ELSEVIER

# COMMENTARY

In their review Seeman, Corbett, and Van Tol consider a number of aspects of the actions of neuroleptic drugs and propose hypotheses for the unusual actions of the atypical neuroleptics. They make some interesting points, and in this commentary I consider their arguments using broadly the same headings as they do.

## DISSOCIATION CONSTANTS OF NEUROLEPTIC DRUGS AT DOPAMINE RECEPTORS

Many studies have been published about the binding of neuroleptic drugs to dopamine receptors, and although clear patterns of activity have emerged from this work, in terms of the relative potencies of various drugs, the actual values obtained for the dissociation constants ($K_d/K_i$) exhibit some dispersity (e.g., Seeman et al. 1984). Whereas previously these differences were perhaps less important, we are now entering a phase of dopamine receptor research where accurate assessment of these $K_d/K_i$ values matters to compare drug actions at related receptor subtypes and to define the detailed mechanisms of action of agonists and antagonists. In their review Seeman et al. suggest that the $K_i$ value for a neuroleptic drug depends on the nature of the radioligand used for the assays. I wish to discuss the origin of their observations.

The principal tool for determining these $K_d/K_i$ values is the ligand binding assay (saturation and competition), and the dispersity of the $K_d/K_i$ values obtained is likely to be due to interlaboratory differences in assay conditions, leading to problems in the execution of the assays or genuine differences in values related to buffer, pH, and so on. The principal problems that can arise in the execution of the assays concern the lack of equilibration of the radioligands and competitors with the receptor and depletion of the ligands by binding to receptor and tissue. These problems have been recognized for some time (Chang et al. 1975; Wells et al. 1980; Golds et al. 1980; Burgisser et al. 1981; Seeman et al. 1984) and have been comprehensively reviewed (Hulme and Birdsall 1992).

The equilibration of ligands with the receptor depends on the rates of association and dissociation of the ligands and their concentrations. Typically, the binding of a single concentration of radioligand is tested and shown to reach equilibrium in the time chosen for the experiments. At lower radioligand concentrations the approach to equilibrium may be limited by the radioligand dissociation rate. Hulme and Birdsall (1992) recommend that assays be conducted for incubation times at least five times the half-time for dissociation. This rarely is considered, and for high-affinity radioligands such as [$^3$H]-spiperone it may lead to some artefacts, such as an overestimation of the $K_d$. The use of low receptor concentrations and the inclusion of competing ligands can introduce further complications. Although lack of equilibration may be a problem in some studies, from the data given by Seeman and Van Tol (1995) it does not seem to be a problem in their studies as extended incubations (up to 6 hours) did not affect $K_d$ estimates. It seems prudent, however, to use incubation times that are longer than the typically used times of about 1 hour to ensure equilibration at all radioligand concentrations, assuming there is no degradation of receptor in the longer assays.

Depletion of the added ligands does seem to be a significant problem in some studies in that it confounds definition of the actual free ligand concentrations that are required for the application of equations defining binding equilibria. Depletion can occur via binding to receptors in the assay or via binding to tissue in a nonspecific manner but in a way that disturbs equilibria. This nonspecific low-affinity binding will be undetected in filtration assays as it will be washed away. This may simply mean that the free ligand concentration is not the same as the added ligand concentration, so that $K_d$ values are overestimated (Chang et al. 1975), but there can be a more subtle effect on the definition of nonspecific radioligand binding (Golds et al. 1980; Seeman et al. 1984). Some analysis programs take account of some aspects of these artefacts. For example, the LIGAND program corrects for the depletion by subtracting the bound radioligand from the added radioligand. Alternatively, this correction can be made manually. However, the nonspecific binding of radioligand to tissue cannot be assessed accurately in a filtration assay, and so this correction will not be made. Also if depletion is high, any corrections will be inaccurate. The only way to avoid these kinds of errors completely is to use conditions that minimize ligand depletion, and this requires either very low tissue concentrations or a radioligand with a dissociation constant so that depletion is unlikely to be a problem. Practical considerations dictate that in a 1-ml assay using a radioligand with a specific activity of about 60 Ci/mmol it will be necessary to use at least 10 pM receptor

NEUROPSYCHOPHARMACOLOGY 1997–VOL. 16, NO. 2

Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/97/$17.00
PII S0893-133X(96)00191-7

