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Morphine-Induced c-*fos* mRNA Expression in Striatofugal Circuits: Modulation by Dose, Environmental Context, and Drug History

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Opiates and psychostimulants produce many shared behavioral and neurobiological adaptations, such as behavioral sensitization and the induction of immediate early genes in the caudate-putamen (CPu). Previous studies indicate that factors such as dose, the environmental context surrounding drug administration and drug history can influence both morphine- and psychostimulant-induced behavioral sensitization. In addition, these factors can modulate the ability of psychostimulants to engage striatofugal circuits in the CPu. The present study, therefore, sought to examine whether these factors have similar influences over the ability of morphine to engage cortico-striatofugal circuits. We report that, when given in the home cage, morphine produced a small, but significant increase in the number of c-*fos* + striatonigral cells and c-*fos* + cells in cingulate cortex, but had no effect on the number of c-*fos* + striatopallidal cells. When given in a novel test environment, however, morphine dramatically increased the number of c-*fos* + striatonigral cells in a dose-dependent fashion, and this effect was maintained following repeated treatment. Unexpectedly, morphine treatment in a novel environment produced a dose-dependent *reduction* in the number of c-*fos* + striatopallidal cells and c-*fos* + cells in cingulate cortex, relative to exposure to novelty alone—effects that were reversed by repeated morphine treatment. We suggest that alterations in c-*fos* expression patterns in striatofugal circuits following morphine administration may be involved in drug-experience-dependent plasticity.
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INTRODUCTION

Although drugs of abuse, such as opiates and psychostimulants, can vary in their initial molecular mechanisms of action, they share the ability to induce many behavioral and neurobiological adaptations. These shared adaptations are an important topic of study because, presumably, some subset of them forms the core processes that mediate addiction. One well-studied example of a shared adaptation between opiates and psychostimulants is behavioral sensitization—a progressive and persistent increase in the psychomotor response following repeated, intermittent drug exposure. Although the detailed molecular underpinnings of behavioral sensitization are not yet clear, one neurobiological adaptation that is thought to contribute to sensitization is the activation of immediate early genes (IEGs) in mesocorticolimbic brain regions, including the caudate-putamen (CPu; Nestler *et al*, 1993; Hyman and Malenka, 2001).

Previous studies have determined that several factors such as dose, the environmental context surrounding drug administration, and drug history can exert strong influences over the behavioral and IEG activation effects of psychostimulant drugs (Badiani *et al*, 1998; Ostrander *et al*, 2003; Uslaner *et al*, 2003a, b). For example, when given in a novel test environment, psychostimulants produce a larger acute response and more robust behavioral sensitization compared to drug administration in the home cage (Badiani *et al*, 1995a, b). These factors can also have a large impact on which subclasses of medium spiny projection neurons in the CPu are engaged (ie, show IEG expression) by psychostimulants. When given in the home cage, psychostimulants induce IEGs predominately in cells that co-express mRNA for dopamine D1Rs, preprodynorphin, and preprotachykinin, and are part of the striatonigral pathway (Berretta *et al*, 1992; Cenci *et al*, 1992; Johansson *et al*, 1994; Ruskin and Marshall, 1994). When given in a novel test

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environment, however, psychostimulants also induce IEGs in cells that co-express mRNA for dopamine D2Rs and preproenkephalin and are part of the striatopallidal pathway (Jaber *et al*, 1995; Badiani *et al*, 1999; Uslaner *et al*, 2001).

The ability of morphine to induce psychomotor activation and promote behavioral sensitization is also modulated by factors such as dose, environmental context, and drug history (Badiani *et al*, 2000; Paolone *et al*, 2003). However, it is not yet known whether these factors have similar influences on which striatofugal circuits are engaged by morphine. To this end, we used dual *in situ* hybridization histochemistry to characterize the ability of morphine to induce one marker of neuronal activity, c-*fos* mRNA, in cells of the striatopallidal pathway, as a function of dose, environmental context and drug history. We report here that each of these factors influence the ability of morphine to engage the striatofugal circuits thought to be involved in drug experience-dependent behavioral plasticity.

METHODS

Subjects

Male Sprague–Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 200–225 g upon arrival were housed individually in clear square plastic cages containing shredded paper bedding, and were given a one-week acclimation period prior to any experimental manipulation. The rooms were temperature- and humidity-controlled and maintained on a 14 : 10 h light : dark cycle, with food and water available *ad libitum*. All experimental procedures were approved by the University of Michigan Committee on the Use and Care of Animals.

Drugs

Morphine sulfate (University of Michigan Hospital, Ann Arbor, MI) was dissolved in sterile 0.9% saline and administered by intraperitoneal (i.p.) injection in a volume of 1 ml/kg. Drug weight refers to the weight of the salt.

Groups and Test Procedures

Experiment 1: Morphine treatment in a novel test environment or in the home cage. Rats were randomly assigned to one of two groups ($n = 9$–10/group). One group of animals (Home) was transferred from the main animal colony to a testing room and placed individually into clear rectangular tubs ($8.5'' \times 17.5'' \times 9''$) containing a clear plastic insert in the center of the cage ($2.5'' \times 9'' \times 9''$) and ground corncob bedding on the floor. They remained in these cages for the duration of the experiment. Animals in the other group (Novel) remained in the main animal colony room. At 7 days after re-housing, Home groups received an i.p. injection of saline or morphine (2.5, 5 or 10 mg/kg) in their home cage (ie, the clear rectangular tubs). Animals in the Novel groups were transported to a testing room, where they received an injection of saline or morphine, and were then immediately placed into clear rectangular cages physically identical to those in which animals in the Home groups lived (but distinct from their own home cages). Behavior was recorded for 50 min and the total number of crossovers (defined by two consecutive beambreaks of sets of infrared photocells spaced 9″ apart across the length of the tub) was used as an index of locomotor activity. At 50 min following injections, animals were decapitated and their brains were removed, frozen in isopentane and stored at −70°C.

Experiment 2: Repeated morphine treatment in a novel test environment. Rats were randomly assigned to one of two groups. During treatment, animals were transported daily to a testing room and placed in cages identical to those described in Experiment 1, where they received daily injections of saline or morphine (10 mg/kg, i.p.) for 9 consecutive days. Behavior was recorded for 120 min during each test session. Following a 14-day withdrawal period, saline- and morphine-treated rats were subdivided into groups and returned to the testing room, where they received a challenge injection of saline or morphine (10 mg/kg) ($n = 7$–9/group). During the challenge test, behavior was recorded for 50 min. At 50 min following injections, animals were decapitated and their brains were removed, frozen in isopentane and stored at −70°C.

