

Acute Amphetamine Exposure Selectively Desensitizes κ -Opioid Receptors in the Nucleus Accumbens

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In the present study, we investigated the effects of psychostimulant exposure on κ -opioid peptide (KOP) receptor signaling in the rat mesolimbic system. A single subcutaneous (s.c.) injection of amphetamine (2.5 mg/kg) reduced the KOP receptor-mediated inhibition of glutamate release in the nucleus accumbens shell, as a consequence of KOP receptor desensitization. This effect was blocked by dopamine (DA) receptor antagonists or the nonselective opioid antagonist, naltrexone (1 mg/kg, s.c.), and mimicked by the KOP receptor agonists U69593 (0.32 mg/kg, s.c.) and dynorphin (1 μ M), indicating that an amphetamine-induced release of dynorphin is producing a long-lasting desensitization of the KOP receptor. Despite the fact that amphetamine also increases dynorphin release in the ventral tegmental area (VTA), KOP receptor function in this region was not affected by amphetamine; there was no difference in the KOP receptor-mediated change in firing rate or resting membrane potential measured in VTA neurons from saline- or amphetamine-treated animals. This study demonstrates that amphetamine can produce regionally selective adaptations in KOP receptor signaling, which may, in turn, alter the effects of subsequent drug exposure.

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INTRODUCTION

The nucleus accumbens (NAc) and ventral tegmental area (VTA) are two important components of the neural circuitry underlying motivation and reinforcement (Everitt and Wolf, 2002; Kelley *et al*, 2005), and long-lasting changes in these nuclei are thought to underlie drug addiction (Nestler, 2004). The NAc receives a predominantly dopaminergic projection from the VTA (Swanson, 1982). Likewise, a subset of the GABAergic medium spiny neuron (MSNs) in the NAc project back to the VTA (Conrad and Pfaff, 1976; Phillipson, 1979). These VTA-projecting MSNs express the neuropeptides substance P, which targets the neurokinin 1 (NK1) receptor, and dynorphin, which targets the κ -opioid peptide (KOP) receptor (Fallon *et al*, 1985; Anderson and Reiner, 1990). These neuropeptides are not only released in the VTA, but are also presumably released within the NAc itself, as the axons of MSNs collateralize extensively within the nucleus (Chang and Kitai, 1985).

KOP receptors are distributed throughout this circuit and clearly influence reward-related behaviors (Shippenberg

and Elmer, 1998; Todtenkopf *et al*, 2004). For example, KOP receptor agonists injected into either the VTA or the NAc produce conditioned place aversion (Bals-Kubik *et al*, 1993). In the rat VTA, KOP receptors directly hyperpolarize a subset of dopamine (DA) neurons (Margolis *et al*, 2003), specifically those that project to the prefrontal cortex (Margolis *et al*, 2006). Within the NAc, KOP receptor agonists inhibit the release of glutamate (Yuan *et al*, 1992; Hjelmstad and Fields, 2001, 2003). DA signaling in the prefrontal cortex and glutamate signaling in the NAc have both been implicated in drug relapse (Cornish and Kalivas, 2000; McFarland and Kalivas, 2001; McFarland *et al*, 2003). Thus, the localization as well as behavioral actions of KOP receptors makes them an inviting therapeutic target for the treatment of addiction.

In addition to altering influencing behavioral responses to psychostimulants, the KOP system shows adaptations following psychostimulant administration (Shippenberg *et al*, 2001). For example, following a single injection of amphetamine, dynorphin mRNA levels are increased, while KOP receptor binding is decreased (Turchan *et al*, 1998). In light of data showing that even a single injection of amphetamine can produce behavioral sensitization in rats (Vanderschuren *et al*, 1999), these changes in KOP signaling may have profound effects on behavior. Thus, determining the functional implications of this plasticity is crucial to our understanding of the role of KOPs in addiction. Here, we investigated the effect of *in vivo* administration of

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amphetamine on KOP receptor function in both the NAc and the VTA. We find that amphetamine treatment produces a long-lasting functional downregulation of the KOP receptor on glutamate terminals in the NAc shell but has no impact on postsynaptic KOP receptor function in the VTA. Furthermore, we show that this decrease in function in the NAc is due to desensitization of the KOP receptor.

MATERIALS AND METHODS

Slice Preparation

All animal procedures were in accordance with protocols approved by the Ernest Gallo Clinic and Research Center's institutional animal care and use committee. Three- to four-week old male Sprague-Dawley rats were given subcutaneous (s.c.) D-amphetamine sulfate (2.5 mg/kg in saline), U69593 (0.32 mg/kg), or saline (0.9% NaCl) injections and returned to their cage for 2 h or 1–5 days (for the time course analysis). Flupenthixol dihydrochloride (0.5 mg/kg), SCH-23390 hydrochloride (0.1 mg/kg), and naltrexone hydrochloride (1 mg/kg) were diluted in saline and administered s.c. 15 min before the amphetamine injection. While naltrexone is most selective for μ -opioid peptide (MOP) receptors, it also binds KOP receptors in the low-nanomolar range (Goldstein and Naidu, 1989).

