



Published in final edited form as:

Brain Res. 2006 October 30; 1117(1): 25–37.

Low affinity binding of the classical D₁ antagonist SCH23390 in rodent brain: Potential interaction with A_{2A} and D₂-like receptors

Sarah K. Leonard, Penelope Ferry-Leeper, and Richard B. Mailman*

Departments of Pharmacology, Psychiatry, Neurology, and Medicinal Chemistry, CB #7160, 7011 NC Neurosciences Hospital, University of North Carolina, School of Medicine, Chapel Hill, NC 27599-7160, USA

Abstract

Whereas structurally dissimilar D₁ antagonists competing for [³H]-SCH23390 binding recognize primarily one site in striatum, two distinct affinity states are observed in both amygdala and hippocampus. The binding profile of SCH23390 is similar in both of these regions, with the high affinity site ($K_D \sim 0.4$ nM) consistent with D₁/D₅ receptors. The appearance of the low affinity site ($K_D \sim 300$ nM) is dependent upon the absence of MgCl₂, but independent of D₁ expression (i.e., still present in D₁ knockout mice). Although the density of high affinity state receptor is lower in hippocampus or amygdala of D₁ knockout mice, some residual binding remains, consistent with the known expression of D₅ receptors in these regions. Remarkably, in hippocampus, the affinity of the low affinity site is shifted rightward in the presence of the D₂ antagonist domperidone and is largely absent in the hippocampus of D₂ knockout animals. Additionally, this site is also shifted rightward in the presence of the A_{2A} ligands SCH58261, CSC, or NECA, or in the absence of A_{2A} receptors. The affinity of SCH23390 for this low affinity site is greater than seen for SCH23390 binding to D₂ receptors in heterologous expression systems, consistent with the hypothesis that both D₂ and A_{2A} receptors are involved in the low affinity binding site. Therefore, we suggest that the heteromerization of D₂ and A_{2A} receptors reported previously *in vitro* also may occur in the brain of both rats and mice.

Keywords

Dopamine receptor; Adenosine receptor; Knockout mice; Heteromerization; Radioreceptor binding

1. Introduction

Dopamine receptors comprise a subfamily of G protein-coupled receptors (GPCRs) encoded by five distinct genes (D₁, D₂, D₃, D₄, D₅). Functionally, D₁-like receptors (D₁ and D₅) are characterized by their ability to stimulate adenylyl cyclase (AC) (Garau et al., 1978; Keabian and Calne, 1979). Radio-receptor binding studies, autoradiographic, immunohistochemical, and *in situ* data clearly show that D₁ receptors are present in the amygdala (Dawson et al., 1988; Huang et al., 1992; Hurd et al., 2001; Mansour et al., 1992; Savasta et al., 1986; Sunahara et al., 1990), yet D₁-like receptors in the amygdala do not couple to activation of AC (Kilts et al., 1988; Leonard et al., 2003a,b; Mailman et al., 1986). Thus, the mechanisms by which D₁ receptors signal in this region remain unknown.

The current work was sparked by the surprising observation that, in the amygdala, but not in the striatum, the D₁/D₅ antagonist SCH23390 recognizes two clearly different affinity states

* Corresponding author. Fax: +1 919 966 9604. E-mail address: Richard_Mailman@med.unc.edu (R.B. Mailman).

(Leonard et al., 2003b). SCH23390 has proven very useful in ascribing functions and/or behaviors to D₁-like receptor activation due to its >500 fold D₁:D₂ selectivity and low affinity for most other neuroreceptors (see <http://pdsp.cwru.edu/pdsp.asp>). SCH23390 cannot, however, distinguish between D₁ and D₅ receptors. In the current work, we compare SCH23390 binding in the amygdala, striatum, and hippocampus to determine the nature of this unexpected low affinity SCH23390 binding site.

Of these three regions, the density of D₁ receptors is highest in the striatum, followed by the amygdala, and then hippocampus, whereas the hippocampus contains the highest density of D₅ receptors (Boyson et al., 1986;Montague et al., 2001). D₁-like receptors are believed to perform diverse physiological roles in these regions. For example, in the striatum, D₁-like receptors play a role in posture and the initiation of movement (Wang et al., 1998), whereas in the amygdala they modulate drug-reward and fear responses (Callahan et al., 1995;Greba and Kokkinidis, 2000). Hippocampal D₁-like receptors participate in learning and memory, likely through modulation of cAMP synthesis (Matthies et al., 1997;Otmakhova and Lisman, 1996).

Recent work has shown that many GPCRs, including the dopamine receptors, may evoke physiological responses through interactions with other GPCRs. D₁ receptors have been shown to interact with A₁ adenosine and NMDA receptors (Franco et al., 2000;Lee et al., 2002), whereas D₂ receptors may interact with somatostatin 2 (SST2) and A_{2A} adenosine receptors (Franco et al., 2000;Rocheville et al., 2000). We hypothesized that such an interaction may be responsible for the low affinity binding of SCH23390 in the brain.

Dopamine D₂ and adenosine A_{2A} receptors have been shown to interact in an opposing manner on several levels. Behaviorally, antagonism of A_{2A} receptors enhances D₂ receptor-mediated locomotor activity (Latini et al., 1996). Biochemically, D₂ receptor activation inhibits A_{2A} agonist-activated cAMP accumulation (Hillion et al., 2002). The A_{2A} agonist CGS21680 also decreases the affinity of D₂ receptors for dopamine, although dopaminergic agonists have not been shown to have any effect on A_{2A} ligand–receptor interactions (Diaz-Cabiale et al., 2001;Ferre et al., 1991). Recent evidence shows that D₂ and A_{2A} receptors colocalize within the same cells, and upon agonist treatment, coaggregate and cointernalize, suggesting the presence of D₂/A_{2A} heteromers (Hillion et al., 2002). BRET and FRET studies support this hypothesis, and in concert with chimeric receptor experiments, indicate that specific regions of the D₂ receptor are critical for heteromerization (Canals et al., 2003). It appears that the heteromerization of A_{2A} and D₂ receptors may be responsible, at least in part, for the molecular and functional interplay of these two receptors. It is expected, however, that only a portion of the D₂ or A_{2A} receptor populations are heteromerized at any given time since both receptors perform a variety of functions when expressed independently in cells, or in mice in which one, or both, of the receptors was ablated (Chen et al., 2001).

D₂ and A_{2A} receptors colocalize in several brain regions, and both are highly enriched in the striatum (~600–900 fmol D₂/mg protein). D₂ receptors are expressed at moderate levels in the amygdala (~200–300 fmol/mg protein), and low levels in the hippocampus (~100 fmol/mg protein), whereas the A_{2A} receptors are expressed at low levels in both regions. We now report that both D₂ and A_{2A} receptors may play a role in the low affinity binding of SCH23390 in the hippocampus, but not in the amygdala, and suggest that this binding may result from the physical interaction of D₂ and A_{2A} receptors.

2. Results

2.1. The absence of MgCl₂ reveals a significant second affinity state in hippocampus and amygdala, but not striatum

Competition radioreceptor binding assays were performed in which unlabeled SCH23390 was used to compete for [³H]-SCH23390 binding sites (classically termed a “cold” saturation assay). Data from striatal tissue (Fig. 1A) were consistent with single-site kinetics reported by many laboratories including our own (Schulz et al., 1985). Conducting this experiment in the absence of added MgCl₂ led to an increase in apparent receptor density (Fig. 1A) without a significant change in the affinity or kinetics of this antagonist (Fig. 1A and Table 1).

Similar results were observed in amygdala and hippocampal tissue, but the appearance of complex binding kinetics in membranes prepared from these brain regions suggests multiple (or interactive) binding sites (Figs. 1B and C, and Table 1). MgCl₂ in a concentration range of 2–10 mM is commonly used in pharmacological assays because it increases the affinity of agonists for GPCRs and is required for many functional effects. In the presence of 4 mM MgCl₂, competition binding experiments in tissue from both the amygdala and hippocampus revealed shallow binding curves with Hill slopes much less than 1, suggestive of more than one binding site. In the absence of added MgCl₂, the difference between the affinities of the two sites is amplified and the data clearly demonstrate the presence of an additional low affinity binding component.

In order to determine if either the greater apparent density of binding sites or the appearance of the low affinity binding site (s), in the absence of MgCl₂, is characteristic of D₁ or D₅ receptors alone, we examined the binding of SCH23390 in HEK cells transiently transfected with D₁ or D₅ cDNA. As was observed in brain tissue, the apparent receptor density was markedly higher in the absence of MgCl₂. Conversely, the affinity of SCH23390 at D₁ and D₅ receptors was unaffected by MgCl₂ (Fig. 1D).

2.2. The effects of MgCl₂ on apparent receptor density vary by brain region

We next compared the effects of MgCl₂ quantitatively since the striatum, hippocampus, and amygdala contain different total and relative expression levels of D₁ and D₅ receptors. As shown in Fig. 2, the absence of added MgCl₂ caused a greater increase in SCH23390 binding in hippocampus (122%) than in amygdala (68%) or striatum (33%). In D₁ and D₅ expressing cell lines, there was a 25% increase in hD₁ binding, whereas there was a 97% increase in hD₅ binding in the absence of MgCl₂. This effect is not specific to MgCl₂ as 4 mM MgSO₄ or CaCl₂ had the same effect on total specific binding. MgSO₄ and CaCl₂ decreased the percentage of sites in the low affinity state, without completely precluding the appearance of the low affinity site in hippocampus, unlike the effect observed with the addition of MgCl₂ (data not shown).

