crude homogenates of rat striatum, having a stereospecific difference of 38 fmol/mg and using the filtration assay. The results for haloperidol and molindone were obtained on the P_2 fraction, having a stereospecific difference of 185 fmol/mg and using the dialysis method. Six determinations for each point.

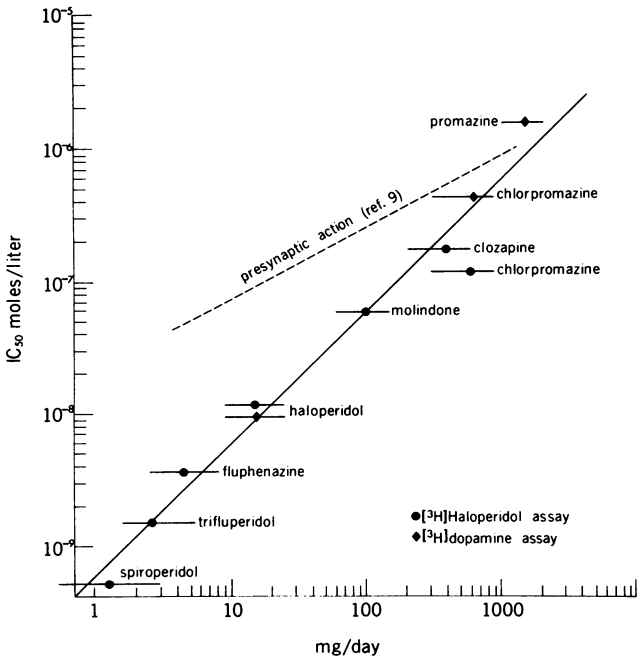

FIG. 5. The IC_{50} values for the various neuroleptics in blocking the binding of [^{3}H]haloperidol (●) or of [^{3}H]dopamine (♦) to rat striatal fractions are plotted against the average clinical doses (in mg/day) for controlling schizophrenia (see ref. 9 for details). The solid line represents a 1:1 relation between the blocking molarity and the clinical dose. (The interrupted line is taken from ref. 9 for comparison; it indicates the presynaptic blocking action of neuroleptics on stimulated release of [^{3}H]dopamine from rat striatal slices.) The IC_{50} values are from an average of the values in Table 1 (SSB columns).

electron microscopy (results not presented). Third, previous work (29, 37) indicates that, with the exception of reserpine, neuroleptics are more potent in causing the release of [^{3}H]dopamine from striatal synaptosomes than in blocking the uptake. High concentrations of (−)-butaclamol did exert some competition in both the [^{3}H]dopamine and [^{3}H]haloperidol assays; (−)-butaclamol, however, is only maximally 99.5% pure, and the possible presence of 0.5% of (+)-butaclamol might account for the 200-fold difference in potency between the enantiomers. Finally, the effect of temperature on binding was minimal, unlike the temperature dependence of dopamine uptake (28, 29).

Fig. 5 illustrates the IC_{50} values for the neuroleptics in blocking the stereospecific binding of [^{3}H]dopamine or [^{3}H]haloperidol plotted against the daily clinical doses for controlling schizophrenia (9). Also included in Fig. 5 is the correlation (from ref. 9) between the clinical doses and the neuroleptic concentrations that block the stimulated release of [^{3}H]dopamine from rat striatal slices. The neuroleptic concentrations that block the stereospecific binding of [^{3}H]dopamine or [^{3}H]haloperidol are lower than those reported in ref. 9, and these concentrations correlate better with the clinical doses. These conclusions were made in our earlier preliminary communication (4). We are now using these assays for examining post-mortem brains of schizophrenics.

Note Added in Proof. We were recently informed by Dr. S. H. Snyder that similar results have been obtained in his laboratory in collaboration with Drs. D. R. Burt, I. Creese, and S. J. Enna.

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