The Role of Dopamine D4 Receptor in the Induction of Behavioral Sensitization to Amphetamine and Accompanying Biochemical and Molecular Adaptations

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ABSTRACT

Our studies examined the role of dopamine D4 receptors in the induction of behavioral sensitization to amphetamine (Amp) and accompanying neurochemical and molecular adaptive responses using a highly selective D4 antagonist, PNU-101387G. Behavioral sensitization to an acute challenge of Amp (2 mg/kg, s.c.) was observed in rats pretreated with five daily doses of Amp (2 mg/kg/d, s.c.) followed by 7-day withdrawal. Interestingly, coadministration of PNU-101387G with Amp during pretreatment completely blocked the sensitized response to an acute Amp challenge. The behavioral sensitization and its blockade by the D4 antagonist were observed in the absence of significant differences in cerebellar Amp levels among the various pretreatment groups. Accompanying behavioral sensitization were two postsynaptic neuroadaptive responses: reduction in the ability of Amp to induce c-fos gene expression in the infralimbic/ventral prelimbic cortex and NT/N mRNA in the accumbal shell. However, concurrent blockade of D4 receptors during Amp pretreatment prevented the refractoriness in c-fos and NT/N responsiveness to acute Amp. We observed also a presynaptic neuroplastic response associated with the behavioral sensitization: a significant augmentation in the ability of Amp to increase extracellular dopamine concentrations in the nucleus accumbens shell. As with the behavioral sensitization and associated postsynaptic adaptive responses, concurrent administration of PNU-101387G with Amp during pretreatment blocked the augmentation in Amp-induced dopamine release. Taken together, these data demonstrate that dopamine D4 receptors play an important role in the induction of behavioral sensitization to Amp and accompanying adaptations in pre- and postsynaptic neural systems associated with the mesolimbic/cortical dopamine projections.

Repeated administration of psychostimulants, like d-Amp, to rodents produces a progressive and long-lasting increase in behaviors such as locomotor activity and stereotypy, a phenomenon generally known as “behavioral sensitization” or “reverse tolerance” (Segal, 1975, Post and Contel, 1983). The enduring hypersensitivity to psychostimulants is observed also in humans and is thought to underlie drug addiction as well as stimulant psychosis (reviewed by Robinson and Berridge, 1993; Lieberman et al., 1997). Hence, recent research has focused on identification and characterization of neuroadaptive responses leading to behavioral sensitization to stimulants.

The mesolimbic dopamine system plays a critical role in the development of sensitization to Amp (reviewed by Kalivas and Stewart, 1991; White and Wolf, 1991; Robinson, 1991) which acts primarily by releasing dopamine. Studies of microinjections of the stimulant in discrete brain regions indicate that the initiation of sensitization may be produced by the dopamine-releasing effect of Amp in the origin of the mesolimbic dopamine neurons, the VTA (Kalivas and Weber, 1988; Vezina, 1993; Cad er et al., 1995). Activation of D1 (D1, D5) but not D2 (D2, D3, D4) receptors in the VTA is proposed to initiate sensitization since administration of a D1-like antagonist (but not D2-like antagonist) directly into the VTA blocks the development of sensitization to peripherally administered Amp (Stewart and Vezina, 1989; B i jou et al., 1996). However, the involvement of D2-like receptors in the initiation of methamphetamine-induced behavioral sensitization is evident in a number of studies in mice using peripheral administration of nonselective D2 antagonists (Ujike et al., 1989; Kuribara, 1994; Kuribara, 1996). It is possible that in addition to the VTA D1 receptors, D2-like receptors expressed at a site(s) outside the VTA participate in the induction of sensitization to Amp. This is supported by a recent

ABBREVIATIONS: PNU-101387G; (S(-)-4-[4-[2-isochroman-1-yl]ethyl]piperazin-1-yl]benzenesulfonamide); amphetamine: Amp, NT/N, neurotensin/neuromedin; VTA, ventral tegmental area; LMA, locomotor activity; IL, infralimbic cortex; vPL, ventral prelimbic cortex; NA, nucleus accumbens; NA-s, nucleus accumbens-shell; NA-c, nucleus accumbens-core.
The D2-like receptors are encoded by three distinct genes termed, D2, D3 and D4 (Bunzow et al., 1988; Sokoloff et al., 1990; Van Tol et al., 1991). The studies cited above examined the role of D2 receptors in induction of sensitization using antagonists that did not distinguish among members of the D2 receptor family. Hence, the role of each subtype of D2-like receptors in stimulant sensitization remains unknown. Recently, a D4 receptor selective antagonist, PNU-101387G (previously termed U-101387G) has become available and shows pharmacological properties distinct from those of non-selective D2-like antagonists (Merchant et al., 1996b). We used PNU-101387G as a tool to understand the role of D4 receptors in the induction of behavioral sensitization to Amp.

