

# Altered Behavioral Response to Dopamine D<sub>3</sub> Receptor Agonists 7-OH-DPAT and PD 128907 Following Repetitive Amphetamine Administration

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Behavioral sensitization, the progressive and enduring enhancement of certain behaviors following repetitive drug use, is mediated in part by dopaminergic pathways. Increased locomotor response to drug treatment, a sensitizable behavior, is modulated by an opposing balance of dopamine receptor subtypes, with D1/D2 dopamine receptor stimulation increasing and D3 dopamine receptor activation inhibiting amphetamine-induced locomotion. We hypothesize that tolerance of D3 receptor locomotor inhibition contributes to behavioral sensitization. In order to test the hypothesis that expression of behavioral sensitization results in part from release of D3 receptor-mediated inhibition, thereby resulting in decreased response to D3 receptor agonists, we examined the effect of repetitive amphetamine administration on the behavioral response to the D3 receptor preferring agonists 7-OH-DPAT and PD 128907. D3-selective effects have recently been described for both drugs at a low dose. At 1 week following completion of a repetitive treatment regimen, amphetamine-pretreated rats displayed a decreased response to D3-selective doses of both 7-OH-DPAT and PD 128907, when compared to animals receiving saline pretreatment. Moreover, in addition to the quantitative alteration in response, there was a change in the inter-relation between response to amphetamine and D3 agonist. A highly significant inverse relation between locomotor inhibitory response to PD 128907 and the locomotor-stimulant response to amphetamine was observed prior to amphetamine treatment. In contrast, 10 days following repetitive amphetamine treatment, the relation between response to PD 128907 and amphetamine was not detected. The observed behavioral alteration could not be accounted for by changes in D3 receptor binding in ventral striatum. These findings suggest a persistent release of D3 receptor-mediated inhibitory influence contributes to the expression of behavioral sensitization to amphetamine.

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## INTRODUCTION

Behavioral sensitization, the progressive and enduring enhancement of certain behaviors following repetitive stimulant drug administration, is an example of persistent behavioral plasticity (Segal and Mandell, 1974; Paulson *et al*,

1991), mediated at least in part by dopaminergic systems (Kuczenski and Leith, 1981; Vezina and Stewart, 1989, 1990; Hitzemann *et al*, 1980; Kalivas and Weber, 1988; Stewart and Vezina, 1989; Kalivas and Stewart, 1991). Much is known regarding the immediate and short-term molecular and cellular consequences of stimulant drug exposure. In contrast, however, a far more limited number of persistent, long-standing cellular, or molecular adaptations to drug use have been described (Sorensen *et al*, 1982; Cha *et al*, 1997; Hope *et al*, 1994; Chen *et al*, 1997). Significantly, a clear link between known cellular adaptations and long-standing behavioral change has remained elusive.

Because tolerance is the expected homeostatic response to repetitive drug exposure (Ramsay and Woods, 1997; Jaffe,

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1992), and because a significant proportion of neuronal pathways within the brain are inhibitory in function (Greengard, 2001), behavioral sensitization likely results at least in part from tolerance of inhibitory pathways. Rodent locomotion, a sensitizable behavior, is regulated by the opposing balance of D3 and D1/D2 receptor activity, with D1/D2 activation increasing and D3 receptor stimulation inhibiting locomotion (Xu *et al*, 1997; Flores *et al*, 1996; Waters *et al*, 1993; Sautel *et al*, 1995; Ekman *et al*, 1998; Menalled *et al*, 1999; Pritchard *et al*, 2003). We have previously described evidence supporting the hypothesis that tolerance of D3 receptor-mediated locomotor inhibition contributes to locomotor sensitization (Richtand *et al*, 2000, 2001a, b). In order to further test this hypothesis, we determined the behavioral response of male Sprague-Dawley rats to D3 receptor agonists 7-OH DPAT and PD 128907, following treatment with repetitive saline or amphetamine (AMPH). D3 agonist doses were chosen based on the previous data demonstrating D3-selective effects of these drugs at the low doses employed in our study (Pritchard *et al*, 2003; Zapata *et al*, 2001). Our evaluation had two primary goals. First, by independently determining the behavioral response to D3 agonist and AMPH at different times in the same animals, we sought to determine the relation between the two measures. Secondly, we wished to test our prediction that sensitized animals would have a diminished behavioral response to both 7-OH-DPAT and PD 128907, at D3-selective doses of both compounds. Here, we report a highly significant inverse correlation between locomotor inhibition resulting from PD 128907, and the enhanced locomotor response to an initial AMPH injection. As predicted, locomotor inhibition resulting from D3 agonists 7-OH-DPAT and PD 128907 was persistently decreased 1 week following completion of a repetitive AMPH treatment regimen. Significantly, the relation between inhibitory response to D3 agonist and locomotor response to AMPH was not detectable following repetitive AMPH treatment. These findings demonstrate a persistent alteration in response to D3 receptor agonist following repetitive AMPH administration, which cannot be accounted for by alteration in D3 receptor binding, and suggest that the release of D3 receptor-mediated inhibitory influence contributes to the expression of behavioral sensitization to amphetamine. Implications of these findings for understanding long-term adaptations to stimulant drugs are discussed.

## MATERIALS AND METHODS

### Behavioral Studies

Male Sprague-Dawley Rats (275–300 g, Harlan Labs, Indianapolis, IN) were housed two per cage in a vivarium maintained on a 12 h (05.00 h on; 17.00 h off) light/dark schedule for 2 weeks prior to use in behavioral studies. D-amphetamine sulfate, ( $\pm$ )-7-hydroxy-N,N-di-n-propyl-2-aminotetralin (7-OH-DPAT) (Sigma, St Louis, MO) and (+)-PD 128907 (Tocris, Ballwin, MO) were dissolved in 0.9% saline. Drug concentrations are described as free base (AMPH), hydrochloride (PD 128907), or hydrobromide (7-OH-DPAT) salt. All injections were subcutaneous in a final volume of 1 ml/kg.

