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Characterization of [^{3}H]zetidoline binding to rat striatal membranes

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The binding of [^{3}H]zetidoline, a novel neuroleptic agent, to rat brain striatal membranes was investigated in-vitro. The optimal binding conditions for [^{3}H]zetidoline differed from those for [^{3}H]spiperone in pH, temperature and time. [^{3}H]Zetidoline has high affinity for striatal dopamine receptors. Its binding is saturable, stereo-specific, has a low non-specific component and is reversible and tissue specific. The Scatchard analysis gave a biphasic curve, indicating that [^{3}H]zetidoline interacts with more than one population of receptor sites (B'_{max} = 67 fmol mg^{-1} protein, K'_d = 0·11 nM; B''_{max} = 500 fmol mg^{-1} protein, K'_d = 2·49 nM). Kinetic analysis of rates of association and dissociation yielded a K_d value in agreement with that measured at equilibrium. Inhibition studies indicated that only dopamine and dopaminergic agents are able to displace [^{3}H]zetidoline from its binding sites, and in a different rank order from that for displacement of [^{3}H]spiperone. (−)-Sulpiride was especially effective in inhibiting [^{3}H]zetidoline specific binding. Furthermore, like that of [^{3}H]benzamides, [^{3}H]zetidoline binding appears to be highly Na^+-dependent and Li^+ only partially substitutes Na^+.

Zetidoline is a selective antidopaminergic agent of a new chemical class (Fontanella et al 1981) which has been found to be highly effective in many pharmacological and biochemical animal models predictive for antipsychotic activity in man (Barone et al 1982a, b; Galliani et al 1983).

In most of these tests, zetidoline proved to be more potent than chlorpromazine and somewhat less potent than haloperidol. Zetidoline differs from most of the classical neuroleptics since it has weaker anti-adrenergic, anticholinergic and cataleptogenic effects and in having no ability to inhibit dopamine-induced stimulation of striatal adenylate-cyclase (Barone et al 1982a, b). Moreover, it is weak in displacing [^{3}H]spiperone from striatal dopamine binding sites in-vitro (Barone et al 1982a, b) and has no sedative effects in psychiatric patients (Silverstone et al 1984). In a recent double-blind comparison with haloperidol, zetidoline was found to be significantly less likely to produce Parkinsonian side-effects. Finally, recent neurophysiological and biochemical studies (Kemali et al 1983; Saletu et al 1983) suggest that zetidoline and sulpiride induce effects consistent with both CNS-inhibiting and activating properties in psychiatric patients. To further define the binding of zetidoline to the dopaminergic receptors, we studied the characteristics of ^{3}H-labelled zetidoline (Fig. 1) binding to rat striatal receptors in-vitro.

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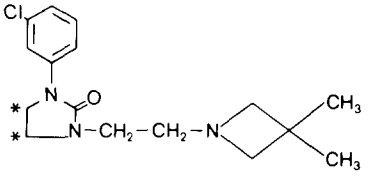

FIG. 1. Chemical structure of zetidoline. Asterisks indicate the ^{3}H in the labelled compound.

MATERIALS AND METHODS

Radioligands

[4,5-^{3}H]Zetidoline, specific activity 24·5–27 Ci mmol^{-1}, was synthesized by New England Nuclear (Boston, Mass., USA) by catalytic reduction of the unsaturated 1-(3-chlorophenyl)-3-(2-(3,3-dimethyl-1-azetidinyl)-ethyl)-imidazolin-2-one precursor, by the method developed by L. Fontanella and G. Tarzia (Lepetit, Italy). Radiochemical purity was always greater than 98·5–99%, as judged by autoradiography and by TLC on silica-gel G in acetone-ammonium hydroxide 99:1 v/v [Benzene ring ^{3}H]-spiperone (specific activity 24·5 Ci mmol^{-1}, New England Nuclear) was used as reference radioligand. The two ^{3}H-ligands were stored in absolute ethanol at −20 °C and dilutions made in ethanol just before use.

Preparation of membranes

Male Wistar rats (150–200 g, Charles River, Calco–Italy) were decapitated and the striata and, for

binding studies on tissue specificity, olfactory tubercles, frontal, parietal and occipital cortex, hippocampus, corpora quadrigemina, cerebellum, midbrain, pons-medulla and hypophysis were quickly dissected out (Glowinski & Iversen 1966) and immediately processed or frozen on dry-ice and stored at −75 °C until use. Fresh or frozen tissue was homogenized in a Polytron PT 10 microhomogenizer (20 s, setting 6) in 100 volumes (w/v) of cold 50 mM Tris-HCl buffer, pH 7.4 (at 25 °C) and centrifuged twice at 50 000*g* for 10 min at 2 °C, with resonication of the intermediate pellet in the same volume of cold buffer. The final pellet, containing the crude synaptosomal membranes, was resonicated and processed according to how it was to be used.