2.3. Structurally diverse D₁ antagonists recognize multiple affinity sites in the amygdala

As we have previously reported, the two-site binding is not unique to SCH23390, but also is seen with a number of other D₁ antagonists (Leonard et al., 2003a,b). As shown in Fig. 3 and Table 2, competition binding experiments with a number of antagonists versus [³H]-SCH23390 yielded shallow binding curves that could be fitted well by a two-site model. These compounds were chosen because they are all D₁ receptor antagonists, yet bind to other neuroreceptors with markedly different affinities (e.g., see <http://kidb.bioc.cwru.edu/pdsp.php>). Detailed binding statistics are shown in Table 2. There was no significant difference in the percentage of receptor in the high affinity state (R_H) with each of the competing ligands ($p=0.176$), and these sites were not guanine nucleotide sensitive. The affinity of each of the ligands in striatum was consistent with that of the high affinity state in the amygdala, except in the case of clozapine.

Whereas the affinity of clozapine in striatum was in agreement with published reports, the affinity in amygdala was much higher, but we were unable to reconcile this observation with what is known about the affinity of SCH23390 and clozapine for other receptors.

2.4. D₁ and D₅ receptors are not required for the low affinity binding site

We tested the hypothesis that these low affinity binding sites in the hippocampus and amygdala were not dependent upon the D₁ receptor by conducting competition binding experiments in mice lacking D₁ receptors. These experiments indicated that the density of receptors in the high affinity state was lower than in wildtype mice, consistent with the absence of D₁ receptors (Fig. 4, *left panels*), but that SCH23390 still binds to the hippocampus and amygdala of D₁ knockout mice at two different affinities (Fig. 4, *right panels*). The quantification of these data is shown in Table 3.

To determine if the low affinity site required the presence of D₅ receptors, similar experiments were conducted in the hippocampus of D₅ knockout mice. Interestingly, not only were two sites still seen, but the percentage of low affinity sites labeled by SCH23390 was actually increased in the absence of D₅ receptors (Fig. 5). These data suggested that the low affinity site was not the D₁ or D₅ receptor itself, yet is a site that can bind D₁-like ligands.

2.5. The role of 5-HT, D₂, and A_{2A} receptors in the low affinity binding site

In order to investigate the identity of the low affinity site, we masked candidate receptors with an appropriate high affinity ligand and then conducted competition binding as done previously. SCH23390, although a relatively selective ligand, has affinity for several serotonin receptors. The presence of serotonin (50 nM), however, did not affect either the affinity, or relative quantity, of receptor in the low affinity population (data not shown). In addition, the presence of ketanserin (100 nM) or mianserin (100 nM) did not have any effect on the low affinity site seen in the SCH23390 competition binding, although the presence of these 5-HT_{2A&C} receptor antagonists did reduce the amount of binding in the high affinity state (Fig. 6A). This observation is consistent with reports that both ketanserin and mianserin have moderate affinity for D₁-like receptors and that a portion of the high affinity SCH23390 binding in hippocampus may include binding to 5-HT_{2C} receptors (Nicklaus et al., 1996).

Another candidate we investigated was the D₂ receptor, which has micromolar affinity for SCH23390, and whose affinity can be affected by modulating proteins. In particular, adenosine A_{2A} receptors can interact with D₂ receptors (Franco et al., 2000), and agonist binding to A_{2A} receptors affects the affinity of D₂ receptors for dopamine (Diaz-Cabiale et al., 2001; Ferre et al., 1991). We thus sought to test the hypothesis that D₂ or A_{2A} receptor occupation would modulate SCH23390 binding to the low affinity state. In these experiments, we conducted SCH23390 competition binding in the presence of the high affinity D₂ antagonist domperidone (30 nM), the high affinity A_{2A} antagonist SCH58261 (100 nM), the lower affinity A_{2A} antagonist CSC (250 nM), or the A_{2A} agonist NECA (250 nM). These compounds were compared to a control compound, idazoxan (100 nM), that is a high affinity α_{2A} adrenergic antagonist. As is shown in Fig. 6, there was a decrease in affinity of the low affinity state when either D₂ or A_{2A} receptors were occupied. Conversely, idazoxan had no effect. SCH58261 did not compete for [³H]-SCH23390 labeled HEK hD₁ receptors or for [³H]-spiperone labeled CHO-D_{2L} receptors with significant affinity, suggesting that this high affinity A_{2A} antagonist was not directly modulating D₁ or D₂ receptors (Fig. 7).

To explore further the role of D₂ receptors in this phenomenon, we examined the binding of SCH23390 in D₂ knockout mice. Strikingly, there was a sharp reduction in the percentage of SCH23390 binding to the low affinity state in the hippocampus of D₂-null mice (Fig. 8A). In contrast, SCH23390 binding was not altered in the amygdala of D₂ knockout mice (Fig. 8B).

In similar experiments with hippocampus tissue from A_{2A} knockout mice, the affinity of the low affinity site was shifted rightward considerably, whereas the high affinity site was unchanged as compared to their wildtype littermates (Fig. 9). Ligand occupation of A_{2A} receptors, or the absence of A_{2A} receptors in A_{2A} knockout mice, resulted in an affinity for the low affinity site consistent with that of SCH23390 for D₂ receptors in transfected cells.

3. Discussion

3.1. MgCl₂ decreases maximal D₁-like receptor binding

Whereas studies have found that Mg²⁺ increases agonist affinity for GPCRs (and is required for functional activation), it has not been reported to affect either the total binding or affinity of antagonists. In contrast to the finding of Schetz and Sibley (1997) that 4 mM MgCl₂ had no effect on D₁ receptor binding density in transfected CHO cells, we found an increase in receptor density in the absence of added MgCl₂ in all three brain regions we studied, as well as in D₁ or D₅ transfected HEK cells. As a percentage of total binding, the increase we observed was much greater for hD₅ than for hD₁ receptors, indicating that D₅ receptors may be more sensitive to MgCl₂, at least in this cell background. Accordingly, brain regions with a greater ratio of D₅:D₁ receptors showed greater increases in binding, such that the hippocampus showed the greatest percent increase, followed by amygdala, and finally striatum (Ciliax et al., 2000; Khan et al., 2000; Montague et al., 2001). An alternate interpretation of these data is that the increase in binding in the absence of MgCl₂ is related to the absolute amount of D₁-like receptor in the region, instead of different D₁ or D₅ receptor sensitivities. This interpretation is also consistent with our data in that the actual increase in binding was highest in the striatum followed by the amygdala and then the hippocampus (i.e., paralleling the rank order of D₁ and D₅ receptor expression in these brain regions).

MgCl₂ has the greatest effect in reducing the percentage of receptors in the low affinity state, although the other salts tested (MgSO₄, CaCl₂) reduced the percentage of receptors in the low affinity state without completely eliminating the latter (data not shown). This suggests that both magnesium and chloride ions may influence binding to the low affinity site.

3.2. Multiple SCH23390 binding sites in the hippocampus and amygdala

The shapes of the competition curves of a number of antagonists against [³H]-SCH23390 are significantly different between striatum and amygdala. As expected, the competition curves in striatum had kinetics consistent with interaction primarily at a single population of binding sites. Conversely, in amygdala, competition of several antagonists consistently gave concentration–response curves that were “shallow” (i.e., $n_H < 1$) relative to those in striatum. When these competition binding experiments were performed in the amygdala in the absence of MgCl₂, analysis of the data using non-linear regression clearly indicated that a model with two sites fit the data better than a one-site model.

The absence of MgCl₂ allowed for the separation of a low affinity SCH23390 binding site from the well-described high affinity D₁ receptor site in amygdala and hippocampus, but not in D₁ or D₅ expression systems. Upon careful examination, a small portion of low affinity binding was also noted in striatum. As noted, GPCR–ligand binding experiments are typically conducted in the presence of ≥ 1 mM MgCl₂ because Mg²⁺ increases agonist affinity. In blood and cerebrospinal fluid, the free concentration of Mg²⁺ is 0.5–1.0 mM and may be higher intracellularly or in the local region of the receptor (Blair et al., 1989; Jeong et al., 2006). Our remaining experiments were conducted in the absence of MgCl₂ because the low affinity state we observed was easily distinguished from the high affinity site under these conditions.

