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Comparison of ^{3}H-Spiperone Binding in Caudate Nuclei of Rabbits and Rats[1]

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HERNANDEZ, L. L. AND D. A. POWELL. *Comparison of ^{3}H-spiperone binding in caudate nuclei of rabbits and rats.* PHARMACOL BIOCHEM BEHAV **26**(2) 253–258, 1987.—^{3}H-Spiperone binding and inhibition by competing ligands were studied in caudate nucleus homogenates from Sprague-Dawley rats and New Zealand albino rabbits. ^{3}H-Spiperone binding sites were very similar in the two species and had pharmacological profiles characteristic of D2-dopamine receptors, in accordance with previous reports. However, differences occurred between the species in the potencies with which dopaminergic ergots and other drugs interacted with these binding sites and with regard to the compounds used to define specific binding. These findings suggest that slight differences in relative proportions of subtypes of ^{3}H-spiperone binding sites, or of neuroleptic potencies at these subtypes, may exist between rabbits and rats.

Dopamine (DA) receptors ^{3}H-Spiperone (spiroperidol) Apomorphine 6,7-ADTN Ergots Neuroleptics Caudate nucleus Rabbits Rats

IN previous studies we have attempted to relate behavioral changes due to brain lesions or aging in rabbits to changes in striatal dopamine content or receptor density [4, 8, 16]. However, few experiments have examined the characteristics of dopamine receptor binding in rabbit striata, although those that have done so have found results similar to those found in rats [16, 20, 22]. We have found that the number of specific ^{3}H-spiperone binding sites, defined using unlabelled apomorphine as the blank, decrease in rabbit caudate with aging; the resultant decline in neostriatal function may parallel age-related impairments in somatomotor response learning, whereas forebrain concentrations of norepinephrine and serotonin are reliably correlated with learned cardiovascular response magnitude in both old and young animals [9,16]. However, the number of ^{3}H-spiperone sites found in young rabbit caudate ($B_{max} \cong 7$ pmol/g tissue) was somewhat less than is typically reported in rat caudate using neuroleptics to define specific binding, suggesting that apomorphine may inhibit only a subset of ^{3}H-spiperone sites in rabbit caudate [16]. Since multiple binding sites for ^{3}H-spiperone have been documented in rat striata (e.g., [17,18]), we undertook the present experiments to directly compare ^{3}H-spiperone binding and inhibition by competing ligands in rabbit and rat caudate nucleus homogenates, to assess the similarities and differences between the two species and to better characterize the binding sites(s) present in rabbit tissue.

METHOD

Tissue Preparation

Animals were sacrificed by decapitation, brains rapidly removed and caudate nuclei dissected. Tissue samples were frozen at −80°C for up to two weeks prior to assay, when they were thawed, weighed and homogenized for 15 sec in 100 volumes (based on wet weight of tissue) of ice cold Tris buffer (50 mM, pH 7.4) using a Brinkman polytron (setting No. 6). Homogenates were centrifuged at 27,000×g, 10°C for 10 minutes; the crude membrane pellets were washed twice by resuspension in cold Tris buffer and recentrifugation. The tissue pellet was resuspended in 100 volumes of ice cold Tris buffer (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, 2.65 mM $CaCl_2$, 1 mM $MgCl_2$, 50 μM l-ascorbic acid and 10 μM pargyline, and was preincubated at 37°C for 5 min in a shaking incubator. The sample was then recentrifuged and membranes resuspended in 100 volumes of fresh buffer including salts, ascorbic acid and pargyline.

Procedure and Data Analyses

Caudate nuclei from each New Zealand albino rabbit were assayed individually; caudates from 4–8 Sprague Dawley rats were pooled to yield an equivalent tissue weight per sample (approximately 100 mg tissue). Rabbits were either male or female; all rats were male. One rabbit and one pooled rat sample were assayed in triplicate during each ex-

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periment; experiments were replicated on 4–5 different occasions. All reagents were freshly prepared on the day of each experiment.