[3H]Zetidoline binding conditions

Many variables in the method usually described (Creese & Snyder 1978; Martres et al 1978) for in-vitro binding studies with 3H-neuroleptics were investigated to find the optimal binding conditions for [3H]zetidoline. Membranes were washed in cold 50 mM Tris-HCl buffer up to 6 times. Pre-washed striatal membranes were incubated with 0·8 nM [3H]zetidoline or 0·2 nM [3H]spiperone at pH values from 6 to 10, with different Tris concentrations in the buffer and with or without various salt concentrations and mixtures, for 1–90 min at 0, 4, 25 or 37 °C.

Characterization of [3H]zetidoline binding

[3H]Zetidoline binding to striatal membranes was characterized by Scatchard analysis (Scatchard 1949), as modified by Weder et al (1974) of saturation studies, compared with [3H]spiperone. These incubations were made in the standard buffer usually described for the in-vitro binding of 3H-neuroleptics: 50 mM Tris, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0·1% ascorbic acid. Incubation pH, time and temperature were optimal as determined in the preliminary studies for each radioligand: pH 7·8 (determined at 25 °C), 45 min at 0 °C (melting ice) for [3H]zetidoline; pH 7·4, 15 min at 37 °C for [3H]spiperone. The 3H-ligands were incubated in triplicate with (non-specific binding) or without (total binding) 3 μM (+)-butaclamol, at concentrations ranging between 0·03 and 16 nM. Specific binding was the total minus the non-specific binding. The incubations were stopped by rapid filtration under vacuum of the entire contents of each test tube (2 ml, 0·25–0·5 mg protein) through Whatman GF/B glass fibre filters followed by two washes with 5 ml of ice-cold incubation buffer. The filters were put into plastic vials containing 10 ml Biofluor (New England Nuclear) and the total radioactivity bound to the membranes trapped in the filters was measured by liquid scintillation spectrometry (Packard 460 C ß-counter).

To assess the tissue specificity of [3H]zetidoline binding and its distribution in the rat brain, crude synaptosomal membranes from ten different cerebral areas were incubated with 0·8 nM [3H]zetidoline or [3H]spiperone (0·2 nM). Displacement studies of [3H]zetidoline and [3H]spiperone binding were carried out in-vitro under the optimal conditions for each radioligand. The resulting IC50 values, the concentrations of the drugs that cause 50% inhibition of the specific 3H-ligand binding, were assessed from at least 6 and sometimes 9 concentrations run in triplicate. The corresponding inhibition curves were transformed into straight lines by Log-Probit analysis (Tallarida & Murray 1981). In-vitro binding data were calculated on an Apple II microcomputer with the 'Recept program' described by Benfenati & Guardabasso (1984). Saturation, tissue specificity and displacement studies were repeated four times. The total protein in the membrane preparation was determined by the Bio-Rad Protein Assay Kit (Bradford 1976).

Reference compounds

Test drugs were gifts from the following sources: spiperone, haloperidol, *cis*- and *trans*-flupenthixol, thioridazine, pimozide, domperidone, fluphenazine and ketanserin, Janssen, Belgium; (+)- and (−)-sulpiride, Ravizza, Italy; (+)- and (−)-butaclamol, Ayerst, USA; molindone, Endo, USA; (±)-ADTN, Burroughs-Wellcome, USA; lysuride, Schering, USA; methiothepine, Hoffman-La-Roche, Switzerland; metergoline, Farmitalia, Italy; phentolamine, Ciba-Geigy, Switzerland; prazosin, Pfizer, USA; clonidine, Boehringer–Ingelheim, West Germany; phenoxybenzamine-HCl and chlorpromazine, Smith Kline and French, USA; clozapine, Sandoz, Switzerland; zetidoline (1-(3-chlorophenyl)-3-[2-(3,3-dimethyl-1-azetidinyl)-ethyl]- imidazolidin-2-one [4,5-3H]), imidoline, metoclopramide and the dopamine agonist (±)-*N*-n-propyl-3-(hydroxyphenol)-piperidine (3-PPP) were synthesized in our laboratories by G. Tarzia and E. Occelli. Apomorphine, dopamine, ergotamine, ergocriptine, bromocriptine, yohimbine, (−)-adrenaline, (−)-noradrenaline and propranolol were purchased from Sigma.

