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Pharmacological Characterisation of the D_2 Dopamine Receptor Expressed in the Yeast *Schizosaccharomyces pombe*

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ABSTRACT. The rat $D_{2(long)}$ dopamine receptor has been expressed in the fission yeast *Schizosaccharomyces pombe* at levels of about 1 pmol/mg of protein. The recombinant receptor, analysed in ligand binding experiments, exhibits properties typical of a D_2 dopamine receptor and the affinities of antagonists agree with values obtained for the receptor expressed in mammalian systems although the affinities of some antagonists are lower. Substituted benzamide antagonists show lower affinities in the absence of sodium ions whereas clozapine and classical antagonists mostly show higher affinities. Agonist binding is insensitive to the effects of GTP indicating lack of a stable interaction with G-proteins. BIOCHEM PHARMACOL **56**;5:577–582, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. receptor; dopamine; fission yeast; recombinant; ligand binding

Receptors for the neurotransmitter dopamine belong to the large super family of G-protein linked receptors and using molecular biology techniques five dopamine receptor subtypes have been identified. These have been divided in to two subfamilies, the $D_{1\text{-like}}$ (D_1, D_5) and the D_2-like (D_2, D_3, D_4) receptors on the basis of structural and functional criteria [1–3]. Each receptor contains seven putative membrane spanning regions linked by extra cellular and intracellular loops. The D_2-like subfamily is of particular interest as these receptors have a high affinity for the anti-psychotic drugs. Isoforms of the D_2-like receptors exist based on differences in the third intracellular loop. For example, for the D_2 receptor, $D_{2(short)}$ and $D_{2(long)}$ isoforms have been described which are identical except for a 29 amino acid insertion in the third intracellular loop of $D_{2(long)}$. These isoforms may couple differentially to G-proteins although there is some disagreement about the precise specificity of these interactions [3].

Much new information on the properties of the D_2 like receptors has been obtained from the expression of these receptors in heterologous systems such as mammalian and insect cells. In order to extend the characterisation of these receptors it would be desirable to express the receptors in a system where the expression may be high and the conditions for the growth of the cells are less stringent. Yeast potentially offers such a system and yeast has been used for the expression of a number of recombinant proteins in high yield (see for example Ref. 4). Yeast offers an attractive host for the heterologous expression of recombinant receptor proteins as yeast contains G-protein linked receptors for mating factors but does not possess any of the receptors for the neurotransmitters. Members of the G-protein linked receptor super family including dopamine receptors have been expressed in yeast [5–10]. The D_2 dopamine receptor has been expressed in the yeast *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the expression levels are higher in the latter organism [10]. Preliminary analysis of the properties of the recombinant receptors showed, however, that these were somewhat different from those of the native receptors [8–10]. In order to examine these differences in more detail and to explore the use of yeast as a potentially highly expressing system, we have expressed the $D_{2(long)}$ dopamine receptor in *S. pombe*. The aim is to obtain a highly expressing recombinant system for the production of large quantities of receptor and in the present report we describe the full characterisation of this receptor expressed in this recombinant system.

MATERIALS AND METHODS

Materials

[^{3}H]Spiperone (~20 Ci/mmol) was obtained from Amersham. (+)-butaclamol, (−)-butaclamol, sulpiride, haloperidol, pindolol and clozapine were obtained from RBI. Clebopride (Almirall), DO 710 (Dr. A. Mann, Strasbourg), flupenthixol (Lundbeck), mianserin (Beecham), nemonapride (Yamanouchi) were generous gifts. All other chemicals were obtained from commercial sources and were of the highest purity available.

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Methods

EXPRESSION OF D_2 DOPAMINE RECEPTORS IN *S. POMBE*. The cDNA for the rat $D_{2(long)}$ dopamine receptor in the pBluescript vector (Stratagene) (D_2Lp32) was a generous gift from Drs. P. Vernier and J. Mallet (CNRS, Gif sur Yvette) and the yeast expression plasmid pREP 1 [11] was a generous gift from Dr. K. Maundrell (Glaxo, Geneva).

