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Dopamine receptors in human and calf brains, using [^{3}H]apomorphine and an antipsychotic drug

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

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ABSTRACT **In order to develop a better dopamine receptor radioligand, [^{3}H]apomorphine was prepared and tested for dopamine-like binding properties in both calf and human brain tissues. Specific binding of [^{3}H]apomorphine was defined as that binding which occurred in the presence of 1 μM (−)-butaclamol (an inactive neuroleptic) minus that occurring in the presence of 1 μM (+)-butaclamol (active neuroleptic). The specific binding was saturable, the number of sites being double that of specific [^{3}H]dopamine binding, and occurred primarily in dopamine-rich regions of postmortem human brains. The binding had a dissociation constant of 0.9 nM for human caudate (2 nM for calf caudate) and was blocked by dopamine and norepinephrine, but not by isoproterenol or (−)-propranolol, distinguishing it from a beta-adrenergic receptor. Since there was little desorption of [^{3}H]apomorphine, the ligand permits extensive washing during routine assays for dopamine receptors, and facilitates biochemical purification of the receptor.**

In order to examine the possible abnormalities in brain dopamine receptors in such diseases as schizophrenia (1–3), Parkinson's disease (4), neuroleptic-induced tardive dyskinesia (5), and others, it is necessary to have a reliable assay for measuring these receptors in small amounts of human brain tissue. The recent introduction of radioreceptor assays for dopamine receptors using [^{3}H]dopamine or [^{3}H]haloperidol (6–10) provides a more direct measurement of brain dopamine receptors than the indirect approach using dopamine-sensitive adenylate cyclase (11–14). There are a number of practical difficulties, however, in [^{3}H]dopamine or [^{3}H]haloperidol. These two ligands, for example, rapidly desorb in a matter of seconds (7, 8). For consistent results, therefore, it is essential to use large amounts of tissue and to separate (i.e., filter) the bound and unbound radioligands quickly and reproducibly; this critical filtration step varies (in speed and other details) between investigators, even in the same laboratory. The specific binding of [^{3}H]dopamine may also be affected by residual endogenous dopamine in the tissue and by the avid uptake of dopamine (in the nM region; ref. 15) by presynaptic terminals in the tissue homogenate.

In searching for an alternate radioligand for monitoring dopamine receptors, it seemed appropriate to choose and prepare [^{3}H]apomorphine, since this compound has been well characterized as a dopamine agonist (16–18). This report describes a reliable radioreceptor assay for dopamine receptors, using [^{3}H]apomorphine.

MATERIALS AND METHODS

The experiments were done on crude homogenates of either calf or human brain tissues. In order to retain all the dopamine receptors, the homogenates were not purified or subfractionated.

Preparation of Calf Caudate Homogenates. Calf brains were obtained fresh from the Canada Packers Hunisett plant (Toronto). The caudates were removed within 2 hr after death, pooled, sliced into small cubes, and suspended in buffer at an approximate concentration of 50 mg of wet weight per ml of buffer. The buffer contained 15 mM Tris·HCl (pH 7.4), 5 mM Na_2EDTA, 1.1 mM ascorbate, and 12.5 μM nialamide. A preliminary crude homogenate of the suspension was made with a glass homogenizer with a Teflon piston (0.13–0.18 mm clearance); this piston, rotating at 500 rpm, was passed up and down 20 times. The crude homogenate was first incubated at 37° for 60 min, and then stored in 3-ml aliquots (1-ml aliquots for human brain tissue) at −20° for future use. Before using, the samples were thawed, resuspended in the glass-Teflon homogenizer (10 up-and-down passes by hand), and centrifuged at 39,000 × g for 15 min at 4°; the supernatant was discarded and the pellet was resuspended in 10 ml of buffer (3 ml for human brain). The suspension was finally homogenized by a Polytron homogenizer (Brinkmann Instrument Co.) at a setting of 7 (full range = 10; setting of 6 for human brain tissue) for 20 sec, using a PT-10 homogenizer probe and a 50-ml polycarbonate tube to contain the suspension. Homogenization at settings higher than 7 led to a loss of protein through the glass fiber GF/B filters used in the radioreceptor assays. The protein (19) concentration of the homogenate was generally around 0.2–0.3 mg per tube. Except where indicated, the homogenates were always kept chilled on ice.

