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Critical reevaluation of spiperone and benzamide binding to dopamine D_2 receptors: evidence for identical binding sites

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Abstract

There are several inconsistencies in the literature as regards the characteristics of benzamide and butyrophenone binding to dopamine D_2-like receptors. The variations observed in B_{max}, K_d and K_i values have led to hypotheses, such as the existence of a specific 'benzamide binding site' and that dopamine D_2 receptors exist in a monomer-dimer equilibrium, where benzamides are supposed to bind receptor monomers and butyrophenones receptor dimers. We have previously suggested that the discrepant results may instead be related to methodological difficulties associated with the use of very high-affinity radioligands (e.g. ligand depletion and failure to achieve equilibrium). The present study was designed to reinvestigate and critically reevaluate the binding characteristics of [^{3}H]spiperone, [^{3}H]nemonapride, [^{125}I](*S*)-3-iodo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethylsalicylamide ([^{125}I]NCQ-298) and [^{3}H]raclopride to cloned human dopamine D_{2A} and rat striatal dopamine D_2 receptors in order to establish whether they label the same receptor population. We found that the K_d values of [^{3}H]spiperone, [^{125}I]NCQ-298 and [^{3}H]nemonapride were about 20 pM and that of [^{3}H]raclopride about 1 nM. We did not find any significant differences between the B_{max} values determined with the various radioligands. Furthermore, the K_i values of spiperone and NCQ-298 (derived from cross-competition studies) for dopamine D_2 receptors labelled with either [^{3}H]spiperone or [^{125}I]NCQ-298 were in good agreement with the corresponding K_d values. In conclusion, our results clearly demonstrate that when studied under correct experimental conditions, all four radioligands label an identical receptor population.

Keywords: Dopamine D_2 receptor; [^{3}H]Spiperone; [^{3}H]Raclopride; [^{125}I]NCQ-298; [^{3}H]YM-09151-2; Nemonapride; Receptor binding, in vitro

1. Introduction

Dopamine receptors are the major target for drugs used in the treatment of many neuropsychiatric diseases, including schizophrenia. There is a good correlation between clinical potency of various antipsychotics and their affinity for dopamine D_2 receptors which supports the dopamine hypothesis of schizophrenia (Seeman, 1987).

Neuroleptics from several chemical classes have been used as radioligands to selectively label dopamine D_2 receptors in various parts of the brain. The most commonly used radioligands are the butyrophenone [^{3}H]spiperone and the substituted benzamides, e.g. [^{3}H]sulpiride, [^{3}H]raclopride, [^{3}H]nemonapride ([^{3}H]YM-09151-2), [^{125}I]epidepride and [^{125}I]NCQ-298 ([^{125}I](*S*)-3-iodo-*N*-[(1-ethyl-2-pyrrolidinyl)methyl]-5,6-dimethylsalicylamide) (Hall et al., 1991a, b; Kessler et al., 1991; Köhler et al., 1985; Niznik et al., 1985; Zahniser and Dubocovich, 1983). There are, however, several inconsistencies in the literature as regards the characteristics of benzamide and butyrophenone binding to dopamine D_2-like receptors. Both the K_i and B_{max} values determined using various radioligands vary considerably (Hall et al., 1991a, b; Niznik et al., 1985; Seeman et al., 1992). For example, the reported K_d value for the most extensively used dopamine D_2 receptor radioligand, [^{3}H]spiperone, varies 1000-fold, from 13 to 1600 pM (Seeman et al., 1984). The discrepancies in B_{max} and K_i/K_d values have led to speculations that substituted benzamides label an unique 'benzamide neuroleptic subtype' of dopamine receptors or that there are various subclasses of dopamine D_2-like receptors (Ögren and Högberg, 1988; Seeman et al., 1992). It has also been suggested that dopamine D_2 receptors exist as both monomers and dimers and that butyrophenones, like

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spiperone, label dimers whereas benzamides, like raclopride, label monomers (Seeman et al., 1992).