3.3. Low affinity binding of SCH23390 does not require D₁ or D₅ receptors

Whereas SCH23390 is widely known as a selective D₁-like receptor antagonist, the differences we have demonstrated between SCH23390 binding sites in the amygdala and hippocampus versus the striatum do not implicate the D₁ or D₅ receptor or complexes involving either of these receptors in the low affinity binding site. Indeed, we have shown that this site is not due to an additional mode of binding at D₁ receptors since the presence of D₁ receptors is not required for SCH23390 binding to this low affinity site. The remaining high affinity binding in the hippocampus and amygdala of D₁ knockout mice is probably due to binding to the D₅ receptor and is proportional to the known expression of D₅ receptors in these regions. Furthermore, the low affinity SCH23390 binding site remains in the hippocampus of D₅ knockout mice. The independence of the low affinity state from D₁ and D₅ receptors could be determined conclusively with similar experiments conducted in tissue from D₁/D₅ double knockout animals, though these are not yet available.

3.4. Low affinity binding of SCH23390 appears to involve both D₂ and A_{2A} receptors

The percentage of binding sites in each affinity state was found to be dependent on the brain region and the competing drug. When SCH23390 was used to compete against [³H]-SCH23390 in rat hippocampus, amygdala, or striatum, 59, 74, or 92% of the binding, respectively, was in the high affinity state. Although the percentage of sites in the low affinity state is very low in the striatum, a two-site analysis was warranted because of the presence of this site in both the amygdala and hippocampus. We note the general guideline, however, that at least 20% of the sites should be in each of the affinity states in order to resolve accurately two sites. Nonetheless, the actual estimated amount of receptor in the low affinity state is directly proportional to D₂-like receptor density in these regions (Boyson et al., 1986). In addition, we have shown that the presence of the low affinity state in hippocampus appears to require the presence of available D₂ receptors. In the hippocampus, either a D₂ antagonist or the absence of D₂ receptors reduced the low affinity SCH23390 binding site. In contrast, in the amygdala, the binding of SCH23390 is unaltered by the absence of D₂ receptors, and thus the identity of this site remains to be determined. It is plausible, however, that D₃ receptors compensate for the lack of D₂ receptors in the amygdala of D₂ knockout mice. In fact, a recent report demonstrated that D₃ and A_{2A} receptors interact in a similar manner as do D₂ and A_{2A} receptors (Torvinen et al., 2005).

Despite the apparent dependence of the low affinity site on the presence of D₂ receptors in hippocampus, this is not simply a “free-standing” D₂ receptor. Whereas D₂-like receptors appear to be involved, the reported affinity of SCH23390 for D₂ receptors, as well as those determined in our experiments in hD_{2L} expressing CHO cells, is much lower (~1 M) than the affinity we observed in brain. These data suggest that the affinity of SCH23390 for D₂-like receptors in amygdala and hippocampus may be altered by a D₂-receptor associated protein. Several proteins have been discovered recently that bind to and modify the function of D₂-like receptors and could be responsible for this change (Binda et al., 2002;Griffon et al., 2003;Jeanneteau et al., 2004). Additionally, D₂ and A_{2A} receptors have been shown to interact behaviorally, biochemically, and physically. One consequence of this interaction is manifest in the observation that CGS21680, an A_{2A} receptor agonist, can decrease the affinity of dopamine for D₂ receptors (Diaz-Cabiale et al., 2001;Ferre et al., 1991).

Our observation that ligands of both D₂ and A_{2A} receptors alter the low affinity SCH23390 binding site is in accordance with these previous observations and suggests a mode of interaction whereby the binding of SCH23390 to D₂ receptors is modulated by physical interaction with A_{2A} receptors. The A_{2A}-D₂ interaction could result in a modified D₂ receptor binding site with greatly increased affinity for SCH23390. This hypothesis is supported by the

almost complete absence of the low affinity site in the hippocampus of mice lacking the D₂ receptor and by the striking shift in affinity in A_{2A} knockout mice.

If this site is truly due to a modulation of D₂ receptor binding by interaction with A_{2A} receptors, it represents the largest dimerization-influenced change in pharmacology in native tissue to date and provides a method whereby it may be possible to quantify the amount of heteromerized A_{2A}-D₂ receptors in relation to their total respective expression levels. Though we are unable to provide an accurate estimate of the amount of receptors bound by SCH23390 at the low affinity site, the relative amount of binding in the low affinity site suggests that only a small percentage of D₂ receptors are heteromerized, and therefore modified by A_{2A} receptors, at any given time. In striatum, where both A_{2A} and D₂ receptors are enriched, we found that less than 10% of the total bound receptor was in this state. Despite the fact that this reflects the relative affinities of the two sites, as well as the relative abundance of the two sites, this estimate supports the recent assertion of Chen et al. (2001) that A_{2A} receptors function independently of D₂ receptors although they also modulate D₂ receptor signals.

Our data provide insight into the A_{2A} receptor-mediated allosteric modulation of D₂ receptor binding. Others have investigated the regions of the two receptors (D₂ and A_{2A}) that interact to modulate the pharmacology of the D₂ receptor through chimeric approaches (Canals et al., 2003; Torvinen et al., 2004). Together, these approaches may lay the foundation for modeling of the A_{2A}-modified D₂ receptor pharmacophore.

3.5. Implications

These findings have clear implications for ongoing research of dopamine receptor pharmacology. In the majority of D₁-like receptor studies, SCH23390 inhibition of function is considered evidence that the function is D₁-like receptor-mediated (Bourne, 2001; Caine et al., 1995). As we have shown, however, SCH23390 is binding to at least one site in addition to D_{1/5} receptors. Classic D₁-like agonists (dopamine, SKF-38393) have been demonstrated to activate IP₃ turnover through G_{αq} even in D₁ knockout animals (Friedman et al., 1997; Jin et al., 1998, 2001; Undie and Friedman, 1992). This function has been attributed to an as-of-yet unidentified D₁-like receptor based on data that indicate that the site is blocked by high concentrations of SCH23390. It is clear that the low affinity binding of SCH23390 demonstrated here is independent of D₁ receptors, however, it is intriguing to hypothesize that the low affinity SCH23390 binding may provide an alternate explanation for the previously unidentified receptor linked to activation of PI hydrolysis. Whether or not this is true, our findings present clear caution that pharmacological tools are at best selective, but never specific, over a broad concentration range.

4. Experimental procedures

4.1. Materials

Chlorpromazine and fluphenazine (GlaxoSmithKline, Philadelphia, PA); clozapine and thioridazine (Novartis Inc., East Hanover NJ); and piflutixol (Lundbeck GmbH & Co., Hamburg Germany) were received as gifts from the original manufacturers. CSC and NECA were a gift from Dr. Kenneth A Jacobson (NIDDK, NIH, Bethesda, MD). The following drugs were purchased from RBI/Sigma Inc. (St. Louis, MO): butaclamol; SCH23390; idazoxan; ketanserin; mianserin; and SCH58261. HEPES (*N*-2-hydroxy-ethylpiperazine-*N*-2-ethylsulfonic acid) was obtained from Research Organics Inc. (Cleveland, OH). Magnesium chloride hexahydrate, calcium chloride dihydrate, and magnesium sulfate were purchased from Mallinckrodt (Mallinckrodt Laboratory Chemicals, Phillipsburg, NJ). [³H]-SCH23390 was synthesized by a previously published method (Wyrick and Mailman, 1985). All other compounds and reagents were purchased from standard commercial sources.

4.2. Cell transfection and culture

Human D₁ and D₅ receptors were cloned from a HeLA cDNA library. The constructs were amplified by PCR and ligated into the pcDNA3 vector containing a hemagglutinin tag (YPYDVP-DYA). The receptor DNAs were expressed in HEK-293 cells according to the Lipofectamine protocol (GibcoBRL-Life Technologies) using 0.5 g (hD₁) or 0.75 g (hD₅) DNA per 100 mm culture dish of HEK cells at 80% confluency. Transfected plates of HEK cells were harvested 48 h after transfection. Cells were rinsed in ice-cold PBS and lysed at 4 °C for 10 min using 3 mL of hypo-osmotic buffer (10 mM HEPES, pH 7.4 with KOH). Cells were lifted with a cell scraper, and the resulting membranes were washed three times before being resuspended in storage buffer (20 mM HEPES, 250 mM sucrose, pH 7.4 with KOH). One milliliter aliquots were flash frozen and stored at –80 °C until use in binding assays. Stably transfected CHO hD₂ cells were grown and harvested as previously described (Gay et al., 2004).

4.3. Knockout animals

Breeding stocks of both D₁ (Drago et al., 1994) and D₂ (Jung et al., 1999) knockout mice were obtained and were bred in a C57/BLJ6 background while housed in an IACUC approved facility. All procedures were reviewed and approved by the UNC-CH Institutional Animal Care and Use Committee. Both male and female adults were sacrificed by cervical dislocation, and brains were removed for immediate dissection. Whole brains from D₅ knockout mice (Holmes et al., 2001) were a gift from Dr. David Sibley at NINDS, Bethesda, Maryland. Hippocampus from A_{2A} knockout mice (Chen et al., 1999) and wildtype littermates were the kind gift of Dr. Jiang-Fan Chen and Michael Schwarzschild of the Boston University School of Medicine, Boston, MA.