To prepare slices, animals were anesthetized with isoflurane, decapitated, and the brain removed and placed into an ice-cold Ringer's solution ($\sim 3^{\circ}\text{C}$) containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO_4 , 1.0 NaH_2PO_4 , 2.5 CaCl_2 , 26.2 NaHCO_3 , and 11 glucose saturated with 95% O_2 /5% CO_2 . Coronal slices (350 μm thick) containing the NAc, or 150 μm thick horizontal slices containing the VTA were cut using a vibratome (Leica Instruments, Germany). Slices were submerged in Ringer's solution and allowed to recover for > 1 h at 34°C .

Electrophysiology

Individual slices were transferred to an Olympus upright microscope with differential interference contrast optics and infrared illumination. Extracellular field or whole-cell patch-clamp recordings were made at 28°C . Picrotoxin (100 μM) was added to the Ringer's solution to block GABA_A receptor-mediated inhibitory postsynaptic potentials. Field recordings were made by placing a 3–5 M Ω electrode filled with Ringer's solution into the medial shell of the NAc, which can be visually distinguished from the neighboring core region in a coronal slice. Whole-cell voltage-clamp recordings from NAc MSNs were made using 2.5–4 M Ω pipettes containing (in mM) 123 Cs-gluconate, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.2 ethylene glycol tetraacetic acid (EGTA), 8 NaCl, 2 MgATP, 0.3 Na_3GTP (pH 7.2, osmolarity adjusted to 280). Cells were identified as MSNs by their appearance and by their relatively hyperpolarized resting potential. Excitatory postsynaptic field potentials (fEPSPs) and excitatory postsynaptic currents (EPSCs) were evoked (0.067 Hz) with a bipolar-stimulating electrode placed along the dorsal edge of the NAc.

VTA whole-cell recordings were made using an internal solution where the Cs-gluconate was replaced with

K-gluconate. I_h currents were recorded in voltage clamp, stepping the neuron from -60 to -40 , -50 , -70 , -80 , -90 , -100 , -110 , and -120 mV. Firing rate and membrane potential were monitored in the current-clamp ($I=0$) mode.

All recordings were made using a Multiclamp 700A (Axon Instruments) amplifier and were filtered at 2 kHz and collected at 5 kHz using Igor Pro (Wavemetrics Inc., Lake Oswego, OR). Series resistance was monitored online by measuring the peak of the capacitance transient in response to a -4 mV voltage step applied before each stimulus. Amplitudes were calculated by comparing a 2 ms period at the peak of the response and a similar period just before the stimulus artifact. Firing rates were analyzed by computing the instantaneous firing rate (calculated as the inverse of the interspike interval after each action potential) and binned into 30 s bins for statistical analysis or 5 s bins for display purposes. For each VTA neuron, significance was determined comparing the last 3 min of baseline with the last 3 min of drug application. Unless otherwise noted, statistical analyses were performed using the Student's *t*-test, and significance was defined at $p < 0.05$. Results are presented as means \pm SEM.

All drugs were applied by bath perfusion. Stock solutions were made and diluted immediately before application. U69593 was mixed in 50% ethanol at a concentration of 10 mM; nor-binaltorphimine (nor-BNI), dynorphin, and DAMGO were diluted in H_2O and picrotoxin was mixed in dimethyl sulfoxide. Chemicals were obtained from Sigma Chemical (St Louis, MO) or Tocris (Ballwin, MO).

Western Blot

VTA slices or NAc tissue blocks were prepared in artificial cerebrospinal fluid as described above. Tissue was placed in ice-cold 50 mM Tris-HCl (pH 7.4), with protease inhibitors and homogenized with a Polytron Homogenizer. The homogenized tissue was centrifuged at 30 000g for 20 min at 4°C , resuspended in same buffer and recentrifuged at 30 000g for 20 min. The pellets were resuspended and stored at -80°C until use.

Membrane proteins (20 $\mu\text{g}/\text{lane}$) were solubilized by boiling in 2% sodium dodecyl sulfate containing 50 mM dithiothreitol for 10 min and were subjected to 4–12% NuPAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were incubated in blocking buffer (5% nonfat dry milk, 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h at room temperature (RT) and then incubated in blocking buffer with a polyclonal anti-KOP receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:2000 dilution) overnight at 4°C . The membrane was washed multiple times in the 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 buffer, and then incubated in horseradish peroxidase-conjugated goat anti-rabbit antibody (Invitrogen, Carlsbad, CA; 1:5000 dilution) for 2 h at RT. The membrane was again washed multiple times in 20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 buffer, and visualized with enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Western blots were quantified using densitometry normalized to β -actin levels.