**Table 1.** Dissociation Constants of Radioligands Determined at $D_2$ Dopamine Receptors

| | [$^3$H]-raclopride (nM) | [$^3$H]-spiperone (pM) | [$^3$H]-nemonapride (pM) |
|---|---|---|---|
| Seeman and Van Tol 1995 (1.5 ml) ($D_2$ short/long, human) | 1.6 | 69 | 82 |
| Seeman and Van Tol 1995 (8 ml) | 1.3 | 25 | 27 |
| Malmberg et al. 1996 ($D_2$ long, human) | 1.16 | 24 | 11 |
| Hoare and Strange in press ($D_2$ long, rat) | — | 17 | — |

to achieve a specific signal of 1500 dpm. In many cases higher receptor concentrations are used, especially if the specific activity of the radioligand is lower [e.g., Castro and Strange (1993) where the receptor concentration was about 40 pM]. For radioligands such as [$^3$H]-spiperone and [$^3$H]-nemonapride whose dissociation constants are less than 50 pM (see below for accurate values), this will lead to significant depletion in a saturation analysis where radioligand concentrations across the $K_d$ are used. For radioligands with $K_d$ values of about 1 nM (e.g., [$^3$H]-raclopride) depletion will be less important as the radioligand concentrations used in a saturation experiment will be correspondingly higher. The depletion with the higher-affinity radioligands can be overcome by using a larger assay volume so that the concentration of receptor is reduced but the absolute amount is unchanged. For example with [$^3$H]-spiperone the $K_d$ for the rat $D_{2(long)}$ dopamine receptor expressed in L cells was found to be 112 pM when a 1-ml assay volume was employed (receptor concentration of about 50 pM), but if a 10-ml assay volume (receptor about 3 pM) was used, the $K_d$ was found to be 17 pM (incubation times were extended, and this is especially important in the large-volume assays with lower receptor concentrations, as the association reaction is a bimolecular reaction) (Hoare and Strange in press). An independent assessment of the $K_d$ for [$^3$H]-spiperone was obtained from kinetic measurements of the association and dissociation rate constants and gave values between 8 and 12 pM (Hoare and Strange in press). These values for the dissociation constant of [$^3$H]-spiperone are considerably lower than most estimates reported in the literature. It should be pointed out, however, that estimates of $K_d$ for [$^3$H]-spiperone that differ by less than a factor of 3 from these values can be routinely obtained in 1-ml assays if correction is made for the depletion of the radioligand (e.g., Castro and Strange 1993; Woodward et al. 1994, 1996).

These issues have been addressed by Malmberg et al. (1996) who determined the $K_d$ and $B_{max}$ values for the human $D_{2(long)}$ receptor for several radioligands under carefully defined conditions that minimized depletion (large assay volumes for high affinity ligands, receptor 2–3 pM) and lack of equilibration (extended incubation times). They reported $K_d$ values of 1.16 nM ([$^3$H]-raclopride), 24 pM ([$^3$H]-spiperone), and 11 pM ([$^3$H]-nemonapride). I believe it is reasonable to take these values as accurate estimates of the dissociation constants for these three radioligands at the $D_{2(long)}$ receptor. Seeman and Van Tol (1995) report values significantly higher in 1.5-ml assay volumes (about 10 pM receptor), but if an 8-ml assay volume (about 6 pM receptor) is used, then values closer to these estimates are seen (Table 1). It seems likely that these differences between values cited by Seeman and Van Tol (1995) are the result of ligand depletion, although it is difficult to tell from the published information whether any correction was made for depletion. Although these authors are aware of the potential problem of ligand depletion, they perform control experiments measuring the free radioligand concentration that they interpret as indicating that no depletion occurred. These experiments used rather high radioligand concentrations (about 250 pM for [$^3$H]-spiperone and [$^3$H]-nemonapride), where with about 20 pM receptor (my estimate), depletion would have been minimal anyway. The problem of depletion is much greater in a saturation analysis where concentrations of radioligand across the $K_d$ will be required. For example, in an experiment with $D_2$ dopamine receptors (about 30 pM) expressed in CHO cells and an added [$^3$H]-spiperone concentration of about 50 pM, the free [$^3$H]-spiperone concentration in the presence of receptor was about 27 pM, indicating a significant depletion (S. Hoare and P. G. Strange, unpublished observations).

