Behavioral Sensitization, Alternative Splicing, and D3 Dopamine Receptor-Mediated Inhibitory Function

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Behavioral sensitization, the progressive and enduring augmentation of certain behaviors following repetitive drug use, alters rodent locomotion in a long-standing manner. The same dopamine pathways playing an important role in drug dependence and psychosis also play a critical role in sensitization. Individual dopamine receptor subtypes have markedly different functional responses to stimulation, with D3 dopamine receptor stimulation inhibiting rodent locomotion. The D3 receptor has highest affinity of the dopamine receptor subtypes for dopamine, and is occupied to a greater degree following stimulant drug administration. D3 receptor activity may be regulated through the expression of an alternatively spliced, truncated receptor isoform (termed 'D3nf') altering receptor localization and function via dimerization with the full-length subunit. The expected physiological response to repetitive drug administration is tolerance. Tolerance of D3 receptor inhibition of locomotion would contribute to sensitization to stimulant drugs. We hypothesize that repetitive D3 receptor stimulation contributes to the development of behavioral sensitization through decreased responsivity of D3-receptor-mediated locomotor inhibition. Increased D3nf expression may direct altered receptor localization and subsequent release of D3-receptor-mediated inhibition, contributing to the expression of sensitization. These hypotheses follow directly from the affinities of the receptor subtypes for dopamine; dopamine concentrations following stimulant administration; the effects of individual dopamine receptor subtype stimulation on locomotion; and the expected homeostatic response of the system to perturbation by drug. Clarifying these mechanisms underlying sensitization may suggest new interventions for neuropsychiatric conditions in which dopamine plays an important role, including psychosis, drug dependence, and Parkinson's disease. This information may also elucidate a previously unrecognized mechanism regulating receptor trafficking and desensitization.
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INTRODUCTION

Although David Segal has sadly passed away, he leaves as one legacy to those who were fortunate to have had the opportunity to know and admire him, an enduring interest in the paradox of behavioral sensitization. Behavioral sensitization, the progressive and long-lasting augmentation of certain behaviors following repetitive stimulant drug administration (Segal and Mandell, 1974), is a well-studied model of behavioral plasticity. Following repeated, intermittent treatment with stimulant drugs such as amphetamine (AMPH) or cocaine, sensitized behaviors may occur more intensely, with shorter latency, and/or at lower dose than before sensitization (Segal *et al*, 1980). The paradox of sensitization is that the expected response of a biological system to repetitive stimulation is tolerance, rather than augmentation of the behavioral response. David's view of this was that it captured the essence of science: that both the work, and the joy, of science begin with the unexpected result, in determining a context in which the unexpected finding fits logically and make sense with the other pieces of the puzzle, that is, in making the paradox unparadoxical. Here we describe a theoretical model of behavioral sensitization, which attempts to do that, by viewing sensitization as tolerance of inhibitory systems, rather than an increase following repeated drug exposure. Evidence for the role of the dopamine D3 receptor in this adaptation is presented, and RNA alternative splicing is suggested as a possible underlying mechanism.

DEVELOPMENT *VS* EXPRESSION OF BEHAVIORAL SENSITIZATION

Behavioral sensitization may be separated into two distinct components, both temporally and spatially, termed 'development' (or 'initiation'), and 'expression.' Initiation of

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sensitization involves both D1 dopamine (DA) receptor and glutamate receptor activation within ventral tegmentum and substantia nigra (Bjijou et al, 1996; Wolf et al, 1995; Kalivas and Alesdatter, 1993). Projections from prefrontal cortex and amygdala also participate in the initiation of sensitization (Wolf, 1998). Expression of sensitization, in contrast, is believed to result from changes in the nucleus accumbens. In general, changes in the ventral tegmentum believed to be related to the initiation of sensitization are transient, such as decreased sensitivity of somatodendritic D2 subtype dopamine autoreceptors (Kamata and Rebec, 1984; White and Wang, 1984; Henry et al, 1989; Ackerman and White, 1990). In contrast, neurochemical changes in the nucleus accumbens accompanying sensitization are persistent (Wolf, 1998), such as increased D1 receptor electrophysiological responsiveness (Henry and White, 1991; Higashi et al, 1989) and upregulation of cAMP signal transduction (Nestler et al, 1990; Terwilliger et al, 1991). Some of the changes in the nucleus accumbens, such as augmentation of the increase in extracellular dopamine elicited by stimulants (Robinson et al, 1988; Kalivas and Duffy, 1990), require a withdrawal time interval to be manifest. The augmentation in behavioral response is an enduring alteration, persisting for at least 1 year in rats (Paulson et al, 1991). Evidence from several labs suggests sensitization is dependent on new protein synthesis (Karler et al, 1993; Sorg and Ulibarri, 1995).

