Systems/Circuits

Restoration of Kv7 Channel-Mediated Inhibition Reduces Cued-Reinstatement of Cocaine Seeking

Jeffrey Parrilla-Carrero,[1,2]* William C. Buchta,[1,2]* Priyodarshan Goswamee,[1,2] Oliver Culver,[1,2]‡ Greer McKendrick,[1,2]‡ Benjamin Harlan,[1,2] Aubin Moutal,[3] Rachel Penrod,[1,2] Abigail Lauer,[4] Viswanathan Ramakrishnan,[4] Rajesh Khanna,[3] Peter Kalivas,[1,2] and Arthur C. Riegel[1,2]

[1]Department of Neuroscience, [2]Neurobiology of Addiction Research Center, Medical University of South Carolina, Charleston, South Carolina 29425, [3]Department of Pharmacology, University of Arizona, Tucson, Arizona 85724, and [4]Department of Public Health Sciences., Medical University of South Carolina, Charleston, SC 29425

Cocaine addicts display increased sensitivity to drug-associated cues, due in part to changes in the prelimbic prefrontal cortex (PL-PFC). The cellular mechanisms underlying cue-induced reinstatement of cocaine seeking remain unknown. Reinforcement learning for addictive drugs may produce persistent maladaptations in intrinsic excitability within sparse subsets of PFC pyramidal neurons. Using a model of relapse in male rats, we sampled >600 neurons to examine spike frequency adaptation (SFA) and afterhyperpolarizations (AHPs), two systems that attenuate low-frequency inputs to regulate neuronal synchronization. We observed that training to self-administer cocaine or nondrug (sucrose) reinforcers decreased SFA and AHPs in a subpopulation of PL-PFC neurons. Only with cocaine did the resulting hyperexcitability persist through extinction training and increase during reinstatement. In neurons with intact SFA, dopamine enhanced excitability by inhibiting Kv7 potassium channels that mediate SFA. However, dopamine effects were occluded in neurons from cocaine-experienced rats, where SFA and AHPs were reduced. Pharmacological stabilization of Kv7 channels with retigabine restored SFA and Kv7 channel function in neuroadapted cells. When microinjected bilaterally into the PL-PFC 10 min before reinstatement testing, retigabine reduced cue-induced reinstatement of cocaine seeking. Last, using cFos-GFP transgenic rats, we found that the loss of SFA correlated with the expression of cFos-GFP following both extinction and re-exposure to drug-associated cues. Together, these data suggest that cocaine self-administration desensitizes inhibitory Kv7 channels in a subpopulation of PL-PFC neurons. This subpopulation of neurons may represent a persistent neural ensemble responsible for driving drug seeking in response to cues.

Key words: afterhyperpolarization; cocaine; dopamine; Kv7 ion channels; prefrontal cortex; spike-frequency adaptation

Significance Statement

Long after the cessation of drug use, cues associated with cocaine still elicit drug-seeking behavior, in part by activation of the prelimbic prefrontal cortex (PL-PFC). The underlying cellular mechanisms governing these activated neurons remain unclear. Using a rat model of relapse to cocaine seeking, we identified a population of PL-PFC neurons that become hyperexcitable following chronic cocaine self-administration. These neurons show persistent loss of spike frequency adaptation, reduced afterhyperpolarizations, decreased sensitivity to dopamine, and reduced Kv7 channel-mediated inhibition. Stabilization of Kv7 channel function with retigabine normalized neuronal excitability, restored Kv7 channel currents, and reduced drug-seeking behavior when administered into the PL-PFC before reinstatement. These data highlight a persistent adaptation in a subset of PL-PFC neurons that may contribute to relapse vulnerability.

Introduction

The prelimbic prefrontal cortex (PL-PFC) plays an important role in relapse to cocaine seeking. This brain region filters input from sensory and limbic areas and is critical for initiating appetitive behaviors via its projections to the basal ganglia (Graybiel, 2008). Chronic exposure to drugs of abuse causes changes in the PL-PFC and other learning- and memory-related brain struc-

tures that contribute to relapse (Hyman et al., 2006; Williams and Adinoff, 2008). Specifically, after extinction (Ext) from chronic cocaine self-administration (cocaine-SA), activated glutamatergic efferents from the PL-PFC innervating the nucleus accumbens drive cue-induced reinstatement of drug seeking (Kalivas and McFarland, 2003; See, 2009; Stefanik et al., 2016).

