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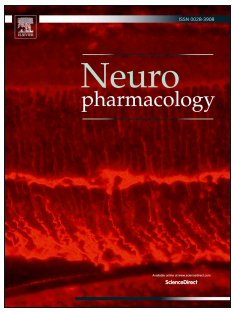

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Suppression of inhibitory G protein signaling in forebrain pyramidal neurons triggers plasticity of glutamatergic neurotransmission in the nucleus accumbens core

Running title: GIRK channel ablation and striatal plasticity

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ABSTRACT

Cocaine and other drugs of abuse trigger long-lasting adaptations in excitatory and inhibitory neurotransmission in the mesocorticolimbic system, and this plasticity has been implicated in several key facets of drug addiction. For example, glutamatergic neurotransmission mediated by AMPA receptors (AMPAR) is strengthened in medium spiny neurons (MSNs) in the NAc core and shell during withdrawal following repeated *in vivo* cocaine administration. Repeated cocaine administration also suppresses inhibitory signaling mediated by G protein-gated inwardly rectifying K^+ (GIRK) channels in pyramidal neurons of the prelimbic cortex, an important source of glutamatergic input to the NAc core that has been implicated in cocaine-seeking and behavioral sensitization. Here, we tested the hypothesis that suppression of GIRK channel activity in forebrain pyramidal neurons can promote plasticity of glutamatergic signaling in MSNs. Using novel conditional knockout mouse lines, we report that GIRK channel ablation in forebrain pyramidal neurons is sufficient to enhance AMPAR-dependent neurotransmission in D_1R-expressing MSNs in the NAc core, while also increasing motor-stimulatory responses to cocaine administration. A similar increase in AMPAR-dependent signaling was seen in both D_1R- and D_2R-expressing MSNs in the NAc core during withdrawal from repeated cocaine administration in normal mice. Collectively, these data are consistent with the premise that the cocaine-induced suppression of GIRK-dependent signaling in glutamatergic inputs to the NAc core contributes to some of the electrophysiological and behavioral hallmarks associated with repeated cocaine administration.

Keywords: Kir3; prelimbic cortex; AMPA receptor; plasticity; cocaine; nucleus accumbens; mice

1. INTRODUCTION

Durable molecular and cellular adaptations evoked by repeated exposure to drugs of abuse such as cocaine complicate the development of treatment strategies for addiction (Koob and Volkow, 2010; Luscher, 2013). These adaptations are thought to underlie the observation, for example, that exposure to stress or re-exposure to drug can reinstate cocaine-seeking behavior in humans and animal models, even after a prolonged period of abstinence (Shaham et al., 2003).

The nucleus accumbens (NAc) is a node within the mesocorticolimbic circuitry that is implicated in goal-directed behavior, and that mediates the rewarding and reinforcing effects of cocaine and other drugs of abuse (Hyman et al., 2006; Kalivas and Volkow, 2005; Koob and Volkow, 2010). Adaptations in cortical and limbic glutamatergic inputs to NAc medium spiny neurons (MSNs), which account for 90-95% of the neurons in this brain region, have been implicated in a number of behaviors linked to cocaine addiction including locomotor sensitization, craving, and relapse (Conrad et al., 2008; Kalivas, 2009; Lobo and Nestler, 2011; Luscher, 2013; Shaham and Hope, 2005; Wolf, 2016).

Cocaine-induced plasticity of glutamatergic transmission in the NAc impacts signaling via AMPA glutamate receptors (AMPAR) (Bowers et al., 2010; Pierce and Wolf, 2013; Wolf, 2016). Repeated *in vivo* exposure to cocaine increases dendritic spine number on NAc MSNs (Churchill et al., 1999; Li et al., 1997; Robinson and Kolb, 2004), and the cell surface levels of AMPAR in the NAc (Boudreau and Wolf, 2005), while also enhancing behavioral responses to subsequent infusion of AMPA into the NAc (Cornish and Kalivas, 2000; Pierce et al., 1996; Suto et al., 2004). Indeed, AMPA administered to either the NAc core or shell evoked enhanced locomotor activity in rats previously given repeated cocaine injections, as compared to saline-treated controls (Pierce et al., 1996). Interestingly, the potentiation of AMPAR-dependent signaling in NAc MSNs has been shown to emerge during extended periods of withdrawal following repeated cocaine administration, and subsequent exposure to stress or cocaine can depotentiate AMPAR-dependent signaling in these neurons (Jedynak et al., 2016; Kourrich et al., 2007).

Previously, we reported adaptations in AMPAR-dependent signaling in NAc core and shell MSNs from mice lacking G protein-gated inwardly rectifying K^+ (GIRK/Kir3)

channels (Arora et al., 2010), prevalent postsynaptic mediators of metabotropic inhibitory signaling in the central nervous system (Lujan et al., 2014; Luscher and Slesinger, 2010). Constitutive ablation of the integral neuronal GIRK channel subunit, GIRK2, correlated with increased density of excitatory synapses and increased synaptic expression of AMPAR in both the NAc core and shell, along with increased amplitude and frequency of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in NAc MSNs. More recently, we reported that repeated non-contingent cocaine administration evoked a persistent suppression of GIRK-dependent signaling in layer 5/6 pyramidal neurons of the prelimbic cortex (PrLC) (Hearing et al., 2013), a key glutamatergic projection to the NAc core (Krettek and Price, 1977; Sesack et al., 1989), suggesting that reduced GIRK-dependent inhibitory signaling in glutamatergic inputs to the NAc may underlie some of the cellular and behavioral effects associated with repeated cocaine administration. In support of this contention, viral RNAi-mediated suppression of GIRK-dependent signaling in layer 5/6 of the mouse PrLC yielded a “pre-sensitized” state characterized by an enhanced acute motor-stimulatory effect of cocaine (Hearing et al., 2013).