For saturation experiments tubes were prepared on ice containing various concentrations of ^{3}H-spiperone (New England Nuclear, 22.6–22.9 Ci/mmol; 0.05–4.0 nM in the final incubation mixture) with or without 1.0 μM apomorphine HCl or 0.1 μM (+)butaclamol to define specific binding; each tissue sample was assayed with each unlabelled ligand. One hundred μl of cold tissue suspension was added to each tube, and tubes were incubated at 37°C for 10 min in a shaking incubator (200 μl final incubation volume containing membranes from 1.0 mg of tissue, i.e., approximately 55 μg protein for rabbit tissue and approximately 80 μg protein for rat tissue). Incubation was terminated by adding 4 ml of ice cold Tris buffer (including salts, ascorbic acid and pargyline) to each tube, followed by rapid vacuum filtration through Whatman GF/B filters or Schlecher and Schuell (S&S) No. 32 filters previously soaked in 0.1% TWEEN-80. Filters were washed twice with 4 ml buffer per wash and air dried; radioactivity was determined by liquid scintillation counting at least 12 hours after the addition of 8 ml Scintillene LSC cocktail to each filter. In control experiments it was determined that soaking the S&S filters in TWEEN-80 reduced butaclamol-inhibitable ^{3}H-spiperone binding to the filters to negligible levels comparable to those obtained using the Whatman filters (i.e., approximately 2.5% of total binding in the presence of caudate tissue).

For inhibition experiments, tubes were prepared containing ^{3}H-spiperone (0.5 nM final concentration) and 6–7 concentrations of unlabelled inhibitor; specific binding was defined with both 1 μM (+)butaclamol and with 1 μM unlabelled spiperone for each tissue. One hundred μl of cold tissue suspension was added (200 μl final incubation volume) and incubation, filtration and liquid scintillation counting carried out as for saturation experiments. In control experiments, 10 nM ketanserin was also included in the incubation mixture to assess the degree of ^{3}H-spiperone binding to serotonin-2 sites (cf. [2,12]).

Protein content was determined using the method of Lowry *et al.* [13]. Nonlinear regression and Hill [10] analyses were applied to saturation data to determine B_{max}, K_d and n_H; data from inhibition experiments were analyzed by the Hill [10] method and linear regression to determine n_H and IC_{50}s. Inhibition constants (K_is) were calculated from IC_{50}s using the Cheng and Prosoff [1] correction, and K_ds for rabbits and rats estimated in saturation experiments using (+)butaclamol to define specific binding.

RESULTS

Saturation Experiments

Specific ^{3}H-spiperone binding appeared to be saturable in both rabbit and rat caudates as defined with either 1 μM apomorphine or 0.1 μM (+)butaclamol as the blank (Fig. 1); specific binding represented 50 to 85% of total binding in all experiments. Scatchard plots of the data were linear and Hill coefficients did not differ from 1.0, indicating that under these assay conditions binding occurred to a single site in both rabbit and rat tissue. Non-linear regression analyses revealed that a one-site model accounted for at least 99.5% of the variance in the data in each case, and yielded estimates of B_{max} and K_d, respectively, of (a) 400.4 fmol/mg protein and 0.69 nM in rabbit caudate vs. (+)butaclamol; (b) 295.6 fmol/mg and 0.72 nM in rabbit vs. apomorphine; (c) 407.4 fmol/mg and 0.83 nM in rat caudate vs. (+)butaclamol; and (d) 422.6 fmol/mg and 0.76 nM in rat vs. apomorphine. Ninety-five percent confidence intervals constructed about each parameter revealed that there were significantly fewer ^{3}H-spiperone binding sites in rabbit caudate as defined with apomorphine than in rabbit caudate as defined with butaclamol. Moreover, there were significantly fewer binding sites in rabbit vs. apomorphine than in rat vs. either butaclamol or apomorphine. B_{max} values for rabbit vs. butaclamol, rat vs. butaclamol and rat vs. apomorphine did not differ significantly from each other, and K_d values were the same in rats and rabbits vs. either butaclamol or apomorphine.