The development of behavioral sensitization to Amp is thought to reflect neuroadaptive biochemical and genomic responses in both pre- and postsynaptic systems triggered by the first exposure to the psychostimulant. Presynaptically, one of the more consistent adaptive responses appears to be an augmentation in the capacity of Amp to release dopamine in the dorsal neostriatum and the nucleus accumbens (reviewed in Kalivas and Stewart, 1991). Postsynaptic neuroplasticity accompanying behavioral sensitization to Amp is evident by alterations in the expression of transcription factors (e.g., c-fos and Fos-like antigens, CREB, NGFI-A), and genes encoding neuropeptides (e.g., dynorphin, enkephalin, substance P) (Graybiel et al., 1990; Konradi et al., 1994; Wang and McGinty et al., 1995a; Jaber et al., 1995). Hence, our studies examined the effects of an Amp challenge on 1) c-fos mRNA expression in the medial prefrontal cortex, 2) expression of the gene encoding the putative "endogenous neuroleptic," neurotensin, in the nucleus accumbens-shell and 3) extracellular dopamine levels in both, the shell and core sectors of nucleus accumbens in rats pretreated with Amp (i.e., behaviorally sensitized rats). To establish the role of D4 dopamine receptors in the induction of behavioral sensitization to Amp and accompanying neuroadaptive responses, we examined the effects of administration of the D4 selective antagonist, PNU-101387G, with Amp during the pretreatment phase on Amp challenge-induced behavioral, neurochemical and genomic responses.

**Materials and Methods**

**Animal treatment.** For the behavioral studies, adult, male, Sprague-Dawley rats (200 g at the beginning of the study) from Charles River Laboratory (Kalamazoo, MI) were maintained in a controlled environment with 12-hr light/dark cycle (lights on at 6:30 A.M.) and free access to laboratory food and tap water. Rats were housed three/cage and after at least 5 days of acclimation they were divided into the following four pretreatment conditions: 1) vehicle (2.5% methylcellulose, 1 ml/kg, i.p.) plus vehicle (0.9% saline, 1 ml/kg, s.c.), 2) vehicle (2.5% methylcellulose, 1 ml/kg, i.p.) plus Amp (2 mg/kg, s.c.) or 3) PNU-101387G (10 mg/kg, s.c.) plus vehicle (0.9% saline, 1 ml/kg, s.c.), placed back in the activity chambers and monitored for 1 hr. Table 1 shows the pretreatment and challenge treatment groups used in the study. At the end of the behavioral recording, rats were killed, trunk blood collected, brains rapidly removed and frozen on dry ice. Brains were used for in situ hybridization histochemistry for c-fos and NT/N mRNA detection as described below. Amp concentrations were determined in the plasma (isolated from the trunk blood) and cerebellum from each animal.

**Measurement of locomotor activity.** Locomotor activity was monitored using Digiscan Animal Activity Monitoring System running DigiPro Windows software (Accuscan Instruments, Columbus, OH). The apparatus consisted of eight transparent Plexiglas activity monitoring cages (40.5 × 40.5 cm). For the measurement of horizontal activity, each cage was equipped with 2 sets of 16 photocell arrays located at right angles to each other, projecting horizontal infrared beams 2.5 cm apart and 3.75 cm above the cage floor. Another set of 16 horizontal beams placed 14 cm above the cage floor recorded vertical activity. Each beam break (regardless of the nature of activity) in the lower array was recorded as a horizontal activity count. Similarly, beam breaks in the upper array recorded vertical activity counts. The DigiPro Windows software uses a mathematical algorithm to compute total distance traveled (in cm) and takes into account factors such as the distance between interrupted beams, the

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<th>Chronic Pretreatment</th>
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Rats were treated once daily for 5 days (chronic pretreatment) with vehicle, Amp, PNU-101387G or a combination of PNU-101387G and Amp. After a 7-day withdrawal period (during which rats received sham-injections daily to reduce the effects of handling or injections), rats were habituated to locomotor activity chambers and given an acute challenge of vehicle, PNU-101387G or Amp. Note that each animal received two injections during pretreatment as well as acute challenge with appropriate drug or vehicle combinations. n = 6 except VV where n = 9.
length of the animal in the horizontal plane and the direction of ambulatory activity (along the walls vs. diagonal). To help isolate test animals from environmental effects, each cage was placed inside a sound attenuation chamber equipped with a dim light and a fan for a constant background noise. All rats were tested between 7:30 A.M. and 3:30 P.M. Eight activity chambers were used simultaneously with allocation of rats (from each treatment group) to the cages in a Latin square design. On the challenge day, rats were placed individually in the LMA chambers and allowed to habituate for 30 min. Animals were then removed briefly from the activity chambers to receive an acute challenge of an appropriate substance. After dosing, animals were replaced in the respective chambers and their activity was monitored for an additional 60 min. Cumulative counts for horizontal activity, vertical activity and total distance were computed for the 60-min period after the challenges. After the behavioral recordings, animals were killed, brains removed and frozen for in situ hybridization histochemistry.