Thus we may conclude that accurate values of radioligand dissociation constants can be obtained only if the assay conditions are defined rigorously. The values for ligand dissociation constants also are influenced by other factors, such as the buffer and pH of the assay and the precise receptor source. For example the $K_d$ for substituted benzamides is influenced by $Na^+$ ions, and the $K_d$ for some ligands is influenced by pH via effects on the receptor and ligand dependent on their respective

**Table 2.** Dissociation Constants (nM) for Neuroleptic Drugs at $D_2$ Dopamine Receptors

| | Malmberg et al. 1993 | Seeman and Van Tol 1995; Seeman et al. (review) |
|---|---|---|
| Chlorpromazine | 0.55 | 1.30 |
| Clozapine | 35.00 | 59.00 |
| Haloperidol | 0.53 | 0.47 |
| Remoxipride | 54.00 | 80.00 |
| Risperidone | 1.30 | 1.60 |
| Sertindole | 0.38 | 1.40 |
| (-)-Sulpiride | 2.50 | — |
| Thioridazine | 1.20 | 1.60 |

The data shown were derived from competition experiments versus [$^3$H]-raclopride in Malmberg et al. (1993) [$D_2$(short)] and for $D_2$ (short/long) from Seeman and Van Tol (1995) and Seeman et al. (review). Note that values for $D_2$ (long) are slightly higher for some drugs, such as substituted benzamides (Castro and Strange 1993; Malmberg et al. 1993)].

p$K_a$ values (Strange 1994; D'Souza and Strange 1995). Ligands also have different affinities for the different $D_2$-like receptor subtypes ($D_2$, $D_3$, $D_4$), and there may be some differences in affinity for the different isoforms of the $D_2$-like receptors.

I have discussed the determination of accurate $K_d$ values for radioligands at some length as these values underpin the determination of $K_i$ values for other ligands in competition assays. In a typical competition experiment a range of competing drug concentrations is used with a single radioligand concentration, and the $IC_{50}$ for inhibition of radioligand binding is used to determine $K_i$ using the assumption of two competing ligands at one saturable site, often referred to as the Cheng–Prusoff correction (Cheng and Prusoff 1973). This equation uses the concentration of the radioligand and its $K_d$ to derive $K_i$ from $IC_{50}$. Artefacts can be generated in competition assays in the same way as in saturation assays (Hulme and Birdsall 1992). For example, the time for equilibration of radioligand with the receptors will be increased by the presence of the competing ligand, although this rarely is considered. Radioligand depletion and possibly competing ligand depletion also can occur, although the concentration of radioligand used in typical competition assays usually is such that depletion is not a major problem; it should not, however, be ignored. A very-high-affinity competitor such as spiperone (at the $D_2$ receptor) may be depleted, and it is impossible to determine the extent of this problem. We have also experienced problems in the accurate dilution of very hydrophobic ligands such as flupenthixol. The major artefact in competition assays for $D_2$-like dopamine receptors, however, seems to be the dispersity of the values used for the $K_d$ of the radioligand, and as I have tried to show, $K_d$ values have typically been overestimated for the very-high-affinity radioligands such as [$^3$H]-spiperone and [$^3$H]-nemonapride. Because $K_d$ is used directly in the $K_i$ calculation, this will affect all the $K_i$ values derived for competitors. For example, if we consider the data of Figure 1 in Seeman and Van Tol (1995) for haloperidol competitions versus [$^3$H]-raclopride, [$^3$H]-spiperone, and [$^3$H]-nemonapride, then estimates of the $IC_{50}$ values for the three experiments are 3, 12, and 23.8 nM, respectively. These can then be corrected for radioligand concentration (2.4 nM, 224 pM, 186 pM, respectively; these concentrations were used directly in calculations as the receptor concentration was about 10 pM) using the $K_d$ values reported by Seeman and Van Tol (1995) to give $K_i$ values of 1.2, 2.8, and 7.3 nM and using the $K_d$ values reported by Malmberg et al. (1996) to give $K_i$ values of 0.98, 1.16, and 1.32 nM, respectively. These latter values are quite close and largely independent of the radioligand used for the determinations. Similar values were obtained with large-volume assays by Seeman and Van Tol (1995) (0.47, 0.63, and 0.89 nM, respectively). Malmberg et al. (1993) report a $K_i$ value for haloperidol of 0.66 nM for $D_{2(long)}$ using [$^3$H]-raclopride. These calculations show that the principal source of the apparent dependence of the ligand $K_i$ values on the radioligand used for competition assays as described by Seeman and Van Tol (1995) and discussed in detail in the review is the use of inappropriate $K_d$ values for the radioligands. Once the correct $K_d$ value for radioligand is used, values for $K_i$ values for competitors are rather similar and independent of radioligand. Data obtained with [$^3$H]-raclopride are least subject to the artefacts described here and the data reported with this radioligand by Seeman and Van Tol

(1995) are generally in good agreement with those of Malmberg et al. (1993). I have given some values for these dissociation constants in Table 2.