Collectively, these findings raise the intriguing possibility that decreased GIRK channel activity in afferent glutamatergic inputs to the NAc may contribute to the plasticity of AMPAR-mediated neurotransmission in the NAc seen following repeated cocaine administration. We tested key elements of this premise using a transgenic Cre-dependent recombination approach and novel conditional GIRK knockout mouse lines. We found that the persistent genetic suppression of GIRK-dependent signaling in forebrain pyramidal neurons enhanced AMPAR-dependent neurotransmission in D_1R-expressing MSNs in the NAc core, in a manner comparable to that seen following repeated cocaine administration in wild-type mice, and yielded enhanced motor stimulatory responses to cocaine.

2. MATERIAL and METHODS

2.1. Animals. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Minnesota. The generation and characterization of *Girk2*$^{flox/flox}$ mice was described previously (Kotecki et al., 2015). B6.Cg-Tg(Drd1a-tdTomato)6Calak/J (D_1R-tdTomato) and Tg(Camk2a-cre)T29-1Stl/J (CaMKIICre) lines were purchased from The Jackson Laboratory (Bar Harbor, ME), and the Tg(Drd2-EGFP)S118Gsat (D_2R-EGFP) strain was obtained from the Mutant Mouse Regional Resource Center. Mice were maintained on a 12 h light/dark cycle (lights on at 0700), with food and water available *ad libitum.*

2.2. Reagents. Baclofen, CGP54626, tetrodotoxin (TTX), picrotoxin, kynurenic acid, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX), and cocaine were purchased from Sigma (St. Louis, MO).

2.3. qRT-PCR and immunoblotting. Quantitative analysis of GIRK subunit mRNA and protein levels from brain micropunches (2-mm diameter, 2-mm thick) was performed as described (Marron Fernandez de Velasco et al., 2015).

2.4. Slice electrophysiology. Coronal slices (250-270 μm) of the mouse PFC, basolateral amygdala (BLA), and NAc (5-7 wk) were prepared as described (Marron Fernandez de Velasco et al., 2015; McCall et al., 2016). Defined NAc MSN subpopulations identified via expression of tdTomato (D_1R-expressing) or EGFP (D_2R-expressing) were targeted for analysis. Somatodendritic currents and mEPSCs were acquired using an EPC10 HEKA amplifier and Patchmaster 2x73.2 software (HEKA Elektronic; Bellmore, NY), as described (Marron Fernandez de Velasco et al., 2015; McCall et al., 2016). All command potentials factored in a junction potential of -15 mV. mEPSC traces were filtered at 2 kHz and digitized at 10 kHz. Amplitude and frequency values were extracted with Mini Analysis (Synaptosoft, Inc.; Decatur, GA) software. Series resistance, membrane resistance, and apparent cell capacitance were tracked throughout each

experiment. If series resistance was unstable (>20% variation), the experiment was excluded from analysis.

2.5. Locomotor activity. Locomotor activity was assessed in automated open field activity chambers (Med-Associates, Inc; St. Albans, VT), as described (Pravetoni and Wickman, 2008). In brief, subjects (8-10 wk) were acclimated over a 3-d period, during which the animals were handled (days 1-3) and exposed to i.p. injection of saline (days 2 and 3), followed by placement in open field environments. Subjects thereafter received a total of 5 saline or cocaine (15 mg/kg i.p.) injections, with one injection administered per day, every other day. Total distance traveled was measured during the 60-min period following handling or injection of saline or cocaine. For studies involving CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ mouse lines, a 6th injection of cocaine (15 mg/kg i.p.) was given 10-14 d after the 5th cocaine injection.

2.6. Statistical analysis. Data are presented throughout as the mean±SEM. Statistical analyses were performed using Prism 6 (GraphPad Software, Inc.; La Jolla, CA). Electrophysiology, qRT-PCR, and immunoblotting data were analyzed for genotypic effects with Student's two-tailed *t*-test, with Welch's correction if appropriate. Locomotor activity data were analyzed using 2-way ANOVA with repeated measures, and the Holm-Sidak *post hoc* test was used for pair-wise comparisons, if warranted. A paired two-tailed *t*-test was used to compare the motor-stimulatory effect of cocaine after the 1st and 6th injections in CaMKIICre(-):*Girk1*$^{fl/fl}$, CaMKIICre(+):*Girk1*$^{fl/fl}$, CaMKIICre(-):*Girk2*$^{fl/fl}$, and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice. Differences were considered significant if $P<0.05$.

3. RESULTS

3.1. Generation of conditional Girk1$^{-/-}$ mice

GIRK channels in mouse layer 5/6 PrLC pyramidal neurons are formed by GIRK1 and GIRK2; genetic ablation of either subunit correlates with decreased GIRK-dependent inhibitory signaling and increased excitability in these neurons (Hearing et al., 2013). Recently, we described the development of a conditional *Girk2* knockout mouse (Victoria et al., 2016). To permit cell-specific ablation of GIRK1, we generated a conditional *Girk1* mutant (*Girk1*$^{fl/fl}$) by flanking a short internal coding sequence exon of the mouse *Girk1/Kcnj3* gene with *loxP* sites (**Fig. S1A-C**). GIRK1 mRNA (**Fig. S1D**) and total protein (**Fig. S1E**) levels were normal in micropunches of the hippocampus, cortex, and cerebellum of *Girk1*$^{fl/fl}$ mice, indicating that "floxing" the *Girk1* gene did not significantly alter baseline *Girk1* expression. We next crossed the *Girk1*$^{fl/fl}$ line with transgenic mice expressing Cre recombinase under the control of the CaMKIIα promoter (CaMKIICre mice), to ablate *Girk1* in forebrain pyramidal neurons. Total GIRK1 protein level in micropunches containing the PFC was significantly lower in CaMKIICre(+):*Girk1*$^{fl/fl}$ mice than in CaMKIICre(-):*Girk1*$^{fl/fl}$ mice littermate controls (t_8=5.0, P<0.01; **Fig. 1A**).