BEHAVIORAL CHARACTERISTICS OF SENSITIZATION

In humans, sensitized behaviors following repetitive stimulant drug administration frequently include psychotic symptoms of paranoia, ideas of reference, and auditory and visual hallucinations in otherwise healthy individuals (Ellinwood, 1967; Griffith et al, 1972; Utena, 1966; Sato et al, 1983). Human symptoms following repetitive stimulant drug use evolve gradually from intense curiosity, progressing to intense exploration of the environment, which may be displayed in repetitive stereotyped searching, sorting, and examining behaviors. This curious 'suspiciousness' of the environment later evolves into paranoia and psychotic thought (Ellinwood et al, 1973; Segal et al, 1981; Segal and Schuckit, 1983). Because pretreatment with AMPH facilitates the later acquisition of drug self-administration in rodents (Piazza et al, 1989), it has also been theorized that sensitization may underlie the development of drug craving, and thus initiate addictive behaviors seen in human drug dependence (Robinson and Berridge, 1993).

Specific behaviors sensitized in rodents depend upon drug dose, with stimulant-induced locomotion enhanced following sensitization with low doses of stimulant drug. At higher drug doses, sensitization of stereotyped behaviors emerges. The initial increase in exploratory locomotion, and later repetitive-focused stereotyped movements observed in rats with progressively increasing drug dose, is in some respects analogous to the evolution of the human behavioral pattern of response to stimulants. Although this description highlights common features shared by rodent behavioral sensitization and the development of psychosis in man (Segal and Mandell, 1974; Segal et al, 1981; Bell, 1973; Segal and Schuckit, 1983; Sato et al, 1983; Lieberman et al, 1997),

the rodent brain and behavioral repertoire is far more limited and less complex than its human counterpart, and there is no direct correspondence between rodent behavioral sensitization and human psychiatric disease. There is, however, strong evidence that behavioral sensitization is related in large part to plasticity of limbic neurochemical systems, which also play an important role in a range of psychiatric and substance use disorders. Thus primitive, highly conserved structures modulating emotional homeostasis are likely shared by systems contributing to behavioral sensitization, psychosis, and drug dependence.

DOPAMINE AND BEHAVIORAL SENSITIZATION

Brain DA systems play an important role in behavioral sensitization, as demonstrated in lesion studies (Creese and Iversen, 1975; Creese and Iversen, 1972; Fog et al, 1970; Roberts et al, 1975; Pijnenburg et al, 1975; Costall and Naylor, 1974), and microinjection studies of both dopamine agonists (Hitzemann et al, 1980; Kalivas and Weber, 1988; Vezina and Stewart, 1990) and antagonists (Stewart and Vezina, 1989). Dopamine-mediated effects occur through interaction with two pharmacologically distinct receptor families, D1 family (including D1 and D5 receptors) and D2 family (D2, D3, and D4 receptors).

D3 dopamine receptor mRNA and protein expression are limited primarily to olfactory tubercle, nucleus accumbens, and islands of Calleja (located ventral to the ventral pallidum and nucleus accumbens), phylogenetically ancient limbic brain regions linked to motivated and emotional behaviors (Levesque et al, 1992; Bouthenet et al, 1991; Richtand et al, 1995; Landwehrmeyer et al, 1993; Gurevich and Joyce, 1999; Levant, 1998). Protein and mRNA expression are highly colocalized, suggesting that receptor expression occurs primarily on perikarya, proximal dendrites, and short axons as opposed to long axon terminals from other brain regions (Levesque et al, 1992).