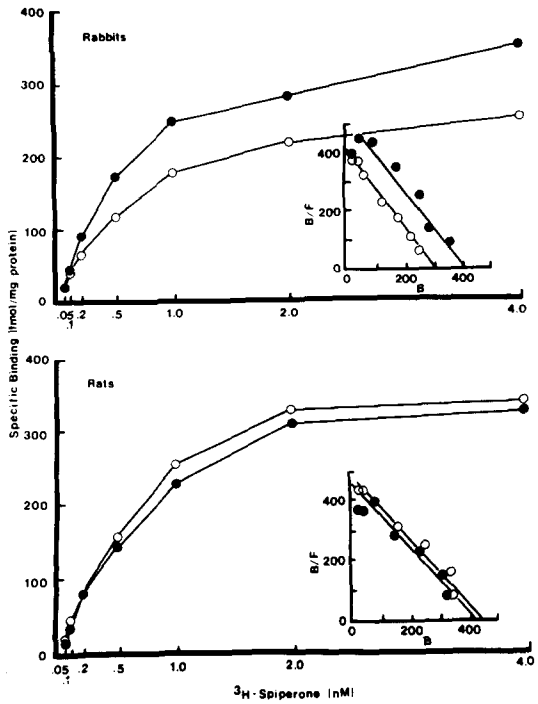

FIG. 1. Specific ^{3}H-spiperone binding in rabbit and rat caudate nuclei using 1.0 μM apomorphine (open symbols) or 0.1 μM (+)butaclamol (filled symbols) as the blank. Insets: Scatchard plots of the data. B: Specific binding (fmol/mg protein). F: Concentration of ^{3}H-spiperone (nM).

Inhibition Experiments

During inhibition experiments maximum specific binding of 0.5 nM ^{3}H-spiperone, as defined with 1.0 μM (+)butaclamol or 1.0 μM unlabelled spiperone, represented 80–95% of total binding in various experiments. Matched-pair *t*-tests revealed that in rat, but not rabbit, caudate specific binding defined by spiperone was a slightly but significantly higher percentage of total binding than was specific binding defined by butaclamol in the same tissue [means 85.3% and 83.1%, respectively; $t(11)=2.51$, $p<0.03$]. Also, maximum specific binding in rabbit caudates was reliably higher than that in rat caudates assayed simultaneously, for both definition ligands [means 289.7 fmol/mg protein for rabbit and 221.3 fmol/mg for rat vs. (+)butaclamol,

t(11)=3.80, $p<0.003$; 281.8 fmol/mg for rabbit and 223.3 fmol/mg for rat vs. spiperone, t(11)=4.11, $p<0.002$], in agreement with the saturation data suggesting somewhat higher affinity in rabbits than rats (cf. Fig. 1, 0.5 nM ^{3}H-spiperone vs. butaclamol blank).

Figure 2 shows the inhibition data for each dopaminergic compound tested, when specific binding was defined with 1.0 μM (+)butaclamol. As shown in Table 1, the non-ergot dopamine agonists apomorphine and (±)6,7-ADTN competed with ^{3}H-spiperone with low Hill coefficients; 95% confidence intervals constructed about these coefficients confirmed that n_H values were significantly less than 1.0 in both rabbit and rat tissues, when specific binding was defined by either (+)butaclamol or unlabelled spiperone. Apomorphine was about twice as potent as 6,7-ADTN in inhibiting ^{3}H-spiperone binding; however for both apomorphine and ADTN, aggregate K_i values were similar in rabbits and rats and for the two definition ligands. The ergot agonists bromocriptine and lisuride inhibited ^{3}H-spiperone binding with Hill coefficients that did not differ significantly from 1.0, and also showed K_is that were similar in rabbits and rats; lisuride was about five times as potent as bromocriptine in inhibiting ^{3}H-spiperone binding, and in both species was the most potent inhibitor tested except unlabelled spiperone itself. However, matched-pair t-tests showed that in rats, but not rabbits, K_i values for both bromocriptine and lisuride were significantly lower when specific binding was defined with butaclamol than when it was defined with unlabelled spiperone (Table 1).

