

# Investigation of Cooperativity in the Binding of Ligands to the D<sub>2</sub> Dopamine Receptor

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## ABSTRACT

The D<sub>2</sub> dopamine receptor exists as dimers or as higher-order oligomers, as determined from data from physical experiments. In this study, we sought evidence that this oligomerization leads to cooperativity by examining the binding of three radioligands ([<sup>3</sup>H]nemonapride, [<sup>3</sup>H]raclopride, and [<sup>3</sup>H]spiperone) to D<sub>2</sub> dopamine receptors expressed in membranes of Sf9 cells. In saturation binding experiments, the three radioligands exhibited different B<sub>max</sub> values, and the B<sub>max</sub> values could be altered by the addition of sodium ions to assays. Despite labeling different numbers of sites, the different ligands were able to

achieve full inhibition in competition experiments. Some ligand pairs also exhibited complex inhibition curves in these experiments. In radioligand dissociation experiments, the rate of dissociation of [<sup>3</sup>H]nemonapride or [<sup>3</sup>H]spiperone depended on the sodium ion concentration but was independent of the competing ligand. Although some of the data in this study are consistent with the behavior of a cooperative oligomeric receptor, not all of the data are in agreement with this model. It may, therefore, be necessary to consider more complex models for the behavior of this receptor.

There is increasing evidence that G protein-coupled receptors (GPCRs) exist as oligomers (Devi, 2001; Angers et al., 2002). This has been demonstrated using biochemical techniques such as coimmunoprecipitation, functional complementation studies, and energy transfer techniques, and this oligomerization may be functionally important (Gazi et al., 2002).

Ligand binding studies may be used to obtain evidence about the oligomeric state of GPCRs. If GPCRs exist as monomers with single binding sites, simple ligand binding patterns should be observed. In saturation binding studies, all radioligands should exhibit the same maximal binding capacities (B<sub>max</sub> values), independent of the assay conditions. All ligands should compete fully with one another, and dissociation constants determined in saturation and competition studies should be the same. Dissociation rates of radioligands should be independent of the method used to initiate dissociation.

If, however, GPCRs exist as homo-oligomers, this should be manifested in terms of the properties of the receptor (Monod et al., 1965; Koshland et al., 1966). Ligand binding, receptor activation, and ligand dissociation experiments could be ex-

pected to exhibit cooperative effects. Some studies report data suggesting cooperative effects in the binding of ligands to GPCRs. Ligand binding studies on muscarinic acetylcholine receptors are inconsistent with independent monomeric receptors (Potter et al., 1991). Maximal binding capacities are different for different radioligands, and these data have been interpreted in terms of the binding of ligands to oligomers (Park et al., 2002). Hill coefficients different from 1 have been reported in ligand binding assays for some receptors (Sinkins and Wells, 1993). Ligand dissociation experiments on  $\beta$ -adrenergic (Limbird and Lefkowitz, 1976), adenosine A<sub>1</sub> (Franco et al., 1996), muscarinic acetylcholine (Hirschberg and Schimerlik, 1994), CXCR3 chemokine (Cox et al., 2001), and dopamine D<sub>2</sub> receptors (Chatterjee et al., 1988) have provided preliminary evidence for noncompetitive effects of ligands.

The D<sub>2</sub> dopamine receptor is of some interest in this regard. This receptor mediates some of the important physiological actions of dopamine such as control of movement, behavior, and prolactin secretion and is an important site of action of the drugs used to treat schizophrenia and Parkinson's disease. There are several reports for this receptor of ligand binding studies inconsistent with the behavior of a monomeric receptor with a single binding site. Maximal binding capacities for different radioligands have been reported to be different (Terai et al., 1989; Seeman et al., 1992; Ng et al., 1994), and these maximal binding capacities change when

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**ABBREVIATIONS:** GPCR, G protein-coupled receptor; NMDG, N-methyl D-glucamine; ANOVA, analysis of variance; CHO, Chinese hamster ovary.

the sodium ion concentration is altered (Theodorou et al., 1983; Armstrong and Strange, 2001). Noncompetitive interactions between ligands have also been reported (Hall et al., 1990; Armstrong and Strange, 2001). These observations have been made in both native tissue and recombinant systems, so the observations are not peculiarities of recombinant systems. It has been suggested that these data may be interpreted in terms of monomer/dimer equilibria (Seeman and Van Tol, 1994; Strange, 1994) or cooperative interactions within a preformed dimer (Armstrong and Strange, 2001). There is some evidence that the contact site between monomers may include the fourth membrane-spanning segment (Guo et al., 2003).

In a study on the D<sub>2</sub> dopamine receptor expressed in CHO cells, such ligand binding data were interpreted in terms of negatively cooperative interactions between the two binding sites in a constitutive receptor dimer (Armstrong and Strange, 2001). It is important, however, to try to understand the generality of these observations. We have therefore expressed the D<sub>2</sub> receptor in another system, Sf9 insect cells, using the baculovirus system and examined the properties of the receptor using ligand binding studies with a broader range of ligands and assay techniques, including equilibrium and kinetic ligand binding analyses. The Sf9 insect cell system is a useful one for expressing GPCRs because the insect cells do not themselves have similar GPCRs. In addition, mammalian GPCRs are expressed with fidelity in this system. We have shown for the D<sub>2</sub> receptor that its properties in this system are very similar to those of native receptors (Cordeaux et al., 2001; Gazi et al., 2003b; Nickolls and Strange, 2003). Although this system can be used to express GPCRs at very high levels, for the D<sub>2</sub> dopamine receptor, expression levels are similar to those seen in native and other recombinant systems. For all of these reasons, therefore, data obtained with this system are relevant to the native system. In the present report, therefore, we describe ligand binding studies on the D<sub>2</sub> receptor expressed in Sf9 cells to investigate further the occurrence of cooperativity for this oligomeric receptor.

## Materials and Methods

**Materials.** [<sup>3</sup>H]Spiperone (15–30 Ci/mmol), [<sup>3</sup>H]nemonapride (83–85 Ci/mmol), and [<sup>3</sup>H]raclopride (60–86 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Beaconsfield, Bucks, UK). Butaclamol, spiperone, nemonapride, and raclopride were purchased from Sigma Chemical (Gillingham, Dorset, UK). Soluene 350 was purchased from PerkinElmer. All other materials were obtained from commercial sources and were of the highest available purity.

**Cell Culture and Infections.** Sf9 insect cells were grown in suspension in TC-100 medium supplemented with 10% (v/v) fetal calf serum and 0.1% (v/v) pluronic F-68 or in SF-900 II medium supplemented with L-glutamine. The cells were maintained at a density of 0.5 to 2.5 × 10<sup>6</sup> cells/ml and were passaged every 2 to 3 days. For infections, cells were seeded at a density of 0.3 to 0.6 × 10<sup>6</sup> cells/ml and then infected once they reached the log phase of growth (i.e., at a density of 1 × 10<sup>6</sup> cells/ml). Infections were carried out with baculovirus containing the DNA for the human D<sub>2</sub>-short receptor (Nickolls and Strange, 2003) with a multiplicity of infection of 1 to 3 to vary expression levels, and the cells were harvested 48 h after infection.