Because D3 receptors exhibit functional as well as physical interactions with both D1 and D2 receptors (Scarselli et al, 2001; Zeng et al, 2006), it is of interest that receptor colocalization studies indicate that D1 and D3 receptors are coexpressed in islands of Calleja, nucleus accumbens, and caudate (Schwartz et al, 1998; Ridray et al, 1998; Surmeier et al, 1996; Le Moine and Bloch, 1996). In human brain, most D3 mRNA-expressing cells also express D2 mRNA (Gurevich and Joyce, 1999), whereas in rodent brain, in contrast, D2 and D3 receptors appear to have predominantly complementary rather than overlapping patterns of expression (Bouthenet et al, 1991). In the nucleus accumbens shell, colocalized D1 and D3 receptors exert synergistic effects on substance P gene expression (Ridray et al, 1998; Schwartz et al, 1998). In contrast, D1 and D3 receptor activation in the islands of Calleja exert opposite effects on c-fos expression, presumably through opposing actions on adenylate cyclase activity (Ridray et al, 1998; Schwartz et al, 1998). Similarly, D2 receptors in the nucleus accumbens core and D3 receptors in the nucleus accumbens shell region play opposing roles in neurotensin expression (Diaz et al, 1994).

D3 receptor occupation by agonist appears to inhibit neuronal firing (Liu et al, 1994a). Following stimulant drug

administration, D1 and D3 receptors exert opposing effects on intracellular signaling pathways and gene expression through opposite regulatory effects on extracellular signal-regulated kinase (ERK) activity and expression of Fos family genes (Zhang *et al*, 2004). Behaviorally, although acute concurrent D1/D2 dopamine receptor activation stimulates rodent locomotion (Dalia *et al*, 1998; Dreher and Jackson, 1989), D3 receptor stimulation inhibits both novelty-stimulated locomotion (Pritchard *et al*, 2003; Xu *et al*, 1997; Menalled *et al*, 1999; Ekman *et al*, 1998; Accili *et al*, 1996) and AMPH-stimulated locomotion (Waters *et al*, 1993). One model, as presented in this paper, suggests the loss of a D3 receptor-mediated 'brake' on these behaviors would be expressed as the emergence of augmented locomotor response to novelty and AMPH in rodents, and might also be expressed as paranoid psychotic symptoms in humans.

D3 DOPAMINE RECEPTOR THEORY OF SENSITIZATION

Dopamine receptor subtypes differ widely in affinity for dopamine and the dopamine concentration range over which receptor occupancy varies and alterations in cellular signaling occur. The D3 receptor has highest affinity for dopamine, with low-affinity state $K_i = 30$ nM (Sokoloff *et al*, 1992), close to basal extracellular (3–5 nM; Kalivas and Duffy, 1993; Parsons and Justice, 1992) and synaptic dopamine concentrations (50 nM; Ross, 1991). In contrast, D1 and D2 receptor affinity for dopamine are far lower: D2 K_i (nM) = 2000 and D1 K_i (nM) = 2300 (Sokoloff *et al*, 1992). Following stimulant drug administration, dopamine concentrations are elevated for prolonged periods (Jones

et al, 1996a, b), with average concentrations of 750 nM (Zetterstrom *et al*, 1983). At that dopamine concentration, calculated low-affinity state receptor occupancy is 96% for D3 receptor, compared to 25% for D1 and 27% for D2 receptor. These differences in receptor occupancy suggest the likelihood that tolerance would develop differentially for each of the three-receptor systems, with greatest tolerance of D3-receptor-mediated inhibitory effects. The D3 dopamine receptor behavioral action as a 'brake' on locomotion suggests that tolerance of this inhibition would contribute to sensitization (Richtand *et al*, 2001a, b).