As also shown in Table 1, the substituted benzamide neuroleptic (±)sulpiride similarly showed a lower K_i in rats when specific binding was defined with butaclamol than when it was defined with spiperone, although sulpiride was much less potent than bromocriptine or lisuride; unlike the ergot agonists, sulpiride also showed a lower K_i vs. butaclamol than vs. spiperone in rabbits. Moreover, sulpiride was about twice as potent in rats as in rabbits, when specific binding was defined with either unlabelled ligand, and in rats vs. spiperone only, the Hill coefficient was significantly less than 1.0 (Table 1).

Like the ergot agonists, the thioxanthene neuroleptic α-flupenthixol showed a lower K_i vs. butaclamol than vs. spiperone as the blank, in rats but not in rabbits; the potency of α-flupenthixol was similar in both species and Hill coefficients did not differ from 1.0 (Table 1). (+)Butaclamol and spiperone inhibited ^{3}H-spiperone binding with K_i values that were similar between the two species and vs. the two definition ligands; among all of the compounds tested unlabelled spiperone was the most potent inhibitor of ^{3}H-spiperone binding (Table 1).

Separate control experiments for serotonin-2 binding showed that inclusion of 10 nM ketanserin in the incubation mixture did not affect (+)butaclamol inhibition of ^{3}H-spiperone binding defined with either butaclamol or spiperone, in either rabbit or rat caudate (data not shown). Similarly, ketanserin had no effect on spiperone inhibition of ^{3}H-spiperone binding in rats. However, in two experiments in rabbits, ketanserin reduced the mean K_i for spiperone inhibition of 0.50 nM ^{3}H-spiperone binding from 0.57 nM (±0.06 nM SEM) to 0.46±0.07 nM vs. (+)butaclamol as the blank and from 0.55±0.07 nM to 0.45±0.06 nM vs. spiperone as the blank. Matched-pair t-tests showed that this difference was significant when butaclamol was the blank, $t(1)=13.28$, $p<0.05$, but, due to the small number of experiments, was only marginally significant when spiperone was the blank, $t(1)=9.00$, $p=0.070$. Neither maximum specific binding nor nonspecific binding defined by either ligand was reduced in rabbits in the presence of ketanserin, however.

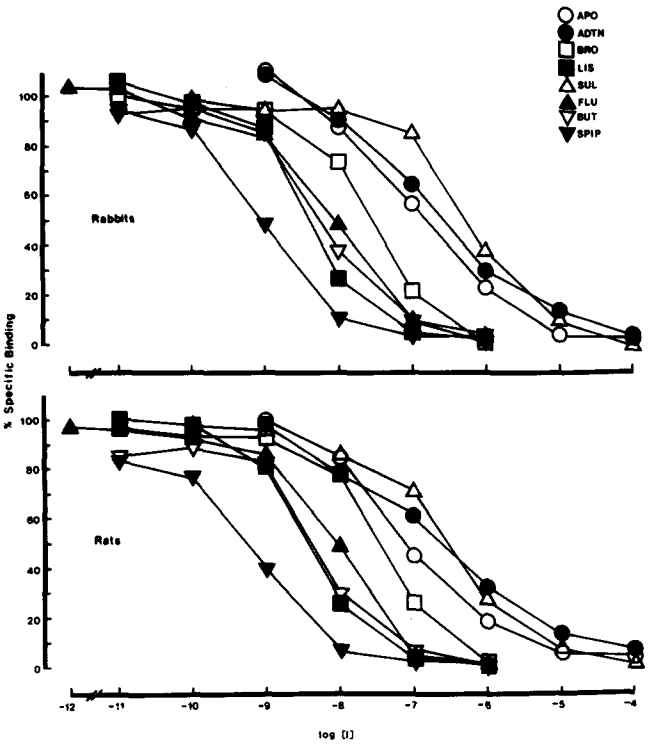

FIG. 2. Inhibition of specific binding of 0.50 nM ^{3}H-spiperone by dopaminergic compounds, in rabbit and rat caudate nuclei. The data shown represent the percentage of maximum specific binding defined using 1.0 μM (+)butaclamol as the blank. [I]: Molar concentration of inhibitor.