**Membrane Preparation.** Cells were collected by centrifugation for 10 min at 1700g. For every 50 ml of cell suspension used, 15 ml

of ice-cold buffer (20 mM HEPES, 1 mM EDTA, and 1 mM EGTA, pH 7.4) was used to resuspend the cell pellet. The cells were homogenized at 4°C using an Ultra Turrax at setting 4 to 5 for 20 s. The homogenate was centrifuged for 10 min at 1700g at 4°C, and the supernatant was collected and centrifuged at 48,000g (4°C) for 1 h. The resulting pellet was resuspended in buffer, homogenized again, and 150-μl aliquots were stored at –80°C. The method of Lowry et al. (1951) was used to determine the protein content of membrane preparations, using bovine serum albumin as a standard.

**Ligand Binding Assays.** Saturation and competition binding assays were performed as described in Armstrong and Strange (2001) incubating 15 to 20 μg of membranes in assay buffer (20 mM HEPES, 1 mM EDTA, and 1 mM EGTA; pH 7.4 with KOH), with or without 100 mM NaCl where indicated, in a final volume of 1 ml at 25°C for 3 h. Some assays were conducted using 5 μg of membrane protein to reduce radioligand depletion, and assay buffer was supplemented with 0.1% bovine serum albumin to prevent the loss of protein by absorption to plastic (preparation 3; Tables 1 and 2). (+)-Butaclamol (3 μM) was used to determine the nonspecific binding. In the saturation assays, the range of radioligand concentrations was from 0.05 to 4 nM for [<sup>3</sup>H]spiperone and [<sup>3</sup>H]nemonapride and from 0.02 to 10 nM for [<sup>3</sup>H]raclopride. In the competition assays, [<sup>3</sup>H]spiperone (0.3 nM) and [<sup>3</sup>H]nemonapride (0.8 nM) were used, and the range of nonradioactive ligand was from 1 fM to 0.1 mM.

The dissociation binding assays were performed by incubating the membranes in assay buffer with the radioligand ([<sup>3</sup>H]nemonapride or [<sup>3</sup>H]spiperone at ~0.25 nM), with or without the presence of 100 mM NaCl, in a final volume of 900 μl until equilibrium was reached (3 h, 25°C) and then incubated with the competing ligand, spiperone or nemonapride (1 μM) or raclopride (100 μM) as indicated, in a final volume of 1 ml, for the times indicated (up to 240 min generally but in the case of [<sup>3</sup>H]nemonapride in the presence of Na<sup>+</sup>, some assays were left for 24 h to check for full reversibility). Assays were then terminated by filtration through GF/C filters and washing with phosphate-buffered saline (140 mM NaCl, 10 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 8 mM Na<sub>2</sub>HPO<sub>4</sub>), and radioactivity on the filters was determined as described by Armstrong and Strange (2001). (+)-Butaclamol (3 μM) and assay buffer were used to determine the nonspecific and total binding, respectively.

**Comparison of Filtration and Centrifugation Assays for Ligand Binding.** [<sup>3</sup>H]Spiperone or [<sup>3</sup>H]nemonapride (0.2–0.3 nM) were incubated in triplicate with membranes from Sf9 cells expressing D<sub>2</sub> dopamine receptors (~1 pmol/mg) (50 μg of protein for filtration assays, 100 μg of protein for centrifugation assays) in assay buffer (20 mM HEPES, pH 7.4, with KOH), with 100 mM NaCl or 100 mM NMDG where indicated, in a final volume of 1 ml at 25°C for 3 h. Total radioligand binding was determined with no further additions, whereas nonspecific radioligand binding was determined with the addition of (+)-butaclamol (3 μM) where indicated. One set of assays was terminated by filtration as described above. A second set of assays was performed in Eppendorf centrifuge tubes and terminated by centrifugation at 19,000g for 10 min. After centrifugation, samples (100 μl) of the supernatant were taken, and radioactivity was determined. The pellets were rinsed superficially with phosphate-buffered saline three times as described by Golds et al. (1980). Approximately half of the tube was then cut off, and Soluene 350 (100 μl) was added to the part containing the pellet and left overnight before adding the remnant of tube and dissolved pellet to scintillation fluid (2 ml). Radioactivity was then determined as described above. As discussed under *Results*, there is excellent agreement between levels of specific binding for [<sup>3</sup>H]spiperone or [<sup>3</sup>H]nemonapride determined in parallel filtration and centrifugation experiments, showing that the centrifugation protocol used here is collecting all of the ligand binding sites.

**Data Analysis.** Data were analyzed using Prism software (GraphPad Software Inc., San Diego CA). In saturation experiments, the free radioligand concentration was determined by subtraction of total bound radioligand from the added radioligand concentration.

TABLE 1

Saturation ligand binding analyses for [<sup>3</sup>H]nemonapride, [<sup>3</sup>H]raclopride, and [<sup>3</sup>H]spiperone binding to D<sub>2</sub> dopamine receptors expressed in Sf9 cells

Saturation binding studies were performed as described under *Materials and Methods* section with [<sup>3</sup>H]nemonapride, [<sup>3</sup>H]raclopride, and [<sup>3</sup>H]spiperone using different preparations of Sf9 cell membranes expressing the D<sub>2</sub> dopamine receptor, under control conditions or with 100 mM sodium ions. Experiments with preparation 3 included 0.1% bovine serum albumin as described under *Materials and Methods*. All saturation analyses fitted best to one-binding site models from which  $B_{\max}$  (pmol/mg) and  $pK_d$  values were determined and are expressed as mean  $\pm$  S.E.M. from three or more experiments.

Radioligand	$B_{\max}$			$pK_d$		
	Preparation 1		Preparation 2		Preparation 3	
	Control	Na <sup>+</sup>	Control	Na <sup>+</sup>	Control	Na <sup>+</sup>
[ <sup>3</sup> H]Nemonapride	0.92 $\pm$ 0.04	1.53 $\pm$ 0.04*	4.13 $\pm$ 0.17	5.28 $\pm$ 0.26*	3.37 $\pm$ 0.26	6.13 $\pm$ 0.28*
[ <sup>3</sup> H]Raclopride	N.D.	0.32 $\pm$ 0.18	N.D.	2.32 $\pm$ 0.29	N.D.	2.20 $\pm$ 0.60
[ <sup>3</sup> H]Spiperone	0.98 $\pm$ 0.06	0.99 $\pm$ 0.06	4.03 $\pm$ 0.75	4.23 $\pm$ 0.23	2.64 $\pm$ 0.19	3.54 $\pm$ 0.15*

N.D., not determined because no saturable high-affinity binding could be detected.

\* Different from control ( $P < 0.05$ ).

Data for specific radioligand binding and free radioligand concentration were fitted to equations describing one- or two-binding site models, and the best fit was determined using an  $F$  test. Competition experiments were fitted to one- and two-binding site models, and the best fit was determined using an  $F$  test. For the kinetic experiments, data were fitted to a model of a single exponential decay with full dissociation of the bound radioligand, which provided the best fit to the data in all cases.

Statistical significance of differences between parameters was determined at the 0.05 level, using ANOVA or Student's  $t$  test, as appropriate.  $K_i$  and  $K_d$  values were first converted to the respective normally distributed negative logarithm ( $pK_i$  or  $pK_d$ ). Mean values are quoted with the respective standard error.

## Results

**Saturation Binding Analyses for D<sub>2</sub> Dopamine Receptors Expressed in Sf9 Cells Using Different Radioligands.** Saturation ligand binding studies were performed with three different radioligands ([<sup>3</sup>H]nemonapride, [<sup>3</sup>H]raclopride, [<sup>3</sup>H]spiperone) to D<sub>2</sub> dopamine receptors expressed in membranes of Sf9 cells. The assays were performed with different buffers to determine the effects of sodium ions on saturation binding for the three radioligands. Different preparations of Sf9 cell membranes expressing D<sub>2</sub> receptors at different levels (Table 1) were tested in these experiments to assess the effect of expression level on the data obtained. Representative data from one preparation are shown in Fig. 1.