Preparation of Homogenates from Human Brain Regions. Postmortem human brains were obtained from a hospital pathologist who performed the postmortem autopsies of individuals who died suddenly. HB19 was a 19-year-old man (homicide); RR24 was a 24-year-old man (respiratory failure); JR66 was a 66-year-old man (myocardial infarction). The autopsies were generally done between 4 and 18 hr after death, and the entire brains were frozen until ready for frozen dissection, using the manual of Riley (20). Putamen sample 75-62-29 was from a 29-year-old man (car accident); sample 75-63-67 was from a 67-year-old man (myocardial infarction); sample 75-61-45 was from a 45-year-old schizophrenic woman (suicide); sample 75-65-24 was from a 24-year-old man (suicide).

The homogenates from the human brain regions were prepared in the same way as those from the calf caudate, except that the preliminary crude homogenates of human brain were frozen in 1-ml aliquots, and after centrifugation they were resuspended in only 3 ml of buffer. Thus, the protein concentrations of both the human and calf homogenates were kept approximately equal. Optimum homogenization with the Polytron was obtained at a setting of 6 (for 20 sec) using a 16 × 100 mm glass test tube to hold the small 3-ml volume. Polytron settings greater than 6 (for 3 ml) caused a loss in binding sites; for calf homogenates, the optimum occurred at 7 (for 10 ml). The minimum amount of human tissue sufficient to carry out a receptor determination in sextuplicate was 50 mg of wet weight.

Apomorphine Binding Assays. [^{3}H]Apomorphine was prepared twice. The first preparation involved exposing 50 mg of apomorphine·HCl (Merck Sharp & Dohme, Canada. Ltd.) to 25 Ci of ^3H_2O (catalytic exchange procedure done by New England Nuclear in 1973) and purifying on silica gel thin-layer radiochromatography, using chloroform/methanol. The specific activity of this first preparation, determined in this laboratory, was 0.04 Ci/mmol, which was too low for experimental studies in the nM concentration range.

The second batch of [^{3}H]apomorphine was custom prepared and purified by New England Nuclear Corp., Boston. The final compound was 96% pure, as determined on ethanol:acetic acid:water (6:3:1, vol/vol/vol) thin-layer silica gel G radiochromatography. The specific activity was 14.1 Ci/mmol. The sample was stored in ethanol at −20° and used without further purification.

The [^{3}H]apomorphine binding assays were done using 12 × 75 mm glass test tubes, in which the following aliquots were placed (using Eppendorf Brinkmann pipettes with polypropylene tips): 0.1 ml of (+)-butaclamol (an active neuroleptic, refs. 21–24; final concentration was 1 μM) or (−)-butaclamol (the inactive enantiomer, final concentration was 1 μM); 0.1 ml of buffer with or without different concentrations of various other drug competitors; 0.2 ml of [^{3}H]apomorphine (final concentration ranging from 0.05 to 20 nM, but with the majority either at 1.5 or 3.3 nM); 0.2 ml of brain homogenate. Each determination was always done in sextuplicate, with six test tubes containing (+)-butaclamol and six containing (−)-butaclamol. After the sample was incubated for 30 min (22°), an aliquot of 0.5 ml was removed (polypropylene pipette tip) from the mixture and filtered under reduced pressure through a glass fiber filter (GF/B, Whatman, 24 mm diameter) using a Millipore stainless steel mesh support for the filter; the filtration took less than 1 sec. The filter was then washed twice with 5 ml of buffer per wash. The buffer was used at room temperature since minor differences in binding were found with the buffer at 4° or 37°. The wash buffer was delivered by gravity from a syringe Re-pipette over a period of 4 sec. The filters were not blotted or dried but placed directly into liquid scintillation vials; 8 ml of Aquasol (New England Nuclear Corp.) were added and the samples were monitored for ^{3}H (25) after they were stored at 4° for at least 6 hr to allow temperature equilibration and to permit the glass fiber filters to become uniformly translucent. Specific binding of apomorphine was defined as that amount bound in the presence of 1 μM (−)-butaclamol minus that bound in the presence of 1 μM (+)-butaclamol, in accordance with previous work (7, 9) on dopamine receptors. The results were calculated in terms of femtomoles of apomorphine specifically bound per mg of homogenate protein. Apomorphine is sufficiently hydrophobic and surface-active to warrant measuring the unbound (free) concentration (26–28) in each experiment. The average loss of [^{3}H]apomorphine to the glass and polypropylene surfaces was 14.3%, while the average nonspecific binding (29, 30) of [^{3}H]apomorphine to the tissue particles lowered the aqueous concentration a further 28.7%.