4.4. Brain dissection

Frozen rat brains (Pel-Freez Biologicals, Rogers, AR) were thawed in saline at 4 °C, and the amygdala, striatum, and hippocampus were removed as described below. No differences were detected when frozen brain tissue was compared to fresh tissue from adult male Sprague–Dawley rats obtained from Charles River Laboratories (Wilmington, MA, USA). Brains were sliced in a 0.8 mm brain block (Heffner et al., 1980). Whole amygdala was microdissected with a 1.5 mm punch (Leonard et al., 2003a,b; Palkovits and Brownstein, 1983). Striatum and hippocampus regions were removed from slices according to the atlas of Paxinos and Watson (1986). Tissue was used immediately or stored at –80 °C until the day of the assay.

4.5. Radioreceptor competition assays

The competition assays were performed in 50 mM HEPES buffer, pH adjusted to 7.4 with KOH. Briefly, tissue was homogenized in buffer using a Teflon-glass homogenizer (eight manual strokes) and centrifuged for 15 min at 27,000×g. The tissue pellet was resuspended in ice-cold buffer at a final concentration of 10 mg/ mL wet weight. Non-specific binding of [³H]-SCH23390 (ca. 0.85 nM) was defined using unlabeled SCH23390 (10 M). Assay tubes contained a total volume of 1 mL; 50 L labeled ligand, 100 L competing ligand or buffer; 100 L tissue homogenate (0.45–0.65 mg protein/mL as measured by the BCA method (Pierce, Rockford, IL)); and 750 L buffer. Assay tubes were incubated for 15 min at 37 °C, and the binding was terminated by filtering with 15 mL of ice-cold buffer on a cell harvester (Skatron, Inc., Sterling, VA). Radioactivity was quantified using an LKB-1219 liquid scintillation counter. In experiments comparing the effects of MgCl₂, MgSO₄, or CaCl₂, 4 mM of the indicated salt was included in the binding buffer.

4.6. Data analysis

Radioreceptor data were analyzed by non-linear regression using Prism Version 3.02 (GraphPad Inc., San Diego CA). Initial analysis used a sigmoidal model with variable slope. Curves were concluded to represent one population of sites when the data were consistent with normal steepness (i.e., $0.85 < n_H < 1.15$), and when there was no evidence by visual inspection of a second affinity site of low density. In the remaining cases, the data were resolved using a two-site model, although it was recognized that even more complex models could not be excluded. In all cases, the analysis was not accepted as valid if the plotted curves of the regressed lines did not incorporate essentially all of the data points. Significant differences between group means were analyzed as appropriate with InStat version 3.05 (GraphPad Inc., San Diego CA), and the particular methods are specified in the individual figure legends. K_D values were calculated from the IC_{50} values in the radioreceptor experiments using the Cheng and Prusoff (1973) equation for a single populations of competitive sites,

$$K_D = IC_{50} / (1 + [R^* / K_D^*])$$
, where R^* is the concentration of radioligand used in each experiment, and K_D is equal to 0.4 nM (the historical value for this laboratory). It should be noted that Prism supposes a single K_D in its calculation of K_L from resolved IC_{50} s. In the present work, it is likely that the K_D of SCH23390 actually is higher than 0.4 nM, and this would result in a slight underestimation of the true K_L .

Acknowledgements

The authors would like to thank Daniel Mooney and John M. Pettito for making the initial observations in this work (Ann. N.Y. Acad Sci., 985: 1–4, 2003), Dr. David Sibley (NINDS, Bethesda, MD) for providing D₅ receptor knockout tissue, and Dr. Michael Schwarzschild (Massachusetts General Hospital, Charlestown, MA) and Dr. Jiang-Fan Chen (Boston University School of Medicine, Boston, MA) for providing A_{2A} receptor knockout tissue. This work was supported by NIH grant MH40537 and Training grant GM07040.

References

- Binda AV, Kabbani N, Lin R, Levenson R. D₂ and D₃ dopamine receptor cell surface localization mediated by interaction with protein 4.1N. *Mol. Pharmacol* 2002;62:507–513.
- Blair JL, Warner DS, Todd MM. Effects of elevated plasma magnesium versus calcium on cerebral ischemic injury in rats. *Stroke* 1989;20:507–512. [PubMed: 2648653]
- Bourne JA. SCH 23390: the first selective dopamine D₁-like receptor antagonist. *CNS Drug Rev* 2001;7:399–414. [PubMed: 11830757]
- Boyson SJ, McGonigle P, Molinoff PB. Quantitative autoradiographic localization of the D₁ and D₂ subtypes of dopamine receptors in rat brain. *J Neurosci* 1986;6:3177–3188. [PubMed: 3534157]
- Caine SB, Heinrichs SC, Coffin VL, Koob GF. Effects of the dopamine D-1 antagonist SCH 23390 microinjected into the accumbens, amygdala or striatum on cocaine self-administration in the rat. *Brain Res* 1995;692:47–56. [PubMed: 8548319]
- Callahan PM, Bryan SK, Cunningham KA. Discriminative stimulus effects of cocaine: antagonism by dopamine D₁ receptor blockade in the amygdala. *Pharmacol Biochem Behav* 1995;51:759–766. [PubMed: 7675856]
- Canals M, Marcellino D, Fanelli F, Ciruela F, de Benedetti P, Goldberg SR, Neve K, Fuxe K, Agnati LF, Woods AS, Ferre S, Lluís C, Bouvier M, Franco R. Adenosine A_{2A}-dopamine D₂ receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J Biol Chem* 2003;278:46741–46749. [PubMed: 12933819]
- Chen JF, Huang Z, Ma J, Zhu J, Moratalla R, Standaert D, Moskowitz MA, Fink JS, Schwarzschild MA. A(2A) adenosine receptor deficiency attenuates brain injury induced by transient focal ischemia in mice. *J Neurosci* 1999;19:9192–9200. [PubMed: 10531422]
- Chen JF, Moratalla R, Impagnatiello F, Grandy DK, Cuellar B, Rubinstein M, Beilstein MA, Hackett E, Fink JS, Low MJ, Ongini E, Schwarzschild MA. The role of the D₂ dopamine receptor (D₂R) in A_{2A} adenosine receptor (A_{2A}R)-mediated behavioral and cellular responses as revealed by A_{2A} and D₂ receptor knockout mice. *Proc Natl Acad Sci U S A* 2001;98:1970–1975. [PubMed: 11172060]

- Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 1973;22:3099–3108. [PubMed: 4202581]
- Ciliax BJ, Nash N, Heilman C, Sunahara R, Hartney A, Tiberi M, Rye DB, Caron MG, Niznik HB, Levey AI. Dopamine D₅ receptor immunolocalization in rat and monkey brain. *Synapse* 2000;37:125–145. [PubMed: 10881034]
- Dawson TM, Barone P, Sidhu A, Wamsley JK, Chase TN. The D₁ dopamine receptor in the rat brain: quantitative autoradiographic localization using an iodinated ligand. *Neuroscience* 1988;26:83–100. [PubMed: 2971144]
- Diaz-Cabiale Z, Hurd Y, Guidolin D, Finnman UB, Zoli M, Agnati LF, Vanderhaeghen JJ, Fuxe K, Ferre S. Adenosine A_{2A} agonist CGS 21680 decreases the affinity of dopamine D₂ receptors for dopamine in human striatum. *NeuroReport* 2001;12:1831–1834. [PubMed: 11435907]
- Drago J, Gerfen CR, Lachowicz JE, Steiner H, Hollon TR, Love PE, Ooi GT, Grinberg A, Lee EJ, Huang SP. Altered striatal function in a mutant mouse lacking D_{1A} dopamine receptors. *Proc Natl Acad Sci U S A* 1994;91:12564–12568. [PubMed: 7809078]
- Ferre S, von Euler G, Johansson B, Fredholm BB, Fuxe K. Stimulation of high-affinity adenosine A₂ receptors decreases the affinity of dopamine D₂ receptors in rat striatal membranes. *Proc Natl Acad Sci U S A* 1991;88:7238–7241. [PubMed: 1678519]
- Franco R, Ferre S, Agnati L, Torvinen M, Gines S, Hillion J, Casado V, Lledo PM, Zoli M, Lluís C, Fuxe K. Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. *Neuropsychopharmacology* 2000;23:S50–S59. [PubMed: 11008067]
- Friedman E, Jin LQ, Cai GP, Hollon TR, Drago J, Sibley DR, Wang HY. D₁-like dopaminergic activation of phosphoinositide hydrolysis is independent of D_{1A} dopamine receptors: evidence from D_{1A} knockout mice. *Mol Pharmacol* 1997;51:6–11. [PubMed: 9016340]
- Garau L, Govoni S, Stefanini E, Trabucchi M, Spano PF. Dopamine receptors: pharmacological and anatomical evidences indicate that two distinct dopamine receptor populations are present in rat striatum. *Life Sci* 1978;23:1745–1750. [PubMed: 723447]
- Gay EA, Urban JD, Nichols DE, Oxford GS, Mailman RB. Functional selectivity of D₂ receptor ligands in a Chinese hamster ovary hD2L cell line: evidence for induction of ligand-specific receptor states. *Mol Pharmacol* 2004;66:97–105. [PubMed: 15213300]
- Greba Q, Kokkinidis L. Peripheral and intraamygdalar administration of the dopamine D₁ receptor antagonist SCH 23390 blocks fear-potentiated startle but not shock reactivity or the shock sensitization of acoustic startle. *Behav Neurosci* 2000;114:262–272. [PubMed: 10832788]
- Griffon N, Jeanneteau F, Prieur F, Diaz J, Sokoloff P. CLIC6, a member of the intracellular chloride channel family, interacts with dopamine D(2)-like receptors. *Brain Res Mol Brain Res* 2003;117:47–57. [PubMed: 14499480]
- Heffner TG, Hartman JA, Seiden LS. A rapid method for the regional dissection of the rat brain. *Pharmacol Biochem Behav* 1980;13:453–456. [PubMed: 7422701]
- Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluís C, Franco R, Ferre S, Fuxe K. Coaggregation, cointernalization, and codesensitization of adenosine A_{2A} receptors and dopamine D₂ receptors. *J Biol Chem* 2002;277:18091–18097. [PubMed: 11872740]
- Holmes A, Hollon TR, Gleason TC, Liu Z, Dreiling J, Sibley DR, Crawley JN. Behavioral characterization of dopamine D₅ receptor null mutant mice. *Behav Neurosci* 2001;115:1129–1144. [PubMed: 11584926]
- Huang Q, Zhou D, Chase K, Gusella JF, Aronin N, DiFiglia M. Immunohistochemical localization of the D₁ dopamine receptor in rat brain reveals its axonal transport, pre- and postsynaptic localization, and prevalence in the basal ganglia, limbic system, and thalamic reticular nucleus. *Proc Natl Acad Sci U S A* 1992;89:11988–11992. [PubMed: 1281547]
- Hurd YL, Suzuki M, Sedvall GC. D₁ and D₂ dopamine receptor mRNA expression in whole hemisphere sections of the human brain. *J Chem Neuroanat* 2001;22:127–137. [PubMed: 11470560]
- Jeanneteau F, Diaz J, Sokoloff P, Griffon N. Interactions of GIPC with dopamine D₂, D₃ but not D₄ receptors define a novel mode of regulation of G protein-coupled receptors. *Mol Biol Cell* 2004;15:696–705. [PubMed: 14617818]