We have previously demonstrated that [^{125}I]NCQ-298, when studied under correct experimental conditions, is an exellent high-affinity radioligand that labels the same receptor population as [^{3}H]raclopride (Jerning et al., 1995). We therefore suggested that the discrepancies between the B_{max} and K_d/K_i values of various neuroleptics frequently reported using high-affinity radioligands, including [^{3}H]spiperone and [^{125}I]NCQ-298, may simply be related to the methodological difficulties associated with the use of radioligands with K_d values in the picomolar range (Jerning et al., 1995; Mohell et al., 1993). Thus, the so-called 'ligand depletion' (due to too high receptor concentration in the assays) as well as the failure to achieve equilibrium often lead to incorrect determination of various receptor binding characteristics (Hulme and Birdsall, 1992). Therefore, in the present studies we have reinvestigated the characteristics of [^{3}H]spiperone, [^{3}H]-nemonapride, [^{125}I]NCQ-298 and [^{3}H]raclopride binding to cloned human dopamine D_{2A} and rat striatal dopamine D_2 receptors using identical and well-defined experimental assay conditions in order to establish whether they label the same receptor population.

2. Materials and methods

2.1. Materials

[^{3}H]Raclopride (specific activity 79 and 82 Ci/mmol), [^{3}H]nemonapride (specific activity 81 Ci/mmol) and [^{125}I]NCQ-298 (specific activity 2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA, USA). [^{3}H]Spiperone (specific activity 82, 95 and 113 Ci/mmol) was purchased from Amersham Life Science (Buckinghamshire, UK). The unlabelled compounds were obtained from the following sources: (+)-butaclamol, Research Biochemicals (Natick, MA, USA); spiperone, Janssen Pharmaceutica (Beerse, Belgium); raclopride and NCQ-298 were synthesized at Astra Arcus (Södertälje, Sweden). All other chemicals were of analytical grade.

Mouse fibroblast (Ltk^-) cells expressing human dopamine D_{2A} (long isoform) receptors were obtained from Dr. O. Civelli (Vollum Institute, Portland, OR, USA) and Sprague-Dawley rats were obtained from B & K Universal (Sollentuna, Sweden).

2.2. Membrane preparations

The cells expressing cloned human dopamine receptors were grown and the cell membranes prepared as described previously (Malmberg et al., 1993). The membranes were stored in aliquots at −70°C. On the day of the experiment, the frozen membranes were thawed, homogenized with a Branson 450 sonifier or an Ultra-Turrax and suspended in appropriate binding buffer to a final concentration of 1–2 μg protein/ml for the [^{3}H]spiperone, [^{3}H]nemonapride and [^{125}I]NCQ-298 binding (2–3 pM receptors). The corresponding concentration for the [^{3}H]raclopride binding was 40–80 μg protein/ml (80–100 pM receptors). Protein concentration was determined by the method of Markwell et al., 1978, with bovine serum albumin as standard.

Male Sprague-Dawley rats (150–220 g) were decapitated and the striata were dissected out on ice and weighed. The tissues were homogenized in 0.05 M Tris-HCl, pH 7.7 with an Ultra-Turrax followed by centrifugation for 10 min at 48 000 × g and 5°C. The pellet was resuspended and recentrifuged. The final pellet was homogenized and resuspended in appropriate buffer. The final concentration of homogenate was 0.05 mg original wet weight/ml (2 pM receptors) for the [^{3}H]spiperone and [^{125}I]NCQ-298 assays and 1 mg original wet weight/ml (25 pM receptors) for the [^{3}H]raclopride assays.

