excluded. The dose–respose experiments are consistent with this hypothesis as the peak shifted progressively to the right with higher doses of the inhibitor. This indicates that less free thrombin was available early in the time course when higher doses were administered. Previous studies of the metabolism of free thrombin in the rabbit also suggest that thrombin reacts with AT III on the vessel wall [8]. The reaction appears to occur here because heparinoids present on the vessel wall are thought to greatly accelerate the rate of reaction between AT III and thrombin [8].

The ultimate fate of most of the drug-inhibited thrombin involves the formation of the TAT complex which clears in the liver [7]. This was demonstrated by the effective blockade of the clearance when unlabelled TAT was injected at the time of the peak and by SDS–polyacrylamide gel electrophoresis of blood samples obtained after injecting drug–thrombin complexes (Figs. 3 and 4). The shift in counts from the lung to the liver is also indicative of this pathway. The data presented in Fig. 3 are not plotted in the typical manner utilized in clearance studies; namely, as percent of the ligand remaining in the circulation (see Ref. 7 for a review of the mouse clearance model). The data in Fig. 3 were plotted as counts per minute in the circulation because the level of thrombin reappearing, as judged from the raw data, actually exceeded the initial number of counts recorded when the blood was drawn from the retroorbital venous plexus at 5 sec. This phenomenon is an artifact of this sampling procedure. Thrombin clearance in the lung is extremely rapid [7, 8]; hence, the blood drawn from the retroorbital venous plexus already will have lost a fraction of the thrombin as the blood makes its first pass through the lung. Thus, the reappearance peak may show higher counts than the initial blood sample.

The clearance of reversibly inhibited thrombin has obvious clinical implications. Since the clearance of thrombin is primarily dependent on AT III, careful monitoring of both the drug and AT III levels appears imperative especially when the drug is being discontinued. If AT III levels were suboptimal, AT III concentrates could be administered to ensure adequate inhibitory capacity. Since, however, the amount of thrombin generated in thrombotic conditions is usually quite small, adequate AT III levels should not be a major obstacle for the use of these drugs in most situations. In this regard, compound No. 805 has been reported to be an effective therapy for preventing thrombosis in AT III deficient rats [13].

In summary, reversibly inhibited thrombin is removed from the circulation in two steps, binding to endothelial cells primarily in lung followed by clearance in the liver as the TAT complex.

Departments of Pathology and Biochemistry
Duke University Medical Center
Durham, NC 27710, U.S.A.

MARK A. SHIFMAN
SALVATORE V. PIZZO*

* Address all correspondence to: Dr. Salvatore Pizzo, Box 3712, Department of Pathology, Duke Medical Center, Durham, NC 27710, U.S.A.

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[^{3}H]Haloperidol binding to more than one site in rat brain striatum

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In order to re-examine the suitability of [^{3}H]haloperidol as a selective ^{3}H ligand for D_2 dopamine receptors [1], we tested the properties of [^{3}H]haloperidol binding to rat brain striatum. The D_2 receptor is defined as that dopaminergic site with high affinity (nanomolar) for neuroleptics and low affinity (micromolar) for dopamine agonists [2].

This report demonstrates that [^{3}H]haloperidol binds to more than one population of sites, when 0.1 μM (+)-butaclamol is used to define specific binding, rather than to a single D_2 receptor site as previously thought [1]; we find that the baseline of 3 μM (±)-sulpiride permits the detection of a single population of sites.

This study on [^{3}H]haloperidol was instigated by recent observations that [^{3}H]spiperone is not as selective for D_2 dopamine receptors as originally thought [3] but also labels serotonin receptors, adrenoceptors and spirodecanone sites [4–6].

Materials and methods

Preparation of homogenates for the binding of [^{3}H]*haloperidol.* Male Wistar rats (Canadian Breeding Laboratories) were housed in pairs with 12-hr light–dark cycles and fed *ad lib.* for 1 week prior to use, at which time they weighed 180–200 g.

Rats were killed by cervical dislocation between 9:00 a.m. and 12:00 noon. The brains were removed and rinsed with ice-cold saline (0.9% NaCl). Striatal tissue was removed by free-hand dissection, and tissue pools from about fifteen to twenty rats were used for a single determination (N = 1) of receptor density by Scatchard analysis or for competition-type experiments.