D3 DOPAMINE RECEPTOR ALTERNATIVE SPLICING

The single gene coding for the D3 receptor is organized to allow for the production of different transcripts via alternative splicing of D3 receptor heteronuclear-RNA (hn RNA or pre-mRNA) in the cell nucleus, as illustrated in Figure 1 (Griffon *et al*, 1996; Fu *et al*, 1995; Giros *et al*, 1991; Park *et al*, 1995). At least seven distinct alternatively spliced D3 receptor variants are produced, including the full-length D3 receptor (called 'D3'), and a shorter receptor isoform, D3S, lacking 21 amino acids within the third cytoplasmic loop (Fishburn *et al*, 1993). Both D3 and D3S exhibit high-affinity dopamine binding. Five additional alternatively spliced variants have also been described, which do not bind dopamine, and are believed to function instead through the regulation of receptor dimerization (Snyder *et al*, 1991). These include D3 (TM3-del) (Giros *et al*, 1991; Snyder *et al*, 1991), D3 (TM4-del) (Nagai *et al*, 1993), D3 (O2-del) (Giros *et al*, 1991), rD3in (Pagliusi *et al*, 1993), and D3nf (Schmauss *et al*, 1993; Liu *et al*, 1994b). D3nf is the best characterized of the non-dopamine-binding splice
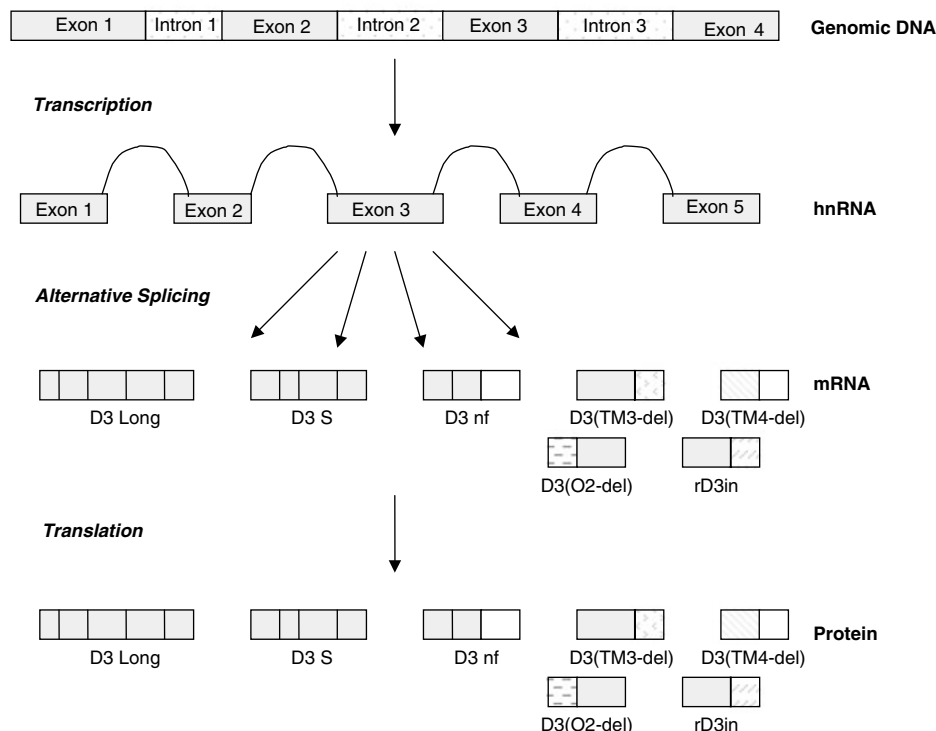

Figure I D3 receptor alternative splicing.

variants. D3nf is formed through a deletion of 98 bp in the third cytoplasmic loop, causing a coding frame shift resulting in the creation of a novel 55-amino-acid peptide and appearance of a new premature stop codon. The prematurely truncated protein thus lacks transmembrane domains 6 and 7 (Schmauss *et al*, 1993; Liu *et al*, 1994b), and does not bind dopamine (Elmhurst *et al*, 2000). D3nf mRNA and protein are expressed in rat, monkey, and human brain (Nimchinsky *et al*, 1997; Liu *et al*, 1994b). Importantly, the highly conserved nature of D3nf splicing and protein expression, from rat to human, suggest D3nf likely plays an important, although currently unknown, biological function. Evidence from three laboratories demonstrates that D3nf binds to the full-length D3 receptor subunit (Nimchinsky *et al*, 1997; Elmhurst *et al*, 2000; Karpa *et al*, 2000). D3nf expression inhibits dopamine binding to full-length D3 receptor (Elmhurst *et al*, 2000), and also redirects full-length D3 receptor localization away from the plasma membrane, and instead into an intracellular compartment (Karpa *et al*, 2000). Importantly, D3 mRNA expression is decreased in the cortex of schizophrenia patients (Schmauss *et al*, 1993), whereas increased D3nf splicing efficiency was observed in the cortex of post-mortem tissue from schizophrenia patients (Schmauss, 1996). These findings suggest that increased D3nf expression may contribute to functional states of altered dopaminergic activity. Collectively, these observations suggest that, in a manner analogous to the way that dimerization plays an important role in the modulation of cell signaling for the homologous insulin- and gonadotropin-releasing hormone receptors (Kahn *et al*, 1981; Gregory *et al*, 1982), D3nf dimerization with full-length D3, D2, or D1 receptor could regulate dopamine signaling. Thus, alterations in D3 receptor isoform expression could modulate the effects of dopamine stimulation in altered functional states such as behavioral sensitization.

