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Mechanisms of Locomotor Sensitization to Drugs of Abuse in a Two-Injection Protocol

Emmanuel Valjent[1,2,3,5], Jesus Bertran-Gonzalez[1,2,3], Benjamin Aubier[1,2,3], Paul Greengard[4], Denis Hervé[1,2,3] and Jean-Antoine Girault*[,1,2,3]

[1]Inserm UMR-S 839, Paris, France; [2]Université Pierre et Marie Curie (UPMC-Paris 6), Paris, France; [3]Institut du Fer à Moulin, Paris, France; [4]Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, USA

A single exposure to psychostimulants or morphine is sufficient to induce persistent locomotor sensitization, as well as neurochemical and electrophysiological changes in rodents. Although it provides a unique model to study the bases of long-term behavioral plasticity, sensitization mechanisms remain poorly understood. We investigated in the mouse, a species suited for transgenic studies, the mechanisms of locomotor sensitization showed by the increased response to a second injection of drug (two-injection protocol of sensitization, TIPS). The first cocaine injection induced a locomotor sensitization that was completely context-dependent, increased during the first week, and persisted 3 months later. The induction of sensitized responses to cocaine required dopamine D1 and glutamate NMDA receptors. A single injection of the selective dopamine transporter blocker GBR12783 was sufficient to activate extracellular signal-regulated kinase (ERK) in the striatum to the same level as cocaine and to induce sensitization to cocaine, but not to itself. The induction of sensitization was sensitive to protein synthesis inhibition by anisomycin after cocaine administration. Morphine induced a pronounced context-dependent sensitization that crossed with cocaine. Sensitization to morphine injection was prevented in knockin mutant mice bearing a Thr-34-Ala mutation of DARPP-32, which suppresses its ability to inhibit protein phosphatase-1 (PP1), but not mutation of Thr-75 or Ser-130. These results combined with previous ones show that TIPS in mouse is a context-dependent response, which involves an increase in extracellular dopamine, stimulation of D1 and NMDA receptors, regulation of the cAMP-dependent and ERK pathways, inhibition of PP1, and protein synthesis. It provides a simple and sensitive paradigm to study the mechanisms of long-term effects of drugs of abuse.

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INTRODUCTION

Repeated exposure to drugs of abuse results in a progressive and long-lasting enhancement of the locomotor response, a phenomenon termed psychomotor (or locomotor) sensitization (Robinson and Berridge, 1993). One remarkable aspect of this phenomenon is its duration as sensitized responses can be observed after several weeks, months, or up to at least a year of drug-free period (Robinson and Berridge, 1993). In rodents, sensitization has been shown to correlate with enhanced predisposition to self-administer psychostimulants (Schenk and Partridge, 2000; Vezina *et al*, 2002) and reinstatement of extinguished self-administration (De Vries *et al*, 1998; Suto *et al*, 2004). Although the existence of sensitization in humans is disputed, locomotor sensitization has been proposed to correspond to certain aspects of drug addiction such as compulsive drug-seeking behavior (Robinson and Berridge, 1993; Vanderschuren and Kalivas, 2000; Vezina and Leyton, 2009). Irrespective of its exact correlates in human behavior, locomotor sensitization is an extremely interesting phenomenon because it provides a simple readout to understand the mechanisms by which drugs of abuse induce long-lasting neuronal alterations.

The induction of sensitization depends on the temporal pattern of drug exposure. Repeated intermittent treatments with moderate doses of drugs are more effective to induce sensitization than continuous exposure to high or escalating drug doses (Robinson and Berridge, 1993; Vanderschuren and Kalivas, 2000; Vezina and Leyton, 2009). Moreover, contextual environment affects the development of behavioral sensitization (Badiani and Robinson, 2004), because the expression of sensitized locomotor responses is markedly strengthened when tested in the context previously associated with drug injections (Badiani and Robinson, 2004). However, most administration schedules use multiple drug exposures, which also promote the parallel

*Correspondence: Dr JA Girault, Inserm UMR-S 839, Institut du Fer à Moulin, 17 rue du Fer à Moulin, 75005 Paris, France,
Tel: +33 1 45 87 61 52, Fax: +33 1 45 87 61 59,
E-mail: jean-antoine.girault@inserm.fr
[5]Current address: Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden
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development of tolerance and dependence that could interfere with behavioral sensitization, thereby adding a level of complexity in the interpretation of the behavioral responses (Stewart and Badiani, 1993). Moreover, repeated injections of drugs of abuse trigger biochemical and cellular responses different from those induced by single exposures, making it very difficult to establish causality links. Therefore, in spite of a large amount of work from many laboratories, the mechanisms underlying sensitization are not yet fully understood.

Remarkably, studies in rats and mice show that a single exposure to psychostimulants (amphetamine or cocaine) or morphine induces behavioral sensitization in both a context-dependent and a context-independent manner (Jackson and Nutt, 1993; Robinson *et al*, 1982; Guan *et al*, 1985; Robinson, 1991; Vanderschuren *et al*, 2001, 1999; Weiss *et al*, 1989). It should be noted that although the changes in responsiveness are induced by the first injection, their existence is usually revealed by a second injection of the same (or different) drug. Hence, we refer to this protocol as two-injection protocol of sensitization (TIPS). TIPS is a very simple paradigm to explore the mechanisms by which drugs of abuse exert long-lasting effects on the brain. Induction of TIPS involves stimulation of NMDA receptors in the ventral tegmental area (VTA) (Kalivas and Alesdatter, 1993) and is sufficient to induce long-term potentiation in this region (Ungless *et al*, 2001). Although the recent use of this paradigm provided interesting clues on the role of specific signaling pathways *in vivo* (Corbille *et al*, 2007; Stipanovich *et al*, 2008; Valjent *et al*, 2005), TIPS has not been fully characterized and cannot be compared with other paradigms. Here, we explore the mechanisms of TIPS in mouse using pharmacological and genetic models. We show that it is more sensitive than the repeated injections paradigm to alterations in signaling mechanisms and that, in combination with other approaches, it provides an excellent model for the molecular dissection of long-lasting effects of drugs of abuse.

MATERIALS AND METHODS

Mice

Male 8-week-old C57BL/6J mice were purchased from Charles River (L'Arbresle, France). Heterozygous mice with a disrupted *drd1a* gene had a hybrid 129 and C57Bl/6 genetic background. They were generated by Drago and colleagues (Laboratory of mammalian genes and development, NIH, Bethesda) and backcrossed in our laboratory for up to five generations with C57BL/6J mice (purchased from Charles River). Mice expressing dopamine- and cAMP-regulated phosphoprotein with an Mr 32 000 (DARPP-32) with a point mutation of important phosphorylated residues (Thr-34, or Thr-75, or Ser-130) were generated at the Rockefeller University, as described (Svenningsson *et al*, 2003; Zhang *et al*, 2006). C57Bl/6J-Swiss Webster hybrid transgenic mice carrying a bacterial artificial chromosome (BAC) that expresses enhanced green fluorescent protein (BAC-EGFP) under the control of D1 dopamine receptor (D1R) promoter (*drd1a*-EGFP) were generated by the GENSAT (Gene Expression Nervous System Atlas) program at the Rockefeller University (New York, NY). All mice were kept in our animal house in stable conditions of temperature (22°C) with a constant cycle of 12 h light and 12 h dark and free access to food and water. All experiments were performed in accordance with the guidelines of the French Agriculture and Forestry Ministry for handling animals (decree 87-848, license B 75-05-22).

Drugs

Cocaine–HCl (20 mg/kg, i.p.), morphine (5 mg/kg, s.c.), SCH23390 (0.1 mg/kg, i.p.), raclopride (0.3 mg/kg, i.p.), and MK801 (0.15 mg/kg, i.p.) were purchased from Sigma-Aldrich. Drugs were dissolved in 0.9% (w/v) NaCl (saline). Haloperidol (0.5 mg/kg, i.p., Sigma-Aldrich) was dissolved in saline containing 5% (vol/vol) acetic acid and the pH was adjusted to 6.0 with 1 M NaOH. GBR12783 (7.5, 15, and 30 mg/kg, i.p., a gift from Jean-Pol Tassin, CNRS UMR7148, Collège de France) was dissolved in water and injected i.p. Anisomycin (100 mg/kg, i.p.) (Sigma-Aldrich) was diluted in saline and dissolved in 1 N HCl. This amount of anisomycin has been shown to yield $>90\%$ protein synthesis inhibition in the brain during the first 2 h (Flood *et al*, 1973). The pH was adjusted to approximately 7 with 1 N NaOH.

Behavioral Analysis

Locomotor activity measurement. Locomotor activity (LA) was measured in a circular corridor with four infrared beams placed at 90° angles (Imetronic, Pessac, France) in a low luminosity environment. Counts were incremented by consecutive interruption of two adjacent beams (ie mice moving through 1/4 of the circular corridor). All mice were habituated to the test apparatus, handling, and procedure for 3 consecutive days before the actual experiment. In this habituation procedure mice were placed for 30 min in the activity box, received a first injection of saline, were placed back in the box for 30 min, received a second injection, and were placed for 1 h in the box. For the acute drug injection (day 4), the handling was identical, except that the second saline injection was replaced by a drug injection, before mice were placed back in the LA box for 1 or 3 h (acute response for cocaine and morphine, respectively).

Challenges with a second drug injection were performed at the indicated times after the first drug injection. This protocol in which mice received the two injections of drug in the same environment (ie, the LA box) was referred to as context-dependent sensitization. All other experiments were performed using this context-dependent paradigm, unless otherwise indicated. In time-course experiments all experimental groups were pretreated with saline or cocaine the same day and the challenge injection was done for each group at the indicated times.

Results were expressed as LA which corresponds to the number of interruptions of two adjacent beams (i.e. 1/4 turns in the circular corridor) per 5 or 60 min. To compare the effects at various times after the first injections, LA in response to cocaine second injection (LAcoc-coc) was normalized to the mean LA of saline-pretreated mice (LAsal-coc) and the sensitization ratio was calculated by dividing the normalized locomotor response to the second injection by the normalized response to the first injection: sensitization ratio = (LAcoc-coc/LAsal-coc)/(LAcoc/LAsal).