Behavioral characterization was carried out in Residential Activity Chambers, designed as previously described (Richtand *et al*, 2000; Welge and Richtand, 2002). Each chamber consists of a lighted, ventilated, sound-attenuated cabinet (Cline Builders, Covington, KY) housing a 16"  $\times$  16"  $\times$  15" Plexiglas enclosure. A fan in each enclosure provides air circulation and constant background noise. Lights inside the chambers were coordinated with the vivarium light cycle, and behavioral testing was performed during the 'lights-on' portion of the cycle between 05.00 and 17.00 h. Locomotion was monitored with a 16  $\times$  16 photo-beam array (San Diego Instruments, San Diego, CA) located 1.5 inches above the enclosure floor. Locomotor activity is expressed as crossovers, defined as the number of times the animal enters into any of the five divisions subdividing the enclosure as previously described (Pritchard *et al*, 2003).

### Behavioral Data Analysis

Crossovers were collected by computer into 3-min interval bins. For all analyses with the exception of parametric regression model (Figure 2), crossovers were summed for analysis of behavioral observation periods of 60, 120, or 180 min, depending on the time period of behavioral response following drug treatment. The test interval for each experiment was dependent on the individual drug administered in that given experiment. Crossover data are analyzed using analysis of variance (ANOVA) techniques for independent observations. Because the data represent counts bounded at zero and typically skewed to the right, the geometric mean provides a better estimate of central tendency for the data distribution than the arithmetic mean. Results are expressed as the geometric mean number of crossovers,  $\pm$  standard error, for each group. Planned *t*-type comparisons were performed for hypotheses of interest, with the level of significance set at  $p \leq 0.05$ . In the case of each planned comparison corresponding to a theory-derived hypothesis that one mean would differ from another in a predictable direction, a one-tailed test was performed. In the case of comparisons for which we had no *a priori* reason to believe that the null hypothesis was untrue (eg when comparing pretreatment groups following saline injection for the purpose of demonstrating lack of a measurable difference in conditioned locomotion response or change in baseline activity between pretreatment groups), two-tailed tests were used, since the relevant alternative hypothesis in such cases is a nonzero absolute, rather than directional, difference.

ANOVA does not provide information about systematic temporal trends in the data. For one of the experiments reported here (Figure 2), we therefore also utilized parametric regression models, as we have previously described (Welge and Richtand, 2002), in order to identify temporal patterns in longitudinal crossover measurements. Briefly, for longitudinal modeling the log of the number of crossovers per 3-min interval was modeled as a polynomial function (of order  $\leq 3$ ) of time, separately for each treatment group. The intercepts and linear components of change were allowed to vary randomly among animals according to a bivariate normal distribution with a null mean vector and unknown covariance matrix. Time intervals over which the pointwise 95% confidence intervals

for the experimental groups did not overlap were interpreted as regions of significant difference.

### D3 Receptor Binding Assay

Brains were frozen on powdered dry ice for the determination of D3 receptor binding and stored at  $-70^{\circ}$  prior to frozen dissection and receptor binding assay. D3 receptor binding was quantified using [ $^3$ H]PD 128907 binding assay as previously described (Bancroft *et al*, 1998). Under the  $Mg^{2+}$ -free *in vitro* assay conditions employed to eliminate high-affinity agonist D2 receptor binding, specific [ $^3$ H]PD 128907 binding is not detectable in caudate/putamen, indicating negligible labeling of striatal D2 receptors and therefore greater than 300-fold D3/D2 selectivity (Bancroft *et al*, 1998). Ventral striatum (nucleus accumbens and olfactory tubercle) were dissected from each brain and pooled in order to provide sufficient material for accurate determination of both  $K_D$  and  $B_{max}$  values for each experimental group. Two independent saturation analyses were performed on each sample. Tissue was homogenized in 20 vol (w/v) of buffer (50 mM Tris, 1 mM EDTA; pH 7.4 at  $23^{\circ}C$ ) using a PRO homogenizer (setting four for 10 s). The crude homogenate was centrifuged twice (15 min at 48000g), and pellet resuspended each time in 20 vol of buffer. The final pellet was resuspended in buffer to yield a final tissue concentration of 10 mg original wet weight/ml. Binding assays were performed in duplicate in polystyrene tubes in a final volume of 0.5 ml. In all, 10 concentrations of (+)-[N-propyl-2,3- $^3$ H]PD 128907 (114 Ci/mmol; Amersham, Arlington Heights, IL) were used (0.02–2 nM). Nonspecific binding was defined by 1  $\mu$ M spiperone. Binding was initiated with the addition of membrane homogenate, tubes incubated for 3 h at  $23^{\circ}C$ , and the reaction terminated by rapid filtration through Whatman GF/B filters pretreated with 0.5% polyethyleneimine using a Brandel cell harvester. Filters were subsequently washed 3 times with 3 ml ice-cold buffer (50 mM Tris-HCl; pH 7.4), and placed in scintillation vials. After the addition of scintillation cocktail, vials were shaken, equilibrated for 2 h, and radioactivity determined by scintillation counter. Protein concentrations were determined using the BCA colorimetric assay method (Pierce, Rockford, IL). Specific [ $^3$ H]PD 128907 binding is expressed as fmol/mg protein. Binding data were analyzed using the nonlinear least-squares curve-fitting program LIGAND.

## RESULTS

### Persistent Locomotor Augmentation Following Repetitive AMPH Pretreatment

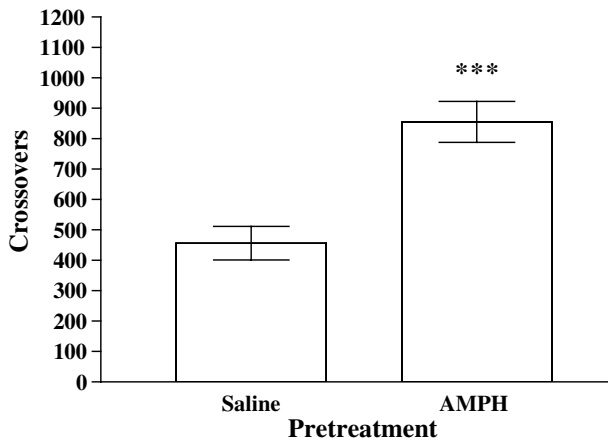
The effect of environmental context on the ability of amphetamine to induce behavioral sensitization is dose dependent (Browman *et al*, 1998; Badiani *et al*, 1997). In order to clearly establish the occurrence of behavioral sensitization in response to treatment under our conditions in which animals received repetitive amphetamine pretreatment in a home-cage environment, and behavioral testing following sensitization in different surroundings, we determined amphetamine-stimulated locomotion under

conditions similar to those employed in subsequent studies (Table 1, Experiment 1). Animals were single housed and treated in home cages for 5 consecutive days with either saline or AMPH (1 mg/kg) ( $n=8$  rats/group). At 5 days following the final injection, animals were moved from their home cages into individual Residential Activity Chambers for both housing and behavioral testing for the remainder of the study. In order to determine if there were differences in conditioned behavioral response to prior treatment experience, on days 5 (experiment day 10) and 6 (experiment day 11) following this 5-day pretreatment regimen all animals were injected with saline and locomotion recorded following injection. We tested our prediction that there would be no difference between the groups in locomotor response to saline injection, using a two-tailed test. There was no significant difference between the pretreatment groups in locomotor response to saline injection on days 10 or 11 (day 10,  $p=0.180$ , day 11,  $p=0.884$ ), demonstrating a lack of measurable conditioning effect or change in baseline activity distinguishing the two pretreatment groups.