3.2. Loss of GIRK channels in pyramidal neurons in CaMKIICre:Girk$^{fl/fl}$ mice

To probe the efficacy of GIRK channel ablation in forebrain pyramidal neurons, we measured somatodendritic inhibitory currents evoked by the $GABA_B$ receptor ($GABA_BR$) agonist baclofen in layer 5/6 PrLC pyramidal neurons of CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ mice. The baclofen-induced somatodendritic current in layer 5/6 PrLC pyramidal neurons consists of GIRK-dependent and GIRK-independent components (Hearing et al., 2013). Most (~60%) of the baclofen-induced current in neurons from male mice is mediated by GIRK channel activation, whereas the non-GIRK conductance predominates in neurons from female mice (Marron Fernandez de Velasco et al., 2015). Thus, these and subsequent studies focused on male mice only.

Layer 5/6 PrLC pyramidal neurons from Cre(+) mice exhibited significantly smaller baclofen-induced currents than those seen in Cre(-) controls in both CaMKIICre:*Girk1*$^{fl/fl}$

(t_{16}=7.9, P<0.001) and CaMKIICre:*Girk2*$^{fl/fl}$ (t_{15}=3.8, P<0.01) mouse lines (**Fig. 1B,C**), comparable to current amplitudes reported in layer 5/6 PrLC pyramidal neurons from *Girk1*$^{-/-}$ and *Girk2*$^{-/-}$ mice (Hearing et al., 2013). Resting membrane potentials were also depolarized in neurons from Cre(+) subjects from both CaMKIICre:*Girk1*$^{fl/fl}$ (-61.9±0.8 vs. -64.3±0.4 mV; t_{16}=2.5, P<0.05) and CaMKIICre:*Girk2*$^{fl/fl}$ (-64.9±1.3 vs. -70.3±0.6 mV; $t_{19.2}$=3.8, P<0.01) mouse lines. Consistent with the reported pattern of Cre-dependent recombination in CaMKIICre(+) mice, we also observed reduced baclofen-induced currents in pyramidal neurons of the basolateral amygdala from CaMKIICre(+):*Girk1*$^{fl/fl}$ mice (**Fig. S2**), and in CA1 pyramidal neurons of the dorsal hippocampus from CaMKIICre(+):*Girk2*$^{fl/fl}$ mice (Victoria et al., 2016). Thus, CaMKIICre(+):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice exhibit a loss of GIRK-dependent signaling in multiple forebrain pyramidal neuron populations.

3.3. Glutamatergic signaling in NAc MSNs in CaMKIICre:Girk$^{fl/fl}$ *mice*

To test whether the genetic suppression of GIRK-dependent inhibitory signaling in forebrain pyramidal neurons is sufficient to enhance excitatory neurotransmission in NAc MSNs, we crossed CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ lines with D_1R-tdTomato and D_2R-EGFP transgenic mice (**Fig. 2A**). These lines permitted the targeted electrophysiological evaluation of AMPAR-dependent signaling in D_1R- and D_2R-expressing MSN neuron sub-populations, which differ in their intracellular responses to dopamine, gene expression profiles, and influence on cocaine-associated behaviors (Lobo and Nestler, 2011; Smith et al., 2013). MSNs in the NAc core were targeted as this NAc sub-region receives a prominent glutamatergic projection from PrLC pyramidal neurons that has been implicated in cocaine-seeking behavior (Krettek and Price, 1977; Moorman et al., 2014; Sesack et al., 1989).

We observed significantly higher mEPSC frequencies in D_1R-expressing MSNs from CaMKIICre(+):*Girk1*$^{fl/fl}$ (t_{22}=3.6, P<0.01) and CaMKIICre(+):*Girk2*$^{fl/fl}$ (t_{14}=2.9, P<0.05) mice (**Fig. 2B-D**). mEPSCs were completely blocked by the non-selective ionotropic glutamate receptor antagonist kynurenic acid (2 mM), as well as the more selective AMPAR antagonist NBQX (10 μM; not shown). mEPSC frequencies were normal in D_2R-expressing MSNs from the forebrain pyramidal neuron-specific mutant lines,

though a non-significant trend toward elevated frequency was seen in D_2R-expressing MSNs from CaMKIICre(+):*Girk1*$^{fl/fl}$ mice (t_{20}=1.7, P=0.10). mEPSC amplitudes did not differ between Cre(+) and Cre(-) mice in either D_1R- or D_2R-expressing MSNs (**Fig. 2E**). Collectively, these data indicate that the loss of GIRK channel activity in forebrain pyramidal neurons is sufficient to enhance AMPAR-dependent signaling in D_1R-expressing MSNs in the NAc core.

3.4. Glutamatergic signaling in NAc MSNs following repeated cocaine

We next sought to compare the impact of GIRK subunit ablation in forebrain pyramidal neurons to the effect of repeated cocaine treatment on AMPAR-dependent signaling in NAc MSNs. While repeated cocaine administration has been shown to increase mEPSC frequency and amplitude in NAc MSNs, these adaptations emerge only after an extended period (10-14 d) of withdrawal (Jedynak et al., 2016; Kourrich et al., 2007). Thus, we measured mEPSCs in D_1R- and D_2R-expressing NAc core MSNs, 10-14 d after repeated cocaine or saline treatment (**Fig. 3A**).

Repeated cocaine treatment resulted in the development of behavioral sensitization in both D_1R-tdTomato and D_2R-EGFP mouse lines, as assessed by comparing motor activity following the 1st and 5th cocaine injections (**Fig. 3B**). Cocaine-treated mice exhibiting a ≥30% increase in motor activity on the 5th relative to the 1st injection were used in subsequent slice electrophysiological experiments. Higher mEPSC frequencies were observed in both D_1R-expressing (t_{20}=2.7, P<0.05) and D_2R-expressing (t_{25}=2.3, P<0.05) NAc core MSNs of cocaine-treated mice, relative to saline-treated controls (**Fig. 3C-D**). mEPSC amplitudes, however, were unaffected by cocaine treatment in either the D_1R-expressing (t_{20}=0.2, P=0.87) or D_2R-expressing (t_{25}=1.8, P=0.08) NAc core MSNs **(Fig. 3E)**. Thus, adaptations in AMPAR-dependent signaling in NAc core MSNs observed during withdrawal from repeated cocaine treatment are overlapping but distinct from those seen in drug-naïve CaMKIICre(+):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice.