Context-dependent vs independent sensitization. The influence of the context was studied in a 'third world' protocol (Robinson *et al*, 1998). After 3 days of habituation to the LA boxes (as described above), the mice received an injection of saline, were either placed back in the home cage or in a Y maze (context A), or in a LA box (context B). One hour later they received an injection of cocaine, and 1 h later another injection of saline, before returning to the home cage. The three injections were used to dissociate the effect of the drug from the 'context' because of handling by the experimenter. Control mice received three injections of saline. Seven days later all the mice received cocaine and were tested for LA in the circular corridor (context B).

Cross-sensitization. For cross-sensitization experiments, after 3 days of habituation, groups of mice received saline, cocaine, or morphine in the LA boxes. Seven days later, the group pretreated with morphine was challenged with cocaine, whereas mice pretreated with cocaine were challenged with morphine. Saline pre-exposed mice were challenged with either cocaine or morphine.

Role of dopaminergic and glutamatergic transmission in cocaine sensitization. A D1/D5-selective antagonist, SCH23390 (0.1 mg/kg), a D2/D3-selective antagonist, raclopride (0.3 mg/kg), a D2/D3-preferential antagonist, haloperidol (0.5 mg/kg), or a noncompetitive NMDA antagonist, MK801 (0.15 mg/kg) were given 15 min before the first or the second injection of cocaine to test the contribution of the D1/D2 types and the NMDA receptors in the induction or expression of cocaine sensitization, respectively. The effects of the specific dopamine reuptake inhibitor GBR12783 (7.5, 15, and 30 mg/kg) were tested by a first injection of this drug and a challenge with the same dose. To compare the effect of dopamine uptake inhibitor and cocaine on LA, mice received either saline, GBR12783 (15 mg/kg), or cocaine (20 mg/kg) in the LA boxes. Seven days later they were challenged with cocaine (20 mg/kg).

Role of protein synthesis in cocaine sensitization. To evaluate whether newly synthesized proteins are necessary for sensitization to cocaine induced by a single exposure, a protein synthesis inhibitor, anisomycin (100 mg/kg) was injected 2, 6, 8, or 24 h after the first cocaine administration. Mice were then tested by a cocaine challenge 7 days later.

Immunoblotting

Mice were treated as for behavioral analysis, placed in the activity boxes, and at the indicated times after drug injection, they were decapitated and their heads immediately frozen in liquid nitrogen (12 s). The frozen heads were cut into 210 μm thick slices with a cryostat and 10 frozen microdisks (1.4 mm diameter) were punched out bilaterally from the dorsal striatum and stored at −80°C. Micropunches were homogenized by the addition of a hot solution (maintained in a boiling water bath) of 1% (weight/volume) SDS and 1 mM sodium orthovanadate in water, immediate sonication and incubation at 100°C for 5 min to inactivate phosphatases and proteases. Equal amounts of protein (100 μg) were separated by SDS–polyacrylamide gel electrophoresis (10% acrylamide weight/volume) before electrophoretic transfer onto a nitrocellulose membrane (Hybond Pure, Amersham, Orsay, France). Membranes were blocked for 1 h at room temperature (RT) in Tris-buffered saline (TBS: 100 mM NaCl, 10 mM Tris, pH 7.5) with 0.05% (vol/vol) Tween-20. Membranes were then incubated overnight at 4°C with anti-diphospho-Thr183-Tyr185-ERK2 (P-ERK, extracellular signal-regulated kinase), mouse monoclonal antibody (1 : 1000, Sigma-Aldrich) and anti-ERK1/2 rabbit polyclonal antibody (Millipore, Molsheim, France). Bound antibodies were detected with anti-rabbit IgG IRdye800CW-coupled and anti-mouse IgG IR-dye700DX-coupled antibodies (1 : 4000, Rockland Immunochemicals, Gilbertsville, PA). Fluorescence on the immunoblots was analyzed at 680 and 800 nm using the Odyssey infrared imager (Li-Cor, Lincoln, NE) and the relevant immunoreactive bands were quantified using Odyssey software. For evaluating effects on ERK2 activation, the ratio of signals obtained with phospho-specific antibodies and antibodies reacting independently of its phosphorylation state (total) was determined for each sample. The results normalized for each blot were expressed as percentages of saline-treated controls.

Immunohistofluorescence of Brain Sections

Procedures were as described earlier (Valjent *et al*, 2000, 2005). In brief, mice were rapidly anesthetized by i.p. injection of pentobarbital (500 mg/kg, Sanofi-Aventis, France) before intracardiac perfusion of 4% (weight/volume) paraformaldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer, pH 7.5, delivered with a peristaltic pump at 20 ml/min during 5 min. Brains were postfixed overnight in the same solution and stored at 4°C; 30 μm thick sections were cut with a vibratome (Leica, France) and stored at −20°C in a solution containing 30% (vol/vol) ethylene glycol, 30% (vol/vol) glycerol, and 0.1 M sodium phosphate buffer, until they were processed for immunofluorescence. After permeabilization, free-floating sections were incubated with rabbit antibodies for diphospho-ERK (cat-9101; Cell Signaling Technology, Beverly, MA, dilution 1 : 400) and a mouse monoclonal antibody for DARPP-32 (dopamine- and cAMP-regulated phosphoprotein Mr ~ 32 000, dilution 1 : 1000) overnight at 4°C. After three rinses in TBS, sections were incubated for 45 min at RT with the secondary fluorescent goat antibodies (1 : 400 Cy3-coupled anti-rabbit IgG and 1 : 400 Cy5-coupled anti-mouse IgG, Jackson ImmunoReasearch Europe Ltd) in TBS. Sections were then rinsed twice in TBS and twice in TB and mounted on a slide under coverslips using Vectashield (Vector Laboratories, AbCys, Paris, France).

Triple-labeled images from each region of interest were obtained bilaterally using sequential laser scanning confocal microscopy (SP2, Leica). Neuronal quantification was performed in 375 μm × 375 μm confocal images by counting P-ERK-Cy3 immunofluorescent nuclei (Bertran-Gonzalez *et al*, 2008). Images were then combined with EGFP fluorescence and counted cells were classified as EGFP + and EGFP− neurons. Cell counts were done by an observer unaware of the treatment received by the mice.

Statistical Analysis

Behavioral, biochemical, and immunofluorescence data were analyzed by two-way ANOVA followed by Bonferonni test. Locomotor sensitization results were analyzed with two-way ANOVA with matching data (ie, comparing the

response of each mouse to the first and second drug injection). Results are expressed as means ± SEM and the p threshold for significance was 0.05. Statistical analysis was performed with PRISM 3.0 software (San Diego, CA).

RESULTS

Time-Dependent Locomotor Sensitization to Cocaine in a Two-Injection Protocol in Mice

We evaluated the time course of TIPS in mice by testing the locomotor effects induced by a challenge injection of cocaine (20 mg/kg, i.p.) 2 days–3 months after a single injection of cocaine or saline vehicle (Figure 1a). A clear sensitization of the locomotor effects of cocaine was observed at all time points in cocaine-pre-exposed animals (Figure 1a; Supplementary Figure 1). To evaluate more precisely the time course of sensitization and take into account possible variations over time in responses of saline-pretreated mice, we compared the sensitization ratios (see Materials and Methods) at these different time points (Figure 1b). The sensitization ratio increased between 2 and 7 days, and decreased thereafter to remain stable at 2 and 3 months (Figure 1b). These results show that a single cocaine exposure induces a behavioral sensitization that is long-lasting and increases during the first week. In subsequent experiments we tested the sensitized responses at 7 days.

Context Dependence of Locomotor Sensitization to Cocaine in a Two-Injection Protocol

Preliminary experiments showed that sensitization was significant only when mice received the first injection in the LA box. To test precisely the effect of the context on cocaine TIPS, we used a protocol inspired from the 'third world' described by Robinson *et al* (1998). Mice received a first injection of saline or cocaine either in the 'neutral context' of the home cage, in a Y maze (context A), or in the LA boxes (context B). They were all challenged with a test injection of cocaine 7 days later in the activity boxes (context B) (Figure 2a). To avoid association of drug effects with the 'context' of handling and injection by the experimenter, every mouse received three injections per session, the second injection being saline or cocaine, all the others saline. When the mice received the first injection in

