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# Drugs of Abuse and Stress Impair LTP at Inhibitory Synapses in the Ventral Tegmental Area

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## Abstract

Synaptic plasticity in the ventral tegmental area (VTA) is modulated by drugs of abuse and stress and is hypothesized to contribute to specific aspects of addiction. Both excitatory and inhibitory synapses on dopamine neurons in the VTA are capable of undergoing long-term changes in synaptic strength. While the strengthening or weakening of excitatory synapses in the VTA has been widely examined, the role of inhibitory synaptic plasticity in brain reward circuitry is less established. Here, we investigated the effects of drugs of abuse, as well as acute stress, on long-term potentiation of GABAergic synapses onto VTA dopamine neurons ($LTP_{GABA}$). Morphine (10 mg/kg i.p.) reduced the ability of inhibitory synapses in midbrain slices to express $LTP_{GABA}$ both at 2 and 24 hours after drug exposure but not after 5 days. Cocaine (15 mg/kg i.p.) impaired $LTP_{GABA}$ 24 hours after exposure, but not at 2 hours. Nicotine (0.5 mg/kg i.p.) impaired $LTP_{GABA}$ 2 hours after exposure, but not after 24 hours. Furthermore, $LTP_{GABA}$ was completely blocked 24 hours following brief exposure to a stressful stimulus, a forced swim task. Our data suggest that drugs of abuse and stress trigger a common modification to inhibitory plasticity, synergizing with their collective effect at excitatory synapses. Together, the net effect of addictive substances or stress is expected to increase excitability of VTA dopamine neurons, potentially contributing to the early stages of addiction.

### Keywords

addiction; GABA; synaptic plasticity; patch clamp

## Introduction

The ability of addictive drugs to usurp brain reward circuits relevant to natural rewards and establish addictive behaviors is hypothesized to be due in part to changes in synaptic plasticity of the mesolimbic dopamine system triggered by drugs of abuse. Initial studies seeking to identify common adaptations in the brain produced by drugs of abuse ascertained that these substances all increased dopamine release probability of ventral tegmental area (VTA) dopamine neurons that project to the nucleus accumbens (Wise, 1996; Koob *et al*., 1998; Nestler, 2001). Other investigators found that *in vivo* administration of addictive drugs is sufficient to induce a long-lasting change in synaptic transmission of excitatory inputs onto dopamine neurons in the VTA (Ungless *et al*., 2001; Saal *et al*., 2003; Faleiro *et al*.,

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2004; Argilli *et al*., 2008; Chen *et al*., 2008; Engblom *et al*., 2008; Mameli *et al*., 2009). Long-term potentiation (LTP) of excitatory synapses is a mechanism that underlies the ability of neural circuits to form memories, in addition to many other brain functions (Malenka & Bear, 2004). Thus, addictive drugs may co-opt brain reward circuitry to 'overlearn' the value of a drug to the organism (Kauer & Malenka, 2007; Niehaus *et al*., 2009). Since the net output of a neuron depends upon the balance between excitatory and inhibitory inputs onto the cell, some studies have considered whether inhibitory synaptic transmission is modified by addictive drugs. Cocaine, morphine and ethanol have all been found to affect inhibitory synaptic plasticity in the VTA (Melis *et al*., 2002; Liu *et al*., 2005; Nugent *et al*., 2007). Long-lasting potentiation of GABAergic synapses onto dopamine neurons in the VTA ($LTP_{GABA}$) is blocked by *in vivo* administration of morphine (Nugent *et al*., 2007); however, it is unknown whether other drugs of abuse, many of which potentiate excitatory synapses onto VTA dopamine neurons (Saal *et al*., 2003), can analogously alter $LTP_{GABA}$.

In addition to addictive drugs, acute stress can also affect synaptic plasticity. In the hippocampus, acute stress impairs LTP and simultaneously facilitates LTD (Shors *et al*., 1989; Kim *et al*., 1996; Xu *et al*., 1997). Acute stress, as well as various drugs of abuse, potentiates excitatory synaptic strength at dopamine neurons in the VTA (Saal *et al*., 2003; Dong *et al*., 2004) suggesting that stress and drugs of abuse converge on a common pathway that modifies dopamine neuron output and thereby reward circuitry. Stress can increase dopamine release (Inglis & Moghaddam, 1999) and can facilitate behaviors such as drug-seeking and drug-taking that may develop into addiction, perhaps by augmenting the rewarding effects of drug administration (Piazza & Le Moal, 1998; Miczek *et al*., 2008). Here we examine whether *in vivo* administration of addictive drugs or exposure to acute stress regulate inhibitory inputs onto dopamine neurons in the VTA. Our findings suggest that drugs of abuse and stress may utilize common mechanisms to modulate synaptic plasticity of inhibitory synapses.

## Methods

### Animals and *in vivo* manipulations

All procedures were carried out in accordance with the guidelines of the National Institutes of Health for animal care and use, and were approved by the Brown University Institutional Animal Care and Use Committee. Sprague-Dawley rats were maintained on a 12-hour light/dark cycle and provided food and water ad libitum. For drug administration, rats were given a single injection i.p. at the same time of day of either saline (volume matched with experimental injections), morphine (10 mg/kg), cocaine (15 mg/kg) or nicotine (0.5 mg/kg), housed singly in a new cage for 2 hours and then returned to the home cage. For morphine, cocaine, nicotine and stress experiments, only one brain slice per rat was used for each experiment so that reported *n* numbers represent the number of animals. Acute stress was administered by a modified Porsolt forced swim task (Saal *et al*., 2003), wherein rats were placed in a 2 L plastic beaker containing approximately 1 L of cold water (6 °C) for 5 min. Following the swim task, animals were dried off, wrapped in a dry cloth for 5 min, placed singly in a warmed cage that was heated underneath by a heating pad for 2 hours and then returned to the home cage. RU486 (40 mg/ml in DMSO) or DMSO (as vehicle) were administered i.p. 30 min prior to the cold water swim.

### Preparation of brain slices

Preparation of slices was performed as previously described (Jones *et al*., 2000; Nugent *et al*., 2007). Sprague-Dawley rats (15–21 days old) were deeply anesthetized using isoflurane and quickly decapitated. Unless stated otherwise, brain slices were prepared 24 hours after

drug administration or stress exposure. The brain was rapidly removed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 $NaHCO_3$, 2.5 KCl, 1.2 $NaH_2PO_4$, 2.4 $CaCl_2$, 1.2 $MgSO_4$, 11.1 glucose and 0.4 ascorbic acid, saturated with 95% $O_2$/5% $CO_2$ (pH 7.4). Horizontal midbrain slices containing the VTA were cut at a thickness of 250 μm using a vibratome (Leica Microsystems), stored for at least one hour at 34°C, and transferred to a recording chamber where the slice was submerged in warmed ACSF.