Dopamine Binding Assays. Details are given in refs. 7 and 9.

Neuroleptic (Haloperidol) Binding Assays. The [^{3}H]haloperidol binding assays were done by the same procedure as for the [^{3}H]-apomorphine assays, except that the butaclamol concentrations were 100 nM and the radioactive aliquot contained [^{3}H]haloperidol (8.4 Ci/mmol; IRE Belgique, Mol-Donk, Belgium; final *total* concentration of 2.14 nM). Only one wash with 5 ml of buffer was used. The buffer was kept at room temperature, since this reduced the nonspecifically bound

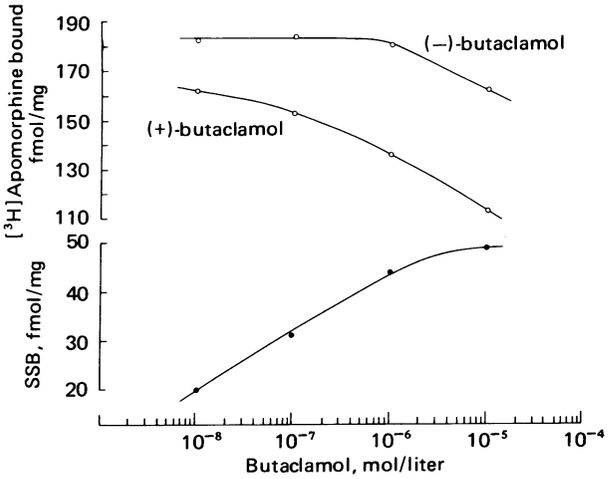

FIG. 1. (Top) Effect of butaclamol on the total binding of [^{3}H]apomorphine to calf caudate crude homogenate. (+)-Butaclamol is 250 to 1000 times more effective than (−)-butaclamol in reducing the binding. The nonspecific action of (−)-butaclamol starts at about 1 μM. (Bottom) The specific component (SSB) of [^{3}H]apomorphine binding was defined as that amount bound in the presence of (−)-butaclamol minus that amount bound in the presence of (+)-butaclamol (7). The total [^{3}H]apomorphine concentration was 1.6 nM (free concentration was 0.91 nM). The GF/B filters had been washed once with 5 ml of buffer.

[^{3}H]haloperidol to both filters and biomembranes. Both the wash volume and the filtration speed of the wash were found to be very critical in determining the amount of [^{3}H]haloperidol specifically bound. The optimum speed of the 5-ml wash was between 2 and 3 sec. Haloperidol is strongly hydrophobic (29, 30); in the present experiments 50% of the total haloperidol was nonspecifically adsorbed.

RESULTS

Properties of Specific [^{3}H]Apomorphine Binding. An example of both the total and specific components of [^{3}H]apomorphine binding is given in Fig. 1 for different concentrations of (+)- and (−)-butaclamol. The (+)-enantiomer of butaclamol was 250 to 1000 times more effective than the (−)-enantiomer in reducing the total binding of [^{3}H]apomorphine, in quantitative agreement with the enantiomeric potency ratio *in vivo* (21–23) and *in vitro* on the dopamine and neuroleptic receptor assays (7). The nonspecific action of (−)-butaclamol started at about 1 μM. Since maximum specific binding was almost attained at 1 μM, it was decided to adhere to 1 μM (+)- and (−)-butaclamol for the definition of the specific component of [^{3}H]apomorphine binding; 1 μM is also the butaclamol concentration used in the [^{3}H]dopamine receptor assay (7).

The number of specific [^{3}H]apomorphine binding sites detected was directly proportional to the amount of protein added to the filter in the range of 50–400 μg per filter. One of the major advantages of [^{3}H]apomorphine was that little desorption occurred within 15 min. Consistent with these results was the fact that specific binding of [^{3}H]apomorphine was not reduced by slow washing (4–8 sec) or by large wash volumes up to 30 ml. The specific binding of [^{3}H]haloperidol, in contrast, was very sensitive to these factors (see *Materials and Methods*).

The characteristics of [^{3}H]apomorphine and [^{3}H]dopamine specific binding are shown in Fig. 2 and Table 1.

