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D1 and D2 Dopamine Binding Site Up-Regulation and Apomorphine-Induced Stereotypy

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CHIPKIN, R. E., R. D. McQUADE AND L. C. IORIO. *D1 and D2 dopamine binding site up-regulation and apomorphine-induced stereotypy*. PHARMACOL BIOCHEM BEHAV **28**(4) 477–482, 1987.—Treatments with drugs to up-regulate specific receptors is a strategy often employed in mechanism of action studies. In this type of experiment, changes in the numbers of receptors and concomitant changes in an animal's sensitivity to the drug have been used as evidence for the participation of the binding site in the behavior. In these studies, to test for the role of D1 and D2 receptors in apomorphine-induced stereotypy (AIS), dopamine binding sites were up-regulated by appropriate pre-treatments and the ability of these pre-treatments to alter AIS was subsequently investigated. In the first experiment, 19 days of pre-treatment with SCH 23390 or haloperidol selectively increased by 35 and 40% the numbers of striatal D1 and D2 binding sites, respectively, without affecting their affinities. However, when challenged with apomorphine, only the animals pre-treated with the D2 antagonist showed behavioral supersensitivity. In the second experiment, reserpine pre-treatment (30 mg/kg IP, 24-hr pre-test) increased the numbers of D1 binding sites by 18%, but did not significantly alter the numbers of striatal D2 binding sites. Behaviorally, these rats were supersensitive to apormorphine's stereotypy-inducing effects; however, they also showed an increased sensitivity to the ability of either haloperidol or SCH 23390 to block AIS. Moreover, this blockade was only attenuated by a D2 (but not a D1) agonist. Collectively, these data suggest that AIS is mediated by both D1 and D2 binding sites, but that D2 binding sites have a more important role.

Haloperidol SCH 23390 D1 binding sites D2 binding sites Reserpine apomorphine-induced stereotypy

ONE strategy often used to determine the mechanism of action of a compound is to alter the numbers of its receptors and determine subsequent behavioral changes. This is premised on the assumption that the changes in the numbers of binding sites and the animal's sensitivity to the drug are linked. Using this concept, others have previously shown that dopamine receptors are involved in the mechanism of action of apomorphine-induced stereotypy (AIS). For example, Tarsy and Baldessarini [23] have demonstrated that repeated treatments with either the dopamine antagonist chlorpromazine or the amine depletor reserpine (but not the sedative/hypnotic phenobarbital) induced supersensitivity to apomorphine's stereotypy-inducing effects. Moreover, since chronic treatment with neuroleptics also increased the number of dopamine binding sites (e.g., [8]), it has been theorized that apomorphine is acting through dopaminergic receptors.

The classification of dopamine binding sites into D1 and D2 subtypes has been facilitated by the discovery of selective agonists and antagonists. Recently, Creese and Chen [6] and Porceddu *et al.* [19] have demonstrated that repeated treatments with the selective D1 antagonist SCH 23390 increased the number of D1 binding sites without altering the number of D2 binding sites. Likewise, repeated administration of the D2 selective antagonist haloperidol increased the number of D2 binding sites without affecting the numbers of D1 binding sites. In either case binding site affinity was not altered.

The purpose of these studies was to investigate the roles of D1 and D2 receptors in AIS. The strategy was to selectively increase the numbers of D1 or D2 binding sites by appropriate treatments and then to determine the effects of these changes on AIS. In the first set of studies, 19 days of repeated treatment with either SCH 23390 or haloperidol was used to selectively up-regulate D1 and D2 binding sites, respectively. In the second set of studies reserpine pretreatment (30 mg/kg IP 24 hr pre-test) was used to produce dopaminergic supersensitivity because this model is less time consuming than repeatedly treating animals and because it has previously been reported [23] that reserpinized rats are supersensitive to dopamine agonists (such as apomorphine).

METHODS

Animals

Male, Sprague-Dawley rats (Charles River Breeding Laboratories, MA), weighing between 140–170 grams at the start of the experiment were used throughout.