## Electrophysiology

Midbrain slices were continuously perfused with ACSF (no ascorbic acid) at 28–32°C at 1.5–2 ml/min containing: 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10μM), strychnine (1μM), and 1, 3-dipropyl-8-cyclopentylxanthine (DPCPX; 1μM) to block AMPA-, glycine-, and A1 adenosine receptors, respectively. A1 receptors were blocked to remove endogenous levels of adenosine-mediated inhibition of GABA release (Floran *et al*., 2002). Patch pipettes were filled with (in mM): 125 KCl, 2.8 NaCl, 2 $MgCl_2$, 2 ATP-$Na^+$, 0.3 GTP-$Li^+$, 0.6 EGTA, and 10 HEPES. Whole-cell patch-clamp recordings were made from neurons visually identified in the VTA. Dopamine neurons were identified by the presence of a large $I_h$-current (>60 pA) during a voltage step from −50 mV to −100 mV. A recent study showed that expression of $I_h$ alone is not sufficient to identify DA cells unequivocally (Margolis *et al*., 2006), but see (Chen *et al*., 2008). Therefore in each set of our experiments, a subset of the neurons recorded from and reported here are possibly non-dopaminergic neurons. $GABA_A$ receptor-mediated IPSCs were stimulated at 0.1 Hz (100 μsec) using a bipolar stainless steel stimulating electrode placed 200–500 μm rostral to the recording site in the VTA. Cells were voltage-clamped at −70mV and the cell input resistance and series resistance were monitored throughout the experiment and were discarded if these values changed by more than 10% during the experiment. IPSCs were amplified using an AxoClamp 2B amplifier (Axon Instruments) and Brownlee Precision model 210 postamplifier, low-pass filtered at 3 kHz and digitally sampled to computer at 30 kHz using an analog-to-digital interface (National Instruments). Custom-designed software for use with LabVIEW (National Instruments) was used to measure the peak IPSC amplitudes.

## Materials

3-isobutyl-1-methylxanthine (IBMX; 100 μm) was used to inhibit phosphodiesterase-mediated degradation of cGMP and applied via perfused ACSF for at least 10 minutes prior to induction of $LTP_{GABA}$ by SNAP or BAY 41-2272. Brain slices from control animals (vehicle-injected) were interleaved with brain slices from experimental animals (morphine, cocaine or nicotine injected). Cocaine was provided by Brown University Animal Care Facility. SNAP (S-nitroso-N-acetylpenicillamine) was obtained from Cayman Chemical. IBMX and DPCPX were obtained from Tocris Bioscience. Morphine, nicotine, BAY 41-2272, RU 486, DNQX, strychnine and all other chemicals were obtained from Sigma-Aldrich.

## Analysis

Data are presented as means ± s.e.m. Data from Figures 1–3, 5 and 7 were analyzed with a two-factor analysis of variance (ANOVA), using the experimental condition (saline vs. drug or stress vs. RU486) as the between-subjects factor and time (in minutes) as the within-subjects factor. Data from Figures 4 and 6 were analyzed by one-way ANOVA to assess drug effect (SNAP or BAY 41-2272) on IPSC amplitude over time course of experiment (in minutes). Main effects and interactions from ANOVAs were determined with a significance level of $p < 0.05$. Magnitude of LTP was calculated by averaging 30 consecutive IPSCs 5 min prior to drug application and 20–25 min after drug application.

## Results

$LTP_{GABA}$, a potentiation of inhibitory GABAergic synapses onto dopamine neurons in the VTA, requires NO as a retrograde messenger to activate presynaptic soluble guanylate cyclase (sGC). Cyclic GMP (cGMP) produced by sGC then activates cGMP-dependent protein kinase (PKG), resulting in the observed persistent enhancement of GABA release (Nugent *et al*., 2007; Nugent *et al*., 2009). $LTP_{GABA}$ can be elicited by synaptic stimulation or alternatively by direct bath application of an NO donor (Nugent *et al*., 2007; Nugent *et al*., 2009). The maintenance of $LTP_{GABA}$ does not require the persistent presence of NO, as an NO scavenger fails to reverse the potentiation (Nugent *et al*., 2009). In agreement with our previous studies, bath-applied SNAP, an NO donor, induced $LTP_{GABA}$ in midbrain slices prepared from saline-treated animals (Supplementary Fig. S1); however, in brain slices prepared from animals 24 hours after a single *in vivo* morphine exposure, SNAP was unable to potentiate GABA IPSCs and induce $LTP_{GABA}$ (Supplementary Fig. S1).

### Time course of morphine blockade of $LTP_{GABA}$

How soon after morphine exposure is $LTP_{GABA}$ impaired and how long does this blockade persist? A more detailed understanding of the process by which morphine alters $LTP_{GABA}$ could provide valuable information about the effects of morphine on the VTA and the brain reward circuit. We previously found that acute, *in vitro* application of μ-opioids had no effect on SNAP-induced $LTP_{GABA}$ whereas 24 hours after *in vivo* exposure to morphine $LTP_{GABA}$ was completely blocked (Nugent *et al*., 2007). We therefore next tested the effect of a single *in vivo* injection of morphine at an early time point, preparing brain slices 2 hours following drug or saline exposure. SNAP potentiated GABA IPSCs in slices from saline-treated animals; however, the SNAP-induced potentiation was already significantly reduced in slices from morphine-treated animals even 2 hours after morphine (Fig. 1*B*). We next asked whether $LTP_{GABA}$ remained impaired 5 days following a single morphine injection. Animals were injected once with either morphine or saline and brain slices were prepared 5 days following the single injection. In both groups, SNAP potentiated GABA IPSCs (Fig. 1*C*) indicating that although the $LTP_{GABA}$ blockade by morphine was observed at 2 or 24 hours after morphine, the blockade persisted for less than 5 days. The temporal window during which $LTP_{GABA}$ is altered by morphine exposure appears to take place between 2 hours and 5 days following drug administration.

### Cocaine and nicotine impair $LTP_{GABA}$ at different times

A single administration of several diverse addictive drugs increases the strength of excitatory synapses on VTA dopamine neurons, increasing the AMPA/NMDA ratio (Saal *et al*., 2003). Is morphine unique in obstructing $LTP_{GABA}$ or do other addictive drugs similarly alter inhibitory synaptic plasticity in the VTA? Based on their differing mechanisms of action from that of morphine, we chose to examine the effects of cocaine, a psychostimulant that enhances brain dopamine levels by blocking reuptake, and nicotine, which acts on nicotinic acetylcholine receptors (nAChRs) to increase dopamine neuron excitability. *In vivo* administration of cocaine impaired (but did not prevent) the expression of $LTP_{GABA}$ in brain slices prepared 24 hours after cocaine injection (Fig. 2*A*–*C*). Since $LTP_{GABA}$ was not completely blocked 24 hours after cocaine exposure, we tested whether cocaine might have a greater impact on $LTP_{GABA}$ at 2 hours after administration. However, cocaine did not significantly impair the expression of $LTP_{GABA}$ 2 hours after exposure as compared to saline-injected controls (Fig. 2*D*–*F*). In contrast to the significant attenuation observed 24 hours after cocaine, the induction and persistence of $LTP_{GABA}$ were unaffected by *in vivo* exposure to nicotine 24 hours after administration (Fig. 3*A*–*C*). To determine whether there was a time-dependent effect of nicotine, we tested the effect of nicotine on $LTP_{GABA}$ at 2 hours after administration. Surprisingly, two hours after exposure, $LTP_{GABA}$ was impaired

in nicotine-treated animals as compared to saline-treated controls (Fig 3*D–F*). Taken together, the reduction in inhibitory plasticity induced by morphine, cocaine and nicotine, drugs with different mechanisms of action, demonstrates that drugs of abuse that potentiate excitatory synapses can also inhibit plasticity at inhibitory synapses. However, drugs of abuse do not necessarily impair $LTP_{GABA}$ at the same time point after a single administration.