Let us now consider the explanation for the radioligand dependence of $K_i$ values proposed by Seeman and Van Tol (1995) and in the review. They propose that there is a relationship between the tissue–buffer partition coefficient for the radioligand and the $K_i$ value for a competing neuroleptic drug. The tissue–buffer partition coefficient is defined as the gradient of the line relating nonspecific radioligand binding and radioligand concentration [i.e., nonspecific binding/radioligand concentration (pmol/g/nM)]. Although this may be a reflection of tissue–buffer partition and may contribute to radioligand depletion, it is not a true partition coefficient, and it also will contain a significant contribution from filter binding. Nevertheless, a linear relation is shown between the $K_i$ determined for neuroleptics versus the three different radioligands and this "tissue–buffer partition coefficient." A linear relationship of this nature does not prove a causal relationship, and I can see no theoretical reason for relating the $K_i$ values and the "tissue–buffer partition coefficient." As I hope I have shown, I believe that the different $K_i$ values are a result of the use of inappropriate $K_d$ estimates, and I would suggest that the apparent linear relation reported is coincidental.

Seeman and Van Tol (1995) and Seeman et al. also extrapolate the linear relation to zero partition and call this value the radioligand-independent dissociation constant or the dissociation constant of the neuroleptic in the absence of any competing radioligand. Surely, the Cheng–Prusoff correction is designed to obtain this value by correcting for the concentration of the radioligand; I believe the terms used by Seeman and Van Tol (1995) and Seeman et al. are misleading and add unnecessary complexity. Seeman et al. support their case by showing that the $K_d$ value for the competing neuroleptic determined in saturation analyses with the [$^3$H]-labeled compound lies close to the intercept at zero tissue–buffer partition. Although this is indeed true for the cases shown, the difference between the $K_i$ values at the $D_2$ receptor determined versus [$^3$H]-raclopride and the $K_d$ of the radiolabeled drug differ by at most 2.5-fold ([$^3$H]-chlorpromazine $K_d$ 0.75 nM, $K_i$ 1.8 nM; [$^3$H]-haloperidol $K_d$ 0.35 nM, $K_i$ 0.7 nM; [$^3$H]-sertindole $K_d$ 1 nM, $K_i$ 1.5 nM), and these differences probably lie within experimental error and any differences in the binding of the radiolabeled and unlabeled drug.

For clozapine defining these quantities is important, especially for the $D_4$ receptor for reasons cited below, but it is impossible to use [$^3$H]-raclopride here because of its low affinity for the $D_4$ receptor, and so Seeman et al. use [$^3$H]-clozapine and obtain a $K_d$ value of 1.6 nM. This contrasts with the $K_i$ value obtained versus [$^3$H]-spiperone of 22.3 nM. According to the arguments outlined earlier, this $K_i$ value will be overestimated by about a factor of 2 or 3. The value of 1.6 nM for the $K_d$ of [$^3$H]-clozapine, therefore, seems rather too low, and I would wish to see a full pharmacologic characterization of the properties of the binding of this radioligand to the D4-COS cells. Some cell lines express endogenous receptors. For example, functional endogenous muscarinic receptors have been described on CHO cells (Wang et al. 1995); clozapine has an affinity of about 3 nM for muscarinic receptors, and studies using [$^3$H]-clozapine have shown that it mostly labels muscarinic receptors in brain tissue (Flamez et al. 1994; Kusumi et al. 1995). I conclude that $K_i$ values for clozapine at the $D_2$ and $D_4$ receptors are approximately 40 and 8 nM, respectively (in the presence of $Na^+$).

In summary, the determination of accurate values of dissociation constants for neuroleptic drugs at $D_2$-like dopamine receptors can be accomplished using radioligand binding assays, but care needs to be taken in setting up for the assays. In Table 2 I have given some data for dissociation constants of neuroleptics at $D_2$ receptors that I believe are good estimates of the values.