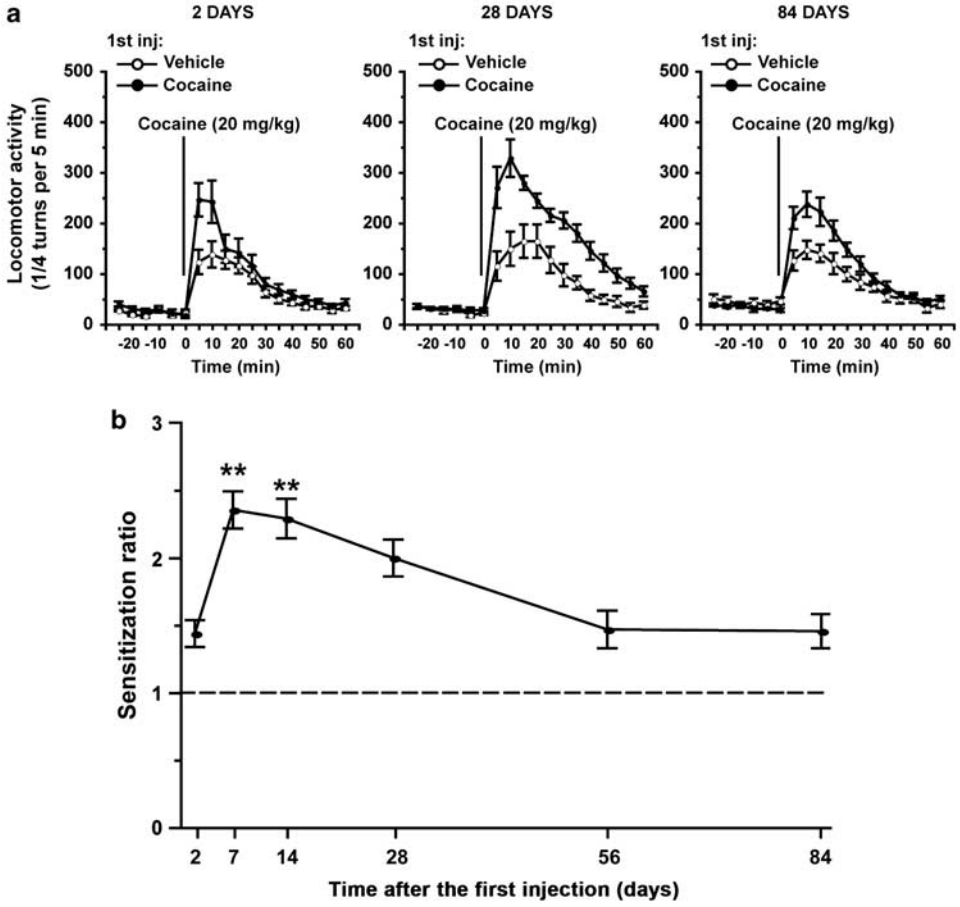

Figure 1 Locomotor sensitization to cocaine in the two-injection protocol is time-dependent. (a) Mice were injected with vehicle (open circles) or cocaine (filled circles), and challenged with cocaine (20 mg/kg) 2, 28, or 84 days later. Locomotor activity was measured by 5 min intervals. Data (means ± SEM) were analyzed using repeated-measures ANOVA with the between-subjects factors of pretreatment and the within-subjects factors of time: 2 days (effect of pretreatment $F_{(1,14)} = 8.69$, $p < 0.05$; effect of time $F_{(17,238)} = 28.63$, $p < 0.01$; interaction $F_{(17,238)} = 2.60$, $p < 0.01$); 28 days (effect of pretreatment $F_{(1,15)} = 21.04$, $p < 0.01$; effect of time $F_{(17,255)} = 48.78$, $p < 0.01$; interaction $F_{(17,255)} = 6.57$, $p < 0.01$); 84 days (effect of pretreatment $F_{(1,14)} = 3.69$, NS; effect of time $F_{(17,238)} = 62.72$, $p < 0.01$; interaction $F_{(17,238)} = 6.47$, $p < 0.01$). (b) Sensitization ratios: (LAcoc-coc/LAsal-coc)/(LAcoc/LAsal) (see Materials and Methods). Data (means ± SEM) were analyzed using one-way ANOVA: $F_{(5,44)} = 10.61$, $p < 0.01$. *Post hoc* comparison (Bonferroni test), $^{**}p < 0.01$, different from ratio at day 2.

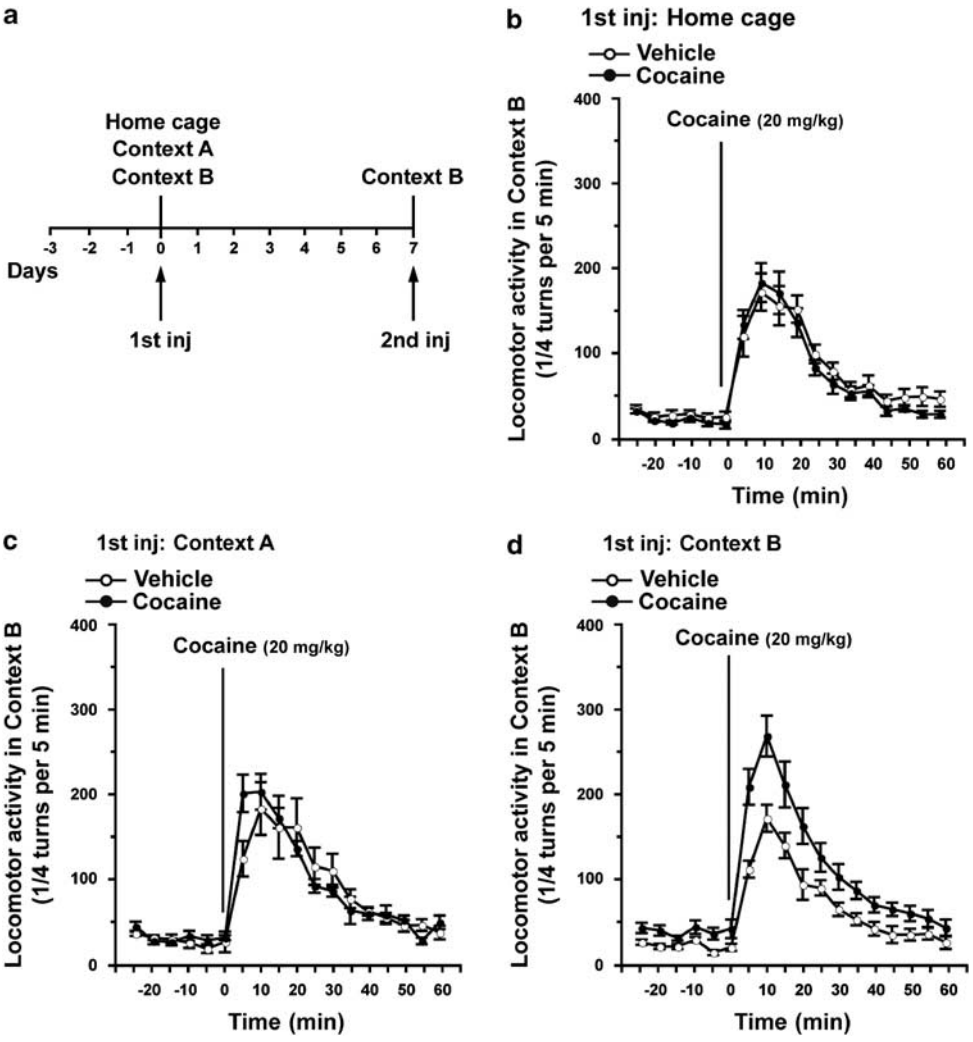

Figure 2 Locomotor sensitization to cocaine in the two-injection protocol is context-dependent. (a) After 3 days of habituation to the locomotor activity boxes (days 3–1), mice were injected with vehicle or cocaine (1st injection) either in their home cages, in a different testing apparatus (Context A), or in the locomotor activity boxes (Context B). Seven days later all groups were challenged with cocaine (2nd injection) in the locomotor activity boxes (Context B). (b–d) Locomotor activity response after the 2nd injection (day 7) measured in Context B of mice pretreated in their home cage (b), Context A (c), or Context B (d). Data (means ± SEM, $n = 7$–10 per group) were analyzed using two-way repeated-measures ANOVA with the between-subjects factor of pretreatment and the within-subjects factor of time: (b) effect of pretreatment $F_{(1,\ 154)} = 0.30$, NS; effect of time $F_{(11,154)} = 41.04$, $p < 0.0001$; interaction $F_{(11,154)} = 0.65$, NS. (c) Effect of pretreatment $F_{(1,143)} = 0.01$, NS; effect of time $F_{(11,143)} = 33.61$, $p < 0.0001$; interaction $F_{(11,143)} = 2.20$, $p < 0.05$. (d) Effect of pretreatment $F_{(1,176)} = 14.42$, $p < 0.01$; effect of time $F_{(11,176)} = 48.96$, $p < 0.0001$; interaction $F_{(11,176)} = 2.81$, $p < 0.01$.

the home cage, no sensitization was observed (Figure 2b). Similarly, no significant sensitization was observed when mice received the first injection in a different novel context that is context A (Figure 2c). In contrast, a clear behavioral sensitization was observed when the mice received the two cocaine injections in the activity boxes (context B, Figure 2d). These results show that behavioral sensitization induced by a single cocaine exposure in mice is strongly influenced by the environmental context. For this reason in the following experiments the context-dependent paradigm was used.

Role of Dopamine Receptors in Cocaine Sensitization in a Two-Injection Protocol

To characterize the involvement of dopamine receptors in the induction of cocaine TIPS in mice, we first used the dopamine D1/D5 receptor antagonist, SCH23390 and the dopamine D2-type receptor preferential and selective antagonists, haloperidol and raclopride, respectively. Mice pretreated with SCH23390 (0.1 mg/kg) before the first cocaine exposure failed to develop locomotor sensitization when tested 1 week later with cocaine (Figure 3a). In contrast, pretreatment with haloperidol (0.5 mg/kg) or raclopride (0.3 mg/kg) did not impair significantly the induction of behavioral sensitization to cocaine (Figure 3a). Although SCH23390 is selective for D1-type *vs* D2-type dopamine receptors, it has some effects on other receptors including 5-HT2C receptors (Millan *et al*, 2001), which have been shown to be involved in locomotor sensitization with repeated cocaine treatments (Lanteri *et al*, 2008). Therefore, we also tested the role of D1R using mutant mice devoid of these receptors. These mice displayed a basal locomotor hyperactivity, and cocaine