Effect of Drugs and Neurotransmitters on Apomorphine Binding. The pharmacological specificity of the [^{3}H]apomorphine binding sites was studied. An example of the procedure

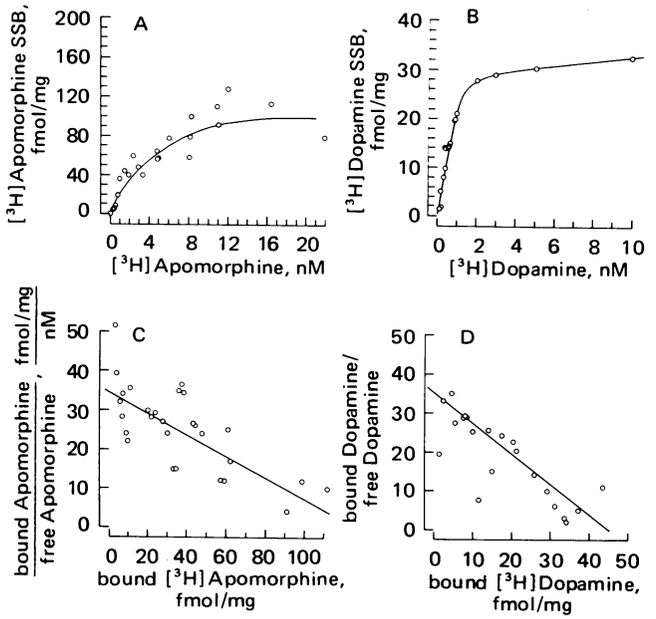

FIG. 2. Characteristics of specific [^{3}H]apomorphine and [^{3}H]dopamine binding to homogenate. Specific binding was that which occurred in presence of 1 μM (−)-butaclamol minus that in presence of 1 μM (+)-butaclamol. (A) Adsorption isotherm for [^{3}H]apomorphine, done by adding increasing amounts of [^{3}H]apomorphine rather than by varying the amount of nonradioactive apomorphine. (B) Adsorption isotherm for [^{3}H]dopamine (since many of the experimental points were done at the same concentration, an average binding value was graphed in these cases). (C) Scatchard analysis of data in panel (see Table 1). (D) Scatchard analysis of [^{3}H]dopamine binding data (see Table 1).

is shown in Fig. 3 for nonradioactive apomorphine, which reduced the binding of [^{3}H]apomorphine in the presence of either (+)- or (−)-butaclamol; the specific component of [^{3}H]apomorphine binding (bottom of Fig. 3) was reduced by 50% at 1.3 nM (free apomorphine concentration of 0.74 nM). Such $IC_{50\%}$ values are listed in Table 2, together with those for [^{3}H]dopamine. From these data it is possible to derive the dissociation constants of the inhibiting drugs (31).

The neurotransmitter most effective in inhibiting the specific binding of [^{3}H]apomorphine was dopamine (Fig. 4; Table 1). Norepinephrine had one-fourth the potency of dopamine, while epinephrine had one-fifth the potency of dopamine. All of the other neurotransmitters had no effect below 1 μM. This order of potencies meets the criteria of a dopamine receptor, but not those of a beta-adrenergic receptor (32, 33).

Another type of experiment was done that indicated that

Table 1. Dissociation constants and total number of specific binding sites in calf caudate

	[^{3}H]Dopamine	[^{3}H]Apomorphine	[^{3}H]Haloperidol
N, fmol/mg of protein	45 ± 5	124 ± 16	140 ± 15
K_D, nM	1.3 ± 0.3	3.5	2.7
K_D^{free}, nM	1.3 ± 0.3	2.0	1.3

Summary of specific binding characteristics of [^{3}H]apomorphine, [^{3}H]dopamine, and [^{3}H]haloperidol of crude homogenate of calf caudate. The apparent dissociation constant, K_D, corrected for nonspecific binding, is K_D^{free}. N is the total number of binding sites determined by Scatchard analysis.

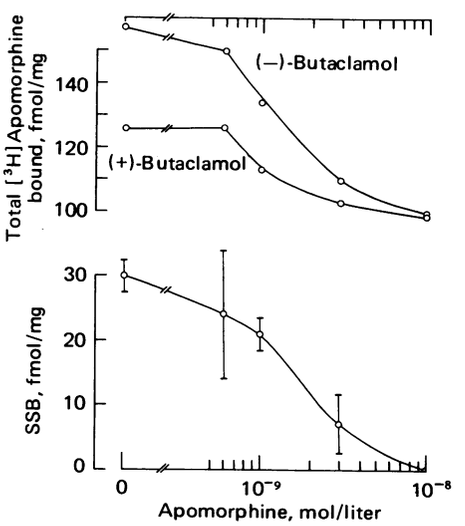

FIG. 3. Illustration of the procedure for testing the potency of a compound for its ability to compete for binding to specific [^{3}H]apomorphine sites. Here, nonradioactive apomorphine reduced the specific component of [^{3}H]apomorphine binding (data at bottom, derived from data at top) by 50% at 1.3 nM. Calf caudate: total [^{3}H]apomorphine concentration was 1.4 nM.