## EFFECTS OF NEUROLEPTIC DRUGS ON SYNAPTIC ACTIONS OF DOPAMINE

Seeman et al. consider the synaptic actions of the neuroleptic drugs and attempt to calculate the concentrations of these drugs that would be required to block about 75% of the receptors: the apparent blockade that creates the antipsychotic effect (Farde et al. 1992; Sedvall and Farde 1995). It would be very useful to make such a calculation, but I believe that there are so many uncertainties that the results of these calculations would be very inaccurate. For example, it would be necessary to know the synaptic concentration of dopamine. The data of Kawagoe et al. (1992) show that this is about 6 nM at a distance of 2 μm from the synapse but higher (about 97 nM) at the synapse itself. Dopamine may act synaptically and in a paracrine fashion, and it is unclear which action is relevant here. There also is considerable uncertainty in the levels of drugs in different cellular compartments. There is the issue of protein binding of the drugs in plasma [addressed by Seeman (1992)], but also antipsychotic drugs may be concentrated in the brain (Baldessarini et al. 1993) and the concentrations of these drugs in different cellular and fluid compartments of the brain are entirely unknown.

What is clear is that, whereas most antipsychotic drugs are able to occupy 75% or more of the $D_2$-like receptors in brain at their normal doses, clozapine occupies fewer $D_2$-like receptors [up to 50% depending on the probe used (Karbe et al. 1991; Farde et al. 1992; Nordstrom et al. 1995; Sedvall and Farde, 1995)]. Given the uncertainties mentioned, it is very difficult to infer the percentage occupancy of $D_4$ receptors by clozapine from plasma concentrations.

## MECHANISMS OF ACTION OF ATYPICAL NEUROLEPTIC DRUGS

Seeman et al. propose a number of hypotheses for the actions of the atypical neuroleptic drugs and discuss the information supporting or refuting these hypotheses. Much research remains to be done to understand the actions of these drugs. Maybe there is no unifying mechanism; rather there may be different ways of being "atypical."

I do, however, wish to comment on two aspects of their discussion. The first of these concerns the division of neuroleptics into those with dissociation constants at the $D_2$ receptor in the nM range (largely the typical neuroleptics) and those with dissociation constants at least 10 times higher (largely the atypicals). Seeman et al. argue that this division has mechanistic relevance associated with the competition of the drugs with dopamine at synapses and extend the argument to differences in the blockade of striatal and limbic receptors based on differences in dopamine levels in the two parts of the brain. If we consider a situation where the drug and dopamine are at equilibrium at the receptors, then the ability of the drug to compete with dopamine at the synapse will depend on the concentration of dopamine and the concentration of drug relative to its dissociation constant. Although it is difficult to estimate the synaptic concentrations of drugs from the plasma-free concentrations, it is generally the case that the lower affinity drugs are used at higher doses and achieve higher free concentrations in plasma (see Table 4 of Seeman 1992). Therefore, the lower-affinity drugs will compete with endogenous dopamine as well as the higher-affinity drugs if an equilibrium model is assumed. This does not, however, take into account the dynamic nature of synaptic action. For a given drug dose the drug level in the brain will be relatively stable. Although there may be a background stable level of dopamine, the dopamine levels may change depending on synaptic activity. These changes in dopamine levels will perturb the equilibrium, and if the drug has a high affinity for the receptors, then its dissociation rate is likely to be slow and, over a short time, occupancy will not adjust to take into account the extra dopamine. For a drug with a lower affinity, dissociation will probably be quicker and receptor occupancy by dopamine will change. This has actually been shown by Young et al. (1991); Logan et al. (1991) and Dewey et al. (1993) in studies using labeled probes on experimental animals where the lower-affinity probe raclopride responded kinetically to changes in dopamine levels but the higher-affinity probe N-methyl spiperone did not. If there are different dopamine levels in limbic and striatal brain regions, then this might affect occupancy of receptors by all drugs at equilibrium but is unlikely to alter the temporal differences of the drug actions outlined.