RESULTS

(*a*) *Optimal assay conditions*

Figs 2–4 show optimal conditions for [3H]zetidoline and [3H]spiperone binding. they are achieved by

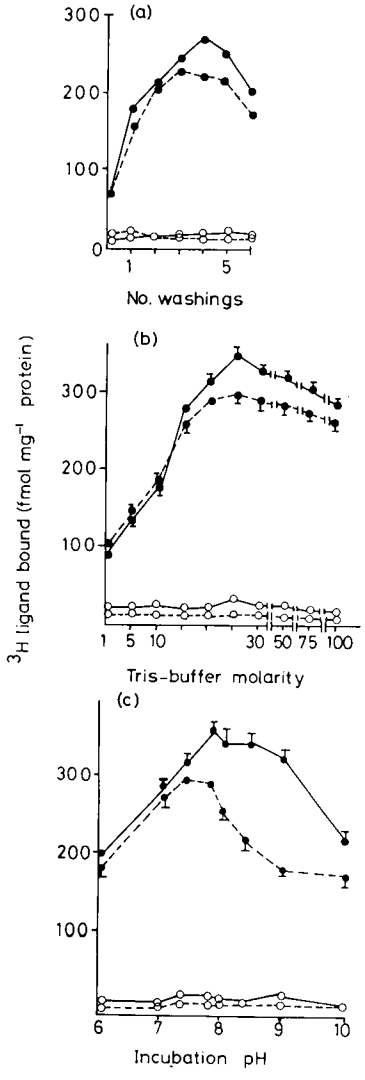

FIG. 2. Effect of pellet washing (a) Tris-buffer molarity (b) and buffer pH (c) on [^{3}H]zetidoline (solid line) and [^{3}H]spiperone (broken line) total (closed circles) and non-specific (open circles) binding. Each point represents the mean (± s.e.m. for b, c) of 4 experiments in triplicate.

incubating membranes washed 3 or 4 times (Fig. 2a) in 20–50 mM Tris with the same standard ion composition and concentration generally described for ^{3}H-neuroleptics (Fig. 2b). The optimal binding conditions for the two ^{3}H-ligands differ on: (i) pH range, which for [^{3}H]zeditoline is 7·8–8·4, while for [^{3}H]spiperone it is 7·0–7·8 (Fig. 2c); (ii) temperature and time, [^{3}H]zetidoline binds better at 0 °C for 45 min, while the best conditions for [^{3}H]spiperone were, as expected (Creese & Snyder 1978; Martres et al 1978), 37 °C for 15 min (Fig. 3a, b); (iii) Na^+ ion

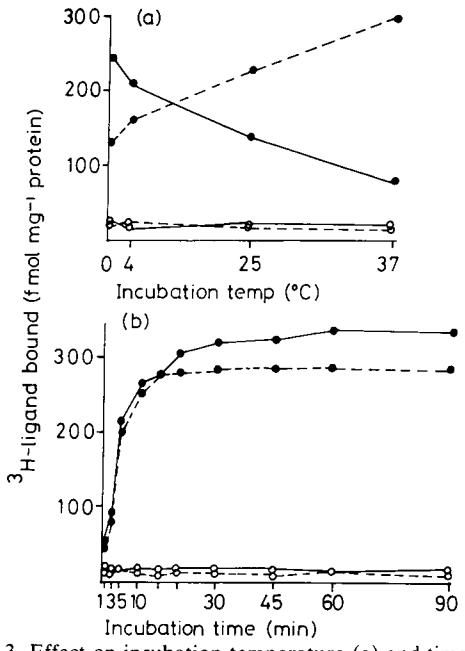

FIG. 3. Effect on incubation temperature (a) and time (b) on [^{3}H]zetidoline (solid line) and [^{3}H]spiperone (broken line) total (closed circles) and non-specific (open circles) binding. Each point represents the mean of 4 experiments in triplicate.

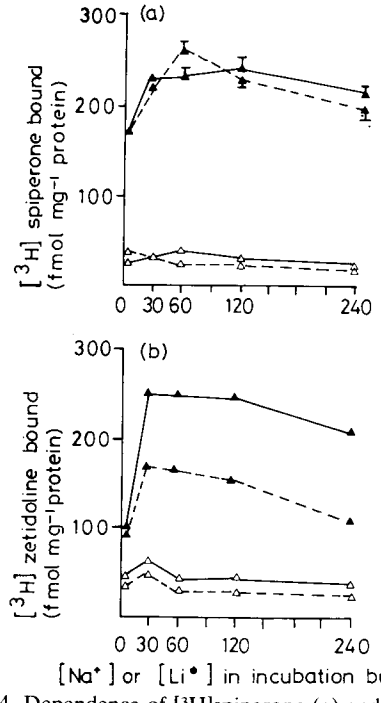

FIG. 4. Dependence of [^{3}H]spiperone (a) and [^{3}H]zetidoline (b) bonding upon the concentration of Na^+ (solid line) and Li^+ (broken line). Closed and open triangles represent total and non-specific binding respectively. Each point is the mean (± s.e.m. for a) of 4 experiments in triplicate.