The freshly dissected striatal tissue pools were placed in a 40 ml volume of cold TEAN buffer (15 mM Tris–HCl, pH 7.4; 5 mM Na_2EDTA; 0.02% (1.1 mM) abscorbate; and 12.5 μM nialamide), homogenized with a Brinkmann Polytron (setting = 7) for 15 sec, and then repeatedly centrifuged and resuspended in buffer (44,000 *g*) three times. The final pellet was resuspended in enough TEAN buffer to make the final tissue concentration contain 1 mg protein/ml volume. The tissue was stored frozen (−20°) until use. Immediately before using, the frozen tissue homogenate was thawed, re-homogenized with a Polytron (setting = 7) for 10 sec, and then used for the receptor assay.

Binding of [^{3}H]*haloperidol to rat striatal receptors.* The binding of [^{3}H]haloperidol (Scatchard analysis) was done using concentrations of [^{3}H-G]haloperidol (8.5 Ci/mmole) (I.R.E., Belgium, or N.E.N.) ranging from 0.07 to 13 nM. Triplicate determinations were done at each concentration. Specific binding of [^{3}H]haloperidol was defined as that inhibited by the presence of sulpiride (3 μM) or 0.1 μM (+)-butaclamol. Comparisons of Scatchard analyses using sulpiride or (+)-butaclamol baselines were always done on tissues pooled from the same rats and experimentally done on the same day. Rosenthal dissection of Scatchard curves was done graphically by the method of Rosenthal [7].

The binding of [^{3}H]haloperidol to the homogenate was done in glass test tubes (12 × 75 mm), in which the following aliquots were placed (using Eppendorf–Brinkmann pipettes with polypropylene tips) in the order listed: 0.2 ml TEAN buffer (or 0.2 ml drug solution), 0.2 ml of [^{3}H]haloperidol and 0.2 ml of tissue homogenate containing 1 mg protein/ml suspension. After the samples were incubated at room temperature (22°) for 45 min, an aliquot of 0.5 ml was removed from the mixture and filtered under vacuum through a glass fiber filter (Whatman GF/B; 24 mm diameter) on a Millipore stainless steel mesh support; the filtration took less than 1 sec. The filter was then washed with 10 ml of TEAN buffer (2–3 sec). The filters were not blotted or dried but were placed directly into liquid scintillation vials, 8 ml of Aquasol (New England Nuclear Corp., Boston, MA) was added, and the samples were assayed for ^{3}H (at 42% efficiency) after storage at 4° for at least 6 hr to allow temperature equilibrium and to permit the glass filters to become uniformly translucent.

Results

Figure 1 shows the inhibition (by various drugs) of the binding of 2 nM [^{3}H]haloperidol to rat striatal homogenates. (+)-Butaclamol inhibited 50% of the total binding at high affinity (IC_{50} = 3.6 nM). Spiperone, domperidone and sulpiride had biphasic actions with the transition points occurring at the 70% level of total binding. The IC_{50} values for the high-affinity component of these curves correspond quite well with those for D_2 receptors (see Table 1). Comparison (Fig. 1) of the (+)-butaclamol data with the biphasic results for spiperidone, domperidone and sulpiride suggested that [^{3}H]haloperidol was binding to at least two sites, with the high-affinity components for spiperone, domperidone and sulpiride corresponding to the D_2 site (30% of total binding when [^{3}H]haloperidol = 2 nM).