For [<sup>3</sup>H]spiperone, the  $K_d$  and  $B_{\max}$  values were mostly unaffected by the addition of sodium ions to buffers (Table 1 and Fig. 1). In one case, the  $B_{\max}$  and  $K_d$  values for this radioligand were increased in the presence of Na<sup>+</sup>, but the effects were small. For the two substituted benzamide ligands ([<sup>3</sup>H]raclopride and [<sup>3</sup>H]nemonapride), however, the addition of sodium ions did have a clear effect on the binding parameters (Table 1 and Fig. 1). For [<sup>3</sup>H]nemonapride, the  $pK_d$  was increased by the addition of sodium ions to buffers, as was the  $B_{\max}$ . The effect of sodium ions on  $B_{\max}$  depended on the preparation of membranes used, but the  $B_{\max}$  was between 25 and 80% greater in the presence of sodium ions. In the absence of sodium ions, the  $B_{\max}$  for [<sup>3</sup>H]nemonapride was similar to the  $B_{\max}$  for [<sup>3</sup>H]spiperone with or without sodium ions in all preparations. For [<sup>3</sup>H]raclopride, it was difficult to obtain saturation curves in the absence of sodium ions, whereas in the presence of sodium ions, clear saturation curves could be recorded. The  $B_{\max}$  for [<sup>3</sup>H]raclopride in the presence of sodium ions was less than that seen for [<sup>3</sup>H]nemonapride, and the extent of the difference varied between preparations, representing 20 to 40% of the  $B_{\max}$  value for [<sup>3</sup>H]nemonapride under the same conditions. For each radioligand, saturation curves fitted well to one-binding site models in all cases. When experiments were performed with the addition of 100 mM NMDG to act as a cation substitute, the data were very similar to those in the absence of sodium ions (data not shown) so that the effects of sodium ions are not caused by changes in ionic strength.  $K_d$  values and effects of sodium ions for the different radioligands were consistent between the different preparations.

Taken together, these data are not consistent with a monomeric receptor with a single class of binding sites. To determine whether the different radioligands were labeling independent populations of sites or whether a more complex



model was required, we performed competition studies between the different ligands.

**Competition Binding Analyses with Different Ligands.** Competition experiments were performed for nonradioactive nemonapride, spiperone, and raclopride versus the binding of [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]spiperone in the absence and presence of sodium ions, using two membrane preparations from Sf9 cells expressing D<sub>2</sub> receptors at different levels (Fig. 2 and Table 2). For each ligand/radioligand pair, the lower plateau of the competition curves indicated that all of the specific radioligand binding [defined by (+)-butaclamol] had been inhibited.

With [<sup>3</sup>H]spiperone, nemonapride competition curves fitted a one-binding site model in the presence of sodium ions, whereas in the absence of sodium ions, a two-binding site model provided the best fit to data. Raclopride/[<sup>3</sup>H]spiperone competition curves were fitted best by one-binding site models in the absence of sodium ions, whereas in the presence of sodium ions, competition curves indicated a single affinity in one preparation, but in a second preparation, a small proportion of a higher-affinity binding site was also seen.

For [<sup>3</sup>H]nemonapride, spiperone competition curves in the absence of sodium ions were fitted well by a one-binding site model, whereas in the presence of sodium ions, a two-binding site model provided the best fit. Raclopride/[<sup>3</sup>H]nemonapride competition curves were fitted well by a one-binding site model in the absence and presence of sodium ions. Binding parameters for the different experiments are given in Table 2.

There are differences between the behavior of ligands in competition assays and saturation assays. For example when values for dissociation constants for ligands determined in competition assays ( $K_i$ ) are compared with values obtained in saturation assays ( $K_d$ ), then  $K_i$  values for some ligands are different from the corresponding  $K_d$  value [e.g., nemonapride (control) and raclopride ( $\text{Na}^+$ )]. Some ligands exhibit complex competition curves, whereas their behavior in saturation assays may be described by one-binding site models. These discrepancies are particularly striking for spiperone, whose binding is generally sodium ion-insensitive in saturation experiments but strongly affected by sodium ions based on the competition experiments versus [<sup>3</sup>H]nemonapride in terms of

$K_i$  values and the shape of the competition curve. Such differences could result from negative cooperativity between the ligands in the competition assays, which would be absent in saturation assays. The kinetic data for [<sup>3</sup>H]spiperone and [<sup>3</sup>H]nemonapride presented below show that equilibration of the radioligand may be slow under some conditions (e.g., [<sup>3</sup>H]nemonapride with  $\text{Na}^+$ ). This could affect  $K_i$  values obtained in competition experiments. For example, in a raclopride/[<sup>3</sup>H]nemonapride competition study, the slow kinetics of the radioligand and the faster kinetics of the competitor may lead to an overestimation of the affinity of the competitor. We do not expect this difference to be more than approximately 3-fold, because the data obtained here are similar to those reported by Malmberg et al. (1996), where experiments were conducted under conditions likely to reduce these potential artifacts.

These competition data do not provide any evidence that the differences in  $B_{\text{max}}$  seen in saturation analyses result from different populations of receptors with different affinities for the different radioligands. Competition experiments between pairs of ligands that label different numbers of receptors in saturation assays are in some cases characterized by full competition with Hill coefficients of 1. One example here would be the binding of [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]raclopride in the presence of sodium ions, in which the former radioligand labels considerably more sites (~2–3 times more). Despite this discrepancy, raclopride can fully inhibit [<sup>3</sup>H]nemonapride binding in competition experiments with Hill coefficients of 1. These data, therefore, might indicate negatively cooperative interactions between these ligands when binding to one set of receptors. Some ligand pairs provide evidence for more complex behavior. For example, [<sup>3</sup>H]spiperone/nemonapride in the absence of sodium ions and [<sup>3</sup>H]nemonapride/spiperone in the presence of sodium ions. In both examples, competition curves exhibit two affinities, but these two affinities would be expected to be detected in saturation analyses, so this cannot be taken as evidence for separate binding sites but could indicate cooperative interactions.

We therefore performed radioligand dissociation studies to determine whether more evidence could be obtained for non-competitive interactions between the different ligands.

TABLE 2  
Competition ligand binding analyses

Competition binding studies were performed for the indicated competing ligands, versus [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]spiperone, using different preparations of Sf9 cell membranes expressing the D<sub>2</sub> dopamine receptor as in Table 1, in buffers with and without 100 mM sodium ions, as described under *Materials and Methods*. Data were fitted to one- and two-binding site models, and the parameters for the best fits are presented ( $K_i$  for a one-binding site model, and two  $K_i$  values ( $K_H$ ,  $K_L$ ) and the respective percentages of sites (% $R_H$ , % $R_L$ ) for a two-binding site model). Parameters are expressed as mean  $\pm$  S.E.M. from three or more experiments. Experiments with preparation 3 included 0.1% bovine serum albumin as described under *Materials and Methods*.