dependency, specific [³H]spiperone binding is reduced by 25% when there is no Na^+ in the incubation buffer and the maximum specific binding is obtained when 30–120 mM Na^+ is present (Fig. 4a). In addition, 30 mM Li^+ completely restores [³H]spiperone specific binding (Fig. 4a). Similarly, 30–120 mM Na^+ in the incubation buffer gives maximal [³H]zetidoline binding (Fig. 4b); but the absence of Na^+ causes a reduction of 80% that is only partially (50%) overcome by 30–120 mM Li^+ (Fig. 4b). Higher concentrations of Li^+ are unable to overcome the loss of binding caused by the absence of Na^+ in the incubation buffer. Replacement of Na^+ by K^+ (30–120 mM) or Cs^+ (30–120 mM) has some effect on the specific binding of [³H]zetidoline (data not shown). Similarly, the presence or the absence of divalent cations, such as Ca^{2+}, Mg^{2+} and Mn^{2+} only modestly affects [³H]zetidoline and [³H]spiperone binding (data not shown).

(b) Saturability of [³H]zetidoline binding

Saturable [³H]zetidoline binding (i.e., the binding inhibited by 3 μM (+)-butaclamol) after 45 min incubation reached a plateau at approximately 4 nM (Fig. 5a, inset). Fig. 5b (inset) shows that the specific and saturable [³H]spiperone binding, measured under the optimal assay conditions, differs from that of [³H]zetidoline especially for the lower concentration plateau (1 nM). The non-saturable binding, evaluated in the presence of 3 μM (+)-butaclamol, increased linearly at least up to 16 nM ³H-ligand. At 2 nM [³H]zetidoline, saturable binding was more than 80% of the total (Fig. 5a, inset). By increasing the number of membrane washings before the incubation and after the filtration of the incubates, the percentage of specific binding attained was 95% (data not shown).

(c) Binding parameters for [³H]zetidoline

For both ³H-ligands, Scatchard analysis (calculated from a general hyperbolic regression by computer iteration) repeatedly gave biphasic curves indicating two populations of sites (Fig. 5a, b). [³H]Zeditoline apparently labels one population of binding sites with high affinity (K'_d = 0·11 nM) and low capacity (B'_{max} = 67 fmol mg^{-1} protein) and another population of sites with lower affinity (K''_d = 2·49 nM) and higher capacity (B''_{max} = 500 fmol mg^{-1} protein). [³H]Spiperone has K'_d = 0·06 nM and B'_{max} = 465 fmol mg^{-1} protein for the first population, and a K''_d = 0·74 nM and B''_{max} = 790 fmol mg^{-1} protein for the second. When [³H]zetidoline was incubated at 37 °C for 15 min, the parameters were: B'_{max} = 22·5 fmol mg^{-1} protein; K'_d = 0·44 nM; B''_{max} = 360 fmol mg^{-1} protein; K''_d = 15·5 nM.

The Hill coefficients calculated from the same data were significantly different from one: 0·81 ± 0·035