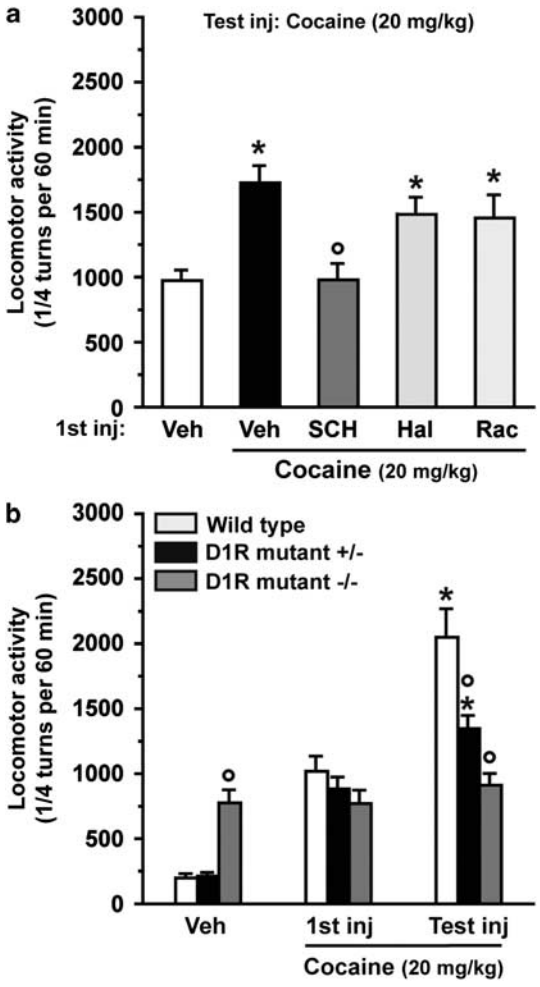

Figure 3 Selective role of dopamine D1 receptors in locomotor sensitization to cocaine in the two-injection protocol. (a) Mice were treated with vehicle (Veh) or cocaine in combination with vehicle, SCH23390 (SCH, 0.1 mg/kg), haloperidol (Hal, 0.5 mg/kg) or raclopride (Rac, 0.3 mg/kg). Locomotor responses were measured during 60 min after a cocaine exposure 7 days later. Data (means ± SEM, $n=8$ per group) were analyzed using one-way ANOVA, $F_{(4,35)}=7.13$, $p<0.01$). *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$, different from vehicle pretreated group, $^{\circ}p<0.05$, different from cocaine pretreated group. (b) Locomotor responses were measured during 60 min after an injection of saline (Veh) or a first injection of cocaine and a test injection of cocaine 1 week later (Test inj) in wild type ($n=9$), heterozygous (D1R+/− $n=13$), and homozygous (D1R−/−, $n=8$) mutant mice. Data were analyzed using repeated-measures ANOVA with the between-subjects factors of genotypes and the within-subjects factors of treatment: genotype $F_{(2,27)}=4.16$, $p<0.01$; treatment $F_{(2,54)}=97.22$, $p<0.01$; interaction $F_{(2,54)}=24.05$, $p<0.01$. *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$, different from first injection, $^{\circ}p<0.05$, different from wild type.

failed to further enhance LA after the first or the second cocaine administration 7 days later (Figure 3b). Interestingly, heterozygous mice that showed a normal acute cocaine-induced LA, also displayed a decreased sensitization when re-exposed to cocaine 7 days later (Figure 3b). Together these results provide strong evidence that the induction of locomotor sensitization by a single cocaine exposure requires dopamine D1 receptors.

Specific Blockade of Dopamine Transporter Is Sufficient to Induce ERK Phosphorylation in The Striatum

As dopamine D1 receptors seemed necessary for the induction of locomotor sensitization to cocaine, we investigated whether increasing the extracellular concentration of dopamine was sufficient to induce sensitization using a selective blocker of the plasma membrane dopamine transporter, GBR12783 (Bonnet and Costentin, 1986). To choose a dose of GBR12783 that had biological effects comparable to those of cocaine, we first examined whether this compound was capable of inducing ERK phosphorylation in the striatum, an effect common to drugs of abuse (Valjent *et al*, 2004) and which seems critical for some of their long-lasting effects (Valjent *et al*, 2000, 2005). Immunoblot analysis of diphospho-ERK, which corresponds to the activated form of the enzyme, showed that treatment of mice with GBR12783 increased ERK phosphorylation in the dorsal striatum (Figure 4a). The effects of 15 mg/kg GBR12783 were of the same amplitude as those of 20 mg/kg cocaine (Figure 4a). As we have shown earlier that cocaine induces ERK phosphorylation only in the striatonigral neurons, identified by the expression of the *drd1a* promoter (Bertran-Gonzalez *et al*, 2008), we analyzed in which neuronal subpopulation the effects of GBR12783 took place using confocal immunofluorescence microscopy in *drd1a*-EGFP transgenic mice (Figure 4b and c). The injection of GBR12783 induced a strong ERK phosphorylation in the dorsal striatum and nucleus accumbens core and shell (Figure 4b and c). This activation occurred selectively in D1R-expressing striatonigral medium-sized spiny neurons (MSNs), as shown by colocalization with DARPP-32 immunoreactivity and EGFP (Figure 4b). Quantification showed that virtually all P-ERK immunoreactive neurons also contain EGFP, in all striatal regions (Figure 4c, EGFP + *vs* EGFP− bars). However, not all EGFP-positive neurons contained diphospho-ERK immunoreactivity (Figure 4b). These results show that blockade of the dopamine transporter selectively induced ERK phosphorylation in a subpopulation of D1R-expressing striatonigral neurons. Thus, the effects of GBR12783 on ERK in the striatum are very similar to those of cocaine (Bertran-Gonzalez *et al*, 2008 and this study).

Selective Blockade of the Dopamine Transporter Is Sufficient for the Induction but not the Expression of Sensitization in a Two-Injection Protocol

We then tested the ability of GBR12783 to induce behavioral sensitization. The first administration of GBR12783 (7.5, 15, or 30 mg/kg) induced a robust increase in LA. When mice pretreated with GBR12783 were challenged 1 week later with the same drug, a slight increase in the locomotor response was observed (<50%), which did not reach the statistical significance threshold (Figure 5a). This suggested that increased extracellular dopamine levels were not consistently sufficient to recapitulate the locomotor sensitization induced by cocaine. To test whether this was due to a lack of induction or a lack of expression of sensitization, we pretreated mice with cocaine (20 mg/kg) or GBR12783 (15 mg/kg) and challenged them 1 week later with cocaine (20 mg/kg, Figure 5b). These two doses were chosen because

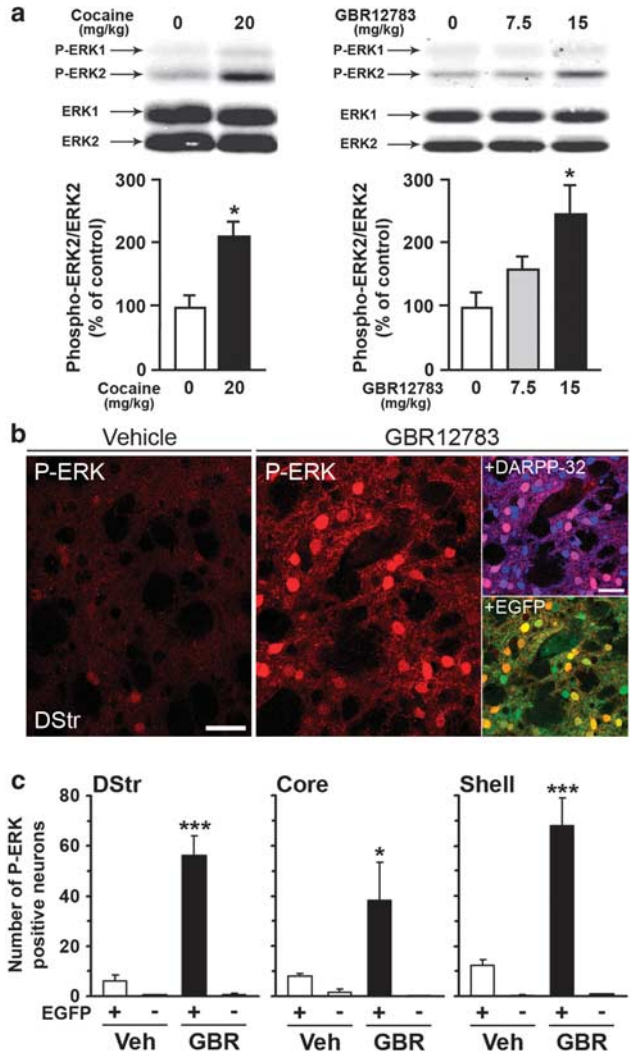

Figure 4 A selective dopamine reuptake inhibitor induces strong ERK phosphorylation in D1R-expressing striatal neurons. (a) Phosphorylated ERK1/2 (P-ERK-1/2) or total ERK1/2 were analyzed by immunoblotting in the dorsal striatum of mice treated with vehicle (0), 20 mg/kg cocaine or GBR12783 (7.5 and 15 mg/kg). Immunoreactive bands were quantified by Li-Cor, and results were expressed as ratios of phosphorylated/total protein and normalized to the mean of saline-treated mice (percentage of control). Note that P-ERK1 was barely detectable in most experiments. Data (means ± SEM, $n = 5$–7 mice per group) were analyzed using unpaired Student's *t*-test (cocaine effect, $^{*}p < 0.01$) or one-way ANOVA followed by Bonferroni test (effect of GBR12783, $F_{(2,22)} = 5.53$; $p < 0.05$). $^{*}p < 0.05$ compared with saline. (b) The selective DAT blocker, GBR12783 (15 mg/kg), was administered to *drd1a*-EGFP BAC transgenic mice, in which EGFP expression is driven by D1R promoter. Single confocal sections showing P-ERK (red) and DARPP-32 (blue, upper panel) immunofluorescence combined with D1R-mediated EGFP fluorescence (green, lower panel) in the dorsal striatum (DStr) of *drd1a*-EGFP mice 15 min after GBR12783 injection. Scale bar 40 μm. (c) Quantification of P-ERK immunoreactive neurons between EGFP-positive (+) or EGFP-negative (−) neurons in the dorsal striatum (DStr), nucleus accumbens core (Core), and shell (Shell) of *drd1a*-EGFP mice 15 min after vehicle (veh) or GBR12783 (GBR) injections. Data (means ± SEM, $n = 3$–4 mice per group) were analyzed using two-way ANOVA: (DStr) Interaction $F_{(1,12)} = 36.86$, $p < 0.0001$; EGFP+ *vs* EGFP−: $F_{(1,12)} = 56.16$, $p < 0.0001$; Veh *vs* GBR: $F_{(1,12)} = 37.76$, $p < 0.0001$; (Core) Interaction $F_{(1,10)} = 6.049$, $p < 0.05$; EGFP+ *vs* EGFP−: $F_{(1,10)} = 12.07$, $p < 0.01$; Veh *vs* GBR: $F_{(1,10)} = 4.88$, $p > 0.05$; (Shell) Interaction $F_{(1,12)} = 24.79$, $p < 0.001$; EGFP+ *vs* EGFP−: $F_{(1,12)} = 51.66$ $p < 0.0001$; Veh *vs* GBR: $F_{(1,12)} = 25.88$, $p < 0.001$. *Post hoc* comparison (Bonferroni test), $^{*}p < 0.05$, $^{***}p < 0.001$ group Veh *vs* GBR.