### Stress blocks $LTP_{GABA}$ similarly to drugs of abuse

Like addictive drugs, acute stress also potently potentiates glutamatergic synapses onto VTA dopamine neurons (Saal *et al*., 2003). At the behavioral level, reinstatement of drug-taking can be triggered by a stressor, by a single episode of drug administration, or by environmental cues associated with prior drug taking or stress (Piazza & Le Moal, 1998; Shaham *et al*., 2000), suggesting a possible link between stress/drug effects on VTA synapses and stress/drug effects on relapse. Since morphine, cocaine and stress all potentiate excitatory synapses, we next investigated the effect of an acute stress on plasticity of inhibitory synapses on VTA dopamine neurons. Indeed, a modified Porsolt forced swim task administered 24 hours prior to brain slice preparation completely blocked the ability of SNAP to induce $LTP_{GABA}$ (Fig. 4*B,D*). As previously demonstrated by Saal and colleagues, the potentiation of excitatory synapses by stress depends on glucocorticoid receptor activation and was completely blocked by pretreatment with the glucocorticoid antagonist RU486 (Saal *et al*., 2003). Similarly, we found that RU486 administered 30 minutes prior to the swim task entirely prevented the effect of stress on $LTP_{GABA}$, as SNAP effectively potentiated GABAergic IPSCs and induced $LTP_{GABA}$ (Fig. 4*C,D*). These data indicate that the stress-induced blockade of inhibitory synaptic plasticity occurs through the glucocorticoid receptor pathway, and that both brief stress and a single exposure to drugs of abuse can impair inhibitory synaptic plasticity at VTA dopamine neurons.

### Morphine blockade of $LTP_{GABA}$ is independent of the stress pathway

Do morphine and acute stress block $LTP_{GABA}$ via a common mechanism? To determine whether morphine might be acting through an acute stress pathway, RU486 was administered 30 minutes prior to morphine injection. In brain slices prepared 24 hours later, bath-application of SNAP failed to potentiate GABAergic IPSCs (Fig. 5). This result shows that the glucocorticoid receptor antagonist cannot rescue impairment of inhibitory plasticity, suggesting that the morphine blockade of $LTP_{GABA}$ is likely independent of the effects of stress.

### Soluble guanylate cyclase activator overcomes morphine blockade of $LTP_{GABA}$

We next examined in more detail the locus of morphine's action on GABAergic nerve terminals. *In vivo* morphine exposure appears to prevent $LTP_{GABA}$ by disrupting the signaling cascade between NO generation and cGMP generation (Nugent *et al*., 2007). Normally, NO binds to the heme moiety of soluble guanylate cyclase leading to activation and production of cGMP. To determine whether *in vivo* exposure to morphine might be affecting the guanylate cyclase, we used a sGC activator, BAY 41-2272, which activates sGC independently of NO (Stasch *et al*., 2001). First, BAY 41-2272 was bath-applied to directly stimulate sGC in VTA brain slices in order to test whether chemical activation of guanylate cyclase by this drug could produce $LTP_{GABA}$ as expected. BAY 41-2272 increased the amplitude of GABAergic IPSCs (Fig. 6) in naïve brain slices through an increase in cGMP levels. Thus, BAY 41-2272 mimicked synaptic- or SNAP-induced $LTP_{GABA}$, and confirmed the requirement of cGMP in the signaling cascade.

Morphine most likely interferes with $LTP_{GABA}$ downstream of NO generation and upstream of cGMP production, as an NO donor is unable to potentiate synapses in slices from

morphine treated animals whereas a cGMP analog induces $LTP_{GABA}$ (Nugent *et al*., 2007). If morphine prevents NO from activating sGC, BAY 41-2272 should potentiate GABAergic transmission by directly activating sGC. In agreement with this idea, BAY 41-2272 not only potentiated GABA IPSCs from saline-treated animals (Fig. 7*A*,*C*), but also potentiated GABA IPSCs from morphine-treated animals (Fig. 7*B*,*C*). The ability of BAY 41-2272 to induce $LTP_{GABA}$ in slices from morphine-treated animals indicates that sGC is still present at significant levels in VTA inhibitory synapses and is capable of generating cGMP that can overcome the upstream block of the signaling cascade and trigger $LTP_{GABA}$.

### Presynaptic protein RIM1α is not necessary for $LTP_{GABA}$

The expression of $LTP_{GABA}$ occurs via a sustained increase in GABA release after initial activation of the NO-cGMP-PKG pathway. Since RIM1α has been linked to other forms of synaptic plasticity, we considered the possibility that the active zone protein RIM1α is necessary for $LTP_{GABA}$ by observing $LTP_{GABA}$ in RIM1α-deficient mice. RIM1α knockout mice have decreased neurotransmitter release at GABAergic synapses in the hippocampus during paired stimulation (Schoch *et al*., 2002) and mossy fibre LTP, a presynaptic form of LTP, is abolished in RIM1$\alpha^{-/-}$ mice (Castillo *et al*., 2002). Furthermore, LTP at GABAergic synapses in the cerebellum requires RIM1α for the sustained increase in GABA release (Lachamp 2009). However, at GABAergic synapses on VTA dopamine cells, SNAP induced $LTP_{GABA}$ in both RIM1α-deficient mice (137.4 ± 10.3 % of baseline, n = 8) and wild-type littermates (138.5 ± 5.7 % of baseline, n = 5) indicating that unlike other presynaptic forms of plasticity RIM1α is not necessary for expression of $LTP_{GABA}$. Two-way ANOVA revealed significant effects of time ($F_{1,39} = 7.94$, $p < 0.001$) and genotype ($F_{1,39} = 11.59$, $p < 0.001$), but no significant interaction between the two factors ($F_{1,39} = 0.78$, $p > 0.05$).

## Discussion

Our data demonstrate that morphine, cocaine and nicotine all cause a loss of LTP at inhibitory synapses onto dopamine neurons in the VTA (Fig. 8). Different drugs of abuse were previously shown to enhance excitatory synaptic strength onto VTA dopamine neurons (White *et al*., 1995;Zhang *et al*., 1997;Ungless *et al*., 2001;Saal *et al*., 2003;Faleiro *et al*., 2004;Borgland *et al*., 2004). Our results suggest that drug-induced changes in inhibitory synaptic plasticity in the VTA also represent a significant neuroadaptation in the brain reward circuit. Remarkably, acute stress blocked $LTP_{GABA}$ in a manner similar to that caused by morphine, cocaine and nicotine (Fig. 8), again in parallel with previous results at excitatory synapses (Saal *et al*., 2003). The potentiation of excitatory synapses along with the loss of $LTP_{GABA}$ at GABAergic synapses will act in concert to increase dopamine cell excitability. Taken together, these observations suggest that the effects of drugs of abuse and stress on both excitatory and inhibitory synapses in the VTA are intertwined, resulting in adaptations in reward circuitry that could contribute not only to reward learning but perhaps to drug seeking, use and relapse.

### Time course of drug induced block of $LTP_{GABA}$

Previously we found that SNAP-induced $LTP_{GABA}$ was blocked 24 hours after *in vivo* administration of morphine, but was completely unaffected by an acute *in vitro* application (Nugent *et al*., 2007). How quickly after *in vivo* treatment with drugs of abuse is $LTP_{GABA}$ blocked? As early as 2 hours after morphine injection, the magnitude of $LTP_{GABA}$ was reduced compared to saline controls. Five days after a single morphine injection, the magnitude of $LTP_{GABA}$ was no longer significantly different from saline injected controls, indicating that morphine initiates a synaptic change that is evident within 2 hours, persists for 24 hours, but is reversed within 5 days. Interestingly, $LTP_{GABA}$ was not impaired 2

hours after cocaine exposure, suggesting that the effect of cocaine is time-dependent and requires more than 2 hours to impact the expression of $LTP_{GABA}$. Even after 24 hours, cocaine did not entirely block $LTP_{GABA}$, although we found a significant attenuation. This raises the possibility that psychostimulants may not affect $LTP_{GABA}$ as much as morphine or nicotine, or alternatively that the block of $LTP_{GABA}$ by cocaine peaks either at a time between 2 and 24 hours, or later than 24 hours. Cocaine has been reported to decrease GABAergic input onto VTA dopamine neurons following repeated cocaine exposure, by Liu and colleagues, and a decrease in maximal IPSC amplitude was not detected until 5 days of cocaine treatment, although the authors determined that the cocaine-induced reduction was postsynaptic (Liu *et al*., 2005). Thus, cocaine reduces GABAergic transmission in the VTA by at least two distinct mechanisms.

