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Methamphetamine-Evoked Depression of $GABA_B$ Receptor Signaling in GABA Neurons of the VTA

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SUMMARY

Psychostimulants induce neuroadaptations in excitatory and fast inhibitory transmission in the ventral tegmental area (VTA). Mechanisms underlying drug-evoked synaptic plasticity of slow inhibitory transmission mediated by $GABA_B$ receptors and G protein-gated inwardly rectifying potassium (GIRK/Kir_3) channels, however, are poorly understood. Here, we show that 1 day after methamphetamine (METH) or cocaine exposure both synaptically evoked and baclofen-activated $GABA_BR$-GIRK currents were significantly depressed in VTA GABA neurons and remained depressed for 7 days. Presynaptic inhibition mediated by $GABA_BRs$ on GABA terminals was also weakened. Quantitative immunoelectron microscopy revealed internalization of $GABA_{B1}$ and GIRK2, which occurred coincident with dephosphorylation of serine 783 (S783) in $GABA_{B2}$, a site implicated in regulating $GABA_BR$ surface expression. Inhibition of protein phosphatases recovered $GABA_BR$-GIRK currents in VTA GABA neurons of METH-injected mice. This psychostimulant-evoked impairment in $GABA_BR$ signaling removes an intrinsic brake on GABA neuron spiking, which may augment GABA transmission in the mesocorticolimbic system.

INTRODUCTION

Changes in the motivation for drugs and natural rewards are central to the development of addiction (Koob and Volkow, 2010). The mesocorticolimbic dopamine (DA) system is the major brain reward circuit involved in translating motivations into goal-directed behaviors. Within this system, natural rewards increase activity of the ventral tegmental area (VTA) DA neurons, which primarily project to the nucleus accumbens (NAc), amygdala, and medial prefrontal cortex (mPFC). Addictive drugs converge on the mesocorticolimbic DA system, however, producing long-lasting changes in DA levels and excitability of DA neurons (Koob and Volkow, 2010; Lüscher and Malenka, 2011). One of the key pathways for controlling DA neuron excitability is through activation of a slow GABA-dependent inhibitory current, mediated by $GABA_B$ receptors ($GABA_BRs$) and G protein-gated inwardly rectifying potassium (GIRK/Kir_3) channels (Johnson and North, 1992; Cruz et al., 2004; Labouèbe et al., 2007) and through an auto-inhibitory pathway mediated by D2 dopamine receptors (D2Rs) and GIRK channels (Johnson and North, 1992; Beckstead et al., 2004). In vivo exposure to psychostimulants leads to reduced sensitivity of D2 autoreceptors and increased DA neuron excitability (White and Wang, 1984; Henry et al., 1989; White, 1996), implicating GIRK channels in the response to addictive drugs (Lüscher and Slesinger, 2010). Consistent with this, mice lacking GIRK channels self-administer less cocaine (Morgan et al., 2003) and show reduced withdrawal after chronic exposure to morphine (Cruz et al., 2008). Furthermore, *Girk2* transcripts in the mesocorticolimbic dopamine pathway are increased in some human cocaine addicts (Lehrmann et al., 2003). Although GIRK channels are implicated in the response to addictive drugs, the cellular mechanisms underlying drug-evoked changes in GIRK signaling are not well understood.

Accumulating evidence suggests that acquisition of addictive behaviors is learned and, similar to other learning and memory models, involves persistent changes in synaptic strength within the reward circuit and changes in DA neuron signaling (Koob and Volkow, 2010; Lüscher and Malenka, 2011). Early

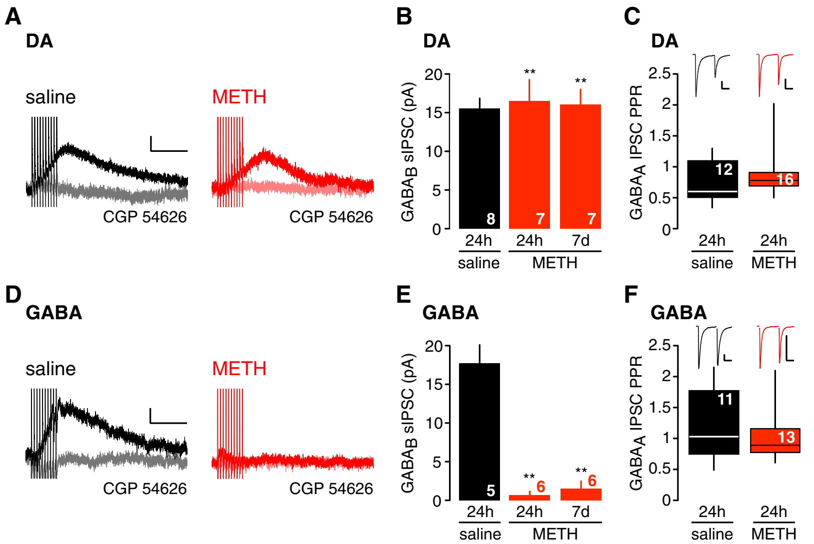

Figure 1. Absence of Slow Inhibitory Postsynaptic Currents in VTA GABA Neurons 24 Hr and 7 Days following In Vivo METH Exposure

The slow inhibitory postsynaptic current (sIPSC) recorded from DA (A) and GABA (D) neurons in the VTA 24 hr following a saline (0.9%) or METH (2 mg/kg) injection. The $GABA_B$ receptor antagonist CGP 54626 (2 μM) inhibited the sIPSC (light gray or light red trace). The $GABA_BR$-sIPSC is reduced in the VTA GABA neuron 24 hr following METH injection. Scale bars: 5 pA, 200 ms.

(B and E) Bar graphs show mean amplitudes for sIPSC following saline or METH (24 hr and 7 days later) in DA (B) (DA saline: 15.8 ± 1.5 pA; DA METH: 16.5 ± 2.8 pA; DA 7 days METH: 16.1 ± 2.0 pA) and GABA (E) neurons (GABA saline: 17.8 ± 2.6 pA; GABA METH: 0.7 ± 0.5 pA; GABA 7 days METH: 1.5 ± 1.0 pA). The sIPSC is significantly depressed 24 hr and 7 days following a single injection of METH in GABA neurons (**$p < 0.05$ [one-way ANOVA]).

(C and F) Box plots show $GABA_A$ receptor-mediated IPSC paired-pulse ratio (PPR) plotted for DA (C) and GABA (F) neurons in saline and METH -injected mice (DA saline: 0.73 ± 0.10 pA; DA METH: 0.89 ± 0.09 pA; GABA saline: 1.20 ± 0.17 pA; GABA METH: 1.02 ± 0.11 pA (ns, $p > 0.05$ [Mann-Whitney test]). Line shows mean. Insets show representative traces for each condition.

Scale bars: 100 pA, 20 ms. N (number of recordings) indicated on all graphs. All bar graphs show mean ± SEM.

drug-evoked neuroadaptations are thought to occur within the VTA and are critical for remodeling the reward circuit and facilitating the development of addiction. Lesion of VTA DA neurons blocks drug-dependent addictive behaviors (Roberts and Koob, 1982). Neuro-adaptations that occur 24 hr following exposure to addictive drugs in vivo have been described. Systemic injection of a psychostimulant strengthens excitatory synapses in the VTA (White et al., 1995; Zhang et al., 1997; Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008) through recruitment of GluA2-lacking AMPA receptors to the synapses (Bellone and Lüscher, 2006; Argilli et al., 2008). Neuro-adaptations in fast GABA transmission have also been reported; fast inhibitory currents mediated by $GABA_A$ receptors are impaired 24 hr after a single injection of morphine (Nugent et al., 2007), and the amplitudes of GABA-mediated synaptic currents are reduced in mice receiving several injections of cocaine (Liu et al., 2005). Chronic amphetamine enhances $GABA_B$ receptor transmission in the VTA during early withdrawal, but the cellular mechanism underlying this change is unknown (Giorgetti et al., 2002). Following chronic cocaine or morphine treatment, D1R stimulation decreases $GABA_B$-GIRK currents in DA neurons, but this occurs from a change in presynaptic GABA release (Bonci and Williams, 1996). In this study, we sought to characterize the early modulation of $GABA_B$ signaling by a single exposure to psychostimulants. We discovered that ~24 hr following intraperitoneal (i.p.) injection of methamphetamine (METH) or cocaine, $GABA_B$ receptor signaling in VTA GABA neurons is strongly and persistently impaired. This drug-evoked depression of $GABA_BR$-GIRK signaling involves dephosphorylation of the $GABA_B$ receptor and changes in $GABA_BR$ and GIRK channel trafficking. As a consequence, VTA GABA neuron firing is not affected by the $GABA_BR$ agonist baclofen, suggesting GABAergic function may be augmented in the VTA with psychostimulants.