[^{3}H]apomorphine was not binding to a beta-adrenergic receptor. We repeated the experiment in Fig. 1 using (−)-propranolol (an active beta-adrenergic blocking drug; refs. 32 and 33) and (+)-propranolol instead of the (+)- and (−)-butaclamols. Both propranolols were inactive up to 100 nM.

[^{3}H]Apomorphine Binding to Human Brain Tissues. Table 3 lists the number of specific [^{3}H]apomorphine sites in various human brain regions, as assayed at a total concentration of 3.3 nM [^{3}H]apomorphine (K_D was 1.5 nM for human caudate; K_D^{free} was 0.9 nM).

Significant amounts of specific [^{3}H]apomorphine binding occurred in the three dopamine-rich regions (4) (caudate, putamen, and nucleus accumbens) and in the septum. [^{3}H]Apomorphine binding in all the other human brain regions was not significantly different from background.

Table 2. Inhibition of binding by drugs

	[^{3}H]Apomorphine $IC_{50\%}$, nM	[^{3}H]Dopamine $IC_{50\%}$, nM
Apomorphine	1.3	2
Dopamine	3.5	9
l-Norepinephrine	15	46
Epinephrine	20	68
Isoproterenol	1400	
Fluphenazine		250
Haloperidol	500	300
Trifluperidol		1500
Chlorpromazine	1500	2000
Spiroperidol		4000
Serotonin	5 μM	0.1 mM
Carbachol	n.s.e. at 1 μM	
Glycine	n.s.e. at 1 μM	
Glutamate	n.s.e. at 1 μM	
Gamma-aminobutyrate	n.s.e. at 1 μM	

Concentrations ($IC_{50\%}$ values) of drugs and neurotransmitters that reduced the specific binding of [^{3}H]apomorphine (1.5 nM; free concentration = 0.86 nM) or [^{3}H]dopamine (4.5 nM) by 50% (crude homogenate of calf caudate). n.s.e. indicates no significant effect. 1 μM (−)- or 1 μM (+)-butaclamol was in all test tubes.

DISCUSSION

The results indicate that [^{3}H]apomorphine meets the criteria of a valid dopamine receptor ligand, on the grounds that the specific component of [^{3}H]apomorphine binding: *(i)* is saturable; *(ii)* has a K_D in the nM range, as do most receptors; *(iii)* is properly defined as the displacement difference between two enantiomers (34); *(iv)* is blocked by dopamine at very low concentrations (3.5 nM); *(v)* is localized to dopamine-rich regions of the human brain; *(vi)* has the identical order of sensitivity to various neuroleptics and catecholamines as the [^{3}H] dopamine ligand; *(vii)* is readily distinguished from the beta-adrenergic receptor by the fact that isoproterenol and epinephrine are poor inhibitors compared to norepinephrine or dopamine, and by the fact that the enantiomers of propranolol do not yield a specific component of [^{3}H]apomorphine binding; and finally, *(viii)* is not less than the number of specific [^{3}H] dopamine sites.

It is not clear whether [^{3}H]apomorphine is labeling the postsynaptic or the presynaptic sites in these homogenates. Nothing is known about the uptake process of apomorphine in presynaptic terminals, although it is known that apomorphine does have an action on the presynaptic tyrosine hydroxylase (35). The fact that the number of [^{3}H]apomorphine sites is greater than the number of [^{3}H]dopamine sites (Table 1) may suggest that nondopaminergic sites are also being measured. Presynaptic uptake and concentration of [^{3}H]apomorphine is probably not appreciable, since specific [^{3}H]apomorphine