DISCUSSION

The present results are generally in good agreement with previous studies in rabbits and rats showing that the majority of striatal ^{3}H-spiperone binding sites are very similar in the two species and have pharmacological profiles characteristic of D2-dopaminergic receptors as distinguished by Kebabian and Calne [11] (e.g., [17, 18, 20, 22]). Our data also suggest that there may be multiple subtypes of these ^{3}H-spiperone sites in both rabbits and rats, also in accordance with previous reports (e.g., [2, 17–19, 22]). The results of inhibition experiments suggest that the non-ergot dopamine agonists apomorphine and 6,7-ADTN inhibited ^{3}H-spiperone binding in both species by interacting with more than one subtype of ^{3}H-spiperone binding site, since Hill coefficients were less than 1.0. Zahniser and Dubocovich [22] found similar results for the non-ergot agonists N,N-di-n-propyldopamine, dopamine and dopamine+GTP in rabbits and rats. Non-ergot dopamine agonists have been found in several species and different neural tissues to distinguish between two ^{3}H-spiperone binding sites that are not recognized as different by dopamine antagonists or by ergopeptine agonists (e.g., [2, 5, 21, 22]); these sites both appear to be of the D2 type and may be interconverting, GTP-dependent configurations of the

TABLE 1

INHIBITION OF ^{3}H-SPIPERONE (0.50 nM) BINDING IN STRIATAL HOMOGENATES

Inhibitor	SB def.	N	Rabbits K_i (nM) (SEM)	Rabbits n_H (SEM)	Rats K_i (nM) (SEM)	Rats n_H (SEM)
Apomorphine (10^{-4}–10^{-9} M)	But[1]	4	94.53 (11.71)	0.740* (0.066)	54.67 (13.03)	0.599* (0.053)
	Spi[1]	4	94.80 (13.68)	0.732* (0.065)	48.02 (15.83)	0.597* (0.029)
6,7-ADTN (10^{-4}–10^{-9} M)	But	4	168.95 (48.19)	0.628* (0.063)	139.98 (48.68)	0.488* (0.034)
	Spi	4	177.83 (46.95)	0.650* (0.047)	113.08 (46.28)	0.504* (0.044)
Bromocriptine (10^{-6}–10^{-11} M)	But	5	15.22 (4.15)	1.044 (0.096)	19.95‡ (2.33)	0.902 (0.145)
	Spi	5	16.53 (3.56)	1.002 (0.099)	22.76 (1.90)	0.852 (0.130)
Lisuride (10^{-6}–10^{-11} M)	But	4	2.88 (1.01)	1.208 (0.119)	2.60‡ (0.64)	1.155 (0.060)
	Spi	4	2.87 (0.81)	1.202 (0.067)	3.03 (0.75)	1.059 (0.069)
(±)Sulpiride (10^{-4}–10^{-10} M)	But	4	334.51‡ (38.22)	0.904 (0.041)	171.37†‡ (10.20)	0.760‡ (0.080)
	Spi	4	389.95 (49.35)	0.966 (0.110)	207.44† (9.08)	0.701* (0.080)
α-Flupenthixol (10^{-6}–10^{-12} M)	But	4	5.79 (2.01)	0.960 (0.129)	6.30‡ (0.84)	1.234 (0.058)
	Spi	4	6.01 (1.64)	0.976 (0.198)	7.10 (0.90)	1.166 (0.085)
(+)Butaclamol (10^{-6}–10^{-11} M)	But	4	4.33 (1.14)	0.922 (0.102)	3.78 (1.03)	0.824 (0.063)
	Spi	4	4.02 (0.74)	1.008 (0.158)	3.06 (0.28)	0.798 (0.070)
Spiperone (10^{-6}–10^{-11} M)	But	4	0.55 (0.15)	0.808 (0.085)	0.40 (0.06)	0.697 (0.140)
	Spi	4	0.56 (0.13)	0.867 (0.060)	0.33 (0.02)	0.695 (0.142)