The D_2 receptor sequence was cut from the pBluescript plasmid by digestion with the restriction enzymes Sal I and Xma I and ligated in to the equivalent sites on pREP 1. The resulting construct was checked by restriction digest. The pREP 1_{D2} plasmid was used to transform *S. pombe* (*h*$^-$ *ura4*$^-$D18, *leu1-32*, *ade6-704*) and colonies were isolated after growth on solid MMA medium [12]. These colonies were grown in Edinburgh Minimal liquid medium with supplements and 2 μM thiamine until at mid-log phase when the cells were transferred to medium lacking thiamine. The cells were then grown for 16 hr, harvested and a crude membrane homogenate prepared as described below. This was used in [^{3}H]spiperone binding assays as described below and highly expressing colonies (~0.5 pmol/mg, 0.5 nM [^{3}H]spiperone) were used for subsequent studies.

PREPARATION OF MEMBRANES FROM *S POMBE* CELLS EXPRESSING THE D_2 DOPAMINE RECEPTOR. *S. pombe*-D_2 cells were grown in noninducing medium to mid-log phase and then transferred to inducing medium for 16 hr. The cells were harvested by centrifugation (4000 g, 10 min, 4°), washed by resuspension and centrifugation in 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7.4 (PBS)† and frozen in liquid nitrogen. The cells were then thawed and resuspended in 0.1 w/v of buffer (20 mM HEPES, 1 mM EDTA, 1 mM EGTA, pH 7.4). Sterile acid washed glass beads (0.4 mM diam.) were then added up to the meniscus of the slurry and vortex mixed (15 times for 30 sec) at 4°. Three milliliters of buffer was then added and removed and kept at 4° and the beads were washed with the same buffer and the washes amalgamated. These were centrifuged (180 *g*, 5 min, 4°), to remove cell debris and unbroken cells and the supernatant was centrifuged (48,000 *g*, 10 min, 4°). The pellet was resuspended in buffer containing protease inhibitors (0.01 w/v of antipain, aprotinin, chymostatin, leupeptin, pepstatin all at 0.5 mg/mL in water; 0.1 μM phenylmethylsulphonylfluoride).

LIGAND BINDING ASSAYS. Fifty micrograms of membranes of *S. pombe*-D_2 cells were incubated with [^{3}H]spiperone (0.5 nM for competition experiments, 0.03–4 nM for saturation experiments) and other drugs where appropriate in a final volume of 1 mL of buffer containing 0.1% BSA at 25° for 1 hr at which time binding was at equilibrium. Where Na^+ free buffers were used adjustment of pH was achieved by adding potassium hydroxide solution. The assays were terminated by filtration through GF/C glass fiber filters and the filters were washed with three 4-mL portions of PBS before radioactivity was determined. Specific [^{3}H]spiperone binding was defined as that binding inhibited by 1 μM (+)-butaclamol in competition experiments and as the difference in binding in parallel assays containing 1 μM (+)- and (−)-butaclamol in saturation experiments. Ligand binding data were analysed using the computer program Graphpad/Inplot using one and two site binding models where appropriate.

† *Abbreviation:* NMDG, *N*-methyl D-glucamine hydrochloride.

RESULTS

Construction of S. Pombe *Strains Expressing the D_2 Dopamine Receptor*

The sequence of the D_2 dopamine receptor was cut from the plasmid D_2Lp32 and inserted in the expression vector pREP1 [11] between the *Sal*I and *Xma*I restriction sites. The pREP1-D_2 was then isolated, the identity confirmed by diagnostic restriction digest and *S. pombe* cells were transformed with this plasmid. Colonies were isolated and screened for the expression of the D_2 receptor after induction by growth in thiamine-free medium using the binding of [^{3}H]spiperone. Several of these colonies were found to express the D_2 receptor and [^{3}H]spiperone binding could be detected at levels between 100 and 500 fmol/mg of protein. One of these colonies that expressed at a high level (~500 fmol/mg) was chosen for further characterisation and all subsequent work was carried out on this organism.

Time Course of Expression of D_2 Dopamine Receptors in S. Pombe

The time course of induction of the expression of the D_2 receptor was studied by growing cells in noninducing medium for 16 hr and then transferring the cells to inducing medium (thiamine free) for different periods of time such that the cells would be at a similar density at the end of the culture period. Saturation assays with [^{3}H]spiperone were then performed and the data for the maximal number of binding sites (B_{max}) and dissociation constant (K_d) are shown in Fig 1. Expression begins by about 10 hr and then rises to reach a peak at 16–20 hr after which time expression declines substantially. The dissociation constant for [^{3}H]spiperone binding was about 0.2 nM and did not change significantly during the culture period (ANOVA on pK_d, $P > 0.05$).