*In vivo* administration of nicotine impaired $LTP_{GABA}$ 2 hours after administration, but did not result in persistent blockade of $LTP_{GABA}$ when examined 24 hours after initial exposure. The distribution of pharmacologically distinct nicotinic acetylcholine receptors (nAChRs) in the VTA might partially explain the time dependent effect of nicotine on GABAergic plasticity. Glutamatergic synapses onto VTA dopamine neurons express α7-containing nAChRs that are resistant to desensitization (Pidoplichko *et al*., 1997; Wooltorton *et al*., 2003) whereas non α7-containing nAChRs present on GABAergic nerve terminals rapidly desensitize in the presence of nicotine (Mansvelder *et al*., 2002). The observed potentiation of glutamatergic synapses 24 hours following *in vivo* exposure to nicotine may involve α7-containing nAChRs, while $LTP_{GABA}$ might be affected by the desensitizing non α7-containing nAChRs present at GABAergic synapses.

### Acute stress blocks $LTP_{GABA}$

We demonstrated that acute stress potently blocks $LTP_{GABA}$ at synapses on VTA dopamine neurons. Importantly, a glucocorticoid receptor antagonist prevented the blockade of $LTP_{GABA}$ by acute stress, but had no effect on morphine-induced blockade of $LTP_{GABA}$. These results indicate that the effect of morphine on GABAergic plasticity was not simply due to stress associated with drug administration and that while drugs of abuse and acute stress produce a similar effect on $LTP_{GABA}$, the mechanisms leading to the reduction in GABAergic plasticity are distinct. As observed at excitatory synapses (Saal *et al*., 2003; Dong *et al*., 2004), drugs of abuse and stress act in a similar manner to converge in a reduction of GABAergic transmission. Reduced plasticity of GABAergic synapses impairs the ability to persistently increase GABA release and is permissive for increased excitation of a dopamine neuron by excitatory synapses or other neuromodulators. During drug withdrawal, a coordinated increase in excitatory synaptic strength and impairment of inhibitory synaptic plasticity might contribute to stress-induced relapse as a result of increased dopamine neuron firing rate and dopamine release in VTA projection areas. Infusion of GABA agonists into the VTA or infusion of D1/D2 receptor antagonists into the dorsal prefrontal cortex both prevent footshock-induced reinstatement of cocaine-seeking behavior (McFarland *et al*., 2004) suggesting that dopamine released by afferents from the VTA projecting to the prefrontal cortex is necessary for stress-induced relapse. McFarland and colleagues hypothesized that footshock stress activates the central extended amygdala, which subsequently activates motor circuitry via dopaminergic input to the prefrontal cortex. The increased dopamine concentration in the prefrontal cortex initiates reinstatement via glutamatergic projections to the nucleus accumbens core (Yap & Miczek, 2008). One effect of stressful stimuli is to increase the release of corticotropin-releasing factor (CRF) in the brain, which triggers glutamate release in the VTA and in turn dopamine release (Wang *et al*., 2005). In cocaine-experienced animals, footshock stress causes release of CRF in the VTA, triggering local glutamate release and subsequent dopamine release to reinstate cocaine-seeking behavior (Wise & Morales, 2010). Activation of the CRF receptor, CRF-

R1, increases the firing rate of VTA dopamine neurons (Korotkova *et al*., 2006; Wanat *et al*., 2008) leading to an increase in dopamine release in brain regions that are innervated by the VTA (Inglis & Moghaddam, 1999; McFarland *et al*., 2004). The increase in dopamine neuron firing frequency occurs via an enhancement of the hyperpolarization activated cation current, $I_h$ (Wanat *et al*., 2008); however, a loss of potentiation of GABAergic synapses on dopamine neurons would be expected to further facilitate an increase in firing rate. Stress or CRF infusions can reinstate drug-seeking in animals that previously had extinguished this behavior, while CRF antagonists have some efficacy in reducing drug seeking behavior following stress (Sarnyai *et al*., 2001). Although CRF does not appear to be involved in drug-primed reinstatement (Sarnyai *et al*., 2001), the neuronal adaptations induced by exposure to drugs of abuse may increase the probability of relapse triggered by environmental stress.

### Mechanism of morphine-induced block of $LTP_{GABA}$

Soluble guanylate cyclase is a critical component of the $LTP_{GABA}$ signaling cascade and a possible target of the events initiated by exposure to drugs of abuse. When sGC was inhibited by ODQ, neither synaptic stimulation nor exogenous NO donors were able to induce $LTP_{GABA}$; however, a cGMP analog induced $LTP_{GABA}$ by circumventing sGC generation of cGMP (Nugent *et al*., 2007). Morphine-induced blockade of $LTP_{GABA}$ appears to occur at the level of sGC activation because an NO donor is unable to initiate $LTP_{GABA}$ whereas the sGC activator BAY 41-2272 produced $LTP_{GABA}$. Since BAY 41-2272 requires the reduced prosthetic heme group of sGC (Stasch *et al*., 2001), our results show that *in vivo* treatment of morphine does not prevent $LTP_{GABA}$ by oxidation or removal of the heme group in sGC, as BAY 41-2272 would be ineffective if the heme group was oxidized or absent. We conclude that sGC is still capable of producing cGMP in morphine-treated animals, but through an incompletely understood mechanism, sGC is not sufficiently activated by NO to generate levels of cGMP necessary to potentiate GABAergic transmission. We do not yet know whether morphine has a direct or indirect effect on sGC to diminish the capacity of NO to activate the cyclase. The block of $LTP_{GABA}$ was observed within 2 hours after morphine injection, and this time course limits the possible mechanisms of morphine's action. Post-translational modification of sGC or an associated protein appears most likely. It will be interesting to determine whether nicotine, cocaine, and stress also act on sGC to block the potentiation of $LTP_{GABA}$. If all three drugs and stress interfere with sGC function, this might suggest that they all act on a common intermediate.

The NO-cGMP-sGC-PKG signaling pathway, while more extensively studied in the cardiovascular system, provides important functions in the nervous system, including effects on synaptic plasticity (Kleppisch & Feil, 2009). NO and cGMP are known modulators of neurotransmitter release in the hippocampus, cerebellum, cortex and striatum (Prast and Philippu, 2001) although the molecular mechanisms are varied and not completely understood. Presynaptic neurotransmitter release could be regulated by cGMP through effects on vesicle docking, fusion and recycling (Kleppisch and Feil, 2009). Recent data indicate that PKG is required for induction of $LTP_{GABA}$, but not for its maintenance (Nugent *et al*., 2009). Based on its involvement in other forms of presynaptically-maintained plasticity, the possible role of RIM1α in $LTP_{GABA}$ was investigated. In the cerebellum, a form of LTP expressed by GABAergic interneurons via a presynaptic cAMP/PKA signaling pathway was abolished in RIM1α knockout mice (Lachamp *et al*., 2009). However, in the VTA we found that RIM1α is not necessary for induction or maintenance of $LTP_{GABA}$. Although activation of PKA and PKG both potentiate GABAergic IPSCs (Melis *et al*., 2002; Nugent *et al*., 2009), our results indicate the kinases do not converge upon RIM1α as a common cellular mechanism to increase GABA transmission. Further investigation is needed to determine the downstream molecular targets of NO-sGC-cGMP signaling in VTA

dopamine neurons and potential role of these signaling components in the brain reward circuit.