In situ hybridization histochemistry. One half (left or right, randomly chosen) of each brain was cut into 20-μm sections and thaw-mounted onto gelatin-coated slides. These were stored at -80°C until used. Sections were processed as detailed previously (Merchant et al., 1992). Briefly, slides were air dried, fixed in 4% paraformaldehyde, rinsed in PBS, acetylated in triethanolamine buffer containing acetic anhydride, dehydrated through a graded alcohol series, delipidated with chloroform, dehydrated in 100% ethanol, hydrated with 95% ethanol and air dried. A c-fos antisense deoxyligonucleotide probe complementary to the rat c-fos mRNA was end-labeled using terminal deoxynucleotidyl transferase and [35S]-labeled dATP to a specific activity of 7.5 to 15 x 10⁶ dpm/μmol probe. For NT/N mRNA hybridization, a cRNA probe was transcribed in vitro and labeled with [35S]UTP to a specific activity of 40 to 80 x 10⁶ dpm/μmol. Hybridization was carried out overnight at 37°C for the c-fos deoxyligonucleotide probe or 48°C for the NT/N riboprobe at a concentration of 2 pmol probe/ml of hybridization buffer as detailed before (Merchant et al., 1992). After the incubation, high stringency washes were conducted in 1X SSC at 65°C for c-fos and 0.1X SSC at 55°C for NT/N mRNA hybridization. Slides were dehydrated through a graded alcohol series, air-dried and apposed to KODAK Biomax-MR film for autoradiography. After film developing, slides were coated with liquefied NTB-2 emulsion (Kodak), and developed in Kodak D-19 developer after an appropriate exposure time. Sections were counterstained with Harris Hematoxylin from Master-Tech (Lodi, CA).

Analysis of hybridization signal was carried out using dark and bright-field optics at 40x (for the number of labeled cells) or 100x (for the number of grains/cell) magnification. The number of labeled cells and the average density of autoradiographic grains per cell was determined as detailed before (Merchant et al., 1996a). Three atlas-matched (Paxinos and Watson, 1986) sections were used for each region from each animal. The infralimbic/ventral prelimbic region (Bregma 2.7 mm) and the shell sector of the nucleus accumbens (Bregma 1.6 mm) (as shown in schematics in figs. 4 and 6) were analyzed for counting the total number of c-fos or NT/N mRNA expressing cells. The average grain density per cell was determined by analyzing 70 to 100% of the labeled cells in each region. Data from the three sections were averaged to obtain the value for each animal. Group averages were computed for statistical analysis of the hybridization signal.

Plasma amphetamine detection. Trunk blood was centrifuged (1000 x g for 20 min) to separate plasma and frozen at -20°C for later analysis of Amp content using HPLC and fluorescence detection. The assay of Amp was based on the primary amine character of the chemical. Primary amines can be measured sensitively because they form fluorescent adducts with ω-phenylaldehyde and a thiol. To 1 ml of plasma 100 μl of 10 N NaOH and 5 μg of internal standard were added. The solution was mixed with 2 ml of acetonitrile and centrifuged. The organic layer was removed and dried using a stream of nitrogen until 100 μl of solution remained. The concentrated solution was filtered through a quaternary amine column, and to the effluent was added 100 μl of the OPA/BSH reaction mixture (5 mg of ω-phenylaldehyde, 2 μl of t-butylthiol, 100 μl methanol and 898 μl of 4N Na₂Borate). A 100-μl sample was then injected onto an HPLC column. Mobile phase 90% methanol, 10% H₂O and 1 g/ml t-butyl ammonium perchlorate was pumped through a 25 cm ODS column at a rate of 1.5 ml/min. A fluorescence detector was used where EX was 350 and EM was 420 μm. Calculations were based on the results of a standard curve normalized to the internal standard.

Cerebellar amphetamine detection. Frozen brains were weighed in a 1.5 ml Eppendorf tube and 500 μl of acetone containing 10 μg/ml of internal standard was added. The internal standard was a phenylcyclohexyl amine analogue of Amp. The mixture was pulse sonicated to homogeneity and centrifuged. The supernatant was transferred to another Eppendorf tube, which was placed in a Speed-Vac concentrator and concentrated to about 100 μl. The concentrated solution was filtered through a quaternary amine column and to the effluent was added 100 μl of the OPA/BSH reaction mixture. HPLC was conducted as for the plasma level detection. Data are expressed as milligrams of d-amphetamine per gram of tissue (mg/g).