Drugs

SCH 23390 maleate was synthesized at Schering Re-

TABLE 1

EFFECT OF REPEATED TREATMENT WITH SCH 23390, HALOPERIDOL OR VEHICLE ON D1/D2 BINDING IN RAT STRIATUM

Treatment	Dose	Duration	D1 (mean ± SE) K_d (nM)	D1 (mean ± SE) B_{max} (pmoles/mg protein)	D2 (mean ± SE) K_d (nM)	D2 (mean ± SE) B_{max} (pmoles/mg protein)
Vehicle	—	19 days	0.39 ± 0.19 (5)*	0.85 ± 0.21 (5)	0.14 ± 0.03 (3)	0.51 ± 0.07 (3)
SCH 23390	25 PO	19 days	0.47 ± 0.26 (5)	1.17 ± 0.31† (5)	0.13 ± 0.01 (3)	0.52 ± 0.04 (3)
Haloperidol	3 PO	19 days	0.47 ± 0.14 (4)	0.98 ± 0.23 (4)	0.12 ± 0.01 (3)	0.73 ± 0.04† (3)

*N=Number in parentheses.
†Significantly different from vehicle-treated animals, $p<0.05$, two-tailed paired t-test.

TABLE 2

EFFECT OF RESERPINIZATION ON D1/D2 BINDING IN RAT STRIATUM

Treatment	Dose	D1 (mean ± SE) K_d (nM)	D1 (mean ± SE) B_{max} (pmoles/mg protein)	D2 (mean ± SE) K_d (nM)	D2 (mean ± SE) B_{max} (pmoles/mg protein)
Vehicle	—	0.60 ± 0.02 (4)*	1.55 ± 0.03 (4)	0.07 ± 0.01 (3)	0.53 ± 0.01 (3)
Reserpine	30 IP	0.75 ± 0.07 (4)	1.83 ± 0.09† (4)	0.08 ± 0.01 (3)	0.60 ± 0.03 (3)

*N=Number in parentheses.
†Significantly different from vehicle-treated animals, $p<0.05$, two-tailed paired t-test.

search Labs. Haloperidol was a gift of McNeil Pharmaceuticals. Reserpine and apomorphine hydrochloride were purchased from Sigma Chemical Co. (St. Louis). All doses refer to the base.

Drug Regimens

Repeated dosing studies. SCH 23390 (25 mg/kg PO), haloperidol (3 mg/kg PO) or vehicle (0.4% aqueous methylcellulose, 10 ml/kg PO) were given daily for 19 days. The doses were chosen as identical multiples (5×) of their minimal effective doses obtained at peak times in the rat conditioned avoidance test [11]. In the rat these doses have activity for at least 6 hours. Drug testing (see below) took place on day 20 after a 24 hr wash-out period.

Reserpine study. Between 2 and 4 p.m. on the day before a study, rats were treated with either 0.4% aqueous methylcellulose or reserpine (30 mg/kg IP). Previous studies in this lab have demonstrated that rats administered this dose exhibited typical behavioral signs associated with reserpinization (e.g., ptosis, diarrhea, urination). Testing (see below) took place the next day between 1 and 4 p.m.

Apomorphine stereotypy. A modification of the method of Costall and Naylor [5] was used. After the treatments with the drugs or vehicle, the animals were divided into groups of 4–6 rats and injected (SC) with apomorphine at logarithmically spaced doses. In the chronic dosing study, stereotypy was scored at 10, 20, 30, 60 and 75 min post-apomorphine because possible changes in the duration of apomorphine's action needed to be tested. Since in reserpinized rats pilot studies showed no change in apomorphine's duration, the stereotypy ratings were done at 5, 10, 15, 20, 25 and 30 min post-apomorphine. The following scoring system was used: 0=no stereotypy; 1=discontinuous sniffing; 2=continuous sniffing; 3=continuous sniffing and discontinuous biting, gnawing or licking; 4=continuous sniffing and continuous biting, gnawing or licking. The score for each rat at each time point was then summed with a maximum total score of 20 or 24 possible. The mean and standard error for the group were calculated and used to construct the dose response curves. The data were analyzed using a Mann-Whitney U-test. The criterion for significance was set at $p<0.05$. Alternatively, in some studies differences between the dose-response curves for various treatments were analyzed using Finney's Parallel Line Bioassay Test [7], with a criterion for significance set at $p<0.05$.