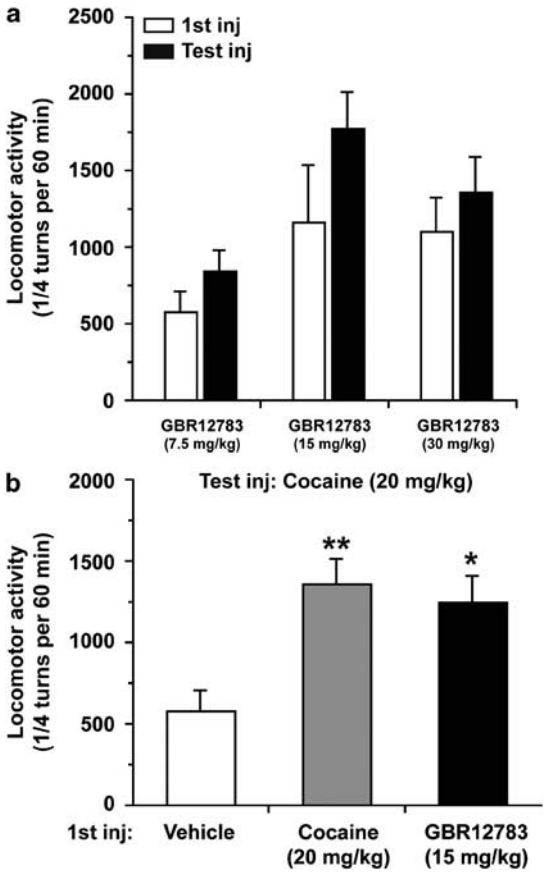

Figure 5 A specific dopamine reuptake inhibitor is sufficient for the induction, but not the expression of locomotor sensitization in the two-injection protocol. (a) Locomotor activity was measured during 60 min after a first (open bars) or a second injection, 7 days later (Test inj, black bars) of GBR12783 (7.5, 15, and 30 mg/kg, i.p.). Data (means ± SEM, $n = 9$–10 per group) were analyzed using a paired Student's *t*-test. (b) Locomotor activity was measured during 60 min after cocaine exposure (20 mg/kg) in mice pretreated 7 days before with vehicle (white bar), cocaine (gray bar, 20 mg/kg), or GBR12783 (black bar, 15 mg/kg). Data (means ± SEM, $n = 10$ per group) were analyzed using one-way ANOVA, $F_{(2,26)} = 8.418$, $p < 0.01$. *Post hoc* comparison (Bonferroni test), $^{*}p < 0.05$, $^{**}p < 0.01$, vehicle *vs* drugs (cocaine or GBR12783). Note that the locomotor activity in the 30 min preceding the cocaine injection, but after receiving a vehicle injection (see Materials and Methods), was not significantly different between the three groups of mice, which differed by the treatment received a week before (Veh–Veh: 134.7 ± 38.1; Coc–Veh: 161.7 ± 30.0; GBR–Veh: 162.2 ± 14.3).

they gave similar results in terms of ERK phosphorylation (Figure 4), an index of their ability to increase extracellular dopamine. Remarkably, the mice pretreated with 15 mg/kg GBR12783 expressed a sensitized locomotor response to cocaine comparable to that of mice pretreated with 20 mg/kg cocaine (Figure 5b). Thus, GBR12783 induced a locomotor sensitization to cocaine as strong as that induced by cocaine itself, but was not sufficient to allow the full expression of this sensitization. Importantly, earlier exposure to GBR12783 or cocaine (7 days before) did not modify the locomotor response to a vehicle injection (see legend to Figure 5b). Altogether, these results strongly suggest that dopamine is sufficient to induce context-dependent locomotor sensitization, but that other monoamines may be involved in its full expression.

Role of NMDA Receptors in Behavioral Sensitization to Cocaine in a Two-Injection Protocol

In addition to involving dopamine neurotransmission, there is substantial evidence that sensitization induced by repeated treatment with psychostimulants requires glutamate transmission (Vanderschuren and Kalivas, 2000). The noncompetitive NMDA antagonist MK801 has the interesting property of enhancing LA and inducing sensitization to its own effects, but also of preventing psychostimulant-induced behavioral sensitization (Wolf and Khansa, 1991). We used MK801 to investigate the role of NMDA receptors in cocaine TIPS. Mice pretreated with MK801 (0.15 mg/kg) before the first cocaine exposure failed to develop locomotor sensitization when tested 7 days later with cocaine (Figure 6a). In contrast, when NMDA receptors were blocked before the test injection of cocaine, mice previously exposed to cocaine expressed a locomotor sensitization that was comparable to that observed in mice challenged only with cocaine (Figure 6b). These results show that NMDA receptors are essential for the induction of sensitization, not for its expression.

Induction of Sensitization Requires Protein Synthesis During a Restricted Time Period

Regulation of gene expression and protein synthesis is widely acknowledged to be necessary for the long-lasting synaptic plasticity, as well as for behavioral changes induced by drugs of abuse. Sensitization induced by repeated injections of cocaine or amphetamine is blocked by protein synthesis inhibitors injected at the periphery (Karler *et al*, 1993) or in the VTA (Sorg and Ulibarri, 1995). We took advantage of TIPS to precisely determine the time window during which protein synthesis was critical for induction of sensitization (Figure 7). The protein synthesis inhibitor, anisomycin (100 mg/kg, i.p.), or vehicle was administered at various time points within 24 h after the first injection of cocaine (1st inj) and mice were then challenged with cocaine 7 days later (Test inj). In mice that received anisomycin immediately (data not shown), 2 or 6 h after the first injection of cocaine, the sensitization was markedly impaired (Figure 7). In contrast, the injection of anisomycin 8 or 24 h after the first injection of cocaine did not alter locomotor sensitization (Figure 7). These data show that synthesis of proteins is necessary for cocaine locomotor sensitization, and that the critical protein synthesis occurs before 8 h after the exposure to cocaine.

Morphine Sensitization Is Context-Dependent and Crossed with Cocaine in a Two-Injection Protocol

Locomotor sensitization is a common property of psychostimulant drugs, and we found that TIPS was also induced by amphetamine and methylphenidate (data not shown). Sensitization is also dramatically induced by opiates. Moreover, the existence of a cross-sensitization between morphine and psychostimulants strongly suggests that locomotor sensitization to various drugs involves, at least in part, a common neural pathway (Cunningham *et al*, 1997). For this reason we investigated the sensitization to morphine using TIPS and compared it with the response to cocaine. In this protocol, the locomotor effects of morphine (5 mg/kg, s.c.) were enhanced in mice pre-exposed to morphine 7, 56, and 86 days before the challenge injection (Figure 8a). Interestingly, the effects appeared slightly more pronounced 2 months than a week after the first injection. We tested the context-dependence of TIPS, by comparing the sensitization in mice that had received the first injection of morphine in their home cage and in mice that had received it in the activity box. Behavioral sensitization to morphine was only observed when the test injection was done in the same environment as the first injection (Figure 8b).

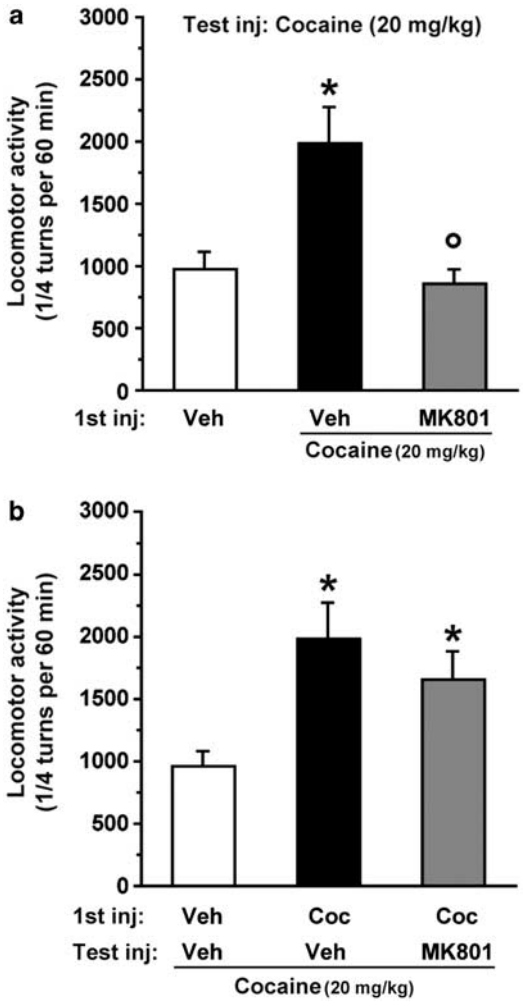

Figure 6 Induction, but not expression, of locomotor sensitization is prevented by blockade of NMDA receptors in the two-injection protocol. (a) Locomotor responses were measured during 60 min after cocaine exposure in mice pretreated with vehicle (Veh), or cocaine in combination with vehicle or MK801 (0.15 mg/kg). Data (means ± SEM, $n = 8$ per group) were analyzed using one-way ANOVA, $F_{(2,21)} = 10.46$, $p < 0.01$). (b) Locomotor responses were measured during 60 min after a test injection of cocaine in combination with vehicle (Veh) or MK801 (0.15 mg/kg) in mice pretreated with vehicle or cocaine (1st inj, as indicated). Data (means ± SEM, $n = 8$ per group) were analyzed using one-way ANOVA, $F_{(2,21)} = 5.45$, $p < 0.05$. *Post hoc* comparison (Bonferroni test), $^{*}p < 0.05$, different from vehicle-only pretreated group. $p < 0.005$, different from vehicle and cocaine pretreated group.

