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GIRK channel activity in prelimbic pyramidal neurons regulates the extinction of cocaine conditioned place preference in male mice

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Abstract

Drug-induced neuroadaptations in the prefrontal cortex (PFC) have been implicated in drug-associated memories that motivate continued drug use. Chronic cocaine exposure increases pyramidal neuron excitability in the prelimbic subregion of the PFC (PL), an adaptation that has been attributed in part to a suppression of inhibitory signalling mediated by the $GABA_B$ receptor ($GABA_BR$) and G protein-gated inwardly rectifying K^+ (GIRK/Kir3) channels. Although reduced GIRK channel activity in PL pyramidal neurons enhances the motor-stimulatory effect of cocaine in mice, the impact on cocaine reward and associated memories remains unclear. Here, we employed Cre- and CRISPR/Cas9-based viral manipulation strategies to evaluate the impact of GIRK channel or $GABA_BR$ ablation in PL pyramidal neurons on cocaine-induced conditioned place preference (CPP) and extinction. Neither ablation of GIRK channels nor $GABA_BR$ impacted the acquisition of cocaine CPP. GIRK channel ablation in PL pyramidal neurons, however, impaired extinction of cocaine CPP in male but not female mice. Since ablation of GIRK channels but not $GABA_BR$ increased PL pyramidal neuron excitability, we used a chemogenetic approach to determine if acute excitation of PL pyramidal neurons impaired the expression of extinction in male mice. While acute chemogenetic excitation of PL pyramidal neurons induced locomotor hyperactivity, it did not impair the extinction of cocaine CPP. Lastly, we found that persistent enhancement of GIRK channel activity in PL pyramidal neurons accelerated the extinction of cocaine CPP. Collectively, our findings show that the strength of GIRK channel activity in PL pyramidal neurons bi-directionally regulates cocaine CPP extinction in male mice.

KEYWORDS
cocaine, conditioned place preference, extinction, $GABA_B$ receptor, Kir3

1 | INTRODUCTION

Drugs with addictive potential induce neuroadaptations throughout the mesocorticolimbic system. These neuroadaptations can provoke negative affective states and promote the formation of long-term memories of environmental stimuli associated with drug use.[1,2] Over time, exposure to just the stimuli (e.g., context or cues) is sufficient to trigger physiological and psychological states that motivate continued drug use and relapse after abstinence.[1,3,4] From a therapeutic perspective, treating negative affective states and enhancing inhibitory control over drug-conditioned responses is crucial for preventing relapse.

In individuals with substance use disorders, the prefrontal cortex (PFC) is hyperactivated by exposure to drug-associated stimuli, and the level of hyperactivity correlates with drug craving.[1] In rodents, activity in the prelimbic subregion of the medial PFC (PL) is important for cue-induced drug seeking[5]; activity of PL pyramidal neurons projecting to the nucleus accumbens core is particularly relevant in this context.[6] PL pyramidal neurons encode reward-predictive cues,[7] and they undergo synaptic potentiation following drug exposure that correlates with drug-seeking behaviour.[8,9] These neurons also regulate affect-related behaviours and cognitive functions relevant to addiction,[10,11] including extinction learning,[12] a process whereby the salience of a memory is reduced when a stimulus is repeatedly presented without reinforcement. Extinction of drug reward memories is a learning process that could be targeted by behavioural and/or pharmacological interventions to prevent relapse.[4] The success of such an approach, however, depends on the elucidation of drug-induced plasticity mechanisms and neural correlates that underlie addiction-related behaviours. Given the overlap between functions regulated by the PL and those disrupted in addiction, considerable effort has been directed at identifying drug-induced plasticity in this brain region.

Repeated cocaine exposure can drive a persistent increase in the intrinsic excitability of PL pyramidal neurons.[9,13,14] Suppression of inhibitory G protein-dependent signalling mediated by the GABAB receptor ($GABA_BR$) and its prominent somatodendritic effector—the G protein-gated inwardly rectifying K^+ (GIRK) channel—may contribute to this phenomenon.[15] Indeed, repeated cocaine treatment in mice correlates with a durable suppression of $GABA_BR$-GIRK somatodendritic currents and increased internalization of $GABA_BR$ and GIRK channels, in layer 5/6 PL pyramidal neurons.

Previously, we reported that genetic suppression of GIRK channel activity in PL pyramidal neurons of drug-naive mice potentiates the acute motor-stimulatory effect of cocaine.[16] GIRK channel ablation in PL pyramidal neurons also impacted measures of affect and impaired working memory and diminished cognitive flexibility in male, but not female, mice.[17] Because GABAergic signalling in the PL has been implicated in the expression and extinction of context-evoked cocaine memories,[12,18,19] we sought to determine the impact of PL pyramidal neuron-specific ablation of GIRK channels or $GABA_BR$ on cocaine conditioned place preference (CPP) and extinction. We also investigated the behavioural impact of acute chemogenetic excitation of, or GIRK channel overexpression in, PL pyramidal neurons. Our findings inform the neuronal populations and molecular mechanisms that mediate extinction learning in the PFC and provide further support for the GIRK channel as a potential target for treatment of substance use disorders.

2 | MATERIALS AND METHODS

2.1 | Animals

All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee. The generation of *Girk1*$^{fl/fl}$ mice was described previously.[20] CaMKIICre (B6.Cg-Tg [Camk2a-cre] T29-1Stl/J, RRID:IMSR_JAX:005359) and Cre-dependent Cas9GFP (B6J.129(B6N)-Gt (ROSA)26Sortm1(CAG-cas9*,-EGFP)Fezh/J, RRID: IMSR_JAX:026175) knock-in lines were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained by backcrossing against the C57BL/6J strain. Heterozygous CaMKIICre(+) and homozygous Cas9GFP(+/+) lines were crossed to yield CaMKIICre(+): Cas9GFP(+/+) mice. Male C57BL/6J mice were purchased from The Jackson Laboratory. Mice were group housed, maintained on a 14:10-h light/dark cycle and were provided ad libitum access to food and water.

2.2 | Chemicals

Baclofen was purchased from Sigma Millipore (Burlington, MA), CGP54626 and clozapine-N-oxide (CNO) were purchased from Tocris Bioscience (Bristol, UK), and cocaine hydrochloride was obtained through Boynton Health Pharmacy at the University of Minnesota (Minneapolis, MN).

2.3 | Viral vectors

Generation and characterization of the guide RNA (gRNA) targeting *Gabbr1* ($GABA_B1$; referred to throughout as GB1) was described previously[21]; this gRNA targets a shared sequence in the two most abundant splice isoforms—*Gabbr1a* and *Gabbr1b*. pAAV-CaMKIIα-GIRK2c (eGFP), pAAV-CaMKIIα-hM3Dq(mCherry) and pAAV-CaMKIIα-mCherry plasmids were generated by the VVCC using standard cloning techniques and pAAV-CaMKIIα-hChR2(C128S/D156A)-mCherry (RRID:Addgene_35502, a gift from Karl Deisseroth) as the backbone, as described previously.[16,20] AAV8-CaMKIIα-Cre (mCherry) and AAV8-CaMKIIα-eGFP were purchased from the University of North Carolina Vector Core (Chapel Hill, NC). All other viral vectors were packaged in AAV8 serotype by the VVCC; viral titers were between 0.2 and 4×10^{14} genocopies/ml.

