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^{3}H-Domperidone: A Selective Ligand for Dopamine Receptors

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Summary. ^{3}H-Domperidone, a potent antagonist of dopamine but less lipophilic than neuroleptic drugs, was studied as a potential ligand for cerebral dopamine receptors. It labeled with high affinity an apparently homogeneous population of non-interacting sites in a particulate fraction of mouse striatum. Association occurred rapidly and dissociation was relatively slow ($t^1/_2 \simeq 4$ min); this allowed extensive washing of membranes which reduced the non-specific binding to values as low as 5% of the total binding. Consistent Kd values of 0.7 nM were obtained by analysing by various methods either the kinetics of binding or the saturation of binding sites at equilibrium.

The relative potencies of various dopamine receptor agonists or antagonists to inhibit ^{3}H-domperidone binding, paralleled their pharmacological activity. On the other hand, a variety of non-dopaminergic agents failed to inhibit ^{3}H-domperidone binding. The findings indicate that striatal dopamine receptors are selectively labeled by this ^{3}H-ligand.

In various non-striatal regions of mouse brain the saturable binding of ^{3}H-domperidone was almost entirely inhibited by apomorphine indicating its selectivity for dopamine receptors in spite of the low density of the latter. This was not the case for the binding of ^{3}H-spiperone, evaluated on the same preparations, indicating that ^{3}H-domperidone probably represents the most selective ligand presently available.

Key words: ^{3}H-Domperidone – Dopamine receptors.

Introduction

An important approach to characterize the interaction of drugs with dopamine receptors is the use of labeled agonists and antagonists. For this purpose ^{3}H-haloperidol was first proposed. Its high affinity binding to striatal membranes provides a good assay for neuroleptics, since the inhibition of the binding by these drugs correlates well with their clinical properties (Creese et al., 1975; Snyder et al., 1975; Seeman et al., 1975; Burt et al., 1976; Seeman, 1977; Leysen et al., 1978a; Titeler et al., 1978). However, neuroleptics are generally highly fat soluble: this account for their easy penetration into brain but also explains their accumulation in cell-membranes which results in a high percentage of non-specific binding, i.e. of binding not associated with dopamine receptors. The use of the butaclamol stereoisomers, only one of which is active, allows determination of stereospecific ^{3}H-haloperidol binding (Creese et al., 1975; Seeman et al., 1975, 1978; Burt et al., 1976). However, the fraction of ^{3}H-haloperidol bound to receptors is still evaluated over a rather large "blank" value, a drawback particularly important when assays are performed on brain regions with a lower density of receptors than the striatum. The same difficulties are encountered in binding studies with agonists like ^{3}H-dopamine (Burt et al., 1975, 1976; Creese et al., 1975; Seeman et al., 1975; Titeler et al., 1978) or ^{3}H-apomorphine (Seeman et al., 1975, 1976; Titeler et al., 1978). Again, the percentage of non-specific binding is high (generally more than 50%). In addition it appears that ^{3}H-apomorphine preferentially labels presynaptic receptors which are poorly recognized by neuroleptics (Nagy et al., 1978).

Recently ^{3}H-spiperone has been proposed as the ligand of choice for postsynaptic dopamine receptors in view of its slow rate of dissociation and the high percentage of specific binding as compared to ^{3}H-haloperidol (Leysen et al., 1978a; Leysen and Gommeren, 1978). However, it is now clear that ^{3}H-spiperone labels heterogeneous sites as shown by a higher number of binding sites, biphasic dissociation curves and a significant inhibitory potency of non-dopaminergic agents (Creese and Snyder, 1978; Leysen et al., 1978a, b; Titeler et al., 1978). Most of these data

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A portion of this work has been already presented in abstract form at the Symposium on Receptors of Dopamine Antagonists (Beerse, Belgique 1978; Martres et al., 1978

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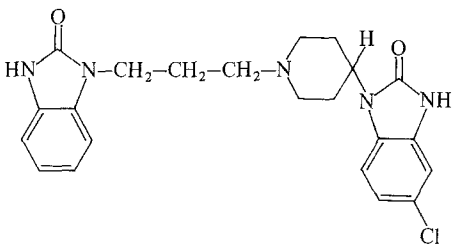

Fig. 1. Chemical structure of domperidone

can be explained by the good affinity of spiperone for serotonin receptors which renders its use difficult in studies of dopamine receptors in brain regions in which the density of the latter is low as compared to that of serotonin receptors.