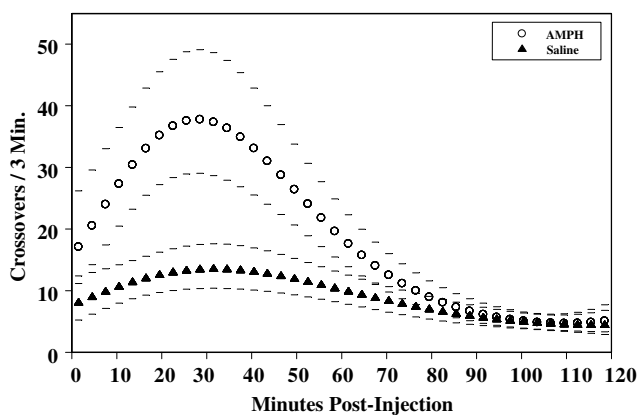
We tested our prediction that AMPH pretreatment resulted in an enduring enhancement of AMPH-stimulated locomotion, using a one-tailed test. Crossovers from 0 to 120 min following AMPH (0.5 mg/kg) injection were determined in both pretreatment groups on experiment day 12, 7 days following the last pretreatment injection. The 0–120 min time interval includes the period of locomotor stimulation following amphetamine injection under the conditions of this study. The two pretreatment groups differed significantly in mean crossovers from 0 to 120 min following AMPH injection (one-tailed  $p=0.0002$ , Figure 1). Animals in the AMPH pretreatment group exhibited an 89% higher rate of crossovers than animals in the saline pretreatment group. The time course of behavioral response for both pretreatment groups was also compared utilizing a mixed-effect linear regression model fitted to the logtransformed counts within each 3-min interval from 0 to 120 min following injection, as previously described (Welge and Richtand, 2002). The fitted model, accounting for 42% of the variance in crossover activity, is shown in Figure 2. The difference in behavioral response between groups is apparent during the first 3 min of recorded activity following AMPH injection, is maximal at 30 min following injection, and persists for approximately 90 min. Thus, under these conditions of AMPH dose, injection route and schedule, and testing environment, AMPH pretreatment results in robust behavioral sensitization, relative to saline-pretreated animals.

**Table 1** Injection Schedule for Behavioral Sensitization, Experiment 1

Group	Days		
	1–5	10–11	12
Saline ( $n=8$ )	Saline	Saline	AMPH
AMPH ( $n=8$ )	AMPH	Saline	AMPH



**Figure 1** Geometric mean  $\pm$  SEM of total crossovers for saline ( $n=8$ ) or AMPH ( $n=8$ ) pretreatment groups following AMPH (0.5 mg/kg) injection to both pretreatment groups 1 week following completion of pretreatment. Bars represent mean  $\pm$  SEM of total crossovers for each treatment group from 0 to 120 min after drug administration. \*\*\*Denotes crossovers significantly increased in the AMPH pretreatment group ( $p=0.0002$ , one-tailed).



**Figure 2** Fitted values by pretreatment group (saline = ▲, AMPH = ○) and 95% confidence intervals (dashed lines) for crossovers as a function of time in response to an injection of AMPH (0.5 mg/kg) 1 week following pretreatment with AMPH ( $n=8$ ) or saline ( $n=8$ ). Analysis is by mixed-effect regression model as previously described (Welge and Richtand, 2002).

### Behavioral Response to D<sub>3</sub> Receptor Agonist is Diminished in Amphetamine-Sensitized Rats

In order to test the hypothesis that a persistent release of D<sub>3</sub> receptor-mediated inhibitory influence follows repetitive AMPH administration, we determined the behavioral response to D<sub>3</sub> receptor preferential doses of 7-OH-DPAT and PD 128907 1 week following repetitive AMPH treatment using the same treatment regimen (Table 2, Experiment 2). D<sub>3</sub> agonist doses were chosen based upon recent studies demonstrating D<sub>3</sub>-selective locomotor inhibitory effects in wild-type, but not D<sub>3</sub> knockout, mice for both drugs at the doses used (Pritchard *et al*, 2003). In dose-ranging studies of 7-OH-DPAT and PD 128907 in rat, we have been unable to detect locomotor inhibition at doses lower than those used in the current study (Richtand *et al*, unpublished

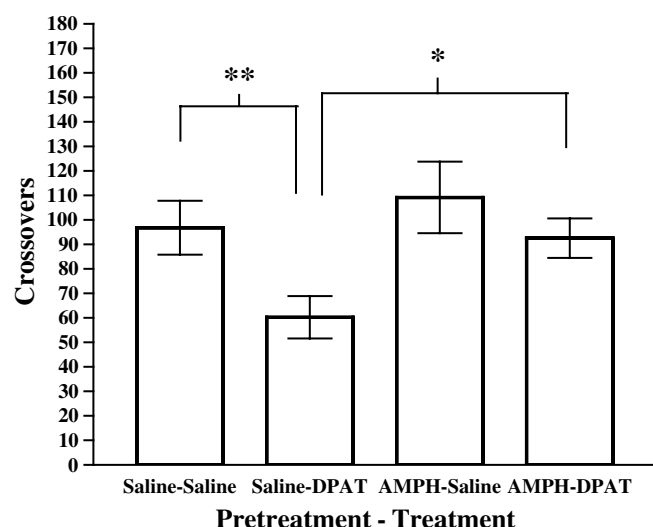
**Table 2** Injection Schedule for Behavioral Sensitization and Treatment with 7-OH-DPAT, Experiment 2

Group	Days		
	1–5	10–11	12
Saline–Saline ( $n=18$ )	Saline	Saline	Saline
Saline–DPAT ( $n=18$ )	Saline	Saline	7-OH DPAT
AMPH–Saline ( $n=19$ )	AMPH	Saline	Saline
AMPH–DPAT ( $n=19$ )	AMPH	Saline	7-OH DPAT

observations). This suggests that dose ranges for mice and rats are comparable, consistent with previously published studies evaluating D<sub>3</sub>-selective doses delivered via subcutaneous injection in adult male Sprague–Dawley rats (Levant *et al*, 1996).