Seeman et al. discuss the role of the $D_4$ dopamine receptor as a possible site of action of atypical neuroleptic drugs. The original stimulus to this theory was the observation of Van Tol et al. (1991) that clozapine had a selectivity for $D_4$ versus $D_2$ receptors. This was then followed by the observations of Seeman et al. (1993) that $D_4$ receptors were higher in the striatum of patients with schizophrenia. As I have argued, I believe that the affinities of clozapine for the $D_2$ and $D_4$ receptors are about 40 and 8 nM, respectively, showing only a modest selectivity. I have argued elsewhere (Strange 1994) that the techniques used by Seeman et al. (1993) to determine $D_4$ receptors in postmortem brain (striatum) have limitations, and indeed studies using $D_4$-selective ligands in human brain (Lahti et al. 1996) and $D_4$ receptor–specific antibodies in primate brain (Mrzljak et al. 1996) have now shown that there are few if any $D_4$ receptors in the striatum. Others have used direct and indirect methods to reach the same conclusion (Reynolds and Mason 1994, 1995). Seeman et al. (1995 and review) have argued that the method used by Reynolds and Mason (1995) has technical limitations, but apparently these do not invalidate the conclusions reached (GP Reynolds personal communication). The lack of $D_4$ receptors in the human striatum also is supported by the observation of very low levels of mRNA for this subtype in the human striatum or cortex (Matsumoto et al. 1996). The existence of humans with nonfunctional $D_4$ receptors, but with no marked psychiatric problems (Nothen et al. 1994), suggests that the $D_4$ receptor does not play a major functional role. Therefore, although clozapine does show some modest selectivity for the $D_4$ receptor and there are $D_4$ receptors in certain brain regions (Mrzljak et al. 1996), the functional role of these receptors is unclear.

Seeman et al. argue that perhaps the increased numbers of $D_4$-like receptors that they report in the human striatum in schizophrenia (Seeman et al. 1993) are due to an alteration of the properties of the $D_2$ receptor. They cite their observations on the densities of $D_2$ receptors in the brain and recombinant mammalian and insect cells (Ng et al. 1995), where [$^3$H]-nemonapride exhibits a higher density of $D_2$ receptors than [$^3$H]-spiperone, which they suggest is consistent with the existence of monomers and dimers of receptors (Seeman et al. 1992). These are intriguing findings, but Seeman et al. make no comment on the inability of others to replicate these findings qualitatively (Vile et al. 1995; Malmberg et al. 1996) or quantitatively (Terai et al. 1989; Hidaka et al. 1995). We also have examined recombinant $D_2$ receptors expressed in insect cells and find no difference between the number of receptors determined with the two radioligands (Y Cordeaux and PG Strange, unpublished observations). Elsewhere I have set out some ideas that can reconcile these observations (Strange 1994) based on a variable monomer–dimer equilibrium, and it is indeed important to note that for other G-protein–coupled receptors there are suggestions that these may exist in oligo-

mers (Potter et al. 1991; Wreggett and Wells 1995). To validate these ideas for the $D_2$-like dopamine receptors, however, careful biophysical experiments are required.

## ACKNOWLEDGMENTS

I thank my colleagues in Canterbury for helpful discussions during the preparation of this Commentary and especially Sam Hoare for doing the experiments on radioligand depletion.

Philip G. Strange Ph.D.
Department of Biosciences
University of Kent at Canterbury

## REFERENCES

Baldessarini RJ, Centorrino F, Flood JG, Volpicelli SA, Huston-Lyons D, Cohen BM (1993): Tissue concentration of clozapine and its metabolites in the rat. Neuropsychopharmacology 9:117–124

Burgisser E, Hancock AA, Lefkowitz RJ, De Lean A (1981): Anomalous equilibrium binding properties of high affinity racemic radioligands. Mol Pharmacol 19:205–216

Castro S, Strange PG (1993): Differences in the ligand binding properties of the short and long versions of the $D_2$ dopamine receptor. J Neurochem 60:372–375

Chang K, Jacobs S, Cuatrecasas P (1975): Quantitative aspects of hormone receptor interaction of high affinity. Effect of receptor concentration and measurement of dissociation constants of labeled and unlabeled hormones. Biochim Biophys Acta 406:294–303

Cheng YC, Prusoff WH (1973): Relationship between the inhibition constant ($K_i$) and the concentration of inhibitor that causes 50% inhibition ($I_{50}$) of an enzyme reaction. Biochem Pharmacol 22:3099–3108

Dewey SL, Smith GS, Logan J, Brodie JD, Fowler JS, Wolf AP (1993): Striatal binding of the PET ligand [$^{11}$C]-raclopride is altered by drugs that modify synaptic dopamine levels. Synapse 13:350–356

D'Souza UM, Strange PG (1995): pH dependence of ligand binding to $D_2$ dopamine receptors. Biochemistry 34:13636–13541