The inhibition by other neuroleptics (haloperidol, fluphenazine, clozapine, piflutixol, alpha-flupenthixol) appeared to have similar affinities for each of these two sites, since the Hill coefficients had values approaching unity. Some of these drugs started to show plateaus at levels below 50% of total binding, suggesting that a third component may exist for [^{3}H]haloperidol binding. This

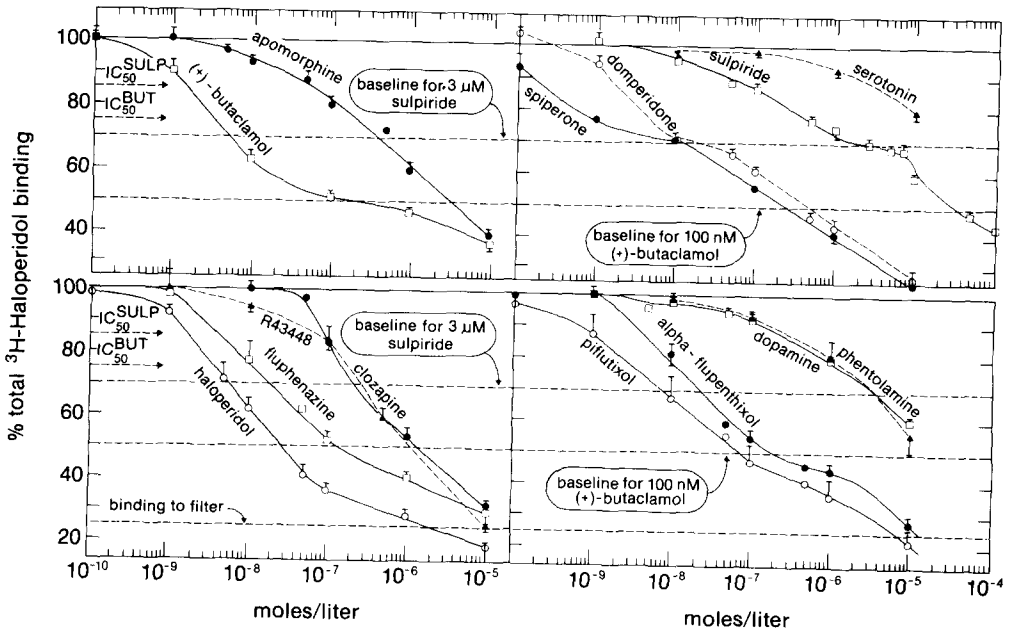

Fig. 1. Competition-type experiments using 2 nM [^{3}H]haloperidol (rat striatum). Values are means ± S.E. for three to ten independent experiments in triplicate. Standard error bar is omitted when error is smaller than symbol size. The absolute amount of total binding (cpm) per filter was 550. Filter background was 25% of total binding.

Table 1. Inhibition of 2 nM [^{3}H]haloperidol binding to rat striatum*

	IC_{50} (nM)	Hill coefficient
Spiperone	0.32 ± 0.1†	0.5
	48 ± 2.5	
Domperidone	2.4 ± 2.5†	0.7
	105 ± 9	
Haloperidol	3.7 ± 0.9	1.1
Piflutixol	6 ± 3	0.7
(+)-Butaclamol	3.6 ± 0.6	1.1
α-Flupenthixol	16 ± 3	1.2
Fluphenazine	14 ± 4	1.3
Clozapine	166 ± 12	1.3
Sulpiride	114 ± 30†	0.5
	8,250 ± 170	
Apomorphine	178 ± 43	0.7
Dopamine	1,500 ± 153	0.5
(−)-Butaclamol	>100	
R43448	>100	
Phentolamine	>1,000	
Serotonin	10,000	

* IC_{50} Value was defined as that drug concentration required to inhibit 50% of specific binding [defined by 100 nM (+)-butaclamol]. Results are expressed as means ± S.E.; N = 3–10 independent determinations for each of the above experiments. The concentration range of drugs is shown in Fig. 1.

† Values of IC_{50} for high-affinity site as defined by 3 μM sulpiride.