2.4 | Intracranial viral manipulations

Intracranial infusion of virus (400 nl per side) into the PL (+2.00 mm AP, ±0.45 mm ML, −1.60 mm DV) of mice (7–8 weeks) was

performed as described.[16] Viral load and coordinates were optimized to yield extensive coverage of the PL along anterior/posterior and medial/ventral axes, with limited spread into the adjacent anterior cingulate, medial orbital and infralimbic cortices. After surgery, animals were allowed 2–3 weeks (chemogenetic or GIRK2 overexpression studies) or 4–5 weeks (GIRK1 or GB1 ablation studies) for full recovery and gene expression before behavioural and/or electrophysiological assessments. The scope and accuracy of viral targeting were assessed using fluorescence microscopy as previously described.[16] Brightfield and fluorescent images were overlaid and evaluated using the mouse brain atlas,[22] and only data from mice in which >70% of viral-driven bilateral fluorescence was confined to the PL were included in the final analysis.

2.5 | Slice electrophysiology

Somatodendritic currents evoked by baclofen (200 μM) were recorded in layer 5/6 PL pyramidal neurons from 11- to 13-week-old mice, as described.[23] Peak current amplitudes were analysed using Clampfit v. 10.7 software (Molecular Devices; San Jose, CA). For rheobase assessments, cells were held at 0 pA in current-clamp mode and given 1-s current pulses, beginning at −60 pA and increasing in 20-pA increments. Rheobase was identified as the injection step at which initial spiking was elicited.

2.6 | Behavioural studies

Mice underwent a behavioural test battery that included elevated plus maze (EPM), forced swim test (FST) and cocaine CPP. For EPM, mice were acclimated to the testing room (1 h) and handling (5 min), 1 day prior to testing. On test day, mice were transferred to the testing room 1 h before evaluation. Mice were then placed in the centre of a lit (~250 lx) EPM (L/W/H: 75 × 10 × 53 cm), facing an open arm and away from the experimenter, and their subsequent activity was recorded for 5 min by video camera. Mice that fell off the maze were excluded from analysis. Time spent in the open arms, closed arms, maze centre and total distance travelled were extracted using ANY-maze 5.2 software (Stoelting Co; Wood Dale, IL). FST was conducted 2–3 days after EPM studies. Mice were transferred to the testing room 1 h before evaluation. Mice were then placed in a 4-L beaker filled with 1.5 L of 23–25°C water, and video was recorded for 6 min using a video camera and ANY-maze 5.2 software (Stoelting Co). The latency to first immobile bout, and percent time immobile during the final 4 min of testing, were analysed by hand using ANY-Maze software.

Cocaine CPP studies were performed 3–4 days after FST experiments, except for chemogenetic studies, wherein EPM and FST performance was not assessed. CPP was performed in two-compartment chambers (Med Associates; Fairfax, VT; L/W/H: 16.76 × 12.7 × 12.7 cm per compartment) housed within sound-attenuating cubicles. The chambers contained custom wall inserts that were constructed from polycarbonate sheets and designed to exhibit two visually distinct (vertical or horizontal striped walls) compartments using black and white electrical tape. Both compartments contained identical overhead lighting (single 2.8-W light bulb), but different flooring (wire mesh or metal rods) to permit tactile discrimination. Mice were acclimated to the testing room (30 min), and handling (5 min), 1–2 days before the CPP paradigm. On Day 1 (baseline testing), mice were placed in the chamber for 20 min with the door separating the compartments open; time spent in each compartment was recorded using Med-PC IV software (Med Associates). On Days 2–4 (conditioning), mice were subjected to two 20-min conditioning sessions, one in the morning (0800–1100) and one in the afternoon (1300–1600). In the morning session, mice were given a saline injection (IP) and confined to the compartment that was preferred on Day 1. In the afternoon sessions, mice were given cocaine (15 mg/kg, IP) and confined to the opposite compartment. On Day 5 (preference testing), mice were placed in the chamber for 20 min with the door open; time spent in each compartment was recorded. On Days 8–9 (extinction training), mice underwent two 20-min extinction training sessions that mirrored the conditioning sessions on Days 2–4, with the exception that saline replaced cocaine in afternoon sessions. On Day 10 (extinction testing), mice were placed in the chamber for 20 min with the door open; time spent in each compartment was recorded. In chemogenetic studies, mice were injected with CNO (2 mg/kg, IP) 30 min prior to extinction testing on Day 10. In GIRK overexpression studies, mice underwent a modified CPP procedure involving only 1 day of extinction training (Day 8) prior to extinction testing (Day 9). For all studies, time spent in each compartment during testing on Day 1 (baseline), Day 5 (preference) and Day 9 or Day 10 (extinction) was analysed. Preference scores were determined by calculating the ratio of time spent in the cocaine-paired side to total time spent in both sides. Movement during the extinction test was measured as the total number of beam breaks within both compartments. Only data from animals that formed a preference for the drug-paired side, designated by greater time spent in the drug-paired side than the saline-paired side during the Day 5 preference test, were included in analyses.

2.7 | Experimental design and statistical analysis

Data are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software; San Diego, CA); the specific statistical tests employed are described in Section 3 and figure legends. When both male and female subjects were used in a study, sex was included as an analysis variable. In all studies, differences were considered significant when $p < 0.05$.

3 | RESULTS

3.1 | GIRK channel ablation in PL pyramidal neurons

Repeated cocaine exposure in mice increases the excitability of layer 5/6 PL pyramidal neurons.[15] This neuroadaptation has been

linked to a suppression of inhibitory signalling mediated by $GABA_BR$ and GIRK channels. GIRK channels formed by GIRK1 and GIRK2 subunits mediate ~50% of the composite $GABA_BR$-dependent somatodendritic current in layer 5/6 PL pyramidal neurons; the GIRK channel component of the $GABA_BR$-dependent current is selectively suppressed by repeated cocaine.[15] To evaluate the behavioural consequences of this cocaine-induced neuroadaptation, we used an established viral Cre ablation approach and conditional *Girk1* knockout (*Girk1*$^{fl/fl}$) mice to suppress GIRK channel activity in PL pyramidal neurons in drug-naïve subjects.[16,17] AAV8-CaMKIIα-Cre (mCherry) or AAV8-CaMKIIα-mCherry vectors were infused into the PL of *Girk1*$^{fl/fl}$ mice (Figure 1A). Following a 4- to 5-week recovery period, we evaluated the electrophysiological impact of Cre and control treatment on mCherry-positive layer 5/6 PL neurons.

Consistent with prior reports,[16,17] Cre-mediated ablation of GIRK1 reduced the composite somatodendritic current evoked by the $GABA_BR$-selective agonist baclofen by ~40% in layer 5/6 PL pyramidal neurons from both male and female subjects (Figure 1B,C); two-way analysis of variance (ANOVA) revealed a main effect of viral treatment ($F(1,21) = 16.53$, $^{***}p = 0.0006$), but no main effect of sex ($F(1,21) = 0.56$, $p = 0.46$) or interaction between sex and viral treatment ($F(1,21) = 0.03$, $p = 0.86$). Moreover, rheobase was decreased in layer 5/6 PL pyramidal neurons from Cre-treated male and female subjects (Figure 1D), consistent with an increase in baseline excitability; two-way ANOVA revealed a main effect of viral treatment ($F(1,21) = 17.69$, $^{***}p = 0.0004$), but there was no main effect of sex ($F(1,21) = 0.49$, $p = 0.49$) or interaction between sex and viral treatment ($F(1,21) = 0.17$, $p = 0.69$). Thus, viral Cre ablation of GIRK1 similarly suppresses GIRK channel activity and enhances baseline excitability in layer 5/6 PL pyramidal neurons from both male and female mice.