Recently it has been shown that domperidone (5-chloro-1 {1-[3-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl) propyl]-4-piperidinyl}-1,3-dihydro-2H-benzimidazol-2-one; Fig. 1) is a potent antagonist of dopamine at receptors located outside the "blood-brain barrier". For instance, domperidone strongly antagonizes apomorphine-induced emesis, but is devoid of significant neuroleptic properties, a feature possibly related to its lower lipophilicity, as compared to most neuroleptics (Niemegeers and Janssen, 1978; Costall et al., 1979). We reasoned that due to this character ^{3}H-domperidone might less readily than ^{3}H-neuroleptics accumulate non-specifically in membrane components, and might constitute a selective ligand for dopamine receptors.

Methods

Striata of two male mice (20–25 g, Charles River, Saint-Aubin-lès-Elbeuf, France) were homogenized in 2 ml (25 vol) of ice-cold 50 mM Tris-HCl buffer, pH 7.4, by sonication for 30 s (Ultra-sound generator, Annemasse, France). After a 3-fold dilution, the homogenate was centrifuged ($5 \cdot 10^3$ g × min) in a Sorvall RC-3 (rotor HG 4L). The resulting supernatant was diluted 2-fold and centrifuged again ($2 \cdot 10^5$ g × min) in a Spinco L50 (rotor R30). The final pellet was superficially washed with 2 × 3 ml buffer and resuspended by gentle sonication in 2 ml of cold, freshly prepared 50 mM Tris-HCl buffer, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Burt et al., 1976). In incubations with dopamine or apomorphine, the buffer was supplemented with 10 µM pargyline and 0.1% ascorbic acid, which did not modify the characteristics of the binding. Neuroleptics were dissolved in 0.07% lactic acid, and the resulting solution was diluted with the incubation medium. On the day of use, the methanolic solution of ^{3}H-domperidone (10 Ci/mmole) was diluted to the appropriate concentration in the solution used for incubations, containing 0.02% lactic acid. A 100 µl sample of the tissue suspension (1.0–1.5 mg protein/ml unless otherwise stated) was routinely preincubated for 20 min at 30° C in order to eliminate endogenous dopamine possibly remaining in the extract. 100 µl of solution containing both ^{3}H-domperidone and various drugs were added and the incubations (total volume of 200 µl) carried on for 30 min. At the end of incubations 3 ml cold buffer were added and membranes were recovered by rapid filtration under vacuum through Whatman GF/B filters. Tubes were rinsed with 3 ml of ice-cold buffer used in incubations, and the filters were washed with 2 × 15 ml (in some experiments specified under Results 4 × 15 ml) of buffer. The filters were counted by liquid scintillation spectrometry in 10 ml of toluene – triton × 100 – PPO – POPOP (21–11–16.5 g–0.45 g) at an efficiency of 45%. In all experiments, binding of ^{3}H-domperidone on filters was determined by adding the ^{3}H-ligand just after dilution of the membrane suspension and by filtering immediately. It represented about 2% of total radioactivity (or only 1% after 4 × 15 ml washes) and was substracted from all reported values.

Protein was determined by the method of Lowry et al. (1951), and results were expressed in fmoles per mg protein in the incubation. Saturation curves were analyzed according to Parker and Waud (1971) i.e. by fitting, by means of the least squares method, a hyperbola directly to the data without linear transformation.