Characterisation of the Recombinant D_2 Dopamine Receptor Expressed in S. Pombe

The D_2 dopamine receptor expressed in *S. pombe* was characterised more fully in competition and saturation binding assays with [^{3}H]spiperone for cells grown in inducing medium (thiamine free) for 16 hr. Saturation analyses were performed under a variety of conditions (without Na^+, with Na^+, without Na^+ but with NMDG† to compensate for changes in ionic strength). All saturation curves

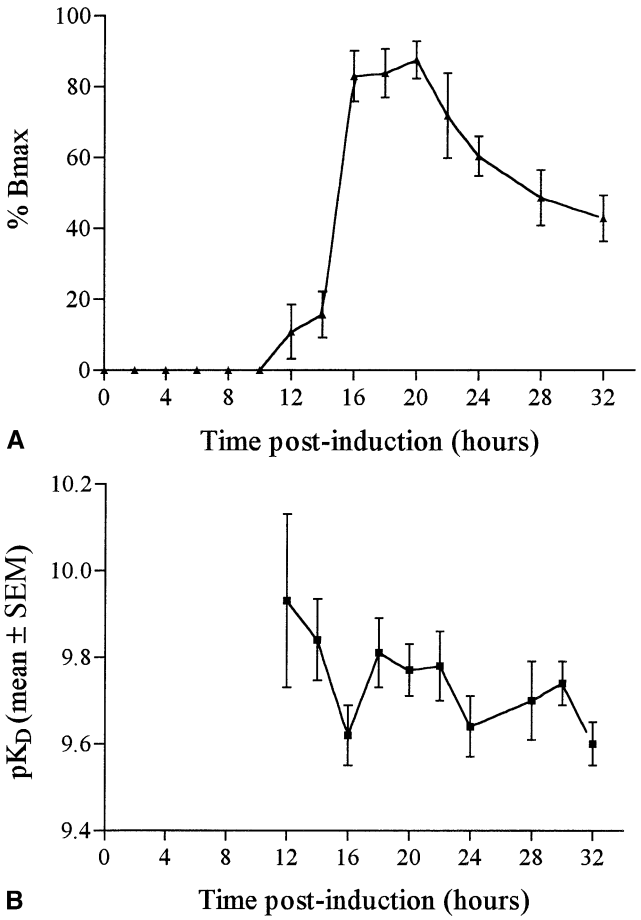

FIG. 1. Time course of expression of recombinant D_2 dopamine receptors expressed in *S. pombe*. Recombinant yeast were grown under inducing conditions for different periods of time as described in the Materials and Methods and Results sections. Saturation analyses with [^{3}H]spiperone were performed and the values for B_{max} and pK_d are given as mean ± SEM for three experiments. For the B_{max} values data in individual experiments were expressed as % of the maximal value in the time course (generally about 18 hr) and the % values expressed as a mean from the three experiments. The actual expression value for 16 hr was 927 ± 134 fmol/mg of protein.

fitted well to one binding site models and values for B_{max} and K_d are given in Table 1. B_{max} values were not significantly different under the three conditions. There was a small effect of the removal of Na^+ ions to increase significantly the affinity of the receptors to bind [^{3}H]spiperone independent of effects of ionic strength.

Competition experiments with a range of antagonists and agonists were performed under a variety of conditions as for the saturation analyses and uniformly gave competition curves that conformed to one site binding models. These experiments showed that the recombinant receptor expressed in *S. pombe* was able to bind typical dopamine antagonists such as haloperidol and flupenthixol with high affinity and that the binding of butaclamol was stereoselective with the (+)-isomer binding with higher affinity than the (−)-isomer. The derived K_i values are given in Tables 1 and 2 and representative data are shown in Figs. 2 and 3. Agonist competition curves were insensitive to the effects of GTP (100 μM). Antagonist affinities were in most cases altered by the replacement of Na^+ by NMDG indicating that this was due to the removal of Na^+ and not due to changes in ionic strength. Affinities of classical antagonists (butaclamol and flupenthixol but not haloperidol) were slightly increased by the removal of Na^+ ions in agreement with the data for [^{3}H]spiperone above. Affinities for atypical antagonists such as sulpiride were mostly decreased substantially by the removal of Na^+ ions with the exception of clozapine whose affinity was slightly increased.