To examine the behavioural consequences of this manipulation, we prepared a separate cohort of *Girk1*$^{fl/fl}$ mice treated with Cre or control vectors. After a 4- to 5-week recovery period, we evaluated subject performance in a test battery that began with the elevated plus maze (EPM) and forced swim test (FST) (Figure 1E). Two-way ANOVAs revealed no main effect of sex or interaction between sex and viral treatment for distance travelled (main effect: $F(1,55) = 3.77$, $p = 0.057$; interaction: $F(1,55) = 3.15$, $p = 0.081$) or percent time spent in the open arms (main effect: $F(1,55) = 0.067$, $p = 0.80$; interaction: $F(1,55) = 0.31$, $p = 0.58$) of the EPM. Similarly, there were no main effects of sex or interactions between sex and viral treatment for latency to first immobile bout (main effect: $F(1,57) = 0.042$, $p = 0.84$; interaction: $F(1,57) = 1.04$, $p = 0.31$) or percent time spent immobile (main effect: $F(1,57) = 1.48$, $p = 0.23$; interaction: $F(1,57) = 0.091$, $p = 0.76$) during the FST. As viral Cre-mediated ablation of GIRK channels in PL pyramidal neurons was reported to elicit deficits in affective and cognitive behaviours in male but not female mice,[17] we also analysed our behavioural data independently by sex to increase our ability to detect viral treatment effects. Viral Cre-mediated ablation of GIRK channels in PL pyramidal neurons did not impact total distance travelled (Figure 1F) or percent time spent in the open arms of the EPM (Figure 1G), nor did it impact latency to the first immobile bout (Figure 1H) or percent time spent immobile in the FST (Figure 1I), in either male or female mice.

We next examined the impact of GIRK channel ablation in PL pyramidal neurons on the acquisition and extinction of cocaine CPP (Figure 1J). Three-way repeated measures ANOVA revealed an interaction between sex, session, viral treatment ($F(2,66) = 4.90$, $p = 0.010$). To enhance our ability to detect viral treatment effects, we conducted sex-specific two-way repeated measures ANOVA analyses. For males (Figure 1K), we observed an interaction between viral treatment and session ($F(2,40) = 5.69$, $p = 0.007$). Bonferroni's multiple comparison test revealed no differences between Cre- and control-treated mice in terms of baseline side preference ($p > 0.99$; baseline session), and cocaine CPP after conditioning was comparable for both groups ($p = 0.92$; preference session). Extinction of cocaine CPP was significantly impaired, however, in male Cre-treated subjects relative to controls ($^{**}p = 0.0062$). For females (Figure 1L), we observed a main effect of session ($F(2,26) = 33.39$, $p < 0.0001$), but there was no main effect of viral treatment ($F(1,13) = 1.06$, $p = 0.32$) or interaction between viral treatment and session ($F(2,26) = 0.89$, $p = 0.42$). Collectively, these data suggest that loss of GIRK channels in PL pyramidal neurons does not impact the acquisition of cocaine CPP in either male or female mice, but it does impair extinction of cocaine CPP in male mice.

3.2 | $GABA_BR$ ablation in PL pyramidal neurons

To determine whether loss of $GABA_BR$ recapitulated the behavioural impact of GIRK channel ablation, we used a viral CRISPR/Cas9 approach to ablate $GABA_BR$ in PL pyramidal neurons (Figure 2A). This approach involved infusion of viral vectors harbouring guide RNAs (gRNAs) targeting either the $GABA_BR1$ subunit (U6-gRNA [*Gabbr1*]-hSyn-NLSmCherry) or bacterial β-galactosidase (U6-gRNA [LacZ]-hSyn-NLSmCherry) into the PL of CaMKIICre:Cas9GFP mice, which express Cas9GFP in Cre-positive cells.[24] The *Gabbr1* gRNA was characterized previously and found to selectively ablate $GABA_BR$-dependent signalling in dopamine neurons of the ventral tegmental area in mice.[21]

Following a 4- to 5-week recovery period, we first evaluated the electrophysiological impact of viral treatment on layer 5/6 PL neurons expressing both mCherry (which marked neurons infected with the gRNA-containing vector) and eGFP (which marked neurons expressing Cas9GFP). *Gabbr1* gRNA treatment abolished somatodendritic currents evoked by the $GABA_BR$ agonist baclofen in these neurons, in both male and female mice (Figure 2B,C); two-way ANOVA revealed a main effect of viral treatment ($F(1,24) = 141.9$, $^{****}p < 0.0001$), but there was no main effect of sex ($F(1,24) = 0.57$, $p = 0.46$) or interaction between sex and viral treatment ($F(1,24) = 2.76$, $p = 0.11$). In contrast to GIRK channel ablation in PL pyramidal neurons (Figure 1D),[16,17] $GABA_BR$ ablation did not impact rheobase (Figure 2D); two-way ANOVA revealed no main effect of viral treatment ($F(1,26) = 1.37$, $p = 0.25$) or sex ($F(1,26) = 1.30$,