## Comparison of the temporal effects of drugs on excitatory and inhibitory synapses

At excitatory synapses, *in vivo* cocaine or amphetamine enhanced the AMPA/NMDA ratio in brain slices prepared 2 hours after exposure and this increase was of similar magnitude to that observed 24 hours after cocaine (Faleiro *et al*., 2004; Argilli *et al*., 2008). Nicotine and morphine both enhanced the AMPA/NMDA ratio at 24 hours, and other time points have not yet been examined. It is intriguing that psychostimulants, morphine and nicotine all potentiate glutamatergic synapses 24 hours later (Saal *et al*., 2003), while the effects of nicotine and cocaine on inhibitory synapses appear strongly dependent on the temporal window examined. An intriguing question that remains unresolved is whether repeated drug administration would prolong the impairment of $LTP_{GABA}$. At excitatory synapses onto VTA dopamine neurons, daily cocaine administration over a period of 7 days still caused potentiation, but the magnitude of the increase was similar to that of a single injection of cocaine (Borgland *et al*., 2004). Furthermore, the increased AMPA/NMDA ratio induced by repeated cocaine administration persisted for at least 5 days following the final cocaine injection, but not 10 days, (a time course seen after a single cocaine administration (Ungless *et al*., 2001), suggesting that repeated injections enhanced neither the extent nor the duration of the potentiation (Borgland *et al*., 2004). A more recent study found that even an escalating dose of cocaine over 14 days does not further elevate the AMPA/NMDA ratio (Chen *et al*., 2008). These findings suggest that a single injection of cocaine saturates plasticity at excitatory synapses and repeated drug administration has no additive effect on AMPA/NMDA ratio (Chen *et al*., 2008; Mameli *et al*., 2009). If our results parallel those found at excitatory synapses then $LTP_{GABA}$ may remain impaired following chronic drug administration. Alternatively, it is possible that the transient impairment of $LTP_{GABA}$ may be unaffected by repeated drug administration, perhaps due to a compensatory mechanism involved in restoring inhibitory synaptic plasticity following a single exposure to morphine (Fig. 1). At excitatory synapses in the VTA, induction of a form of LTD mediated by type 1 metabotropic glutamate receptors reverses cocaine-evoked elevation of the AMPA/NMDA ratio (Bellone & Luscher, 2006; Mameli *et al*., 2009). A parallel to this endogenous reversal of cocaine-induced potentiation might exist at inhibitory synapses to restore normal $LTP_{GABA}$ function.

It is appealing to speculate that addictive drugs might begin to seize control of reward circuits by simultaneously enhancing excitation and reducing inhibition in the local VTA circuit. Perhaps the robust neuroadaptations that occur in response to drugs of abuse and stress are related to the combined ability to alter both excitatory and inhibitory synapses. It is striking that even a single exposure to addictive drugs, or to a stressful stimulus, is sufficient to catapult the local VTA circuit into a different state that persists for several days. The effects must involve the majority of fast synapses in the VTA, since in the randomly sampled dopamine neurons in our studies and those of others, striking synaptic alterations are observed. One puzzling issue is how different drugs (and stressful stimuli) with entirely non-overlapping mechanisms of action converge to both potentiate excitatory synapses and inhibit $LTP_{GABA}$ over a period of hours after administration. This observation suggests that drugs or stress may act on a common substrate that in turn influences synaptic strength beginning within hours after the stimulus, and lasting as long as several days (Nestler, 2005). Several such intermediates have been suggested. As mentioned above, peptides such as CRF and orexins influence synaptic function in the VTA and interact with behavioral stress and with drugs (Bonci & Borgland, 2009). The involvement of transcription factors and growth factors (e.g. bFGF, BDNF, Δ FosB, and CREB) has also been proposed in recent years, and these potent regulators of cell physiology and structure might reasonably play the

role of intermediates (Forget *et al*., 2006; Russo *et al*., 2009). Our present findings strengthen the argument that a common mediator may exist and provide clues to the time course of activation of such a mediator, at least in the VTA. Moreover, if $LTP_{GABA}$ is controlled by multiple addictive drugs and stress, members of the $LTP_{GABA}$ signaling cascade may represent valuable targets for therapeutic intervention to treat drug addiction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| **ACSF** | artificial cerebrospinal fluid |
| **cGMP** | cyclic GMP |
| **IPSC** | inhibitory postsynaptic current |
| **LTP** | long-term potentiation |
| **nAChR** | nicotinic acetylcholine receptor |
| **NO** | nitric oxide |
| **PKG** | cGMP-dependent protein kinase |
| **sGC** | soluble guanylate cyclase |
| **SNAP** | S-nitroso-N-acetylpenicillamine |
| **VTA** | ventral tegmental area |

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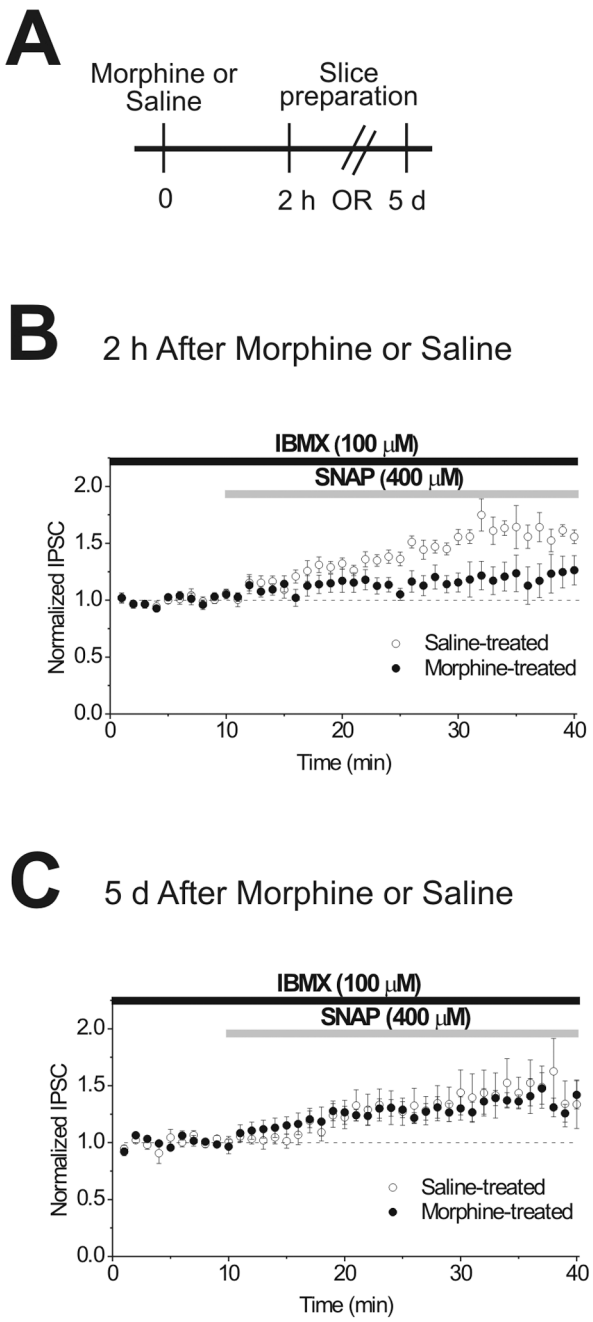


**Figure 1. $LTP_{GABA}$ is impaired by morphine 2 hours following injection, but recovers after 5 days**

***A***, Diagram of injection protocol. ***B***, Averaged experiments show that SNAP potentiates IPSCs in slices prepared from saline-treated animals 2 hours after injection ($n = 7$), but is impaired in morphine-treated animals ($n = 9$). We analyzed the effect of morphine on $LTP_{GABA}$ using two way ANOVA with the drug treatment (saline vs. morphine) as the between subjects factor and time (in minutes) as the within subjects factor. This analysis revealed significant effects of drug treatment ($F_{1,39} = 99.2$, $p < 0.001$), time ($F_{1,39} = 7.00$, $p < 0.001$) and an interaction between these two factors ($F_{1,39} = 0.11$, $p < 0.001$). ***C***, Averaged experiments show that SNAP potentiation of IPSCs is not significantly different in slices

prepared from saline ($n = 6$) or morphine-treated animals ($n = 8$) 5 days after a single injection of morphine. Statistical analysis revealed a significant effect of time ($F_{1,39} = 5.29$, $p < 0.001$), but no significant effects of drug treatment ($F_{1,39} = 0.63$, $p > 0.05$) or interaction between the two factors ($F_{1,39} = 0.37$, $p > 0.05$).