2.3. Radioligand binding assays

[^{3}H]Spiperone, [^{3}H]nemonapride, [^{125}I]NCQ-298 and [^{3}H]raclopride binding assays were carried out in duplicate at 30°C for 4–6 h. The assay buffer for cloned dopamine D_{2A} receptors contained (in mM): 50 Tris-HCl, 120 NaCl, 5 KCl, 4 $MgCl_2$, 1 EDTA; pH 7.6 at room temperature. For rat striatal membranes, the composition of the buffer was (in mM): 50 Tris-HCl, 120 NaCl, 5 KCl, 1 $MgCl_2$, 0.01 pargyline and 0.01% ascorbic acid. In addition, different protease inhibitors were added: 0.05 mM phenylmethane sulfonyl fluoride (PMSF), 70 mg/l bacitracin and 0.1 mM EDTA. In order to remove endogenous dopamine, the striatal membranes were preincubated for 10 min at 37°C. The total incubation volumes were 10 ml for the [^{3}H]spiperone and [^{3}H]nemonapride binding, 2 ml for the [^{125}I]NCQ-298 binding and 0.5 ml for the [^{3}H]raclopride binding. In competition experiments, 30–36 pM [^{3}H]-spiperone or [^{125}I]NCQ-298 was incubated with 10–12 concentrations of competing drug. 1 μM (+)-butaclamol was used to define the nonspecific binding. The incubations were terminated by rapid filtration through Whatman GF/B glass fibre filters and subsequent washing with cold buffer (50 mM Tris-HCl, pH 7.4) using a Brandel or a Skatron cell harvester. Scintillation cocktail was added and the radioactivity determined in a Packard 2500TR liquid scintillation counter.

2.4. Calculations

The data were analysed by nonlinear regression using the LIGAND program (Munson and Rodbard, 1980). The K_d values obtained from saturation studies were used to calculate the K_i values by the LIGAND program. The Hill coefficients were calculated for each individual experiment. The B_{max} values achieved with the various radioligands were compared by analysis of variance (ANOVA).

3. Results

3.1. Comparison of the binding characteristics of various dopamine D_2 radioligands

In the first series of experiments, the receptor binding characteristics of the two benzamide radioligands [^{125}I]NCQ-298 and [^{3}H]raclopride were compared with those of [^{3}H]spiperone using cloned human dopamine D_{2A} and rat striatal dopamine D_2 receptors. The experimental assay conditions for the various radioligands were kept as similar as possible. In order to avoid ligand depletion at all radioligand concentrations, the high-affinity ligands (spiperone and NCQ-298) were incubated with a low receptor concentration of 2–3 pM (0.05 mg original wet weight/ml for rat striatum and 1–2 μg of protein/ml for cloned dopamine D_2 receptors). Consequently, in order to obtain enough counts (cpm) for reliable results, the incubation volume for the tritiated ligand (spiperone) had to be increased to about 10 ml, while a 2-ml volume for the iodinated ligand (NCQ-298) could be used. The incubation time and temperature were the same for all radioligands,