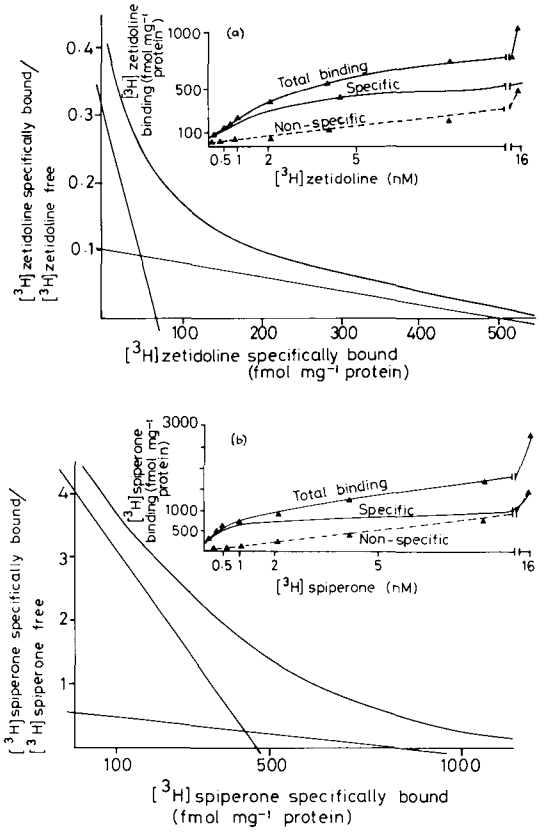

FIG. 5. [³H]Zetidoline (a) and [³H]spiperone (b) binding to rat striatal membranes. The figures summarize four different experiments, done in triplicate, at 0 °C for 45 min, pH 7·8 for [³H]zetidoline or at 37 °C for 15 min, pH 7·4 for [³H]spiperone. Saturation curves (inset) were obtained in the presence of ³H-ligands alone (total binding) or with 3 μM (+)-butaclamol (non-specific binding). The specific binding was the total minus the non-specific binding. The concentrations of free ligand are the differences between radioactivity added and radioactivity bound to the membranes (specific plus non-specific binding). Scatchard analysis, done on the specific binding saturation curves, gave biphasic curves, indicating two populations of binding sites for both ³H-ligands. Experimental points are not reported, since Scatchard curves are the resultants of a general hyperbolic regression by computer iteration and not the interpolations of experimental points. Binding parameters are:

for [³H]zetidoline: B'_{max} = 67 fmol mg^{-1} protein; K'_d = 0·11 nM
B''_{max} = 500 fmol mg^{-1} protein; K''_d = 2·49 nM

for [³H]spiperone: B'_{max} = 465 fmol mg^{-1} protein; K'_d = 0·06 nM
B''_{max} = 790 fmol mg^{-1} protein; K''_d = 0·74 nM

for [3H]zetidoline and 0·47 ± 0·020 for [3H]spiperone.

(d) Kinetic parameters of [3H]zetidoline binding

Saturable [3H]zetidoline binding at 0 °C reached equilibrium at 45 min (Fig. 6). The rate constant for association (k_{on}), calculated from the initial slope of the association curve (up to 3 min) and the known concentration of the ligand and of binding sites according to Bennett (1978), was 1·86 × 10^7 M^{-1} min^{-1}. The dissociation kinetics were studied by rapid filtration (8–10 s) following a 1000-fold dilution of labelled zetidoline in the incubation medium by addition of 800 nM cold ligand at a time when equilibrium of binding was sure to have been achieved (Fig. 6). The dissociation of [3H]zetidoline from binding sites was checked every 15 s for the 1st min, every 30 s until the 5th min and every 2·5 min until the 40th min after the addition of cold ligand. The dissociation clearly appeared to follow first-order kinetics (not shown), and plots of the data on a semi-log representation produced a dissociation rate constant (k_{off}) of 4·27 × 10^{-2} min^{-1}. The rate k_{off}/k_{on}, representing the K_d (dissociation constant), was 2·30 nM, which is very close to the value determined by Scatchard analysis for the higher capacity component (K_d = 2·49 nM).

(e) Effects of various drugs on [3H]zetidoline binding

Two different series of compounds were investigated as inhibitors of [3H]zetidoline and [3H]spiperone binding. Both ligands were tested at concentrations (0·8 and 0·2 nM) corresponding to 1/5 of the saturating ones, which allows labelling of both binding sites. The first series included various antagonist and agonist for dopamine receptors, while the second series included 5-HT agents and α- and β-adrenergic compounds (Table 1). Spiperone is the most potent inhibitor of both 3H-ligands. When incubated under the respective optimal conditions, the various inhibitors displace [3H]zetidoline and [3H]spiperone with a different rank order. Haloperidol, (−)-sulpiride, zetidoline and imidoline show greater affinity for [3H]zetidoline than for

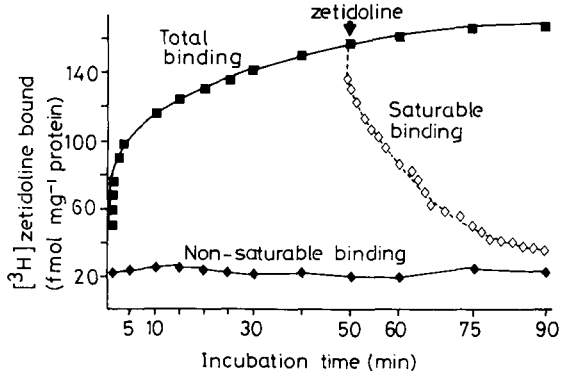

FIG. 6. Time course of [3H]zetidoline binding to rat striatal membranes: association and dissociation. For the determination of association, membranes were incubated at 0 °C, pH 7·8 for increasing periods with 0·8 nM [3H]zetidoline with (non-saturable binding) or without (total binding) 3 μM cold zetidoline. After 50 min incubation, a 1000-fold excess of cold zetidoline (800 nM) was added to half of the tissue and [3H]zetidoline remaining bound to the membranes was measured by filtration of aliquots taken at various times (dotted line). The points are means of 4 experiments.

Table 1. In-vitro inhibition of [3H]zetidoline and [3H]spiperone binding to rat striatal membranes by various agents.