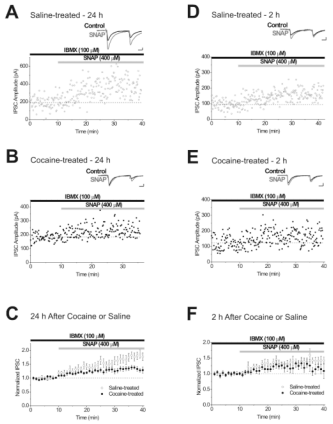


**Figure 2. A single *in vivo* administration of cocaine reduces $LTP_{GABA}$ 24 hours after exposure, but not at 2 hours**

***A***, Single experiment illustrating $LTP_{GABA}$ in a brain slice prepared 24 hours after saline-treatment. Insets, Representative IPSCs evoked before (Control) and 15 min after (SNAP) drug application. In this and all subsequent figures, ten IPSCs were averaged for illustration. ***B***, Single experiment illustrating impaired ability of SNAP to induce $LTP_{GABA}$ in a slice from an animal injected with cocaine 24 hours earlier. ***C***, At 24 hours after exposure, averaged experiments show SNAP potentiation of IPSCs in slices from cocaine-treated animals ($n = 10$) is reduced compared to those from saline-treated animals ($n = 10$). Two-way ANOVA analysis revealed significant effects of drug treatment ($F_{1,39} = 99.2$, $p < 0.001$), time ($F_{1,39} = 10.2$, $p < 0.001$) and an interaction between these two factors ($F_{1,39} = 1.85$, $p < 0.001$). ***D***, Single experiment illustrating $LTP_{GABA}$ in a brain slice prepared 2 hours after saline-treatment. ***E***, Single experiment illustrating $LTP_{GABA}$ in a slice from an animal injected with cocaine 2 hours earlier. ***F***, At 2 hours after exposure, averaged experiments show no significant difference in SNAP-induced potentiation of IPSCs in slices from cocaine-treated animals ($n = 9$) compared to saline-treated animals ($n = 7$). There were significant effects of drug treatment ($F_{1,39} = 24.8$, $p < 0.001$) and time ($F_{1,39} = 3.70$, $p < 0.001$), but not a significant interaction between these two factors ($F_{1,39} = 0.50$, $p > 0.05$). Scale bars: 10 ms, 50 pA.

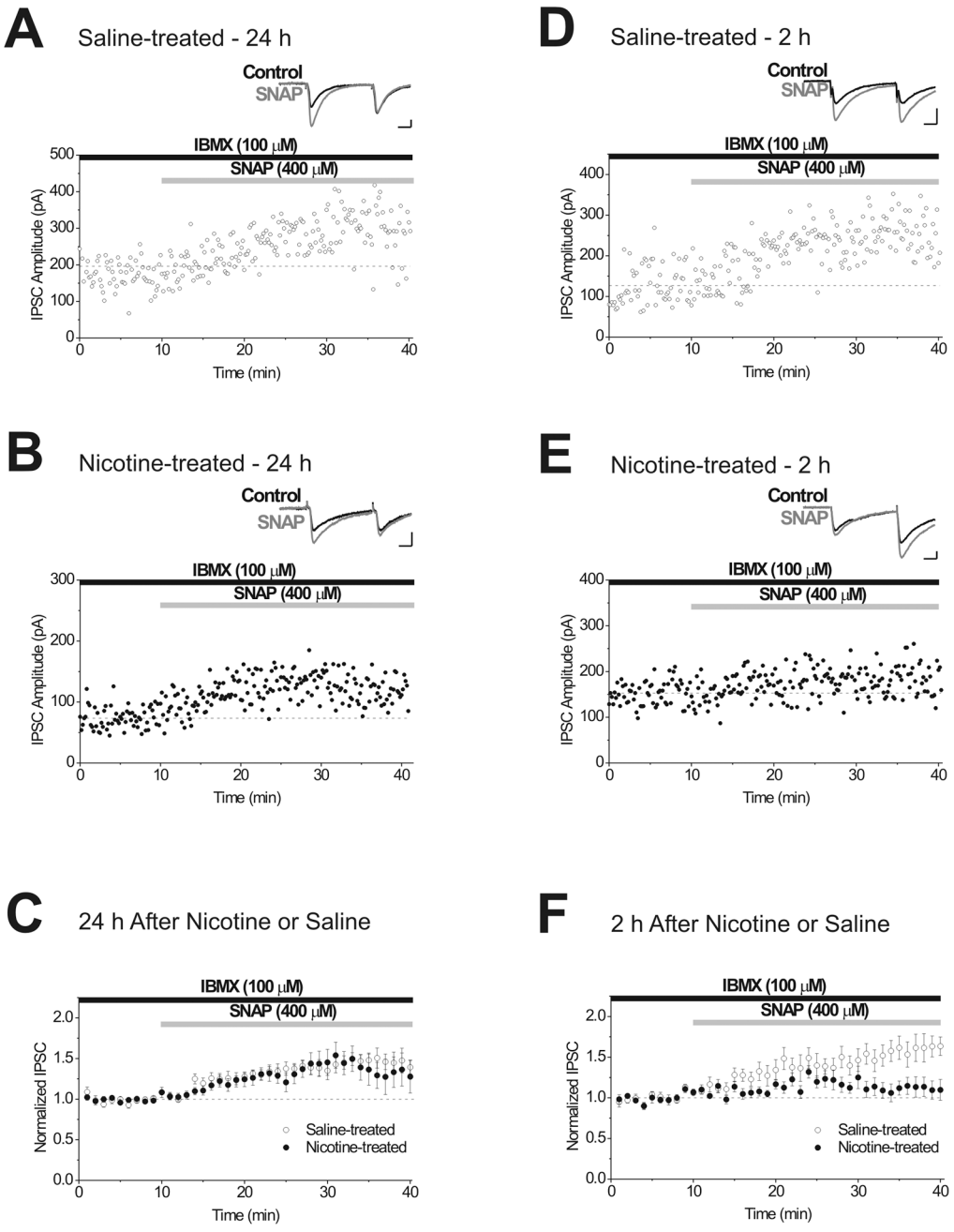


**Figure 3. A single *in vivo* administration of nicotine reduces $LTP_{GABA}$ 2 hours after exposure, but not at 24 hours**

**A**, Single experiment illustrating $LTP_{GABA}$ induced by bath application of SNAP onto a brain slice prepared 24 hours after saline-injection. Insets, Representative IPSCs evoked before (Control) and 15 min after (SNAP) drug application. ***B***, Single experiment illustrating that $LTP_{GABA}$ is unaffected in a slice prepared 24 hours after nicotine-injection. ***C***, At 24 hours after exposure, averaged experiments show no significant difference in SNAP-induced potentiation of IPSCs from saline ($n$ = 11) or nicotine-treated animals ($n$ = 13). Statistical analysis revealed a significant effect of time ($F_{1,39} = 8.44$, $p < 0.001$), but no significant effects of drug treatment ($F_{1,39} = 3.55$, $p > 0.05$) or interaction between the two factors

($F_{1,39} = 0.36$, $p > 0.05$). **D**, Single experiment illustrating $LTP_{GABA}$ induced by bath application of SNAP onto a brain slice prepared 2 hours after saline-injection. ***E***, Single experiment illustrating that $LTP_{GABA}$ is impaired 2 hours after nicotine-injection. ***F***, At 2 hours after exposure, averaged experiments show SNAP potentiation of IPSCs in slices from nicotine-treated animals ($n = 7$) is reduced compared to those from saline-treated animals ($n = 7$). There were significant effects of drug treatment ($F_{1,39} = 102.0$, $p < 0.001$), time ($F_{1,39} = 5.1$, $p < 0.001$) and an interaction between these two factors ($F_{1,39} = 1.99$, $p < 0.001$). Scale bars: 10 ms, 50 pA.