[1]Definition ligand for specific binding: But=1.0 μM (+)Butaclamol, Spi=1.0 μM Spiperone.
*n_H less than 1.0, α=0.05.
†Different from rabbits, α=0.05.
‡Different from SB def.=Spi, α=0.05.

same receptor which may have different pre- and post-synaptic localization (e.g., [2, 3, 7, 14]).

Although the inhibition data suggest that these two dopamine agonist binding sites are similar in the two species, the saturation data further suggest that together, apomorphine sites comprise only about 75% of ^{3}H-spiperone binding sites in rabbit caudate [16]. It should be noted that one possible explanation for the lower number of apomorphine sites in rabbit caudate is the relatively low concentration of apomorphine used to define specific binding: 1 μM represents only about a 10-fold excess over the K_i of apomorphine (Table 1). This concentration was chosen to replicate the condition used in our previous experiment, which showed a significant reduction in apomorphine-inhibitable ^{3}H-spiperone binding sites in aged rabbits [16]; a similarly low concentration of (+)butaclamol (~10–20 times its K_i) also was used in saturation experiments for comparison. Since inhibition experiments indicated that apomorphine interacts with more than one ^{3}H-spiperone binding site (i.e., its Hill coefficients were less than 1; Table 1) it is possible that 1 μM apomorphine was too low a concentration to completely occupy all ^{3}H-spiperone binding sites labelled in saturation experiments by higher ^{3}H-spiperone concentrations, resulting in an apparent reduction in number of these sites (Fig. 1). However, neither the K_i nor the Hill coefficients for apomorphine differed between rabbits and rats (Table 1), and

therefore it appears unlikely that the low apomorphine concentration used as the blank can account for the species difference in the saturation curves.

Examination of Fig. 1 also suggests that 4.0 nM ^{3}H-spiperone may not have completely saturated all binding sites in rabbit caudate, although the decelerative slope of the curve is clear and specific binding of 2.0 vs. 4.0 nM ^{3}H-spiperone did not differ significantly using either definition ligand. However, these data may represent a small component of binding to a low-affinity receptor (e.g., D1 receptor or a non-dopaminergic receptor) in rabbit caudate; if so, this may suggest that a species difference also exists for non-D2 ^{3}H-spiperone binding sites. This possibility is also supported by the inhibition data showing that inclusion of ketanserin in the incubation mixture increased the potency of spiperone to inhibit ^{3}H-spiperone binding in rabbits but not rats. Ketanserin binds to both S2 and α-1 adrenergic spiperone sites in frontal cortex of rats and mice (e.g., [12,15]) although in caudate nucleus non-S2 binding should be negligible. In any case, the slightly higher binding of 4.0 nM over 2.0 nM ^{3}H-spiperone in rabbit caudate occurred using both definition ligands (Fig. 1), and therefore it cannot account for the much larger difference in binding defined by (+)butaclamol vs. apomorphine in rabbits, nor for the lack of this difference in rats. Further investigations will be necessary before the nature of this and other observed species differences can be determined.

