

Amphetamine-Sensitized Animals Show a Marked Increase in Dopamine D₂ High Receptors Occupied by Endogenous Dopamine, Even in the Absence of Acute Challenges

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KEY WORDS amphetamine; dopamine; D₂ receptor; high-affinity state of D₂; striatum; behavior

ABSTRACT While a range of dopamine D₂-related behaviors are exaggerated in amphetamine-sensitized animals, studies of the dopamine D₂ receptor have reported either no change or a decrease in dopamine D₂ receptor density—especially when measured using radioligand. We hypothesized that a decrease in D₂ receptors may actually be “apparent” and that these receptors may still be present, but are noncompetitively “occupied” by endogenous dopamine. Animals sensitized to amphetamine (and their saline controls) were examined 4 weeks after their last injection. We first measured the [³H]raclopride binding in vivo, and observed that sensitized animals showed a 29% lower level of raclopride binding in vivo, suggesting an apparently lower level of dopamine D₂ receptors. To assess the reason for this we examined the density of receptors (using Scatchard analysis in vitro) measured by [³H]raclopride in the presence and absence of guanylimidodiphosphate. This guanine nucleotide converts the dopamine-bound high-affinity state of D₂ receptors into low-affinity states, thereby making measurable the absolute density of the sites. As previously reported, the amphetamine-sensitized animals showed a 31% lower number of D₂ receptors in conventional binding (B_{max} 15.6 vs. 22.7 pmol/g). However, with the addition of guanylimidodiphosphate there was an equalization of both groups (B_{max} 25.9 vs. 25.6 pmol/g), revealing an additional 10.3 pmol/g in the sensitized animals, but only 2.9 pmol/g in saline controls. There were no changes in the dissociation constant of [³H]raclopride for the receptors. The nearly four-fold increase of dopamine D₂ receptors in the high-affinity state occupied by dopamine may explain why amphetamine-sensitized animals show almost an order of magnitude greater response to dopamine-releasing challenges or dopamine agonists, even though the absolute receptor number is unchanged and the “apparent” receptor number is decreased. **Synapse 46:235–239, 2002.** © 2002 Wiley-Liss, Inc.

INTRODUCTION

A single dose of amphetamine or repeated doses over several days leads to behavioral sensitization of animals several weeks later (Vanderschuren et al., 1999; Robinson and Becker, 1986). Depending on the dose (generally 1–10 mg/kg) and the withdrawal period, these animals show enhanced responses to amphetamine, direct-acting dopamine agonists, and other stressors on behaviors, including enhanced rotation, locomotion, drinking, self-stimulation, startle reaction,

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and cage climbing (Bailey and Jackson, 1978; Robinson and Becker, 1986). Most of these enhanced behaviors can be blocked by dopamine D₂-receptor blocking drugs in the sensitized animals.

These sensitization-enhanced behaviors, however, are not accompanied by any increase in the density, or any change in the dissociation constant of the radioligand for the dopamine D₂ receptors in the rat brain. In fact, virtually all of the 30 or more studies using sensitizing doses of 2–20 mg/kg amphetamine have found a reduction or no change in the density of dopamine D₂ receptors (Muller and Seeman, 1979; Riffée et al., 1982; Sibley et al., 1982; Robinson and Becker, 1986), including studies in rats and monkeys *in vivo* (Kaneno and Shimazono, 1981; Ginovart et al., 1999). While the reduction is often thought to represent a downregulation in the face of heightened transmission, it cannot explain why direct-acting dopamine agonists show enhanced behavior in sensitized rats.

In the absence of any evidence for an increased postsynaptic D₂ receptor density to explain amphetamine-induced sensitization, many studies have examined other alternatives to account for the sensitization process, including striatal dopamine concentration, the rate of striatal dopamine synthesis, the resting rate of dopamine release, the rate of dopamine release after an amphetamine challenge, dopamine autoreceptor subsensitivity, and altered norepinephrine activity. However, none of these processes were significantly altered in the amphetamine-sensitized animals, with the important exception of amphetamine-induced release of dopamine, which is consistently enhanced (Robinson and Becker, 1986; Vanderschuren et al., 1999). Even studies that have focused on the release of dopamine find that the increased release occurs in response to a challenge with amphetamine and there is little evidence to indicate that there are high baseline levels of dopamine in animals at rest (although it has to be acknowledged that microdialysis is not ideal for measuring baseline states).