Measurement of extracellular dopamine concentrations by in vivo microdialysis. Dr. Yamamoto carried out these studies at Case Western Reserve University. Rats were pretreated with Amp and withdrawn as described above. On day 4 of the 7-day withdrawal period, all rats were anesthetized with a combination of xylazine (6 mg/kg, i.m.) and ketamine (70 mg/kg, i.m.). Rats were placed in a stereotaxic apparatus, the skull was exposed and a 1-mm hole was drilled into the skull above the nucleus accumbens shell (1.8 mm anterior to Bregma, 0.7 mm lateral to the mid-line suture) or core (1.7 mm anterior to Bregma and 1.6 mm lateral to the mid-line suture) (Paxinos and Watson, 1986). Dura was carefully removed and a 21-gauge stainless steel guide cannula was placed stereotaxically into the hole up to the surface of the cortex without penetrating the brain. The guide cannula with a stylet obturator was secured to the skull with cranioplastic cement and three set screws. The tip of the microdialysis probe was located 8.2 or 7.2 mm from the cortical surface for the accumbal shell or core, respectively.

A concentric-shaped dialysis probe was constructed as previously described (Yamamoto and Davy, 1992; Yamamoto and Pehek, 1990). The exposed portion of the dialysis membrane (MW cutoff = 13,000) extended 1.5 mm beyond the tip of the 26-gauge stainless steel tubing. The microdialysis perfusion flow rate was 2.0 μl/min. A stainless steel spring tether connected the animal to a liquid swivel. The perfusion medium was a modified Dulbecco’s phosphate-buffered saline containing 138 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, 1.2 mM CaCl₂ and 0.5 mM d-glucose, pH 7.4.

On day 13 after commencing the pretreatment, dialysis probes were inserted and rats were placed in circular cages that allowed free movement. After a 4-hr stabilization period, 3 base-line samples (30-min fractions) were collected and assayed for dopamine by HPLC with electrochemical detection (Stephans and Yamamoto, 1995). Rats were then administered an acute challenge of d-amphetamine sulfate (2 mg/kg, s.c.) and the perfusate was collected every 30 min for a total of 150 min. At the end of 150 min, rats were killed by decapitation, brains removed and the placement of the probe was determined by histological examination of the tissue slices. Only those rats with probe placement into the nucleus accumbens shell or core were included in the analysis.

Statistical analysis. There were six to nine animals in each treatment group. A multifactorial analysis of variance was performed for each measure (except for locomotor activity where the time-dependent effects were analyzed by repeated measure analysis of variance). After a significant difference (P < .05), a multiple comparisons post hoc test (Fisher’s PLSD or Tukey) was applied to identify groups differing significantly from each other.
Results

Amphetamine levels in the plasma and cerebellar tissue. Table 2 contains the plasma levels of Amp 1 hr after an acute Amp challenge to rats pretreated with vehicle, Amp, PNU-101387G (U) or PNU-101387G plus Amp (U+A). Amp levels were not measurable in groups that did not receive an acute Amp challenge (data not shown). The A/A group had significantly higher Amp levels in the plasma than the V/A group. The groups pretreated with PNU-101387G alone or in combination with Amp showed significantly lower plasma Amp levels after Amp challenge (U/A or U+A/A) than A/A or V/A groups. Cerebellar Amp levels were determined to investigate whether the increased plasma levels of Amp in the Amp-pretreated group also reflects increased brain levels of the drug. As shown in table 2, cerebellar Amp levels did not increase in Amp-pretreated rats compared to the vehicle-pretreated group (i.e., A/A = V/A). Similarly, pretreatment with PNU-101387G in combination with Amp had no effect on cerebellar Amp levels after the psychostimulant challenge.

Locomotor activity. A repeated measure analysis of variance followed by Fisher’s PLSD test showed a significant treatment x time interaction for horizontal and vertical activity measures \( F(55, 325) = 2.11, P < .0001; F(55, 325) = 2.54, P < .0001 \) as well as total distance counts \( F(55, 325) = 2.13, P < .0001 \). Acute Amp induced motor stimulation in vehicle-pretreated animals (V/A) primarily as an increase in vertical activity. However, in Amp-pretreated rats, an Amp challenge (A/A) produced greater increases in locomotor activity (behavioral sensitization) with a significant effect in horizontal activity and total distance counts (fig. 1). Challenges with either vehicle or PNU-101387G produced similar levels of horizontal and vertical activity in all groups regardless of pretreatment condition. However, pretreatment with PNU-101387G alone led to a sensitized response to Amp challenge (U/A) similar to that seen in Amp-pretreated rats. Despite this, coadministration of PNU-101387G with Amp during pretreatment blocked the behavioral sensitization (in both, the horizontal activity counts and total distance traveled) to Amp challenge (i.e., U+A/A group). Factorial analysis of variance with cumulative counts over the one hour period also showed the same pattern of results (fig. 2).