3.5. GIRK channel expression in NAc MSNs

Loss of GIRK channels in NAc MSNs could drive adaptations in excitatory neurotransmission in a cell-autonomous manner. To probe for GIRK channel expression in the mouse NAc, we used established qRT-PCR and immunoblotting approaches targeting the primary neuronal GIRK subunits (GIRK1, GIRK2, and GIRK3). While all 3 GIRK mRNAs were detected in NAc micropunches, the levels of GIRK1 (t_8=6.7, P<0.001), GIRK2 (t_9=10.8, P<0.001), and GIRK3 (t_9=9.6, P<0.001) were approximately 5 to 10-fold lower in NAc as compared to mPFC samples (**Fig. 4A**). Similarly, GIRK1 (t_9=20.3, P<0.001), GIRK2 (t_{12}=21.8, P<0.001), and GIRK3 (t_8=5.5, P<0.001) total protein levels were significantly lower in the NAc than the mPFC (**Fig. 4B**).

We also probed for evidence of GIRK channel expression by measuring somatodendritic inhibitory currents evoked by baclofen. In a small fraction of D_1R- (1/14; **Fig. 4C**) and D_2R-expressing (1/8; **Fig. 4D**) MSNs in the NAc core, baclofen elicited an outward current with concomitant decrease in input resistance. Thus, while GIRK channels may be expressed in a small fraction of NAc core MSNs, the enhanced excitatory neurotransmission seen in NAc core MSNs in CaMKIICre(+):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice is not likely attributable to the loss of GIRK channels in these neurons themselves.

3.6. Cocaine-induced motor activity in CaMKIICre:Girk$^{fl/fl}$ mice

Elevated glutamatergic neurotransmission in D_1R-expressing MSNs in the NAc core has been implicated in behavioral sensitization and cocaine-seeking behavior (Lobo and Nestler, 2011). To test whether mice lacking GIRK1 or GIRK2 in forebrain pyramidal neurons exhibit altered motor-stimulatory responses to cocaine, we used a standard repeated cocaine treatment paradigm (**Fig. 5A**). Mice lacking either GIRK1 or GIRK2 in forebrain pyramidal neurons exhibited normal baseline motor activity (**Fig. 5B,C**).

When challenged with cocaine, Cre(+) subjects in both the CaMKIICre:*Girk1*$^{fl/fl}$ ($F_{1,14}$=13.1, P<0.01) and CaMKIICre:*Girk2*$^{fl/fl}$ ($F_{1,16}$=8.6, P<0.01) lines exhibited higher activity levels than their Cre(-) counterparts (**Fig. 5B,C**); increased cocaine-induced activity was evident after the first cocaine injection and carried across all subsequent injections. In addition, a significant main effect of cocaine injection number (considering 1st, 5th, and 6th/T injections) was observed for both CaMKIICre:*Girk1*$^{fl/fl}$ ($F_{2,28}$=23.8,

P<0.001) and CaMKIICre(+):*Girk2*$^{fl/fl}$ ($F_{2,32}$=22.6, *P*<0.001) lines. No interaction between genotype and cocaine injection number was detected, however, for either the CaMKIICre:*Girk1*$^{fl/fl}$ ($F_{2,28}$=1.1, *P*=0.35) or CaMKIICre:*Girk2*$^{fl/fl}$ ($F_{2,32}$=0.7, *P*=0.50) lines. Notably, motor activity responses measured after 6th/T cocaine injection were significantly larger than the responses to the 1st cocaine injection in each of the 4 genotypes evaluated in this study, indicative of the capacity of each group to exhibit behavioral sensitization. Thus, the loss of GIRK-dependent signaling in forebrain pyramidal neurons yields enhanced motor-stimulatory responses to cocaine, but does not occlude behavioral sensitization evoked by repeated cocaine treatment.

4. DISCUSSION

Repeated non-contingent cocaine administration triggers a persistent reduction in GIRK-dependent signaling in layer 5/6 PrLC pyramidal neurons, including neurons that project to the NAc core (Hearing et al., 2013). This adaptation is evident within a day of cocaine withdrawal and persists for weeks. In contrast, enhanced AMPAR-dependent neurotransmission in NAc core MSNs emerges after an extended period of withdrawal from repeated cocaine exposure (Jedynak et al., 2016). In this study, we asked whether a reduction in GIRK-dependent signaling in forebrain pyramidal neurons could trigger plasticity of excitatory neurotransmission in the NAc core. We show that persistent genetic suppression of GIRK-dependent signaling in forebrain pyramidal neurons triggers elevated glutamatergic neurotransmission in D_1R- but not D_2R-expressing MSNs in the NAc core. A similar enhancement in glutamatergic neurotransmission in D_1R-expressing MSNs in the NAc core of wild-type mice was seen during an extended period of withdrawal from repeated cocaine administration.

Converging lines of evidence support the contention that drug-induced adaptations in D_1R-expressing NAc MSNs play a prominent role in the sensitizing effects of drugs of abuse (Lobo and Nestler, 2011; Smith et al., 2013). For example, activation of D_1R-expressing MSNs enhances the sensitivity of a rodent to cocaine and other drugs of abuse (Ferguson et al., 2011; Hikida et al., 2010; Lobo et al., 2010). Given the different timelines over which cocaine-induced adaptations in GIRK- and AMPAR-dependent signaling occur, our data suggest that the cocaine-induced suppression of GIRK-dependent signaling in glutamatergic inputs to the NAc core may promote or facilitate the adaptations in excitatory neurotransmission in D_1R-expressing MSNs that have been linked to key aspects of addiction, including reinstatement of cocaine-seeking behavior.