Compounds	IC50 (nM) on [3H]zetidoline	rank order	[3H]spiperone	rank order
Dopamine antagonists				
Spiperone	0·160	(1)	0·315	(1)
Haloperidol	1·75	(2)	3·40	(6)
(−)-Sulpiride	2·15	(3)	71·6	(11)
Zetidoline	2·65	(4)	50·6	(10)
Imidoline	3·10	(5)	130	(13)
cis-Flupenthixol	4·30	(6)	1·90	(3)
Chlorpromazine	4·55	(7)	12·1	(8)
Thioridazine	5·50	(8)	27·0	(9)
Domperidone	5·90	(9)	4·50	(7)
Molindone	6·80	(10)	90·0	(12)
Pimozide	7·20	(11)	0·950	(2)
Metoclopramide	17·0	(12)	280	(14)
Fluphenazine	22·0	(13)	2·3	(5)
(+)-Butaclamol	89·8	(14)	2·15	(4)
(+)-Sulpiride	228	(15)	5760	(17)
Clozapine	360	(16)	430	(15)
trans-Flupenthixol	2 400	(17)	470	(16)
(−)-Butaclamol	80 900	(18)	6780	(18)
Dopamine agonists				
Apomorphine	66·0		72·0	
(±)-ADTN-Br	70·0		300	
Dopamine	525		3 480	
(±)-3-PPP	870		1 300	
Mixed 5- HT/dopamine agents				
Methiothepine	0·96		0·67	
Lysuride	17·5		1·41	
Ergotamine	19·0		2·20	
Metergoline	44·0		92·0	
Ergocriptine	98·0		3·50	
Bromocriptine	180		8·00	
Ketanserin	1 250		900	
α-ß-adrenergic agents				
Yohimbine	460		1 850	
Phentolamine	5 500		3 900	
(−)-Adrenaline	8 520		23 300	
(−)-Noradrenaline	9 000		23 800	
Propranolol	18 000		53 000	
Phenoxybenzamine	31 500		740	
Prazosin	36 000		36 000	
Clonidine	36 000		36 000	

Assays were performed using 0·8 nM [3H]zetidoline and 0·2 nM [3H]spiperone. Incubations were at the best conditions for each 3H-ligand: 0 °C for 45 min, pH 7·8 for [3H]zetidoline and 37 °C for 15 min, pH 7·4 for [3H]spiperone. Specific binding was that displaced by 3 μM (+)-butaclamol. In incubations with dopamine, adrenaline, noradrenaline or apomorphine, the buffer was supplemented with 10 μM pargyline which did not modify the characteristics of the binding. All drugs were tested at least at 6 and sometimes at 9 concentrations in triplicate. The resulting inhibition curves were transformed into straight lines according to Log-Probit analysis to give the IC50 values. The data are the mean of 4 different experiments.

[3H]spiperone binding, while the contrary is true for pimozide, fluphenazine and (+)-butaclamol. These differences in the inhibition of the two 3H-ligands are temperature-dependent, since they disappear when the incubations are performed at the same temperature (Table 2). Differences of lesser degree are seen in displacement data for both 3H-ligands by 5-HT agents. This class of compound is generally a great deal less active against [3H]zetidoline than against [3H]spiperone binding. Compounds with adrenergic activity are very poorly active or inactive in displacing either 3H-ligand. The rank order for inhibition for the neurotransmitters was dopamine > (−)-adrenaline > (−)-noradrenaline, for both [3H]zetidoline and [3H]spiperone.

Table 2. Temperature-dependence of the displacement of [3H]zetidoline and [3H]spiperone binding in-vitro.

	IC50 (nM) on			
	[3H]zetidoline at		[3H]spiperone at	
	0 °C for 45 min	37 °C for 15 min	0 °C for 45 min	37 °C for 15 min
(−)-Sulpiride	2·15	27	3·90	71·6
Pimozide	7·20	0·105	3·80	0·950
Metoclopramide	17·0	155	5·20	280
(+)-Butaclamol	89·8	0·42	21·0	2·15
Fluphenazine	22·0	1·57	18·5	2·30
cis-Flupenthixol	4·30	0·95	6·7	1·90

Incubations were in standard buffer (Tris-HCl 50 mM, 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, 0·1% ascorbic acid, pH 7·4 at 25 °C). See legend to Table 1.

(*f*) *Stereoselectivity of [3H]zetidoline binding*

Sulpiride, flupenthixol and butaclamol displaced the two labelled ligands in highly stereospecific fashion, with only (−)-sulpiride, *cis*-flupenthixol and (+)-butaclamol active. The (−) enantiomer of sulpiride was 100 times more active than (+)-sulpiride, while (+)-butaclamol and *cis*-flupenthixol were 900 times and 600 times more potent in displacing [3H]-zetidoline binding than their respective enantiomers (Table 1).