Dual *In Situ* Hybridization Methods

The brains were sectioned using a cryostat and 16 μm coronal sections were thaw-mounted onto Superfrost/Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at −70°C until processed for dual *in situ* hybridization (dISH). The method was a modification of that described by Curran and Watson (1995). Slides containing four tissue sections were processed using a ^{35}S-UTP and -CTP-labeled riboprobe complementary to c-*fos* mRNA (680-mer, courtesy of Dr T Curran, St Jude Children's Research Hospital, Memphis, TN) and a digoxigenin-UTP-labeled riboprobe complementary to preproenkephalin mRNA (693-mer, courtesy of Dr J Douglass, Amgen, Thousand Oaks, CA). The radioactive riboprobe was generated by incubating linearized c-*fos* DNA (1 μg) at 37°C for 1.5 h in 1 × transcription buffer, 100 μCi of α-^{35}S-UTP (100 Ci/mM; 20 mCi/ml; Amersham, Arlington Heights, IL), 160 μCi of α-^{35}S-CTP (800 Ci/mM; 40 mCi/ml; Amersham), 400 μM GTP, 400 μM ATP, 8 mM dithiothreitol (DTT), 10 U RNase inhibitor, and 50 U of T7 RNA polymerase. The nonradioactive riboprobe was generated by incubating linearized preproenkephalin DNA (1 μg) at 37°C for 1.5 h in 1 × transcription buffer, 320 μM digoxigenin-UTP (dig-UTP; Boehringer, Manheim, Germany), 80 μM UTP, 400 μM GTP, 400 μM ATP, 400 μM CTP, 10 mM DTT, 10 U RNase inhibitor, and 50 U of T7 RNA polymerase. The resulting probes were incubated at room temperature with 83 U of RNase-free DNase for 15 min and then separated from free nucleotides on Micro Bio-Spin Chromatography columns (BioRad, Hercules, CA).

Prior to hybridization, tissue sections were fixed in 4% phosphate-buffered paraformaldehyde for 1 h at room temperature, rinsed three times in 2 × SSC, placed into a solution of 0.1 M triethanolamine/0.25% acetic acid for 10 min, rinsed in water and dehydrated in a series of graded

alcohols (50–100%). The ^{35}S-labeled and dig-UTP-labeled probes were diluted in hybridization buffer (50% formamide, 10% dextran sulfate, 3 × SSC, 50 mM sodium phosphate, pH 7.4, 1 × Denhardt's solution, and 10 mg/ml yeast tRNA) to give an approximate concentration of $3–4 \times 10^6$ d.p.m./80 μl and 2.5 μl/80 μl, respectively. Slides were coverslipped with diluted probe (80 μl) and placed in hybridization trays lined with filter paper dampened with 50% formamide/50% water. The trays were sealed and incubated at 55°C for 16 h. Coverslips were floated off in 2 × SSC and the slides were rinsed three times in 2 × SSC. The slides were then incubated in RNase A (200 μg/ml) at 37°C for 1 h, rinsed in 2 ×, 1 ×, 0.5 ×, incubated in 0.1 × SCC at 65°C for 1 h, and then cooled to room temperature. The slides were rinsed in 0.1 M sodium phosphate buffer (pH 7.4), incubated with shaking for 1 h at room temperature in a blocking solution (0.25% carageenan, 0.5% Triton-X, 0.1 M sodium phosphate buffer), and then incubated overnight with shaking at room temperature with an antibody against digoxigenin, conjugated to alkaline phosphatase (sheep anti-dig AP, Fab fragments; Boehringer) that was diluted 1 : 10 000 in a blocking solution. Slides were then incubated two times at room temperature with shaking (1 h each) in 0.1 M sodium phosphate buffer, two times (30 min each) in Tris-buffered saline and rinsed in alkaline substrate buffer (ASB) (100 mM Tris base, 50 mM NaCl, 50 mM $MgCl_2$, pH 9.5). The color reaction was carried out in the dark at room temperature in ASB containing 5% polyvinyl alcohol, 0.025% levamisole, and 2% NBT/BCIP (Boehringer). After ~4 h, the color reaction was stopped by washing the slides extensively in water, incubating in 0.1 M glycine, 0.2% Triton X-100, pH 2.2 for 10 min at room temperature, and rinsing in water. Slides were then fixed in 2.5% glutaraldehyde for 2 h, rinsed in water, and air-dried.

Slides were exposed to an X-ray film for 3–4 days (Kodak Biomax MR, Rochester, NY) and then dipped in emulsion (Ilford KD-5, Polysciences, Warrington, PA) and stored in light-tight boxes at 4°C for 17–21 days. Slides were developed (Kodak D-19) for 2.5 min at 17°C, rinsed in water, and fixed (Kodak Rapid Fix) for 5 min at 17°C. Slides were then washed extensively in water, dehydrated in a series of graded alcohols (50–100%), washed extensively in xylene, and coverslipped with Permount mounting medium.

Control experiments using sense probes or tissue pretreated with RNase A (200 μg/ml at 37°C for 1 h) were performed to ensure probe specificity, and no binding was observed with either control.

Quantification of Gene Expression and Data Analysis

Initial quantification of c-*fos* mRNA expression was conducted on autoradiographs in multiple regions of the CPu across its entire rostrocaudal extent. The dorsomedial portion of the CPu at levels 0.0 mm to bregma and 0.4 mm posterior to bregma (see Figure 1; Paxinos and Watson, 1998) was selected for further analysis because, consistent with previous reports, the magnitude of c-*fos* expression induced by morphine was the greatest in this region. Cingulate cortex was also quantified at these levels (see Figure 1) because this region of frontal cortex is a major

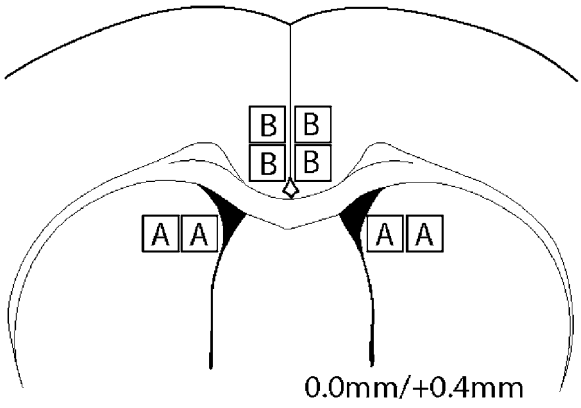

Figure 1 Illustration of a coronal section showing the regions in the CPu and cingulate cortex that were quantified. The boxes labeled 'A' represent the area of the dorsomedial CPu where the number of single-labeled c-*fos*+ and Enk+ cells and the number of double-labeled c-*fos*/Enk+ cells was counted. The boxes labeled 'B' represent the area of cingulate cortex where the number of single-labeled c-*fos*+ cells was counted. Cell counts for each region were averaged across two levels (0.0 mm and 0.4 mm from bregma) to give a total area of 1 mm^2.