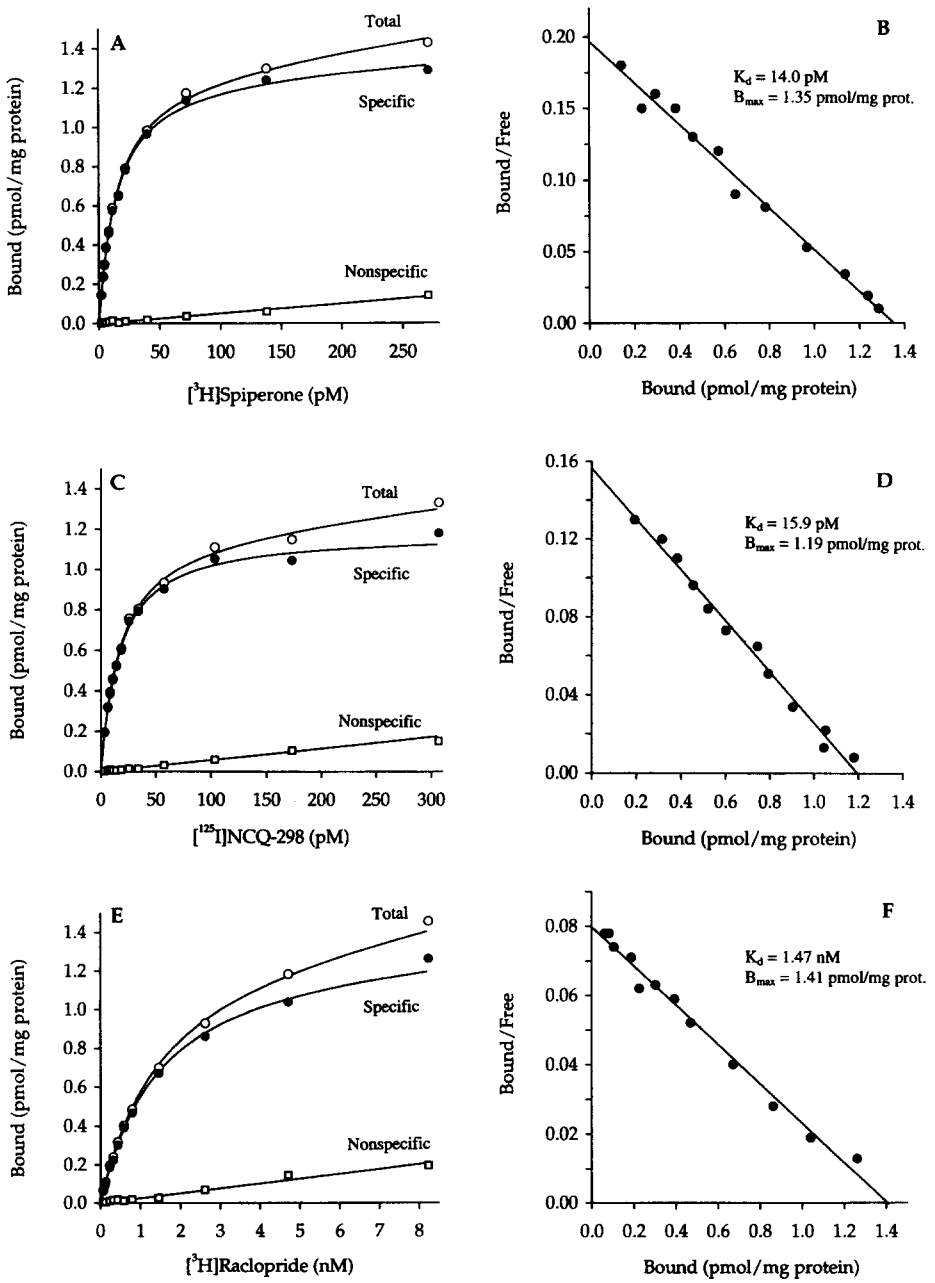

Fig. 1. Representative saturation binding curves for [^{3}H]spiperone (A), [^{125}I]NCQ-298 (C) and [^{3}H]raclopride (E) binding to cloned human dopamine D_{2A} receptors and the corresponding Scatchard plots (B, D and F) of the specific binding. The values determined from the Scatchard analysis were in good agreement with those obtained from the LIGAND program.

Table 1
K_d and B_{max} values of [^{3}H]spiperone, [^{125}I]NCQ-298 and [^{3}H]raclopride binding to cloned human dopamine D_{2A} (A) and rat striatal dopamine D_2 (B) receptors

(A)	K_d (pM)	B_{max} (pmol/mg protein)	n
[^{3}H]Spiperone	15±1	1.41±0.17	4
[^{125}I]NCQ-298	16±0	1.13±0.07	4
[^{3}H]Raclopride	1240±70	1.34±0.03	4
(B)	K_d (pM)	B_{max} (pmol/g wet weight)	n
[^{3}H]Spiperone	19±1	28.4±2.0	5
[^{125}I]NCQ-298	18±1	28.1±3.8	3
[^{3}H]Raclopride	1030±20	25.9±1.2	4

The radioligand binding studies were performed and dissociation constants (K_d) and receptor densities (B_{max}) were calculated as described in Materials and methods. The results are mean±S.E.M. values of n experiments. There were no statistically significant differences between the B_{max} values determined with the various radioligands ($P > 0.05$).

including [^{3}H]raclopride (see Materials and methods for further details).