Ligands/Radioligands and Parameters	Preparation 1		Preparation 3	
	Control	$\text{Na}^+$	Control	$\text{Na}^+$
Nemonapride/[ <sup>3</sup> H]spiperone				
$pK_H$ or $pK_i$ (%Rh)	10.87 $\pm$ 0.08 (47 $\pm$ 6)	11.47 $\pm$ 0.18*	10.48 $\pm$ 0.09 (45 $\pm$ 5)	10.94 $\pm$ 0.04*
$pK_L$ (%RL)	8.22 $\pm$ 0.05 (53 $\pm$ 6)		7.84 $\pm$ 0.04 (55 $\pm$ 5)	
Raclopride/[ <sup>3</sup> H]spiperone				
$pK_H$ (%Rh)				8.93 $\pm$ 0.28 (28 $\pm$ 7)
$pK_L$ or $pK_i$ (%RL)	6.28 $\pm$ 0.15	7.95 $\pm$ 0.12*	6.22 $\pm$ 0.09	7.69 $\pm$ 0.14* (72 $\pm$ 7)
Spiperone/[ <sup>3</sup> H]nemonapride				
$pK_H$ or $pK_i$ (%Rh)	11.33 $\pm$ 0.11	11.44 $\pm$ 0.12 (32 $\pm$ 3)	11.31 $\pm$ 0.20	10.91 $\pm$ 0.17 (48 $\pm$ 6)
$pK_L$ (%RL)		9.31 $\pm$ 0.10* (68 $\pm$ 3)		9.36 $\pm$ 0.09* (52 $\pm$ 6)
Raclopride/[ <sup>3</sup> H]nemonapride				
$pK_i$	N.D.	N.D.	6.61 $\pm$ 0.08	7.72 $\pm$ 0.07*

N.D., not determined.

\*  $P < 0.05$  relative to control.

**Radioligand Dissociation Studies.** Two radioligands ( $[^3\text{H}]$ nemonapride and  $[^3\text{H}]$ spiperone) were tested in dissociation experiments using different nonradioactive ligands to initiate dissociation (Fig. 3). The radioligand was allowed to

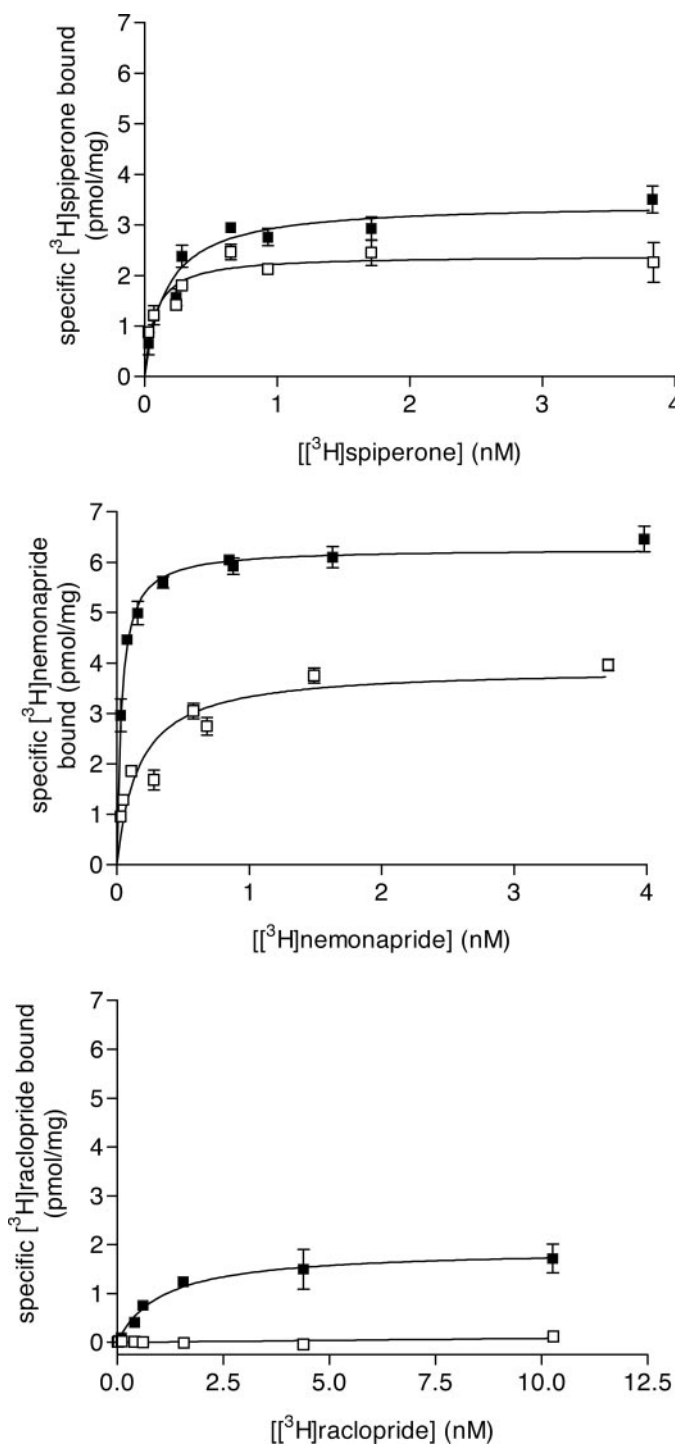
bind to the receptors, and the time course of the dissociation in the presence of the different nonradioactive competitors was determined. Experiments were conducted in the presence and absence of sodium ions. The corresponding nonradioactive ligand was used in both cases to initiate dissociation, and experiments were also performed using nonradioactive raclopride, because on the basis of saturation and competition experiments, this ligand seems to provide evidence for cooperativity.

The dissociation of both radioligands was sodium ion-dependent. For  $[^3\text{H}]$ nemonapride, dissociation occurred much more slowly in the presence of sodium ions than in their absence, but when dissociation assays were allowed to proceed for 24 h, all of the radioligand dissociated. The time course of dissociation was unaffected by the nature of the nonradioactive competitor used to initiate dissociation (nemonapride or raclopride). For  $[^3\text{H}]$ spiperone, dissociation was slower in the absence of sodium ions than in the presence of sodium ions, but all of the bound radioligand dissociated at long incubation times. As for  $[^3\text{H}]$ nemonapride, the dissociation of  $[^3\text{H}]$ spiperone was not affected by which nonradioactive competitor was used to initiate dissociation.

These ligand dissociation experiments, therefore, show that the nonradioactive ligand used does not influence the dissociation rate of the radioligand, but they highlight the ability of sodium ions to regulate the binding of all ligands to the  $\text{D}_2$  receptor. The data shown in Fig. 3 are from one preparation of membranes, but in common with the saturation and competition analyses, we did find some variation in the kinetic properties of different preparations of receptor.

**Assessment of Radioligand Depletion.** In the saturation binding assays, binding of radioligand to receptors may lead to the depletion of the added radioligand so that the free radioligand concentration will not be the same as the added concentration. This is likely to be a problem for the two high-affinity radioligands ( $[^3\text{H}]$ spiperone and  $[^3\text{H}]$ nemonapride) at the higher expression levels (Golds et al., 1980; Strange, 2001). In the lower-expressing preparation ( $\sim 1$  pmol/mg), however, the amount of receptor added to each assay was  $\sim 20$  pM, and depletion should be much less. In this preparation, depletion of  $[^3\text{H}]$ spiperone and  $[^3\text{H}]$ nemonapride does not exceed 20% at 100 pM free radioligand. Depletion of  $[^3\text{H}]$ raclopride is much less in all preparations. To minimize the depletion of  $[^3\text{H}]$ spiperone and  $[^3\text{H}]$ nemonapride for the more highly expressing preparations ( $\sim 4$  pmol/mg, preparation 3), a set of assays was performed using  $5 \mu\text{g}$  of membrane protein, equivalent to  $\sim 20$  pM receptor, and here protein loss was minimized by the inclusion of 0.1% albumin in assays. Data obtained under these conditions are similar to those obtained with other preparations.