source of glutamatergic input into the dorsomedial CPu (Spencer, 1976; Girault *et al*, 1986; McGeorge and Faull, 1989). Quantification was carried out by an experimenter blind to the experimental conditions at 20 × magnification using a Leica microscope (Letiz DMR, Wetzler, Germany) on sections from 7–10 animals/group. The total number of single- and double-labeled cells in the dorsomedial CPu and single-labeled cells in cingulate cortex were counted in two 250 μm^2 grids in each hemisphere at each level (for a total of four grids/region/level; see Figure 1). Cell counts for a given region were averaged across the two levels. ^{35}S-labeled cells (containing c-*fos* mRNA) appeared as silver grains under darkfield conditions and dig-labeled cells (containing preproenkephalin mRNA) appeared as purple precipitate under brightfield conditions (see Figure 3). Only ^{35}S-labeled cells that contained dense clusters of silver grains (ie, at least 10 silver grains/cell) and dig-labeled cells that were uniformly darkly stained (ie, at least 10 times above background staining) were considered to be positively labeled. The total number of preproenkephalin mRNA-positive cells did not differ across groups (data not shown).

The majority of cells in the CPu are medium spiny projection neurons that either co-express mRNA for dopamine D2 receptors and preproenkephalin and are part of the striatopallidal pathway (these will be called Enk+ cells) or co-express mRNA for dopamine D1 receptors, preprodynorphin and preprotachykinin and are part of the striatonigral pathway (these will be called Enk− cells). For example, we have found that under our *in situ* conditions, preproenkephalin mRNA and preprotachykin mRNA co-localize in only 4% of cells in the CPu (Uslaner *et al*, 2003a). Morphine reportedly induces c-Fos in very few interneurons in the CPu (Garcia and Harlan, 1997; Garcia *et al*, 2003), and therefore, in the present study, the number of c-*fos*/Enk+ cells was subtracted from the total number of c-*fos*+ cells in the CPu for each animal to give the number of c-*fos*/Enk− cells. This number was then used as an indication of

the number of cells in the striatonigral pathway that were activated following each treatment.

Statistical Analysis

For Experiment 1, group differences in locomotor activity and the number of c-*fos*+ cells were tested using planned *t*-tests, one-way analyses of variances (ANOVA) followed by Dunnett's post-hoc tests or two-way ANOVA followed by Bonferroni's post-hoc tests. Relationships between variables were analyzed using Pearson correlation coefficients. For all comparisons, $\alpha < 0.05$.

For Experiment 2, group differences in locomotor activity during treatment were tested using two-way repeated-measures ANOVA followed by Bonferroni's post-hoc tests, and within-group comparisons were made using paired *t*-tests. For the challenge data, group differences in locomotor activity and the number of c-*fos*+ cells were analyzed using planned *t*-tests. Relationships between variables were analyzed using Pearson correlation coefficients. For all comparisons, $\alpha < 0.05$.

RESULTS

Experiment 1: Environmental Context and Dose Modulate Morphine-Evoked Psychomotor Activation and c-*fos* Expression

The purpose of this experiment was to explore how dose and environmental context interact to influence the ability of a single, first injection of morphine (this will be referred to as an 'acute' injection) to modulate psychomotor activation and to engage specific cortico-striatofugal circuits.

Locomotor behavior. Figure 2 (panel a) depicts the effects of environmental context and dose on the ability of an acute injection of morphine to modulate locomotor activity during the first 50 min following treatment. When given in the home cage, morphine treatment did not alter locomotor activity compared to saline treatment, at any dose tested (Figure 2a; $F_{3,23} = 0.31$, $p = 0.82$). Mere exposure to a novel environment increased locomotor activity compared to saline treatment in the home cage (Figure 2a; $t = 3.57$, $p = 0.003$). Morphine administration in a novel test environment decreased this novelty-evoked locomotor activity in a dose-dependent fashion (Figure 2a; $F_{3,27} = 5.93$, $p = 0.003$).

c-fos mRNA expression. Figure 2 (panels b–d) shows the effects of environmental context and dose on the ability of an acute injection of morphine to induce c-*fos* mRNA in cells of the cingulate cortex (panel b) and in cells of the CPu that are part of the striatopallidal pathway and express preproenkephalin mRNA (Enk+ cells; panel d) or those cells that are part of the striatonigral pathway and do not contain preproenkephalin mRNA (Enk− cells; panel c). Figure 3 provides a representative example of a c-*fos*/Enk+ dual *in situ*. As expected from previous studies, saline treatment in the home cage induced very few c-*fos*+ cells. Therefore, this group was used as an index of basal levels of c-*fos* mRNA expression.

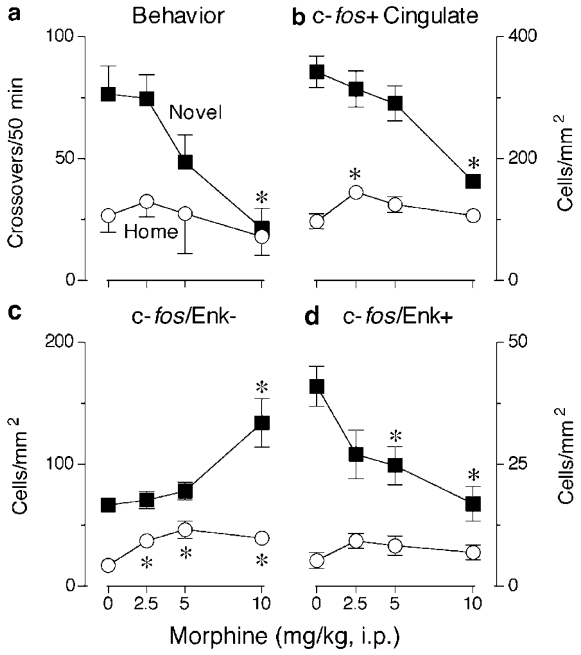

Figure 2 Dose and environmental context modulate morphine-induced locomotor activity and the number of c-*fos*+ cells in cingulate cortex and in specific striatal cell populations. Black squares represent groups given morphine in a novel test environment and white circles represent groups given morphine in the home cage. (a) The mean (±SEM) number of crossovers during the first 50 min following treatment. (b) The mean (±SEM) number of c-*fos*+ cells in cingulate cortex. (c) The mean (±SEM) number of c-*fos*/Enk− cells in the CPu. (d) The mean (±SEM) number of c-*fos*/Enk+ cells in the CPu. *, differs from 0 mg/kg ($p < 0.05$, one-way ANOVA followed by Dunnett's test).