RESULTS

Psychostimulant-Evoked Plasticity in $GABA_BR$ Signaling in VTA

A single injection of psychostimulants enhances glutamatergic synaptic efficacy in the VTA 24 hr later (Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008). We examined whether a single injection of psychostimulant also alters $GABA_BR$-GIRK signaling in the VTA. To test this, we injected C57BL/6 mice with methamphetamine (METH) at 2 mg/kg, a dose that elicits locomotor sensitization when administered repeatedly (Shimosato et al., 2001; Fukushima et al., 2007; Scibelli et al., 2011) and examined $GABA_BR$-GIRK signaling in the VTA 24 hr later. We first investigated the synaptically activated $GABA_BR$-GIRKs, commonly referred to as the slow inhibitory postsynaptic current (sIPSC), in acutely prepared VTA slices. High frequency (66 Hz) stimulation of GABA afferents induces a spillover of GABA that diffuses to perisynaptic $GABA_B$ receptors and elicits a slow outward K^+ current (sIPSC; Figure 1). The $GABA_B$ receptor antagonist, CGP 54626 (2 μM), inhibited the evoked current, confirming the identity of the $GABA_B$ sIPSC (Figure 1), similar to previous studies (Johnson and North, 1992; Bonci and Williams, 1996). In DA neurons, the $GABA_B$ sIPSC did not significantly change 24 hr following METH, compared to saline injection (Figures 1A and 1B). By contrast, the sIPSC was significantly smaller in GABA neurons (Figures 1D and 1E). Moreover, the sIPSC in GABA neurons remained depressed for at least 7 days (Figure 1E). Examination of the paired-pulse ratio for the fast $GABA_A$-mediated IPSC revealed no difference in either DA or GABA neurons (Figures 1C and 1F), suggesting that the depression of the sIPSC in GABA neurons was not due to the inability of GABA terminals to release GABA.

To investigate the effects of METH on synaptic and extrasynaptic $GABA_B$ receptors, the $GABA_B$ receptor agonist baclofen was applied to the bath. As described previously (Labouèbe et al., 2007), saturating doses of baclofen (300 μM for DA and 100 μM for GABA) elicited large and desensitizing $GABA_BR$-activated GIRK currents in DA neurons and small nondesensitizing currents in GABA neurons (Figure 2). All baclofen-activated currents were inhibited with the inwardly rectifying K_+ channel inhibitor Ba^{2+} or the $GABA_B$ receptor antagonist (CGP 54626, not shown). In contrast to the sIPSC recordings, there was an ~40% decrease in the $GABA_BR$-GIRK currents of DA neurons 24 hr following a METH injection (Figures 2A and 2B). However, this decrease in current was not apparent at 7 days following METH injection (Figure 2B). By contrast, the baclofen-activated GIRK ($I_{Baclofen}$) currents in GABA neurons were significantly depressed by ~55% 24 hr following a single METH injection and the reduced $I_{Baclofen}$ persisted for 7 days (Figures 2C and 2D). We next examined whether METH altered $GABA_BR$-GIRK signaling in other brain regions. There was no significant change in the sIPSC or $I_{Baclofen}$ in CA1 hippocampal pyramidal or GABAergic neurons 24 hr following METH (Figure S1 available online). We also measured the sIPSC and $I_{Baclofen}$ in pyramidal and GABAergic neurons of the prelimbic cortex, a target region of VTA DA cells, and observed no significant changes in $GABA_B$-GIRK currents in METH-injected mice (Figure S1). Thus, a single exposure to METH triggered a profound and long-lasting depression in both the sIPSC and $I_{Baclofen}$ in GABA neurons of the VTA.

In addition to postsynaptic $GABA_B$ receptors, presynaptic $GABA_B$ receptors are also involved in reducing GABA release, typically through inhibition of voltage-gated Ca^{2+} channels (Padgett and Slesinger, 2010). To investigate whether in vivo exposure to METH altered $GABA_B$ receptor-dependent presynaptic inhibition, we used an optogenetic strategy to selectively stimulate GABA neurons in the VTA and measure the effect of baclofen on a light-evoked fast inhibitory postsynaptic current (IPSC) recorded in DA neurons (Figure 3). AAV virus expressing a double floxed-stopped channel rhodopsin 2 (ChR2)-eYFP was stereotaxically injected into the VTA of mice expressing Cre recombinase in GABA neurons (GAD65-Cre; Figure 3A; Figure S2). After 21 days, neurons expressing ChR2-eYFP were evident in horizontal slices of the VTA (Figure S2A). Prolonged blue light stimulation (400 ms) elicited tetrodotoxin (TTX)-insensitive photocurrents in GABA neurons, whereas short light pulses (4 ms) evoked picrotoxin- and TTX-sensitive fast IPSCs in DA neurons (Figures S2B and 2SC; Figure 3B). Bath application of baclofen (1 μM) depressed the light-evoked IPSC by ~50% in saline-injected mice. By contrast, baclofen (1 μM) decreased the light-evoked IPSC by only ~20% in METH-injected mice (Figures 3B and 3C). Construction of dose-response curves revealed that $GABA_B$ receptor-dependent inhibition of presynaptic release was shifted significantly to higher agonist concentrations (Figure 3C), reflected by an increase in the IC_{50}, which is the concentration of Baclofen needed to inhibit 50% of the light-induced current (Figure 3D). Similar to the change in postsynaptic $GABA_BR$-GIRK signaling, the reduced sensitivity of presynaptic $GABA_BRs$ persisted for 7 days (Figures 3C and 3D). As a control, we examined $GABA_BR$-dependent presynaptic inhibition of glutamate release onto DA neurons by measuring the amplitude of electrically evoked AMPA EPSC, in the presence of increasing concentrations of baclofen (Figure S3). We found no significant change in the IC_{50} in METH-injected mice, compared to saline controls. Taken together, these results demonstrate that a single in vivo injection of METH triggers a depression in $GABA_B$ receptor signaling in VTA GABA neurons, both presynaptically (inhibition of GABA release) and postsynaptically (activation of GIRK channels).

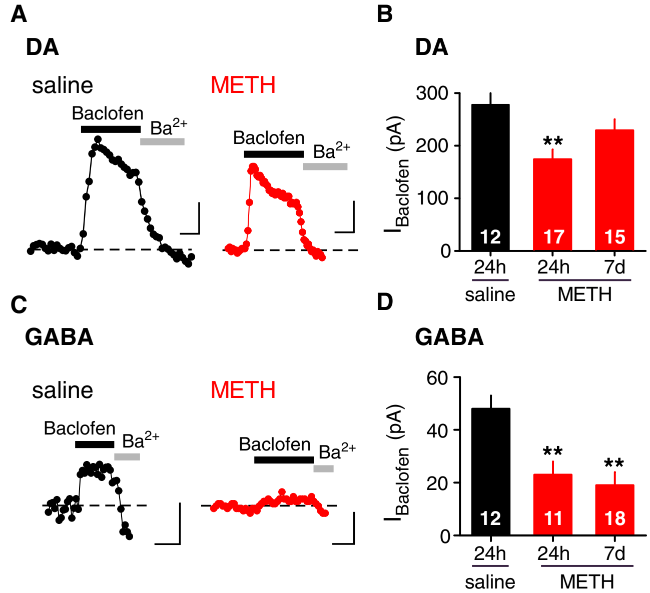

Figure 2. Reduced $GABA_BR$-GIRK Currents in VTA GABA Neurons 24 Hr and 7 Days following In Vivo METH Exposure

The baclofen-activated GIRK currents ($I_{Baclofen}$) recorded from VTA DA (A) and GABA (C) neurons 24 hr following a saline (0.9%) or METH (2 mg/kg) injection. Outward currents recorded at −50 mV are plotted as a function of time. $I_{Baclofen}$ is blocked by the inward rectifier inhibitor Ba^{2+} (1 mM) or by the $GABA_BR$ antagonist CGP 54626 (data not shown).