Farde L, Nordstrom AL, Wiesel FA, Pauli S, Halldin C, Sedvall G (1992): PET analysis of central $D_1$ and $D_2$ dopamine receptor occupance in patients treated with classical neuroleptics and clozapine Archiv Gen Psychiatr 49:538–544

Flamez A, De Backer JP, Wilczak N, Vauquelin G, De Keyser JD (1994): [$^3$H]-clozapine is not a suitable radioligand for the labeling of $D_4$ dopamine receptors in postmortem human brain. Neurosci Lett 175:17–20

Golds PR, Przyslo FR, Strange PG (1980): The binding of some antidepressant drugs to brain muscarinic acetylcholine receptors. Br J Pharmacol 68:541–549

Hidaka K, Matsumoto M, Tada S, Tasaki Y, Yamaguchi T (1995): Differential effects of [$^3$H]nemonapride and [$^3$H]spiperone binding on human dopamine $D_4$ receptors. Neurosci Lett 186:145–148

Hoare S, Strange PG (in press): The regulation of $D_2$ dopamine receptors by amiloride and amiloride analogues. Mol Pharmacol

Hulme EC, Birdsall NJM (1992): Strategy and tactics in receptor binding studies. In Receptor-Ligand Interactions—A practical approach. Oxford: IRL Press, pp 63–176

Karbe H, Wienhard K, Hamacher K, Huber M, Herholz K, Coenen HH, Stocklin G, Lovenich A, Heiss WD (1991): Positron emission tomography with [$^{18}$F]-methyl spiperone demonstrated $D_2$ dopamine receptor binding differences of clozapine and haloperidol. J Neural Trans 86:163–173

Kawagoe KT, Garris PA, Wiedemann DJ, Wightman RM (1992): Regulation of transient dopamine concentration gradients in microenvironment surrounding nerve terminals in rat striatum. Neuroscience 51:55–64

Kusumi I, Matsubara S, Takahashi Y, Ishikane T, Koyama T (1995): Characterisation of [$^3$H]-clozapine binding sites in rat brain. J Neural Trans 101:231–235

Lahti RA, Primus RJ, Gallagher DW, Roberts R, Tamminga CA (1996): Distribution of dopamine $D_4$ receptor in human postmortem brain sections: Autoradiographic studies with [$^3$H]-NGD-94-1. Schizophrenia Res 18:173

Logan J, Dewey SL, Wolf AP, Fowler JS, Brodie JD, Angrist B, Volkow ND, Gatley SJ (1991): Effects of endogenous dopamine on measures of [$^{18}$F]-N-methyl spiroperidol binding in the basal ganglia: Comparison of simulations and experimental results from PET studies in baboons. Synapse 9:195–207

Malmberg A, Jackson DM, Eriksson A, Mohell N (1993): Unique binding characteristics of antipsychotic agents interacting with human dopamine $D_{2A}$, $D_{2B}$, and $D_3$ receptors. Mol Pharmacol 43:749–754

Malmberg A, Jerning E, Mohell N: (1996): Critical reevaluation of spiperone and benzamide binding to $D_2$ dopamine receptors: Evidence for identical binding sites. Eur J Pharmacol 303:123–128

Matsumoto M, Hidaka K, Tada S, Tasaki Y, Yamaguchi T (1996): Low levels of mRNA for dopamine $D_4$ receptors in human cerebral cortex and striatum. J Neurochem 66:915–919

Mrzljak L, Bergson C, Pappy M, Huff R, Levenson R, Goldman-Rakic PS (1996): Localisation of dopamine $D_4$ receptors in GABAergic neurones of the primate brain. Nature 381:245–248

Ng YK, O'Dowd BF, Caron M, Dennis M, Brann MR, George SR. (1994): Phosphorylation and palmitoylation of the human $D_{2L}$ dopamine receptor in Sf9 cells. J Neurochem 63:1589–1595

Nordstrom AL, Farde L, Nyberg S, Karlsson P, Halldin C, Sedvall G (1995): $D_1$, $D_2$ and 5-$HT_2$ receptor occupancy in relation to clozapine serum concentration: A PET study of schizophrenic patients. Am J Psychiatr 152:1444–1449

Nothen MM, Cichon S, Hemmer S (1994): Human dopamine $D_4$ receptor gene: Frequent occurrence of a null allele and observations of homozygosity. Human Mol Gen 3:2207–2212