In all of the experiments, we corrected for ligand depletion by the subtraction of total bound radioligand from the added concentration. Therefore, to check the validity of this assessment of radioligand depletion, we performed a series of control experiments for the two higher-affinity radioligands ( $[^3\text{H}]$ nemonapride and  $[^3\text{H}]$ spiperone), in which ligand binding assays were run at approximately 30% depletion and were terminated using filtration and centrifugation in parallel assays. In the centrifugation assays, it was possible to determine the actual free radioligand concentration after pellet formation and compare this to the calculated free ra-



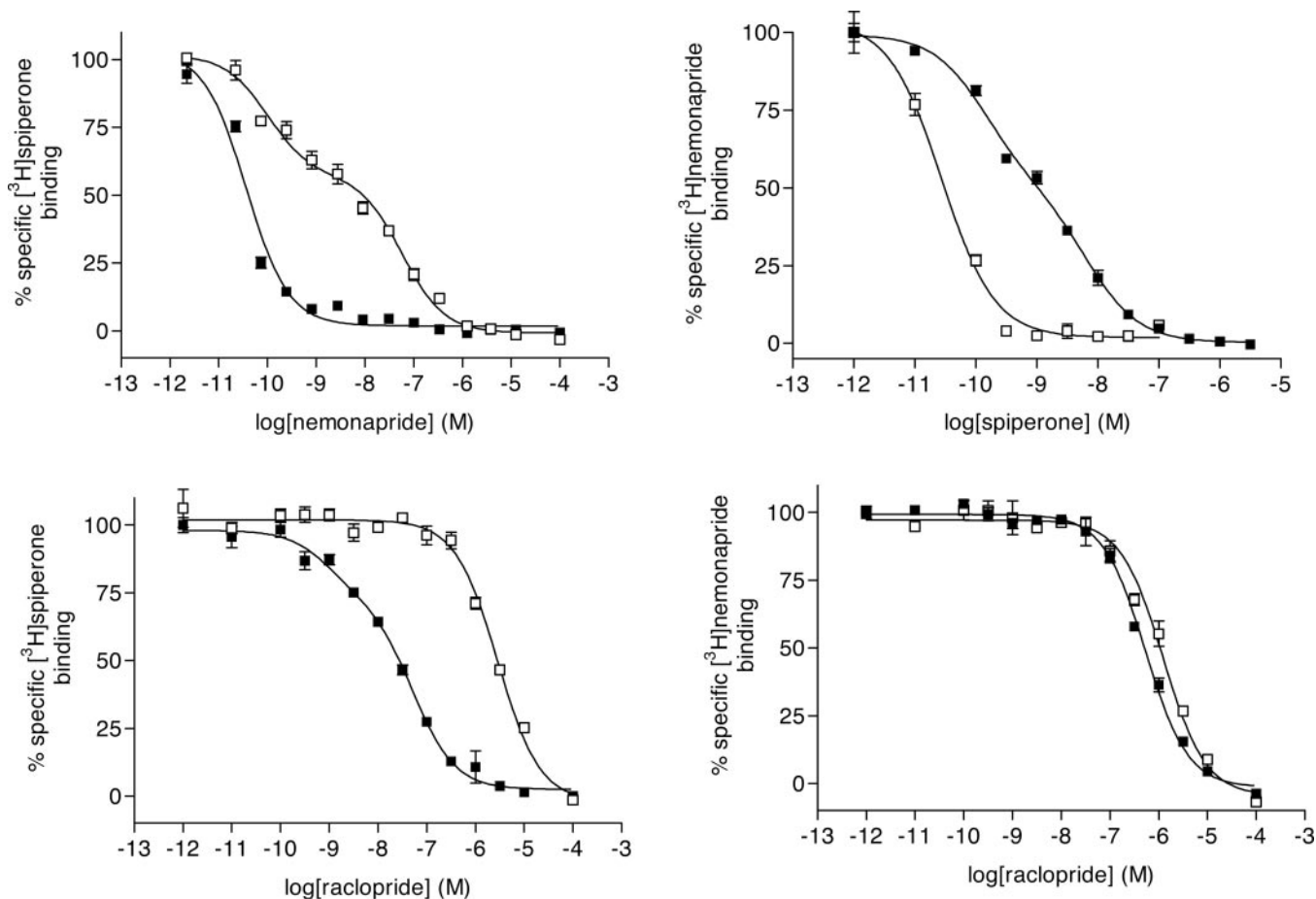
**Fig. 1.** Saturation radioligand binding studies to  $\text{D}_2$  dopamine receptors expressed in Sf9 cell membranes. Saturation binding studies were performed as described under *Materials and Methods* with  $[^3\text{H}]$ spiperone,  $[^3\text{H}]$ nemonapride, and  $[^3\text{H}]$ raclopride in buffers with (■) and without (□) 100 mM sodium ions. Specific radioligand binding is plotted against free radioligand concentration, determined as described in under *Materials and Methods*. Curves shown are for one-binding site models, which provided the best fit to data in all cases. Data from representative experiments on preparation 3 are shown, replicated as in Table 1.

dioligand concentration derived from the subtraction of bound radioligand concentration from added radioligand concentration. To perform the centrifugation experiments, we had to alter the assay buffer slightly to allow the collection of a firm pellet. This required the removal of EDTA and EGTA, because these hinder pellet formation. In addition, it was necessary to assess the effects of Na<sup>+</sup> ions by comparing assays with 100 mM Na<sup>+</sup> or NMDG, because 100 mM monovalent cation was found to be necessary to assist pellet formation. These modified assay conditions do not affect the basic observations seen in this report. We also needed to use 100  $\mu$ g of membrane protein to form a firm pellet. The parallel filtration assays were run at 50  $\mu$ g of membrane protein to avoid saturating the filters, and Table 3 shows the results of these experiments corrected for the different protein concentrations.

It should be noted that in the comparison of filtration and centrifugation assays, we used much higher protein amounts in these assays than in the saturation and competition experiments. It was necessary to use a higher amount of protein in the centrifugation assays (100  $\mu$ g) to obtain good pellets, and a similar amount of protein (50  $\mu$ g) was used in the parallel filtration assays. In the saturation and competition experiments terminated by filtration (Table 1), it was necessary to use lower amounts of protein (<20  $\mu$ g) to avoid massive depletion of the higher-affinity radioligands. We checked whether filtration assays run at different protein

concentrations gave comparable recovery of protein by examining the relationship between specific binding and protein amount. For both [<sup>3</sup>H]spiperone and [<sup>3</sup>H]nemonapride in the absence or presence of Na<sup>+</sup>, there was a linear relationship between specific radioligand binding and protein in the range of 10 to 50  $\mu$ g. Assays run at different protein concentrations may, therefore, be compared.

From the data in Table 3, it can be seen that both the total and specific binding for the two radioligands are very similar, whether determined by centrifugation or filtration, differing by no more than 10%. This shows that both methods for assay termination are valid and that there is no major component of radioligand binding detected in centrifugation assays that has been washed away in filtration assays. There could, for example, have been low-affinity nonspecific radioligand binding that would have depleted added radioligand and hence altered the actual free radioligand concentration, but not be detected in filtration assays, but this does not seem to be the case. In addition, the actual free radioligand concentration was determined in the centrifugation assays and was found to be very similar to the free radioligand concentration determined by subtraction of bound from added (differing by no more than 15%). Given that the total bound radioligand is similar in the two assay formats, the subtraction method for assessment of free radioligand will also be accurate in the filtration assays. Taken together, these observations show that the determinations of  $B_{\max}$ ,  $K_d$ , and  $K_i$  reported here are



**Fig. 2.** Competition binding studies to D<sub>2</sub> dopamine receptors expressed in Sf9 cell membranes. Competition binding studies were performed as described under *Materials and Methods* with [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]spiperone in buffers with (■) and without (□) 100 mM sodium ions. Data from representative experiments are shown, replicated as in Table 2. Best fit curves to one- or two-binding site models are shown.



valid, and accurate corrections can be made for ligand depletion.