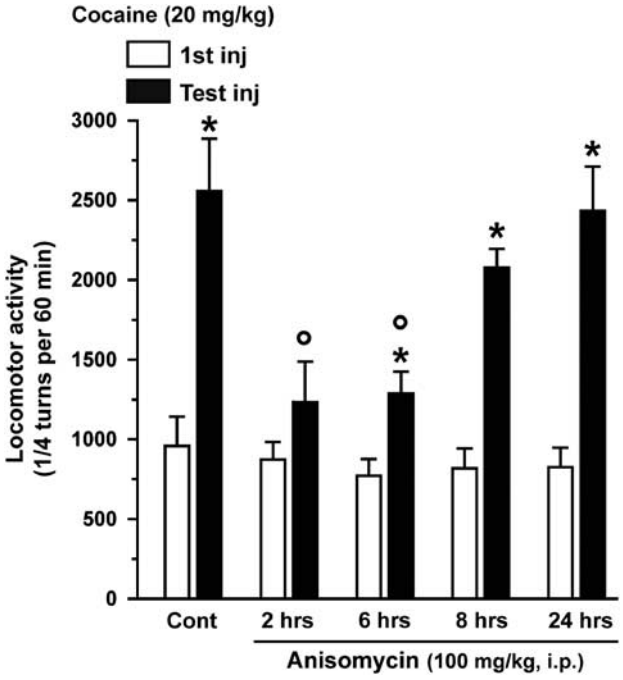

Figure 7 Protein synthesis inhibition prevents locomotor sensitization to cocaine in the two-injection protocol. Mice locomotor responses were measured during 60 min after cocaine first exposure (open bars) or 7 days later (Test inj, filled bars). The cocaine 1st injection was followed by no further treatment (control, Cont) or by anisomycin treatment (100 mg/kg) at the indicated time points. Data (means ± SEM, $n = 8$ mice per group) were analyzed using repeated-measures ANOVA with the between-subjects factors of anisomycin post treatment and the within-subjects factors of cocaine treatment (first *vs* test injection): (effect of cocaine $F_{(1,88)} = 79.46$, $p < 0.01$; effect of anisomycin $F_{(4,88)} = 6.09$, $p < 0.01$; interaction $F_{(4,88)} = 4.92$, $p < 0.01$). *Post hoc* comparison (Bonferroni test), $^{*}p < 0.05$, 1st inj *vs* test inj, $^{\circ}p < 0.05$, anisomycin *vs* control.

We then examined the effects of a single pre-exposure to 20 mg/kg cocaine or 5 mg/kg morphine on LA induced by morphine and cocaine, respectively, a week later (Figure 8c and d). Morphine-pretreated mice were sensitized to the locomotor effects induced by cocaine (20 mg/kg, i.p., Figure 8c). Similarly, cocaine-pre-exposed mice were sensitized to the locomotor effects of morphine (5 mg/kg, s.c., Figure 8d). In both cases, sensitized responses were similar to those observed when the same drug (morphine or cocaine) was used for the two injections (Figure 8c and d).

Point Mutation of Thr-34, but not Thr-75 or Ser-130, in DARPP-32 Alters Sensitization to Morphine in a Two-Injection Protocol

Cellular responses to dopamine in the striatum involve a signaling pathway that associates the stimulation of protein kinases and the inhibition of protein phosphatase-1 (PP1) through dopamine- and cAMP-regulated phosphoprotein with an Mr ~ 32 000 (DARPP-32). To be active as a phosphatase inhibitor, DARPP-32 needs to be phosphorylated on Thr-34 by cAMP-dependent protein kinase (Svenningsson *et al*, 2004), providing a feed-forward amplification loop to some phosphorylation reactions (Fernandez *et al*, 2006; Lindskog *et al*, 2006). Studies in mutant mice have shown that DARPP-32 induces a leftward shift of the dose–response curve in a variety of experiments (Fienberg *et al*, 1998). Interestingly, in DARPP-32 knockout mutant mice sensitization to a single injection of cocaine was reduced (Valjent *et al*, 2005), whereas sensitization to repeated injections of cocaine was increased (Hiroi *et al*, 1999). Similarly, in knockin mutant mice in which Thr-34 was replaced by an alanine (T34A), a mutation, which prevents DARPP-32 from inhibiting PP1, sensitization to a single injection of cocaine was prevented (Valjent *et al*, 2005), whereas sensitization to repeated injections was increased (Zachariou *et al*, 2006). It has also been reported that sensitization to repeated injections of morphine was not significantly altered in null or T34A mutant mice (Borgkvist *et al*, 2007). Owing to the apparent differences between sensitization to single and repeated injections in the DARPP-32 mutant mice, we tested TIPS for morphine in these mice. Locomotor sensitization was measured after a single injection of morphine (2.5 or 5 mg/kg) in wild type or knockin DARPP-32 mutants (Figure 9). T34A mutant mice displayed a decreased locomotor response to the first injection of morphine (Figure 9a), as reported earlier (Borgkvist *et al*, 2007). No sensitization was observed after the second injection of morphine at the two doses tested (Figure 9a). We also examined mice with a mutation of Thr-75 to alanine, which prevents the ability of the protein to inhibit cAMP-dependent protein kinase. These mice had a normal acute response to morphine (Figure 9b). The response to the second injection was higher than the first, although it did not reach statistical significance in the samples studied (Figure 9b). Finally, we examined morphine TIPS in Ser-130-Ala mutant mice, in which the casein kinase 1 phosphorylation site is deleted. This site is involved in the regulation of Thr-34 dephosphorylation and is expected to enhance the function of DARPP-32 as a phosphatase inhibitor (Desdouits *et al*, 1995). These mice displayed a decreased locomotor response to 5 mg/kg morphine, but a normal sensitization (Figure 9c). These results indicate that mutation of Thr-34, but not Thr-75 or Ser-130, dramatically disrupts sensitization to morphine in TIPS, whereas other mechanisms compensate for this defect when repeated injections are used (Borgkvist *et al*, 2007). These results show that TIPS is particularly sensitive to detect behavioral consequences of mutations.

DISCUSSION

In this study we characterized the mechanism by which a single drug exposure induces enduring behavioral sensitization in mice using a two-injection-based protocol (TIPS). Our study extends earlier reports, which showed that a single exposure to psychostimulants or opiates elicits a long-lasting locomotor sensitization (Jackson and Nutt, 1993; Robinson *et al*, 1982; Guan *et al*, 1985; Robinson, 1991; Vanderschuren *et al*, 2001, 1999; Weiss *et al*, 1989). Our findings present similarities and differences with these earlier results, and highlight some interesting divergences with sensitization induced by repeated injections. Sensitization induced by a single cocaine administration clearly involved dopaminergic transmission and required stimulation of both D1 and NMDA receptors. In addition, we identified a time window during which synthesis of new