Fig. 1 shows representative saturation binding curves of [^{3}H]spiperone (Fig. 1A), [^{125}I]NCQ-298 (Fig. 1C) and [^{3}H]raclopride (Fig. 1E) to dopamine D_{2A} receptors. The Scatchard analyses of the specific [^{3}H]spiperone (Fig. 1B), [^{125}I]NCQ-298 (Fig. 1D) and [^{3}H]raclopride binding (Fig. 1F) resulted in linear plots consistent with a noncooperative, single class of binding sites. Table 1 summarises the K_d and B_{max} values of the three radioligands binding to cloned (Table 1A) and to striatal (Table 1B) dopamine D_2 receptors. As can be seen, both [^{3}H]spiperone and [^{125}I]NCQ-298 had very high affinities with K_d values in the low picomolar range (15–19 pM), while [^{3}H]raclopride had a K_d value of about 1 nM. There were no statistically significant differences between the B_{max} values determined with the various radioligands ($P > 0.05$), neither for cloned (Table 1A) nor striatal (Table 1B) dopamine D_2 receptors.

In the second set of experiments, the binding characteristics of [^{3}H]nemonapride were investigated and compared with those of [^{3}H]spiperone and [^{3}H]raclopride using dopamine D_{2A} receptors. The results are summarised in Table 2. [^{3}H]nemonapride bound with a very high affinity (K_d value of 11 pM) and identified the same amount of

Table 2
K_d and B_{max} values of [^{3}H]spiperone, [^{3}H]nemonapride and [^{3}H]-raclopride binding to cloned human dopamine D_{2A} receptors

	K_d pM	B_{max} pmol/mg protein	n
[^{3}H]Spiperone	24±3	2.60±0.20	4
[^{3}H]Nemonapride	11±1	2.73±0.18	4
[^{3}H]Raclopride	1160±30	2.68±0.21	3

The radioligand binding studies were performed and dissociation constants (K_d) and receptor densities (B_{max}) were calculated as described in Materials and methods. The results are mean±S.E.M. values of n experiments. There were no statistically significant differences between the B_{max} values determined with the various radioligands ($P > 0.05$).

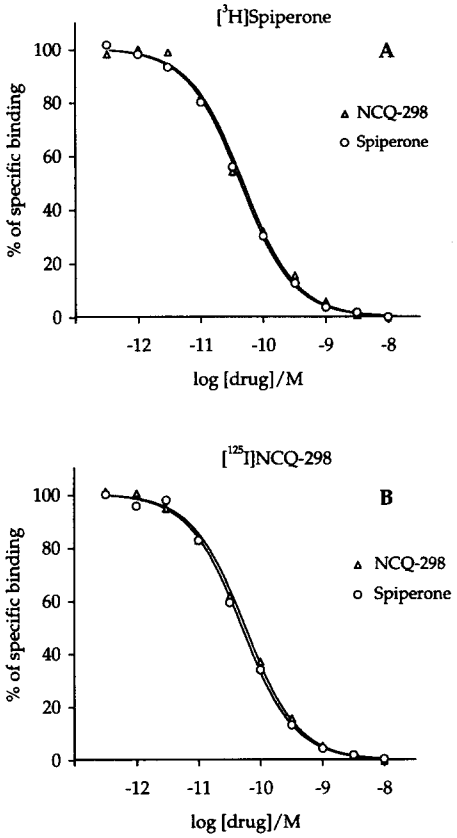

Fig. 2. Inhibition of [^{3}H]spiperone (A) and [^{125}I]NCQ-298 (B) binding to cloned human dopamine D_{2A} receptors by spiperone and NCQ-298. Each figure shows representative inhibition curves performed in parallel.

receptors (B_{max}) as [^{3}H]raclopride and [^{3}H]spiperone ($P > 0.05$).

3.2. Cross-competition studies

In order to show that the radioligands labelled identical binding sites, cross-competition studies were performed. Fig. 2 shows representative competition curves of spiperone and NCQ-298 for [^{3}H]spiperone (Fig. 2A) and [^{125}I]NCQ-298 binding (Fig. 2B) to dopamine D_{2A} receptors. As can be seen, the cold ligands were able to compete with both [^{125}I]NCQ-298 and [^{3}H]spiperone to the level of (+)-butaclamol (defined as 0%). In addition, the Hill coefficients for both ligands were close to one. The results of the cross-competition studies with spiperone and NCQ-298 to cloned and striatal dopamine D_2 receptors are summarised in Table 3. They confirmed that the radioligands label an identical receptor population (Table 3).