Animals were individually housed and treated in home cages for 5 consecutive days with either saline or AMPH (1 mg/kg) (Table 2). At 5 days following the final injection, animals were moved from their home cages into individual Residential Activity Chambers for both housing and behavioral testing for the remainder of the study. In order to determine if there were differences in conditioned behavioral response to prior treatment experience, on days 5 (experiment day 10) and 6 (experiment day 11) following this 5-day pretreatment regimen all animals were injected with saline and locomotion monitored following injection. Locomotion was determined for 60 min following injection, because this corresponds to the time interval of behavioral response to 7-OH-DPAT under the conditions studied. We tested our prediction that there would be no difference between the groups in locomotor response to saline injection, using a two-tailed test. Comparison of crossover activity during the 0–60 min period following saline injection demonstrates no significant difference between the pretreatment groups on days 10 or 11 (day 10  $p=0.799$ , day 11  $p=0.123$ ), demonstrating lack of a measurable difference in conditioned locomotion response or change in baseline activity between pretreatment groups.

At 1 week following the last pretreatment injection (experiment day 12), rats from each pretreatment group were treated with either saline ((pretreatment–treatment) = (Saline–Saline) or (AMPH–Saline)), or 7-OH-DPAT (10  $\mu$ g/kg) ((pretreatment–treatment) = (Saline–DPAT) or (AMPH–DPAT)) and locomotion recorded following injection. Locomotion was determined for 60 min following injection, because this corresponds to the time interval of behavioral response to 7-OH-DPAT under the conditions studied. Locomotor activity for a period of 0–60 min following injection is shown for each of the four groups in Figure 3. We tested our prediction that there would be no difference between the groups in locomotor response to saline injection, using a two-tailed test. The two pretreatment groups did not differ in locomotor response to saline injection (Saline–Saline vs AMPH–Saline group mean comparison  $p=0.508$ ). A one-tailed test was used to test our prediction that 7-OH-DPAT would inhibit locomotion in the saline pretreatment group, while a two-tailed test was used to test our prediction that 7-OH-DPAT would not inhibit locomotion in the AMPH pretreatment group. As predicted, the pretreatment groups differed significantly in



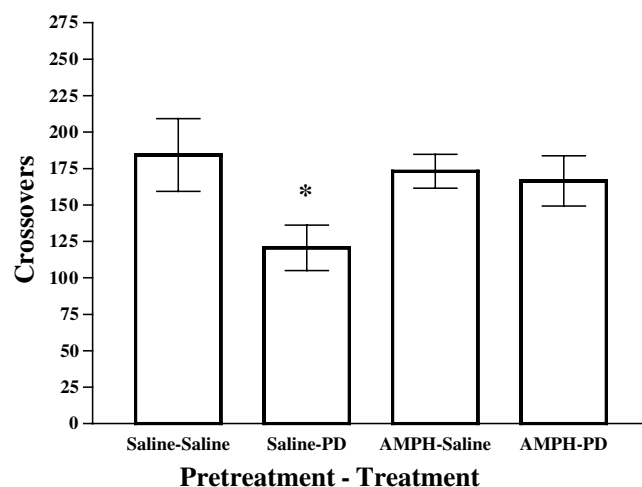
**Figure 3** Geometric mean  $\pm$  SEM of total crossovers for each pretreatment group during the period 0–60 min after 7-OH-DPAT (10  $\mu$ g/kg) or saline injection 5 days following pretreatment with AMPH or saline. Differences between pretreatment groups are denoted by symbols: \*Saline–DPAT group mean vs AMPH–DPAT group mean planned comparison  $p=0.011$ , two-tailed. \*\*Saline–Saline group mean vs Saline–DPAT group mean planned comparison  $p=0.008$ , one-tailed.

**Table 3** Injection Schedule for Behavioral Sensitization and Treatment with PD 128907, Experiment 3

Group	Days			
	1–5	12–14	15	16
Saline ( $n=15$ )	Saline	Saline or PD 128907	Saline	AMPH
AMPH ( $n=14$ )	AMPH	Saline or PD 128907	Saline	AMPH

behavioral response to 7-OH-DPAT. In the saline pretreatment group, treatment with 7-OH-DPAT significantly inhibited locomotion (Saline–DPAT group mean vs Saline–Saline group mean planned comparison  $p=0.008$ , one-tailed). In contrast, locomotor response to 7-OH-DPAT and saline were not significantly different in the AMPH pretreatment group (AMPH–DPAT group mean vs AMPH–Saline group mean comparison  $p=0.329$ ). Saline pretreatment group rats had fewer crossovers than AMPH pretreatment animals following 7-OH-DPAT injection (Saline–DPAT group mean vs AMPH–DPAT group mean planned comparison  $p=0.011$ , two-tailed). These data demonstrate an attenuation of 7-OH-DPAT-induced locomotor inhibition, measured 1 week following the completion of repetitive AMPH pretreatment.