c-fos mRNA in the IL/vPL cortex. Acute administration of PNU-101387G induced c-fos mRNA levels in the IL/vPL cortex in all groups of rats, regardless of the various pretreatment conditions. The effect was evident primarily in deep (V + VI) cortical layers. An acute Amp challenge also induced c-fos gene expression; the effect was seen in all layers of the IL/vPL cortex of vehicle-pretreated rats (V/A). However, a reduction in this response was observed in rats pretreated with Amp (A/A), i.e., a refractoriness to Amp-induced c-fos expression accompanied behavioral sensitization. Pretreatment with PNU-101387G alone did not modulate the Amp challenge-induced (i.e., U/A group) increases in c-fos mRNA levels in deep layers but significantly attenuated the Amp

![Fig. 1. Time course of locomotor activity. Rats were pretreated with vehicle (V), Amp (A), PNU-101387G (U) or PNU-101387G plus Amp (U+A) as described in “Materials and Methods.” On the challenge day, rats were habituated to the locomotor activity boxes, challenged with vehicle, Amp or PNU-101387G and monitored for 60 min for horizontal activity counts (A), vertical activity counts (B) or total distance traveled (in cm; C).](Image)

**TABLE 2**

Effects of various pretreatment treatments on plasma/brain Amp concentrations 1 hr after an acute Amp challenge

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<tr>
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<tr>
<td>Plasma Amp (ng/ml plasma)</td>
<td>178.5 ± 14.1</td>
<td>286.2 ± 17.5 *</td>
<td>114.8 ± 15.1 *</td>
<td>109.3 ± 6.49 *</td>
</tr>
<tr>
<td>Cerebellar Amp (mg/g tissue)</td>
<td>0.753 ± 0.037</td>
<td>0.860 ± 0.085</td>
<td>NT</td>
<td>0.873 ± 0.068</td>
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* P < .05 vs. V/A, *P < .05 vs. A/A (n = 6 per group). NT, Not tested.
response in superficial layers of the IL/vPL cortex. As with the blockade of behavioral sensitization, coadministration of PNU-101387G with Amp during pretreatment blocked the refractoriness in c-fos mRNA response to Amp-challenge (U+A/A) in deep layers of the IL/vPL cortex; the superficial layers showed a partial restoration of Amp-induced c-fos gene induction (figs. 3 and 4).

**NT/N mRNA levels in the NA-s.** Unlike c-fos gene expression in the medial prefrontal cortex, an acute challenge of PNU-101387G produced no changes in NT/N mRNA expression in the NA-s, regardless of the pretreatment condition. As can be seen in figures 5 and 6, there was a significant elevation in NT/N mRNA expression in the NA-s after an acute Amp challenge in vehicle-pretreated rats (V/A). The NT/N induction in the NA-s by Amp was totally abolished when the Amp challenge was given to Amp-pretreated rats (A/A). However, animals that received PNU-101387G plus Amp (U+A/A) during the pretreatment period showed Amp challenge-induced NT/N mRNA induction that was quantitatively similar to that observed in vehicle-pretreated rats (V/A). In contrast to the effects of PNU-101387G pretreatment on Amp-induced behavior or c-fos mRNA levels in superficial IL/vPL cortex, basal or Amp-induced NT/N mRNA levels were not altered in PNU-101387G-pretreated rats.

**Extracellular dopamine levels in the NA-s and NA-c.** Figure 7 schematically shows the placement of the dialysis probe in the shell and core sectors of the nucleus accumbens. Base-line and Amp-induced alterations in extracellular dopamine levels in the NA-s and NA-c of freely moving rats are shown in figure 8. As expected, acute administration of Amp significantly increased extracellular dopamine concentration in both the shell and the core of the nucleus accumbens in vehicle-pretreated rats (V/A). In rats preexposed to Amp, there was an augmentation in Amp challenge-induced extracellular dopamine levels (i.e., A/A group) in the NA-s but not in the NA-c. However, as with the behavioral sensitization and postsynaptic responsiveness to acute Amp challenge, coadministration of PNU-101387G with Amp pretreatment blocked the neural adaptations leading to the augmentation in dopamine releasing capacity of Amp (U+A/A).