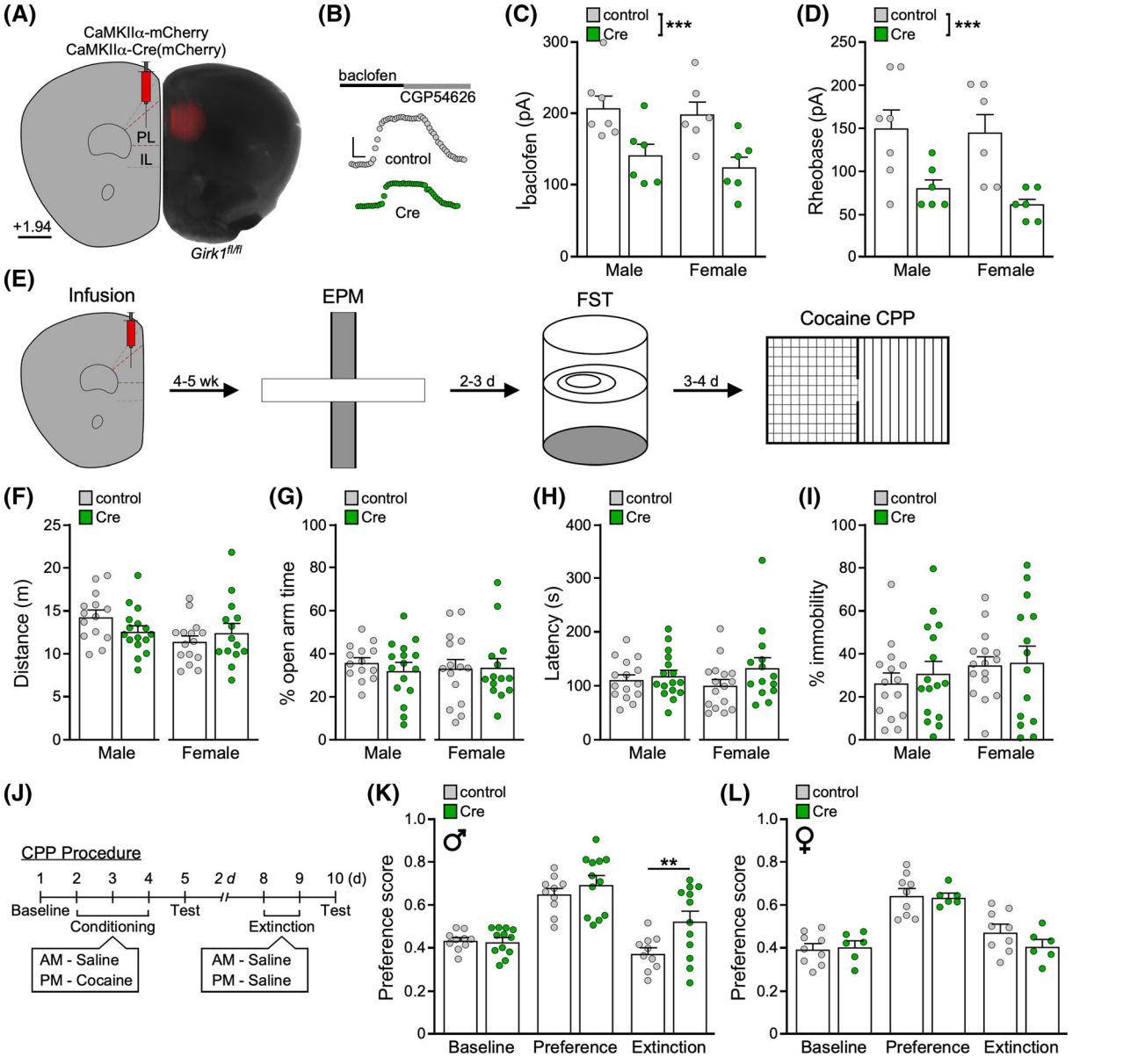

FIGURE 1 Behavioural impact of GIRK channel ablation in PL pyramidal neurons. (A) Example of viral targeting in a *Girk1*$^{fl/fl}$ mouse treated with AAV8-CaMKIIα-Cre (mCherry) vector; scale bar: 650 μm. (B) Somatodendritic currents evoked by baclofen (200 μM) in layer 5/6 PL pyramidal neurons from *Girk1*$^{fl/fl}$ mice treated with CaMKIIα-Cre (mCherry) or control vector (V_{hold} = −60 mV). Currents were reversed by the $GABA_BR$ antagonist CGP54626 (2 μM); scale bars: 100 pA/60 s. (C,D) Baclofen-induced currents and rheobase in layer 5/6 PL pyramidal neurons from *Girk1*$^{fl/fl}$ mice treated with CaMKIIα-Cre (mCherry) or control vector (6–7 recordings/group from 2–3 mice/group); ***$p < 0.001$ (main effect of viral treatment). (E) Timeline of viral infusion and behavioural testing battery. (F) Total distance travelled during the EPM test in Cre- and control-treated male ($t(28) = 1.71$, $p = 0.10$) and female ($t(27) = 0.88$, $p = 0.39$) *Girk1*$^{fl/fl}$ mice (N = 14–16 mice/group; unpaired Student's t test). (G) Percent time spent in the open arms during the EPM test in Cre- and control-treated male ($t(28) = 0.91$, $p = 0.37$) and female ($t(27) = 0.047$, $p = 0.96$) *Girk1*$^{fl/fl}$ mice (unpaired Student's t test). (H) Latency to first immobile bout during the FST test in Cre- and control-treated male ($t(29) = 0.51$, $p = 0.62$) and female ($t(28) = 1.58$, $p = 0.13$) *Girk1*$^{fl/fl}$ mice (unpaired Student's t test). (I) Percent time spent immobile during the FST test in Cre- and control-treated male ($t(29) = 0.63$, $p = 0.53$) and female ($t(19.45) = 0.15$, $p = 0.88$) *Girk1*$^{fl/fl}$ mice (male: Unpaired Student's t test; female: Unpaired Student's t test with Welch's correction). (J) Schematic outlining the CPP procedure. (K,L) Preference scores measured in 20-min baseline, preference and extinction sessions for male and female Cre- and control-treated *Girk1*$^{fl/fl}$ mice conditioned with cocaine. The results from sex-specific two-way repeated measures ANOVA analyses and post hoc tests are presented. Only within-session comparisons between viral treatment groups are shown on the plots in the interest of clarity; **$p < 0.01$ (N = 6–12 mice/group).

$p = 0.26$), or interaction between sex and viral treatment ($F(1,26) = 0.54$, $p = 0.47$). Thus, viral genetic ablation of $GABA_BR$ or GIRK channels suppresses somatodendritic $GABA_BR$-dependent currents in layer 5/6 PL pyramidal neurons from male and female subjects. Only GIRK channel ablation, however, impacts the baseline excitability of these neurons.