Potter L, Ballasteros LA, Bichaglan LH, Ferrendelli CA, Fisher A, Hanchett HE, Zhang R (1991): Evidence for paired M2 muscarinic receptors. Mol Pharmacol 39:211–221

Reynolds GP (1996): Dopamine $D_4$ receptors in schizophrenia. J Neurochem 66:881–882

Reynolds GP, Mason SL (1994): Are striatal dopamine $D_4$ receptors increased in schizophrenia? J Neurochem 63:1576–1577

Reynolds GP, Mason SI (1995): Absence of detectable dopamine $D_4$ receptors in schizophrenia. Eur J Pharmacol 281:R5–R6

Sedvall G, Farde L (1995): Chemical brain anatomy in schizophrenia. Lancet 346:743–749

Seeman P (1992): Dopamine receptor sequences. Therapeutic levels of neuroleptics occupy $D_2$ receptors, clozapine occupies $D_4$. Neuropsychopharmacology 7:261–284

Seeman P, Van Tol HHM (1995): Deriving the therapeutic concentrations for clozapine and haloperidol: The apparent dissociation constant of a neuroleptic at the dopamine $D_2$ or $D_4$ receptor varies with the affinity of the competing radioligand. Eur J Pharmacol 291:59–66

Seeman P, Van Tol HHM (1996): Dopamine $D_4$ receptors in schizophrenia. J Neurochem 66:882–883

Seeman P, Ulpian C, Wreggett KA, Wells JA (1984): Dopamine receptor parameters detected by [$^3$H]-spiperone depend on tissue concentration: Analysis and examples. J Neurochem 43:221–235

Seeman P, Guan HC, Civelli O, Van Tol HHM, Sunahara RK, Niznik HB (1992): The cloned dopamine $D_2$ receptor reveals different densities for dopamine antagonist ligands. Implications for human brain positron emission tomography. Eur J Pharmacol 227:139–146

Seeman P, Guan HC, Van Tol HHM (1993): Dopamine $D_4$ receptors are elevated in schizophrenia. Nature 365:441–445

Seeman P, Guan HC, Van Tol HHM (1995): Schizophrenia: Elevation of dopamine $D_4$-like sites using [$^3$H]-nemonapride and [$^{125}$I]-epidepride. Eur J Pharmacol 286:R3–R5

Strange PG (1994): Dopamine $D_4$ receptors: Curiouser and curiouser. Trends Pharmacol Sci 15:317–319

Terai MK, Hidaka, Nakamura Y (1989): Comparison of[$^3$H]-YM-09151-2 with [$^3$H]-spiperone and [$^3$H]-raclopride for dopamine $D_2$ receptor binding to rat striatum. Eur J Pharmacol 173:177–182

Van Tol HHM, Bunzow JR, Guan HC, Sunahara RK, Seeman P, Niznik HB, Civelli O (1991): Cloning of the gene for a human $D_4$ receptor with high affinity for the antipsychotic clozapine. Nature 350:610–614

Vile J, D'Souza UD, Strange PG (1995): [$^3$H]-nemonapride and [$^3$H]-spiperone label equivalent numbers of $D_2$ and $D_3$ dopamine receptors in a range of tissues and under different conditions. J Neurochem 64:940–943

Wang SZ, Zhu SZ, El Fakahany EE (1995): Expression of muscarinic acetylcholine receptors in Chinese hamster ovary cells. Eur J Pharmacol 291:R1–R2

Wells JW, Birdsall NJM, Burgen ASV, Hulme EC (1980): Competitive binding studies with multiple sites. Effects arising from depletion of the free ligand. Biochim Biophys Acta 632:464–469

Woodward R, Coley C, Daniell S, Naylor LH, Strange PG (1996): Investigation of the role of conserved serine residues in the long form of the rat $D_2$ dopamine receptor using site directed mutogenesis. J Neurochem 66:394–402

Woodward R, Daniell S, Strange PG, Naylor LH (1994): Structural studies on $D_2$ dopamine receptors: mutation of a histidine residue specifically affects the binding of a subgroup of substituted benzamide drugs. J Neurochem 62:1664–1669

Wreggett KA, Wells JW (1995): Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. J Biol Chem 270:22488–22499

Young LT, Wong DF, Goldamn S, Minkin E, Chen C, Matsumura K, Scheffel U, Wagner HN (1991): Effects of endogenous dopamine on kinetics of [$^3$H]-N-methyl spiperone and [$^3$H]-raclopride binding in the rat brain. Synapse 9:188–194