- Jeong SM, Hahm KD, Shin JW, Leem JG, Lee C, Han SM. Changes in magnesium concentration in the serum and cerebrospinal fluid of neuropathic rats. *Acta Anaesthesiol Scand* 2006;50:211–216. [PubMed: 16430544]
- Jin LQ, Cai G, Wang HY, Smith C, Friedman E. Characterization of the phosphoinositide-linked dopamine receptor in a mouse hippocampal–neuroblastoma hybrid cell line. *J Neurochem* 1998;71:1935–1943. [PubMed: 9798918]
- Jin LQ, Wang HY, Friedman E. Stimulated D₁ dopamine receptors couple to multiple Galpha proteins in different brain regions. *J Neurochem* 2001;78:981–990. [PubMed: 11553672]
- Jung MY, Skryabin BV, Arai M, Abbondanzo S, Fu D, Brosius J, Robakis NK, Polites HG, Pintar JE, Schmauss C. Potentiation of the D₂ mutant motor phenotype in mice lacking dopamine D₂ and D₃ receptors. *Neuroscience* 1999;91:911–924. [PubMed: 10391470]
- Kebabian JW, Calne DB. Multiple receptors for dopamine. *Nature* 1979;277:93–96. [PubMed: 215920]
- Khan ZU, Gutierrez A, Martin R, Penafiel A, Rivera A, De La CA. Dopamine D₅ receptors of rat and human brain. *Neuroscience* 2000;100:689–699. [PubMed: 11036203]
- Kilts CD, Anderson CM, Ely TD, Mailman RB. The biochemistry and pharmacology of mesoamygdaloid dopamine neurons. *Ann N Y Acad Sci* 1988;537:173–187. [PubMed: 3059923]
- Latini S, Pazzagli M, Pepeu G, Pedata F. A2 adenosine receptors: their presence and neuromodulatory role in the central nervous system. *Gen Pharmacol* 1996;27:925–933. [PubMed: 8909972]
- Lee FJ, Xue S, Pei L, Vukusic B, Chery N, Wang Y, Wang YT, Niznik HB, Yu XM, Liu F. Dual regulation of NMDA receptor functions by direct protein–protein interactions with the dopamine D₁ receptor. *Cell* 2002;111:219–230. [PubMed: 12408866]
- Leonard SK, Anderson CM, Lachowicz JE, Schulz DW, Kilts CD, Mailman RB. Amygdaloid D₁ receptors are not linked to stimulation of adenylate cyclase. *Synapse* 2003a;50:320–333. [PubMed: 14556237]
- Leonard SK, Petitto JM, Anderson CM, Mooney DH, Lachowicz JE, Schulz DW, Kilts CD, Mailman RB. D₁ dopamine receptors in the amygdala exhibit unique properties. *Ann N Y Acad Sci* 2003b; 985:536–539.
- Mailman RB, Schulz DW, Kilts CD, Lewis MH, Rollema H, Wyrick S. Multiple forms of the D₁ dopamine receptor: its linkage to adenylate cyclase and psychopharmacological effects. *Psychopharmacol Bull* 1986;22:593–598. [PubMed: 2879302]
- Mansour A, Meador-Woodruff JH, Zhou Q, Civelli O, Akil H, Watson SJ. A comparison of D₁ receptor binding and mRNA in rat brain using receptor autoradiographic and in situ hybridization techniques. *Neuroscience* 1992;46:959–971. [PubMed: 1531866]
- Matthies H, Becker A, Schroeder H, Kraus J, Holtt V, Krug M. Dopamine D₁-deficient mutant mice do not express the late phase of hippocampal long-term potentiation. *NeuroReport* 1997;8:3533–3535. [PubMed: 9427321]
- Montague DM, Striplin CD, Overcash JS, Drago F, Lawler CP, Mailman RB. Quantification of D_{1B} (D₅) receptors in dopamine D_{1A} receptor-deficient mice. *Synapse* 2001;39:319–322. [PubMed: 11169782]
- Nicklaus KJ, McGonigle P, Molinoff PB, Otmakhova NA, Lisman JE. D₁/D₅ dopamine receptor activation increases the magnitude of early long-term potentiation at CA1 hippocampal synapses. *J Neurosci* 1996;16:7478–7486. [PubMed: 8922403]
- Otmakhova NA, Lisman JE. D₁/D₅ dopamine receptor activation increases the magnitude of early long-term potentiation at CA1 hippocampal synapses. *J Neurosci* 1996;16:7478–7486. [PubMed: 8922403]
- Palkovits, M.; Brownstein, M. Microdissection of brain areas by the punch technique. In: Cuello, AC., editor. *Brain Microdissection Techniques*. Wiley; New York: 1983. p. 1-36.
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. Academic Press; Sydney: 1986.
- Rocheville M, Lange DC, Kumar U, Patel SC, Patel RC, Patel YC. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. *Science* 2000;288:154–157. [PubMed: 10753124]
- Savasta M, Dubois A, Scatton B. Autoradiographic localization of D₁ dopamine receptors in the rat brain with [³H] SCH 23390. *Brain Res* 1986;375:291–301. [PubMed: 2942221]

- Schetz JA, Sibley DR. Zinc allosterically modulates antagonist binding to cloned D₁ and D₂ dopamine receptors. *J Neurochem* 1997;68:1990–1997. [PubMed: 9109525]
- Schulz DW, Stanford EJ, Wyrick SW, Mailman RB. Binding of [³H]SCH23390 in rat brain: regional distribution and effects of assay conditions and GTP suggest interactions at a D₁-like dopamine receptor. *J Neurochem* 1985;45:1601–1611. [PubMed: 2864398]
- Sunahara RK, Niznik HB, Weiner DM, Stormann TM, Brann MR, Kennedy JL, Gelernter JE, Rozmahel R, Yang YL, Israel Y. Human dopamine D₁ receptor encoded by an intronless gene on chromosome 5. *Nature* 1990;347:80–83. [PubMed: 1975640]
- Torvinen M, Kozell LB, Neve KA, Agnati LF, Fuxe K. Biochemical identification of the dopamine D₂ receptor domains interacting with the adenosine A_{2A} receptor. *J Mol Neurosci* 2004;24:173–180. [PubMed: 15456930]
- Torvinen M, Marcellino D, Canals M, Agnati LF, Lluís C, Franco R, Fuxe K. Adenosine A_{2A} receptor and dopamine D₃ receptor interactions: evidence of functional A_{2A}/D₃ heteromeric complexes. *Mol Pharmacol* 2005;67:400–407. [PubMed: 15539641]
- Undie AS, Friedman E. Selective dopaminergic mechanism of dopamine and SKF38393 stimulation of inositol phosphate formation in rat brain. *Eur J Pharmacol* 1992;226:297–302. [PubMed: 1327844]
- Wang Y, Chan GL, Holden JE, Dobko T, Mak E, Schulzer M, Huser JM, Snow BJ, Ruth TJ, Calne DB, Stoessl AJ. Age-dependent decline of dopamine D₁ receptors in human brain: a PET study. *Synapse* 1998;30:56–61. [PubMed: 9704881]
- Wyrick SD, Mailman RB. Tritium-labeled (+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4, 5-tetrahydro-1H-3-benzazepine (SCH23390). *J Label Compd Radiopharm* 1985;22:189–195.