^{3}H-Domperidone (10 Ci/mmole) and ^{3}H-spiperone (8.3 Ci/mmole) were kindly donated by Janssen Pharmaceutica (Beerse, Belgium). Dopamine hydrochloride and serotonin creatinine sulfate (Calbiochem); apomorphine hydrochloride and methysergide bimaleate (Sandoz); (±) and (–) butaclamol (Ayerst); haloperidol (Lebrun); sulpiride (Delagrange); bufotenine mono-oxalate hydrate, carbamyl choline chloride, γ-aminobutyric acid, 5-methoxytryptamine hydrochloride, noradrenaline hydrochloride and L-DOPA (Sigma); histamine dichlorhydrate (Prolabo); L-glutamic acid sodium salt monohydrate (Koch-Light).

Results

Parameters of ^{3}H-Domperidone Binding in Mouse Striatum

Saturable ^{3}H-domperidone binding (representing the binding inhibited by 500 nM of the non-radioactive ligand) after a 30-min incubation, reached a plateau at approximately 4 nM. In contrast, the non-saturable binding evaluated in the presence of 500 nM domperidone increased linearly at least up to 10 nM ^{3}H-ligand (Fig. 2). At 4 nM ^{3}H-domperidone, the saturable binding represented 75% of the total binding under the standard conditions. Recently we have observed that following a more extensive wash of the filters (4 × 15 ml instead of 2 × 15 ml, see Methods) the percentage was as high as 95%, without a change in saturable binding. A Scatchard plot of the data of Fig. 2 provided a single straight line (Fig. 3, left) leading to a dissociation constant (Kd) of 0.7 nM and a maximal binding (B_M) of 483 fmole · mg protein^{-1}. The value of the Hill coefficient (Fig. 3, right) was not significantly different from unity. In both cases the concentration of free ligand was estimated as the difference of radioactivity added and the radioactivity bound to membranes (saturable plus non-saturable binding). Data from Fig. 2 were also analyzed by an iterative program based on the least squares (Parker and Waud, 1971), and the parameters are reported in Table 1.

Saturable ^{3}H-domperidone binding took place quite rapidly at 30° C, reaching equilibrium at 30 min

(Fig. 4). The rate constant for association (k_{on}), calculated from the initial slope of the association curve (up to 2.5 min) and the known concentrations of the ligand (2.4 nM) and of binding sites (0.70 nM) was $0.29 \cdot 10^9\ M^{-1} \cdot min^{-1}$. The dissociation kinetics were studied by rapid filtration (less than 10 s) following a 100-fold dilution of the incubation medium at a time when equilibrium of binding was achieved (Fig. 4). When the dissociation of ^{3}H-domperidone from binding sites was studied at 30 s intervals, it appeared to follow first-order kinetics (not shown), and plots of the data on a semi-log representation led to a dissociation rate constant (k_{off}) of $0.19\ min^{-1}$.

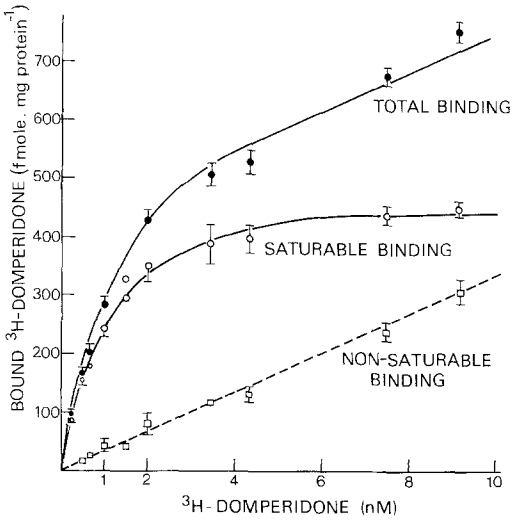

Fig. 2. Saturation of *^{3}H-domperidone* binding in mouse striatum. The particulate fraction (0.2 mg protein) was incubated for 30 min with *^{3}H-domperidone* in increasing concentrations either alone (total binding) or in the presence of 500 nM non-radioactive domperidone (*non-saturable binding*). *Saturable binding* refers to the difference between the two values. Abscissa: total *^{3}H-domperidone* concentration in the incubation (free + total bound). Means ± S.E.M. of 6–8 experiments

In Table 1 the Kd values obtained as the ratio of k_{off} and k_{on} on the one hand and by analysis of the saturation curve at equilibrium on the other hand (Parker and Waud, 1971), are compared.