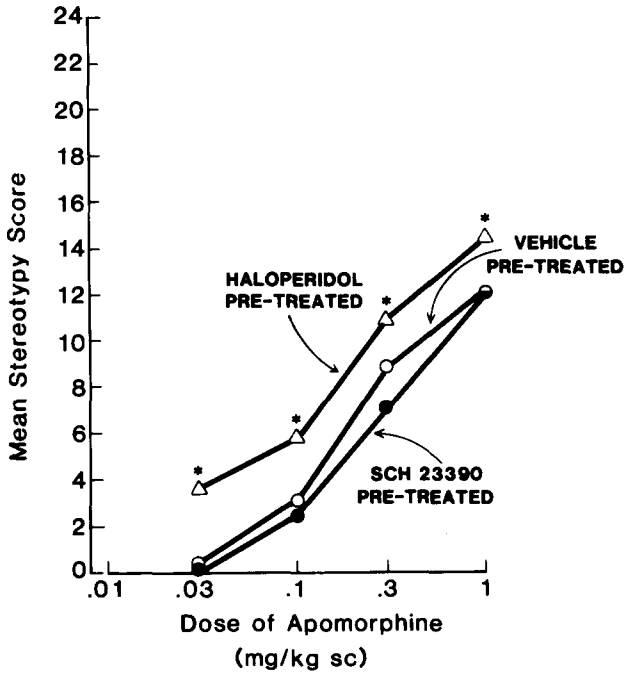

FIG. 1. Effect of pre-treatment with SCH 23390 (●), 25 mg/kg/day PO, haloperidol (△), 3 mg/kg/day PO or vehicle (○) on apomorphine-induced stereotypy. SCH 23390, haloperidol or vehicle were given for 19 days and the rats were tested 24 hr (day 20) following the last injection. Stereotypy scores were determined at 10, 20, 30, 60 and 75 min following apomorphine (SC). N=4–6/dose. *Significantly different from the effect of apomorphine in vehicle pre-treated rats, $p<0.05$, Mann-Whitney U-test.

Intracerebroventricular (ICV) injections. ICV injections were done free hand with a Hamilton microsyringe using a modified Haley and McCormick [9] technique with a collared 27 ga needle that penetrated the skull 4 mm. Control studies with injections of dye showed that >90% of the animals injected in this manner had the dye localized to the lateral cerebral ventricle. The injection volume was 10 μl/rat.

Biochemical Studies

Following the pharmacological treatments described above, the rats were given a one day washout period to allow for the metabolism and excretion of the drugs. The rats were then decapitated, the brains removed and striatal tissue excised. Striatal membranes were then prepared according to the method of Billard *et al.* [2]. The protein concentrations of the resulting membranes were determined by Lowry analysis [13].

D1 Binding Sites

In the studies on animals receiving repeated drug treatments the affinity and number of D1 binding sites were determined by equilibrium saturation analysis. Striatal membranes (0.18 mg protein) were incubated with ^{3}H-SCH 23390 (0.3 nM) in the presence of increasing concentrations of unlabeled SCH 23390 for 20 min at 37°C. The assay was performed in a final volume of 1 ml of 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (Buffer A). The reaction was terminated by filtration over GF/B filters which were subsequently washed with 12 ml of cold 50 mM Tris-HCl, pH 7.4 and counted by liquid scintillation spectroscopy. Data were analyzed according to the method of Scatchard, and K_d and B_{max} values were obtained using linear regression analysis.

Striatal membranes (0.124 mg protein) from reserpinized rats were incubated with increasing concentrations of ^{3}H-SCH 23390 for 20 min at 37°C in a final volume of 1 ml of Buffer A. Non-specific binding was determined in the presence of 100 nM unlabeled SCH 23390. The incubation mixtures were then filtered, washed, counted and analyzed as described above.

In these studies, differences were noted in the B_{max} values for vehicle-treated rats (see Tables 1 and 2) in two separate experiments. This was due to normal variations observed between tissue preparations (Billard and McQuade, unpublished). Since all comparisons are made *within* an individual experiment (and not *between* experiments), this variability does not alter the interpretation. The reasons why the B_{max} values varied between these experiments is unknown.

D2 Binding Sites

D2 binding site affinity and number were determined by similar techniques. Briefly, striatal membranes from chronic neuroleptic-treated rats (0.18 mg protein) and from reserpinized rats (0.12 mg protein) were incubated with increasing concentrations of ^{3}H-spiperone in a final volume of 1 ml of Buffer A for 15 min at 37°C. Non-specific binding was determined in the presence of 1 μM (+)butaclamol. Bound ligand was separated from free by filtration over GF/B filters, which were subsequently counted and the data subjected to Scatchard analysis.

Statistics

The binding studies were performed 3–5 times on pooled striatal membranes from six treated rats. The data were analyzed using a two-tailed paired *t*-test with a criterion for significance of $p<0.05$.