When given in the home cage, only 2.5 mg/kg morphine significantly increased the total number of c-*fos*+ cells in cingulate cortex above baseline (Figure 2b; $F_{3,27} = 3.22$, $p = 0.04$). In addition, morphine produced small, but significant increases in the number of c-*fos*/Enk− cells in the CPu (Figure 2c; $F_{3,27} = 5.91$, $p = 0.003$), although the magnitude of this effect was not dose-dependent. There was no effect of morphine administration in the home cage on the number of c-*fos*/Enk+ cells (Figure 2d; $F_{3,27} = 0.89$, $p = 0.46$).

Mere exposure to novelty significantly increased the total number of c-*fos*+ cells in cingulate cortex above baseline (Figure 2b; $t = 12.02$, $p < 0.0001$), and morphine produced a dose-dependent decrease in the number of these novelty-evoked c-*fos*+ cells in cingulate cortex (Figure 2b; $F_{3,27} = 9.28$, $p = 0.0002$). Mere exposure to novelty also significantly increased the number of c-*fos*/Enk− cells in the CPu (Figure 2d; $t = 7.40$, $p < 0.0001$), and morphine treatment in a novel environment produced a further dose-dependent increase in the number of c-*fos*/Enk− cells (Figure 2c; $F_{3,27} = 8.13$, $p = 0.0005$). Finally, exposure to a novel environment alone significantly increased the number of c-*fos*/Enk+ cells in the CPu (Figure 2d; $t = 7.52$, $p < 0.0001$), but, in sharp contrast to the effect on Enk− cells, morphine treatment in a novel environment *decreased* the number of novelty-evoked c-*fos*/Enk+ cells in a dose-dependent fashion (Figure 2d; $F_{3,27} = 5.55$, $p = 0.004$).

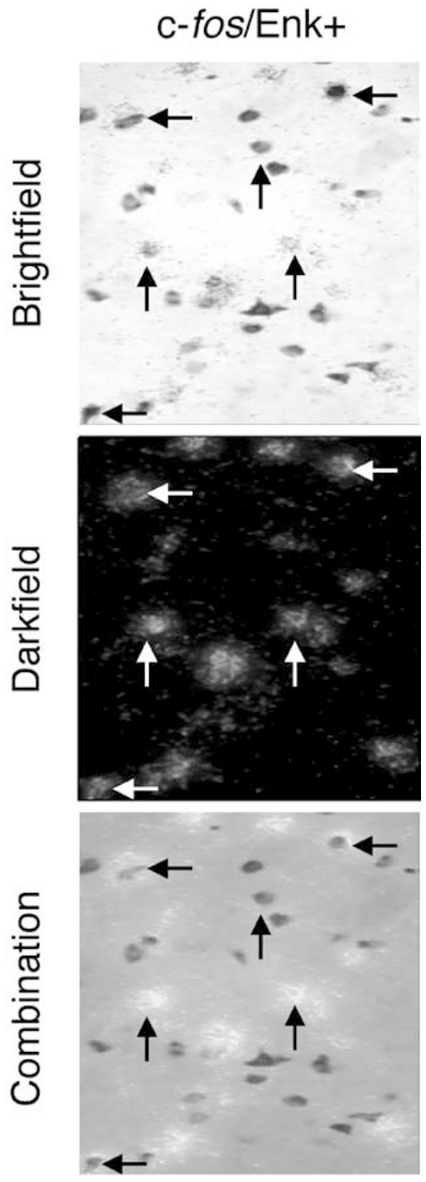

Figure 3 Representative histological plates depicting sections from the dorsomedial CPu that were double-labeled for c-*fos* mRNA and preproenkephalin mRNA. Sections were taken from an animal that received repeated morphine treatment (10 mg/kg, i.p.) in a novel test environment, followed by a 14-day withdrawal period and a morphine challenge (10 mg/kg, i.p.). (Top) Brightfield image in which Enk+ cells are indicated by purple precipitate. (Middle) Darkfield image in which c-*fos*+ cells are indicated by clusters of silver grains. (Bottom) Overlay of brightfield and darkfield images. Up arrows indicated single-labeled cells (c-*fos*+ or Enk+). Left arrows indicate double-labeled cells (c-*fos*/Enk+).

Experiment 2: Drug History Modulates the Ability of Morphine to Produce Psychomotor Activation and Engage Specific Cortico-striatofugal Circuits

We next examined the extent to which repeated, intermittent administration of morphine in a novel test environment—which induces robust psychomotor sensitization—engages specific cortico-striatofugal circuits.

Locomotor behavior. The locomotor response during the treatment phase of this experiment is illustrated in Figure 4. There was a significant interaction in the number of crossovers summed over the entire 120 min test session between the treatment day and group factors (Figure 4a; $F_{1,34} = 19.57$, $p < 0.0001$). Post-hoc tests revealed that there was no difference between animals given saline or morphine (10 mg/kg, i.p.) on day 1 (Figure 4a; $t = 0.56$, $p > 0.05$), but there was a significant difference between these groups on day 9 (Figure 4a; $t = 3.90$, $p < 0.001$), due to an increase in locomotor response over days in the morphine-treated group and a decrease in the saline-treated group.

Figure 5 (panel a) shows the locomotor response to a challenge injection of saline or morphine (10 mg/kg, i.p.) administered 14 days following the last (9th) treatment injection. As there were no differences in either locomotor activity or c-*fos* expression between saline- and morphine-treated animals that were given a saline challenge, data from these two groups were pooled to form a single saline challenge control group (saline group; Figure 5, panels a–d). Similar to the results from Experiment 1, administration of morphine to saline-treated animals (acute morphine group) produced a significant decrease in locomotor activity, relative to the saline group (Figure 5a; $t = 6.05$, $p < 0.0001$). When morphine-treated animals were challenged with morphine (repeated morphine group), however, they responded with a significant increase in locomotor activity compared to the acute morphine group, confirming that this group was sensitized (Figure 5a; $t = 3.66$, $p = 0.002$).