In order to determine if a comparable decrease in behavioral response to the D<sub>3</sub> agonist PD 128907 follows repetitive AMPH pretreatment, a similar experiment was performed, substituting 10  $\mu$ g/kg PD 128907 for 10  $\mu$ g/kg 7-OH-DPAT. We have previously demonstrated that 10  $\mu$ g/kg PD 128907 inhibits novelty-induced locomotion in wild-type mice but is without measurable behavioral effect in D<sub>3</sub> knockout mice (Pritchard *et al*, 2003). Experimental details



**Figure 4** Geometric mean  $\pm$  SEM of total crossovers for each pretreatment group during the period 0–180 min after PD 128907 (10  $\mu$ g/kg) or saline injection 5 days following pretreatment with AMPH or saline. Differences between pretreatment groups are denoted by symbols: \*Saline pretreatment group, PD 128907 vs saline injection planned comparison  $p=0.021$ , one-tailed.

for the AMPH vs saline pretreatment regimen were similar to those described in Experiments 1 and 2; however in this study, animals remained in home cages throughout the study, and were transferred into individual Residential Activity Chambers for behavioral testing only and not for housing. As in the previous study, animals were single housed and treated in home cages for 5 consecutive days with either saline or AMPH (1 mg/kg) (Table 3, Experiment 3). Animals were injected between 7 and 9 days following completion of the pretreatment regimen (study days 12–14) in random order with either saline or PD 128907 (10  $\mu$ g/kg) on a rotating basis, allowing each animal to be used as its own control to measure D<sub>3</sub> agonist-mediated inhibitory effect. Locomotion was determined for 180 min following injection, because this corresponds to the time interval of behavioral response to PD 128907 under the conditions studied. Subsequent data analysis by one-way ANOVA revealed no difference in the order of injection (saline first or PD 128907 first). We tested our prediction that there would be no difference between the groups in locomotor response to saline injection, using a two-tailed test. There was no significant difference between the pretreatment groups in locomotor response to saline ( $p=0.368$ , two-tailed), demonstrating lack of a measurable difference in conditioned locomotion response between pretreatment groups.

Locomotor activity for each of the pretreatment groups for a period of 0–180 min following injection with saline and PD 128907 is illustrated in Figure 4. A one-tailed test was used to test our prediction that PD 128907 would inhibit locomotion in the saline pretreatment group, while a two-tailed test was used to test our prediction that PD 128907 would not inhibit locomotion in the AMPH pretreatment group. The two pretreatment groups did not differ in locomotor response to saline injection (Saline vs AMPH pretreatment group response to saline injection mean comparison  $p=0.368$ , two-tailed). As predicted, however,

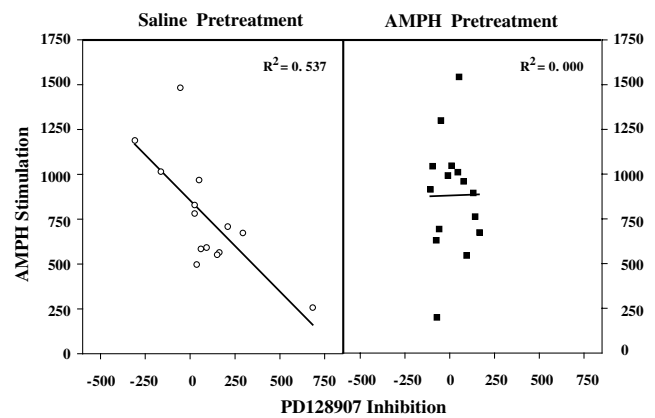
the pretreatment groups differed significantly in behavioral response to PD 128907. In the saline pretreatment group, treatment with PD 128907 significantly inhibited locomotion relative to saline injection (Saline pretreatment group, PD 128907 vs saline injection planned comparison  $p=0.021$ , one-tailed). In contrast, locomotor response to PD 128907 and saline were not significantly different in the AMPH pretreatment group (AMPH pretreatment group, PD 128907 vs saline injection planned comparison  $p=0.754$ ). These data demonstrate an attenuation of PD 128907-induced locomotor inhibition, measured 1 week following the completion of repetitive AMPH pretreatment.

### Behavioral Response to Amphetamine and to D<sub>3</sub> Receptor Agonist PD 128907 are Correlated

The relation between behavioral response to AMPH and behavioral response to D<sub>3</sub> agonist was determined by measuring AMPH-stimulated locomotion in the same animals in which locomotor response to PD 128907 had been previously determined (Table 3 and Figure 4). *AMPH-stimulated locomotion* was determined by injecting all animals with saline on experiment day 15, and the following day injecting all rats with AMPH (Table 3). Locomotion was monitored following each injection, and the relation between *AMPH-stimulated locomotion* and *PD 128907 locomotor inhibition* determined. *AMPH-stimulated locomotion* is defined as (locomotor response to AMPH (day 16))–(locomotor response to saline (day 15)). *PD 128907 locomotor inhibition* is defined as (locomotor response to saline (days 12–14))–(locomotor response to PD 128907 (days 12–14)).

Within the saline pretreatment (ie nonsensitized) group, a plot of *PD 128907 locomotor inhibition vs AMPH-stimulated locomotion* (Figure 5, left panel) resulted in a highly significant inverse linear correlation ( $R^2=0.537$ ). Thus, *rats most inhibited by D<sub>3</sub> agonist had the smallest locomotor response to AMPH*; this relation accounts for over half of the individual variance in locomotor response to AMPH. A similar analysis performed among rats receiving repeated AMPH pretreatment revealed strikingly different results. Within the AMPH pretreatment (ie sensitized) group, a plot of *PD 128907 locomotor inhibition vs AMPH-stimulated locomotion* (Figure 5, right panel) demonstrated no correlation between these two measures ( $R^2=0.000$ ). Thus, in addition to an attenuation of the locomotor response to PD 128907 following AMPH pretreatment, there is an alteration in the relation between locomotor inhibitory response to PD 128907, and locomotor stimulation in response to AMPH.

A relation between locomotor response to novelty and subsequent locomotor response to AMPH and cocaine has previously been described by several (Piazza *et al*, 1989; Hooks *et al*, 1991, 1992), but not all investigators (Djano and Martin-Iverson, 2000). Because locomotor response to novelty might potentially influence locomotor response to saline injection, one of the measures contributing to our defined *PD 128907 locomotor inhibition*, the correlation between AMPH-stimulated locomotion, and responses to PD 128907 and saline (Table 3, days 12–14) were also evaluated individually. Within the saline pretreatment (ie nonsensitized) group, a plot of locomotion response to