When the tissue concentration in the incubation was varied, it appeared that both the apparent Kd (representing the total concentration of ^{3}H-domperidone which corresponds to half-saturation of the binding sites) and the maximal binding varied linearly up to 0.30 mg protein in 0.2 ml (corresponding to a 0.70 nM concentration of sites); the straight line representing the variation of Kd intersected the ordinate axis at a value of 0.60 nM (Fig. 5).

Effects of Drugs on ^{3}H-Domperidone Binding

Various agents were tested for their ability to inhibit the binding of 4 nM ^{3}H-domperidone (Fig. 6 and Table 2). The most potent inhibitors were neuroleptics such as haloperidol, (±) butaclamol and sulpiride. (±) Butaclamol was 3000-fold more potent than its optic isomer (–) butaclamol. ^{3}H-Domperidone binding was also inhibited by dopamine and its potent agonist, apomor-

Table 1. Association and dissociation constants of saturable ^{3}H-domperidone binding in mouse striata. Kinetic parameters were calculated from the data of two independent experiments performed as described in Fig. 4. Constants at equilibrium were calculated as described by Parker and Waud (1971) from data of Fig. 2 taking into account the total bound radioactivity to evaluate the concentration of free ^{3}H-ligand

A) Kinetic parameters:
Association k_{on} $0.29 \cdot 10^9\ M^{-1} \cdot min^{-1}$; Dissociation k_{off} $0.19 \cdot min^{-1}$; $K_d = k_{off}/k_{on}$ 0.65 nM

B) Constants at equilibrium:
Maximal binding B_M 478 ± 12 fmole · mg protein^{-1}; Dissociation constant Kd 0.71 ± 0.05 nM; Hill coefficient 1.03 ± 0.07

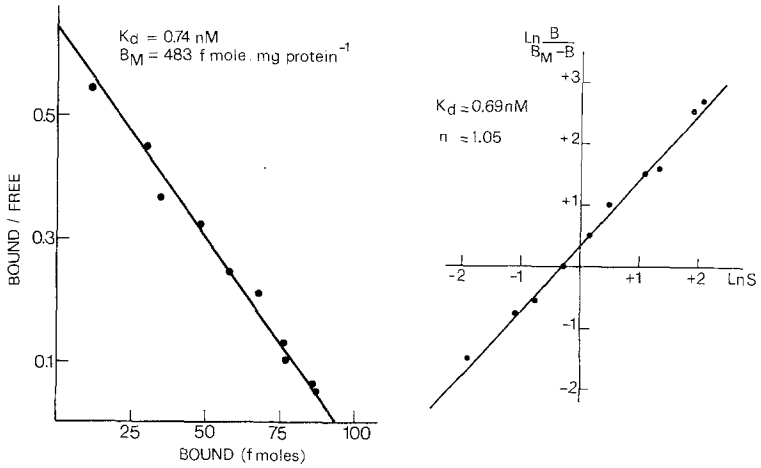

Fig. 3
Saturable binding of ^{3}H-domperidone in mouse striatum: Scatchard (left) and Hill (right) representations of data of Fig. 2 (saturable binding). Concentrations of free domperidone were evaluated by subtracting total bound ^{3}H-domperidone from ^{3}H-domperidone added in the incubation mixture. In the Scatchard representation bound (*B*) and free (*F*) ^{3}H-domperidone are expressed in fmoles. Incubations contained 0.2 mg protein in a volume of 0.2 ml of medium. Data of the Hill representation were calculated taking into account a capacity of sites (B_M) of 483 fmole · mg protein^{-1}. Constants derived from least square analysis

phine (16-fold more potent than the transmitter). Agonists and antagonists of serotonin slightly antagonized the specific ^{3}H-domperidone binding, particularly the antagonist methysergide which exhibited a K_i of about 0.1 μM. Finally, noradrenaline inhibited the binding with a K_i of about 30 μM. Various other non-dopaminergic agents studied had no effect at a concentration up to 1 mM.