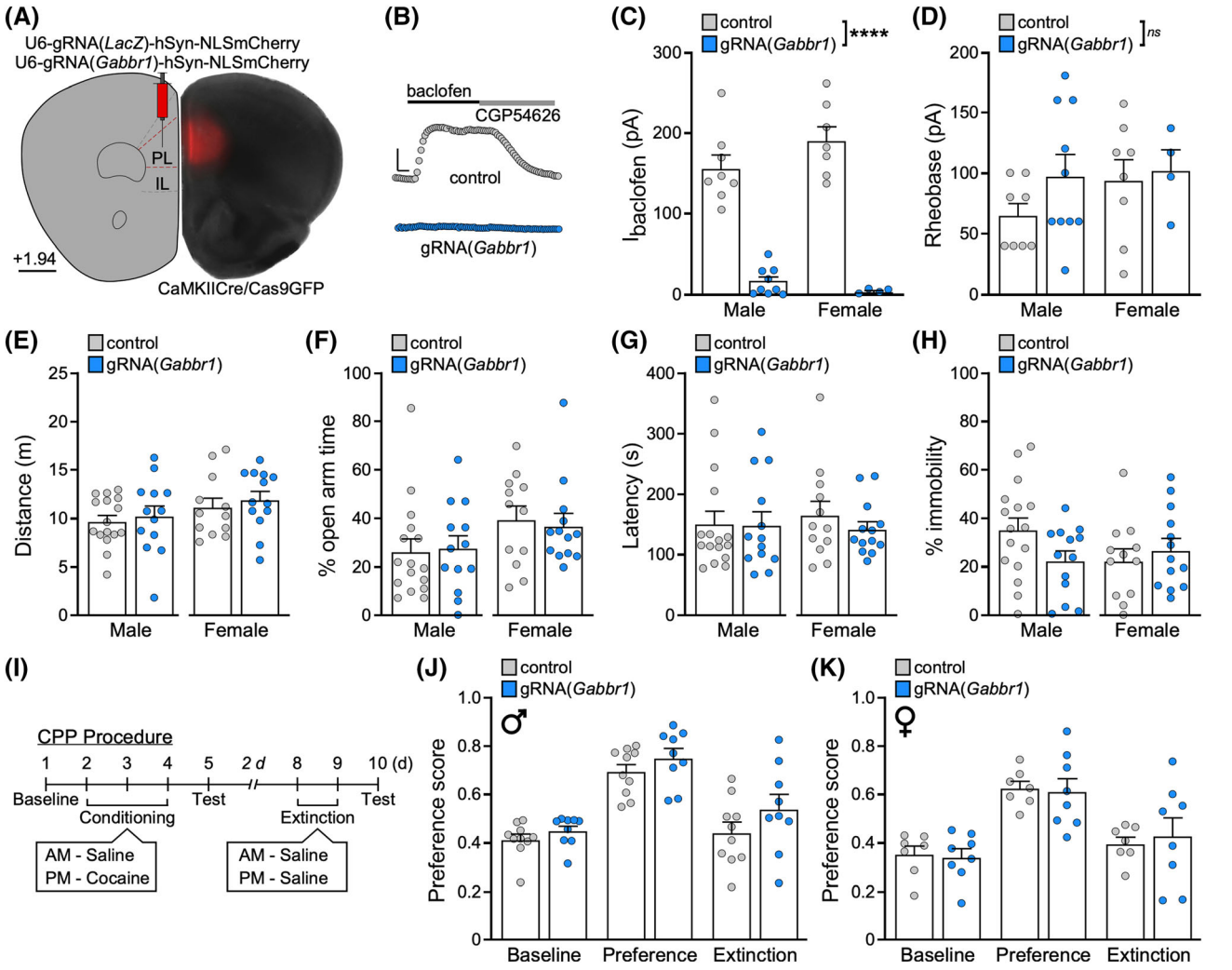

FIGURE 2 Behavioural impact of $GABA_BR$ ablation in PL pyramidal neurons. (A) Viral targeting in a CaMKIICre:Cas9GFP mouse treated with AAV8-U6-gRNA (*Gabbr1*)-hSyn-NLSmCherry vector; scale bar: 650 μm. (B) Somatodendritic currents evoked by baclofen (200 μM) in layer 5/6 PL pyramidal neurons from CaMKIICre:Cas9GFP mice treated with *Gabbr1* or control (LacZ) gRNA. Currents were reversed by the $GABA_BR$ antagonist CGP54626 (2 μM); scale bars: 100 pA/60 s. (C,D) Baclofen-induced currents and rheobase in layer 5/6 PL pyramidal neurons from CaMKIICre:Cas9GFP mice treated with *Gabbr1* or control gRNA (4–10 recordings/group from 2–4 mice/group); $^{****}p < 0.0001$ (main effect of viral treatment). (E) Total distance travelled during the EPM test in *Gabbr1* or control-treated male ($t(27) = 0.47$, $p = 0.64$) and female ($t(23) = 0.63$, $p = 0.53$) CaMKIICre:Cas9GFP mice ($N = 12–16$ mice/group; unpaired Student's *t* test). (F) Percent time spent in the open arms during the EPM test in *Gabbr1* or control-treated male ($t(27) = 0.20$, $p = 0.85$) and female ($t(23) = 0.35$, $p = 0.73$) CaMKIICre:Cas9GFP mice (unpaired Student's *t* test). (G) Latency to first immobile bout during the FST test in *Gabbr1* or control-treated male ($t(27) = 0.05$, $p = 0.96$) and female ($t(23) = 0.93$, $p = 0.36$) CaMKIICre:Cas9GFP mice (unpaired Student's *t* test). (H) Percent time spent immobile during the FST test in *Gabbr1* or control-treated male ($t(27) = 1.95$, $p = 0.062$) and female ($t(23) = 0.70$, $p = 0.49$) CaMKIICre:Cas9GFP mice (unpaired Student's *t* test). (J,K) Preference scores measured in 20-min baseline, preference and extinction sessions during the CPP study for male and female CaMKIICre:Cas9GFP mice treated with *Gabbr1* or control gRNA ($N = 7–10$ mice/group). The results from sex-specific two-way repeated measures ANOVA analyses did not reveal any differences in CPP performance with respect to viral treatment.

The behavioural consequences of $GABA_BR$ ablation in PL pyramidal neurons were evaluated in a separate cohort of mice. As we found for mice lacking GIRK channels in PL pyramidal neurons, two-way ANOVAs revealed no main effect of sex or interaction between sex and viral treatment for distance travelled (main effect: $F(1,50) = 3.30$, $p = 0.076$; interaction: $F(1,50) = 0.018$, $p = 0.89$) or percent time spent in the open arms (main effect: $F(1,50) = 0.58$, $p = 0.45$; interaction: $F(1,50) = 0.13$, $p = 0.72$) of the EPM. Similarly, there were no main effects of sex or interactions between sex and viral treatment for latency to first immobile bout (main effect: $F(1,50) = 0.030$, $p = 0.86$; interaction: $F(1,50) = 0.31$, $p = 0.58$) or percent time spent immobile (main effect: $F(1,50) = 0.74$, $p = 0.40$; interaction: $F(1,50) = 3.48$, $p = 0.068$) during the FST. Sex-specific analyses revealed no impact of $GABA_BR$ ablation in PL pyramidal neurons on total distance travelled (Figure 2E) or percent time spent in the open arms (Figure 2F) of the EPM, or on latency to the first immobile bout (Figure 2G) or percent time spent immobile (Figure 2H) in the FST, for either male or female mice.

In the cocaine CPP study, three-way repeated measures ANOVA revealed a main effect of sex ($F(1,30) = 7.79$, $p = 0.009$). To enhance our ability to detect viral treatment effects, we conducted sex-specific two-way repeated measures ANOVA analyses. For male subjects, we observed a main effect of session ($F(1.806,30.7) = 54.50$, $p < 0.0001$), but neither a main effect of viral treatment ($F(1,17) = 2.40$, $p = 0.14$) nor an interaction between session and viral treatment ($F(2,34) = 0.5695$, $p = 0.57$) was detected (Figure 2J). Similarly, for female subjects, a main effect of session was observed ($F(2,26) = 23.12$, $p < 0.0001$), but there was no main effect of viral treatment ($F(1,13) = 0.003$, $p = 0.96$) or interaction between session and viral treatment ($F(2,26) = 0.21$, $p = 0.82$) (Figure 2K). Thus, $GABA_BR$ ablation in PL pyramidal neurons had no impact on subject performance across the behavioural test battery for either male or female mice.

3.3 | Chemogenetic excitation of PL pyramidal neurons

Ablation of GIRK channels (Figure 1K) but not $GABA_BR$ (Figure 2J) in PL pyramidal neurons impaired the extinction of cocaine CPP in male mice. As GIRK channel ablation increased PL pyramidal neuron excitability (Figure 1D) while $GABA_BR$ ablation was without effect (Figure 2D), we hypothesized that increased PL pyramidal neuron excitability underlies the CPP extinction impairment in male mice. To test this hypothesis, we employed a neuron-specific chemogenetic approach to acutely increase the excitability of PL pyramidal neurons in male mice.[16] AAV vectors harbouring hM3Dq (CaMKIIα-hM3Dq (mCherry)) or control (CaMKIIα-mCherry) were infused into the PL of male C57BL/6J mice (Figure 3A). After a 2- to 3-week recovery period, we tested whether chemogenetic excitation enhanced layer 5/6 PL pyramidal neuron excitability. Bath application of CNO (10 μM) significantly depolarized (Figure 3B; $t[6.95] = 9.05$, $^{****}p < 0.0001$; unpaired Student's t test with Welch's correction) and decreased the rheobase (Figure 3C; $t(12) = 4.55$, $^{***}p = 0.0007$; unpaired Student's t test) of hM3Dq-expressing layer 5/6 PL neurons, relative to controls.