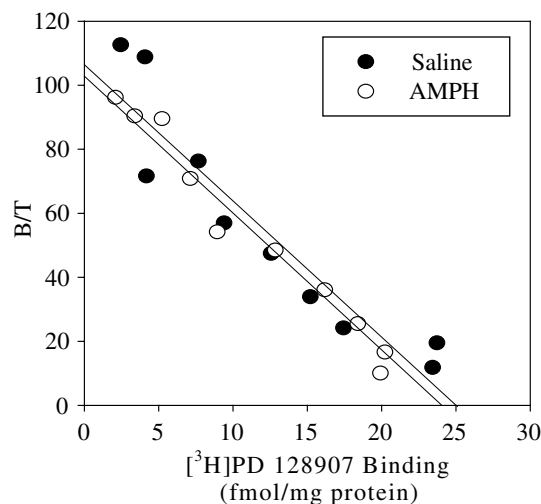
**Figure 5** Correlation between *PD 128907 locomotor inhibition* ((locomotor response to saline (Table 3, days 12–14))–(locomotor response to PD 128907)) vs *AMPH-stimulated locomotion* ((locomotor response to AMPH (day 16))–(locomotor response to saline (day 15))). Left panel, saline pretreatment (days 1–5) group. Right panel, AMPH (1 mg/kg, days 1–5) pretreatment group.

saline injection (Table 3, days 12–14) and subsequent locomotor response to AMPH demonstrated a moderate linear correlation ( $R^2=0.313$ ). Similarly, within the saline pretreatment (ie nonsensitized) group, a plot of locomotion response to PD 128907 injection and subsequent locomotor response to AMPH demonstrated a comparable moderate correlation ( $R^2=0.346$ ). Combining the two terms to plot *PD 128907 locomotor inhibition vs AMPH-stimulated locomotion* resulted in the most highly significant correlation ( $R^2=0.537$ , Figure 5, left panel).

In contrast, within the repeated AMPH pretreatment (ie sensitized) group, a plot of locomotion response to saline injection (Table 3, days 12–14) and subsequent locomotor response to acute AMPH injection failed to demonstrate a correlation between the two measures ( $R^2=0.004$ ). Similarly, within the AMPH pretreatment group a plot of locomotion response to PD 128907 injection and subsequent locomotor response to AMPH failed to identify any correlation between these two measures ( $R^2=0.001$ ). As described above, combining the two terms to plot *PD 128907 locomotor inhibition vs AMPH-stimulated locomotion* within the repeated AMPH pretreatment group similarly demonstrated no correlation ( $R^2=0.000$ ) between these two measures (Figure 5, right panel).

### D<sub>3</sub> receptor Binding is not Altered Following Repetitive Amphetamine Pretreatment

In order to determine whether alterations in D<sub>3</sub> receptor expression or binding affinity might account for the observed alteration in D<sub>3</sub>-mediated function, D<sub>3</sub> receptor binding was evaluated by saturation analysis. Both  $B_{max}$  and  $K_D$  values were determined, in order to identify possible change in affinity state in the absence of change in protein expression. D<sub>3</sub> receptor binding levels were determined 1 week following treatment for 5 days with either saline (1 ml/kg s.c.) or AMPH (1.0 mg/kg s.c.). Animals were randomly assigned among two treatment groups (AMPH 1.0 mg/kg or saline) for five daily treatments on days 1–5. At 1 week following the last pretreatment injection, rats in both



**Figure 6** Rosenthal plot of [<sup>3</sup>H]PD 128907 binding in ventral striatal membranes. Animals were sacrificed 1 week following daily pre-treatment  $\times$  5 days with saline or AMPH (1 mg/kg). Two independent determinations were performed with similar results. Saline-treated animals exhibited [<sup>3</sup>H]PD 128907 binding with a  $K_D$  value of 0.31 nM and a  $B_{max}$  of 25 fmol/mg protein. Amphetamine-treated animals exhibited [<sup>3</sup>H]PD 128907 binding with a  $K_D$  value of 0.27 nM and a  $B_{max}$  of 24 fmol/mg protein.

pretreatment groups were sacrificed by decapitation and brains quickly removed for the determination of D<sub>3</sub> receptor binding. There were no detectable differences between saline and AMPH pretreatment groups in  $B_{max}$  and  $K_D$  values (Figure 6).

## DISCUSSION

### 'D<sub>3</sub> Dopamine Receptor Hypothesis' of Sensitization

The expected physiological response to repetitive drug administration is tolerance (Ramsay and Woods, 1997), that is, drug effects generally become smaller with repeated usage, requiring more drug to achieve the same end point (Jaffe, 1992). We have suggested that tolerance of D<sub>3</sub> receptor inhibition of locomotion contributes to sensitization to stimulant drugs (Richtand *et al*, 2000, 2001a, b). This hypothesis follows directly from the affinities of the receptor subtypes for dopamine; dopamine concentrations following stimulant drug administration; the effects of individual dopamine receptor subtype stimulation on locomotion; and the expected homeostatic response of the system to perturbation by drug. Dopamine receptor subtypes differ widely in affinity for dopamine, and therefore in the dopamine concentration range over which receptor occupancy varies and alterations in cellular signaling occur. The D<sub>3</sub> receptor has the highest affinity for dopamine. Dopamine receptors exhibit differing affinity states for dopamine, termed high-affinity and low-affinity states. The low-affinity  $K_i$  for dopamine for the cloned rat D<sub>3</sub> receptor is in the range of 27–44 nM (Sokoloff *et al*, 1990, 1992; Levesque *et al*, 1992; Burris *et al*, 1995), close to basal extracellular dopamine (3–5 nM (Kalivas and Duffy, 1993; Parsons and Justice, 1992)) and basal synaptic dopamine concentrations ((50 nM (Ross, 1991)). In con-

trast, the low-affinity state  $K_i$ 's for D<sub>1</sub> and D<sub>2</sub> receptors are significantly higher. The low-affinity state  $K_i$  for dopamine for the cloned rat D<sub>2</sub> receptor is in the range of 1705–6500 nM (median 2290 nM) (Levesque *et al*, 1992; Sokoloff *et al*, 1990, 1992; Giros *et al*, 1989), and the low-affinity state  $K_i$  for dopamine for the cloned rat D<sub>1</sub> receptor is between 640 (Monsma *et al*, 1990) and 2300 nM (Zhou *et al*, 1990). The disparity in binding affinity between D<sub>2</sub> and D<sub>3</sub> receptor subtypes is not as great for the high-affinity state receptors. The high-affinity state D<sub>3</sub> receptor exhibits approximately seven-fold greater affinity for dopamine than the high-affinity D<sub>2</sub> receptor (Burris *et al*, 1995), while the high-affinity D<sub>3</sub> receptor has approximately 60-fold greater affinity for dopamine than the high-affinity state D<sub>1</sub> receptor (Seeman, 1999). Because dopamine concentrations are elevated for prolonged periods following stimulant drug administration (Jones *et al*, 1996a,b; Schad *et al*, 2002; Zetterstrom *et al*, 1983), these differing affinities suggest that tolerance would develop differentially for each of these three receptor systems under these conditions, with greatest tolerance of D<sub>3</sub> receptor-mediated effects. The D<sub>3</sub> dopamine receptor behavioral action as a 'brake' on locomotion suggests that tolerance of this inhibition would contribute to sensitization (Richtand *et al*, 2000, 2001a, b).