In Experiment 1 and the challenge test of Experiment 2 we report that acute morphine administration in a novel test environment produces a decrease in locomotor activity compared to saline-treated animals. However, in the treatment phase of Experiment 2 we report that on Day 1 there is no difference in the locomotor response of animals given saline or morphine. In addition, on Day 9 of the treatment phase of Experiment 2, morphine-treated animals have a greater locomotor response than saline-treated animals, whereas in the challenge test of Experiment 2 there are no differences in locomotor activity between these groups. The apparent discrepancies in these findings can be attributed to the biphasic pattern of psychomotor activation elicited by morphine. Acute administration of 10 mg/kg morphine produces an initial depressant effect lasting approximately 60 min, followed by an increase in locomotor activity (Figure 4b). Repeated administration of 10 mg/kg morphine produces both a tolerance to the initial locomotor depressant effect and sensitization to the locomotor stimulant effects (Figure 4c). In order to optimize conditions for studying mRNA expression, animals were killed 50 min following morphine administration during Experiment 1 and the challenge test of Experiment 2, and therefore, only the first phase of the locomotor response to morphine was captured.

c-fos mRNA expression. Figure 5 (panels b–d) depicts the effects of repeated morphine treatment (10 mg/kg, i.p.) on the number of c-*fos*+ cells in cingulate cortex (panel b) and the number of c-*fos*/Enk− cells (panel c) or the number of c-*fos*/Enk+ cells (panel d) in the CPu. Consistent with the results from Experiment 1, acute morphine treatment (10 mg/kg, i.p.) produced a significant decrease in the total number of c-*fos*+ cells in cingulate cortex compared to the saline group (Figure 5b; $t = 4.20$, $p = 0.0003$). Repeated

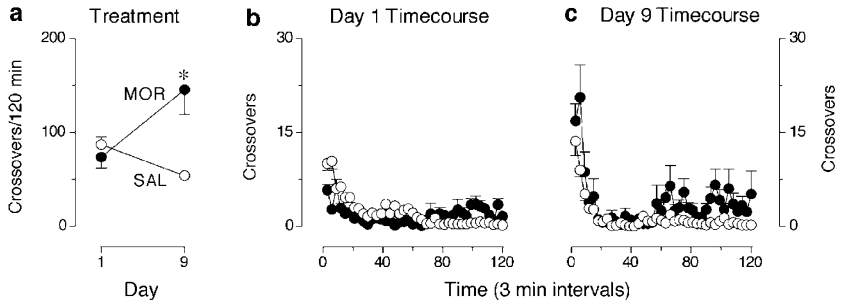

Figure 4 Repeated, intermittent exposure to morphine (10 mg/kg, i.p.) produces locomotor sensitization. (a) The mean (±SEM) number of crossovers summed over 120 min on day 1 and day 9 of treatment. (b) The mean (±SEM) number of crossovers over time (3-min intervals) on day 1. (c) The mean (±SEM) number of crossovers over time (3-min intervals) on day 9. Animals given morphine (black circles) made significantly more crossovers on day 9 than animals given saline (white circles). *differs from saline ($p<0.05$, two-way ANOVA, followed by Bonferroni's test).

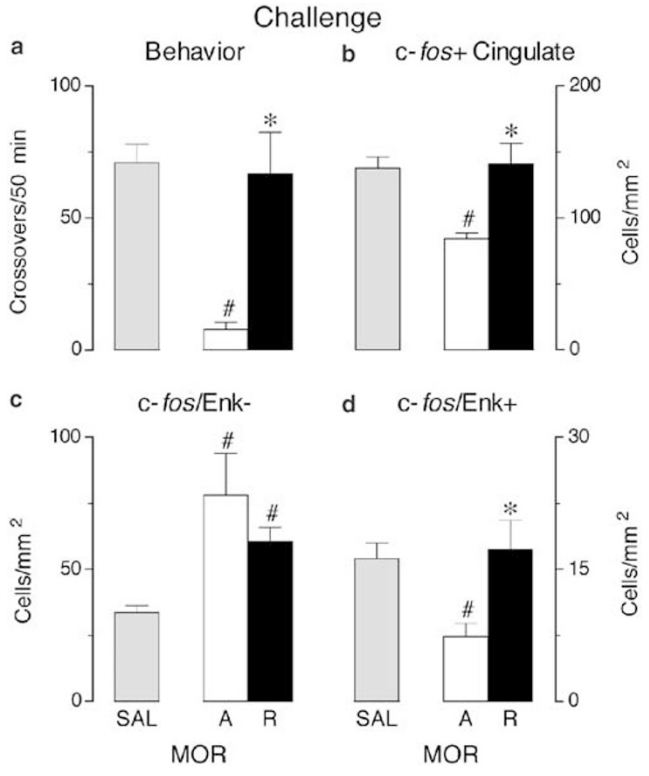

Figure 5 Drug history modulates morphine-induced locomotor activity and the number of c-*fos*+ cells in cingulate cortex and in specific striatal cell populations. There were no differences between saline- and morphine-treated (10 mg/kg, i.p.) animals that were given a saline challenge (saline group), so these groups were combined (gray bars). White bars represent saline-treated animals given a morphine challenge (10 mg/kg, i.p.; acute morphine group) and black bars represent morphine-treated animals given a morphine challenge (repeated morphine group). (a) The mean (±SEM) number of crossovers during the first 50 min following treatment. (b) The mean (±SEM) number of c-*fos*+ cells in cingulate cortex. (c) The mean (±SEM) number of c-*fos*/Enk− cells in the CPu. (d) The mean (±SEM) number of c-*fos*/Enk+ cells in the CPu. *differs from the acute morphine group, #differs from the saline group ($p<0.05$, planned *t*-tests).

morphine treatment upregulated the total number of c-*fos*+ cells in cingulate cortex compared to the acute morphine group (Figure 5b; $t=3.45$, $p=0.003$); however, there were no differences in the number of c-*fos*+ cells in cingulate cortex compared to the saline group (Figure 5b; $t=0.19$, $p=0.85$). Both acute and repeated morphine treatment significantly increased the number of c-*fos*/Enk− cells in the CPu compared to the saline group (Figure 5c; $t=3.86$–5.09, $p<0.001$), but there were no differences in the number of c-*fos*/Enk− cells between these groups (Figure 5c; $t=1.06$, $p=0.3$). Finally, similar to the results from Experiment 1, acute morphine treatment produced a significant decrease in the number of c-*fos*/Enk+ cells compared to the saline group (Figure 5d; $t=3.19$, $p=0.004$). However, repeated morphine treatment upregulated the number of c-*fos*/Enk+ cells compared to the acute morphine group (Figure 5d; $t=2.74$, $p=0.01$), although there were no differences in the number of c-*fos*/Enk+ cells compared to the saline group (Figure 5d; $t=0.31$, $p=0.76$).