To test whether chemogenetic excitation of PL pyramidal neurons impairs the expression of cocaine CPP extinction, a separate cohort of hM3Dq- or control-treated male C57BL/6J mice were evaluated in the cocaine CPP test (Figure 3D). In this test, CNO (2 mg/kg, IP) was administered to both groups only once, 30 min prior to the extinction

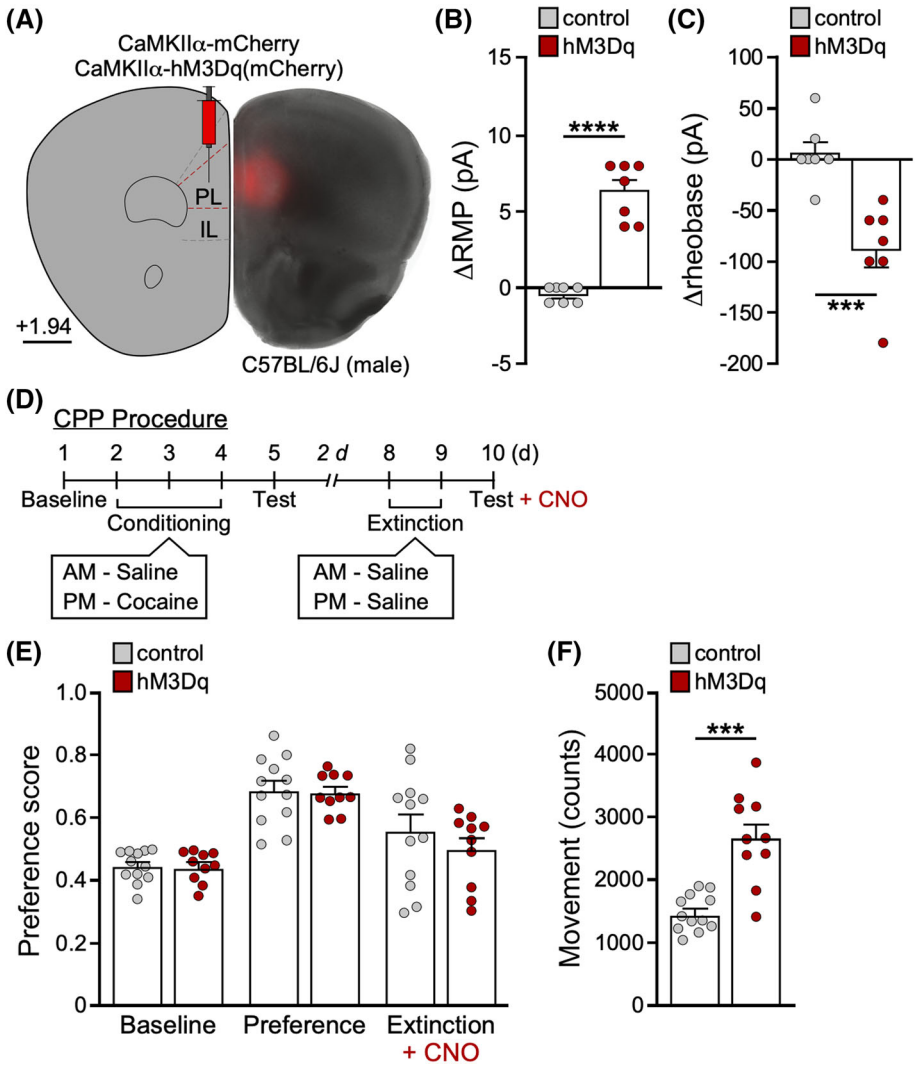

FIGURE 3 Behavioural impact of chemogenetic excitation of PL pyramidal neurons. (A) Example of viral targeting in a C57BL/6J mouse treated with AAV8-CaMKIIα-hM3Dq(mCherry) vector; scale bar: 650 μm. (B) Change in resting membrane potential (ΔRMP) induced by CNO (10 μM) in layer 5/6 PL pyramidal neurons from male C57BL/6J mice treated with CaMKIIα-hM3Dq(mCherry) or control vector (7 recordings/group from 2–3 mice/group); $^{****}p < 0.0001$. (C) Change in rheobase (Δrheobase) induced by CNO (10 μM) in layer 5/6 PL pyramidal neurons from male C57BL/6J mice treated with hM3Dq or control vector (7 recordings/group from 2–3 mice/group); $^{***}p = 0.0007$. (D) Schematic of the cocaine CPP procedure. All subjects were conditioned with cocaine (15 mg/kg, IP) and treated with CNO (2 mg/kg, IP) 30 min before the extinction test. (E) Preference scores measured in 20-min baseline, preference and extinction sessions during the CPP study for male C57Bl/6J mice treated with hM3Dq or control vector. (F) Movement (beam breaks within the CPP apparatus) of male hM3Dq- and control-treated C57BL/6J mice during the extinction test, which was conducted 30 min after CNO injection (2 mg/kg, IP); $^{***}p < 0.001$ ($N = 10$–12 mice/group).

test. Two-way repeated-measures ANOVA revealed a main effect of session ($F(1.51,30.14 = 46.42$, $p < 0.0001$), but there was no main effect of viral treatment ($F(1,20) = 0.42$, $p = 0.52$) or interaction between session and viral treatment ($F(2,40) = 0.90$, $p = 0.42$) (Figure 3E). These data suggest that chemogenetic excitation of PL pyramidal neurons does not disrupt the expression of extinction behaviour in male mice. While chemogenetic excitation of PL pyramidal neurons did not impact CPP extinction in male mice, the manipulation did increase overall movement (Figure 3F; $t[11.41] = 4.96$, $^{***}p = 0.0004$; unpaired Student's t test with Welch's correction), consistent with our prior report.[16]

3.4 | GIRK channel overexpression in PL pyramidal neurons

Given that loss of GIRK channel activity in PL pyramidal neurons impaired extinction in male mice, we next tested whether strengthening GIRK channel activity in these neurons could accelerate extinction of cocaine CPP. GIRK2 overexpression (AAV8-CaMKIIα-GIRK2(eGFP)) or control (AAV8-CaMKIIα-eGFP) vectors were infused into the PL of male C57BL/6J mice (Figure 4A), followed 2–3 weeks later by electrophysiological or behavioural assessments. GIRK2 overexpression increased baclofen-evoked currents in GFP-positive PL neurons (Figure 4B,C; $t[7.23] = 7.30$, $^{***}p = 0.0001$; unpaired Student's t test with Welch's correction). Rheobase was also increased by GIRK2 over-expression (Figure 4D; $t[8.99] = 2.36$, $^{*}p = 0.043$; unpaired Student's t test with Welch's correction), consistent with a reduction in basal neuronal excitability.

Augmentation of GIRK channel activity in PL pyramidal neurons from male C57BL/6J mice did not impact total distance travelled (Figure 4E) or percent time spent in the open arms of the EPM (Figure 4F). While GIRK2 overexpression did not impact latency to the first immobile bout (Figure 4G) in the FST, it did correlate with a modest (but not statistically significant) increase in percent time spent immobile (Figure 4H). The impact of GIRK2 overexpression in PL pyramidal neurons was next assessed in a modified cocaine CPP procedure (Figure 4I). The modified CPP procedure involved only a single extinction training session, in order to reduce the level of extinction so that facilitation of extinction could be detected (i.e., to prevent a floor effect). Two-way repeated measures ANOVA revealed a significant interaction between session and viral treatment ($F(2,42) = 4.38$, $p = 0.019$). While acquisition of CPP did not differ between GIRK2- and control-treated subjects (preference session, $p = 0.206$), extinction was enhanced in GIRK2-treated animals as compared to controls (Figure 4J; $^{**}p = 0.0051$). Thus, strengthening GIRK channel activity in PL pyramidal neurons facilitated extinction of cocaine CPP in male mice.