DISCUSSION

In this paper we describe the expression of the D_2 dopamine receptor in the fission yeast *S. pombe* and the characterisation of the expressed receptor in ligand binding assays. The results show that the rat D_2 dopamine receptor can be expressed in this eukaryotic system at moderate levels and with the properties typical of the D_2 dopamine receptor.

The D_2 receptor is expressed in *S. pombe* in the present experiments at levels of about 1 pmol/mg of protein and these levels are comparable with levels achieved in many mammalian expression systems. The receptor is expressed from an inducible expression vector [11] and, following induction by the removal of thiamine from the culture medium, expression rises from a background of zero expression to reach maximum levels after about 16 hr. Expression declines later based on the B_{max} for [^{3}H]spiperone binding and it is not clear why this occurs but it may reflect nutrient depletion. Despite this loss of receptor expression the dissociation constant for [^{3}H]spiperone binding does not change so it is unlikely that the receptors that are detected are being proteolysed. There is one other preliminary report of the expression of the D_2 dopamine receptor in *S. pombe* [10] and a much higher level is achieved using a very similar construct. We have no explanation for the difference but it many relate to the extent of untranslated sequence flanking the coding region used in the present experiments.

We have extensively characterised the recombinant D_2 receptor in ligand binding assays using the binding of a range of ligands. These studies showed that the receptor is being expressed with the properties expected of a D_2 dopamine receptor: high affinity for a range of dopaminergic drugs and stereo selectivity for drugs such as butaclamol. A good correlation is seen between the affinities for drugs for binding to the receptor expressed in *S. pombe* in the present study and in COS cells [13, 14] (Fig. 4). The recombinant receptor also shows a low affinity for drugs such as mianserin and pindolol which are selective for other receptors (serotonin $5HT_2$, $5HT_{1A}$) for which the radioligand spiperone has a high affinity. The affinities of drugs for the recombinant D_2 receptor are, however, reduced in many cases compared to the affinities seen in mammalian systems [13–15]. Some drugs such as (+)-butaclamol (in the presence of NMDG) have affinities similar to the mammalian systems whereas many others have affinities

TABLE 1. Binding of antagonists to D_2 dopamine receptors expressed in recombinant *S. pombe* cells.

pK_i (K_i, nM)	+NaCl	+NMDG	−
(+)-Butaclamol	8.11 ± 0.07 (7.7)	8.70 ± 0.06 (2.0)	n.d.
(−)-Butaclamol	n.d.	5.38 (4168)	n.d.
Clebopride	7.56 ± 0.09 (27.5)	6.59 ± 0.07 (257)	6.43 ± 0.02 (372)
Clozapine	6.37 ± 0.06 (427)	6.63 ± 0.01 (236)	6.45 ± 0.01 (355)
DO 710	7.22 ± 0.07 (60.2)	5.84 ± 0.08 (1445)	5.67 ± 0.04 (2130)
cis Flupenthixol	7.71 ± 0.11 (19.5)	8.27 ± 0.05 (5.4)	n.d.
Haloperidol	7.69 ± 0.05 (20.4)	7.82 ± 0.02 (15.1)	n.d.
Mianserin	n.d.	5.35 ± 0.15 (4467)	n.d.
Nemonapride	8.89 ± 0.15 (1.3)	8.25 ± 0.01 (5.6)	8.12 ± 0.02 (7.6)
Pindolol	<5	<5	n.d.
(−)-Sulpiride	6.84 ± 0.05 (144)	5.32 ± 0.03 (4786)	5.91 ± 0.08 (1270)
[^{3}H]Spiperone	9.62 ± 0.07 (239 pM) 931.5 ± 72.3 fmol/mg	9.81 ± 0.05 (155 pM) 959.8 ± 38.8 fmol/mg	9.78 ± 0.05 (166 pM) 844.9 ± 44.0 fmol/mg