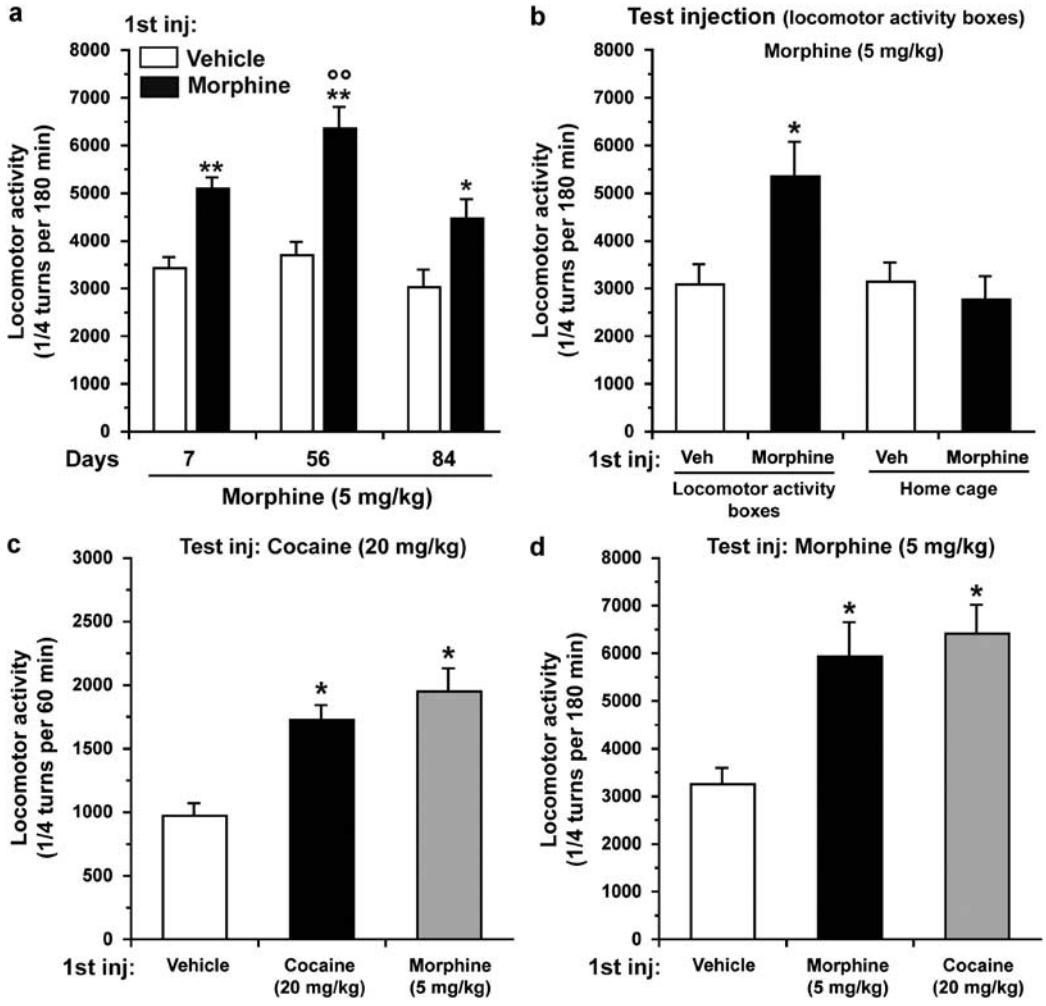

Figure 8 Morphine induces a strong locomotor sensitization and a cross-sensitization with cocaine in the two-injection protocol. (a) Locomotor responses were measured during 180 min after morphine exposure in mice previously treated with vehicle (open bars) or morphine (filled bars), 7 days ($n=8$), 56 days ($n=8$), or 84 days ($n=8$) before. Data are means ± SEM, $n=7$–8 per group. Between-subjects assessment was analyzed using ANOVA with the between-subjects factors of pretreatment and withdrawal day: pretreatment $F_{(1,42)}=53.79$, $p<0.01$, withdrawal time $F_{(2,42)}=8.07$, $p<0.01$, interaction $F_{(2,42)}=2.06$, NS. *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$, $^{**}p<0.01$: different from mice pretreated with vehicle. (b) Locomotor activity measured during 180 min after morphine exposure in mice previously pretreated with vehicle (open bars) or morphine (filled bars) either in the locomotor activity boxes or in their home cage. Data (means ± SEM, $n=7$–8 per group) were analyzed using ANOVA with the between-subjects factors of pretreatment and context: effect of pretreatment $F_{(1,26)}=4.06$, $p<0.05$; effect of context $F_{(1,26)}=5.69$, $p<0.05$; interaction $F_{(1,26)}=7.52$, $p<0.01$. *Post hoc* comparison (Newman–Keuls test), $^{*}p<0.05$, different from mice pretreated with vehicle. (c) Locomotor activity measured during 60 min after cocaine exposure in mice previously pretreated with vehicle (open bars), cocaine (filled bars), or morphine (gray bars). Data (means ± SEM, $n=9$–10 per group) were analyzed using one-way ANOVA ($F_{(2,26)}=15.49$, $p<0.01$). *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$, different from mice pretreated with vehicle. (d) Locomotor activity measured during 180 min after morphine exposure in mice previously pretreated by vehicle (open bars), morphine (filled bars), or cocaine (gray bars). Data (means ± SEM, $n=10$ per group) were analyzed using one-way ANOVA ($F_{(2,27)}=9.19$, $p<0.01$). *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$, different from mice pretreated with vehicle.

proteins was required for the induction of the sensitized responses. The results with DARPP-32 point mutants highlight the sensitivity of TIPS to identify alterations in drug-induced plasticity and provide important clues about their mechanism.

We found that a single injection of cocaine or morphine in mice induced a prolonged locomotor sensitization (as long as the maximal duration tested, ie, 3 months). As in rat (Vanderschuren *et al*, 1999), the sensitization to these two drugs involves mechanisms that are, at least in part, common, as a cross-sensitization was clearly observed. Interestingly, the amplitude of the sensitized response varied with time and peaked 1 week after the first injection of cocaine or later after morphine administration. This delayed increase in the amplitude of the response, sometimes referred to as 'incubation', has been observed in various models (Kolta *et al*, 1985; Paulson and Robinson, 1991, 1995; Vanderschuren *et al*, 1999). The mechanism underlying the delayed increase in these responses is not known. It could reflect a slow modification of synaptic strength or other neuronal properties, yet to be identified. Alternatively, it may be related to the concomitant existence of two processes with opposite effects, such as tolerance and sensitization. If tolerance has a shorter duration, as

suggested by earlier work (Stewart and Badiani, 1993), an apparent progressive increase in sensitization is expected to occur over time, until tolerance has completely disappeared. This explanation could account for the apparent longer 'incubation' of morphine sensitization as compared with cocaine sensitization, as morphine induces a stronger, more prolonged tolerance than cocaine. Interestingly, the phenomenon of incubation has also been reported in a rat model of drug craving and relapse (Grimm *et al*, 2001; Lu *et al*, 2004). In that case it involves ERK activation in the central amygdala (Li *et al*, 2008; Lu *et al*, 2005). It remains to be established whether incubation of craving and incubation of sensitization share the same mechanisms.

RNA and protein synthesis are necessary for the induction of long-lasting changes by drugs of abuse (Kelley, 2004; Nestler, 2004). For example, the immediate early genes that encode the transcription factors cFos, cJun, Zif268, and delta-FosB, which themselves regulate the expression of other genes, have been shown to participate in the neuronal changes induced by repeated exposure to drugs (Nestler, 2004). Zif268 in particular is critical for both TIPS and classical sensitization to cocaine (Valjent *et al*, 2006). TIPS allowed testing the precise timing of sensitivity to protein translation inhibitors. Our results point out the existence of a time window during which *de novo* protein synthesis is particularly important, early after drug administration (ie, before 8 h after cocaine administration). This is in agreement with the critical period for protein synthesis requirement observed in other learning protocols.

In this study, sensitization induced by a single exposure to cocaine or morphine was clearly observed only when the two drug injections were done in the same novel environment that is LA boxes. When the mice were first injected in the home cage or in another context and tested in the activity boxes, their locomotor responses were not significantly increased as compared with saline-pretreated mice. Context dependence of locomotor sensitization to a single drug injection has been reported in rats (Weiss *et al*, 1989) and mice (Jackson and Nutt, 1993), although Vanderschuren and colleagues found that a single injection of amphetamine or opiate was sufficient to induce a context-independent sensitization in rats (Vanderschuren *et al*, 2001, 1999). It should be noted that the context-independent

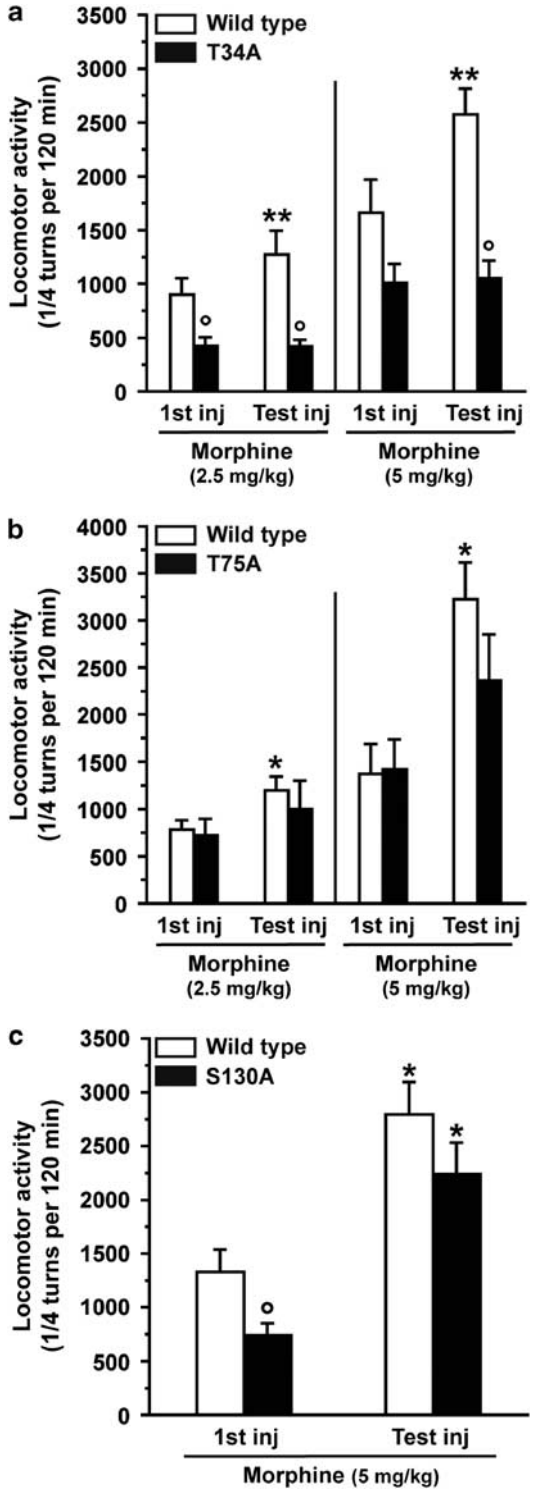

Figure 9 DARPP-32 Thr-34 is required for sensitization to morphine in the two-injection protocol. (a) Locomotor responses were measured during 120 min after the first and second (Test inj, 7 days later) injection of morphine (2.5 and 5 mg/kg) in wild type or T34A-DARPP-32 (T34A) mutant mice ($n=6$–10 mice per group). Data were analyzed using repeated-measures ANOVA with the between-subjects factors of genotypes and the within-subjects factors of treatment: 2.5 mg/kg (genotype $F_{(1,16)}=14.08$, $p<0.01$; treatment $F_{(1,16)}=10.15$, $p<0.01$; interaction $F_{(1,16)}=8.41$, $p<0.01$); 5 mg/kg (genotype $F_{(1,11)}=11.14$, $p<0.01$; treatment $F_{(1,11)}=5.09$, $p<0.05$; interaction $F_{(1,11)}=5.29$, $p<0.01$). *Post hoc* comparison (Bonferroni test), $^{**}p<0.01$ different from first injection, $^{\circ}p<0.05$ different from wild type (b) Locomotor responses were measured during 120 min after the first and second (Test inj, 7 days later) injections of morphine (2.5 and 5 mg/kg) in wild type or T75A-DARPP-32 (T75A) mutant mice ($n=8$–11 mice per group). Data were analyzed using repeated-measures ANOVA with the between-subjects factors of genotypes and the within-subjects factors of treatment: 2.5 mg/kg (genotype $F_{(1,15)}=0.230$, NS; treatment $F_{(1,15)}=4.84$, $p<0.05$; interaction $F_{(1,15)}=0.052$, NS); 5 mg/kg (genotype $F_{(1,17)}=0.21$, NS; treatment $F_{(1,17)}=19.44$, $p<0.01$; interaction $F_{(1,17)}=0.94$, NS). *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$ different from first injection (c) Locomotor responses were measured during 120 min after the first and second (7 days later) injection of 5 mg/kg morphine in wild type ($n=8$) and in S130A-DARPP-32 (S130A) mutant mice ($n=8$). Data were analyzed using repeated-measures ANOVA with the between-subjects factors of genotypes and the within-subjects factors of treatment: genotype $F_{(1,14)}=5.27$, $p<0.05$; treatment $F_{(1,14)}=43.98$, $p<0.01$; interaction $F_{(1,14)}=0.06$, NS. *Post hoc* comparison (Bonferroni test), $^{*}p<0.05$ different from first injection, $^{\circ}p<0.05$ different from wild type.

sensitization reported in these latter studies was of relatively low amplitude. It is not clear at this point whether this partial discrepancy could result from a species difference, or from differences in the experimental protocol such as the type of LA box used in our study. It is important to point out that in our conditions when we used a protocol designed to prevent association between mouse manipulation and drug effects, the small context-independent component disappeared (data not shown and see Figure 2).