4 | DISCUSSION

Drug-induced adaptations in inhibitory signalling pathways have been described throughout the mesocorticolimbic system.[13] We previously reported that repeated cocaine exposure increased the excitability of layer 5/6 PL pyramidal neurons, an adaptation correlated with the suppression of $GABA_BR$-GIRK signalling.[15] Prior studies that modelled the loss of GIRK channel activity in PL pyramidal neurons in drug-naïve mice revealed a potentiated motor-stimulatory effect of cocaine,[16] along with disrupted affective behaviours, working memory and cognitive flexibility in male mice.[17] Here, we sought to determine whether viral ablation of GIRK channels and/or $GABA_BR$ impact affective and cocaine reward-related behaviours.

In rats, withdrawal following chronic cocaine exposure has been associated with increased anxiety- and depression-like behaviour,[25–27] as well as altered reactivity of the dorsal PL to anxiogenic stimuli.[28] mPFC neurons also track behavioural states in EPM and FST models.[29,30] Although these observations suggest a link between cocaine-induced dysfunction of the PL and negative affect, we found that performance in EPM or FST tests in either male or female mice was not impacted by ablation of GIRK channels or $GABA_BR$ in PL pyramidal neurons. This outcome was particularly surprising given the work from Hearing and colleagues that showed, using identical reagents, that GIRK channel ablation in PL pyramidal neurons increased open arm time in the EPM and immobility in the FST in male mice.[17] These different behavioural outcomes may relate to the scope of viral targeting, or subtle differences in the EPM or FST procedures and/or testing environments. With respect to the former consideration, optogenetic and chemogenetic manipulations targeting overlapping but distinct PL subareas, projection neurons and neighbouring brain regions have yielded mixed results in preclinical models of anxiety- and depression-related behaviours.[10,11,31] Therefore, hypofunctional $GABA_BR$-GIRK signalling in discrete PL sub-regions or sub-populations of PL pyramidal neurons may contribute to cocaine-induced alterations in affect-related behaviours.

Prolonged cocaine exposure has been correlated with disruption of multiple cognitive functions associated with the mPFC in mice,[32] and in the extinction of learned fear in rats.[33] While GIRK channel ablation did not impact the acquisition of cocaine CPP, the manipulation impaired extinction in male, but not female, mice. This finding aligns with a recent report demonstrating that chemogenetic inhibition of a local GABAergic interneuron population in the dorsal mPFC (dmPFC, which includes the PL and anterior cingulate cortex) impaired the extinction of cocaine CPP in male mice.[12] These results support the contention that inhibitory signalling in adjacent PL pyramidal neurons, including perhaps GIRK-dependent signalling, is necessary for the extinction of cocaine CPP in male mice. Interestingly, chronic exposure to stress delayed the extinction of opioid-induced CPP in male mice[34] and, similar to repeated cocaine exposure, suppressed $GABA_BR$-dependent signalling in PL pyramidal neurons in male and female mice.[17] These findings, as well as data presented herein, suggest that GIRK channel plasticity in the PL of male mice may contribute to the stress-induced persistence of drug-conditioned behaviour. Whether reduced GIRK channel activity in PL pyramidal neurons impairs extinction by promoting the retrieval of cocaine-associated memories, and/or disrupting the formation of extinction memories, remains an important topic for future research.