The nature of the inhibition of the specific binding of ^{3}H-domperidone by haloperidol was assessed by establishing saturation curves in the presence of 1 nM or 5 nM haloperidol (Fig. 7). The Scatchard representation of these data shows that the maximal number of binding sites (intersection of the straight lines with the abscissa) was not altered in the presence of haloperidol. This representation allowed the determination of the apparent dissociation constant of the ^{3}H-ligand which varied with the concentration of the inhibitor

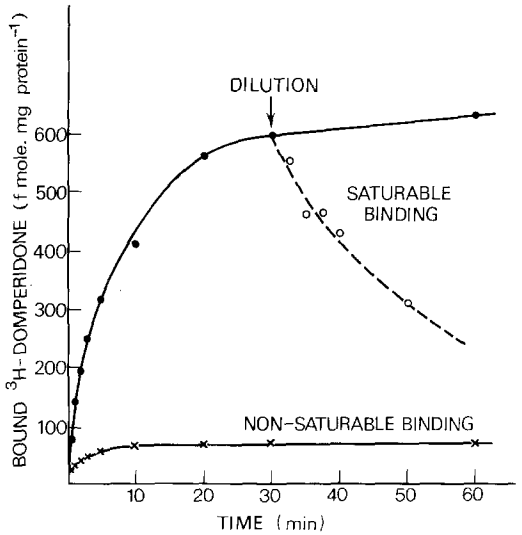

Fig. 4. ^{3}H-Domperidone binding to mouse striatal membranes: association and dissociation. For the measurement of association, membranes were incubated at 30°C for increasing periods with 2.4 nM ^{3}H-domperidone in the presence or the absence of 500 nM non-radioactive domperidone (*non-saturable binding*). In dissociation experiments, the incubation medium was diluted 100-fold after a 30 min-incubation at 30°C and ^{3}H-domperidone remaining bound to the membranes was measured by filtrations performed at various times (dotted line). Means of two independent experiments

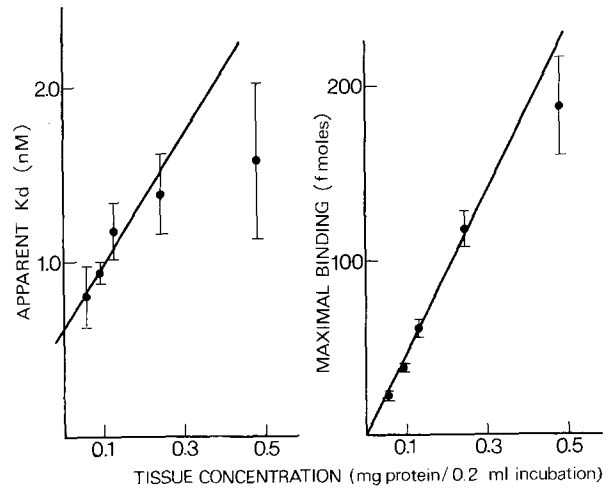

Fig. 5. Effect of tissue concentration on *apparent Kd* and *maximal binding*. Saturation experiments analogous to that described in Fig. 2 were performed with increasing tissue concentrations, and *apparent Kd* and *maximal binding* were evaluated by Parker and Waud (1971) analysis of the data (triplicate determinations). In this analysis, free concentrations of ligand were not used and *apparent Kd* was evaluated by taking into account the total ^{3}H-domperidone added into the incubation. True Kd and B_M were evaluated by linear regression using the first 4 points