RESULTS

Effects of Repeated Antagonist Pre-Treatment on Dopamine Binding Sites

Following 19 days of repeated treatments with SCH 23390 or haloperidol there was no change in the affinities of D1 or D2 binding sites for ^{3}H-SCH 23390 or ^{3}H-spiperone, respectively (Table 1). In contrast, binding site up-regulation was observed after these treatments that was dependent on the selectivity of the pre-treatment drug. Thus, the D1 selective drug, SCH 23390, significantly increased (by 35%) only the number of D1 binding sites, while the D2 selective drug haloperidol significantly up-regulated (by 40%) only the numbers of D2 binding sites (Table 1). These results agree with previous findings [6,19].

Effect of Repeated Antagonist Pre-Treatment on AIS

Consistent with previous studies (e.g., [21]), the dose-response curve for AIS was significantly shifted to the left in haloperidol pre-treated rats relative to vehicle pre-treated rats; this demonstrates that apomorphine was more potent in these animals than in the control rats (Fig. 1). In contrast, the dose-response curve for AIS in SCH 23390 pre-treated rats was not significantly different from that of the vehicle pre-treated rats (Fig. 1).

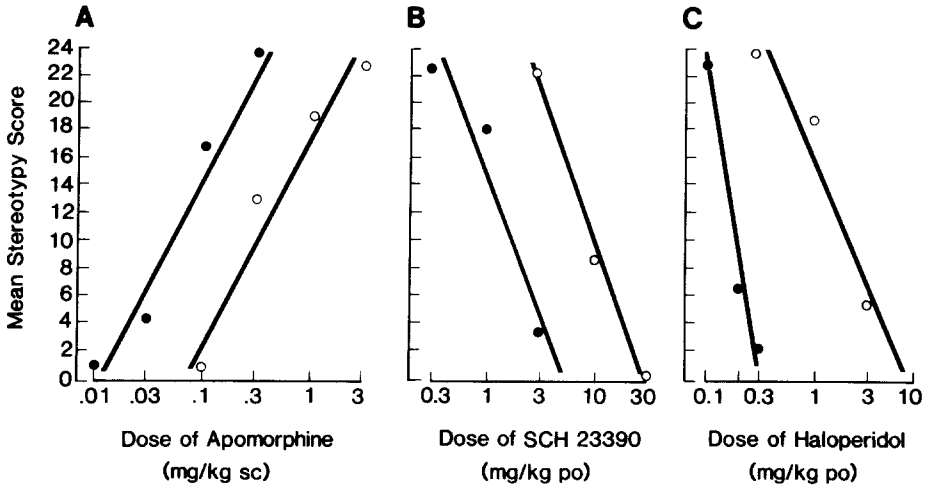

FIG. 2. (A) Stereotypy-inducing effects of apomorphine in reserpinized (●), 30 mg/kg IP 24 hr previously, or vehicle pre-treated (○) rats. (B) Effect of SCH 23390 on apomorphine-induced stereotypy in reserpinized (●) or vehicle pre-treated (○) rats. (C) Effect of haloperidol on apomorphine-induced stereotypy in reserpinized (●) or vehicle pre-treated (○) rats. SCH 23390 or haloperidol were given (PO) 30 min prior to apomorphine (SC). In the antagonist experiments reserpinized rats received 0.3 mg/kg SC of apomorphine whereas the vehicle pre-treated rats received 3 mg/kg SC of apomorphine. Animals were scored for stereotypy every 5 min for 30 min following apomorphine. N=4–6/dose.