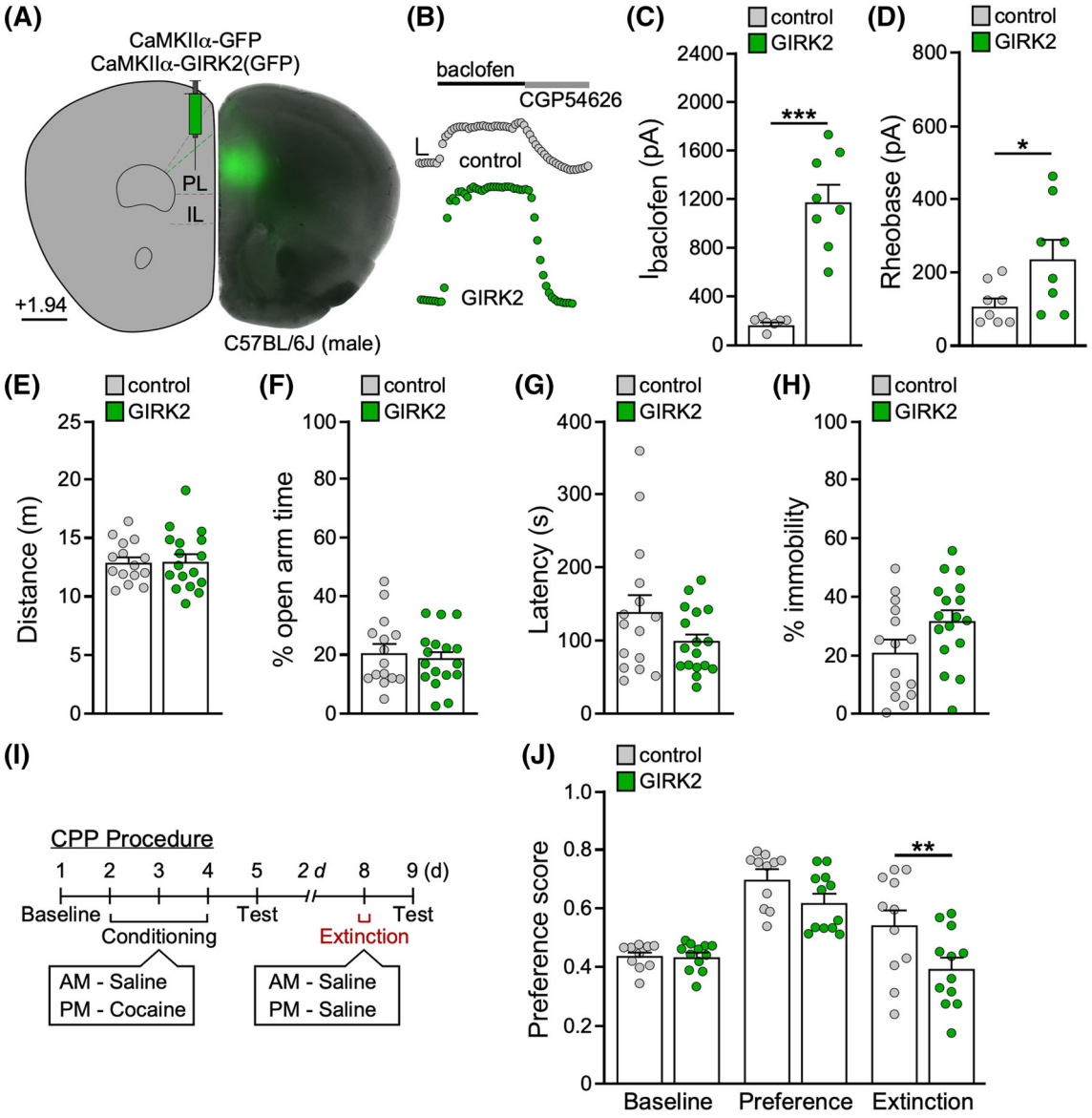

FIGURE 4 Behavioural impact of GIRK channel overexpression in PL pyramidal neurons. (A) Viral targeting in a male C57BL/6J mouse treated with AAV8-CaMKIIα-GIRK2(GFP) vector; scale bar: 650 μm. (B) Currents evoked by baclofen (200 μM) in layer 5/6 PL pyramidal neurons from mice treated with CaMKIIα-GIRK2(GFP) or control vector. Currents were reversed by the $GABA_BR$ antagonist CGP54626 (2 μM); scale bars: 100 pA/50 s. (C,D) Baclofen-induced currents and rheobase in layer 5/6 PL pyramidal neurons from male C57BL/6J mice treated with CaMKIIα-GIRK2(GFP) or control vector (7–8 recordings/group from 3 mice/group); ***$p < 0.001$ and *$p < 0.05$, respectively. (E,F) Distance travelled ($t(30) = 0.19$, $p = 0.85$; unpaired Student's t test) and percent time spent in the open arms ($t(30) = 0.58$, $p = 0.57$; unpaired Student's t test) during the EPM test in male C57BL/6J mice treated with CaMKII⟨-GIRK2(GFP) or control vector ($N = 15–17$ mice/group). (G,H) Latency to first immobile bout ($t(19.50 = 1.56$, $p = 0.14$; unpaired Student's t test with Welch's correction) and percent time spent immobile ($t(30) = 2.00$, $p = 0.054$; unpaired Student's t test) during the FST test by male C57BL/6J mice treated with CaMKIIα-GIRK2(GFP) or control vector. (I) Schematic outlining the modified cocaine CPP procedure, which involved a single day of extinction training prior to the extinction test. (J) Preference scores measured in 20-min baseline, preference and extinction sessions during the CPP study for male C57BL/6J mice treated with GIRK2 overexpression or control vector. Only within-session comparisons between viral treatment groups are shown on the plots in the interest of clarity; **$p < 0.01$ ($N = 11$ mice/group).

GIRK channel ablation in PL pyramidal neurons impaired extinction in male, but not female, mice. GIRK channel activity in, and baseline excitability of, layer 5/6 PL pyramidal neurons is comparable in young adult (60–70 days) male and female mice.[23] Moreover, our electrophysiological validation suggests that viral-mediated ablation of GIRK1 similarly impacts somatodendritic $GABA_BR$-dependent currents in, and the baseline excitability of, layer 5/6 PL pyramidal neurons from male and female mice (Figure 1C,D).[16,17] Thus, the differential impact of GIRK ablation in male and female mice does not appear to be driven by differences in $GABA_BR$-GIRK signalling, the

basal GIRK channel influence on neuronal excitability, or the efficacy of viral-mediated GIRK channel ablation across the sexes. While an explanation for the differential behavioural impact of PL pyramidal neuron-specific ablation of GIRK channels is lacking at present, our findings align nicely with work from Hearing and colleagues showing that this same manipulation impaired cognitive flexibility in an operant task in male, but not female, mice.[17] Diminished cognitive flexibility in male mice lacking GIRK channels in PL pyramidal neurons is a plausible explanation for the noted impairment in extinction, perhaps disrupting the discrimination between an initial cocaine-associated memory and a newer extinction memory.[35] Interestingly, prior studies also support a role for the PL in mediating sex differences in conditioned cocaine-seeking behaviour,[36] as well as extinction of learned fear.[37,38] For example, female rats showed greater learned fear expression during extinction than male rats, and enhanced fear expression in females was associated with sustained PL activation.[38] In addition, microstimulation of the PL increased the expression of learned fear and prevented extinction in male rats.[39]

Since $GABA_BR$ can regulate GIRK channel activity in PL pyramidal neurons,[15,23] and repeated cocaine correlated with internalization of both $GABA_BR$ and GIRK channels in these neurons, we hypothesized that ablation of either $GABA_BR$ or GIRK channel function would yield similar behavioural outcomes. We found, however, that $GABA_BR$ ablation failed to recapitulate the impairment in extinction of cocaine CPP seen in male mice with GIRK channel ablation. Although both manipulations suppressed $GABA_BR$-dependent signalling, key differences between the manipulations could explain their differential influence on physiology and behaviour. Most notably, the loss of GIRK channel activity in PL pyramidal neurons correlated with a decrease in rheobase (increased excitability), whereas loss of $GABA_BR$ had no impact on baseline excitability. This distinction is likely attributable to a loss of basal GIRK channel activity and suggests that there is little tonic $GABA_BR$-dependent signalling in layer 5/6 PL pyramidal neurons, at least in the context of the acutely isolated slice. We cannot formally exclude the possibility, however, that GIRK channels in these neurons are regulated by another inhibitory GPCR that may exhibit tonic activity and/or that is normally engaged during extinction.

Given that GIRK channel ablation increased the basal excitability of layer 5/6 PL pyramidal neurons, we hypothesized that elevated excitability of PL pyramidal neurons underpinned the impairment of CPP extinction in male mice. Acute chemogenetic excitation of PL pyramidal neurons, however, did not alter the expression of extinction in male mice. This finding, combined with the bi-directional influence of persistent GIRK channel manipulations in PL pyramidal neurons on extinction of cocaine CPP, suggest that the CPP extinction impairment is linked to more persistent changes in PL pyramidal neuron excitability and/or changes in excitability evident during extinction training. Indeed, a variety of interventions that occur at various time points before the expression of extinction memory have been shown to modulate extinction.[35] For example, microstimulation of the PL during extinction training was found to impair the extinction of learned fear in male rats.[39] It is also possible, however, that hyperactivity evoked by hM3Dq activation in PL pyramidal neurons masks an extinction impairment via an indiscriminate reduction in apparatus side preference. Alternatively, broad chemogenetic excitation of pyramidal neurons in the PL simply may not affect extinction, because this manipulation does not mimic the endogenous firing patterns and/or engage the specific neuronal ensembles that may be required to overrule extinction of cocaine CPP.

Persistent and recurrent drug memories represent a major obstacle to sustained abstinence.[4,35] Emerging evidence suggests that modulation of the extinction process may represent a promising strategy to selectively weaken drug memories and prevent relapse.[35] Since decreased GIRK channel activity in PL pyramidal neurons impaired the extinction of cocaine CPP in male mice, we sought to determine whether strengthening GIRK-dependent signalling in these neurons would facilitate extinction. We found that augmentation of GIRK channel activity in PL pyramidal neurons, achieved via overexpression of GIRK2, enhanced extinction in male mice. This finding aligns with clinical and preclinical work to support the notion that postsynaptic inhibitory signalling in PL pyramidal neurons can drive extinction learning. For example, GABAergic signalling in the human dorsal anterior cingulate cortex,[40] as well as the homologous rodent PL,[12,41,42] has been suggested to initiate extinction learning in male subjects. Preclinical studies have also shown that systemic delivery of baclofen accelerated the extinction of CPP evoked by methamphetamine and morphine in male rodents.[34,43,44] Furthermore, intra-mPFC baclofen restored behavioural flexibility in aged male rats.[45] Together with our findings, these results suggest that GIRK channel activity in PL pyramidal neurons serves as a key mediator of extinction learning in male mice.

In summary, we report that GIRK channel activity in PL pyramidal neurons bi-directionally regulates the extinction of cocaine CPP in male mice. Although the cocaine-induced weakening of this inhibitory influence may contribute to the persistence of drug-seeking behaviour, therapeutic interventions that restore inhibitory tone may confer resilience to this effect.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Kevin Wickman and Timothy R. Rose were responsible for study concept and design. Timothy R. Rose performed research, analysed data and wrote the first draft of the paper. Ezequiel Marron Fernandez de

Velasco and Eric H. Mitten performed research, and Ezequiel Marron Fernandez de Velasco also analysed data and contributed reagents/analytic tools. All authors critically reviewed content and approved the final version for publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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