There is considerable evidence showing that sensitized responses can be powerfully modulated by the environmental context, whereas discrete cues that predict drug administration do not have this property (Vezina and Leyton, 2009). Context-dependent sensitization may share some mechanisms with the excitatory conditioned response (CR), elicited by vehicle injection paired with the environment in which the drug was injected earlier (Anagnostaras *et al*, 2002). However, in behavioral models using repeated exposure, the CR was not correlated to behavioral sensitization (Hotsenpiller and Wolf, 2002) and locomotor sensitization was still observed following extinction procedures, which eliminate the CR (Anagnostaras and Robinson, 1996; Carey and Gui, 1998; Stewart and Vezina, 1991). Moreover, CR, but not context-dependent locomotor sensitization, was abolished in GluR1 knockout mice (Dong *et al*, 2004). These results do not exclude the existence of common mechanisms, but show that context-dependent sensitization is either, partly, independent of or more robust than CR alone. In fact, the two behavioral responses could share some mechanisms, such as long-term potentiation at glutamatergic synapses on dopamine neurons, which is correlated to sensitization (Ungless *et al*, 2001; Wanat and Bonci, 2008). Accordingly, the context-dependent sensitization induced by single exposure to cocaine was completely prevented in MK801-treated mice (this study), as in the repeated injection protocol (Schenk *et al*, 1993). Similarly, NR1-knockdown mice showed an attenuation of sensitization induced by cocaine (Ramsey *et al*, 2008). However, the precise location at which NMDA receptors are critical appears not to be limited to dopamine neurons themselves as mice with specific inactivation of NR1 in these neurons showed no alteration of short-term sensitization, but a decreased long-term sensitization (Engblom *et al*, 2008; Zweifel *et al*, 2008). In contrast, expression of mutant NMDARs in D1R-containing MSNs prevented cocaine sensitization (Heusner and Palmiter, 2005). Altogether, these observations support the hypothesis that NMDARs located in MSNs, in the striatum, and/or on their terminals in the VTA, as indicated by the effects of local infusion of antagonists (Vezina and Queen, 2000), contribute to the development of sensitization.

The importance of striatal neurons in the establishment of sensitization to cocaine is also supported by our results on the requirement for D1R. The role of these receptors has been already addressed in several studies with conflicting results. Although D1R was consistently reported to be essential for acute responses to psychostimulants, its role in the development of behavioral sensitization is more controversial. Indeed, co-administration of a D1R antagonist (SCH23390) and cocaine did not impair the development of behavioral sensitization under most conditions (Kalivas and Stewart, 1991; Steketee, 1998; White *et al*, 1998), whereas it prevented the induction of sensitization to amphetamine (Karper *et al*, 2002; Vezina, 1996). Interestingly, a recent study showed the blockade of cocaine- and D-amphetamine-induced locomotor sensitization by SCH23390, but concluded that it resulted from the agonist property of SCH23390 at 5-HT2C receptors (Lanteri *et al*, 2008). All these studies used a repeated treatment regimen to induce behavioral sensitization. Here, using TIPS, we found that injection of SCH23390, but not haloperidol or raclopride, before the first cocaine exposure strongly impaired the sensitized locomotor responses measured 7 days later. The fact that sensitization was also altered in homozygous or even heterozygous D1R mutant mice, indicates that D1R was indeed involved in these effects. Accordingly, behavioral sensitization induced by repeated injections of cocaine was reduced in two different lines of D1R knockout mice (Crawford *et al*, 1997; Karlsson *et al*, 2008; Karper *et al*, 2002; Xu *et al*, 2000). Thus, our results support the idea that D1R stimulation is a critical component of the context-dependent sensitization in TIPS in the mouse. The fact that sensitization to repeated cocaine injections seems less reliably dependent on D1R suggests that additional, possibly DA-independent, mechanisms are recruited by chronic treatment.

To explore further the specific contribution of dopamine, we used GBR12783, a specific inhibitor of the dopamine transporter. On repeated administration, this compound induced a context-dependent locomotor sensitization at 10 mg/kg (Boulay *et al*, 1996), but not at 5 mg/kg (Drouin *et al*, 2002). Pre-administration of GBR12783 also enhanced its own rewarding effects and those of cocaine measured in the conditioned place preference paradigm (Le Pen *et al*, 1996, 1998). Interestingly, our data using TIPS indicate that the increased extracellular dopamine levels induced by GBR12783 were not sufficient for revealing the locomotor sensitization. However, when mice pretreated with 15 mg/kg GBR12783 were challenged with cocaine, their sensitization was similar to that induced by cocaine. This result indicates that stimulation of dopaminergic transmission is sufficient for the induction, but not for the full expression of TIPS. It emphasizes the probable role of serotonin and/or norepinephrine in the expression of sensitization, as underlined by recent reports (Lanteri *et al*, 2008).

Although the molecular mechanisms underlying sensitization are still largely unknown, the stimulation of both D1 and NMDA receptors is needed for its induction, as discussed above. Interestingly, activation of the ERK pathway and immediate early genes expression induced by psychostimulants also requires the stimulation of D1 and NMDA receptors (Konradi *et al*, 1996; Valjent *et al*, 2000, 2005) and occurs specifically in D1R-expressing MSNs (Bertran-Gonzalez *et al*, 2008). Here, we show that specific blockade of DA uptake by GBR12783 also activated ERK specifically in a subpopulation of D1R-expressing striatonigral MSNs. Moreover, blockade of ERK activation markedly attenuates locomotor sensitization in response to cocaine (Valjent *et al*, 2005). Thus, converging evidence suggests that the ERK pathway is involved in the establishment of long-lasting changes that take place in MSNs and contribute to sensitization (see Girault *et al*, 2007 for a review). The decrease in sensitization observed in D1R hetereozygous mutant mice (see above) is consistent with

this hypothesis, as in these mice psychostimulant-induced ERK activation was virtually abolished (Corvol, Valjent, Pascoli, Hervé, Girault, unpublished observations), whereas the cAMP-dependent phosphorylation pathway was normal (Corvol *et al*., 2007). The cAMP pathway is also involved in these responses, as cAMP-dependent DARPP-32 phosphorylation is necessary for the phosphorylation of ERK in MSNs in response to drugs of abuse (Valjent *et al*, 2005). However, experiments testing locomotor sensitization in protocols using repeated injections have led to results that contradict this model, showing that sensitization to morphine was normal (Borgkvist *et al*, 2007) and sensitization to cocaine increased (Hiroi *et al*, 1999; Zachariou *et al*, 2006) in mice bearing null mutations of DARPP-32 or a Thr-34-Ala point mutation, which prevents the protein's ability to inhibit PP1 in response to cAMP stimulation. When we tested the effects of these mutations on the sensitization to morphine in TIPS, we found a complete lack of sensitization 7 days after the first injection in Thr-34-Ala mutant mice, but not in Thr-75-Ala and Ser-130-Ala mutants. A dramatic alteration of cocaine TIPS was also observed in KO and Thr-34-Ala mutant mice (Valjent *et al*, 2005). These results for both cocaine and morphine TIPS, which contrast with those obtained with repeated injections (Hiroi *et al*, 1999; Zachariou *et al*, 2006; Borgkvist *et al*, 2007), have interesting implications. First, they suggest that repeated injections overcome the deficit apparent after a single injection, perhaps by activating signaling pathways different from those activated after the first injection. For example, after repeated cocaine or morphine injections, induction of p35 may increase CDK5 activity, resulting in a higher phosphorylation of DARPP-32 on Thr-75 (Bibb *et al*, 2001; Scheggi *et al*, 2004). This phosphorylation leads to inhibition of cAMP-dependent protein kinase, an event that is expected to have opposite consequences to those induced by Thr-34 phosphorylation. Thus, TIPS may allow studying a simpler component of the responses induced by drugs of abuse. The normal phosphorylation of DARPP-32 on Thr-34 dramatically enhances the responsiveness to a single injection of cocaine or morphine, both in terms of acute effects (Valjent *et al*, 2005; Zachariou *et al*, 2006; Borgkvist *et al*, 2007), and of locomotor sensitization (Valjent *et al*, 2005 and this study). This supports the role of DARPP-32 as an amplifier of the D1 signaling pathway. In contrast, repeated injections may unravel a different aspect of the role of this protein, related to a negative feedback control.

TIPS shows that a single administration of cocaine or morphine reliably induces a long-lasting locomotor sensitization in mice when both injections are done in the same contextual environment. As TIPS is strongly context-dependent, it provides a simple paradigm to investigate the role of dopamine in the control of associative learning and its molecular mechanisms. Dopamine is necessary for the induction of this response, which also requires D1 dopamine and NMDA glutamate receptors, as well as PP1 inhibition by DARPP-32, ERK activation, and *de novo* protein synthesis. Evidence suggests that most if not all these biochemical responses occur in striatal MSNs, highlighting the important role of these neurons in locomotor sensitization.

In conclusion, we would like to emphasize that TIPS has the advantage of investigating the long-lasting effects of a single exposure to drugs and provides an ideal paradigm to study the parallelism between molecular and cellular changes and enhanced behavioral response. TIPS allows an easy distinction between the induction of sensitization (first injection) and its expression (second injection), which can be manipulated independently from each other. Its mechanisms are expected to be simpler than those of repeated injections in which responses to any injection are modified by the previous administrations in a manner that is difficult to control, and may combine tolerance and desensitization. Thus, TIPS is appropriate to study the first effects of drugs of abuse and provide a reference to determine how repeated administrations modify these effects. The modifications of sensitization mechanisms between single and repeated injections are important to study to better understand the responses to drugs of abuse. Thus, we suggest that TIPS, in parallel to other approaches, provides an excellent simple paradigm to study the mechanisms of the long-lasting effects of drugs in the brain.

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DISCLOSURE

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