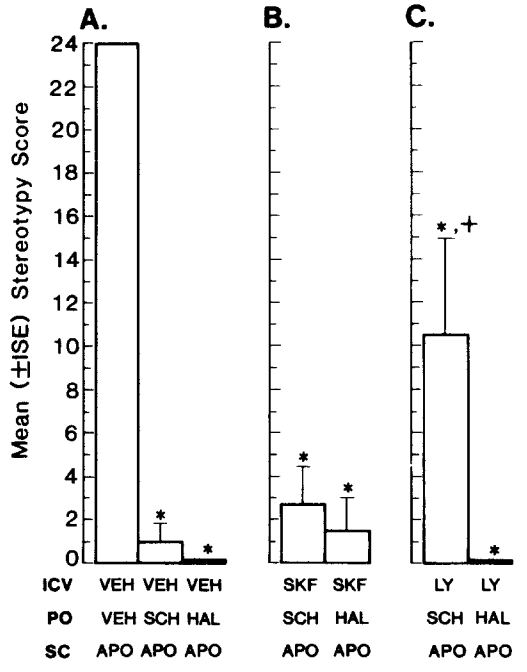

FIG. 3. (A) Effect of vehicle, SCH 23390 (3 mg/kg PO) or haloperidol (1 mg/kg PO) on apomorphine-induced stereotypy (AIS, 0.3 mg/kg SC) in reserpinized (30 mg/kg IP 24 hr previously) rats. (B) Effect of SKF 38393 (30 μg/rat ICV) on the blockade of AIS by SCH 23390 (3 mg/kg PO) or haloperidol (1 mg/kg PO) in reserpinized rats. (C) Effect of LY 171555 (30 μg/rat ICV) on the blockade of AIS by SCH 23390 (3 mg/kg PO) or haloperidol (1 mg/kg PO) in reserpinized rats. N=6/group. ICV injections were given 10 min before the PO treatments and APO was administered 30 min after the PO treatments. Animals were scored for stereotypy every 5 min for 30 min following apomorphine. *Significantly different from vehicle-vehicle-apomorphine group; +Significantly different from vehicle-SCH 23390-apomorphine group, $p<0.05$, Mann-Whitney U-test.

Effect of Reserpine Pre-Treatment on Dopamine Binding Sites

Twenty-four hours following reserpine treatment (30 mg/kg IP) there was no change in the affinities (K_d) of D1 or D2 binding sites for ^{3}H-SCH 23390 or ^{3}H-spiperone, respectively. However, there was a selective and significant increase (by 18%) in the numbers of D1 binding sites (Table 2). On a percentage basis this was less than that seen after repeated pre-treatment with SCH 23390 (see Table 1). In contrast, a much smaller and non-significant increase in the numbers of D2 binding sites was observed in reserpinized rats. Thus, reserpinization appeared to selectively increase the number of D1 (and not D2) binding sites in the striatum.

Effect of Reserpinization on AIS

The stereotypy-inducing effects of apomorphine were potentiated in reserpinized rats. The data in Fig. 2A show that the dose producing equivalent maximal stereotypy scores was ten-fold less in reserpinized than vehicle-treated rats (0.3 vs. 3 mg/kg SC, respectively). These results are similar to those seen by Arnt [1] who observed an enhancement of apomorphine-induced activity in rats following reserpine.

To determine if the ability of D1 or D2 antagonists to block AIS was altered in reserpinized rats, the following study was done. Behaviorally equivalent, maximally effective doses of apomorphine were given to reserpinized or vehicle pre-treated rats, and graded doses of SCH 23390 or haloperidol were compared for their ability to inhibit AIS. The results (Fig. 2B and C) show that there was an enhancement in the ability of both these drugs to block AIS. In the case of haloperidol, there was roughly a ten-fold shift in the dose-response curve, whereas for SCH 23390, the shift was only 5-fold. To determine the role of D1 and D2 binding sites in the potentiation of SCH 23390's or haloperidol's antagonism of AIS, the following study was done. Reserpinized

rats, treated with either vehicle, SCH 23390 or haloperidol, were given ICV injections of either a D1 (SKF 38393) or a D2 (LY 171555) agonist, and the ability of apomorphine to produce stereotypy was investigated. Previous work (not presented) had determined that these doses of SKF 38393 (30 μg/rat ICV) and LY 171555 (30 μg/rat ICV) were not active alone and did not potentiate the ability of apomorphine to produce stereotypy in normal or reserpinized rats. Higher doses of SKF 38393 or LY 171555 could not be tested because they produced tremors and convulsions.

The results of this study are presented in Fig. 3. The data in panel A demonstrate (as above) that these doses of SCH 23390 and haloperidol could fully block AIS. The data in panel B show that pre-treatment with SKF 38393 (30 μg/rat ICV) did not alter the ability of these dopamine antagonists to inhibit AIS. However, as shown in panel C, the D2 agonist LY 171555 significantly attenuated the ability of SCH 23390 to block AIS. In contrast, the D2 agonist did not have an effect on haloperidol's ability to block AIS (Fig. 3C).

DISCUSSION

These experiments have evaluated the involvement of D1 and D2 binding sites in AIS. The results suggest that D1 and D2 receptors are not acting as independent entities in the mechanism of AIS, but rather are interacting in some as yet undefined manner and that D2 binding sites play a greater role in AIS than D1 binding sites.