A bi-directional effect of cocaine on AMPAR-dependent neurotransmission in NAc MSNs was first established in the NAc shell (Kourrich et al., 2007). Extended withdrawal from repeated *in vivo* cocaine administration was shown to trigger an increase in both mEPSC frequency and amplitude in NAc shell MSNs, adaptations that were reversed by a challenge injection of cocaine. Recent findings, including data presented herein, support the contention that AMPAR-dependent signaling in NAc core MSNs is similarly

modulated by cocaine (Conrad et al., 2008; Jedynak et al., 2016; Moussawi et al., 2011). While there is consensus that AMPAR-dependent neurotransmission is increased during withdrawal from repeated cocaine administration, some notable differences across studies are evident. For example, we found that withdrawal following cocaine exposure correlated with increased mEPSC frequency but not amplitude in NAc core MSNs in the mouse, similar to findings in rats trained to self-administer cocaine (Moussawi et al., 2011). In contrast, a recent report showed that withdrawal from repeated non-contingent cocaine exposure in mice correlated with increased in mEPSC amplitude in NAc core MSNs, along with a non-significant trend toward increased mEPSC frequency (Jedynak et al., 2016). While this study did not involve the targeted evaluation of defined MSN cell types, our data does not suggest that D_1R- and D_2R-expressing MSNs differ substantially in this regard. Thus, the manifestation of enhanced AMPAR-dependent neurotransmission in NAc core MSNs that emerges during withdrawal from repeated cocaine may be sensitive to finer details of study design, including mouse strain, animal handling procedures, or recording conditions.

The RNAi-dependent suppression of GIRK1 and GIRK2 in layer 5/6 of the mouse PrLC correlated with an enhanced motor-stimulatory response to an initial injection of cocaine ("pre-sensitization"), akin to the cocaine-induced motor activity phenotypes seen in both CaMKIICre(+):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice. Given that layer 5/6 PrLC pyramidal neurons make a prominent glutamatergic projection to the NAc core (Kalivas, 2009; Koob and Volkow, 2010), our current working hypothesis is that the loss of GIRK-dependent signaling in layer 5/6 PrLC pyramidal neurons underlies the increase in mEPSC frequency observed in D_1R-expressing MSNs in the NAc core. Our analysis of Cre-dependent recombination in CaMKIICre(+):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ lines, however, has revealed deficits in GIRK-dependent signaling in BLA pyramidal neurons (this study) and CA1 pyramidal neurons in the dorsal hippocampus (Victoria et al., 2016), in addition to layer 5/6 PrLC pyramidal neurons. Thus, the adaptations in excitatory neurotransmission seen in the NAc in mice with deficient GIRK-dependent signaling may reflect the combined influence of enhanced excitability in multiple afferent glutamatergic inputs to the NAc core.

Consistent with the reported expression profile of GIRK channel subunits in the rodent central nervous system (Karschin et al., 1996), we found that GIRK mRNA and protein levels were 5-10-fold lower in the NAc than mPFC. In addition, GIRK-like responses to $GABA_BR$ activation were seen in only a small fraction (10%) of D_1R- and D_2R-expressing neurons. While the subset of NAc neurons exhibiting these responses is unknown, our preliminary efforts do not suggest that these responses are linked to the small fraction of MSNs expressing both D_1R and D_2R, or to cholinergic interneurons (data not shown). Collectively, these findings support the contention that adaptations in excitatory neurotransmission seen in NAc core MSNs in our loss-of-function models are driven by enhanced excitability in afferent glutamatergic inputs to the NAc.

While the loss of GIRK-dependent signaling in forebrain pyramidal neurons is sufficient to evoke an electrophysiological hallmark that has been linked to repeated cocaine treatment, this genetic manipulation did not recapitulate the full extent of adaptations observed in the NAc during withdrawal following repeated cocaine exposure. Indeed, enhanced mEPSC frequency was seen in both D_1R- and D_2R-expressing MSNs in the NAc core of cocaine-treated mice, whereas only D_1R-expressing MSNs exhibited this adaptation in our CaMKIICre(+):$Girk^{fl/fl}$ models. Notably, GIRK-dependent signaling in VTA DA neurons is also suppressed by *in vivo* cocaine exposure (Arora et al., 2011), and this adaptation is predicted to enhance DA neurotransmission in the NAc. Selective genetic ablation of GIRK channels in midbrain DA neurons, however, did not enhance the frequency or amplitude of AMPAR-dependent mEPSCs in either NAc core or shell MSNs (McCall et al., 2016). Moreover, the loss of GIRK-dependent signaling in forebrain pyramidal neurons did not preclude the development of behavioral sensitization to cocaine, indicating that other processes also contribute to this phenomenon. Collectively, these lines of evidence support the contention that the cocaine-induced suppression of GIRK-dependent signaling in afferents to the NAc may promote or facilitate some, but not all, of the long-lasting adaptations and behaviors associated with repeated cocaine exposure.

Statement of interest. None

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FIGURE LEGENDS

Figure 1. Loss of GIRK-dependent signaling in pyramidal neurons from CaMKIICre:*Girk*$^{fl/fl}$ mice

A) Representative segments of immunoblots for GIRK1 (upper, cropped at 52 and 72 kDA) and β-actin (lower, cropped at 38 and 52 kDA) in mPFC micropunches from CaMKIICre(-):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk1*$^{fl/fl}$ mice. The histogram shows GIRK1 band intensity normalized to β-actin within sample, as a function of genotype (n=5 samples per group). Symbols: ***P*<0.01 vs. Cre(-) control.

B) Somatodendritic inhibitory currents (V_{hold}= -60 mV) evoked by baclofen (200 μM) in layer 5/6 PrLC pyramidal neurons in slices from CaMKIICre(-):*Girk1*$^{fl/fl}$ (black) and CaMKIICre(+):*Girk1*$^{fl/fl}$ (gray) mice. Currents were accompanied by a decrease in input resistance (not shown) and were reversed by the $GABA_BR$ antagonist CGP54626 (2 μM).

C) Summary of baclofen-induced currents in Cre(-) and Cre(+) layer 5/6 PrLC pyramidal neurons from CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ mouse lines (n=6-11 recordings per genotype). Symbols: **,****P*<0.01 and 0.001, respectively, vs. Cre(-) control.

Figure 2. Excitatory neurotransmission in NAc MSNs of CaMKIICre:*Girk*$^{fl/fl}$ mice

A) Coronal sections (120 μm) through the NAc of D_1R-tdTomato (left) and D_2R-EGFP (right) mice, with core and shell regions delineated with dotted lines. Abbreviations: AC, anterior commissure; CPu, caudate putamen. Scale bar = 100 μm.