Scale bars: 100pA (A) 50 pA (C), 100 s.

(B) Bar graph shows average $I_{Baclofen}$ in DA neurons 24 hr following saline (DA saline: 278 ± 37 pA) or 24 hr and 7 days following METH injection (DA METH: 174 ± 19; DA 7 days METH: 229 ± 21 pA).

(D) Bar graph shows average $I_{Baclofen}$ in GABA neurons 24 hr following saline injection (GABA saline: 48.4 ± 5.3 pA) or 24 hr and 7 days following METH injection (GABA METH: 22.9 ± 4.7; GABA 7 days METH: 19.4 ± 4.5 pA). Note significant decrease in $I_{Baclofen}$ in GABA neurons of METH-injected mice that persists for 7 days (**$p < 0.05$ [one-way ANOVA]).

Psychostimulant-Evoked Plasticity in $GABA_BR$ Involves Dopamine

Cocaine is another psychostimulant that rapidly elevates DA levels within minutes after the injection. In contrast to METH, which is taken up by DA neurons and stimulates reverse transport of DA through the dopamine transporter (DAT), cocaine inhibits DAT from the extracellular side (Sulzer, 2011). We examined whether a single injection of cocaine would evoke a change in $GABA_BR$-GIRK signaling. Like METH, cocaine (15 mg/kg) produced a significant decrease in the sIPSC in GABA neurons but not in DA neurons 24 hr later (Figures 4A–4D). Similarly, $I_{Baclofen}$ was depressed in GABA neurons but not in DA neurons (Figures 4E–4H). Thus, both cocaine and

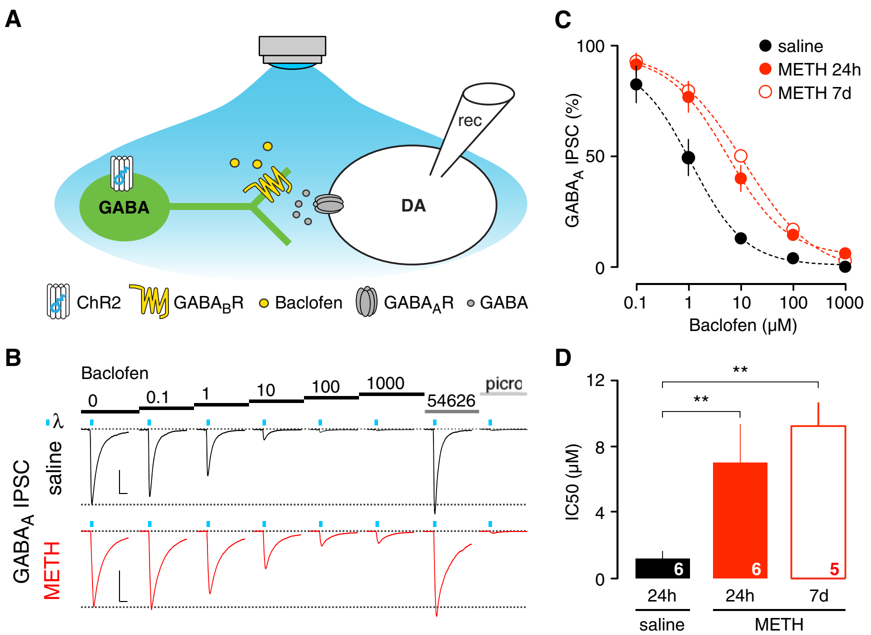

Figure 3. Reduced Sensitivity of Presynaptic $GABA_B$ Receptor-Mediated Inhibition 24 hr and 7 days following In Vivo METH Exposure

(A) Schematic shows channel rhodopsin 2 (ChR2) protein expressed selectively in VTA GABA neurons of GAD65-Cre mice. GABA neuron activity is induced by blue light, resulting in a fast $GABA_A$ receptor-mediated IPSCs recorded from VTA DA neuron. Baclofen impairs GABA release by acting on presynaptic $GABA_B$ receptors.

(B) Example traces of blue light-evoked IPSCs recorded 24 hr following saline or METH injection in presence of increasing concentrations of baclofen (μM). Blue ticks indicate light stimulation (two 4 ms pulses). Basal IPSC amplitude recovers after application of CGP 54626 (2 μM) and is subsequently blocked by picrotoxin (100 μM). Scale bars: 200 pA, 10 ms.

(C) Dose response curves show reduced sensitivity for baclofen-dependent inhibition of fast IPSCs in METH injected mice after 24 hr and 7 days.

(D) Bar graph plots IC_{50} for indicated conditions (saline: 1.2 ± 0.4 μM; 24 hr METH: 7.1 ± 2.4 μM; 7 day METH: 9.4 ± 1.4 μM (**$p < 0.05$ [one-way ANOVA]).

METH trigger a similar neuroadaptation in $GABA_BR$-GIRK signaling in GABA neurons of the VTA, suggesting that elevated DA may be an important step in inducing the $GABA_BR$-GIRK plasticity.

Dopamine stimulates two classes of DA receptors, D1- and D2-like receptors, in the brain (White, 1996). D1-like receptor antagonists block sensitization to psychostimulants (Kalivas and Stewart, 1991), reduce self-administration of cocaine (Caine et al., 2007), and prevent psychostimulant-induced changes in glutamatergic signaling in the VTA (Argilli et al., 2008; Brown et al., 2010). To test the requirement for DA receptors, we injected an antagonist for D1-like (SCH39166) or D2-like (Eticlopride) receptors with METH (Figure 5). In contrast to injection of METH alone, the sIPSC recorded from mice injected with METH and SCH39166 (0.3 mg/kg) was not significantly different from saline (Figures 5A and 5B). By contrast, co-injection of Eticlopride (0.1 mg/kg) with METH did not attenuate the METH-dependent decrease in sIPSC. For macroscopic $GABA_BR$-GIRK currents, co-injection of METH and SCH39166 also partially blocked the METH-dependent decrease in $I_{Baclofen}$ (Figures 5C and 5D). Interestingly, co-injection of Eticlopride with METH attenuated the METH-dependent decrease in $I_{Baclofen}$ (Figures 5C and 5D), in contrast to the effect of Eticlopride on the sIPSC. This could reflect a difference in synaptically and extrasynaptically activated $GABA_B$ receptors. For cocaine, co-injection of METH or cocaine with both SCH39166 and Eticlopride partially recovered the sIPSC, compared to saline-injected controls (Figures 5B and 5D). Together, these pharmacological experiments clearly implicate DA and the D1-like receptor in mediating psychostimulant-dependent depression in $GABA_BR$-GIRK signaling in VTA GABA neurons, similar to the plasticity changes in excitatory synapses in VTA DA neurons following cocaine (Argilli et al., 2008).