The observed number and affinity of ^{3}H-spiperone sites defined by (+)butaclamol agree well with those reported by Thal *et al.* [20] for rabbits, but differ somewhat from those reported by Zahniser and Dubocovich [22] who, using somewhat more sensitive assay procedures, found about 2-fold higher numbers and about 7-fold higher affinities for ^{3}H-spiperone binding in both rabbits and rats. This discrepancy is most probably attributable to differences in the binding assays, notably the tissue preparation procedures and tissue concentrations used in the two studies (e.g., [18]). The present results also agree reasonably well with our own previous report of ^{3}H-spiperone binding defined by apomorphine in rabbit caudate [16], although that study showed somewhat higher affinity and somewhat lower number of sites in young rabbits ($K_d \cong 0.23$ nM, $B_{max} \cong 7$ pmol/g tissue or about 130 fmol/mg protein). This difference is probably attributable to the higher ascorbic acid concentration used in the buffer medium in our earlier experiment (2.8 mM in the incubation mixture); ascorbic acid in this concentration range is known to inhibit both dopamine agonist and antagonist binding in other species (e.g., [6,18]).

The relative potencies of the various drugs tested in inhibition experiments are also in good agreement with those of Thal *et al.* [20] in rabbits and with those of Zahniser and Dubocovich [22], who also found that S-sulpiride was about half as potent in rabbits as in rats in inhibiting ^{3}H-spiperone binding. However, the present results differ somewhat from those of the latter authors in that, in addition to the non-ergot agonist drugs, they estimated Hill coefficients less than 1.0 for (+)butaclamol in rats, and for spiperone and α-flupenthixol in rabbits, which we did not. Conversely, we estimated n_H less than 1.0 for sulpiride in rats ($p<0.05$ vs. spiperone only), whereas they did not. This discrepancy is probably due to the higher sensitivity of the assay procedures used by Zahniser and Dubocovich (i.e., 12-point competition curves and lower tissue concentration in greater volume incubations), which allowed them to resolve two high affinity neuroleptic binding sites [18] not distinguishable with the present procedure. In the case of sulpiride, the different results are probably due to the enantiomeric form used: We used racemic sulpiride, which has an affinity between those of the separated enantiomers used by Zahniser and Dubocovich [22]. Their data showed that S-sulpiride was about 20 times as potent as R-sulpiride in inhibiting ^{3}H-spiperone binding in rat striata whereas it was about 40 times as potent in rabbit striata. Moreover, their saturation experiments indicated that ^{3}H-spiperone labels two sites in both species, only the major one of which is also bound by ^{3}H-S-sulpiride. Thus, this second, lower affinity site for ^{3}H-spiperone (presumably bound by R-sulpride) would more likely be seen in rats than in rabbits using (±)sulpiride.

Although the ^{3}H-spiperone binding sites in rabbits and rats appear to be very similar pharmacologically, the present data also show certain differences between the species. In addition to the apparent difference in numbers of apomorphine-inhibitable ^{3}H-spiperone sites noted in the saturation experiments, and the different potencies and Hill coefficients noted for (±)sulpiride in inhibition experiments, the present results also revealed that the ergot agonists, (±)sulpiride and α-flupenthixol showed apparently higher affinity for ^{3}H-spiperone binding sites in rats when specific binding was defined by butaclamol than when it was defined by unlabelled spiperone; only (±)sulpiride showed this difference in rabbits and, as noted above, was the only compound that showed differential potency in the two species. Maximum specific binding of 0.50 nM ^{3}H-spiperone was also greater vs. 1.0 μM spiperone than vs. 1.0 μM (+)butaclamol in rats but not rabbits. Thus, slight differences in the relative proportions of D2 receptor subtypes, or in the relative affinities of (+)butaclamol and spiperone for these sites, appear to exist between the species. Since ketanserin increased the potency of spiperone to inhibit ^{3}H-spiperone binding only in rabbits (vs. butaclamol), a slight difference in an S2 component of ^{3}H-spiperone binding in caudate, or of butaclamol affinity for S2 receptors in the two species, may also exist; Zahniser and Dubocovich [22] also reported differences in ketanserin binding properties to ^{3}H-spiperone sites in rabbits and rats. However, these findings concur with previous reports that the majority of ^{3}H-spiperone binding sites in rabbit and rat caudate appear to be highly similar and pharmacologically of the D2 type in both species.

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