**Assessment of Membrane Capture in Filtration Assays.** The differences in  $B_{\max}$  for some of the radioligands reported here could result from differential trapping of membrane fragments on the filters in the presence and absence of sodium ions in filtration assays. This is unlikely, because  $B_{\max}$  data for one radioligand, [ $^3\text{H}$ ]spiperone, are generally very similar with and without  $\text{Na}^+$  ions. Nevertheless, this possibility was examined in the parallel filtration and centrifugation experiments outlined above (Table 3). The experiments were performed using the binding of [ $^3\text{H}$ ]spiperone, whose binding is mostly unaffected by  $\text{Na}^+$  ions in filtration assays so that it can act as a control for differences in membrane trapping and the effects of  $\text{Na}^+$  ions. We found that the total and specific [ $^3\text{H}$ ]spiperone bound were very similar in

the presence or absence of sodium ions, whether determined by filtration or centrifugation. Thus, it seems that membrane trapping is not affected by  $\text{Na}^+$ , and the differences in  $B_{\max}$  reported here for other radioligands are not a result of differential membrane trapping.

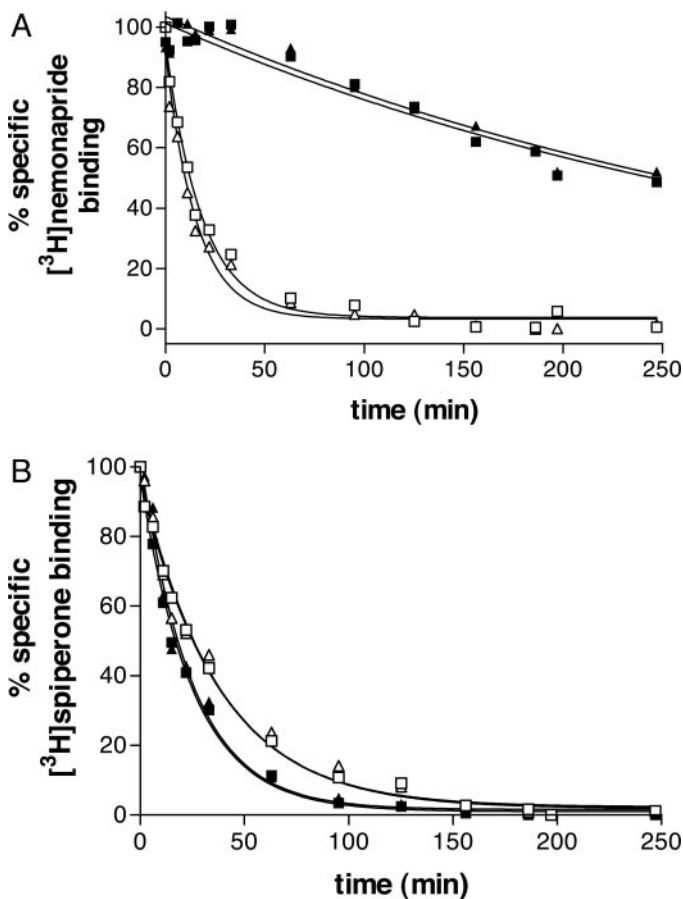
**Effects of Ligand Dissociation on Relative  $B_{\max}$  Values.** The differences in radioligand  $B_{\max}$  could potentially be caused by effects of sodium ions on radioligand dissociation and loss of ligand in filtration assays during the filtration process. To check this point, we conducted parallel filtration and centrifugation experiments with the three radioligands at saturating concentrations in the presence and absence of  $\text{Na}^+$  ions. There should be no losses of bound radioligand during centrifugation assays. The data (Fig. 4) show striking agreement between the values for  $B_{\max}$  for the different radioligands in the two assay formats, so that differences in  $B_{\max}$  are unlikely to be caused by the loss of radioligand during filtration.

## Discussion

This study provides clear evidence taken from different experimental designs that ligand binding to the  $\text{D}_2$  dopamine receptor cannot be described in terms of interactions with a monomeric receptor. The observations may arise from the receptors existing in oligomers, a proposition consistent with cross-linking studies (Guo et al., 2003), the use of variant receptors (Lee et al., 2003), and physical data from both immunological analyses and studies with fluorescence resonance energy transfer, which have shown interaction between  $\text{D}_2$  receptor monomers (Wurch et al., 2001; Gazi et al., 2003a).

Observations analogous to those described here, in which  $B_{\max}$  values for radioligands are increased in the presence of sodium ions, have been reported in other studies on the  $\text{D}_2$  dopamine receptor in native tissues [e.g., brain (Theodorou et al., 1983; Hall et al., 1990) and other recombinant systems (Armstrong and Strange, 2001)]. Noncompetitive interactions between raclopride and spiperone and complex [ $^3\text{H}$ ]nemonapride/spiperone inhibition curves (Hall et al., 1990; Seeman et al., 1992) have been reported in studies on brain tissue. There are also several reports that substituted benzamide ligands such as [ $^3\text{H}$ ]nemonapride label different numbers of receptors in brain compared with [ $^3\text{H}$ ]spiperone (Theodorou et al., 1983; Terai et al., 1989; Hall et al., 1990; Seeman et al., 1992). The phenomena described in the present report are not therefore restricted to recombinant systems but also occur in native tissues such as brain. Given that the  $\text{D}_2$  receptor expressed in Sf9 cells is very similar to the receptor expressed in native systems (see Introduction), the present observations are physiologically relevant.

It is important, therefore, to understand how the present data on  $B_{\max}$  variation and  $K_i/K_d$  differences arise and to suggest models to describe this behavior of the  $\text{D}_2$  receptor. Similar data on different  $B_{\max}$  values for radioligands at the muscarinic acetylcholine receptor were partly a result of unsuspected errors in the specific activities of radioligands caused by an impurity (Wreggett and Wells, 1995; Sum et al., 2001). It seems unlikely that the differences in  $B_{\max}$  reported in the present study result from errors in specific activity. [ $^3\text{H}$ ]Raclopride and [ $^3\text{H}$ ]spiperone exhibit different  $B_{\max}$  values in the present study on receptors expressed in Sf9 mem-



**Fig. 3.** Radioligand dissociation studies on  $\text{D}_2$  dopamine receptors expressed in Sf9 cell membranes. Radioligand dissociation studies were performed as described under *Materials and Methods* with [ $^3\text{H}$ ]nemonapride and nonradioactive nemonapride (■, □) or raclopride (▲, △) as competing ligands (A) or [ $^3\text{H}$ ]spiperone with spiperone (■, □) or raclopride (▲, △) as competing ligands (B) in buffers with (filled symbols) and without (unfilled symbols) 100 mM sodium ions. Data from representative experiments are shown, replicated three times with similar results using membrane preparation 3. The curves shown are the best fit curves to single exponential decay models with full dissociation of bound radioligand with the following half lives (in minutes, mean  $\pm$  S.E.M. of three or more experiments): [ $^3\text{H}$ ]nemonapride:  $-\text{Na}^+$ ,  $12.4 \pm 0.7$  (nemonapride) and  $11.6 \pm 0.9$  (raclopride);  $+\text{Na}^+$ ,  $197.2 \pm 16.9$  (nemonapride) and  $200.8 \pm 16.6$  (raclopride); [ $^3\text{H}$ ]spiperone:  $-\text{Na}^+$ ,  $26.4 \pm 2.4$  (spiperone) and  $23.2 \pm 2.1$  (raclopride);  $+\text{Na}^+$ ,  $16.3 \pm 0.9$  (spiperone) and  $16.9 \pm 1.0$  (raclopride). In all cases, there was no effect of the nonradioactive drug used to initiate dissociation ( $P > 0.05$ , one-way ANOVA with Bonferroni's post test).