**Discussion**

The results demonstrate that dopamine D4 receptors play a critical role in the induction of behavioral sensitization to Amp in the rat. The augmentation in locomotor stimulatory effects of Amp produced by Amp preexposure (pretreatment to Amp followed by a withdrawal period) was blocked in rats coadministered the D4-selective antagonist, PNU-101387G, with Amp during pretreatment. In addition, the D4 antagonist blocked pre- and postsynaptic biochemical/cellular alterations accompanying the behavioral sensitization: 1) an augmentation in the ability of Amp to increase extracellular dopamine levels in the NA shell, 2) a decrease in the capacity of Amp to induce c-fos mRNA expression in the medial prefrontal cortex and 3) an attenuation in Amp-induced NT/N mRNA expression in the accumbal shell. Because the effects of PNU-101387G on biochemical, behavioral and cellular alterations accompanying behavioral sensitization to Amp were observed in the absence of a significant effect on brain (cerebellar) Amp levels, the data strongly implicate a role of dopamine D4 receptors in the initiation of Amp sensitization.

**Behavioral effects.** The behavioral sensitization to Amp observed in our study is likely a context-independent phenomenon because the pretreatment treatments were carried...
Fig. 3. *C-fos* mRNA expression in the infralimbic/ventral prelimbic (IL/vPL) Cortex. Each panel in A is a representative dark-field, low magnification, photomicrograph through the IL/vPL cortex (shown schematically in fig. 4) from the four most critical treatment groups. The groups shown are vehicle/vehicle (V/V), vehicle/Amp (V/A), Amp/Amp (A/A) and PNU-101387G + Amp/Amp (U+A/A). The forceps minor of the corpus callosum (c) is labeled to help with orientation; top of the photomicrographs represents the dorsal side of the brain. Thus the micrographs from the V/A, A/A and A+U/A groups are from the left hemisphere and VV micrograph is from the right hemisphere. The cellular distribution of autoradiographic grains on neurons in layer VI is shown in high magnification photomicrographs in B. Note that acute Amp increased *c-fos* mRNA expression in vehicle-pretreated rats and this effect was significantly reduced in Amp-pretreated animals. Coadministration of PNU-101387G with Amp during pretreatment prevented the reduced capacity of Amp to induce *c-fos* gene expression.
out in the home cages and the rats were exposed to the locomotor activity monitoring chambers for the first time on the challenge day. The potential confounding effects of stress on behavior and c-fos mRNA induction were minimized by 1) habituating the animals to handling and injections during the 7-day withdrawal period and 2) habituating them to locomotor activity boxes before administration of acute challenges. The automated horizontal and vertical activity counts measured in these studies represent two competing behavioral measures, locomotor activation and rearing, respectively. To evaluate alterations in focused stereotypies using the automated system, we examined changes in total distance traveled as an indirect measure. Total distance in the Digiscan system computes horizontal ambulatory movement based on horizontal activity counts (see “Materials and Methods”) such that any disproportionate decrease in total distance (relative to horizontal activity counts) could indicate a shift to focused stereotypies or rearing behavior. Thus automated measures of horizontal activity, vertical activity and total distance counts together offer a comprehensive picture of qualitative and quantitative changes in exploratory behaviors.

The acute stimulatory effect of Amp (V/A group) was seen primarily in the form of increased rearing (vertical activity counts). In several studies since this one, we have observed acute Amp-induced stimulation as an increase in either horizontal or vertical counts or both and likely represents heterogeneity among rats and/or an effect of the relatively short monitoring period (1 hr) necessary for examination of c-fos gene expression. It is possible that because of the greater vertical activity response to acute Amp, the sensitization to Amp was quantitatively and statistically more robust for horizontal activity and total distance measures (A/A compared to V/A). In rats that received PNU-101387G with Amp during pretreatment, the behavioral sensitization in horizontal counts also was lower in the U/A/A group than the A/A group; however, the degree to which total distance was blocked in rats in the U++/A group was no greater than the V/A group (P < .05) and somewhat higher than those observed in the V/V group (P < .0001 vs. V/V; ⋆P < .05, #P < .01 and ***P < .0001 vs. V/A; †P < .05, ††P < .001 vs. A/A).

Interestingly, pretreatment with the D4 antagonist by itself induced cross-sensitization to Amp in all three behavioral measures. Such a cross-sensitization to the effects of methamphetamine has been reported previously in mice pretreated with nonselective D1- or D2-like antagonists (Kuribara, 1994, 1996; Vezina and Stewart, 1989). The mechanism underlying the cross-sensitization between PNU-101387G and Amp is unclear. In recent reports we have demonstrated that the cross-sensitization is a dose-dependent phenomenon for both PNU-101387G (K.M. Merchant, Z.-H. Meng and D.L. Feldpausch, unpublished observations) and nonselective D2 class antagonists, haloperidol, clozapine (Meng et al., 1997). The lack of sensitization to Amp in rats pretreated with PNU-101387G plus Amp demonstrates that the two drugs antagonize the pretreatment effects of each other.
It could be argued that the behavioral sensitization and its modulation by PNU-101387G involved pharmacokinetic factors since the acute Amp challenge produced significantly greater plasma Amp levels in A/A rats compared to the V/A group and this effect was blocked by PNU-101387G given with Amp during pretreatment (i.e., U/A/A group). However, several observations argue against this possibility. First, concurrent with an increase in plasma Amp level, there was no increase in cerebellar Amp levels in the A/A group. Second, Amp effects on NT/N mRNA and immediate early gene expression are dose dependent (Castel et al., 1993; Wang and McGinty, 1995b). Thus a higher Amp concentration in the A/A group is unlikely to lead to the observed reduction in responsiveness of NT/N or c-fos gene expression. Finally, PNU-101387G-pretreated rats also displayed behavioral sensitization to Amp (U/A) although their plasma levels were similar to those seen in vehicle pretreated rats (V/A). Hence, at least the cross-sensitization between the D4 antagonist and Amp could not be due to pharmacokinetic factors regulating Amp concentrations.