B) Representative traces of mEPSCs (V_{hold} = -70 mV) in D_1R-expressing MSNs in the NAc core of CaMKIICre(-):*Girk1*$^{fl/fl}$ and CaMKIICre(+):*Girk1*$^{fl/fl}$ mice; observed events were blocked by the ionotropic glutamate receptor antagonist, kynurenic acid (2 mM).

C) Representative traces of mEPSCs in D_1R-expressing MSNs in the NAc core of CaMKIICre(-):*Girk2*$^{fl/fl}$ and CaMKIICre(+):*Girk2*$^{fl/fl}$ mice; observed events were blocked by kynurenic acid (2 mM).

D) Summary of mEPSC frequencies in D_1R-expressing and D_2R-expressing MSNs in the NAc core of CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ mice. Group sizes ranged from 8-17 per genotype and cell type. Symbols: *,**P<0.05 and 0.01, respectively, vs. Cre(-), within mouse line and MSN subtype.

E) Summary of mEPSC amplitudes in D_1R- and D_2R-expressing MSNs in the NAc core of CaMKIICre:*Girk1*$^{fl/fl}$ and CaMKIICre:*Girk2*$^{fl/fl}$ mice.

Figure 3. Cocaine-induced increase in glutamatergic signaling in NAc core MSNs

A) Depiction of the repeated cocaine treatment regimen. All subjects were exposed to 1 d of handling (H) and 2 d of i.p. saline injections (S_1 and S_2), and then randomly assigned to saline or cocaine treatment groups. Each subject then received 5 injections (1 per day) of either saline or cocaine (15 mg/kg i.p.). After a 10-14 d period, slices of the NAc were prepared for electrophysiological (E) analysis.

B) Total distance traveled in the open field (in m) during the 60-min period immediately after handling or injection on each day of testing, for D_1R-tdTomato and D_2R-EGFP mice in saline (black) and cocaine (gray) treatment groups. No difference in baseline activity (H, S_1, S_2) was observed for D_1R-tdTomato or D_2R-EGFP mice in the saline or cocaine treatment groups. Over the rest of the study, main effects of drug treatment ($F_{1,5}$=52.9, P<0.001) and injection number ($F_{4,20}$=4.6, P<0.01), and an interaction between drug treatment and injection number ($F_{4,20}$=5.5, P<0.01), were observed for D_1R-tdTomato mice. Main effects of drug treatment ($F_{1,6}$=42.7, P<0.001) and injection number ($F_{4,24}$=8.9, P<0.001), as well as an interaction ($F_{4,24}$=8.0, P<0.001), were also observed for D_2R-EGFP mice. Symbols: *,**,***P<0.05, 0.01, and 0.001, respectively, vs. saline (within genotype and injection number); $^{\#\#\#}P$<0.001 vs. injection 1 (within genotype and drug treatment).

C) Representative traces of mEPSCs (V_{hold} = -70 mV) in D_1R-expressing MSNs in the NAc core of mice treated with saline (black, upper) or cocaine (gray, lower).

D) Summary of mEPSC frequency in D_1R-expressing and D_2R-expressing MSNs in the NAc core of saline- and cocaine-treated mice. Group sizes ranged from 10-14 per genotype and treatment. Symbols: *P<0.05 vs. saline (within genotype).

E) Summary of mEPSC amplitude in D_1R-expressing and D_2R-expressing MSNs in the NAc core of saline- and cocaine-treated mice.

Figure 4. GIRK channel expression in the mouse NAc

A) mRNA levels for GIRK1, GIRK2, and GIRK3 in micropunches containing the mPFC (black) and NAc (gray), profiled in samples from 7-8 adult male C57BL/6J mice. GIRK mRNA levels were normalized to GAPDH mRNA in each sample. Symbols: ****P*<0.001 vs. mPFC (within subunit).

B) Total protein levels for GIRK1, GIRK2, and GIRK3 in micropunches containing the mPFC (black) and NAc (gray), profiled in samples from 8 male C57BL/6J mice. GIRK subunit band intensity was normalized to the level of β-actin in each sample. Symbols: ****P*<0.001 vs. mPFC (within subunit).

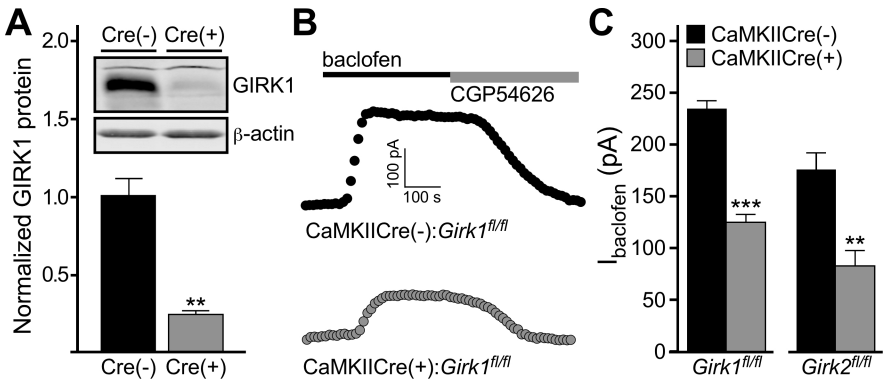
C) Impact of baclofen (200 μM) on somatodendritic holding currents (V_{hold} = -60 mV) in D_1R-expressing (upper traces) and D_2R-expressing (lower traces) MSNs in the NAc core. Representative examples of traces showing no response to baclofen as well as the lone experiment in each cell type that showed a baclofen-induced response are provided. Baclofen-induced currents in the lone responders in each cell type were accompanied by decreased input resistance (not shown), and were reversed by the $GABA_B$R antagonist CGP54626 (2 μM). Scale bars: 100 pA/100 s.