(*g*) *Tissue specificity of [3H]zetidoline binding*

The distribution of [3H]zetidoline binding in the cerebral areas studied was almost exclusively confined to striatum, olfactory tubercles and hypophysis (Fig. 7). Binding in the parietal cortex and in the mid-brain was negligible. In contrast, [3H]spiperone labelled all the brain areas except the cerebellum and pons-medulla.

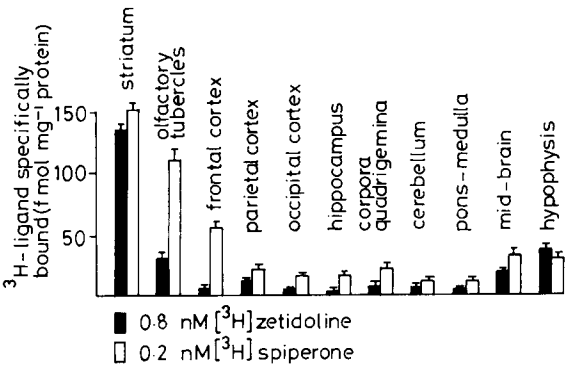

FIG. 7. Comparison of [3H]zetidoline and [3H]spiperone specific binding in several regions of the rat brain. Specific binding was that displaced by 3 μM (+)-butaclamol. Mean ± s.e.m. of 4 independent experiments in triplicate on pooled regions from 8–10 animals.

DISCUSSION

Like that of [3H]spiperone, [3H]zetidoline binding to synaptosomal membranes of the rat striatum fits all the classical prerequisites for binding to a "receptor site": saturability, high affinity, low non-specific binding, stereoselectivity and tissue specificity. Among the neurotransmitters tested, dopamine is the most active in displacing both [3H]zetidoline and [3H]spiperone, indicating that the dopaminergic system is the one most affected by both drugs.

The optimal binding conditions for the two radioligands differ, however, in pH, time, temperature and Na^+-dependence. Maximal [3H]zetidoline binding occurs in a pH range of 7·8–8·4, at 0 °C for 45 min. The corresponding figures for [3H]spiperone are pH 7·0–7·8, at 37 °C for 15 min. Finally, only [3H]zetidoline binding is markedly dependent on Na^+ and Na^+ can be only partially replaced by Li^+.

Scatchard analysis gives biphasic curves for both ligands, indicating that both [3H]zetidoline and [3H]spiperone interact with more than one population of receptor sites. These results confirm the widely recognized ability of spiperone to label sites other than dopamine receptors. Among these, the most important are 5-HT receptors, which although mainly in the cortex (Creese & Snyder 1978), are also present in the striatum (Leysen et al 1978; Howlett & Nahorski 1980; Seeman 1980; List & Seeman 1981). The biphasic [3H]zetidoline binding presumably differs qualitatively from that of [3H]spiperone, since zetidoline essentially does not interact with 5-HT sites in rat brain. This is in agreement with the information that: (1) zetidoline is essentially ineffective in antagonizing the behavioural activities of 5-HT and tryptamine (Restelli et al unpublished observations); (2) zetidoline does not interfere with 5-HT metabolism and turnover in the rat brain-stem or cerebral cortex (Restelli et al); (3) [3H]zetidoline does not bind to cortical areas of the rat brain,

which are very rich in 5-HT receptors (Barone et al 1982a, 1983); (4) in the rat striatum and even more in the prefrontal cortex, zetidoline was practically inactive in displacing [^{3}H]ketanserin bound to 5-HT$_2$ receptors (Barone et al 1984); (5) as shown in the present work, agents which interfere more or less specifically with 5-HT receptors are weak displacers of [^{3}H]zetidoline binding to rat striatal membranes. Moreover, zetidoline does not interact with adrenergic receptors, since adrenergic compounds are inactive against [^{3}H]zetidoline binding.

On the basis of this evidence, the two populations of [^{3}H]zetidoline receptor sites cannot be accounted for as 5-HT or adrenergic receptors. Perhaps [^{3}H]-zetidoline binds to glial receptors which differ in affinity and B_{max} from the neuronal ones. Alternatively, [^{3}H]zetidoline might bind to sites located pre- and post-synaptically. Actually, electrophysiological studies have shown that zetidoline, at doses as low as 100 μg kg^{-1}, antagonizes the depression of firing rate in the substantia nigra of anaesthetized rats caused by apomorphine (Harris et al 1983), proving it to be a potent dopamine antagonist on the central dopamine autoreceptors. Furthermore, in conscious rats, at doses too low to affect the reflex firing of dopaminergic cells in the pars compacta of the nigra, zetidoline was able to inhibit apomorphine-related depression of neuronal firing (Mereu, personal communication).