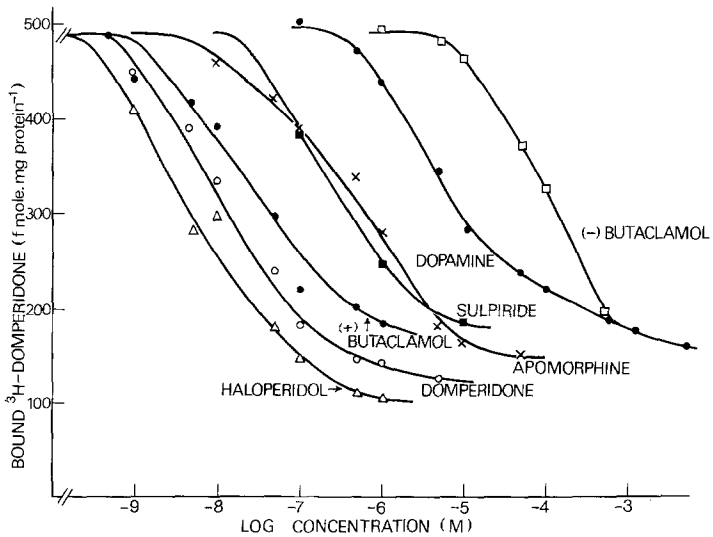

Fig. 6. Inhibition of ^{3}H-domperidone binding by dopaminergic agents. Thirty-min incubations in the presence of 4 nM ^{3}H-domperidone and various dopaminergic agents at increasing concentrations. Means of 1–3 experiments

Table 2. Inhibition of ^{3}H-domperidone binding by various agents. Same conditions as in Fig. 6. K_i values were calculated from the IC_{50} of the various compounds according to the relationship $K_i = IC_{50}/(1 + S/Kd)$ in which Kd = 0.7 nM and S is the concentration of ^{3}H-domperidone (4 nM)

Dopaminergic Agents	K_i (nM)	Non-Dopaminergic agents	K_i (nM)
Haloperidol	0.85	Methysergide	130
Domperidone	1.1	Bufotenine	7,100
(+) Butaclamol	8.3	Noradrenaline	28,000
Sulpiride	81.0	Serotonin	55,000
Apomorphine	109.0	5-Methoxytryptamine	67,000
Dopamine	1,800	Morphine	>100,000
(–) Butaclamol	28,000	γ-Aminobutyric acid	>100,000
		Glutamate	>100,000
		L-DOPA	>100,000
		Histamine	>100,000
		Carbamyl choline	>100,000

(not shown) and led to a K_i value of 1.28 nM for haloperidol.

Comparison of ^{3}H-Domperidone Binding and ^{3}H-Spiperone Binding in Various Regions of Mouse Brain

In all brain regions the saturable binding of ^{3}H-spiperone was higher than that of ^{3}H-domperidone when non-saturable binding was measured in the presence of 500 nM of the corresponding non-radioactive compound. However, in each region the binding inhibited by 10 μM apomorphine did not differ significantly with either ^{3}H-ligand (Fig. 8). Furthermore, only small differences between the binding inhibited by apomorphine and by the non-radioactive ligand were found for ^{3}H-domperidone, whereas several-fold differences were found for ^{3}H-spiperone in various regions like the cerebral cortex.

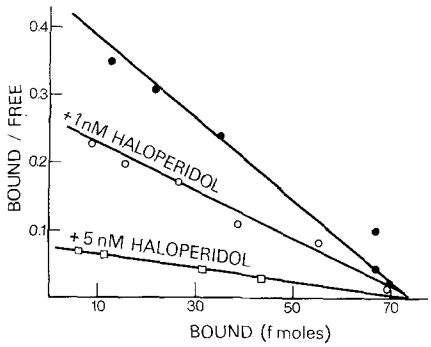

Fig. 7. Saturation of ^{3}H-domperidone binding in the presence of haloperidol: Scatchard representation. The particulate fraction from mouse striatum was incubated for 30 min with increasing concentrations of ^{3}H-domperidone alone or in the presence of either 1 nM or 5 nM haloperidol. Bound (*B*) and free (*F*) concentrations of ^{3}H-domperidone were expressed in fmoles. Incubations contained 0.15 mg protein in a volume of 0.2 ml of medium. Means of two independent experiments with results varying by less than 10%

Discussion

All our experiments with ^{3}H-domperidone indicate that this ligand labels with highaffinity an homogenous population of sites in mouse brain.