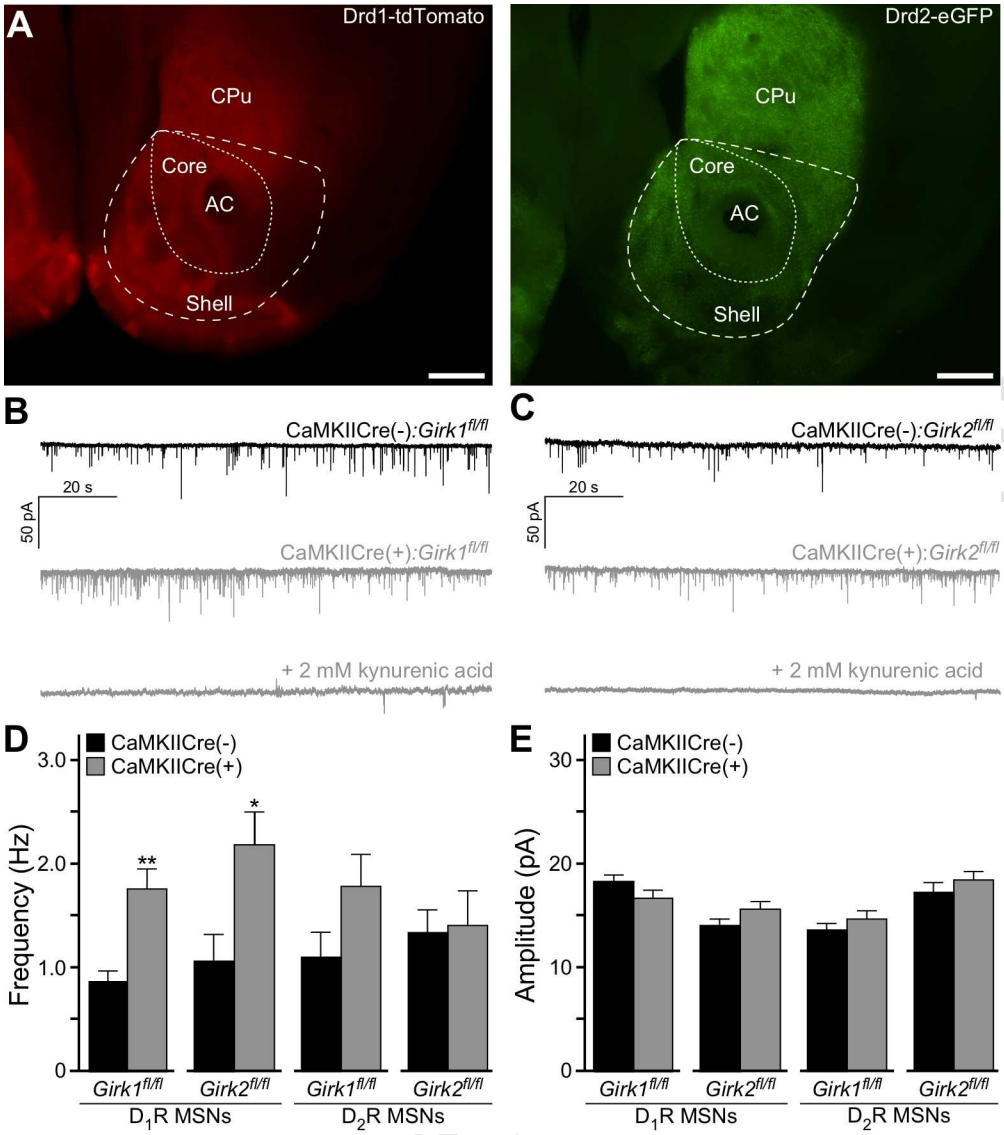
Figure 5. Cocaine-induced locomotor activity in CaMKIICre:*Girk*$^{fl/fl}$ mice

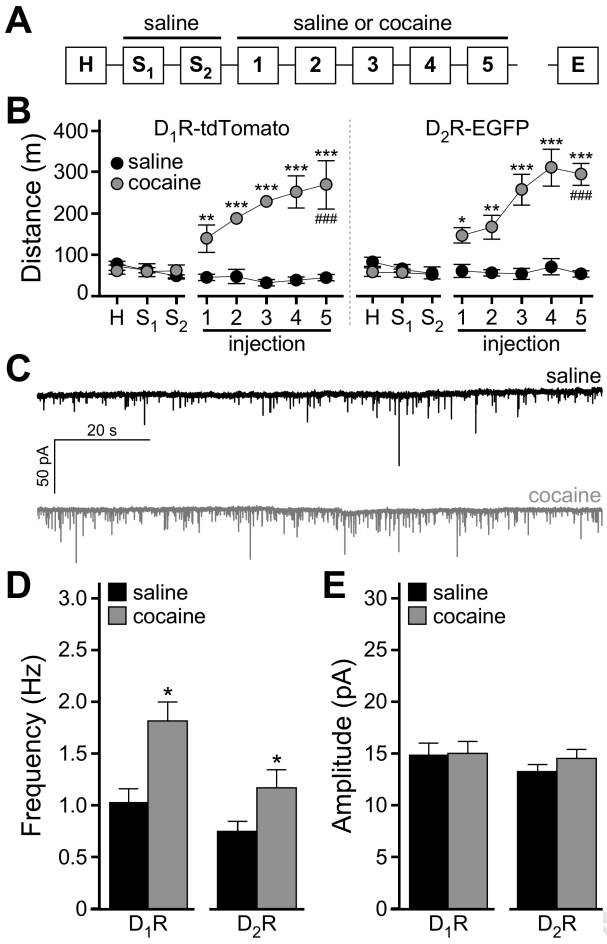
A) Depiction of the repeated cocaine treatment paradigm, which included 1 d of handling (H), 2 d of saline injection (S_1 and S_2), and 5 injections of cocaine (15 mg/kg i.p.) for all subjects. Following a 10-14 d period of abstinence, each subject was given a 6th (test/T) injection of cocaine (15 mg/kg i.p.).