Morphine-Induced Cortical Activation is Positively Correlated With Enk+ Cell Activation

Figure 6 illustrates the relationships between morphine-induced cortical activation (ie, the number of c-*fos*+ cells in cingulate cortex) and morphine-induced activation of either the striatonigral pathway (ie, the number of c-*fos*/Enk− cells; top panels) or the striatopallidal pathway (ie, the number of c-*fos*/Enk+ cells; bottom panels). In all conditions, cortical activation was positively and significantly correlated with activation of the striatopallidal pathway (Dose × Environment: panel e (home groups), $r=0.79$; $p<0.0001$; panel f (novel groups), $r=0.72$; $p<0.0001$; Drug History: panel g, $r=0.86$; $p<0.0001$). There were no significant correlations between cortical activation and activation of the striatonigral pathway (Dose × Environment: panel a (home groups), $r=0.21$; $p=0.31$; panel b (novel groups), $r=-0.30$; $p=0.17$; Drug History: panel c, $r=-0.01$; $p=0.98$).

Morphine-Induced Psychomotor Activation is Positively Correlated with Activation of Enk+ Cells

Figure 7 illustrates the relationships between morphine-induced locomotor activity (ie, the number of crossovers) and morphine-induced activation of either the striatonigral pathway (ie, the number of c-*fos*/Enk− cells; top panels) or the striatopallidal pathway (ie, the number of c-*fos*/Enk+ cells; bottom panels). In all conditions, there were significant positive correlations between locomotor activity and activation of the striatopallidal pathway (Dose × Environment: panel e (home groups), $r=0.55$; $p=0.01$;

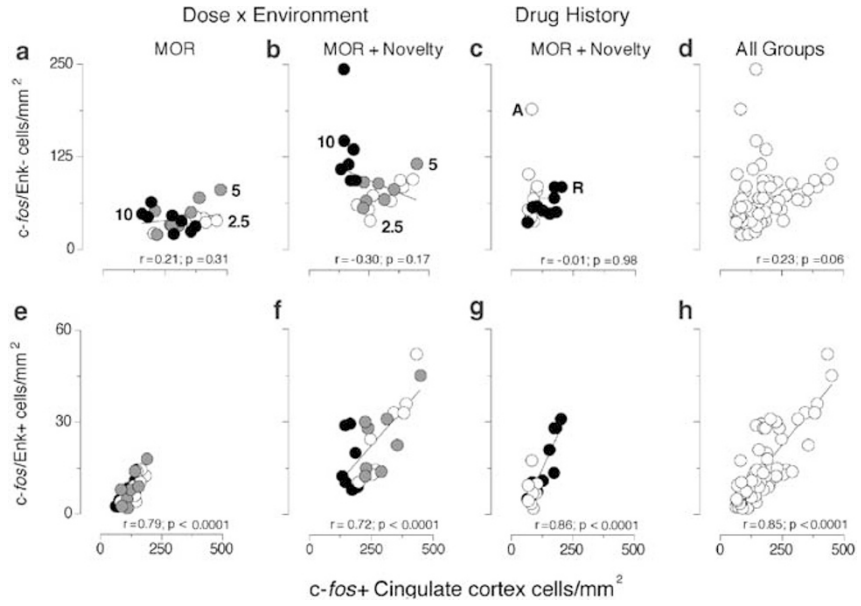

Figure 6 Morphine-induced cortical activation is positively correlated with activation of the striatopallidal pathway. Relationships between the number of morphine-evoked c-*fos*+ cells in cingulate cortex (*x* axes) and the number of morphine-evoked c-*fos*/Enk− cells (*y* axes, top panels) or the number of morphine-evoked c-*fos*/Enk+ cells (*y* axes, bottom panels) as a function of dose (panels a, b, e, f: 2.5 (white circles), 5 (gray circles), and 10 (black circles) mg/kg morphine), environmental context (panels a, b, e, f: morphine administration in the home cage (MOR) and morphine administration in a novel test environment (MOR + novelty)), and drug history (panels c, g: acute morphine treatment (A; white circles) and repeated morphine treatment (R; black circles)).

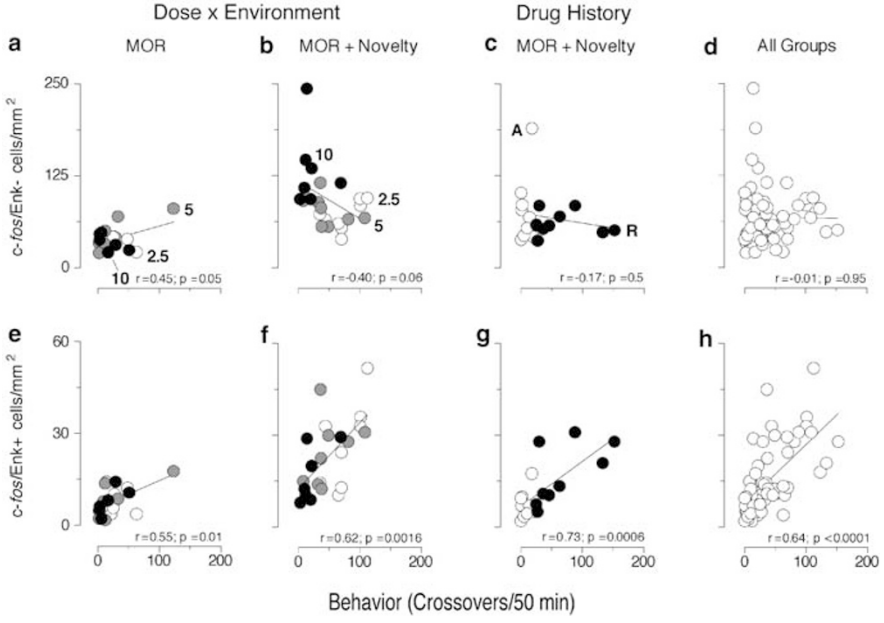

Figure 7 Morphine-induced psychomotor activation is positively correlated with activation of the striatopallidal pathway. Relationship between the number of morphine-induced crossovers (*x* axes) and the number of morphine-evoked c-*fos*/Enk− cells (*y* axes, top panels) or the number of morphine-evoked c-*fos*/Enk+ cells (*y* axes, bottom panels) as a function of dose (panels a, b, e, f: 2.5 (white circles), 5 (gray circles), and 10 (black circles) mg/kg morphine), environmental context (panels a, b, e, f: morphine administration in the home cage (MOR) and morphine administration in a novel test environment (MOR + Novelty)), and drug history (panels c, g: acute morphine treatment (A; white circles) and repeated morphine treatment (R; black circles)).