GTP γ S Binding

[35 S]-GTP γ S (1250 Ci/mmol) was purchased from Perkin-Elmer Life Science (Boston, MA). For [35 S]GTP γ S binding in membranes, brain regions were dissected on ice. Tissue samples were homogenized with Teflon homogenizer on ice in membrane buffer (50 mM Tris-HCl, 3 mM MgCl₂, 1 mM EGTA, pH 7.4) and centrifuged at 30 000g for 20 min at 4°C. Pellets were suspended in membrane buffer and centrifuged again under identical conditions. After the second centrifugation, pellets were homogenized in assay buffer (50 mM Tris-HCl, 3 mM MgCl₂, 0.2 mM EGTA, 100 mM NaCl, pH 7.4). Concentration effect curves of [35 S]GTP γ S binding included 30–3000 nM dynorphin, 30 μ M NaGDP, 0.05 nM [35 S]GTP γ S, 3 μ g protein and assay buffer in a final volume of 200 μ l using 96-well cell culture plates. Assays were incubated at RT for 1.5 h. Reactions were terminated by transferring to 96-well Unifil plate GF/B glass fiber filters with rapid filtration under vacuum followed by 3 washes with 200 μ l cold 50 mM Tris-HCl buffer (pH 7.4). Bound radioactivity was determined by liquid scintillation spectrophotometry at 95% efficiency for [35 S] after overnight extraction of the filters in 40 μ l Microscint scintillation fluid. Data are reported as mean \pm standard error of four separate experiments with each performed in triplicate. Percent stimulation is defined as ((net stimulated binding – basal binding)/basal binding) \times 100. Nonlinear regression analysis of concentration effect curves was performed by Prism (GraphPad Software Inc., San Diego, CA) that determined EC₅₀ values.

RESULTS

To investigate the effect of amphetamine on KOP receptor function, we initially compared the effects of the KOP receptor agonist U69593 on fEPSPs in the NAc shell in slices prepared from amphetamine- and saline-treated rats. In slices cut 2 h following a saline injection, U69593 (1 μ M) inhibited the amplitude of the fEPSP (23.6 \pm 5.9%, n = 6, Figure 1a and b) similar to that seen in naive tissue (Hjelmstad and Fields, 2001). This KOP receptor-mediated inhibition does not readily reverse following removal of the agonist (Hjelmstad and Fields, 2001), however it was completely reversed following application of the KOP receptor selective antagonist nor-BNI (100 nM). In slices prepared from amphetamine-treated rats, the inhibition produced by U69593 was significantly smaller (Figure 1a and b): the average inhibition was reduced to 6.4 \pm 2.9% (n = 8, p < 0.05 compared to the saline-treated group). This change in the KOP receptor-mediated inhibition was not due to a change in the IC₅₀ for U69593 (0.91 μ M in amphetamine-treated animal compared to 0.64 μ M in saline-treated animals, Figure 1c) and occurred even in response to saturating doses of U69593. Moreover, the change in inhibition was also observed with the KOP receptor agonist dynorphin A (500 nM; 6.5 \pm 2.2% for amphetamine-treated group compared to 18.1 \pm 3% for saline-treated group, p < 0.05, Figure 1c).

To investigate the time course of this effect, slices were cut 24 h or 5 days following administration of amphetamine or saline and compared to the 2 h data. The reduced inhibition was present at both of these time points (Figure 1d): in