Using a particulate fraction from mouse striatum, the saturable binding of ^{3}H-domperidone (evaluated as the fraction of the binding inhibited in the presence of a 100-fold excess of the non-radioactive compound) represents 75% of the total binding at a 4 nM concentration of the ^{3}H-ligand. This percentage of "specific" binding can be further raised to 95% of the total when membranes are more extensively washed on the filters. These values are higher than those generally reported for ^{3}H-haloperidol (Creese et al., 1975; Seeman et al., 1975) but in the same range as those for ^{3}H-spiperone, which is usually considered as the ligand of choice for dopamine receptors (Leysen et al., 1978a).

^{3}H-Domperidone associates rapidly to striatal sites, equilibrium being reached after about 30 min at 30° C. Dissociation occurs at this temperature with first-order kinetics and a half-life of about 4 min.

That ^{3}H-domperidone is recognized by an homogenous population of sites is indicated by the monophasic character of the saturation curve at equilibrium (Fig. 2) as well as by Scatchard analysis which reveals a single straight line (Fig. 3). The Hill plot suggests that binding occurs without cooperative interaction since the Hill coefficient is close to unity.

Various methods used to determine the dissociation constant (Kd) of domperidone lead to values in good agreement. Thus analysis of the saturation curve at equilibrium by i) an iterative program based on the least

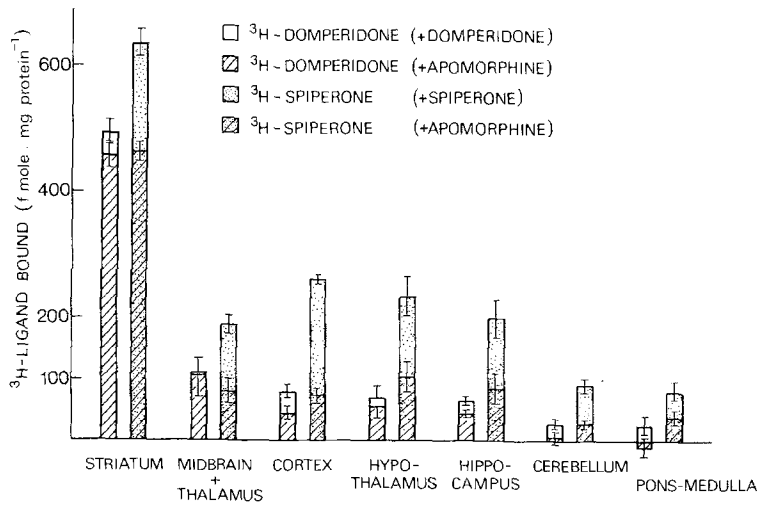

Fig. 8
Comparison of ^{3}H-domperidone binding and ^{3}H-spiperone binding in various regions of mouse brain. Binding was evaluated in the presence of 5 nm of each ^{3}H-ligand, i.e. ^{3}H-domperidone in left columns and ^{3}H-spiperone in right columns. In both cases the saturable binding represents ^{3}H-ligand binding inhibited either by 500 nM of the corresponding non-radioactive ligand (total columns) or by 10 μM apomorphine (hatched part of the columns). Means ± S.E.M. of 3 independent experiments with triplicate determinations on pooled regions from 4–8 animals

squares (Parker and Waud, 1971), ii) Scatchard plot, iii) Hill representation, all give a value of 0.7 nM, when free ligand concentrations are roughly estimated as the difference of total radioactivity in the incubation and total radioactivity bound on membranes. A similar Kd value is also found by the ratio of k_{off} and k_{on} (Table 1). On the other hand, a slightly lower value (Kd = 0.60 nM) which is likely to be more representative (Chang et al., 1975) is obtained by extrapolating the apparent Kd value to a negligible concentration of sites (Fig. 5). This slight difference is probably due to a small overestimation of the concentration of the free ligand: when the latter is estimated by the difference between total radioactivity and radioactivity remaining bound to membranes after filtration and washing, one does not take into account the very low affinity binding probably eliminated during extensive washing of the filters.