Cellular Mechanism Underlying Depression of $GABA_BR$-GIRK Signaling

A reduction in the amplitude of $GABA_B$-GIRK currents could involve a change in G protein coupling (Nestler et al., 1990; Labouèbe et al., 2007), desensitization of $GABA_B$ receptors (Taniyama et al., 1991; González-Maeso et al., 2003), and/or internalization of the receptor channel (Fairfax et al., 2004; Guetg et al., 2010; Maier et al., 2010; Terunuma et al., 2010). To investigate the latter possibility, we used quantitative immuno-electron microscopy to study the subcellular distribution of $GABA_B$ receptors and GIRK channels in saline- and METH-injected mice. In serial ultrathin sections through the VTA, GABA neurons were identified using antibodies against GAD65/67 and secondary antibodies coupled to horseradish peroxidase (HRP), generating a dark reaction product in GABA neurons (Figures 6A and 6B). VTA sections were also labeled with immunogold particles using specific antibodies for $GABA_{B1}$ or GIRK2 (Kulik et al., 2003; Koyrakh et al., 2005). In single ultrathin sections (Figures 6A and 6B), both $GABA_{B1}$ and GIRK2 were expressed predominantly at the plasma membrane of GABA neuron dendrites (Den; arrows) following saline injection. By contrast, 24 hr following a METH injection, there was a reduction in plasma membrane associated $GABA_{B1}$ and an increase at intracellular sites (Figure 6A, crossed arrows). Similarly, there was a reduction in GIRK2 protein on the plasma membrane and an increase in intracellular compartments in GABA neurons following METH treatment (Figure 6B). To quantify these changes, we generated a three-dimensional reconstruction of the dendrite using the serial ultrathin sections (Figures 6C and 6D) and then counted gold particles on the plasma membrane and in the cytoplasm. Calculating the surface and intracellular densities (Figures 6E and 6F) revealed that 24 hr following METH injection there was a significant reduction (∼60%–70%) in plasma membrane-associated

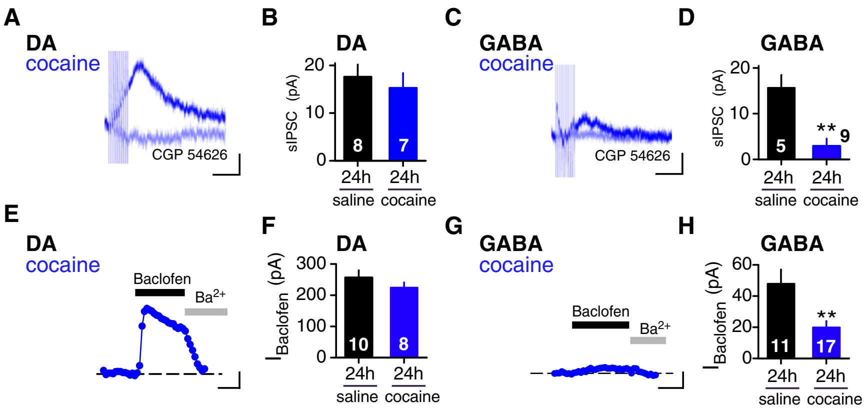

Figure 4. Depression of $GABA_BR$-GIRK Signaling in VTA GABA Neurons 24 hr following a Single Cocaine Injection

(A–D) The sIPSC is reduced in VTA GABA (C) but not in DA (A) neurons 24 hr following a single cocaine (15 mg/kg) injection. Scale bars: 5 pA, 200 ms. Only recordings from cocaine-injected mice are shown. Light blue trace shows sIPSC recorded with CGP 54626 (2 μM).

(B and D) Bar graphs show mean amplitudes for sIPSC 24 hr following saline or cocaine (DA saline: 17.6 ± 2.6 pA; DA cocaine: 15.3 ± 3.1 pA; GABA saline: 15.7 ± 2.8 pA; GABA cocaine: 2.9 ± 1.6 pA).

(E–H) The baclofen-induced GIRK current ($I_{Baclofen}$) is reduced in VTA GABA (G) but not DA (E) neurons 24 hr following cocaine (15 mg/kg) injection. Traces show current recorded at −50 mV with baclofen (100 μM) or Ba^{2+} (1 mM). Scale bars: 50 pA, 100 s.

(F) Bar graph shows average $I_{Baclofen}$ in DA neurons 24 hr following saline or cocaine injection. (DA saline: 269 ± 27; DA cocaine: 225 ± 16 pA).

(H) Bar graph shows average $I_{Baclofen}$ in GABA neurons 24 hr following saline or cocaine injection (GABA saline: 47.7 ± 8.9; GABA cocaine: 20.4 ± 3.8 pA). **$p < 0.05$ (Student's t test).

$GABA_{B1}$ and GIRK2, with a concomitant increase in the intracellular-associated $GABA_{B1}$ and GIRK2 (~50%–65%). By contrast, we did not observe a significant change in immunogold particle labeling of plasma membrane staining for GIRK2 and $GABA_{B1}$ in GAD65/67-negative neurons (GIRK2: 0.924 ± 0.032 particles/μm^2 saline versus 0.843 ± 0.054 METH, n = 21 and $GABA_{B1:}$ 1.042 ± 0.043 saline versus 0.922 ± 0.050 $GABA_{B1}$; $p > 0.05$). Interestingly, the reduction in plasma membrane-associated GIRK2 and $GABA_{B1}$ parallels the ~50% depression in baclofen-induced $GABA_BR$-GIRK currents (Figure 2F). Moreover, the relative decreases in $GABA_{B1}$ and GIRK2 protein on the plasma membrane are very similar, suggesting the $GABA_B$ receptor and GIRK channel may internalize as a signaling complex from the plasma membrane (Boyer et al., 2009). Taken together, these data demonstrate that 24 hr after a single injection of METH, both $GABA_B$ receptor and GIRK channel protein levels are reduced on the plasma membrane of GABA neurons, providing a reasonable explanation for depressed $GABA_BR$-GIRK currents in those neurons.

The quantitative immunogold electron microscopy data suggested that METH treatment induced internalization of the receptor and channel. The phosphorylation status of the $GABA_B$ receptor is important for regulating surface expression of the receptor (Fairfax et al., 2004; Koya et al., 2009; Guetg et al., 2010; Terunuma et al., 2010). We therefore examined whether phosphorylation of the $GABA_B$ receptor could play a role in mediating the METH-dependent depression. We examined the phosphorylation of S783 (p-S783) in $GABA_{B2}$ because dephosphorylation is associated with reduced surface expression of $GABA_B$ receptors in neurons (Terunuma et al., 2010). Protein isolated from tissue punches of the VTA, NAc, hippocampus, or mPFC from saline- and METH-injected mice (24 hr) were examined using a phospho-specific antibody for phosphorylated S783 in $GABA_{B2}$ (Dobi et al., 2010). Remarkably, METH injection led to a ~25% reduction in phosphorylation of $GABA_{B2}$-S783 in the VTA (Figure 7A). This change in p-S783 compares to a METH-induced ~50% reduction in $I_{Baclofen}$ in GABA neurons (Figure 2D). However, the VTA tissue punches contain a mixture of cell types that express $GABA_B$ receptors, which likely account for the smaller change in $GABA_{B2}$-p-S783. By contrast, there was no change in $GABA_{B2}$-p-S783 in the NAc, mPFC, or hippocampus from METH -injected mice (Figures 7B–7D). Examination of p-S892, a different phosphorylation site on $GABA_{B2}$ (Fairfax et al., 2004), revealed no change in phospho-specific labeling of $GABA_{B2}$-p-S892 in METH-injected mice, indicating the effect of METH was unique to $GABA_{B2}$-S783 (Figure 7E). Lastly, there was no apparent change in the levels of $GABA_{B2}$ receptor protein (Figure 7F), suggesting little METH-dependent degradation of receptor.

Dephosphorylation of $GABA_{B2}$-p-S783 has been previously shown to be regulated by protein phosphatase 2A (PP2A; Terunuma et al., 2010), raising the possibility that in vivo exposure to METH enhances the phosphatase activity in VTA GABA neurons. To address this, we examined the effect of acutely inhibiting PP1/PP2A phosphatases with okadaic acid (OA; 100 nM). In saline-injected mice, there was no significant difference in the amplitude of $I_{Baclofen}$ with OA in the pipet, suggesting that basal activity of PP1/PP2A does not significantly regulate $GABA_BR$-GIRKs (Figures 7G–7J). In METH-injected mice, however, intracellular application of OA promoted recovery of the $I_{Baclofen}$ (Figures 7H and 7J). Note the slow time course of activation for $I_{Baclofen}$ in the presence of OA in METH-injected mice. This increase could reflect insertion of $GABA_B$ receptors and GIRK channels on the plasma membrane or restoration of functional G protein coupling. For control, we examined the effect of PKC(19-36), a peptide inhibitor of PKC (Figure 7K). Unlike OA, the presence of PKC inhibitor in the pipet did not restore $I_{Baclofen}$, similar to the effect of METH alone. Taken together, these findings suggest that in vivo exposure to METH triggers a phosphatase-dependent downregulation of $GABA_BRs$ and GIRK channels from the plasma membrane of GABA neurons, which results in reduced $GABA_BR$-GIRK signaling and accumulation of $GABA_B$ receptor complexes in intracellular compartments.