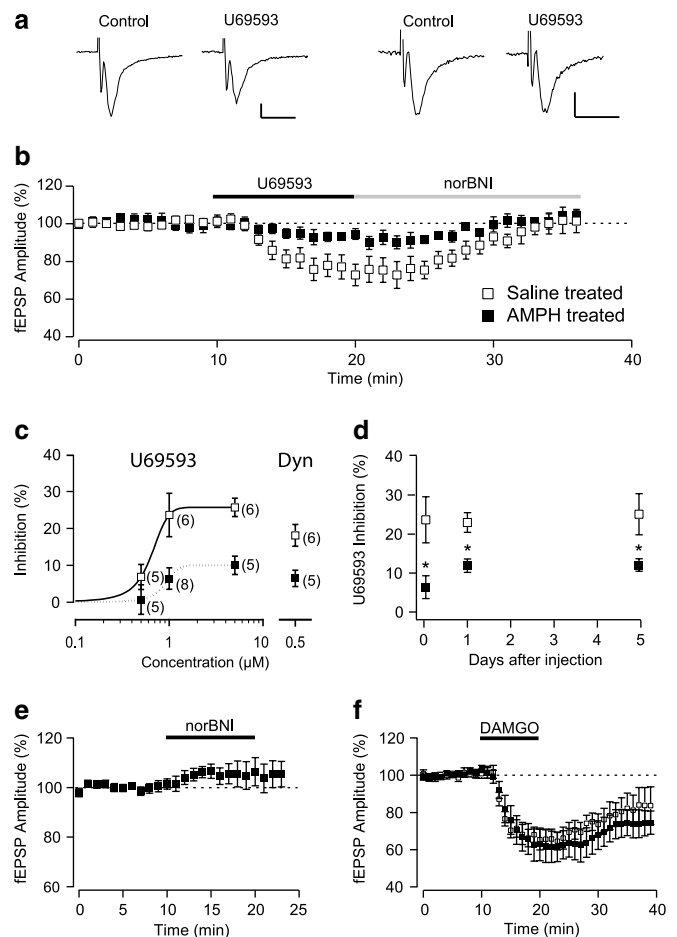


Figure 1 A single injection of amphetamine reduces the KOP inhibition of glutamate release in the NAc. (a) Average of 10 consecutive fEPSPs during control period and in the presence of U69593 from a saline-treated (left) and an amphetamine-treated (AMPH; right) rat. Calibration: 0.2 mV, 10 ms. (b) Averaged field recordings comparing the inhibition of U69593 (1 μ M) on the fEPSP amplitude from slices prepared from saline- and amphetamine-treated rats. (c) Dose–response for U69593-mediated inhibition in slices from amphetamine- and saline-treated rats. Right hand side shows inhibition in response to 500 nM dynorphin (Dyn). Numbers in parentheses show the number of recordings under each condition. (d) Time course of a single amphetamine administration showing the effect lasted for at least 5 days (* p < 0.05 between saline and amphetamine treatment using a two-factor ANOVA with a Holm–Sidak *post hoc* test). (e) The selective KOP receptor antagonist, nor-BNI (100 nM), does not significantly affect fEPSPs in slices from amphetamine-treated rats. (f) The inhibition of fEPSPs by the MOP receptor agonist, DAMGO (1 μ M), is not significantly different between the two groups (p > 0.05).

amphetamine-treated animals the inhibition by U69593 was 11.9 \pm 1.7% (n = 9) at 24 h and 12.1 \pm 1.6% (n = 11) after 5 days, which is significantly less than in the saline-treated group (23.0 \pm 2.6% for 24 h, n = 8; 25.1 \pm 5.3%, n = 5; p < 0.05 for each time point).

Because amphetamine has been reported to increase prodynorphin mRNA expression (Wang *et al*, 1995; Turchan *et al*, 1998), one possibility for the loss of KOP receptor function is that an increase in the release of endogenous dynorphin within the slice competes with the U69593 response. However, application of nor-BNI alone did not significantly alter the amplitude of the fEPSP in

amphetamine-treated animals ($105.4 \pm 4.0\%$, $n = 5$; $p > 0.05$, Figure 1e), implying that this is not the case.

MOP receptors are also present in the NAc shell and like KOP receptors, they modulate glutamate release onto MSNs (Yuan *et al*, 1992; Hjelmstad and Fields, 2001; Hoffman and Lupica, 2001). To investigate whether amphetamine administration also changes MOP receptor function, we tested the effect of the MOP receptor agonist DAMGO on fEPSPs. The inhibition produced by $1 \mu\text{M}$ of DAMGO was not statistically different between the two groups (Figure 1f): DAMGO elicited a $31.1 \pm 4.2\%$ inhibition in saline-treated animal ($n = 5$) vs $31.6 \pm 7.7\%$ in amphetamine-treated animal ($n = 7$, $p > 0.05$).

DA-Dependent Increase in Endogenous Dynorphin

Amphetamine increases extracellular DA. To investigate whether this increase in DA is essential for the loss of KOP receptor function, we injected the nonselective DA receptor antagonist flupenthixol (0.5 mg/kg , s.c.) 15 min before amphetamine administration. In slices prepared from these animals, U69593 produced a $15.6 \pm 2.7\%$ ($n = 9$) reduction in the amplitude of the fEPSP (Figure 2a). Thus, flupenthixol significantly antagonized the effect of amphetamine ($p < 0.05$ compared to amphetamine group, not significant compared to saline group).

We also pretreated the animal with selective D1 receptor antagonist SCH-23390 (0.1 mg/kg , s.c.) 15 min before amphetamine injection. SCH-23390 also blocked the effect of amphetamine on KOP receptor function. U69593 produced a $20.7 \pm 8.0\%$ inhibition of the fEPSP ($n = 8$, $p < 0.05$ compared to amphetamine-treated animal, Figure 2b). These data suggest that the increase of DA levels following the administration of amphetamine activates D1-receptors, which is a necessary step for the downregulation of KOP receptor function.

Activation of D1 receptors in the NAc selectively excites substance P/dynorphin-expressing MSNs (Steiner and Gerfen, 1998) and psychostimulants increase dynorphin peptide levels in the striatum (Bustamante *et al*, 2002). Thus, the reduction in KOP receptor function may reflect agonist-induced desensitization of the receptor caused by the DA-dependent release of dynorphin. To determine the role of endogenous dynorphin release, we tested the effects of the nonselective opioid receptor antagonist naltrexone (1 mg/kg , s.c.), preadministered before the injection of amphetamine. In slices prepared from these animals, the U69593 inhibition of fEPSPs was similar to those from saline-injected animals (Figure 2c, $29.6 \pm 5.8\%$, $n = 9$, $p < 0.05$ compared to the amphetamine-treated group), indicating that the amphetamine-mediated inhibition of KOP receptor function acts through an opioid-dependent mechanism.

We next examined whether the effect of naltrexone was through its antagonism of the KOP receptor. We were unable to test whether the KOP receptor-selective antagonist nor-BNI blocks the effects of amphetamine since its actions persist for days to weeks (Horan *et al*, 1992; Jones and Holtzman, 1992). Instead, we tested whether the systemic administration of a KOP receptor agonist mimics amphetamine's effect. We injected U69593 (0.32 mg/kg , s.c.) and cut slices after 2 h. U69593 inhibited fEPSPs amplitude