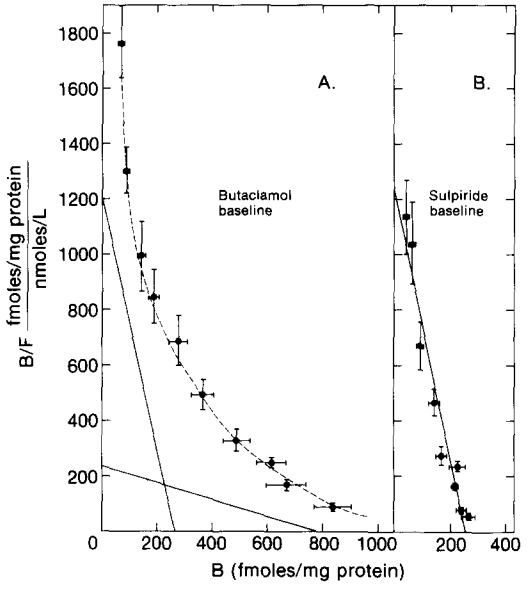

Fig. 2. (A) Scatchard analysis of [^{3}H]haloperidol binding to rat striatum using a 100 nM (+)-butaclamol baseline. Rosenthal dissection of the curve [7] (graphically done) gave a high-affinity site with K_D of 0.22 ± 0.03 nM and B_{max} of 263 ± 29 fmoles/mg protein. The lower affinity site had a K_D of 3.3 ± 0.3 nM and a B_{max} of 783 ± 77 fmoles/mg protein. (B) Scatchard analysis of [^{3}H]haloperidol binding to rat striatum using a 3 μM sulpiride baseline. Only the high-affinity site was revealed: K_D = 0.34 ± 0.06 nM and B_{max} = 296 ± 19 fmoles/mg protein (mean ± S.E.). [^{3}H]Haloperidol concentrations ranged from 0.07 to 13 nM. All points on the diagram represent means ± S.E.; N = 3–4 independent experiments for Fig. 2A and 2B.

third component might not be a receptor site, however, since these high concentrations also inhibited the binding of [^{3}H]haloperidol to the filters.

Figure 2 shows the Scatchard analyses of the [^{3}H]haloperidol binding isotherms. When 0.1 μM (+)-butaclamol was used to define specific binding, [^{3}H]haloperidol appeared to bind at two sites. The high-affinity site had a K_D of 0.22 ± 0.03 nM and a density (B_{max}) of 263 ± 29 fmoles/mg protein (mean ± S.E.). The low-affinity sites had a K_D of 3.3 ± 0.3 nM and B_{max} of 783 ± 77 fmoles/mg protein (as calculated by the method of Rosenthal [7]). When sulpiride was used to determine non-specific binding of [^{3}H]haloperidol only the high-affinity site was revealed (K_D of 0.34 ± 0.06 nM and B_{max} of 296 ± 19 fmoles/mg protein).

Discussion

The results indicate that [^{3}H]haloperidol binds to at least two sites in the rat striatum. Previous literature on the binding of [^{3}H]haloperidol to rat striatal preparations [8–11] has not clearly demonstrated multiple binding sites for [^{3}H]haloperidol. Because a narrow range (less than one-half to two times the K_D of the highest and lowest sets of sites) of [^{3}H]haloperidol concentrations has generally been used for saturation binding isotherms, the multiplicity of binding sites would not have been readily detected. Under such conditions the single K_D is a reflection of the combination of K_D values and mathematically bears little relation to the true K_D values. Generally, the dissociation constants (K_D) for [^{3}H]haloperidol have been reported to range from approximately 1 to 3 nM, these values being approximately intermediate to the values of 0.34 and 3.3 nM found in the present study. One previous study did use higher concentrations of [^{3}H]haloperidol (up to 16 nM) and did report that the Scatchard plot was biphasic [9], but not enough determinations were made at the high-affinity end of the Scatchard plot to determine whether their K_D of 2.2 nM was a composite of the K_D found in our present study.

A more recent study of [^{3}H]haloperidol binding to rat kidney [12] demonstrated two distinct binding sites for [^{3}H]haloperidol. The K_D values for the two binding sites were 0.41 and 5.88 nM, and the receptor density of the high-affinity site was about 40% of the total receptor density, as defined by 1 μM trifluperidol. These receptor affinities and relative densities were similar to those in our study; i.e. K_D values of 0.34 and 3.3 nM in rat striatum with the B_{max} of the high-affinity site being approximately 30% of the total receptor density as defined by 0.1 μM (+)-butaclamol. The paper by Nakajima and Kuruma also described [^{3}H]haloperidol binding to the rat brain striatal P_2 fraction and mentioned that a high-affinity component (K_D = 0.46 nM, B_{max} = 310 fmoles/mg protein) was observed; a lower affinity component of binding to the striatal P_2 fraction was inferred, but it was mentioned that biochemical characteristics of the low-affinity sites were not examined.