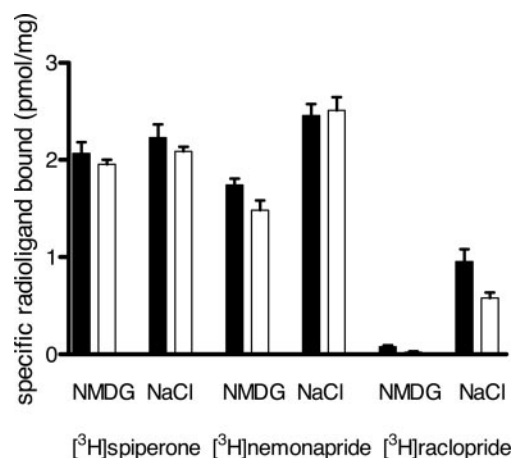
branes. Using the same batches of radioligand, however, similar  $B_{\max}$  values are seen for these two radioligands in binding to D<sub>2</sub> receptors expressed in CHO cells using buffers with sodium ions (B. Knudsen and P. G. Strange, unpublished observations). Also for [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]spiperone in the present study,  $B_{\max}$  values differ only in the presence of Na<sup>+</sup>; in the absence of Na<sup>+</sup>,  $B_{\max}$  values are mostly similar.

We also performed control experiments to check whether  $B_{\max}$  and  $K_d$  data were affected by radioligand depletion or differential trapping of membranes in the presence and absence of sodium ions. From these control experiments, these factors do not seem to have a major influence on the data obtained here. We also showed that the differences in  $B_{\max}$  for the three radioligands could be seen in assays terminated by centrifugation, thus eliminating the possibility that the differences in  $B_{\max}$  were artifacts of filtration assays.

One model to explain the observations would be that the different  $B_{\max}$  values result from separate noninterconverting populations of receptors with different properties. Related to this idea is the possibility that the different radioligands have differential access to receptors at the surface of membrane vesicles and inside these vesicles (Barton et al., 1991). In either case, we would expect evidence for these differences to be obtained in competition experiments between the ligands. However, such evidence is not apparent, because full cross-competition with Hill coefficients of one occurs for some ligand pairs, and in other cases, the complex inhibition curves provide no evidence for different sites. Another possibility might be that the effects seen are related to differential interaction of ligands with free receptor and receptor/G protein complexes; however, this does not seem to be the case. Differences in  $B_{\max}$  for [<sup>3</sup>H]raclopride and [<sup>3</sup>H]spiperone and the complex competition curves seen between spiperone and nemonapride are unaffected by the addition of GTP (data not shown). In addition, the  $B_{\max}$  differences for [<sup>3</sup>H]spiperone and [<sup>3</sup>H]nemonapride are seen when we expressed a D<sub>2</sub> receptor/G<sub>o</sub> fusion protein (Gazi et al., 2003c), which has strong coupling of receptor (R) and G protein (G)

compared with the present preparation in which there is little R/G coupling (Nickolls and Strange, 2003). R/G coupling does not, therefore, contribute to the observations in ligand binding studies reported here.

Given that there is abundant evidence now that GPCRs exist as oligomers, it is tempting to explain the present data in terms of cooperative interactions between ligands in binding to an oligomer. Indeed, the differences in  $B_{\max}$  for the three radioligands and the discrepancies between  $K_d$  and  $K_i$  values may mostly be explained in terms of negatively cooperative interactions. The discrepancies in  $B_{\max}$  among the



**Fig. 4.** Comparison of  $B_{\max}$  values for [<sup>3</sup>H]spiperone, [<sup>3</sup>H]nemonapride, and [<sup>3</sup>H]raclopride in filtration and centrifugation assays.  $B_{\max}$  values for the three radioligands were determined for binding to membranes of Sf9 cells expressing D<sub>2</sub> dopamine receptors in the presence of sodium ions (100 mM) or NMDG (100 mM) as described under *Materials and Methods*. Radioligands were used at saturating concentrations ([<sup>3</sup>H]spiperone, ~2 nM; [<sup>3</sup>H]nemonapride, 3–4 nM; and [<sup>3</sup>H]raclopride, 6–7 nM), and 100  $\mu$ g of membrane protein was used in centrifugation assays and 50  $\mu$ g of membrane protein used in filtration assays. The data shown are mean  $\pm$  S.E.M. from three experiments. ■, filtration experiments; □, centrifugation experiments. Values in filtration and centrifugation assays were not significantly different ( $P > 0.05$ ), whereas effects of Na<sup>+</sup> were significant for [<sup>3</sup>H]nemonapride and [<sup>3</sup>H]raclopride ( $P < 0.05$ ) (one-way ANOVA with Bonferroni's post test).

TABLE 3

Comparison of filtration and centrifugation assays for determination of bound and free radioligand

The binding of two radioligands ([<sup>3</sup>H]spiperone and [<sup>3</sup>H]nemonapride) at the concentrations indicated in the presence of 100 mM Na<sup>+</sup> or NMDG) to membranes of Sf9 cells expressing D<sub>2</sub> dopamine receptors was determined in parallel centrifugation and filtration assays as described under *Materials and Methods*. In the filtration assays, 50  $\mu$ g of membrane was used, and total, nonspecific, and specific radioligand bindings were determined. In the centrifugation assays, 100  $\mu$ g of membrane was used, and the total, nonspecific, and specific radioligand bindings in the pellet were determined as well as the free radioligand in the supernatant after the centrifugation. Bound radioligand is expressed as a concentration (picomolar) in the 1-ml assay volume and for filtration assays has been normalized to 100  $\mu$ g of membranes to allow for comparison with the centrifugation assays. For the free radioligand, the concentration measured (picomolar) is given along with the concentration calculated derived from the amount bound in the pellet in the centrifugation assays. The data are values from one experiment performed in triplicate (data are shown as mean  $\pm$  S.E.M.), which has been replicated three times with similar results. From the three experiments, it is possible to analyze the effects of different conditions. Specific [<sup>3</sup>H]spiperone binding in filtration assays was  $109.8 \pm 3.8\%$  of that in centrifugation assays ( $P < 0.05$ , paired  $t$  test). Total [<sup>3</sup>H]spiperone binding in filtration assays was  $102.0 \pm 3.8\%$  of that in centrifugation assays ( $P > 0.05$ ). Specific [<sup>3</sup>H]spiperone binding in the presence of Na<sup>+</sup> ions was  $106.8 \pm 2.4\%$  of that in the presence of NMDG ( $P < 0.05$ ), and total [<sup>3</sup>H]spiperone binding in the presence of Na<sup>+</sup> ions was  $104.0 \pm 3.0\%$  of that in the presence of NMDG ( $P > 0.05$ ). Calculated free [<sup>3</sup>H]spiperone as a percentage of the measured free [<sup>3</sup>H]spiperone was  $102.5 \pm 5.4$  ( $P > 0.05$ ) and  $109.0 \pm 8.1\%$  ( $P > 0.05$ ) in the nonspecific and total tubes, respectively. Specific [<sup>3</sup>H]nemonapride binding in filtration assays was  $102.2 \pm 5.3\%$  of that in centrifugation assays ( $P > 0.05$ , paired  $t$  test). Total [<sup>3</sup>H]nemonapride binding in filtration assays was  $92.0 \pm 2.4\%$  of that in centrifugation assays ( $P > 0.05$ ). Calculated free [<sup>3</sup>H]nemonapride as a percentage of the measured free [<sup>3</sup>H]nemonapride was  $114.3 \pm 2.3$  ( $P < 0.05$ ) and  $114.5 \pm 4.3\%$  ( $P < 0.05$ ) in the nonspecific and total tubes, respectively.