In light of these conflicting findings, it remains a puzzle how dopamine D₂ receptors may be reduced, yet dopamine D₂-related functions are increased. We propose that this reduction in dopamine D₂ receptors is only apparent. The receptors are actually available in the tissue, but are not revealed in conventional binding studies because they are bound by endogenous dopamine in a noncompetitive fashion. Guanine nucleotide (e.g., guanylimidodiphosphate) is known to convert dopamine D₂ receptors from their high-affinity state to their low-affinity state for the agonist, this process releasing the noncompetitively bound dopamine from the receptors, thereby making those receptors accessible to conventional radioligand competition (Grigoriadis and Seeman, 1985). Therefore, we undertook a series of studies examining D₂ density with [³H]raclo-

pride in the presence and absence of guanylimidodiphosphate.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats, weighing 200–225 g at the start of the experiment, were used. They were housed two per cage with free access to food and water. The housing room was maintained at a constant temperature of 20 ± 2°C on a 12:12 reverse light/dark cycle. Lights were off at 8:00 AM.

Sensitization regimen

Animals (*n* = 8 per group) were randomly divided into groups, with each rat receiving an intraperitoneal injection of either d-amphetamine sulphate (Sigma-RBI, St. Louis, MO) (AMPH) or 0.9% saline (SAL; 1 ml/kg). Since the animals were housed in pairs, cage mates received the same drug treatment. Injections were given on Monday, Wednesday, and Friday for 5 weeks. AMPH-pretreated rats received increasing doses of AMPH for a total of 15 injections over 5 weeks. The dose increased from 1 mg/kg to 5 mg/kg with an increase of 1 mg/kg each week. Immediately after the injection each rat was returned to its home cage. Following the last injection, the animals were left drug-free for 28 days before they were either entered into the *in vivo* raclopride binding study or sacrificed by decapitation for the *in vitro* binding studies. This regimen leads to robust behavioral sensitization in these animals, as documented by monitoring locomotor activity; we have since observed that even lower doses and shorter injections (1–3 mg/kg over 9 injections) lead to similar results—behavioral details and other cognitive effects of these regimens are a part of another report (Tenn et al., submitted).

Raclopride *in vivo* binding studies

The SAL and AMPH preexposed rats were given a tail vein injection of 7.5 µCi [³H]raclopride (RAC) in 0.4 ml of saline. Thirty minutes after the RAC injection, the animals were sacrificed and the brains quickly extracted and the bilateral striata and the cerebellum specifically dissected. Specific (striatum) and nonspecific (cerebellum) binding were determined by scintillation counting. The ratio of specific/nonspecific provided an estimate of the D₂ receptor binding potential—a ratio that is proportional to the ratio of B_{max}/Kd_{app}. The details of this procedure as well as validation against [¹¹C]-raclopride (the ligand used in humans) were previously published (Wadenberg et al., 2000).

Raclopride *in vitro* Scatchard analysis

The brain striata were removed and frozen at –70°C until used. The frozen striata were blotted and weighed

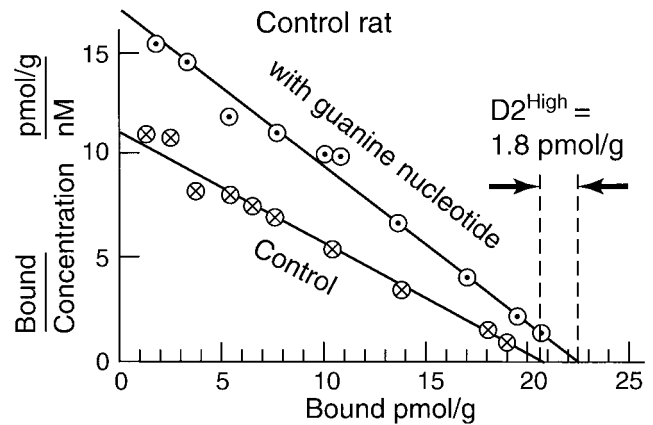
frozen; buffer was added (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM KCl, 1.5 mM CaCl₂, 4 mM MgCl₂, 120 mM NaCl) to yield 4 mg tissue per ml. The suspension was homogenized (using 10 up-down strokes of a glass-Teflon homogenizer with the piston rotating at 500 RPM) but not washed; it had been shown previously (Seeman et al., 1984) that the customary two washes of homogenized tissues resulted in a loss of 30–60% of dopamine receptors. An aliquot of 0.5 ml of buffer (with or without a final concentration of 200 μM guanylimidodiphosphate, and with or without a final concentration of 10 μM S-sulpiride [Ravizza, Milan]) was placed in glass test-tubes (12 × 75 mm), followed by the addition of 0.25 ml of [³H]raclopride (76.8 Ci/mmol; 12 final concentrations, from 0.2–20 nM) and 0.25 ml of the homogenized tissue. After 2 h at room temperature (20°C), the incubates were filtered through buffer-pres soaked glass filters (NO. 7034, Skatron, Sterling, VA) using a 12-well cell harvester (Titertek, Skatron, Lier, Norway) followed by a 15-sec rinse with 7.5 ml of buffer. The filters were then placed in vials with 4 ml of scintillant and monitored 6 h later for tritium in a Beckman L5000 scintillation spectrometer at 55% efficiency. Nonspecific binding for dopamine D₂ receptors was defined as that which occurred in the presence of 10 μM S-sulpiride. The density of [³H]raclopride binding sites and the dissociation constant (K_d) were obtained by Scatchard analysis.