D3 RECEPTOR ALTERNATIVE SPLICING AND BEHAVIORAL SENSITIZATION

The information described above suggests a sensitization model of response to repetitive stimulant drug exposure illustrated in Figure 2: (1) AMPH increases extracellular dopamine, activating opposing second messenger signaling systems downstream of D1 and D3 receptor stimulation. (2) D3 and D1 receptor stimulations interact at the second messenger level (Ridray *et al*, 1998; Schwartz *et al*, 1998), for example, through opposite regulatory effects on ERK activity and Fos gene family expression (Zhang *et al*, 2004). This interaction is depicted in Figure 2 as occurring within the same cell because D3 and D1 receptors are frequently coexpressed (Schwartz *et al*, 1998); however, the interaction could also occur at the systems level (Xu *et al*, 1997; Jung and Schmauss, 1999; Levavi-Sivan *et al*, 1998). The D3 receptor has highest dopamine affinity, and persistent D3 receptor stimulation following dopamine transporter blockade results in homeostatic mechanisms opposing receptor stimulation. This homeostatic response would include (3) a change in D3 hnRNA alternative splicing in the cell nucleus resulting in increased D3nf expression. D3nf is a receptor splice variant whose function opposes D3 receptor stimula-
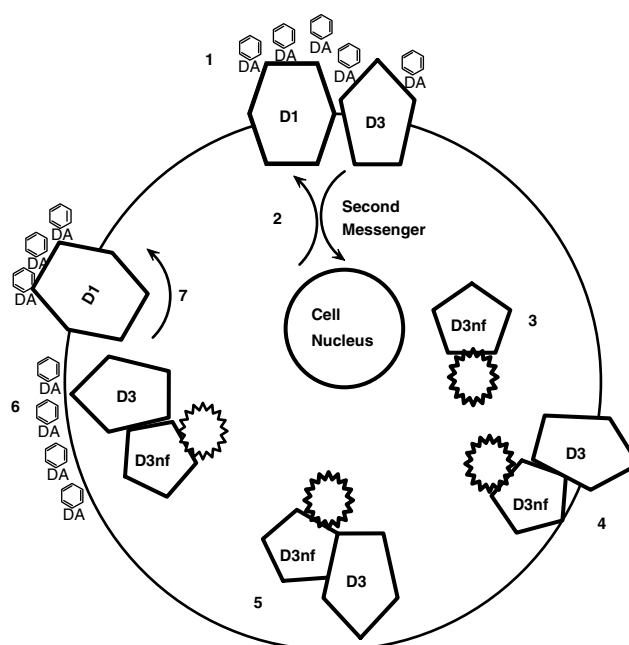
Figure 2 D3/ D3nf mechanism of behavioral sensitization to AMPH. (1) AMPH increases extracellular dopamine. (2) D1 and D3 receptor stimulation activate opposing intracellular signaling systems. D3 receptor has highest dopamine affinity. D3 stimulation results in homeostatic mechanisms opposing receptor stimulation, including (3) increased D3nf expression. (4) D3nf and D3 dimerize, directing the D3/D3nf dimer (5) toward intracytoplasmic trafficking pools and removing D3 receptor from the synaptic membrane. (6) At the next AMPH exposure, D3 receptor is not available to bind dopamine. (7) The result is release of D3 receptor-mediated opposition to D1 receptor stimulation of adenylate cyclase activity. For additional details see in the text.

tion. D3nf shares an NH3-terminus 'dimerization domain' with the D3 receptor, resulting in (4) dimerization between D3nf and D3. D3nf also contains a unique protein sequence at the COOH-tail, which may direct the D3/D3nf dimer (5) toward intracytoplasmic trafficking pools, thereby removing the D3 receptor from a functional position at the synaptic membrane. In this manner, at the next AMPH exposure (6), the D3 receptor is localized in an intracytoplasmic pool, and is not available to bind dopamine at the synapse. The resulting loss of D3 receptor 'brake', at both the cellular and systems level, includes, for example (7), the loss of D3-receptor-mediated opposition to D1 receptor intracellular signaling.