[^{3}H]Zetidoline binds to dopamine receptors in a saturable fashion. Non-specific binding is very low, representing about 5% of the total when membranes are extensively washed. The value of the Hill coefficient, 0·81 ± 0·035, although consistent with an heterogeneity of binding sites, indicates the presence of a main component. [^{3}H]Zetidoline associates to and dissociates from receptor sites with rates yielding a value of the dissociation constant ($k_{off}/k_{on} = k_d$) very close to that shown by the Scatchard analysis for the major component of binding.

Dopamine antagonists, i.e. neuroleptics belonging to several chemical classes, inhibited [^{3}H]zetidoline with a different rank-order from that in which they displaced [^{3}H]spiperone. This finding is clearly due to different optimal binding conditions (temperature, pH and time of incubation) for the two ^{3}H-ligands. [^{3}H]Zetidoline binds better at 0 °C than at 37 °C probably because of both a higher value of k_{on}/k_{off} and a different receptor-site configuration which might be optimal at 0 °C. The reduction of B_{max} for [^{3}H]zetidoline binding at 37 °C strengthens this hypothesis. Differences in binding related to experimental conditions have to be expected in any study of displacers which makes it difficult to qualitatively interpret in-vitro displacement studies dealing with compounds of different chemical classes. However, our data allow us to conclude the following: (1) The selective displacement of labelled zetidoline by the pharmacologically active stereoisomers of sulpiride, butaclamol and flupenthixol indicates that the stereospecificity of [^{3}H]zetidoline binding is high. (2) The regional distribution in the rat brain of [^{3}H]zetidoline binding sites reflects to some degree the density of dopamine nerve endings and thus also supports the idea that dopamine receptors are labelled selectively by [^{3}H]zetidoline. Moreover, most of the classical neuroleptics tested inhibited the binding of labelled zetidoline in a nM range, suggesting that in the rat striatum the same population of sites specifically binds [^{3}H]zetidoline and the reference displacers and they are dopaminergic. (3) The butyrophenones spiperone and haloperidol are the most active against [^{3}H]zetidoline. Interestingly, (−)-sulpiride is as active as haloperidol and more active than zetidoline, flupenthixol, chlorpromazine or any other neuroleptic tested, making it even more probable that zetidoline and sulpiride share, at least in part, common receptor sites. (4) Zetidoline and sulpiride do not inhibit the dopamine-sensitive adenylate cyclase (Barone et al 1982a, b). Both (see the present work and Freedman et al 1981a) have nM affinity for dopamine antagonists and μM affinity for dopamine agonists. (5) Na^+ is clearly needed for optimal interaction of [^{3}H]zetidoline with dopamine receptors and it cannot be completely replaced by Li^+, as it can for [^{3}H]spiperone. This Na^+-dependence of [^{3}H]zetidoline binding is similar to that of the benzamides, [^{3}H]sulpiride (Hall et al 1980; Theodorou et al 1980; & Woodruff 1982) and [^{3}H]sultopride (Mizuchi et al 1982). The Na^+-dependence of [^{3}H]zetidoline binding, as for the benzamides, is Na^+-specific, since no other monovalent cations (Li^+, K^+, Rb^+ and Cs^+) can replace Na^+ and restore 100% of the specific binding. At present, we do not know if the Na^+-dependence of [^{3}H]zetidoline and [^{3}H]benzamide binding are of the same nature. For the Na^+-dependence of benzamides, Stefanini et al (1980, 1981) postulated that it reflects a steric contiguity of the benzamide recognition-sites to the Na^+-channels and/or a reversible modification of the receptor membranes by that which may be preferential for this class of ligands. On the basis of this hypothesis, the striatum would contain at least two populations of dopaminergic sites, Na^+-dependent and Na^+-independent. Theodorou et al (1980, 1982), Hall et

al (1980) and Freedman et al (1981b, 1982a, b) suggested a number of possible explanations for the marked Na^+-dependence of [3H]sulpiride binding to dopamine receptors, indicating the complexity of this problem.

In conclusion, this overall evidence shows that zetidoline selectively binds to a population of dopaminergic receptors different from that labelled by [3H]spiperone. Zetidoline's binding conditions, cerebral distribution and the rank-order of affinity of displacing drugs differ from those of butyrophenones. Moreover, the apparent Na^+-dependence of [3H]zetidoline binding and the high affinity of (−)-sulpiride for zetidoline receptor sites suggest that these two ligands share, at least in part, a common population of binding sites in the rat striatum.

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