RESULTS

The [³H]raclopride binding potential (BP) *in vivo* was determined in the striata of saline- and amphetamine-treated animals after the 28th day of withdrawal. Analyses of the binding potential revealed a significant difference between the two groups [F(1,9) = 16.40, *P* = 0.003]. The amphetamine-sensitized rats showed a 29.4% ± 3.2% decrease in BP as compare to their controls (7.77 ± 1.2 and 5.55 ± 1.0 for saline and amphetamine, respectively).

The density (B_{max}) of dopamine D₂ receptors in saline-treated control rat striata was 22.7 ± 2.2 pmol/g (i.e., pmols per g of original wet tissue; *n* = 6), as shown by the representative data in Figure 1 and for all the tissues in Figure 2. In the presence of guanylimidodiphosphate, the density of D₂ in control tissues increased by 2.9 pmol/g to 25.6 ± 2.2 pmol/g (Fig. 2).

The striata from the amphetamine-sensitized rats revealed a D₂ density of 15.6 ± 1.8 pmol/g. In the presence of guanylimidodiphosphate, the receptor density increased by 10.3 pmol/g to 25.9 ± 1.2 pmol/g (*n* = 6), as shown in Figures 1 and 2. In comparing the density of striatal D₂ high receptors in amphetamine-sensitized animals (10.3 pmol/g) to that of their saline controls (2.9 pmol/g); therefore, the guanine nucleotide raised the apparent density 3.6-fold.



Amphetamine-sensitized rat:
more D₂^{High} states, more synaptic dopamine

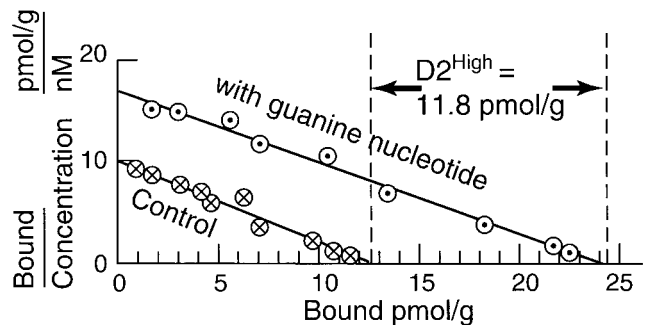


Fig. 1. Representative data for a single experiment using a range of [³H]raclopride concentrations to determine the density of dopamine D₂ receptors in a control rat striatum (top) and in an amphetamine-sensitized rat striatum (bottom) in the absence and presence of 200 μM guanylimidodiphosphate. The absolute density of the high-affinity states for the dopamine D₂ receptor was 1.8 pmol/g in the control striatum, but was 11.8 pmol/g in the amphetamine-sensitized striatum (all points in duplicate).

DISCUSSION

The results show that while *in vivo* binding shows a decrease in the specific/nonspecific binding, suggesting a decrease in receptor density, *in vitro* experiments show that there is no loss of receptors, but that the receptors are bound to endogenous dopamine in a non-competitive fashion.

Control striata showed that guanine nucleotide unmasked or “revealed” 2.9 pmol/g of the dopamine D₂ receptors under standard binding conditions. However, the same experiment done in sensitized animals revealed 10.6 pmol/g. The important point here is that the animals in the sensitized state were examined 1 month after their final injection of amphetamine and they received no new challenges of amphetamine at the time of testing. Thus, this alteration in number of receptors hidden in *in vivo* binding and revealed by GppNHp reflects a constitutive change in these animals once they are sensitized.