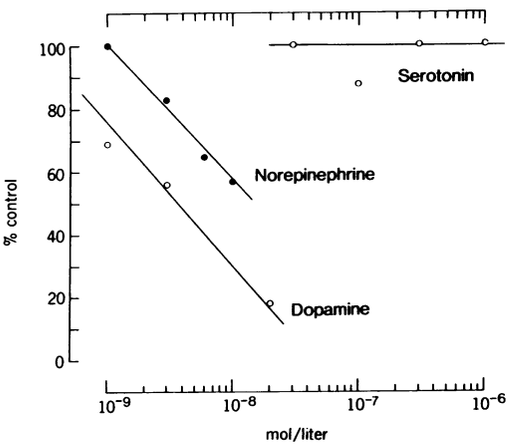

FIG. 4. Effect of three neurotransmitters on the specific binding of [^{3}H]apomorphine. Crude homogenate: of calf caudate total [^{3}H]apomorphine concentration was 1.5 nM.

Table 3. Specific binding of apomorphine and haloperidol in human brain regions

Brain region	[^{3}H]Apomorphine* at 3.3 nM (free = 1.9 nM)			[^{3}H]Haloperidol† at 2.14 nM (free = 1 nM)		
	fmol/mg	*n*	Subject	fmol/mg	*n*	Subject
Caudate	35.0 ± 2.4	15	HB19	104 ± 12	8	JR66
	28.5 ± 4.8	4	RR24			
Putamen	24.5 ± 1.1	14	HB19	62 ± 5	8	JR66
	37.1 ± 3.3	4	RR24	71.9	1	75-62-29
				70.2	1	75-63-67
				65.2	2	75-61-45
				43.7	2	75-64-24
				56.0	2	75-65-24
Nucleus accumbens	20.9	2	HB19	76	2	JR66
Septum	35.2	2	HB19			
Thalamus	6.3 ± 2	5	HB19	24	1	JR66
	5 ± 4	3	JR66			
	1.3	2	RR24			
Hippocampus	7.9 ± 4.4	3	HB19	2.6	1	JR66
	2 ± 1.5	3	JR66			
	7.9 ± 4.3	3	RR24			
Motor cortex	6.6 ± 5	3	HB19	19 ± 10	3	JR66
	1.4	2	RR24			
Frontal cortex	3.7 ± 2	5	HB19	0.4 ± 0.3	3	JR66
	1.8 ± 1.5	3	JR66			
	4.0	2	RR24			
Pallidum, external	3.1 ± 0.4	7	HB19	20	2	JR66
	1.8	1	RR24			
Pallidum, internal	5.1 ± 3	5	HB19	0	1	JR66
	0	1	RR24			
Substantia nigra	5.3	2	HB19			
	0	2	RR24			
Cingulate gyrus	4.2 ± 4.5	7	HB19	0	2	JR66
Cerebellum	5.1 ± 2.5	5	HB19			
	8.4 ± 3.6	3	RR24			

The specific binding results are expressed as fmol of ligand (mean ± SEM) bound per mg of crude homogenate protein. *n* is the number of separate determinations, each done in sextuplicate. Specific binding is defined as that amount bound in the presence of (−)-butaclamol minus that which occurs in the presence of (+)-butaclamol. Significant specific binding of [^{3}H]apomorphine only occurred in the caudate, putamen, nucleus accumbens, and septum, while that in all the other regions was not significantly different from background.

* 1 μM (+)- and (−)-butaclamols.

† 100 nM (+)- and (−)-butaclamols.

binding was little changed by temperature, whereas the presynaptic dopamine uptake process is markedly temperature-dependent (see refs. in ref. 7).

Since the neuroleptic butaclamol is used to define the specific component of [^{3}H]apomorphine binding, it would seem appropriate to conclude that the apomorphine sites are similar or identical to the neuroleptic sites. At least the number of [^{3}H]apomorphine and [^{3}H]haloperidol sites are equal (Table 1). The situation cannot be this simple, however, since the neuroleptics were very weak in blocking the specific binding of both [^{3}H]apomorphine and [^{3}H]dopamine (Table 2). It is possible, however, that [^{3}H]apomorphine and [^{3}H]dopamine label the agonist state of the receptor protein, while [^{3}H]haloperidol labels the antagonist state (36–38). A further complication is that it is known that dopamine receptors and serotonin receptors can bind similar ligands in some cases, such as LSD and (+)-butaclamol (36–39).

The important advantage of the [^{3}H]apomorphine ligand is that its desorption is minimal for 15 min, thus permitting extensive washing for routine assays of human tissues and facilitating future biochemical isolation and characterization of the receptor.

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