panel f (novel groups), $r = 0.62$; $p = 0.0016$; Drug History: panel g, $r = 0.73$; $p = 0.0006$). There were no significant correlations between locomotor activity and activation of the striatonigral pathway (Dose × Environment: panel a (home groups), $r = 0.45$; $p = 0.05$; panel b (novel groups), $r = -0.40$; $p = 0.06$; Drug History: panel c, $r = -0.17$; $p = 0.5$).

DISCUSSION

The present study examined how three factors, dose, the environmental context surrounding drug administration, and drug history influence the ability of morphine to induce c-*fos*+ cells in the CPu and cingulate cortex. Our results demonstrate that each of these factors has a strong influence

on which cortico-striatofugal circuits are engaged by morphine. When given in the home cage, morphine produced a small, but significant, increase in the number of c-*fos*+ striatonigral cells and c-*fos*+ cells in cingulate cortex, but had no effect on the number of c-*fos*+ striatopallidal cells. When given in a novel test environment, morphine dramatically *increased* the number of c-*fos*+ striatonigral cells in a dose-dependent fashion, and this effect was maintained following repeated treatment. Unexpectedly, morphine treatment in this condition led to a dose-dependent *reduction* in the number of c-*fos*+ striatopallidal cells and c-*fos*+ cells in cingulate cortex, relative to exposure to novelty alone—effects that were reversed by repeated morphine treatment.

Morphine-Induced c-*fos* Expression in the Striatopallidal Pathway may be Regulated by Glutamatergic Afferents from Frontal Cortex

The general pattern of immediate early gene (IEG) expression elicited by morphine in the present study (eg, c-*fos* induction primarily in the most dorsomedial portion of the CPu and cingulate cortex) is consistent with the existing literature (Liu *et al*, 1994; Garcia *et al*, 1995; Curran *et al*, 1996; Erdtmann-Vourliotis *et al*, 1999b; Frankel *et al*, 1999b; see Harlan and Garcia (1998) for review). Our results are also consistent with a recent report showing that morphine administration in a stressful environment induces greater c-*fos* expression in the dorsomedial CPu than morphine administration in a calm environment (Mayer *et al*, 2002). Our work extends these previous reports by determining which populations of medium spiny projection neurons are engaged by morphine as a function of dose, the environmental context surrounding drug administration and drug history.

Studies examining the mechanisms underlying morphine-evoked IEG expression in the CPu have established that co-activation of μ opioid and dopamine receptors is required for IEG induction to occur (Liu *et al*, 1994; Sharp *et al*, 1995; Bontempi and Sharp, 1997; Frankel *et al*, 1999a). Morphine is thought to act at μ opioid receptors to disinhibit mesolimbic dopamine neurons, resulting in increased dopamine release in the CPu (Matthews and German, 1984; Di Chiara and North, 1992; Johnson and North, 1992; Spanagel *et al*, 1992; Devine *et al*, 1993). Dopamine is then thought to induce IEG expression via D1 receptor activation of the cAMP/PKA signaling cascade (Lovenberg *et al*, 1991; Cole *et al*, 1994; Konradi *et al*, 1994, 1996). In addition, the ability of morphine to induce IEGs is strongly regulated by glutamate, as NMDA receptor antagonists (Liu *et al*, 1994; Bontempi and Sharp, 1997; D'Souza *et al*, 1999), AMPA receptor antagonists (Garcia *et al*, 2003), and metabotropic glutamate receptor antagonists (Garcia *et al*, 2003) all attenuate morphine-evoked IEG expression in the CPu.

The source of glutamatergic regulation of morphine-induced IEG expression in specific striatal cell populations has not been established. A likely candidate is the frontal cortex, as this region has strong glutamatergic connections to the dorsomedial CPu (Spencer, 1976; Girault *et al*, 1986; McGeorge and Faull, 1989). Using c-*fos* as a marker of neuronal engagement, we examined the relationships between morphine-induced activation of cingulate cortex and morphine-induced activation of both striatal cell populations. We found a strong positive correlation between morphine-induced cingulate cortex activation and activation of Enk+ cells, suggesting that the frontal cortex is a source of glutamatergic regulation of the striatopallidal pathway. Consistent with this idea, stimulation of cortico-striatal afferents preferentially engages Enk+ cells in the CPu, an effect that is blocked by glutamate receptor antagonists (Berretta *et al*, 1997; Parthasarathy and Graybiel, 1997; Sgambato *et al*, 1997).

On the other hand, there was no correlation between morphine-induced cingulate cortex activation and activation of Enk− cells, suggesting that cortical regulation of IEG expression in the striatum may be specific to the striatopallidal pathway. It is not yet clear what is responsible for glutamatergic regulation of IEG expression in the striatonigral pathway. One possibility is that this occurs via thalamic glutamatergic inputs to the CPu. This idea is supported by the finding that intrathalamic infusion of a μ opioid receptor antagonist decreases the ability of acute morphine treatment (which only engages Enk− cells) to induce c-Fos expression in the CPu (Frankel *et al*, 1999a). Further studies are necessary to confirm these roles of cortical and thalamic afferents in modulation of morphine-evoked IEG expression in the CPu.