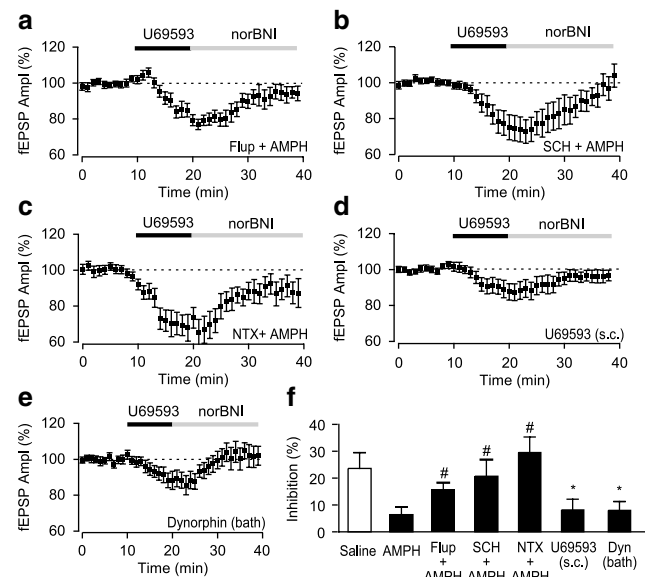


Figure 2 The reduction of KOP receptor function by amphetamine requires DA and can be mediated through enhanced dynorphin release. (a) The diminished KOP receptor-mediated inhibition of glutamate release in amphetamine treated animals is antagonized by systemic administration of flupenthixol (0.5 mg/kg , s.c.; Flup + AMPH). (b) Administration of SCH-23390 (0.1 mg/kg , s.c.) before amphetamine treatment also blocks the effect on KOP receptor function (SCH + AMPH). (c) Administration of naltrexone (1 mg/kg , s.c.) before amphetamine treatment blocks the effect on KOP receptors (NTX + AMPH). (d) Systemic administration of U69593 (0.32 mg/kg , s.c.) mimics the effects of amphetamine in reducing KOP receptor function. (e) Incubating slices in dynorphin (500 nM) for 30 min mimics also the effects of amphetamine administration. (f) Summary of the U69593 inhibition of fEPSPs in slices obtained from animals administered with saline, amphetamine, amphetamine coadministered with flupenthixol, SCH-23390 or naltrexone, systemic U69593, or bath incubation of dynorphin (* $p < 0.05$ compared to amphetamine-treated group; # $p < 0.05$ compared to saline-treated group).

by only $8.2 \pm 4.0\%$ in slices prepared from these animals ($n = 13$, $p < 0.05$ compared to saline-treated group; Figure 2d). Furthermore, incubating NAc slices prepared from naive rats with dynorphin (500 nM for 30 min and then washed with regular Ringer's solution for at least 1 h) similarly reduced the magnitude of the KOP receptor inhibition ($8 \pm 3.3\%$, $n = 10$, Figure 2e).

KOP Receptor Function in the VTA

Substance P/dynorphin-containing MSNs in the NAc shell project to the VTA (Lu *et al*, 1998) and psychostimulant administration also increases dynorphin peptide levels in the midbrain (You *et al*, 1994; Bustamante *et al*, 2002). Therefore, we investigated whether amphetamine alters KOP receptor function in the VTA. To test this, current-clamp recordings were made from I_h -expressing VTA neurons from saline- and amphetamine-treated rats. We found no differences in the responses of VTA neurons to KOP receptor agonists in these two groups. Seven of ten neurons (70%) from saline-treated rat were inhibited by U69593 (Figure 3a). Of the inhibited neurons, five neurons showed a decrease in their firing rate whereas the other two neurons did not fire spontaneously, but were hyperpolarized by U69593. In amphetamine-treated rats, seven of

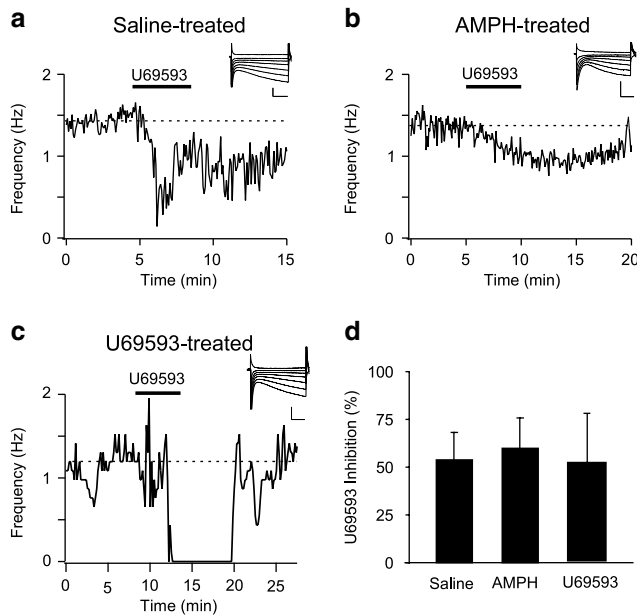


Figure 3 A single dose of amphetamine does not affect KOP receptor function in VTA neurons. (a) A representative recording in a neuron from a saline-treated animal. This neuron expressed an I_h (inset; scale bar, 100 pA and 50 ms) and the firing rate was inhibited by U69593 (500 nM). (b) A representative neuron from amphetamine-treated animal. This neuron expressed an I_h and was also inhibited by 500 nM U69593. (c) Summary of neurons inhibited by U69593 in saline- and amphetamine-treated groups. (c) A representative recording from U69593-treated animal. This neuron expressed an I_h and the firing rate was inhibited by U69593. (d) Summary of U69593 inhibition in saline-, amphetamine-, and U69593-treated groups. Bar graph shows the average inhibition in firing rate for KOP receptor-sensitive neurons.

eleven neurons (64%) were inhibited by U69593 with a reduction in firing rate for five neurons and a hyperpolarization in two neurons (Figure 3b). In those cells that were responsive to U69593, there was no difference between the magnitude of the firing rate inhibition between the saline and amphetamine groups (Figure 3d).