The binding of antagonists was determined as described in the Materials and Methods section in competition experiments versus [^{3}H]spiperone (~0.5 nM) and in saturation experiments with the same radioligand. K_i values or K_d and B_{max} values were determined. Experiments were performed in the standard buffer in the presence of NaCl (120 mM), in the presence of *N*-methyl D-glucamine hydrochloride (NMDG) (120 mM) or, for a few compounds only, in the absence of either of these. All competition and saturation data fitted well to one binding site models. Data are given as mean ± SEM for the pK_i or pK_d of 3 or more experiments and the K_i/K_d values are given in brackets. n.d.: not determined. Unpaired *t*-tests were performed on the pK_i/pK_d values derived in the presence of NaCl or NMDG and these were all significantly different ($P < 0.05$) except for haloperidol ($P > 0.05$). B_{max} values for [^{3}H]spiperone binding were not significantly different under the three conditions (unpaired *t*-test, $P > 0.05$).

that are reduced by factors of between 3- and 10-fold. Such changes in the affinities of drugs for recombinant receptors have been noted for the D_2 receptor expressed in insect cells [16] and in the yeast *S. cerevisiae* [8–10]. It seems likely that the changes are a reflection of the membrane environment of the recombinant receptor and it has been suggested that it is the presence of a different sterol population in the yeast that may mediate the effect [8–10]. It is unlikely that the changes in affinity reflect proteolysis of the receptor as

TABLE 2. Binding of agonists to $D_{2(long)}$ dopamine receptors expressed in recombinant *S. pombe* cells.

pK_i (K_i [μM])	−GTP	+GTP (100 μM)
Apomorphine	5.93 ± 0.02 (1.2)	5.93 ± 0.03 (1.2)
Dopamine	4.36 ± 0.07 (43.6)	4.39 ± 0.02 (40.7)
Quinpirole	4.77 ± 0.08 (16.9)	4.69 ± 0.07 (20.4)

The binding of agonists was determined in competition experiments versus [^{3}H]spiperone in the absence and in the presence of GTP (100 μM) and pK_i values (K_i in brackets) were calculated. Competition data fitted well to one binding site models in all cases and the data are given as mean ± SEM (three or more experiments). There are no significant effect of GTP (paired *t*-test, $P > 0.05$).

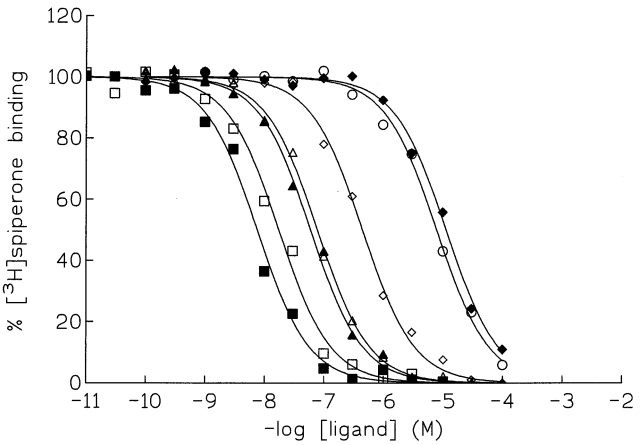

FIG. 2. Binding of antagonists to recombinant D_2 dopamine receptors expressed in *S. pombe*. The binding of (+)-butaclamol, haloperidol and (−)-sulpiride was determined in competition assays vs [^{3}H]spiperone as described in the Materials and Methods section and representative best fit competition curves to a one site binding model are shown; (+)-butaclamol (□, +NaCl; ■, +NMDG); haloperidol (△, +NaCl; ▲, +NMDG); sulpiride (◇, +NaCl; ◆, +NMDG; ○, no addition). Data were replicated as in Table 1.

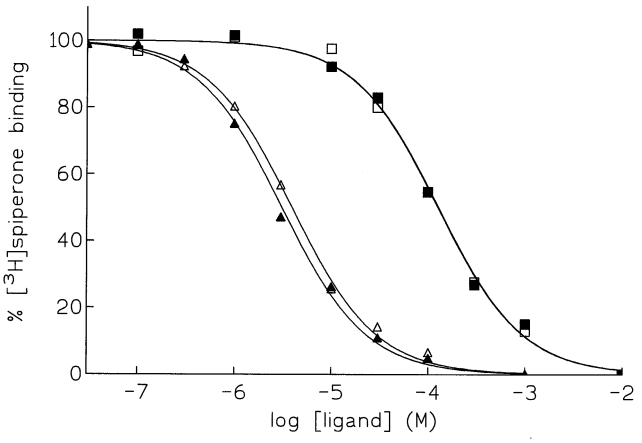

FIG. 3. Binding of agonists to recombinant D_2 dopamine receptors expressed in *S. pombe*. The binding of apomorphine (△ + 100 μM GTP; ▲ control) and dopamine (□ + 100 μM GTP; ■ control) was determined as described in the Materials and Methods section in competition assays versus [^{3}H]spiperone and representative best fit competition curves to a one site binding model, replicated as in Table 2, are shown.