Abbreviations

AC	adenylate cyclase
cAMP	cyclic AMP, adenosine 3' 5'-cyclic monophosphate
CGS21680	2-[4-(2-carboxyethyl)-phenylethylamino]-5' <i>N</i> -ethyl-carboxamido-adenosine
CHO	Chinese Hamster Ovary cell-line
CSC	8-(3-chlorostyryl)caffeine
GPCR	G protein-coupled receptor
HEK	Human Embryonic Kidney cell-line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC₅₀	concentration inhibiting 50% of total binding
K_{0.5}	concentration corrected
IC₅₀	(apparent affinity constant) when $n_H \neq 1.0$

K_H	affinity constant for high affinity state
K_I	affinity constant ($n_H=1.0$)
K_L	affinity constant for low affinity state
NECA	5'- <i>N</i> -ethylcarboxamide-adenosine
n_H	Hill coefficient
R_H	relative amount (percentage) of receptor in the high affinity state
SCH23390	7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1 <i>H</i> -3-benzazepine
SCH58261	7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3e]-1,2,4-triazolo[1,5c]pyrimidine

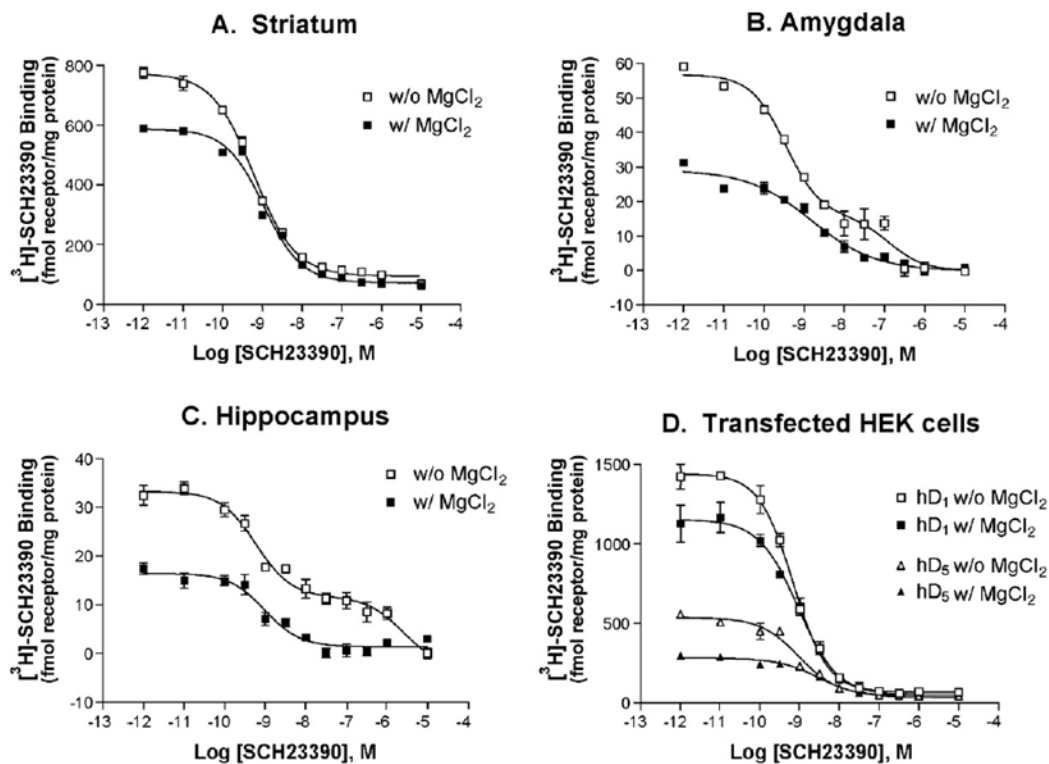


Fig 1. $MgCl_2$ has dramatically different effects on affinity of SCH23390 binding in rat striatum and hD_1 or hD_5 transfected HEK cells versus rat amygdala or hippocampus. Panels A–D show representative SCH23390 vs. [3H]-SCH23390 competition binding experiments in the indicated tissues in the absence (open symbols) or presence (filled symbols) of 4 mM $MgCl_2$. These data represent two (amygdala), three (hippocampus) or four (striatum) separate experiments with triplicate data. See Table 1 for quantification.

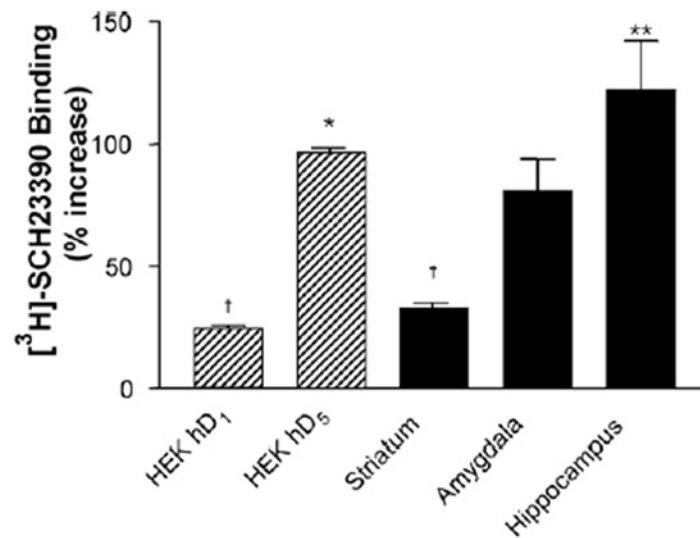


Fig 2.

D₁ and D₅ receptors appear to be affected differently by the absence of MgCl₂. Data reflect the average increase of maximal [³H]-SCH23390 binding in the absence compared to in the presence of 4 mM MgCl₂ of *n* = 2 (amygdala, HEK hD₁, HEK hD₅), *n* = 3 (hippocampus), or *n* = 4 (striatum) replicates. * Indicates significant difference vs. HEK hD₁, *p* < 0.05, ***p* < 0.01; † indicates significant difference vs. HEK hD₅, *p* < 0.05, as determined by one-way ANOVA and Dunnett post hoc analysis.

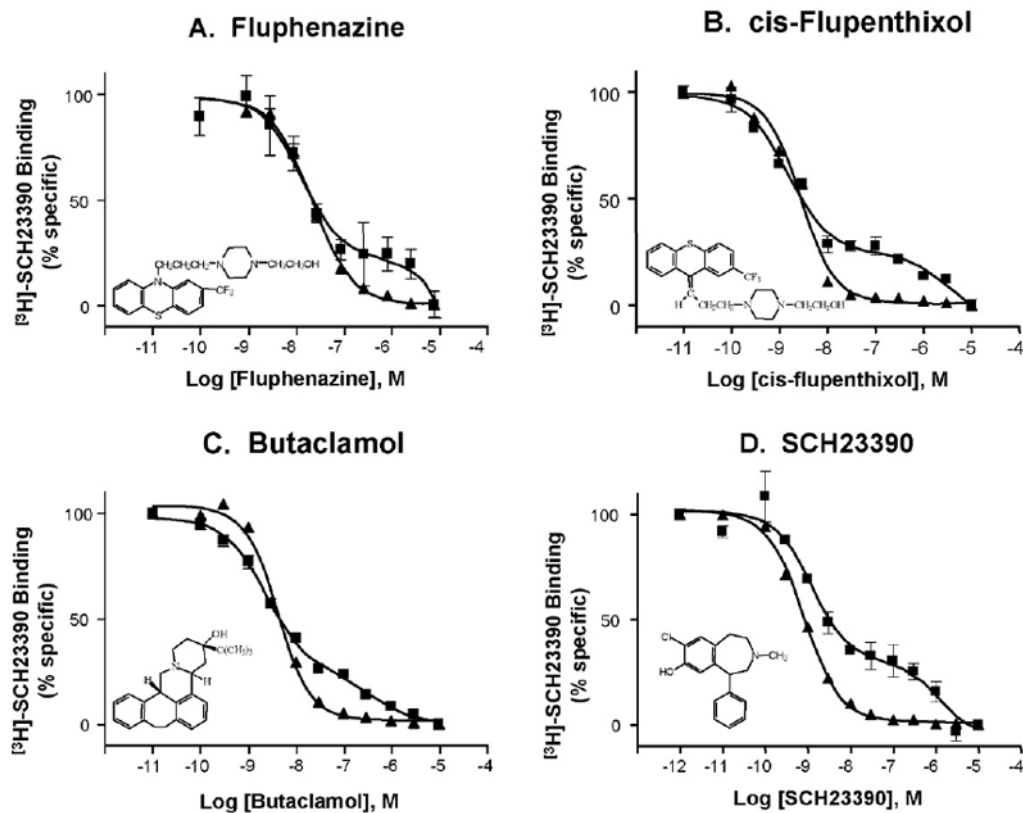
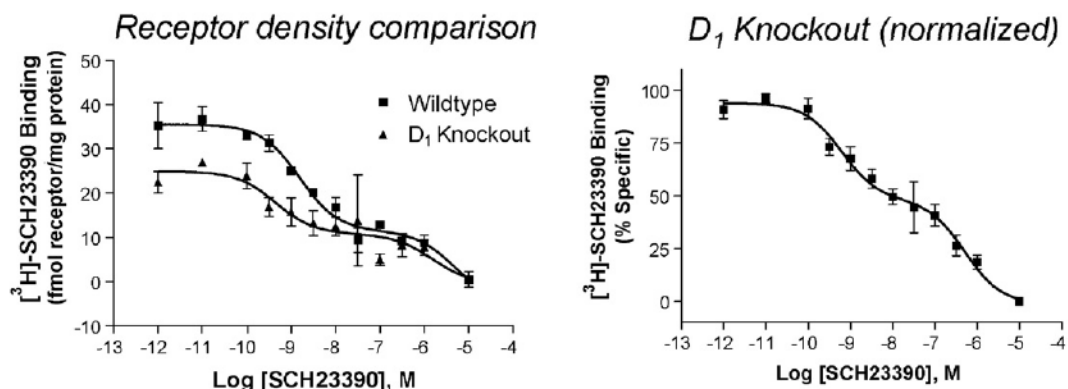