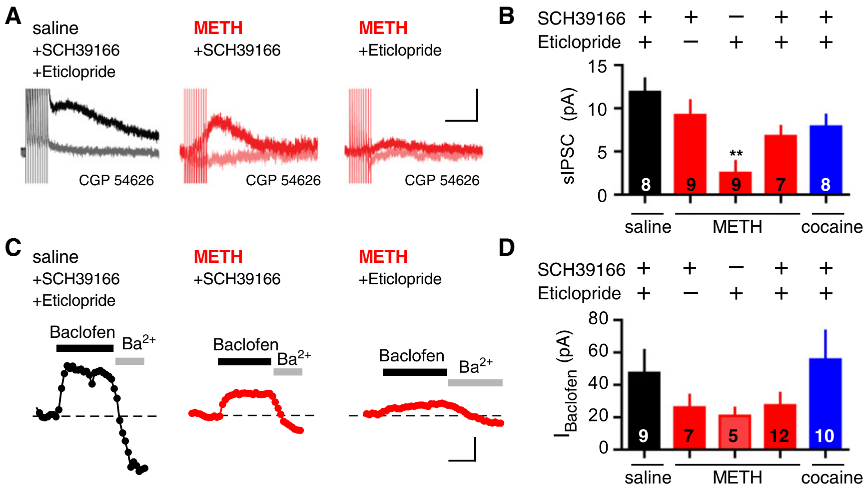

Figure 5. D1-like Receptor Antagonist Blocks METH-Induced Depression in $GABA_BR$-GIRK Signaling

(A) The sIPSC recorded from VTA GABA neurons in a VTA slice from mice co-injected with saline and D1-like receptor antagonist (SCH39166; 0.3 mg/kg)/D2-like receptor antagonist (Eticlopride; 0.1 mg/kg), METH and SCH39166, or METH and Eticlopride. The $GABA_B$ receptor antagonist CGP 54626 (2 μM) inhibited the sIPSC. Scale bar: 5 pA, 200 ms.

(B) Bar graph shows the average sIPSC in VTA GABA neurons (saline + SCH39166/Eticlopride: 11.9 ± 1.6pA; METH + SCH39166: 9.2 ± 1.7 pA; METH + Eticlopride: 2.5 ± 1.4 pA; cocaine + SCH39166/Eticlopride 6.8 ± 1.2 pA (**$p < 0.05$ versus saline [one-way ANOVA]).

(C) $I_{Baclofen}$ recorded from mice co-injected with saline and SCH39166/Eticlopride, METH and SCH39166, or METH and Eticlopride. Scale bar: 50 pA, 100 s.

(D) Bar graph shows the average $I_{Baclofen}$ (saline + SCH39166/Eticlopride: 44.4 ± 12.5 pA; METH + SCH39166: 27.3 ± 4.1 pA; METH + Eticlopride: 23.0 ± 9.7 pA; METH + SCH39166/Eticlopride: 27.3 ± 7.8 pA; cocaine + SCH39166/Eticlopride 55.7 ± 17.8 pA ($p > 0.05$, not significant from saline).

Loss of $GABA_BR$-Dependent Inhibition of VTA GABA Neuron Firing

To investigate the functional consequence of reduced $GABA_BR$-GIRK currents in GABA neurons of METH-injected mice, we examined the effect of baclofen on the induced firing rate of GABA neurons (Figure 8). We predicted that a loss of $GABA_BR$-GIRK signaling would attenuate $GABA_BR$-mediated suppression of firing in GABA neurons. To test this, a series of current steps (20–100 pA) were injected to elicit a train of action potentials in GABA neurons (Figures 8A and 8B). In saline- and METH-injected mice, the input-output (I-O) plot shows a linear increase in firing rate with larger current injections (Figures 8B and 8D). As expected, baclofen (100 μM) significantly suppressed firing in GABA neurons of saline-injected mice, decreasing the slope of the I-O curve (Figures 8A and 8B). By contrast, a saturating dose of baclofen (100 μM) did not significantly change the I-O curve in METH-injected mice (Figures 8B and 8C). These results demonstrate that a loss of $GABA_BR$-GIRK currents in GABA neurons removes an important "brake" on GABA neuron firing in the VTA.

DISCUSSION

Drug-evoked synaptic plasticity can cause persistent modifications of neural circuits that eventually lead to addiction. We report here that a single dose of METH or cocaine is sufficient to significantly weaken the ability of $GABA_B$ receptors to control VTA GABA neuron firing when measured ex vivo 24 hr later. As such, this adaptive change is not likely sufficient to cause addiction but rather represents a building block of the adaptations that underlie addictive behavior with repetitive exposure. Studying the effect of a single injection of drug enabled us to systematically probe the mechanism underlying the plasticity of the slow IPSC. We discovered the methamphetamine-induced loss of the slow IPSC arises from a reduction in the $GABA_BR$-GIRK currents, due to changes in protein trafficking, and is accompanied by a significant decrease in the sensitivity of presynaptic $GABA_B$ receptors in GABA neurons of the VTA. In contrast, GABA neurons of the hippocampus and prelimbic cortex did not show similar changes in $GABA_B$-GIRK signaling, suggesting the $GABA_BR$s in the VTA are uniquely targeted by psychostimulants.

The psychostimulant-evoked reduction of $GABA_B$-GIRK currents in VTA GABA neurons could arise from a change in G protein coupling (Nestler et al., 1990; Labouèbe et al., 2007) or internalization of the receptor-channel (González-Maeso et al., 2003; Fairfax et al., 2004; Guetg et al., 2010; Maier et al., 2010; Terunuma et al., 2010). In support of the latter possibility, quantitative immunogold electron microscopy revealed a significant reduction in surface expression of $GABA_B$ receptors and GIRK channels in GABA neurons of METH-injected mice, coincident with a decrease in phosphorylation of $GABA_BR$s. In cortical and hippocampal neurons, a balance of AMP-activated protein kinase (AMPK)-dependent phosphorylation of $GABA_{B2}$-S783 and PP2A-dependent dephosphorylation governs postendocytic sorting of $GABA_B$ receptors (Terunuma et al., 2010). The persistence of the $GABA_B$-GIRK depression and the rapid recovery with phosphatase inhibitors suggest the balance of surface and internalized $GABA_B$ receptors in GABA neurons might be controlled by a molecular switch in a phosphatase, perhaps akin to the autophosphorylation switch in CaMKII (Lucchesi et al., 2011) or through an endogenous regulator of protein phosphatase activity (Guo et al., 1993). It remains possible that other kinases are also involved; both PKA- and CaMKII-dependent phosphorylation have been implicated in stabilization of $GABA_{B1}$ on the plasma membrane (Couve et al., 2002; Guetg et al., 2010). Interestingly, total protein levels of $GABA_{B2}$ receptors were not significantly changed in METH-injected mice, suggesting that the internalized pool of receptors was not redirected to a degradation pathway, in contrast to activity-dependent degradation of $GABA_B$ receptors observed in cortex (Terunuma et al., 2010). If phosphorylation controls