The physiological significance of these data becomes relevant by understanding the mechanism by which

Amphetamine-sensitized rat has more D₂^{High} states, more synaptic dopamine

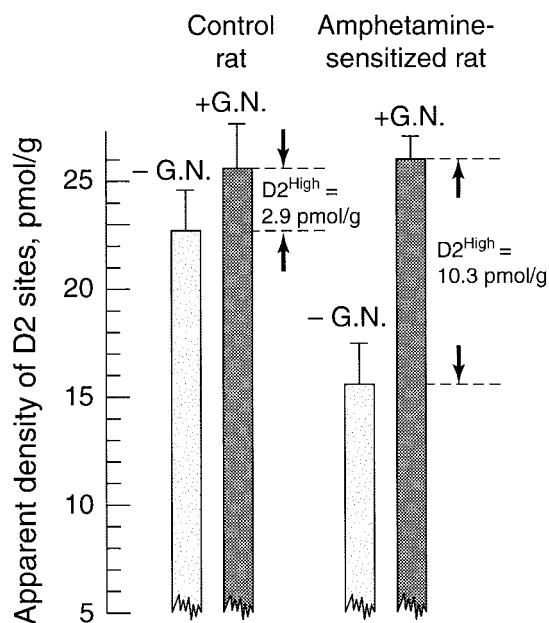


Fig. 2. Although the density of the entire population of dopamine D₂ receptors in the amphetamine-sensitized rat striata went down, compared to control striata, the density of high-affinity states (as defined in Fig. 1) in the sensitized striata increased from a control value of 2.9 pmol/g to 10.3 pmol/g, an increase of 3.6-fold.

the guanine nucleotide reveals apparently additional receptors. Under control tissue conditions, most of the endogenous receptors are not bound to endogenous ligand. However, a proportion of the receptors (~10% for control animals at rest), presumably those that were in the high-affinity state in the live animal, are bound to endogenous dopamine noncompetitively. Guanilylimidodiphosphate has been shown to convert receptors from their high-affinity state to their low-affinity state—even in the presence of bound agonist (Grigoriadis and Seeman, 1985). The dissociation constant of dopamine at the high-affinity state of D₂ is 1.5 ± 0.2 nM (Kapur and Seeman, 2001), and the dissociation constant for dopamine at the low-affinity state is in the micromolar concentration range (Grigoriadis and Seeman, 1985). Thus, in the presence of the guanine nucleotide, when all the receptors are converted to their low-affinity state, any endogenously bound dopamine is released from the receptors, thus making the previously occupied receptors accessible and available for competitive radioligand binding studies. As a result, the addition of the guanine nucleotide leads to an increase in B_{max}, thereby unmasking or “revealing” receptors. We propose that in the case of the amphetamine-sensitized rats, even under baseline resting conditions, a higher proportion (3.6-fold) of the receptors were occupied by endogenous dopamine and, as a

result, with the addition of the guanine nucleotide almost 40% of the total binding sites were revealed.

The present results also agree with previous work by many others (reviewed by Robinson and Becker, 1986) showing that the density of the entire population of dopamine D₂ receptors, as measured conventionally, is lower in the tissues from the sensitized animals (Fig. 2, in the absence of guanine nucleotide). However, the absolute concentration of high-affinity states had not been previously measured by the present method. Although it is customary to determine the proportion of high-affinity states by the competition between a dopamine D₂ radioligand and exogenously added dopamine (Grigoriadis and Seeman, 1985), this method did not reveal any significant difference in the proportion (45%) of high-affinity states between control striata and amphetamine-sensitized striata (results not shown). The advantage of the present method, using guanine nucleotide and [³H]raclopride saturation curves (i.e., the Scatchard method), is that it yields the absolute density of the high-affinity states without altering the endogenous concentration of dopamine.

The 3.6-fold increase in high-affinity states of D₂ could contribute to the markedly increased sensitivity of the sensitized animal to amphetamine and dopamine agonists. For example, as shown previously (List and Seeman, 1980), an increase of 40% in functional D₂ receptors would readily account for a 5-fold leftward shift in the agonist dose–response curve. Hence, an elevation of 360% would markedly shift leftward the behavioral sensitivity to amphetamine or apomorphine.

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