Raclopride competition of [^{3}H]spiperone and [^{125}I]-NCQ-298 binding to dopamine D_{2A} receptors gave K_i values of 1.94 ± 0.07 nM ($n = 3$) and 1.52 ± 0.005 nM ($n = 2$), respectively, which are close to the K_i value (2.3 nM) determined using [^{3}H]raclopride (Malmberg et al., 1993). Thus, the K_i values for cold spiperone, NCQ-298 and raclopride were in good agreement with the corre-

Table 3
Inhibition of [^{3}H]spiperone and [^{125}I]NCQ-298 binding to cloned human dopamine D_{2A} receptors and rat striatal dopamine D_2 receptors by spiperone and NCQ-298

Compound	D_{2A}		Striatum	
	[^{3}H]Spiperone	[^{125}I]NCQ-298	[^{3}H]Spiperone	[^{125}I]NCQ-298
Spiperone				
K_i	13±1	14±1	21±0	20±2
n_H	0.88±0.04	0.90±0	1.04±0.08	0.98±0.04
n	4	2	2	2
NCQ-298				
K_i	19±2	18±1	18±2	13±1
n_H	0.81±0.02	0.90±0.01	0.98±0.07	0.98±0.03
n	3	2	2	2

The competition studies were performed and the K_i values were calculated as described in Materials and methods. The results are mean ± S.E.M. values of n experiments. n_H represents the Hill coefficient.

sponding K_d values of the radioligands presented in Table 1A and 1B.

4. Discussion

Several inconsistences exist in the literature as regards the receptor binding characteristics of various dopamine D_2 receptor radioligands. For example, the benzamide [^{3}H]nemonapride has been reported to identify 40% more binding sites than the butyrophenone [^{3}H]spiperone in canine striatal membranes (Niznik et al., 1985) whereas another benzamide [^{125}I]NCQ-298 was reported to label 50% less binding sites than [^{3}H]raclopride, also a benzamide, in rat striatal membranes (Hall et al., 1991a, b). Moreover, Seeman and coworkers recently reported that [^{3}H]nemonapride and [^{3}H]raclopride consistently label 50–100% more binding sites than [^{3}H]spiperone in various membrane preparations (Seeman et al., 1992). They suggested that the dopamine D_2 receptor may exist in a monomer-dimer equilibria, and that the benzamides nemonapride and raclopride bind to monomers while the butyrophenone spiperone binds to dimers, which can explain the differences in dopamine D_2 receptor density observed. Other studies, however, do not support this concept (Jerning et al., 1995; Urwyler and Coward, 1987; Vile et al., 1995).

A common feature of the ligands involved in these studies is their very high dopamine D_2 receptor affinity. Benzamide radioligands with affinities in the picomolar range include [^{3}H]nemonapride (K_d = 57 pM (Niznik et al., 1985)), [^{125}I]epidepride (K_d = 24 pM (Kessler et al., 1991)), [^{3}H]NCQ-115 (K_d = 214 pM (Hall et al., 1991a, b)) and [^{125}I]NCQ-298 (K_d = 17 pM (Hall et al., 1991a, b; Jerning et al., 1995)). In addition to the substituted benzamides, [^{3}H]spiperone binds with a very high affinity to dopamine D_2 receptors, even though the reported K_d values vary considerably (from 13 to 1600 pM) (Seeman et al., 1984). Consequently, K_i values (inhibition constants) for various neuroleptics determined using [^{3}H]spiperone as radioligand and calculated according to the equation of Cheng and Prusoff (1973) ($IC_{50}/1 + ([L]/K_d)$ also show discrepancies.