One possibility for the difference between the VTA and NAc is that the amphetamine treatment is not increasing dynorphin levels to the same degree in the two brain regions. However, systemic treatment with the KOP receptor agonist U69593, which reduced KOP receptor function in the NAc, did not reduce KOP receptor responses in the VTA: five of nine neurons were inhibited by U69593 (Figure 3c). Three of the five spontaneously firing neurons (60%) were inhibited by U69593. In those three neurons, U69593 caused $52.2 \pm 25.7\%$ inhibition in firing rate (Figure 3d). There was no difference among the magnitude of the inhibition compared to saline- and amphetamine-treated group.

Loss of KOP Receptor Function is due to Desensitization

A previous study found evidence for KOP receptor downregulation in the NAc following psychostimulant treatment (Turchan *et al*, 1998). Therefore, we asked whether the functional changes we have observed in KOP receptor function in the NAc were due to receptor downregulation. We saw no change in the overall KOP receptor levels

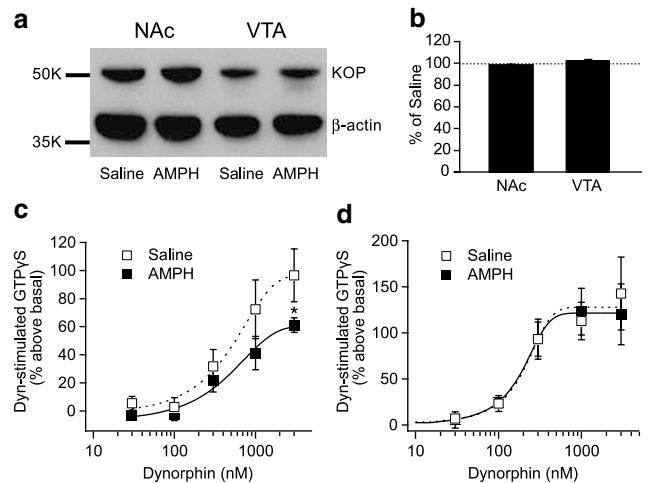


Figure 4 NAc KOP receptors are desensitized following amphetamine treatment. (a) NAc tissue and VTA slices taken from amphetamine- and saline-treated animals exhibited similar expression of KOP receptor. (b) Immunoblots quantified by scanning densitometry showed no significant difference between amphetamine- and saline-treated rats in either the NAc or the VTA. (c) Concentration-effect curves of dynorphin-stimulated [35 S]GTP γ S binding to NAc membranes from rats treated with saline and amphetamine. Data are presented as the mean \pm SEM of four experiments performed in triplicate ($*p < 0.05$ compared to saline group). (d) Concentration-effect curves of dynorphin-stimulated [35 S]GTP γ S binding to VTA membranes from saline- vs amphetamine-treated rats.

in either the NAc or the VTA following amphetamine administration (Figure 4a and b).

We next tested whether KOP receptors are desensitized following amphetamine treatment. Using a GTP γ S binding assay, we found a significant decrease in dynorphin-stimulated GTP γ S in membranes prepared from the NAc of amphetamine vs saline-treated rats, with no significant change in the EC_{50} (Figure 4c). Consistent with our electrophysiological results, we observed no differences in receptor activity in VTA preparations (Figure 4d).

Effects of Amphetamine on Basic Synaptic Properties

The loss of presynaptic inhibition may be accompanied by other presynaptic changes. To determine whether there were changes in basic synaptic function, we made whole-cell recordings from MSNs and analyzed both the paired-pulse ratio and short-term plasticity in response to trains of stimuli. EPSCs from saline-treated animals showed short-term depression following a 25 Hz train. The amplitude of the eighth EPSC was $39.9 \pm 2.5\%$ of the first ($n = 11$, Figure 5a and b) similar to the depression seen in naive tissue (Hjelmstad, 2004). In slices from amphetamine-treated rats, the short-term depression was significantly decreased. On average, the eighth EPSC was $55.9 \pm 5.9\%$ of the first ($n = 12$, $p < 0.05$, Figure 5a and b). In addition, comparing the amplitudes of the first and second EPSC showed a significant increase in the paired-pulse ratio (saline, 0.87 ± 0.04 ; amphetamine, 1.02 ± 0.05 , $p < 0.05$, Figure 5c). Both of these measures of short-term plasticity are consistent with a decrease in the synaptic probability of release. Finally, we tested whether there was a presynaptic change in probability of release by assaying the frequency of spontaneous