For example, it is evident that both D1 and D2 receptors are necessary for the full expression of AIS since it can be blocked by either selective D1 or D2 antagonists. Moreover, these same data can be used to conclude that neither binding site alone is sufficient for the production of AIS, since in the presence of selective blockade, the resulting selective agonism at the other site failed to induce the syndrome. Evidence to support this latter point is also seen in studies showing that neither selective D1 nor D2 agonists given alone mimics all the behavioral effects of AIS [16,20].

Therefore, these data suggest that, at the least, an interaction at D1 and D2 sites is required for AIS. The present studies also examined the participation of these sites by inducing selective up-regulation and observing the consequences on AIS. Thus, in the first experiment it was shown that up-regulation of the D2 binding site but not the D1 binding site was associated with supersensitivity to apomorphine since when the number of either binding site was increased by roughly the same percentage, only the haloperidol treated rats were behaviorally affected. This implies that D2 binding sites are more important in AIS than D1 binding sites.

Likewise, the experiments with reserpinized rats support the predominance of the D2 binding site in AIS. For example, the dose-response curve for haloperidol to block AIS in reserpinized rats was shifted twice as much as that of SCH 23390, despite the fact that the reserpine pre-treatment increased substantially only the number of D1 binding sites. Similarly, it was the D2 agonist that attenuated SCH 23390's effect in reserpinized rats, presumably because the D1 binding site was blocked. Hence, the D2 agonist had unrestricted accessibility to the D2 binding site and partial restoration of AIS was observed. In contrast, in haloperidol-treated reserpinized rats, the antagonist blocked the access of LY 171555 to the D2 binding sites, thus inhibiting any possible effect.

One caveat that should be added regarding the role of D2 sites in stereotypy is that the stereotypy-inducing drug itself (i.e., apomorphine) may be biasing the results. Thus, it has been proposed [12] that apomorphine is only a partial agonist at the D1 site but a full agonist at the D2 site. If this is true, then this would necessarily lead to a predominant role for D2 sites in AIS because of the differences in efficacy. Arguing against this however is the equal affinity of apomorphine for D1 and D2 sites [2] and our behavioral results using the D1 agonist (see Fig. 3). Regardless, whether the modulating role of D2 receptors in AIS is a reflection of the drug or of the fundamental nature of the dopaminergic system may not be resolvable with the drugs that are currently available.

The present results compare favorably with other reports in the literature. Thus, both Meller *et al.* [15] and Morelli and DiChiara [17] report on studies very similar to these which demonstrated that in rats SCH 23390-induced catalepsy was attenuated by D2 (e.g., LY 171555 or bromocriptine) and not D1 (SKF 38393) agonists. Also, Meller *et al.* [14] state that inactivation of D2 binding sites by EEDQ blocks AIS to a greater extent than similar inactivation of D1 binding sites. Thus, the literature is generally consistent with the concept that on striatally mediated, neurological measurements, the D2 binding site is more important than the D1 binding site.

It is interesting to note that despite that fact that there are roughly twice as many striatal D1 binding sites as there are D2 binding sites (Billard and McQuade, personal communication), it is the D2 binding site that is more important in AIS. Therefore, finding the critical differences between D1 and D2 binding site function should provide a comprehensive explanation for these results. There are at least three possibilities. First, although there are both D1 and D2 post-synaptic binding sites, the pre-synaptic binding sites are exclusively D2 [12]. Stimulation of these binding sites decreases the release and turnover of dopamine. Whether an ability to affect these parameters is crucial for controlling AIS is unknown. Second, D1 and D2 binding sites have opposing effects on dopamine stimulated adenylatecyclase (DSAC) [18,22] which in turn controls the phosphorylation of post-synaptic proteins like DARPP 32 [10]. The role of this and other second (and third) messengers as well as the respective balance between D1 and D2 binding sites on DSAC in the possible mediation of AIS is likewise unknown. Third, in the striatum, dopamine regulates cholinergic interneurons via D2 binding sites [3,22]. These binding sites appear to be selectively involved in the D2 mediation of AIS since scopolamine inhibits haloperidol-induced blockade of AIS, but does not alter the effects of SCH 23390 [4]. Thus, AIS has a strong cholinergic component that is mediated via D2 but not D1 binding sites. Presently, the data are insufficient to choose among these hypotheses and all merit further research.

In summary, D1 and D2 binding sites are both involved in AIS. Further, the data suggest that the D2 binding site has a greater role in this behavior.

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