Morphine Induces a Different Pattern of Gene Expression in the Striatopallidal Pathway than that of Psychostimulants

We have previously shown that manipulating factors such as dose, the environmental context surrounding drug administration, and drug history can strongly influence which striatofugal circuits are engaged by psychostimulant drugs (Badiani *et al*, 1999; Uslaner *et al*, 2001, 2003a, b). For example, psychostimulant administration in a novel test environment produces a dose-dependent increase in c-*fos* expression in Enk− cells compared to novelty alone (Uslaner *et al*, 2003a, b), and this increase in c-*fos* expression is maintained following repeated treatment and extended withdrawal (Uslaner *et al*, 2003a). The present study shows that morphine administration in a novel test environment also induces this pattern of striatonigral cellular activation. Although the mechanism of this activation in either case is not yet clear, the fact that antagonists of either dopamine or glutamate receptors block both morphine- and psychostimulant-evoked c-*fos* expression in Enk− cells (Liu *et al*, 1994; Ferguson *et al*, 2003) suggests that this regulation may be occurring through a common mechanism.

In contrast, there are some significant differences in c-*fos* expression in Enk+ cells elicited by these two classes of drugs of abuse. For example, morphine treatment in a novel test environment leads to a dose-dependent *decrease* in novelty-evoked c-*fos* expression in Enk+ cells in the CPu, an effect that is reversed by repeated morphine treatment. By contrast, psychostimulants show a different profile of c-*fos* activation in Enk+ cells. Although high doses of amphetamine (but not cocaine) given in a novel test environment have a similar inhibitory effect on c-*fos* expression as does morphine (Uslaner *et al*, 2003a, b), low

to moderate doses of either amphetamine or cocaine *increase* c-*fos* expression in Enk+ cells (Uslaner *et al*, 2001, 2003a, b), an effect that is maintained following repeated treatment and extended withdrawal (Uslaner *et al*, 2003a).

What might explain the opposing effects of morphine and psychostimulants on Enk+ cell activation? Given the hypothesized role of corticostriatal afferents as an important modulator of striatopallidal activity, one possibility is that these opposing effects are due to differential drug actions on cortical activity and subsequent striatal glutamate release. Acute administration of morphine, which decreases the number of novelty-evoked c-*fos*/Enk+ cells, concomitantly decreases the number of novelty-evoked c-*fos*+ cells in cingulate cortex, as well as firing of cortical neurons (Giacchino and Henriksen, 1996, 1998) and corticostriatal glutamate release (Desole *et al*, 1996; Nicol *et al*, 1996). By contrast, at doses that activate Enk+ cells, psychostimulants increase novelty-evoked c-*fos* expression in cingulate cortex (Badiani *et al*, 1998) and stimulate corticostriatal glutamate release (Smith *et al*, 1995; Reid *et al*, 1997; Gray *et al*, 1999). Furthermore, transection of corticostriatal afferents leads to a selective blockade of psychostimulant-evoked c-*fos* expression in Enk+ cells (SMF and TER unpublished observations). Similarly, repeated morphine treatment, which engages Enk+ cells and upregulates the number of c-*fos*+ cells in cingulate cortex (compared to acute treatment), also upregulates striatal glutamate release (Desole *et al*, 1996). All of this evidence suggests that glutamatergic afferents from the cortex play an important regulatory role in activation of the striatopallidal pathway across drug classes.

Is Striatal c-*fos* Expression Just a Consequence of Locomotor Activity?

Acute psychostimulant treatment in a novel test environment results in marked psychomotor activation and c-*fos* expression in Enk+ cells (Badiani *et al*, 1999; Uslaner *et al*, 2001), whereas acute administration of morphine has the opposite effect on locomotor activity and c-*fos* expression in Enk+ cells. It is tempting to speculate, therefore, that c-*fos* expression in these studies is secondary to changes in locomotor activity. However, drugs that elicit large locomotor responses tend to induce weaker c-Fos expression in the CPu than drugs that elicit little to no locomotor responses (Erdtmann-Vourliotis *et al*, 1999a). And, in fact, in the present study, acute administration of 10 mg/kg morphine in a novel test environment evoked the greatest number of c-*fos*/Enk− cells in the CPu, even though there was little locomotion in this group. These data suggest that striatal c-*fos* expression is not merely a reflection of ongoing locomotor activity, but may actually play a role in mediating that activity.

The Ability of Morphine to Engage the Striatopallidal Pathway is Correlated with Psychomotor Activation

Traditional models of basal ganglia function postulate that dopamine-mediated psychomotor activation occurs via concomitant excitation of the cortico-striatonigral pathway and inhibition of the cortico-striatopallidal pathway. This combination is proposed to have a net effect of inhibiting basal ganglia output nuclei, ultimately leading to disinhibition of the thalamus and consequent motor output (Albin *et al*, 1989; Gerfen *et al*, 1990; Gerfen, 1992). Based on these models, we would predict that in the present study there would be positive relationships between morphine-induced psychomotor activation and engagement of the striatonigral pathway and negative relationships between morphine-induced psychomotor activation and engagement of the striatopallidal pathway. This was not the case. Using c-*fos* as an indicator of neuronal engagement, we found no relationship between morphine-induced locomotor activity and activation of the striatonigral pathway, but a strong *positive* correlation between morphine-induced locomotor activity and activation of the striatopallidal pathway. Consistent with our results, morphine infused directly into the substantia nigra leads to a negative correlation between rotational behavior and c-Fos expression in the dorsomedial CPu (Bontempi and Sharp, 1997). These findings are also in agreement with recent reports that intrastriatal infusions of dopamine agonists, which increase locomotor activity, also increase neuronal firing in basal ganglia output nuclei (Waszczak *et al*, 2001, 2002), and that intravenous infusions of dopamine agonists, at doses that are sufficient to induce rotational behavior and increase c-Fos in both striatofugal pathways, did not decrease the firing rate of basal ganglia output nuclei (Ruskin *et al*, 1999).

The functional role(s) of alterations in IEG expression patterns in striatofugal circuits in morphine sensitization is not yet known. In the present study, acute morphine treatment led to activation of the striatonigral pathway, yet morphine did not elicit a locomotor response. The level of activation of the striatonigral pathway did not change as a function of drug history (ie, both acute and repeated morphine treatment led to the same level of activation), yet repeated morphine treatment results in behavioral sensitization. Importantly, acute morphine treatment also prevented activation of the striatopallidal pathway, an effect that was reversed by repeated morphine treatment. It should be noted, however, that repeated morphine treatment does not activate the striatopallidal pathway beyond that of saline controls, and therefore may reflect a tolerance to the acute inhibitory effects of morphine on this pathway. We propose that a threshold level of activation of the striatonigral pathway must be obtained for morphine sensitization to occur. Once this threshold is reached, activation of the striatopallidal pathway may become an important factor in the development of sensitization. Future studies targeted toward selective manipulations of these striatofugal circuits are necessary to confirm this hypothesis.

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