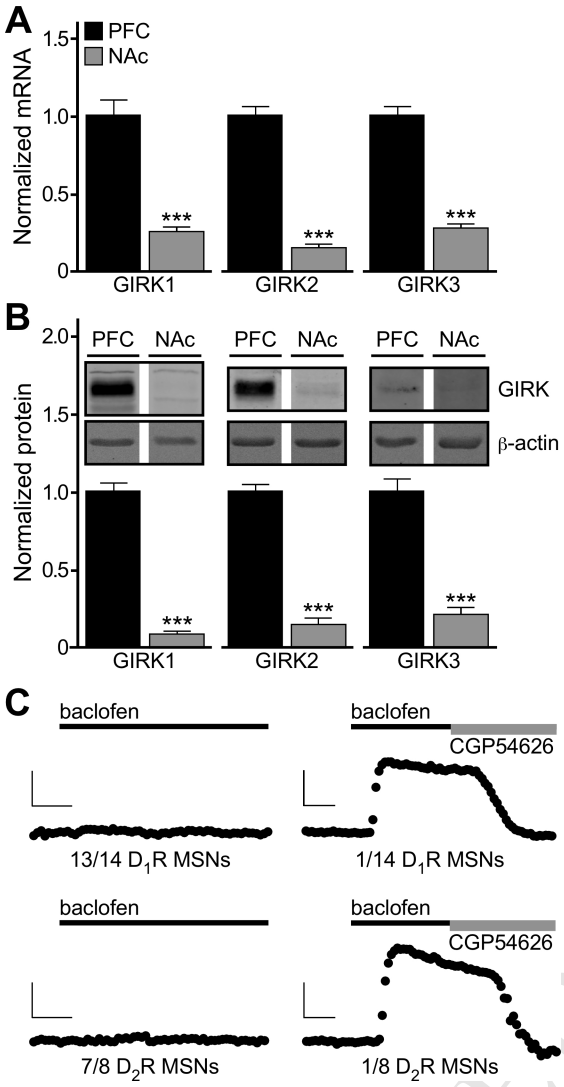
B) Total distance traveled in the open field (in m) during the 60-min period immediately after handling or injection on each day of testing, for CaMKIICre(-):*Girk1*$^{fl/fl}$ (black) and CaMKIICre(+):*Girk1*$^{fl/fl}$ (gray) mice. Symbols: ***P*<0.01 (main effect of genotype); $^{###}$*P*<0.001 (main effect of cocaine injection number); $^{+,+++}$*P*<0.05 and 0.001, respectively, vs. cocaine injection 1 (within genotype; paired two-tailed *t*-test).

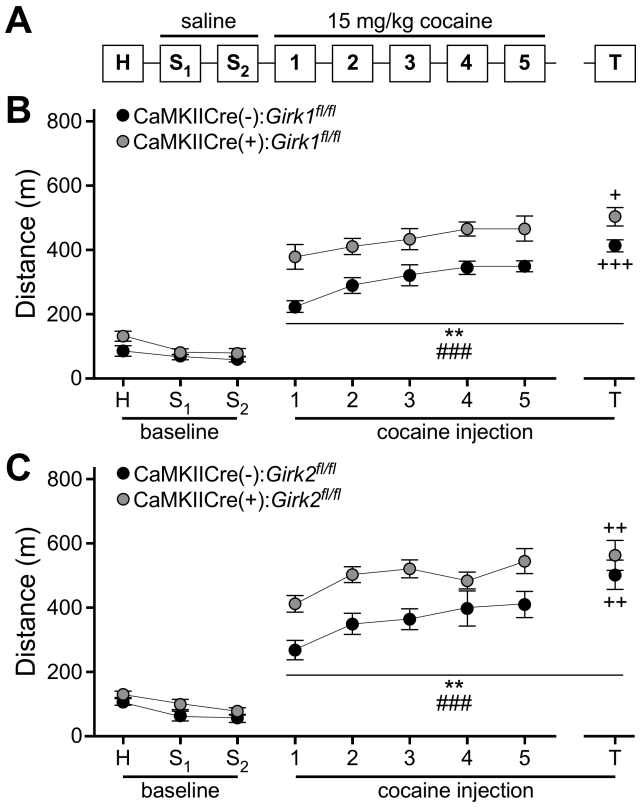
C) Total distance traveled in the open field (in m) for CaMKIICre(-):*Girk2*$^{fl/fl}$ (black) and CaMKIICre(+):*Girk2*$^{fl/fl}$ (gray) mice. Symbols: ***P*<0.01 (main effect of genotype); $^{\#\#\#}$*P*<0.001 (main effect of cocaine injection number); $^{++}$*P*<0.01 vs. cocaine injection 1 (within genotype; paired two-tailed *t*-test).




A
Drd1-tdTomato
CPu
Core
AC
Shell
Drd2-eGFP
CPu
Core
AC
Shell
B
CaMKIICre(-):Girk1fl/fl
20 s
50 pA
CaMKIICre(+):Girk1fl/fl
+ 2 mM kynurenic acid
C
CaMKIICre(-):Girk2fl/fl
20 s
50 pA
CaMKIICre(+):Girk2fl/fl
+ 2 mM kynurenic acid
D
CaMKIICre(-)
CaMKIICre(+)
Frequency (Hz)
3.0
2.0
1.0
0
**
*
Girk1fl/fl
Girk2fl/fl
Girk1fl/fl
Girk2fl/fl
D1R MSNs
D2R MSNs
E
CaMKIICre(-)
CaMKIICre(+)
Amplitude (pA)
30
20
10
0
Girk1fl/fl
Girk2fl/fl
Girk1fl/fl
Girk2fl/fl
D1R MSNs
D2R MSNs







HIGHLIGHTS

- *GIRK channel ablation in pyramidal neurons enhances AMPAR signaling in the NAc core*
- *The AMPAR enhancement induced by GIRK loss is seen in D_1R-expressing NAc core MSNs*
- *Withdrawal from repeated cocaine also enhances AMPAR signaling in NAc core MSNs*
- *GIRK channels are not widely expressed in D_1R- or D_2R-expressing NAc core MSNs*
- *GIRK channel loss in pyramidal neurons enhances cocaine-induced motor activity*