**c-fos mRNA induction.** Acute blockade of D4 receptors by PNU-101387G induced c-fos mRNA expression in layers V and VI of the IL/vPL cortex as seen previously (Merchant et al., 1996b). Acute Amp also increased c-fos mRNA in deep layers of the IL/vPL cortex. However, there appear to be distinct differences with respect to c-fos induction by Amp and PNU-101387G: 1) c-fos induction by acute Amp became refractory after repeated Amp administration but not after repeated PNU-101387G administration. 2) Preexposure to PNU-101387G did not reduce the capacity of a subsequent PNU-101387G challenge to induce c-fos mRNA levels. Thus Amp and PNU-101387G appear to target distinct neuronal populations in the medial prefrontal cortex. These data are relevant because they indicate that PNU-101387G blocked the development of the neuroadaptive c-fos response to Amp not by directly influencing c-fos gene expression in Amp-responsive neurons, but by an indirect mechanism that may involve cortical-cortical interactions. Further indirect support for the possibility that the medial prefrontal cortex is the site at which PNU-101387G exerts its blockade of Amp sensitization (behavioral and associated genomic/biochemical responses) derives from 1) the predominantly cortical localization of the D4 receptor (Mrzljak et al., 1996; Ariano et al., 1997; Defagot et al., 1997) and, 2) our previous results that peripheral administration of PNU-101387G induces c-fos gene expression only in the prefrontal cortex within the forebrain (Merchant et al., 1996b). Regardless of the precise mechanism, changes in c-fos regulation by Amp in the medial prefrontal cortex indicates that this region participates in neuroadaptive postsynaptic responses associated with behavioral sensitization to Amp and their modulation by the D4 receptor. These data are in agreement with the conclusions of Wolf et al. (1995), and Karler et al. (1997) who demonstrated that the medial prefrontal cortex plays a critical role in Amp sensitization and its modulation by D2-like receptors.

Compared to the deep layers, c-fos mRNA induction in the superficial IL/vPL cortex showed several distinct characteristics: 1) Amp, but not PNU-101387G, significantly increased c-fos mRNA expression, 2) pretreatment with PNU-101387G by itself reduced the ability of the Amp challenge to increase...
c-fos mRNA levels and perhaps due to this, the refractoriness to Amp was not blocked by concurrent administration of PNU-101387G with Amp during pretreatment. The reasons underlying these distinctions in PNU-101387G effects remain unclear but indicate that dopamine tone in distinct layers of the IL/vPL cortex may be differentially modulated by the D4 receptor.

The neostriatum has been studied widely and proposed to be a critical site involved in postsynaptic neuroadaptive responses underlying behavioral sensitization to Amp. These studies generally have used a significantly higher dose of Amp (e.g., >5 mg/kg for pretreatment and challenge) than the dose of 2 mg/kg, s.c., used in our studies (Graybiel et al., 1990; Jaber et al., 1995). At 2 mg/kg, acute Amp did not induce c-fos mRNA levels in the dorsal striatum or nucleus accumbens but profoundly increased c-fos mRNA expression in the medial prefrontal cortex. Interestingly, a greater sensitivity of dopamine and glutamate systems in the prefrontal cortex (compared to neostriatum) has been observed also after repeated low doses of methamphetamine using a dosing and withdrawal protocol almost identical to the one used in our study (Stephans and Yamamoto, 1995). Thus the medial prefrontal cortex is not only a critical site but also a more sensitive region to acute and neuroadaptive responses produced by Amp and other psychostimulants.