EVIDENCE FOR D3 RECEPTOR THEORY OF BEHAVIORAL SENSITIZATION

Experimental evidence supporting the D3 receptor model of sensitization comes from three lines of data: (1) the effect of D3 receptor antagonists on the development of sensitization; (2) loss of D3 receptor function following sensitization; and (3) decreased D3 receptor mRNA and binding following sensitization.

If tolerance of persistent D3 receptor stimulation contributes to the development of sensitization, blockade with D3 receptor antagonist should inhibit the development of sensitization to dopamine agonists. Consistent with this

prediction, studies in different laboratories demonstrate inhibition of locomotor sensitization by pretreatment with the D3 receptor antagonists nafadotride (Richtand *et al*, 2000), U99194A, or GR103691 (Chiang *et al*, 2003). Additionally, the effect of U99194A was attenuated by pretreatment with the D3 receptor agonist 7-OH-DPAT (Chiang *et al*, 2003), providing further evidence in support of a model in which D3 receptor stimulation contributes to the development of sensitization.

BEHAVIORAL RESPONSE TO D3 RECEPTOR AGONIST IS DIMINISHED IN AMPH-SENSITIZED RATS

A second, independent line of evidence regarding the D3 hypothesis relates to D3 function in sensitized animals. If persistent release of D3-receptor-mediated inhibitory influence contributes to the expression of sensitization, the behavioral response to D3 agonists should be attenuated in sensitized animals. Consistent with this prediction, D3-receptor-mediated locomotor inhibition is dramatically reduced in sensitized rats (Richtand *et al*, 2003). We determined the behavioral response to D3 receptor agonists 7-OH-DPAT and PD 128907 1 week following repetitive AMPH treatment. Locomotor response to novelty is inhibited by the D3 agonists PD 128907 (shown in Figure 3) and 7-OH-DPAT in animals pretreated with a 5-day saline injection regimen, whereas this D3-receptor-mediated locomotor inhibition is not detectable following completion of a sensitizing AMPH regimen. This study also found, unexpectedly, that the locomotor response to AMPH, and locomotor inhibition by D3 agonist, are negatively correlated within individual animals, accounting for over 50% of the individual variance in locomotor response to AMPH, and that this relationship is abolished following repetitive AMPH treatment. In combination, these findings suggest that release of D3-receptor-mediated inhibition contributes to the expression of behavioral sensitization (Richtand *et al*, 2003).