### Evidence Supporting 'D<sub>3</sub> Dopamine Receptor Hypothesis'

Few studies have tested the D<sub>3</sub> dopamine receptor hypothesis. Consistent with the proposed hypothesis, the D<sub>3</sub> antagonist nafadotride inhibits locomotor sensitization to amphetamine (Richtand *et al*, 2000). Additionally, D<sub>3</sub> receptor binding is reduced in nucleus accumbens following cocaine-induced locomotor sensitization (Wallace *et al*, 1996). Third, other manipulations augmenting responsiveness to stimulant drugs such as exposure to stress in utero- and neonatal hippocampal lesion downregulate D<sub>3</sub> receptor binding in nucleus accumbens (Henry *et al*, 1995; Flores *et al*, 1996). In contrast, other studies failed to detect altered D<sub>3</sub> binding (Stanwood *et al*, 2000) or mRNA expression (Hondo *et al*, 1999) following treatment with cocaine or amphetamine, while studies of D<sub>3</sub> receptor expression in human cocaine-dependent subjects have had widely conflicting results (Staley and Mash, 1996; Mash and Staley, 1999; Segal *et al*, 1997; Meador-Woodruff *et al*, 1995). These divergent findings suggested a need to test more directly the D<sub>3</sub> receptor hypothesis.

### Release of D<sub>3</sub> Receptor-Mediated Inhibition Following Repetitive Amphetamine

Here we report data providing direct support for the hypothesis that expression of locomotor sensitization results in part from release of D<sub>3</sub> receptor-mediated inhibitory influence. We demonstrate decreased locomotor inhibitory response to D<sub>3</sub>-selective doses of both 7-OH-DPAT and PD 128907 following a sensitizing AMPH regimen. Moreover, in addition to the quantitative modification, there is an alteration in the relation between locomotor response to PD 128907 and locomotor response to AMPH. Nonsensitized rats exhibit a linear correlation between PD 128907 locomotor inhibition and AMPH-

*stimulated locomotion*, surprisingly accounting for over half of the individual variance in locomotor response to AMPH. This relation is not detected 7 days following repetitive AMPH treatment. The time course of this altered behavioral response is not presently known. While many examples of acute alteration following stimulant drug use have been described, there are far fewer recognized examples of persistent, enduring change following drug exposure.

### D2 vs D3 Receptor Inhibitory Effects

Our findings must be interpreted in the context of the known narrow selectivity of 7-OH-DPAT and PD 128907 at the D<sub>3</sub> vs D<sub>2</sub> receptor. We have recently described that low 7-OH-DPAT and PD 128907 doses (10 µg/kg) inhibit novelty-induced locomotion in wild-type mice, but are without behavioral effect in D<sub>3</sub> receptor knockout mice (Pritchard *et al*, 2003). Similarly low PD 128907 doses (0.03–0.3 mg/kg) inhibited dopamine release in wild-type, but not in D<sub>3</sub> receptor knockout mice, further demonstrating D<sub>3</sub>-selective effects of PD 128907 at sufficiently low dose (Zapata *et al*, 2001). We have been unable to detect locomotor inhibition in rats at 7-OH-DPAT or PD 128907 doses below 10 µg/kg (Richtand *et al*, unpublished observations). Nonetheless, given the differences between rat and mouse, it is not possible to exclude the possibility that our observed locomotor inhibitory effects of 7-OH-DPAT and PD 128907 may be mediated in part through D<sub>2</sub> autoreceptors, or other receptors. If our findings with 7-OH-DPAT and PD 128907 were mediated via the D<sub>2</sub> receptor this would be consistent with earlier electrophysiological and *in vitro* slice preparation studies hypothesizing that downregulation of inhibitory D<sub>2</sub> autoreceptors on dopaminergic cell bodies and nerve terminals contributes to sensitization (Kamata and Rebec, 1984; White and Wang, 1984). It is difficult, however, to reconcile the known time course of D<sub>2</sub> autoreceptor desensitization, which is transient, with an interpretation of our findings being mediated through the D<sub>2</sub> autoreceptor. Previous electrophysiological studies have clearly delineated a *time-limited, transient* decreased autoreceptor response following a brief 1–4 days withdrawal interval (White and Wang, 1984; Wolf *et al*, 1993). In marked contrast, behavioral sensitization persists even after a much longer 1-week withdrawal interval. Taken together, we believe that these observations strongly suggest that the locomotor inhibitory effects of both 7-OH-DPAT and PD 128907 described in our study are mediated primarily via D<sub>3</sub> receptor stimulation. This stimulation could occur either postsynaptically, or alternatively at a presynaptic D<sub>3</sub> receptor site. Clearly, more work is needed to determine unambiguously the receptor mediating our observed behavioral effects.

### Locus of D3 Receptor-Mediated Inhibitory Effects

The decline in behavioral response to D<sub>3</sub> agonists following behavioral sensitization could be mediated directly at the level of the D<sub>3</sub> receptor, via downstream second messenger signaling, or through cellular and/or systems effects even more distantly removed from the D<sub>3</sub> receptor. Our findings may be related, at least in part, to the well-described increase in sensitivity of nucleus accumbens D<sub>1</sub> receptors

following sensitization (Henry and White, 1991; Hu *et al*, 2002; Beurrier and Malenka, 2002). The majority of D<sub>3</sub> expressing neurons in islands of Calleja and nucleus accumbens shell also express D<sub>1</sub> receptor mRNA (Schwartz *et al*, 1998). While D<sub>1</sub> expression in nucleus accumbens is approximately 10-fold greater than D<sub>3</sub> receptor expression (Richtand *et al*, 1995), decreased D<sub>3</sub> receptor-mediated opposition to D<sub>1</sub> receptor signaling in accumbens could potentially contribute to increased responsiveness of D<sub>1</sub> receptor stimulation following repetitive stimulant drug administration. This interaction could be mediated, either at the cellular or systems level, by opposing effects on adenylate cyclase activity, and/or on other second messenger systems.