We have previously suggested that methodological difficulties, associated with the use of high-affinity radioligands, may explain at least some of the reported discrepancies in K_d and B_{max} values. As pointed out by Hulme and Birdsall (1992) 'undetected ligand depletion and nonattainment of equilibrium are two of the commonest systematic artefacts encountered in receptor binding assays. Both kinds of artefacts cause systematic distortion of kinetic and equilibrium binding curves'. Tritiated radioligands with very high affinity combined with a relatively low specific activity (about 100 Ci/mmol) are not easy to use in such a manner that ligand depletion is avoided (Hulme and Birdsall, 1992; Urwyler and Coward, 1987). Generally, a suitable receptor concentration to use is 10 × less than the K_d value of the radioligand. Thus, for radioligands with K_d values of 20 pM the receptor concentration should be about 2 pM (R_t) in order to assure a free ligand concentration of at least 90% in all concentrations ($R_t < 0.1 \times K_d$). In order to obtain enough counts for reliable results with such low receptor concentration, the incubation volume has to be increased. In assays with iodinated radioligands, however, it is possible to dilute the membranes to 2 pM receptors and keep the incubation volume rather low because the specific activity of an iodionated ligand is about 20-fold higher than that of a tritiated one. Here, we used a volume of 10 ml for the tritiated high-affinity ligands, spiperone and nemonapride, and 2 ml for the iodinated NCQ-298.

Kinetic studies with [^{125}I]NCQ-298 have demonstrated that it dissociates very slowly in accordance with its high affinity (K_d of 20 pM as derived from saturation studies) (Jerning et al., 1995). Therefore, 4 h incubation at 30°C was needed to achieve equilibrium. For a radioligand with a K_d of 10 pM, the estimated incubation time is 9.7 h at 30°C (Hulme and Birdsall, 1992). Furthermore, the time to achieve equilibrium in competition studies is increased when an additional ligand is present. In this study, we used an incubation time of 4–6 h for all radioligands.

The extended incubation time required for high-affinity radioligands then leads to another problem, namely the obvious risk of receptor degradation. This in turn may lead to significant errors in determination of receptor densities (Hulme and Birdsall, 1992). Receptor degradation is usually due to endogenous proteolytic action. In the present study, the proteolytic action in rat striatal membranes was minimized by the addition of EDTA, bacitracin and phenylmethane sulfonyl fluoride. In our previous study, no specific [^{125}I]NCQ-298 binding could be detected in the absence of these protease inhibitors (Jerning et al., 1995). The assays with membranes prepared from cultured cells could be performed in the absence of bacitracin and

phenylmethane sulfonyl fluoride, since no receptor degradation was apparent despite the extended incubation time and increased temperature (data not shown). The reason for this might be a lower concentration of proteases in relation to the receptor density in the cultured cells.

An additional complication with receptor binding assays may be the lack of selectivity of the radioligand. While the benzamides have been shown to be very dopamine D_2 selective it should be noted that [^{3}H]spiperone has been shown to bind to 5-HT_2 receptors in rat striatum (Seeman and Grigoriadis, 1987). Its affinity for 5-HT_2 receptors, however, is considerably lower (about 0.5 nM) than for dopamine D_2 receptors (about 20 pM). Thus, within the concentration range used in this study we did not observe any indication of two binding sites. All radioligands labelled only one binding site as analysed with the LIGAND program which is also illustrated by the straight Scatchard plots (Fig. 1).

The cross-competition experiments shown in Fig. 2 provided additional evidence that benzamides and butyrophenones label the same receptor population. Thus, cold spiperone was able to compete with [^{125}I]NCQ-298 to the level of (+)-butaclamol (0%) and vice versa. The K_i values of spiperone and NCQ-298 were in good agreement with the corresponding K_d values and the Hill coefficients indicate binding to only one receptor site.

In conclusion, [^{3}H]spiperone, [^{3}H]nemonapride and [^{125}I]NCQ-298 display very high dopamine D_2 receptor affinity, in the low picomolar range. Such high-affinity ligands require carefully controlled assay conditions in order to avoid experimental artefacts. We found that under such conditions [^{3}H]spiperone and the benzamide radioligands labelled an identical dopamine D_2 receptor population.

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