LONG-TERM CONSEQUENCES OF STIMULANT DRUG EXPOSURE

The third line of evidence regarding the D3 hypothesis relates to underlying mechanism. If a persistent decline of D3-receptor-mediated inhibition contributes to sensitization, the underlying mechanism should be evident through analysis of the effects of repetitive stimulant exposure on D3 mRNA, binding activity, downstream signaling pathways, or other indices of receptor function. The evidence in this area to date, on balance, has not been conclusive, perhaps in part because direct measures of D3 receptor *function* have been elusive. In support of the D3 receptor hypothesis, multiple studies in different laboratories have observed decreases in measures of D3 receptor expression in sensitized animals. D3 receptor mRNA and protein expression are both decreased in AMPH-sensitized rats (Chiang *et al*, 2003), and D3 binding in the nucleus accumbens is also decreased following locomotor sensitization to cocaine (Wallace *et al*, 1996). D3 receptor binding is also decreased in the nucleus accumbens in rat offspring exposed to stress in late pregnancy, a manipulation causing increased locomotor
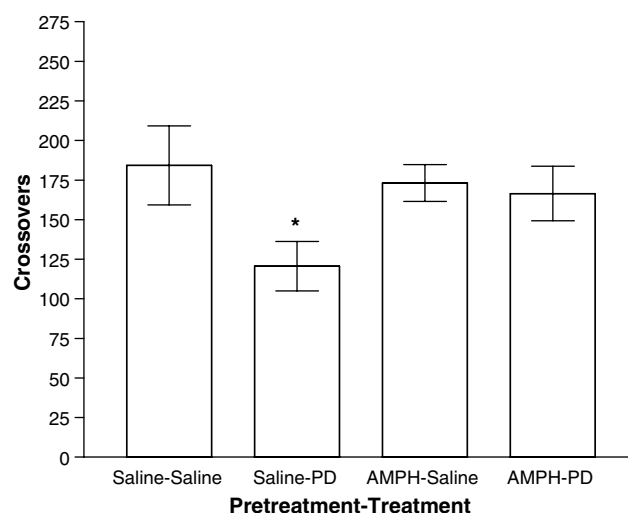
Figure 3 Behavioral response to PD 128907 is diminished in behaviorally sensitized rats. Locomotion following injection with saline and PD 128907 is shown following saline or AMPH pretreatment. *Saline pretreatment group*: *PD 128907 *vs* saline injection planned comparison $p = 0.021$, one-tailed. *AMPH pretreatment group*: PD 128907 *vs* saline injection planned comparison $p = 0.754$. *Saline vs AMPH* pretreatment group response to saline injection mean comparison $p = 0.368$, two-tailed. *Methods*: Male Sprague–Dawley were treated in home cages for 5 consecutive days with either saline or AMPH (1 mg/kg). On days 12–14, rats were injected in random order with either saline or PD 128907 (10 μg/kg) on a rotating basis, allowing each animal to be used as its own control to measure D3-agonist-mediated inhibitory effect. Data analysis by one-way ANOVA revealed no difference in order of injection (saline first or PD 128907 first). Crossover data from 0 to 180 min following injection with saline and PD 128907 were analyzed using ANOVA techniques for independent observations. Results are expressed as geometric mean number of crossovers, ±SE, for each group. Planned *t*-tests were performed for hypotheses of interest. Adapted from Richtand *et al* (2003).

response to novelty, more rapid locomotor sensitization, and increased AMPH self-administration (Henry *et al*, 1995). D3 receptor binding is also decreased in adult rats following neonatal hippocampal lesion (Flores *et al*, 1996), suggesting hyperlocomotor behavioral responses in that model also result from decreased D3 receptor inhibitory function (Wan and Corbett, 1997).

In contrast to the studies described above, we have not detected significant alterations in full-length D3 mRNA expression (Hondo *et al*, 1999) or D3 receptor binding in ventral striatum of AMPH-treated rats (Richtand *et al*, 2003). Studies of D3 expression in human cocaine-dependent subjects have also had conflicting results (Staley and Mash, 1996; Mash and Staley, 1999; Segal *et al*, 1997; Meador-Woodruff *et al*, 1995). In one series studying a small number of subjects ($n = 6$/group), D3 receptor binding and mRNA were both increased in cocaine overdose victims in comparison to age-matched control subjects; however, comparable changes were not observed in cocaine delirium victims (Staley and Mash, 1996; Mash and Staley, 1999; Segal *et al*, 1997). In contrast, another study failed to detect any difference between control and cocaine-abusing subjects in D3 mRNA in autopsy tissue (Meador-Woodruff *et al*, 1995). None of the published studies to date have been designed to distinguish alternatively spliced D3 receptor isoforms. Future studies evaluating D3 receptor isoform expression, subcellular

localization, and intracellular signaling pathways may be able to more clearly elucidate the mechanism underlying the decline in D3 receptor-mediated inhibitory function accompanying behavioral sensitization.

CONCLUSIONS

Behavioral sensitization may be accounted for in part by tolerance of D3-receptor-mediated inhibitory systems. Perturbations in D3-receptor-mediated inhibitory function may contribute to several neuropsychiatric disorders, including drug dependence, schizophrenia, and Parkinson's disease. Because highly selective D3 receptor ligands have been synthesized (Newman *et al*, 2005), identifying the role of the D3 receptor in the pathophysiology of each of these conditions could make novel approaches to drug treatment feasible. Importantly, D3 receptor tissue distribution is relatively limited within limbic brain regions, suggesting the potential for focused treatments with few side effects. Additionally, elucidating the role of alternative splicing pathways in behavioral sensitization may identify a novel mechanism at which to intervene both to prevent sensitization, as well as to potentially reverse sensitization through restoration of D3-mediated inhibitory function.

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