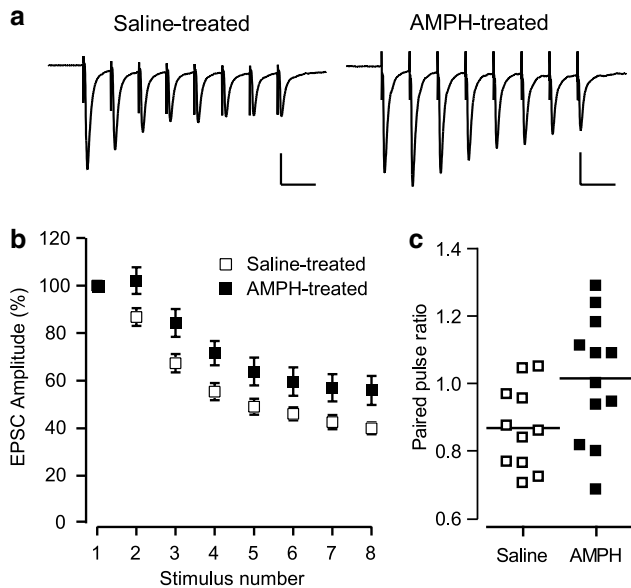


Figure 5 Changes in synaptic short-term plasticity following amphetamine administration. (a) Example of a train of EPSCs (8 stimuli at 25 Hz) recorded in a neuron from a saline-treated (left) or amphetamine-treated (AMPH, right) rat (scale bars, left: 200 pA and 50 ms, right: 100 pA and 50 ms). (b) Average of responses for all neurons from saline- and amphetamine-treated rats. (c) Paired-pulse ratio (second EPSC/first EPSC) in neurons from saline- and amphetamine-treated rats. Horizontal bar represents the group mean.

miniature postsynaptic conductances (mEPSCs). However, we found no significant difference in the mEPSC frequency in neurons recorded from slices prepared from amphetamine- vs saline-treated animals (data not shown).

DISCUSSION

Here we show that a single injection of amphetamine causes a long-lasting, regionally selective downregulation of KOP receptor function. This change in function appears to be a consequence of receptor desensitization rather than receptor downregulation. While fEPSPs from saline-treated animals are inhibited by bath application of U69593, this inhibition is attenuated in amphetamine-treated animals. Amphetamine increases extracellular catecholamine levels through reversal of the plasma membrane-associated DA and norepinephrine transporters (Sulzer *et al*, 2005). Moreover, this increase in DA produces a subsequent increase in dynorphin release, both in the striatum and in the midbrain (You *et al*, 1994; Bustamante *et al*, 2002). Our data indicate that this DA-dependent increase in dynorphin mediates the reduction of KOP receptor function. This reduction in KOP function is not due to a change in the IC₅₀ to U69593 and cannot be explained by occlusion of the receptor due to increased levels of endogenous dynorphin in the slice, since nor-BNI has no significant effect on the EPSP amplitude by itself. Finally, this change shows regional specificity: there is no change in the KOP receptor-mediated hyperpolarization of VTA neurons from amphetamine-treated animals.

Our data indicate that the loss of KOP receptor function in the NAc is caused by a long-lasting desensitization of the

receptor. In support of this, McLaughlin *et al* (2004) showed that repeated administration of the KOP agonist U50488 in mice produces a long-lasting (>7 day) tolerance to the antinociceptive effects of KOPs, paralleled by an increase in the phosphorylation state of a serine residue in the carboxyl terminal of the receptor. Phosphorylation of the rat KOP receptor at this particular site desensitizes the receptor (Appleyard *et al*, 1999; McLaughlin *et al*, 2003). Although many G protein-coupled receptors are internalized after phosphorylation and desensitization, this process appears to be delayed *in vivo* for the KOP receptor as there are no changes in the overall receptor levels or in the levels of phosphorylated receptor for several days following KOP treatment (McLaughlin *et al*, 2004). Indeed, KOPs show poor internalization following agonist application (Liu-Chen, 2004), a step which promotes KOP receptor dephosphorylation and reactivation (McLaughlin *et al*, 2003).

The functional decreases in electrophysiology and KOP receptor G-proteins coupling could be due to the downregulation of KOP receptors levels. However, we found no change in KOP receptor protein level in either the NAc or the VTA. This finding contrasts a previous study (Turchan *et al*, 1998) using autoradiography in brain slices. The reason for this disparity is unclear, but it is not likely due to the assay differences between immunoblot and autoradiography, as we saw no difference in [³H]-U69593 autoradiographic binding in the NAc between saline- and amphetamine-treated rats in NAc (data not shown).

It is curious that amphetamine does not produce a long-lasting desensitization of KOP receptors in the VTA, despite evidence for dynorphin release in the midbrain in response to amphetamine (Bustamante *et al*, 2002). This specific mechanism underlying this difference between the VTA and the NAc is unclear, but may reflect regional differences in KOP receptor trafficking or differences in the trafficking of pre- and postsynaptic KOP receptors.