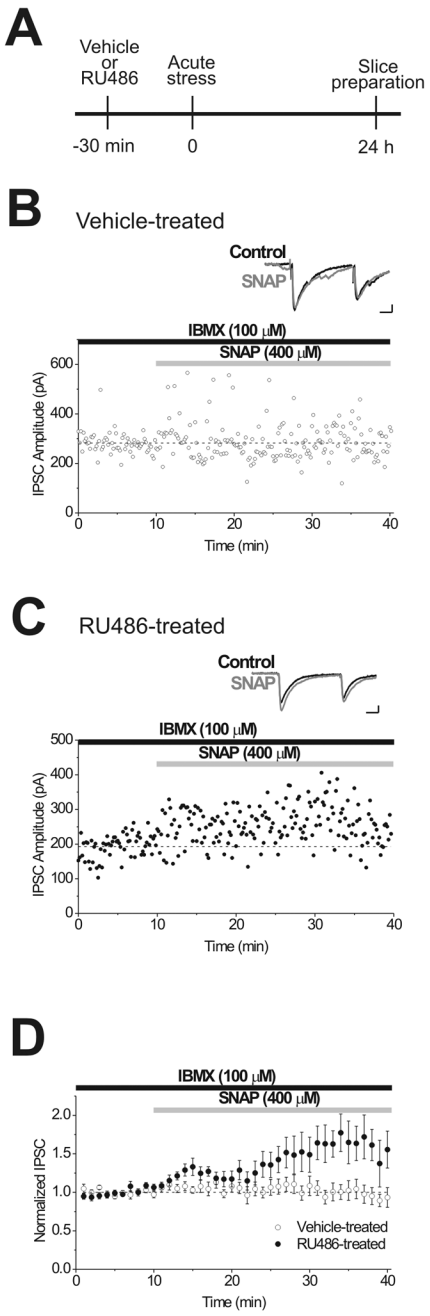


**Figure 4. Acute stress blocks $LTP_{GABA}$ via glucocorticoid receptors**

***A***, Diagram of experimental protocol for injection and acute stress. ***B***, Single experiment illustrating the inability of SNAP to induce $LTP_{GABA}$ in a slice prepared 24 hours after the animal was exposed to stress. Insets, Representative IPSCs evoked before (Control) and 15 min after (SNAP) drug application. ***C***, Single experiment illustrating the recovery of SNAP-induced $LTP_{GABA}$ in a slice from an animal treated with RU486 prior to stress. ***D***, Averaged experiments show that SNAP potentiation of IPSCs is abolished in slices from animals exposed to acute stress ($n$ = 8), whereas SNAP potentiates IPSCs in animals pretreated with RU486 ($n$ = 8). Statistical analysis revealed significant effects of drug treatment ($F_{1,39}$ =

119.0, $p < 0.001$), time ($F_{1,39} = 2.37$, $p < 0.001$) and an interaction between these two factors ($F_{1,39} = 2.38$, $p < 0.001$). Scale bars: 10 ms, 50 pA.

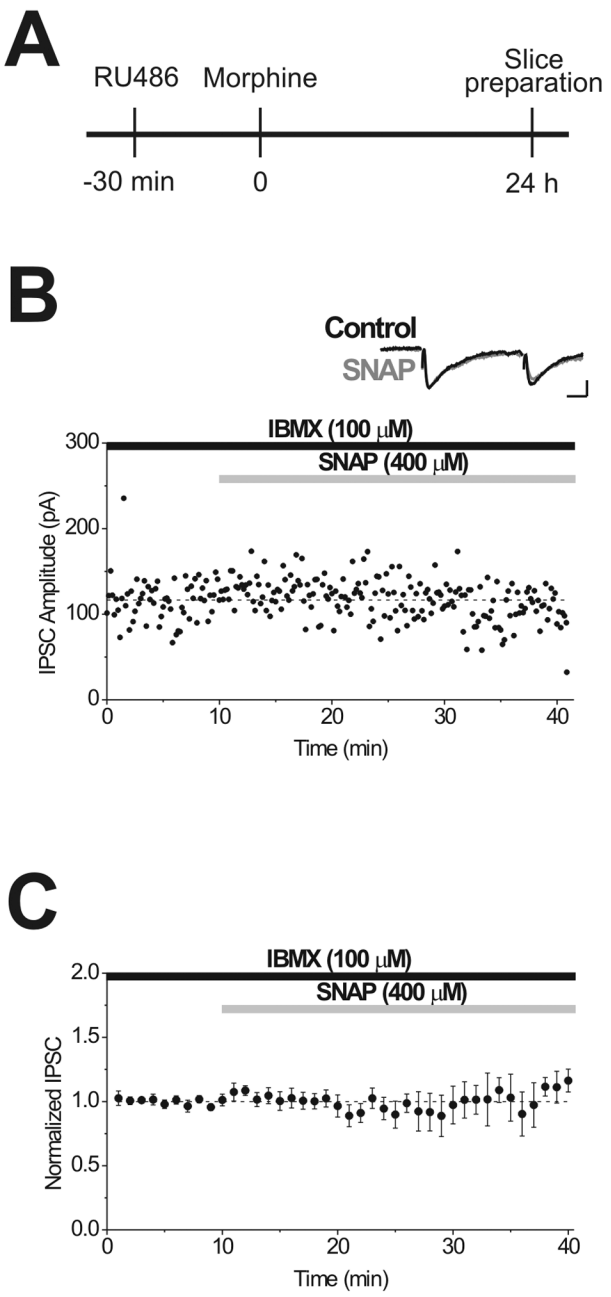


**Figure 5. Morphine-induced blockade of $LTP_{GABA}$ occurs independently of the stress pathway**

***A***, Diagram of injection protocol. ***B***, Single experiment illustrating the inability of SNAP to induce $LTP_{GABA}$ in a slice from an animal injected with RU486 30 min prior to morphine injection. Inset, Representative IPSCs evoked before (Control) and 15 min after (SNAP) drug application. ***C***, Averaged experiments show the inability of SNAP to potentiate IPSCs in slices from morphine-treated animals previously injected with RU486 ($n$ = 8). One-way ANOVA revealed no significant effect of SNAP over time ($p > 0.05$). Scale bars: 10 ms, 50 pA.