These potential interactions highlight a proposed model in which D<sub>3</sub> receptor stimulation contributes to sensitization through interaction with other systems, but is insufficient to account for sensitization independent of other systems. Thus, the proposed model does not contradict the failure of repetitive treatment with D<sub>3</sub>-selective doses of 7-OH-DPAT to induce sensitization (Mattingly *et al*, 1996). For example, increased dopaminergic cell activity because of alterations in glutamatergic activity in ventral tegmentum during early withdrawal stages from cocaine and amphetamine (Henry *et al*, 1989; Zhang *et al*, 1997; White *et al*, 1995) could be expected to result in increased impulse-dependent dopamine release in terminal fields. This in turn would result in preferential decline in D<sub>3</sub> receptor-mediated inhibitory function in those terminal fields, according to our model, illustrating an example of an interaction between the D<sub>3</sub> receptor and other components of the 'sensitization system', which would contribute to the subsequent expression of behavioral sensitization. While this model is highly speculative, it provides a framework for directly testing a hypothesis of sensitization.

### Long-term Consequences of Stimulant Drug Exposure

The D<sub>3</sub> receptor has wide expression throughout limbic brain regions, with densest expression in islands of Calleja and other regions of dorsal and ventral striatum (Diaz *et al*, 2000; Khan *et al*, 1998; Larson and Ariano, 1995; Ariano and Sibley, 1994). D<sub>3</sub> receptor binding characteristics ( $B_{\max}$  and  $K_D$ ) in dorsal and ventral striatum were unchanged by AMPH treatment in our study. In contrast, others have reported increased (Staley and Mash, 1996; Mash and Staley, 1999; Segal *et al*, 1997), decreased (Wallace *et al*, 1996), and unchanged (Stanwood *et al*, 2000; Hondo *et al*, 1999; Meador-Woodruff *et al*, 1995) D<sub>3</sub> receptor binding or mRNA expression following repetitive stimulant exposure. This could reflect differences between the effects of AMPH and cocaine, or in other experimental details between studies such as dose, withdrawal period, etc. Alternatively, this variability may reflect the fact that all of these studies, including ours, measure D<sub>3</sub> receptor binding or mRNA expression, as opposed to D<sub>3</sub> receptor function. In a similar fashion, studies examining the effects of repetitive opiate administration on the closely homologous  $\mu$  opiate receptor were unable to detect consistent alterations in  $\mu$  opiate receptor mRNA or receptor binding levels (reviewed in Sim *et al*, 1996). In contrast, decreased  $\mu$  opioid receptor function was identified in specific brain regions following



chronic opiate treatment using agonist-stimulated GTP $\gamma$ S autoradiography (Sim-Selley *et al*, 2000; Sim *et al*, 1996). Studies of the effects of repetitive stimulant administration on D<sub>3</sub> receptor function within specific brain regions may therefore provide a more informative neuroanatomical test of the D<sub>3</sub> receptor hypothesis of sensitization.

Alternatively, altered D<sub>3</sub> receptor mRNA expression, receptor binding, and/or receptor function in a brain region not analyzed in our study, such as prefrontal cortex (PFC), may mediate the observed behavioral change. Low D<sub>3</sub> expression prevented accurate determination of D<sub>3</sub> receptor binding characteristics in PFC with the methods used in the current study. It has been suggested that release of inhibition of excitatory efferent projections from PFC may contribute to sensitization (reviewed in Wolf, 1998). D<sub>2</sub>-family receptors in PFC inhibit stimulant-induced locomotion and stereotyped behavior, and function of a 'D<sub>2</sub>-family' receptor in PFC is lost following sensitization to cocaine or AMPH (Karler *et al*, 1998a, b). Additional study is needed to determine whether this 'D<sub>2</sub>-family receptor' is of the D<sub>3</sub> receptor subtype, and is identical to the D<sub>3</sub> receptor population mediating the effects described in our study.

### Comparison with Previous Findings

In contrast to our findings, a previous study reported that cocaine sensitization did not prevent the hypolocomotor effects of a low PD128907 dose, while the behavioral response to a higher nonselective dose was diminished, suggesting that altered D<sub>3</sub> receptor sensitivity does not play an important role in sensitization (Prinssen *et al*, 1998). Substantial methodological differences may account for the opposite conclusion of our study, including rodent species (rats *vs* mice) and stimulant drug (AMPH *vs* cocaine). On close inspection, however, the data presented previously (Prinssen *et al*, 1998) are quite similar to our findings. At the lowest, most D<sub>3</sub>-selective dose producing a hypolocomotor response in their study (10  $\mu$ g/kg, identical to the dose used in our study), PD 128907 inhibited locomotion 25–35% in the cocaine 20 mg/kg pretreatment group, compared to approximately 60% inhibition in the saline pretreatment group. Their observed decrease in locomotor inhibition is qualitatively similar to our findings, and raises the possibility that an experimental design with more statistical power would have detected meaningful differences between pretreatment groups in their study.

We observed a negative correlation between locomotor response to saline injection and initial locomotor response to AMPH, in contrast to earlier reports of a positive correlation between heightened locomotor response to novelty and increased locomotor response to AMPH and cocaine in some (Piazza *et al*, 1989; Hooks *et al*, 1991, 1992) but not all (Djano and Martin-Iverson, 2000) studies. Differences in experimental details (eg measurement of response to novelty *vs* our measure, response to saline injection in a novel environment) might account for the differing observations.

In summary, we demonstrate decreased behavioral response to D<sub>3</sub> agonists 7-OH-DPAT and PD 128907 following locomotor sensitization to AMPH. Whether 7-OH-DPAT and PD 128907 inhibit locomotion through a selective D<sub>3</sub> receptor or combined D<sub>2</sub>/D<sub>3</sub> receptor

mechanism, these findings suggest that tolerance of inhibitory effects upon locomotion contribute to the expression of locomotor sensitization to stimulant drugs. Additional study is needed to clarify the role of individual DA receptor subtypes in sensitization, and to further test the hypothesis that persistent adaptive release of D<sub>3</sub> receptor-mediated inhibitory influence contributes to behavioral sensitization.

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