Radioligand	Added Radioligand	Filtration Bound Radioligand			Centrifugation							
					Bound Radioligand			Free Radioligand				
								Total Tubes		Nonspecific Tubes		
		Total	Nonspecific	Specific	Total	Nonspecific	Specific	Calculated	Actual	Calculated	Actual	Actual
[ <sup>3</sup> H]Spiperone + Na <sup>+</sup>	193 $\pm$ 2	64 $\pm$ 2	4 $\pm$ 1	60	70 $\pm$ 2	9 $\pm$ 1	61	123	130 $\pm$ 1	184	210 $\pm$ 1	
[ <sup>3</sup> H]Spiperone - Na <sup>+</sup>	219 $\pm$ 4	66 $\pm$ 2	6 $\pm$ 1	60	66 $\pm$ 3	10 $\pm$ 1	56	153	140 $\pm$ 1	209	200 $\pm$ 2	
[ <sup>3</sup> H]Nemonapride + Na <sup>+</sup>	304 $\pm$ 8	86 $\pm$ 1	4 $\pm$ 1	82	101 $\pm$ 1	11 $\pm$ 1	90	203	190 $\pm$ 1	293	270 $\pm$ 10	
[ <sup>3</sup> H]Nemonapride - Na <sup>+</sup>	317 $\pm$ 4	38 $\pm$ 2	4 $\pm$ 1	34	40 $\pm$ 1	12 $\pm$ 1	28	277	250 $\pm$ 1	305	270 $\pm$ 10	



different ligands are accounted for in this model by the ligands binding to different numbers of monomers in the array with high affinity, the remaining monomers having lower affinity owing to negatively cooperative effects. There is precedent for such a model in the behavior of enzymes. Certain enzymes exhibit "half-of-the-sites reactivity", in which in a symmetrical oligomeric enzyme, only half of the subunits react well (Stallcup and Koshland, 1973). Full inhibition in competition experiments may be achieved in this model via a mixture of competitive and noncompetitive inhibition (Armstrong and Strange, 2001), and cooperative interactions lead to discrepancies between  $K_d$  and  $K_i$  values. It would be desirable to describe this model mathematically so that these predictions may be tested. As indicated below, the data in the Sf9 expression system require an array greater than a dimer. The number of parameters required for such a model precludes a mathematical analysis. Indeed, for muscarinic receptors it was not possible to define parameters even for a tetramer model (Park et al., 2002).

Differences in maximal binding capacity have been reported for the binding of [ $^3$ H]N-methyl scopolamine and [ $^3$ H]quinuclidinyl benzilate for another GPCR, the muscarinic acetylcholine receptor (Sum et al., 2001; Park et al., 2002). Despite this difference, full competition occurs between the two ligands. These observations are analogous to those reported here and have been accounted for in terms of a tetrameric model for this receptor.

The size of the array that would account for the behavior seen in the present study is not defined at present. There is some quantitative variation in the observations reported here, unrelated to overall receptor expression levels, which suggests that the receptor may be expressed differently in different preparations of membrane. This variation is seen in differences in the relative  $B_{\max}$  values for the three radioligands. Likewise, there is some variation in the shape of competition curves in different preparations (e.g., [ $^3$ H]spiperone/raclopride competition and some variability in the kinetic properties of the receptors in different preparations). Therefore, it may be that the receptor is expressed slightly differently in different preparations, and this leads to differences in the behavior. We have also observed such variation in different CHO cell lines expressing the D<sub>2</sub> receptor (B. Knudsen and P. G. Strange, unpublished observations). To account for the relative  $B_{\max}$  values seen in the different preparations of Sf9 membranes expressing D<sub>2</sub> receptors (Table 1), the array must be greater than a dimer and may be as large as a hexamer, although there may be variation in the array size in different preparations.

The detailed properties of the D<sub>2</sub> receptor expressed in CHO cells (Armstrong and Strange, 2001) and in Sf9 cells are also not identical. For example, [ $^3$ H]raclopride binding occurs at a higher level relative to that of [ $^3$ H]spiperone in the CHO cell system compared with the Sf9 system. Indeed, in the Sf9 system in the absence of Na<sup>+</sup> ions, no high-affinity [ $^3$ H]raclopride binding could be detected. Raclopride does bind to the receptors in the competition experiments in the Sf9 system, albeit with an affinity too low to be detected in the saturation assays described. [ $^3$ H]Spiperone binding shows limited sensitivity to sodium ions in equilibrium binding assays in the CHO cell system and Sf9 cell system, and there is some variation in sodium ion sensitivity between preparations. Data in the CHO system could be analyzed in terms of

a cooperative dimer model, whereas for the Sf9 system, a more complex model is required, and it seems that the D<sub>2</sub> receptor is expressed differently in these two systems, possibly in different-sized oligomers. In other systems (native and recombinant), differences in the relative  $B_{\max}$  values for [ $^3$ H]raclopride, [ $^3$ H]spiperone, and [ $^3$ H]nemonapride have been reported (Terai et al., 1989; Hall et al., 1990; Seeman et al., 1992). In addition, the cooperativity exhibited by the muscarinic acetylcholine receptor can be manipulated by detergents, and it has been suggested that this reflects differences in the presentation of the oligomer or oligomers of this receptor (Park et al., 2002). There may, therefore, be variability in the presentation of GPCR oligomers that could give rise to variation in properties.

This model derived from negative cooperativity, however, does not account for all of the data reported in the present study. Although equilibrium ligand binding data suggested negatively cooperative interactions between ligands, this was not manifested in the kinetic experiments, in which the different competing ligands did not alter radioligand dissociation rates. There is precedent for such behavior from studies on allosteric effectors of muscarinic receptors, in which some compounds have been identified that act allosterically in equilibrium binding assays but not in kinetic assays (Lazareno et al., 2002). Generally, however, where cooperativity is seen in equilibrium ligand binding assays, it is also seen in kinetic assays. One possibility is that in the present system, the cooperativity is manifested in effects on ligand association rates, but this has not been examined yet. There are also some discrepancies in the present data when saturation and competition data are compared that do not conform to a negatively cooperative model. It may be, therefore, that the cooperative model is not a complete description of the present data.

There is emerging evidence derived from a number of approaches that GPCRs may form oligomers (Javitch, 2004), and such oligomers should give rise to properties typically seen for oligomeric proteins. The present study, however, shows that a cooperative model cannot fully describe the behavior of the D<sub>2</sub> dopamine receptor, so that a different model may be required. Models that could be considered here include the model based on the conformational spread that has been shown to account for the properties of sensory receptors in bacteria (Bray and Duke, 2004).

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