Feuerstein *et al.* [13] have recently reported changes in K_D as well as B_{max} for [^{3}H]haloperidol binding after nigrostriatal denervation. In view of the fact that they used 0.1 μM (+)-butaclamol to define specific binding, it is possible that their K_D shift represented preferential B_{max} changes in one of the two sites over time, rather than an actual affinity change in one site. The full extent of possible affinity changes may also not be picked up under such experimental conditions.

As mentioned previously, the IC_{50} values for the high-affinity component of inhibition by spiperone, domperidone and sulpiride correspond quite well to those concentrations effective at the D_2 receptor. All three drugs have been used to label selectively the D_2 receptor in the rat striatum [14–18].

The use of 3 μM sulpiride as baseline for [^{3}H]haloperidol

Scatchard analysis suggested that the high-affinity site (0.34 nM K_D) shown under these conditions is the D_2 component of [^{3}H]haloperidol binding. The identity of the 3.3 nM K_D site is difficult to determine. Since R43448, serotonin and phentolamine did not inhibit that component of [^{3}H]haloperidol binding, it was unlikely that [^{3}H]haloperidol was binding to alpha-adrenoceptors or serotonergic receptors, even though haloperidol has been reported to compete with high affinity for alpha-1 adrenergic sites [19]. It is also unlikely that, under our assay conditions, we would be measuring a state of the D_2 receptor that has high affinity for dopamine agonists, as previously reported by Creese and coworkers [20].

The IC_{50} values for inhibition data (Fig. 1) for some neuroleptics (fluphenazine, clozapine, piflutixol, alpha-flupenthixol), but not for haloperidol, are similar to those for [^{3}H]flupenthixol binding (which corresponds to D_1 or dopamine stimulated adenylate cyclase sites [21–23]). However, the data are insufficient, as yet, to determine whether binding was also occurring at the D_1 site; clearly, a reevaluation of some [^{3}H]haloperidol binding studies is necessary, where only one set of sites has been assumed in the past.

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Department of Pharmacology
University of Toronto
Toronto, Ontario
Canada M5S 1A8

DAIGA M. HELMESTE*
PHILIP SEEMAN

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* To whom correspondence should be sent: Psychopharmacology Section, Clarke Institute of Psychiatry, 250 College St., Toronto, Ontario, Canada M5T 1R8.

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Cocaine: comparative effect on dopamine uptake in extrapyramidal and limbic systems

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Dopamine (DA) performs many roles in the central nervous system (CNS) [1–8]. First of all, it is the major neurotransmitter of the nigrostriatal (extrapyramidal) system. A notable advance in modern neurology has been the discovery that DA deficiency in this system leads to Parkinsonism and that replacement therapy via levo-3,4-dihydroxyphenylalanine (*l*-DOPA) corrects the disorder [3, 4].

The role of DA in the limbic system may be equally important though it is less well understood. It has been proposed that this DA system modulates emotional behavior and mood [1, 2, 4–8]. The DA cell bodies for this system are found in the ventral tegmental area of the mesencephalon. Axons of these neurons are projected to the olfactory bulbs, to the nucleus accumbens and associated nuclei (mesolimbic system) and to the prefrontal, cingulate and entorrhinal cortex (mesocortical system) [1]. It has been postulated that DA dysfunction may be responsible for schizophrenia and other emotional disorders [5]. This has been based largely on the fact that many neuroleptics are DA receptor antagonists [3, 5]. A defect in limbic DA neurotransmission would best fit this disorder. Moreover, it has been proposed that affective deterioration in such organic diseases as progressive supranuclear palsy and Parkinsonism may result from degeneration of limbic DA neurons [4, 6–8].

It is evident from animal studies that the extrapyramidal and limbic DA systems respond preferentially to various stimuli and drugs. It has been reported that the mesocortical and mesolimbic DA tracts respond to self-stimulation, long-acting neuroleptics, and foot-shock induced stress,