NT/N mRNA induction. Another molecular alteration accompanying behavioral sensitization to Amp and its blockade by PNU-101387G was a reduction in the inducibility of NT/N mRNA expression in the nucleus accumbens-shell by Amp. Direct injections of NT in the accumbens antagonize acute locomotor stimulation produced by Amp (Ervin et al., 1981). Additionally, immunoneutralization of accumbal NT potentiates locomotor activity and rearing produced by methamphetamine (Wagstaff et al., 1994). These data indicate that accumbal NT may antagonize the behavioral stimulatory effects of Amp. Hence, a reduction in the capacity of Amp to induce NT/N gene expression in the NA-s raises the question whether the reduced NTergic tone (as indicated by mRNA levels) may contribute to enhanced behavioral stimulation (sensitization) produced by Amp challenge in the Amp-pretreated rats. Studies measuring NT release in the accumbal shell accompanying behavioral sensitization will help determine if there indeed is a reduction in the activity of this pathway.

Acute Amp-induced NT/N mRNA expression involves activation of D1 receptors (Castel et al., 1993). Because Amp-pretreated rats showed an augmented extracellular dopamine concentration (our study) and D1 receptor function...
Extracellular dopamine concentrations. Previous studies have shown an increase in the capacity of Amp to induce extracellular dopamine levels in the NA after extended withdrawal from repeated, intermittent Amp treatment (reviewed by Kalivas and Stewart, 1991; Robinson and Berridge, 1993, White and Wolf, 1991). In our study, Amp-induced increases in extracellular dopamine concentration was augmented in rats withdrawn for an intermediate time period (7 days) from an Amp-pretreatment treatment; a protocol that also induced robust behavioral sensitization. Paulson and Robinson (1995) failed to observe behavioral sensitization or augmented dopamine release after 7-day withdrawal. The apparent discrepancy may be due to vast differences in pretreatment protocols (6 wk of escalating dosing by Paulson and Robinson, 1995, vs. 5 days at 2 mg/kg/day in our study) or challenge doses employed. Additionally, our study demonstrated the augmentation in Amp-induced extracellular dopamine levels in the shell but not the core; Paulson and Robinson (1995) did not distinguish between the two sectors of the NA. The regional selectivity in presynaptic dopamine system adaptations are in line with the limbic system attributes of the accumbal shell neurons (Deutch et al., 1993). The increase in dopamine terminal sensitivity to Amp shown here is in complete agreement also with the results of Pierce and Kalivas (1995) who showed that micro-injections of Amp into the accumbal shell, but not the core, of cocaine-pretreated rats produce behavioral sensitization and augmented dopamine release.

Because dopamine transmission in the nucleus accumbens mediates locomotor stimulatory effects of psychostimulants, it is believed that the augmentation in Amp-induced dopamine release in Amp-pretreated animals may contribute to the behavioral sensitization seen in these rats (Robinson and Berridge, 1993). Behavioral evaluation was not carried out in our study during \textit{in vivo} microdialysis. However, the pretreatment and challenge protocols were identical between the behavioral and microdialysis studies (including the vehicles used and the \textit{pH} of each drug solution). Hence, it is likely that the augmentation in Amp-induced extracellular dopamine concentration in Amp-pretreated rats was accompanied by and contributed to behavioral sensitization. This possibility is supported also by the observation that cotreatment of PNU-101387G with Amp blocked both the behavioral sensitization and the augmented dopamine response to the Amp challenge in Amp-pretreated rats.

Role of D4 receptors in Amp sensitization. The selectivity of PNU-101387G for dopamine D4 receptors has been examined extensively and reported by Merchant \textit{et al.} (1996b). PNU-101387G shows moderately high affinity for the rat and human D4 receptor ($K_i = 10 \text{ nM}$). However, its affinity at other dopamine, serotonin, noradrenergic, GABA or glutamate, and several neuropeptide receptors is more than 1.5 \textmu M. It appears that it is the selectivity for the D4 receptor that enables PNU-101387G to produce behavioral, biochemical and physiological effects that are distinct from nonselective blockers of the D2 class of receptors (Merchant \textit{et al.}, 1996b). Immunolabeling studies in nonhuman primates and rats have shown the highest level of expression of the D4 receptor in cortical regions followed by discrete basal ganglia nuclei (Mrzljak \textit{et al.}, 1996; Ariano \textit{et al.}, 1997; De-
Summary and Conclusions

Our study demonstrates that the medial prefrontal cortex, NA shell and the dopamine D4 receptor play a critical role in behavioral, biochemical and genomic events produced by repeated, intermittent Amp exposure. It is likely that the effects of the D4 receptor antagonist are mediated by alterations in prefrontal cortical neuronal activity. Our results simply show an association of the biochemical (augmentation in Amp-induced dopamine release) and genomic responses (reduction in Amp-induced c-fos mRNA in rat medial prefrontal cortex by antipsychotic drug: role of dopamine D2 and D3 receptors. Cerebral Cortex 6:561–570).


and preproenkephalin mRNA expression induced by repeated amphetamine administration in rats. Brain Res 673:262–274.


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