Fig 3. Competition binding of structurally diverse D₁ antagonists versus [³H]-SCH23390 in the absence of MgCl₂ reveals a low affinity binding site in amygdala (squares) but not in the striatum (triangles). Representative data are shown here, and Table 2 contains complete binding statistics. In each panel, the solid triangles (steeper curve) always represent striatum, and solid squares (shallow, biphasic curve) always represent amygdala.

Hippocampus



Amygdala

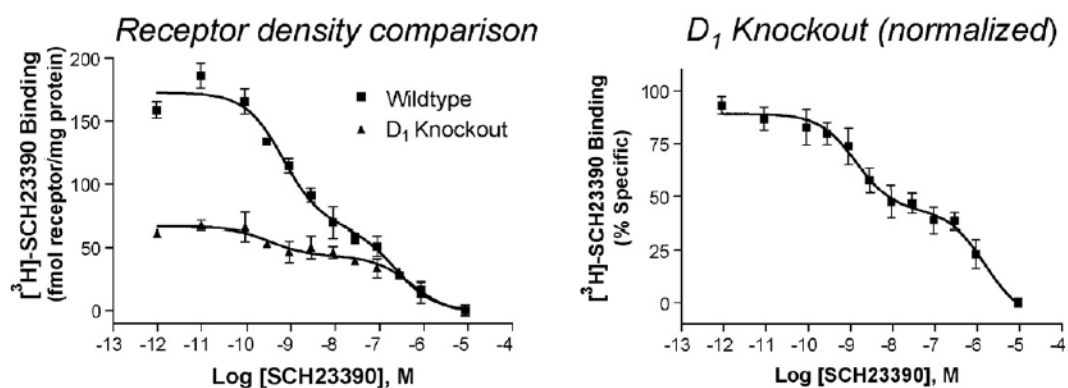


Fig 4.

Two affinity sites are still apparent in competition binding of SCH23390 vs. [^3H]-SCH23390 in hippocampus and amygdala of D_1 knockout mice. *Left*: representative data presented in fmol receptor per mg total protein for comparison of receptor density. *Right*: normalized binding from three separate experiments run in triplicate. See Table 3 for binding affinities.

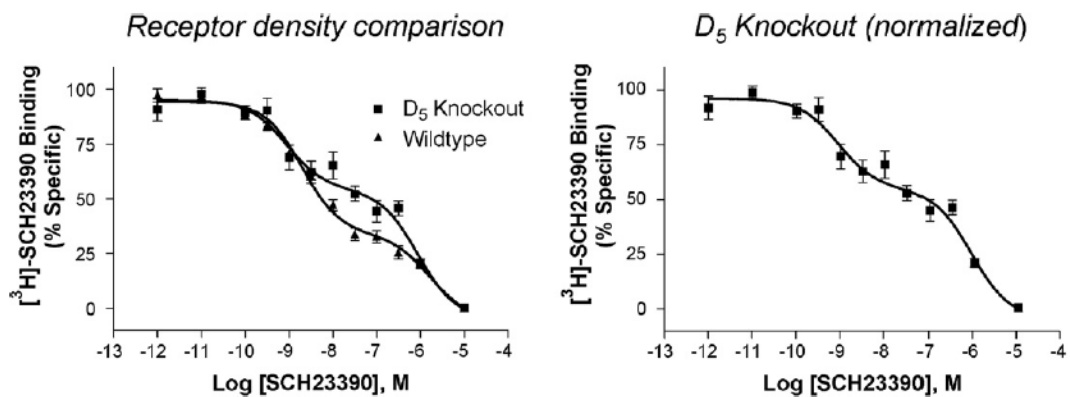


Fig 5.

Competition binding of SCH23390 vs. [³H]-SCH23390 in hippocampus of D₅ knockout mice reveals a statistically significant increase in the proportion of receptor in the low affinity state. Significance determined by two-tailed *t* test, *p*=0.006. *Left*: comparison of binding in WT (*n*=6) vs. D₅ knockout (*n*=3) mice. Total specific binding was not significantly different between WT and D₅ knockout. *Right*: normalized binding from duplicate samples in three separate experiments in D₅ knockout mice. Curves represent approximately 50–60 fmol receptor/mg protein. See Table 3 for binding affinities.

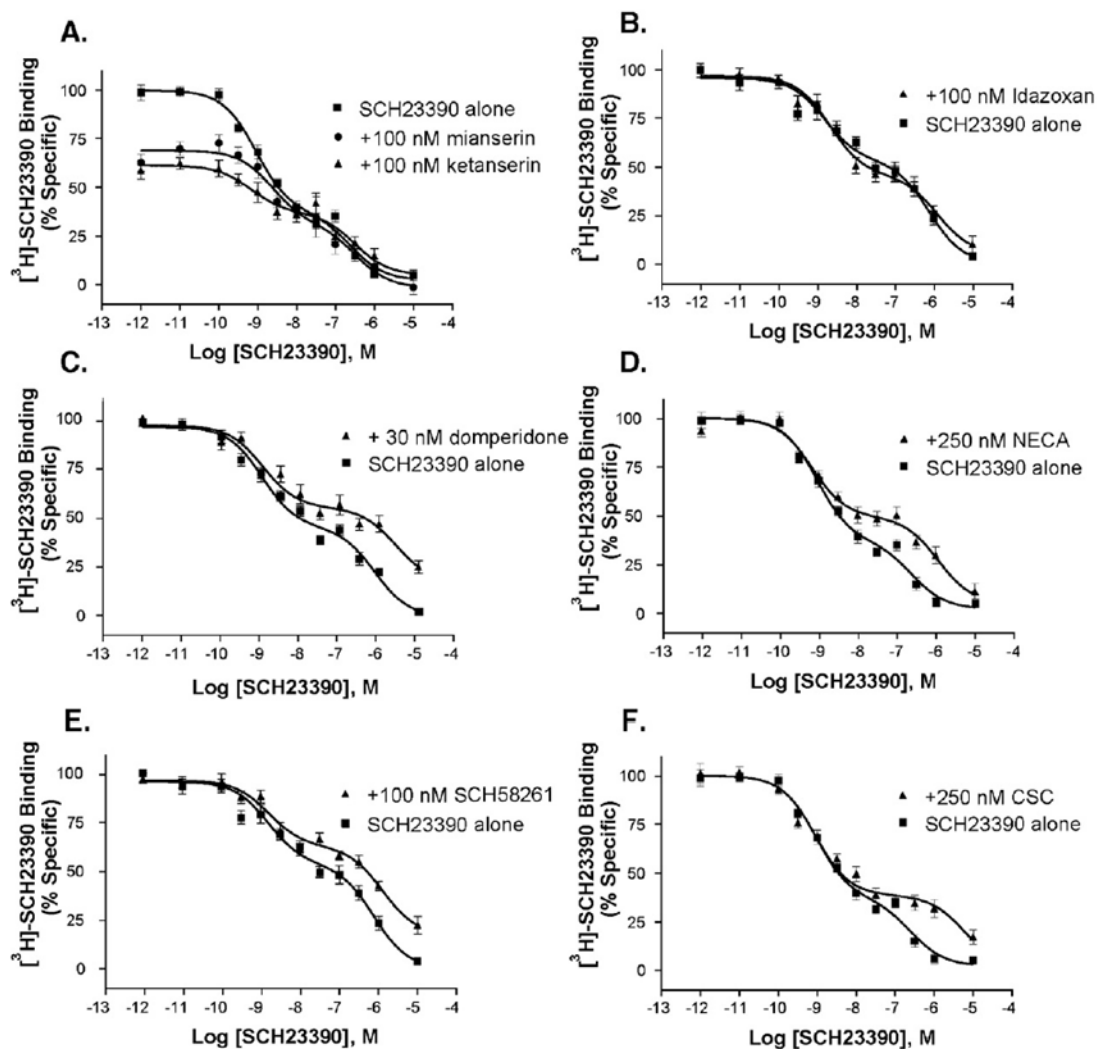


Fig 6.