The capacity of ^{3}H-domperidone binding sites in striatum is linear with tissue concentration up to 1.5 mg protein · ml^{-1}, leading to a value of 530 ± 45 fmole · mg protein^{-1}, i.e. approximately 30 pmoles per g of fresh tissue. This capacity is in the same range as that of in vitro ^{3}H-haloperidol binding in rat striatum (Seeman et al., 1975; Leysen et al., 1978a; Schwarcz et al., 1978). It is also close to the capacity of ^{3}H-pimozide binding sites in the striatum of the living mouse (Baudry et al., 1977; Schwartz et al., 1978). In contrast, the capacity of saturable ^{3}H-spiperone binding sites in mouse striatum is somewhat higher when evaluated either in vitro (Fig. 8) or in vivo (Baudry et al., 1978).

Various approaches indicate that ^{3}H-domperidone selectively labels dopamine receptors in mouse brain. Dopamine antagonists, i.e. neuroleptic drugs belonging to several chemical classes, inhibit ^{3}H-domperidone binding with a potency reflecting their pharmacological activity (Table 2). In the case of haloperidol, the inhibition is competitive, suggesting that the butyrophenone and domperidone are recognized by the same receptor site. This conclusion is also supported by the similarity of the K_i value of haloperidol to its reported Kd value for binding sites in rat striatum (Seeman et al., 1976; Leysen et al., 1978a). The K_i for non-radioactive domperidone is slightly higher than the Kd of the ^{3}H-ligand. This could be due to an non-quantifiable adsorption of the former onto the glass tubes and consequently to an overestimation of the concentration of free non-radioactive domperidone. The stereospecificity of ^{3}H-domperidone binding is demonstrated by the 3000-fold difference in inhibitory potency of the two butaclamol isomers (Table 2).

Dopamine and its agonist, apomorphine, also inhibit with reasonable potency the binding of ^{3}H-domperidone. However, as already reported in the case of ^{3}H-neuroleptic binding in vitro (Burt et al., 1976; Seeman et al., 1976; Titeler et al., 1978) as well as in vivo (Baudry et al., 1977), dopamine receptor agonists are much less active than antagonists, and the inhibition curve is stretched over a larger scale of concentrations. The reason for these features is still a matter of debate, and we are currently investigating this problem. The specificity of ^{3}H-domperidone as a ligand for dopamine receptors is also shown by the lack of effect of a variety of non-dopaminergic agents (Table 2). However, both methysergide and bufotenine, generally considered as serotoninergic agents, are relatively potent inhibitors, as already reported for ^{3}H-haloperidol binding (Burt et al., 1976; Leysen et al., 1978a). It should be stressed that, at least in the case of methysergide, this does not imply a lack of specificity because this agent exhibits significant dopamine antagonist activity in behavioural tests (Leysen et al., 1978b).

The regional distribution of ^{3}H-domperidone binding sites reflects to some degree the density of dopamine nerve terminals and thus also supports the idea that dopamine receptors are labelled (Fig. 8).

Finally, the comparison of the binding of ^{3}H-domperidone with that of ^{3}H-spiperone in various brain regions clearly points out the interest of the former as a selective ligand for dopamine receptors. First, in most regions the inhibition of ^{3}H-domperidone binding by apomorphine and the non-radioactive drug do not differ significantly, indicating that the major fraction of the saturable binding is to dopamine receptors. This is not the case for ^{3}H-spiperone for which the binding inhibited by apomorphine represents only 30 to 70% of the "saturable" binding. This may be due to the high affinity of spiperone to serotonin receptors as shown by in vitro studies (Creese and Snyder, 1978; Leysen et al., 1978a, b). In in vivo experiments the capacity of ^{3}H-spiperone binding sites in mouse brain also is significantly higher than that of ^{3}H-pimozide binding sites (Baudry et al., 1978).

In conclusion ^{3}H-domperidone appears to be useful ^{3}H-ligand for dopamine receptors, especially in brain regions in which their density is rather low.

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