The loss of presynaptic inhibition is selective for the KOP receptor: MOP receptor-mediated inhibition in the NAc in response to a saturating dose of DAMGO is unaffected by acute amphetamine exposure (although we cannot rule out possible changes in the IC₅₀ to DAMGO). Likewise, in our hands, bath application of DA inhibits fEPSPs following amphetamine exposure (data not shown). This is consistent with the finding that chronic cocaine administration produces an enhanced sensitivity to DA (Beurrier and Malenka, 2002). In addition, other investigators have found that presynaptic inhibition by endocannabinoids is unaffected by a single systemic injection of cocaine (Fourgeaud *et al*, 2004). Thus, presynaptic KOP receptor modulation of glutamate release in the NAc appears to be selectively lost following exposure to psychostimulants.

In contrast, several studies have observed postsynaptic changes at glutamatergic synapses in the NAc following psychostimulant exposure. For example, prolonged cocaine treatment (but not following a single injection) decreases the AMPA/NMDA receptor ratio in the NAc shell (Thomas *et al*, 2001). In addition, cocaine self-administration produces a postsynaptic decrease in the AMPA receptor-mediated fEPSP (Schramm-Sapota *et al*, 2006). Finally, many studies have demonstrated changes in glutamate receptor subunit levels in the NAc following exposure to either cocaine or amphetamine (Lu *et al*, 1997, 1999;

Churchill *et al*, 1999; Lu and Wolf, 1999). How these postsynaptic changes integrate with our observations to alter the function of the circuit remains to be determined.

While the changes in KOP receptor function observed in the NAc are robust, the changes in other synaptic properties were more subtle. We observed a significant increase in the paired-pulse ratio of EPSCs and a decrease in the short-term depression during bursts of stimuli following exposure to amphetamine. These changes are both consistent with a decrease in probability of release. However, it is also possible that these observed changes are independent of probability of release, for example, they could be due to alterations in the molecular mechanisms underlying short-term plasticity (Rosenmund *et al*, 2003) or changes in short-term plasticity that are independent of release probability (Casassus *et al*, 2005).

The changes in short-term plasticity observed in our study contrast several studies that did not observe significant changes in the paired-pulse ratio in mice that were either treated with or self-administered cocaine (Thomas *et al*, 2001; Fourgeaud *et al*, 2004; Schramm-Sapota *et al*, 2006). This disparity most likely reflects differences between the pharmacological effects of cocaine and amphetamine. Alternatively, it may reflect differences between mice and rats. Finally, two of the studies measured the paired-pulse ratio using field potentials, as opposed to whole-cell EPSCs, and this method may be less sensitive to changes in release, particularly if those changes occur at only a subset of synapses.

Following systemic administration of either cocaine or amphetamine, expression levels of c-fos mRNA, an indirect measure of activity, are increased in substance P/dynorphin-containing MSNs (Johansson *et al*, 1994). At least a portion of these neurons projects to the VTA (Lu *et al*, 1998). Thus, psychostimulants activate this direct pathway, causing the release of GABA as well as substance P and dynorphin in the VTA. KOP receptor agonists decrease the c-fos staining in these neurons produced by injection of a D1 receptor agonist (Steiner and Gerfen, 1996). This indicates that KOP receptors on glutamate terminals in the NAc have a negative-feedback role, normally decreasing the excitability of substance P/dynorphin MSNs. The loss of this negative feedback would result in a hyperexcitability of these neurons, enhancing the release of dynorphin in downstream structures.

In the rat VTA, KOPs inhibit mesocortical, but not mesolimbic, DA neurons (Margolis *et al*, 2006). Acute amphetamine exposure does not alter KOP receptor function in the VTA. However, the loss of negative feedback in the NAc, along with the enhanced expression of preprodynorphin seen following amphetamine exposure (Wang *et al*, 1995; Turchan *et al*, 1998), will lead to increased KOP receptor activation within the VTA, resulting in reduced mesocortical DA. In fact, microdialysis studies in sensitized animals have observed this predicted decrease in mPFC DA (Sorg *et al*, 1997; Chefer *et al*, 2000) and impairments in cognition observed in amphetamine-sensitized animals can be reversed by D1 receptor agonists injected into the mPFC (Fletcher *et al*, 2005).

The current results are based on a moderately large dose of amphetamine (Grilly and Loveland, 2001) and caution should be exercised in extending these findings to other

doses of amphetamine. For example, it is unclear whether low doses of amphetamine, such as those used to treat ADHD, sufficiently activate the KOP system to produce receptor desensitization. On the other hand, given the very slow recovery from desensitization, small amounts of receptor desensitization may build up over time with repeated dosing.

The observation that an effect of amphetamine administration can be mimicked by KOP receptor agonists appears paradoxical to several studies showing that KOP receptor agonists prevent many of the behavioral effects of amphetamine (Gray *et al*, 1999; Tzaferis and McGinty, 2001). However, these immediate effects of KOP receptor stimulation can be distinguished from their longer-lasting modulatory effects on the endogenous opioid system.

In support of this, although the behavioral effects of cocaine are inhibited by acute activation of KOP receptors, pretreatment with a KOP receptor agonist can enhance cocaine-conditioned place preference (McLaughlin *et al*, 2006). Ultimately, the behavioral effects of opioid receptors in relationship to drugs of abuse depend upon both when and where they are activated. The results here, showing a region-specific decrease in KOP receptor function, highlight this point, and provide insight into how the KOP-mediated behaviors can change with drug experience.

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DISCLOSURE/CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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