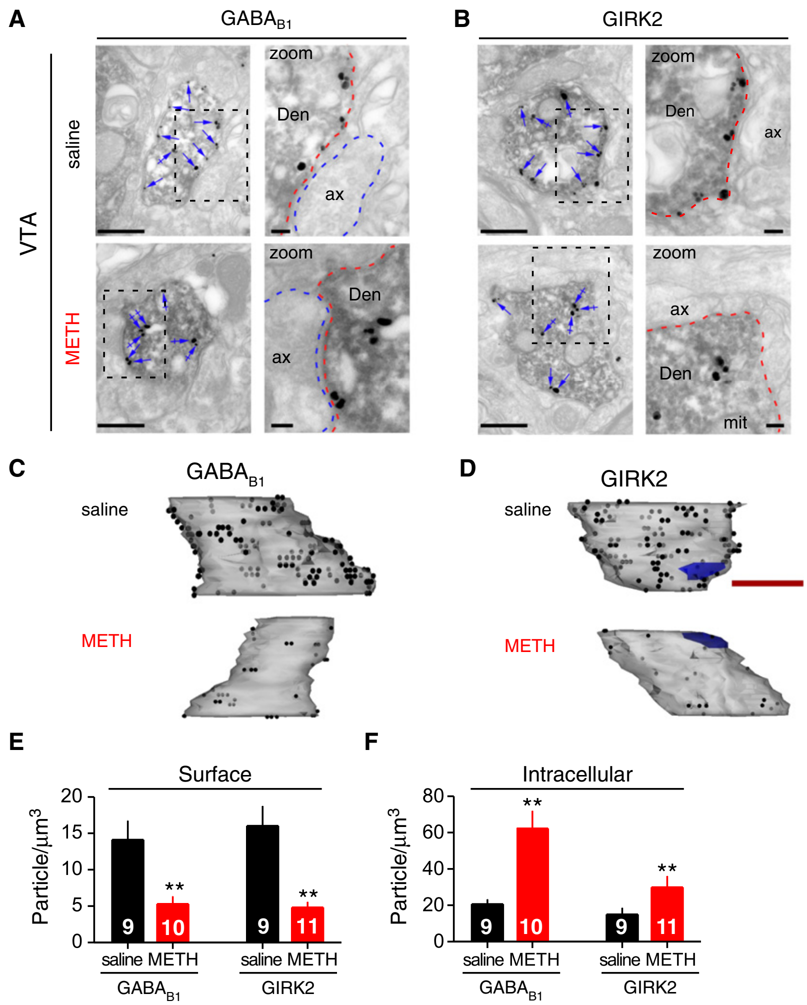

Figure 6. Reduced Surface Expression of $GABA_{B1}$ Receptors and GIRK2 Channels in GABA Neurons of METH-Injected Mice

(A and B) Pre-embedding double-labeling electron microscopy reveals $GABA_{B1}$ and GIRK2 expression in GABAergic neurons of the VTA 24 hr following saline or METH injection. GABA neurons were identified by GAD65/67-HRP immunoreactivity (labeled Den). Immunogold particles identify $GABA_{B1}$ or GIRK2. A reduction in immunogold particles against GIRK2 or $GABA_{B1}$ along the plasma membrane (arrows) was clearly detected in GAD65/67 positive dendrites following METH injection; an increase in immunogold particles against GIRK2 and $GABA_{B1}$ at intracellular sites can also be seen (crossed arrows). Right panels in (A) and (B) show zoom of boxed area.

Den, dendrite; ax, axon; mit, mitochondria. Scale bars: 0.5 µm.

(C) and (D) Three-dimensional reconstructions of dendrites from serial electron micrographs. Note decrease in surface expression of $GABA_{B1}$ receptors (C) and GIRK2 (D) following METH treatment. Black dots represent immunogold particles on the front surface and gray dots show immunogold particles on the back side of the dendrite. Blue regions are excitatory synapses. Note immunogold particles are abundantly distributed over the dendritic plasma membrane in control mice. Scale bars: 0.5 µm.

(E and F) Bar graphs show quantification of immunogold particles in reconstructed GAD65/67-positive dendrites for plasma membrane associated ($GABA_{B1}$ saline: 16.0 ± 2.7; $GABA_{B1}$ METH: 4.8 ± 0.8; GIRK2 saline: 14.1 ± 2.6; GIRK2 METH: 5.3 ± 1.0 particles/µm^3) and intracellular particles ($GABA_{B1}$ saline: 15 ± 3.6; $GABA_{B1}$ METH: 29.9 ± 6.2; GIRK2 saline: 20.7 ± 2.6; GIRK2 METH: 62.3 ± 10.5 particles/µm^3). Immunogold particles against both $GABA_{B1}$ and GIRK2 are significantly reduced at the dendritic plasma membrane and increased at intracellular sites 24 hr after METH injection (**$p < 0.05$ [Student t test]).

surface expression of $GABA_B$ receptors, then what controls the surface expression of GIRK channels? CaMKII-dependent phosphorylation of GIRK2 has been implicated in stabilizing GIRK2 channels on the plasma membrane of hippocampal neurons (Chung et al., 2009). In these neurons, protein phosphatase-1-mediated dephosphorylation promotes GIRK channel recycling and increases surface expression (Chung et al., 2009); therefore, a phosphatase inhibitor would be expected to reduce GIRK expression on the plasma membrane. An alternative explanation is that GIRK channels internalize via association with $GABA_B$ receptors in a macromolecular signaling complex. Previous studies have shown that both GPCRs and GIRK channels are physically close (Lavine et al., 2002; Nobles et al., 2005; Riven et al., 2006; Fowler et al., 2007) and can traffic together through intracellular compartments (Clancy et al., 2007).

Psychostimulants, such as METH and cocaine, generally lead to elevations in DA (Sulzer, 2011) that signals through two classes of GPCRs, D1- and D2-like receptors. Activation of D1-like receptors is required for inducing locomotor sensitization (Kalivas and Stewart, 1991), establishing self-administration of cocaine (Caine et al., 2007), and potentiating excitatory synapses with psychostimulants (Argilli et al., 2008; Brown et al., 2010). Supporting a role for D1-like receptors, co-injection of a D1-like receptor antagonist significantly attenuated the psychostimulant-dependent depression of $GABA_BR$-GIRK currents in VTA GABA neurons. We also observed some effects of the D2-like antagonist and cannot completely rule out a component of D2-like receptor activation in the depression of $GABA_B$-GIRK signaling. Recently, an acute cocaine-induced weakening of baclofen-induced GIRK currents in VTA DA neurons was found to be sensitive to D2-like, but not D1-like, receptor antagonists (Arora et al., 2011). In addition to DA, other neurotransmitters may be involved in the psychostimulant-dependent depression of $GABA_BR$-GIRK signaling. For example, acetylcholine levels in the VTA also increase following a single METH injection (Dobbs and Mark, 2008), and neuropeptides, such as hypocretin/orexin, BDNF, and CRF, could be also involved in the response to addictive drugs (Wang et al., 2005; Borgland et al., 2006; Hyman et al., 2006; Pu et al., 2006). Conditional knockouts or selective pharmacological experiments will be needed to pinpoint the neurotransmitters involved in the

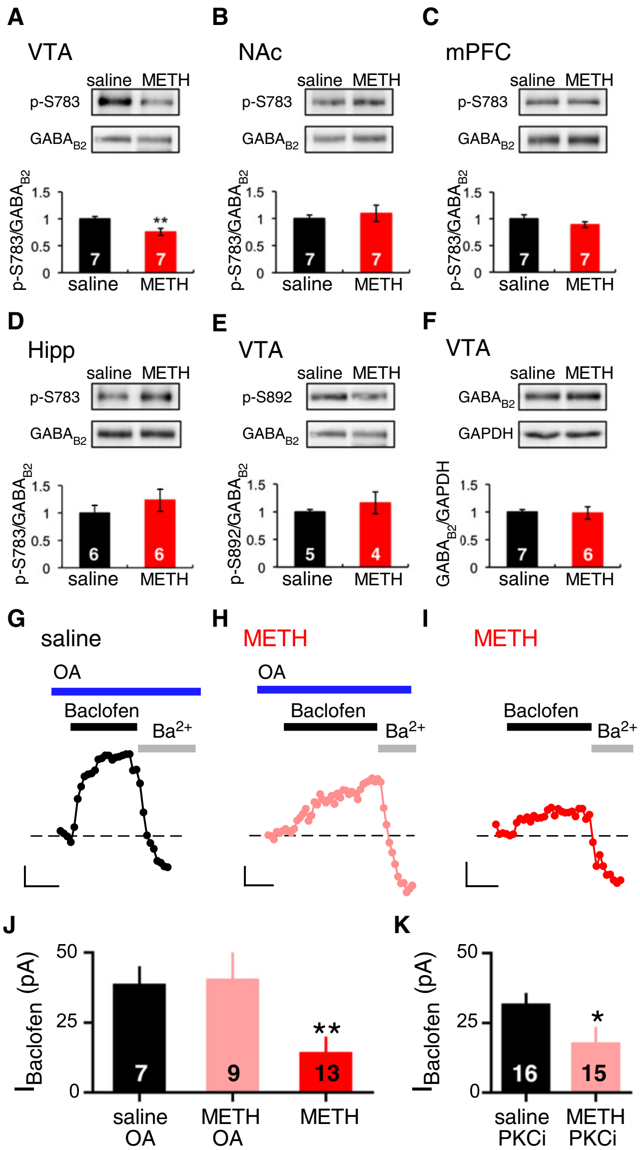

Figure 7. Role of Dephosphorylation of $GABA_BRs$ in METH-Dependent Depression of $GABA_BR$-GIRK Currents in GABA Neurons

(A–D) Western blots using phospho-specific antibody for p-S783 in $GABA_{B2}$ and total $GABA_{B2}$ in tissue punches of VTA, NAc, PFC, and hippocampus from saline and METH-injected mice (6–7 mice per group). Bar graphs show quantification of western blots normalized to $GABA_{B2}$ levels. Note significant decrease in p-S783 in VTA (**, $p < 0.05$ [Student's t test]).