In rat hippocampus, the low affinity binding of SCH23390 is not disrupted by the presence of ketanserin or mianserin (A), and neither site is affected by the α_2 adrenergic receptor antagonist idazoxan (B). In contrast the low, but not the high, affinity binding of SCH23390 is modulated by the presence of the D_2 antagonist domperidone (C), the A_{2A} receptor agonist NECA (D), and the A_{2A} antagonists SCH58261 and CSC (E and F). The indicated concentration of drug was incubated in each tube simultaneously with the SCH23390 competition binding reaction, and each was run in triplicate, three times.

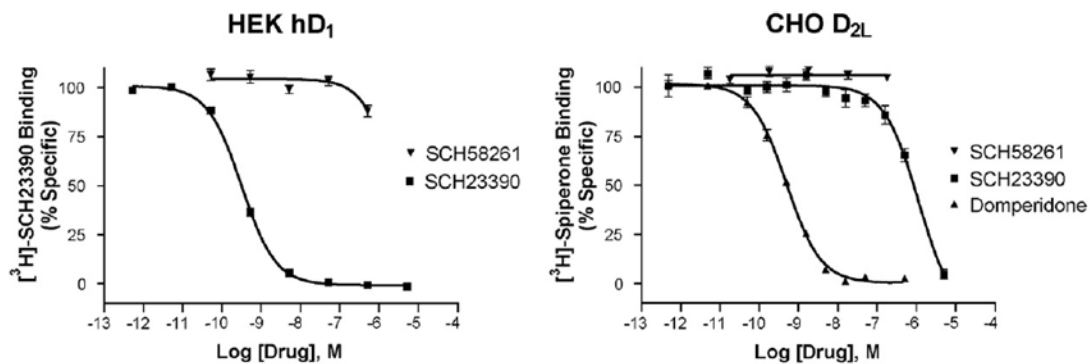


Fig 7. SCH58261 has negligible affinity for D₁ and D₂ receptors as demonstrated by competition binding with SCH23390 and SCH58261 vs. [³H]-SCH23390 in HEK hD₁ membranes (left) or domperidone, SCH23390, and SCH58261 vs. [³H]-spiperone in CHO-D_{2L} membranes (right); *n* = 3. Affinities in nM: D₁: SCH23390 0.29±0.01, SCH58261 3050±700; D₂: domperidone 0.51±0.03, SCH23390 920±170, SCH58261 >10,000.

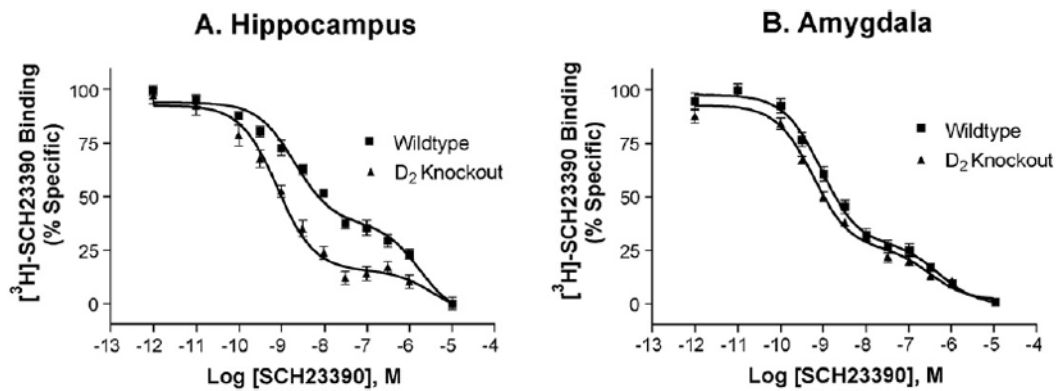


Fig 8.

The low affinity binding of SCH23390 is almost completely lost in D₂ knockout mice but is unaffected by the lack of D₂ receptors in amygdala of D₂ knockout mice. (A) Normalized binding from three separate experiments run in triplicate with hippocampus from D₂ knockout mice. (B) Normalized binding from three separate experiments run in duplicate with amygdala from D₂ knockout mice.

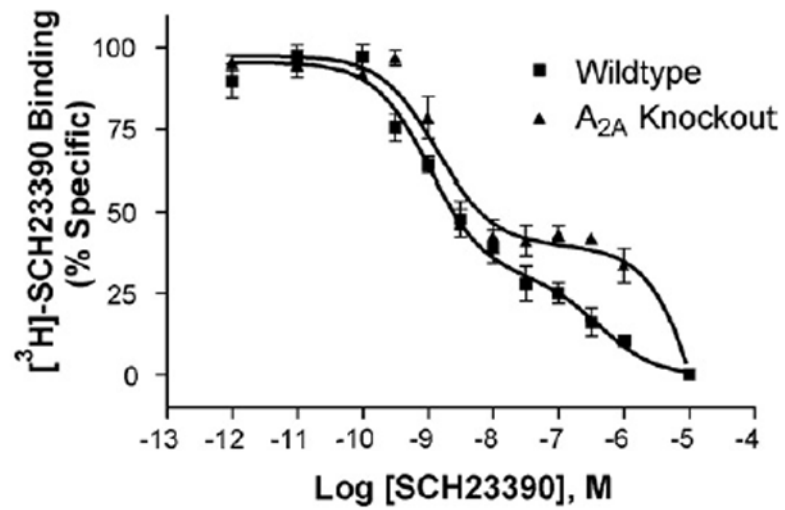


Fig 9.

In the hippocampus of A_{2A} receptor knockout mice, the high affinity SCH23390 binding site is unchanged whereas the low affinity site is shifted rightward dramatically. Wildtype K_H 0.34 ± 0.10 , K_L 162 ± 42 , R_H 67 ± 8 ; A_{2A} knockout K_H 0.42 ± 0.01 , K_L $1322 \pm 255^*$, R_H 50 ± 4 ($n = 3$). * Indicates $p < 0.005$ as compared to WT in two-tailed unpaired t test.

Table 1

Competition binding of SCH23390 against [³H]-SCH23390 in the absence of MgCl₂ demonstrates the presence of a low affinity binding site present in different amounts in different brain regions

		K_H	R_H	K_L	n
Striatum	<i>with MgCl₂</i>	0.28±0.09	90±2	45±26	4
	<i>w/o MgCl₂</i>	0.22±0.03	92±3	135±56	4
Amygdala	<i>with MgCl₂</i>	0.27±0.08	68±15	35±6	2
	<i>w/o MgCl₂</i>	0.42±0.04	75±3	132±41	9
Hippocampus	<i>with MgCl₂</i>	1.02±0.48	91±6	16±13	5
	<i>w/o MgCl₂</i>	0.37±0.08	59±2	343±93	12

Data are presented as the mean± SEM. n is the number of experiments.

Abbreviations: K_H —affinity constant for high affinity binding, R_H —percentage of binding in the high affinity state (relative to the total binding), K_L —affinity constant for low affinity binding.

Table 2

Competition binding with several D₁ receptor antagonists against [³H]-SCH23390 in the absence of MgCl₂ reveals a low affinity binding site in amygdala but not in the striatum

	Amygdala			Striatum
	K_H	R_H	K_L	K_D
Butaclamol	1.6±0.5 (4)	81±3	329±93	3.3±0.9 (5)
Clozapine	8.3±5.0 (3)	44±11	538±267	114±38 (2)
Chlorpromazine	8.6±4.2 (3)	54±23	427±349	19.7±1.9 (3)
Fluphenazine	11.6±4.1 (7)	71±8	1600±440	14.8±2.2 (7)
<i>cis</i> -Flupenthixol	1.1±0.4 (4)	71±2	874±251	1.7±0.6 (4)
Thioridazine	27.5±3.3 (6)	72±6	947±374	43.3±7.2 (6)

Affinity values for each ligand were determined with one- (striatum) or two-site (amygdala) binding analysis in Prism v. 3.0. Data are presented as the mean±SEM of *n* experiments. R_H values were not significantly different between competing ligands ($p=0.176$).

Table 3

Competition binding of SCH23390 vs. [³H]-SCH23390 in hippocampus and amygdala of mice not expressing D₁, D₂, or D₅ receptors

	Hippocampus			Amygdala		
	<i>K_H</i>	<i>R_H</i>	<i>K_L</i>	<i>K_H</i>	<i>R_H</i>	<i>K_L</i>
WT	0.59±0.08 (6)	64±4	451±90	0.30±0.03 (3)	71±5	200±70
D ₁ KO	0.39±0.13 (4)	47±6	269±90	0.45±0.30 (3)	48±9	411±120
D ₂ KO	0.32±0.04 (3)	75±14	>10,000*	0.18±0.04 (3)	71±3	115±50
D ₅ KO	0.32±0.07 (3)	41±2	302±75	N.D.	N.D.	N.D.

Data are presented as the mean±SEM of *n* experiments.

* Indicates significant difference as compared to WT in one-way ANOVA with Dunnett post hoc analysis, no other values were found to be significantly different from WT.