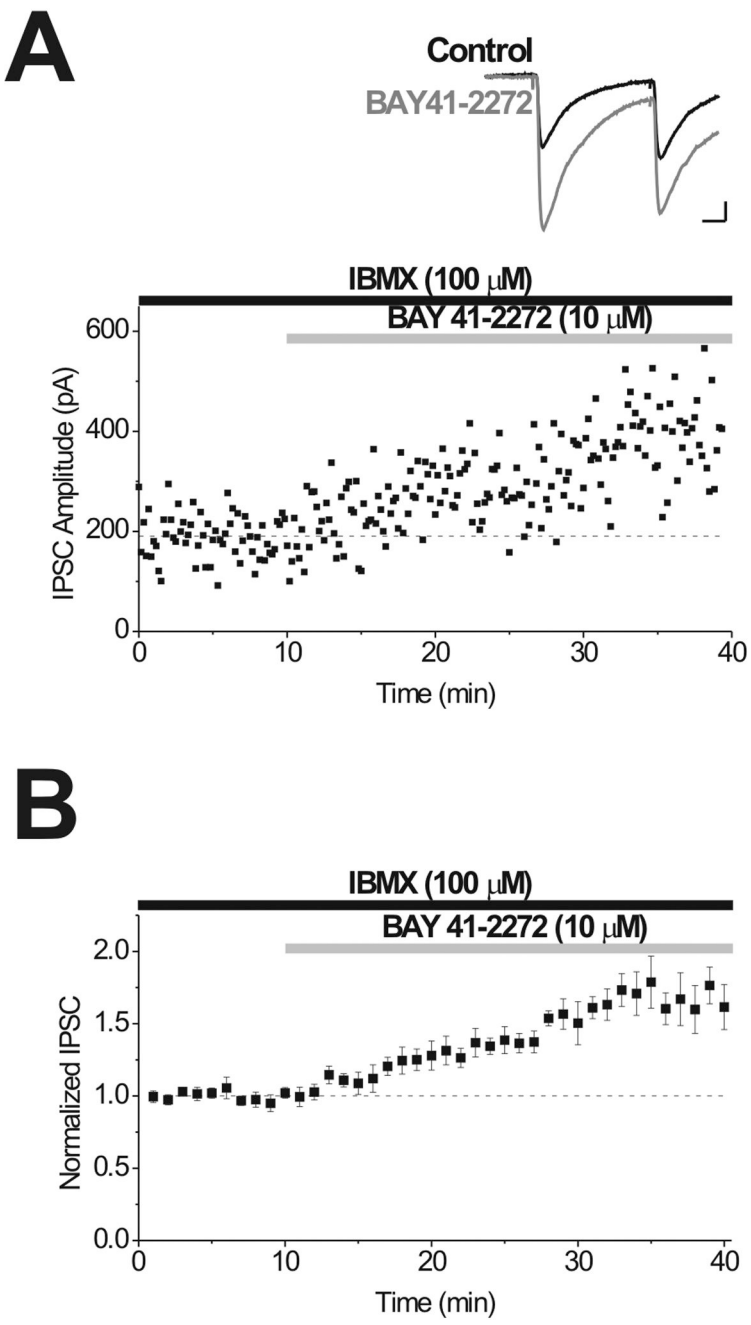


**Figure 6. Activation of sGC by BAY 41-2272 induces $LTP_{GABA}$ in slices from drug-naive animals**

***A***, Single experiment illustrating potentiation of IPSCs by application of 10 μM BAY 41-2272 to induce $LTP_{GABA}$. Inset, Representative IPSCs evoked before (Control) and 15 min after (BAY 41-2272) drug application. ***B***, Averaged experiments showing induction of $LTP_{GABA}$ by BAY 41-2272 ($n = 8$). One-way ANOVA revealed a significant effect of SNAP over time ($p < 0.001$). Scale bars: 10 ms, 50 pA.

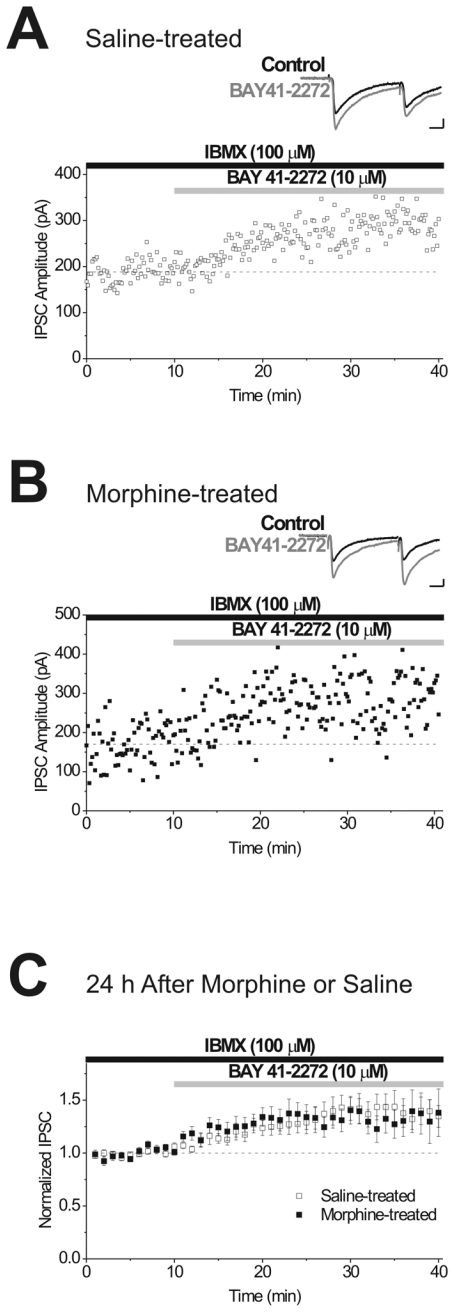


**Figure 7. Activation of sGC overcomes morphine-induced blockade of $LTP_{GABA}$ 24 hours after drug exposure**

***A***, Single experiment illustrating $LTP_{GABA}$ induced by application of 10 μM BAY 41-2272 onto a brain slice from a saline-treated animal. Insets, Representative IPSCs evoked before (Control) and 15 min after (BAY 41-2272) drug application. ***B***, Single experiment illustrating the ability of BAY 41-2272 to induce $LTP_{GABA}$ in a slice from an animal injected with morphine. ***C***, Averaged experiments show BAY 41-2272 potentiation of IPSCs is not significantly different in slices from saline (n = 12) or morphine-treated animals (n = 13). The statistical analysis revealed a significant effect of time ($F_{1,39} = 5.07$, $p < 0.001$), but

no significant effects of drug treatment ($F_{1,39} = 0.04$, $p > 0.05$) or interaction between the two factors ($F_{1,39} = 0.39$, $p > 0.05$). Scale bars: 10 ms, 50 pA.

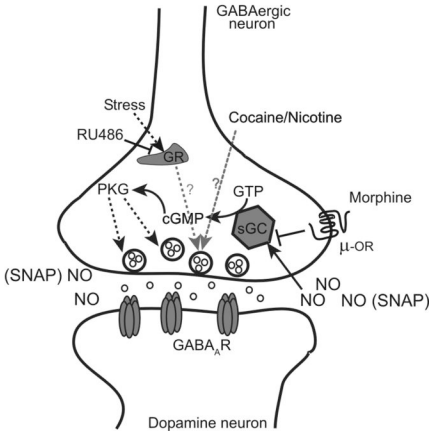


**Figure 8. Proposed model of $LTP_{GABA}$ signaling cascade**

Drugs of abuse and stress alter presynaptic GABA release onto VTA dopamine neurons. *In vivo* injection of morphine and nicotine impairs $LTP_{GABA}$ at 2 hours after drug administration, while cocaine does not. $LTP_{GABA}$ is impaired at 24 hours following morphine and cocaine injection, but not after nicotine. $LTP_{GABA}$ appears to be normal 5 days after morphine exposure. Acute stress potently blocked $LTP_{GABA}$ at the 24 hour time point. The relevant glucocorticoid receptors may be located in the presynaptic neuron as drawn, but may also be located in the postsynaptic dopamine neuron.