(E) Western blot and quantification for p-S892 in $GABA_{B2}$ and total $GABA_{B2}$ in VTA.

(F) Western blot and quantification for total $GABA_{B2}$ and GAPDH in VTA.

(G–I) Intracellular application of OA but not PKC inhibitor recovered $I_{Baclofen}$ in METH-injected mice. Representative recordings of $I_{Baclofen}$ in GABA neurons from saline and METH-injected mice are shown with 100 nM okadaic acid (OA) included in patch electrode (OA_{pipet}) ($V_m = -50$ mV). Scale bars: 10 pA, 100 s.

(J) Bar graph shows average $I_{Baclofen}$ for saline-injected/OA_{pipet} (38.3 ± 6.3 pA), METH-injected/OA_{pipet} (40.2 ± 9.4 pA), and METH-injected (14.1 ± 5.6 pA). (**$p < 0.05$ versus saline using [one-way ANOVA]).

(K) For control, a PKC inhibitor (PKC(19-36), 1 μM) included in the pipet ($PKCi_{pipet}$) did not recover $I_{Baclofen}$ in METH-injected mice (saline+ $PKCi_{pipet}$: 31.5 ± 3.9 pA; $METH^+$ $PKCi_{pipet}$: 17.5 ± 5.7pA; *$p < 0.05$ [Student's t test]).

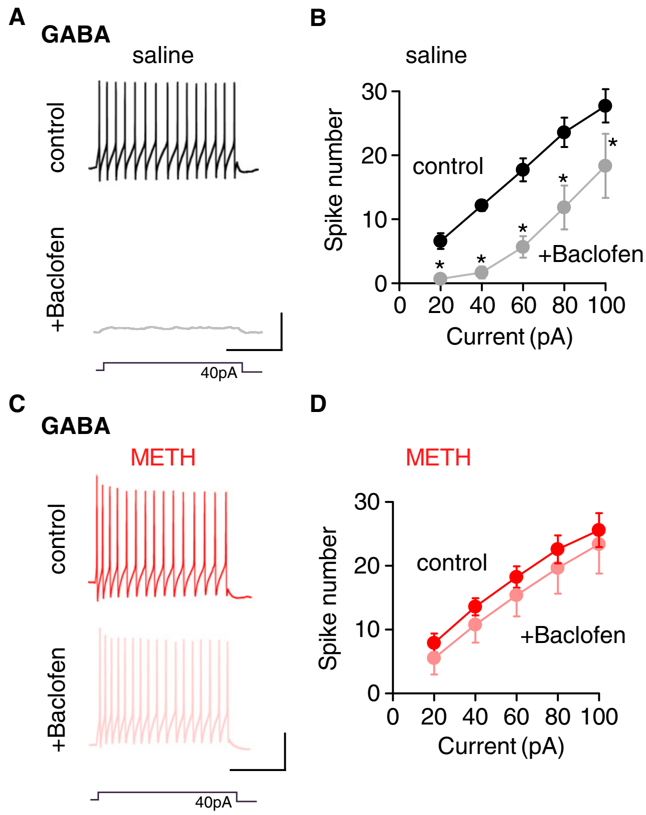

Figure 8. METH-Injected Mice Lack $GABA_BR$-Dependent Inhibition of VTA GABA Neuron Firing

(A and C) Current clamp recordings show spiking of VTA GABA neurons elicited by a current injection (40 pA) in saline (A) and METH- (C) injected mice 24 hr later. Spiking recorded in the absence (top trace) and presence (bottom trace) of 100 μM baclofen. Scale bars: 250 ms, 40 mV.

(B and D) Input-output graphs show spike number in GABA neurons plotted as a function of current injection for saline (B, N = 7) and METH-injected mice (D, N = 7). Average spike number increases with current injections and is significantly suppressed by 100 μM baclofen in saline-injected mice (B). *$p < 0.05$ two-way repeated-measure ANOVA. In METH-injected mice (D), baclofen does not significantly suppress spiking (ns, $p > 0.05$).

psychostimulant-dependent depression of $GABA_BR$-GIRK responses in VTA GABA neurons.

How may the psychostimulant-evoked depression in $GABA_B$-GIRK signaling in VTA GABA neurons alter the physiology of the VTA and contribute to addiction? DA neurons fire in two modes, tonic and phasic, with phasic firing leading to higher DA levels (Cooper, 2002). A balance of NMDAR activation and $GABA_BR$ signaling controls tonic versus phasic firing, and activation of $GABA_B$ receptors plays an important role in reducing phasic firing in VTA DA neurons (Erhardt et al., 2002). The VTA GABA neurons provide a local source of GABA for controlling the firing of VTA DA neurons (Grace and Bunney, 1985; Johnson and North, 1992; Tan et al., 2010). Recent electron microscopy studies have confirmed synaptic contacts between local GABA and DA neurons within the VTA (Omelchenko and Sesack, 2009). In the present study, we demonstrate the functionality of these GABAergic synapses using optogenetic tools. The depression of $GABA_B$R-GIRK signaling in somatodendritic regions along with the reduced sensitivity of $GABA_B$Rs in presynaptic GABA terminals of VTA GABA neurons would markedly impair an intrinsic "brake" on GABA release several days after a single injection of METH. Together, these pre- and postsynaptic neuro-adaptations are predicted to increase GABA-mediated inhibition of VTA DA neurons. In line with this model, other groups have reported psychostimulant-evoked neuro-adaptations in $GABA_B$R-signaling that lead to enhanced GABAergic transmission in the VTA (Giorgetti et al., 2002), the dorsolateral septal nucleus (Shoji et al., 1997), and the NAc (Xi et al., 2003). Similarly, chronic morphine increases the sensitivity of $GABA_B$ receptors on glutamatergic terminals in the VTA, which would further enhance the inhibition of DA neurons mediated by augmented GABA release (Manzoni and Williams, 1999).

An enhanced GABAergic inhibition of VTA DA neurons may represent an attempt to restore balance in activity of the VTA circuit; therefore, this $GABA_B$R-GIRK adaptation may be considered a form of synaptic scaling. Neuro-adaptive changes in $GABA_B$R-GIRK signaling for re-establishing balance in neural circuits have been described in other model systems. In a mouse model of succinic semialdehyde dehydrogenease deficiency, an autosomal recessive disorder of GABA catabolism that leads to elevated synaptic GABA, $GABA_B$R-GIRK currents are significantly depressed in cortical neurons (Vardya et al., 2010). On the other hand, the $GABA_B$R-mediated IPSC in hippocampal pyramidal neurons is enhanced in response to potentiation of excitatory synaptic transmission (Huang et al., 2005). The level of inhibition mediated by $GABA_B$R-GIRK currents may be tightly tuned to changes in neuronal excitability.

The downregulation of $GABA_B$ receptor signaling in VTA GABA neurons occurs in parallel with other plastic changes in VTA DA neurons, such as the redistribution of AMPAR and NMDARs (White et al., 1995; Zhang et al., 1997; Ungless et al., 2001; Borgland et al., 2004; Argilli et al., 2008; Mameli et al., 2011), and alterations of fast GABAergic transmission (Nugent et al., 2007). As proposed above, the drug-evoked depression of $GABA_B$R signaling in GABA neurons removes a "brake" on GABA neuron firing that may enhance GABA-mediated inhibition of DA neurons. If present in vivo, the increase in GABA transmission may reduce reward perception (Koob and Volkow, 2010; Lüscher and Malenka, 2011). However, repeated psychostimulant administration leads to increases in the firing rates of VTA DA neurons (White and Wang, 1984; Henry et al., 1989; White, 1996), partly through reduced sensitivity of D2 autoreceptors (White, 1996). Thus, an increase in GABA-mediated inhibition of VTA DA neurons, whereas efficient at first, may eventually be inadequate to suppress the potentiating effects of psychostimulants on VTA DA neurons and is therefore unable to avert the progression toward addiction. Enhancing this inhibitory pathway, however, might provide a strategy for treating addiction. Clearly, additional experiments will be needed to better understand how the adaptation of $GABA_B$R-GIRK signaling affects VTA GABA neuron function and, more generally, the role of the slow $GABA_B$-mediated inhibition in drug-evoked remodeling of the mesocorticolimbic circuitry.