the affinities of some ligands are unaffected. In the present study, we cannot rule out the possibility that the receptor sequence expressed has acquired a mutation and this is responsible for the differences in ligand affinities. This also seems unlikely in that the same sequence has been used for expression in mammalian cells [13–15] where the properties of the expressed receptors are very similar to those of brain.

We also examined the effect of changing the Na^+ concentration on the properties of the recombinant D_2 receptor. As expected, based on other studies of D_2 receptors, the binding of ligands of the substituted benzamide class was of lower affinity in the absence of Na^+ whereas the binding of clozapine was of higher affinity [17, 18].

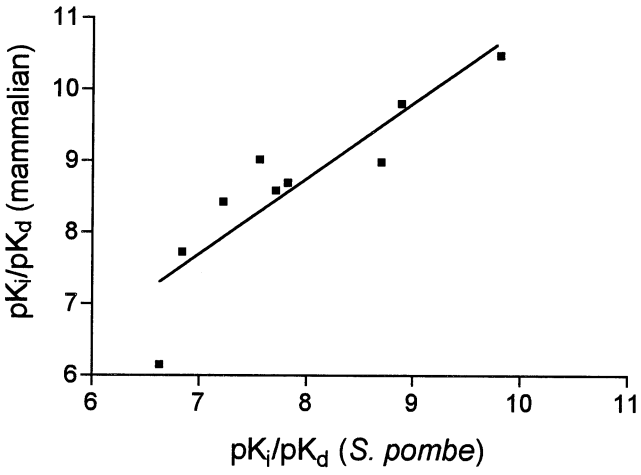

FIG. 4. Correlation between dissociation constants of antagonists for binding to D_2 dopamine receptors expressed in *S. pombe* (the present study) and mammalian cells (COS cells as in Refs. 13, 14). pK_i or pK_d values (in the presence of Na^+ for substituted benzamides and in the absence for all other compounds) were used in a linear regression analysis and for the line shown, $r = 0.890$.

Unexpectedly the affinities of other ligands were also slightly affected by the removal of Na^+ ions, for example the affinities of (+)-butaclamol, flupenthixol and spiperone were increased by a factor of two or more and this effect was not due to the change in the ionic strength. These effects of Na^+ ions must be independent of the coupling of the receptor to G-proteins as the recombinant receptor does not couple to the G-proteins of the cells (see below). It may be that these studies indicate the true Na^+ sensitivity of the receptor independent of G-protein coupling.

We also examined the coupling of the recombinant receptor to G-proteins in the yeast from the sensitivity of the binding of agonists to the effects of GTP [19]. There was no evidence from the experiments for any effect of GTP so that from these experiments we can conclude that the recombinant receptor does not couple stably to the endogenous G-proteins of the cells.

Several recombinant systems have now been used for the expression of D_2 dopamine receptors and a comparison of the different systems can usefully be made. Expression levels in mammalian cells are typically in the range 1–5 pmol/mg of protein [3, 13–15, 19] whereas similar or higher levels have been reported in the insect cell system [16, 20–22]. In yeast, *S. cerevisae* provides moderate [8, 9] or poor (Presland J and Strange PG, unpublished results) levels of expression while *S. pombe* compares well (the present study) or very well [10] with mammalian systems on the basis of expression levels. Yeast, however, has the advantage of ease of culture. As for the nature of the expressed receptors, the mammalian cell systems have the advantage that the properties of the expressed receptors are essentially identical to those reported in brain both in terms of ligand affinities and coupling to G-proteins [see for example Refs. 3, 13–15, 19]. In both the insect cell and yeast systems, there are reports that affinities of expressed receptors for ligands in some [8, 9, 16, 20] but not all studies [21, 22] are lower than expected and there have also been reports of the coupling of the recombinant receptors to G-proteins in some but not all studies [8, 9, 20–22].

In summary, the expression of the D_2 dopamine receptor in recombinant *S. pombe* provides a useful system for the further study of this receptor.

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