In conclusion, we have identified a molecular switch in $GABA_B$ receptor signaling that occurs in response to a single in vivo exposure to psychostimulant—this depression of $GABA_B$R-GIRK signaling persists for days after the injection. This cellular memory trace of drug exposure is encoded in a phosphorylation-dependent depression of $GABA_B$ receptor signaling in VTA GABA neurons, which may augment GABA transmission in the mesocorticolimbic system.

EXPERIMENTAL PROCEDURES

Animals

C57BL/6 mice were purchased from Harlan laboratories or bred in-house and housed under constant temperature and humidity on a 12 hr light-dark cycle (light 6 am–6 pm) with free access to food and water. GAD67-GFP is a knock-in mouse that was kindly provided by Dr. Y. Yanagawa. Pitx3-GFP is a knock-in mouse that was kindly provided by Dr. M. Li. All procedures were performed in the light cycle using IACUC approved protocols for animal handling at the Salk Institute and the University of Geneva.

Drug Treatment

Male and female mice (P15-35) were injected intraperitoneally with 0.9% saline (control), 2 mg/kg methamphetamine (METH), or 15 mg/kg cocaine using a 15-gauge insulin syringe and injection volume <200 μl to minimize stress. Experimental procedures were performed 24 hr–7 days later. Methamphetamine and cocaine were purchased from Sigma (St. Louis, MO, USA).

Electrophysiology in Acute Slices

Twenty-four hours or 7 days following i.p. injections, mice were euthanized and horizontal slices from midbrain (250 μm) were prepared in ice-cold artificial cerebral spinal fluid (see Supplemental Experimental Procedures for details). Neurons were visualized with IR camera Gloor Instrument PCO or Dage-MTI IR-1000) on an Olympus scope (BX50 or BX51) and whole-cell patch-clamp recordings (Axopatch 200B or Multiclamp 700A amplifier) were made from neurons in the VTA, identified as the region medial to the medial terminal nucleus of the accessory optical tract. GABA neurons were identified by the absence of I_h current, a small capacitance (<20 pF) and a fast spontaneous firing rate (5–10 Hz). In contrast DA neurons have an I_h current, large capacitance (20–50 pF) and slow spontaneous firing (1–3 Hz). Pitx3-GFP mice expressing GFP in DA neurons (Zhao et al., 2004) and GAD67-GFP mice expressing GFP in GABA neurons (Tamamaki et al., 2003) were used to confirm electrophysiological identification. The internal solution for measuring baclofen-activated $GABA_B$ currents contained (in mM) potassium gluconate 140, NaCl 4, $MgCl_2$ 2, EGTA 1.1, HEPES 5, Na_2ATP 2, sodium creatine phosphate 5 and Na_3GTP 0.6 (pH 7.3) with KOH. For $GABA_B$ sIPSCs, the internal solution contained (in mM) K-gluconate 140, KCl 5, $MgCl_2$ 2, EGTA 0.2, HEPES 10, Na_2ATP 4, Creatine-phosphate 10, and Na_3GTP 0.3. To measure $GABA_A$ currents, the internal solution contained (in mM) K-gluconate 30, KCl 100, $MgCl_2$ 4, creatine phosphate 10, Na_2 ATP 3.4, Na_3 GTP 0.1, EGTA 1.1, and HEPES 5.

For the sIPSC, the evoked synaptic recordings were isolated in presence of APV (100 μM), NBQX (10 μM), and sulpiride (200 nM) for $GABA_A$R IPSC, and picrotoxin (100 μM) for $GABA_B$R sIPSC. The stimulation electrode consisted of a saline-filled monopolar glass pipet placed caudally to the cell being recorded. $GABA_A$R paired-pulse ratio (PPR) was assessed by applying two pulses at 50 ms interval every 10 s, whereas the $GABA_B$R sIPSCs were evoked by applying a train of 10 electrical pulses at 66Hz once every 20–40 s. For $I_{Baclofen}$, currents were recorded, filtered at 1 kHz and digitized at 5 kHz (Axon pClamp 8). Cells were clamped at −50 or −60 mV (membrane voltages were corrected for liquid junction potential; −15.7 mV). For some recordings, a voltage ramp from +60 mV to −100 mV was delivered at 1 Hz. Cell membrane resistance and approximate access resistance were measured with a 200 ms 10 mV hyperpolarizing step. All electrophysiological chemicals for electrophysiology were purchased from Sigma; drugs were purchased from Tocris Bioscience (Minneapolis, MN). We did not observe any differences with wild-type mice and Pitx3-GFP or GAD67-GFP; therefore, we have pooled the data. Data are expressed as mean ± SEM and statistical significance ($p < 0.05$) determined by one-way analysis of variance (ANOVA) with Holm-Sidak post hoc test, Student's t test, or Mann-Whitney test. All measurements made at ~33°C.

Optogenetic Experiment

AAV5-flox-ChR2-eYFP virus (produced in the Vector Core Facility at the University of North Carolina) was injected into 3-week-old GAD65-Cre mice (kindly provided by Dr. Gero Miesenböck). Anesthesia was induced and maintained with isoflurane (Baxter AG, Vienna, Austria) at 5% and 1%, respectively. The animal was placed in a stereotaxic frame (Angle One; Leica, Solms, Germany) and craniotomies were performed bilaterally over the VTA using stereotaxic coordinates (AP −3.4, ML ± 0.8, and DV 4.4). Injections of AAV-ChR2 flox were carried out using graduated pipets (Drummond Scientific Company, Broomall, PA, USA), broken back to a tip diameter of 10–15 μm, at a rate of ~100 nl min^{-1} for a total volume of 500 nl. In all experiments the virus was allowed 3 weeks to incubate before any other procedures were carried out. Fast $GABA_A$ IPSCs in DA cells were isolated in presence of kynurenic acid (2 mM) and evoked by applying two consecutive 4 ms blue-light (Thorlad–472 nm LED) flashes at 50 ms interval to the slice every 10 s. Recordings were made as described previously.

Antibodies

A rabbit polyclonal antibody anti-Glutamate Decarboxylase 65 & 67 (AB1511; Millipore, Billerica, MA, USA), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho S783-$GABA_{B2}$ (p-S783; Terunuma et al., 2010), anti-phospho S892-$GABA_{B2}$ (p-S892; Couve et al., 2001) were used. A monoclonal antibody anti-$GABA_{B1}$ (Clone N93A/49; NeuroMab, Davis, CA, USA) and anti-$GABA_{B2}$ (Clone N81/37; NeuroMab) were used. A guinea-pig polyclonal antibody anti-GIRK2 (Aguado et al., 2008) was used.

Immunoelectron Microscopy

A similar procedure to that described earlier (Lujan et al., 1996; Koyrakh et al., 2005) was used. See online Supplemental Experimental Procedures for details on procedures and quantitation.

Western Blotting

Tissue punches from VTA, NAc, hippocampus, and mPFC obtained from saline- and METH-injected mice were lysed in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM NaF, 2 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml antipain, and 250 μg/ml 4-(2-Aminoethl) benzenesulfonyl fluoride hydrochloride. Soluble material was then subjected to immunoblotting with anti-$GABA_{B2}$, anti-phospho S783-$GABA_{B2}$ (p-S783), anti-phospho S892-$GABA_{B2}$ (p-S892), anti-GAPDH, and detected by SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). The luminescence images were captured by a Luminescent Image Analyzer (LAS3000; Fujifilm, Tokyo, Japan) and the intensity of bands were measured by Multi Gauge software (version 3; Fujifilm).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2011.12.031.

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