Behaviorally relevant cues activate only a small portion of neurons within a brain region (Cruz et al., 2013, 2015). Across many brain regions, these sparse ensembles of activated neurons contribute to the processing of sensory cues and are thought to store the memory trace for such learned associations (Matsumoto and Hikosaka, 2009; Euston et al., 2012; Pinto and Dan, 2015). These neuronal ensembles of activated neurons can be identified using immediate early gene indicators like cFos, whose expression increases following strong neural activation (Morgan et al., 1987; Morgan and Curran, 1988; Herrera and Robertson, 1996). For example, following cue-induced reinstatement, PL-PFC neurons that project to the accumbens core show robust cFos activation, while cells that project to the accumbens shell do not. This PL-PFC–accumbens core pathway is recruited in a dopamine-dependent manner to drive drug seeking in response to cues (McGlinchey et al., 2016). Dopamine signaling can induce cFos expression (Zhang et al., 2006) and promote the formation and maintenance of activity within cellular neural ensembles (O'Donnell, 2003; Puig and Miller, 2012; Miyawaki et al., 2014). However, the cellular adaptations that occur within these activated ensembles in the PL-PFC remain unclear.

At the cellular level, spike frequency adaptation (SFA) and afterhyperpolarizations (AHPs) are two principle forces regulating neuronal synchronization (Crook et al., 1998; Prescott and Sejnowski, 2008; Prescott et al., 2008). SFA decreases responses to nonpreferred stimuli (Benda et al., 2005; Peron and Gabbiani, 2009). However, SFA is typically dynamic and reduced by many neuromodulators. For example, we recently showed that dopamine release from ventral tegmental area (VTA) terminals in the PL-PFC can reduce SFA (Buchta et al., 2017). In other brain regions, reduced SFA in ensembles of cells can enhance memory storage and behavioral responses to cues encoded by the ensemble (Yiu et al., 2014). Across multiple brain regions and behavioral paradigms, reduced SFA is associated with learning (Moyer et al., 1996; McKay et al., 2009; Sehgal et al., 2014). Relative to nondrug reinforcers such as sucrose, drugs of abuse produce robust, long-lasting neural changes that result in stronger, more persistent behavioral responding for drugs (Ciccocioppo et al., 2004; Tunstall and Kearns, 2016). Thus, reinforcement learning for drugs may produce persistent adaptations in SFA that contribute to enduring cue–reward associations and the subsequent ability of cues to elicit drug-seeking behavior. However, whether SFA is disrupted in the PL-PFC following chronic cocaine self-administration and how this contributes to relapse to drug seeking have not been previously examined.

Here, we used *ex vivo* electrophysiology to examine SFA following cocaine self-administration training, extinction, and reinstatement. We assessed whether previous cocaine self-administration experience modified the responses of activated neurons to dopamine. We also examined the function of the Kv7 potassium channels contributing to SFA and assessed whether dysfunction of these channels contributed to drug-seeking behavior in response to cues. Last, we used cFos-green fluorescent protein (GFP) transgenic rats to identify and record from cFos-GFP^{+} neurons and examine whether SFA changes were specific to GFP^{+} neurons. Our results identify a subset of cFos-GFP[+] PL-PFC neurons that show a persistent loss of SFA and Kv7 chan-

nel inhibition following chronic cocaine self-administration. We further show that pharmacological stabilization of Kv7 channels with retigabine restores SFA and Kv7 channel inhibition, and reduces cue-induced reinstatement. These cells may represent an enduring neural ensemble that maintains drug–cue associations and drives drug seeking in response to cues.

Materials and Methods

Animals
We used adult (i.e., more than postnatal day 65) male Sprague Dawley rats (Charles River Laboratories) and transgenic cFos-GFP Long–Evans rats (bred in-house; breeding pair provided by Dr. Bruce Hope, NIDA-IRP, NIH), each weighing between 250 and 300 g at the beginning of experiments. Rats were individually housed in a temperature- and humidity-controlled environment under a reversed 12 h light/dark cycle (lights on at 6:00 P.M.). Rats were acclimated for a minimum of 1 week before surgeries and had access to food and water *ad libitum*. All procedures, killings, and behavioral experiments were performed during the dark cycle, and in accordance with guidelines obtained from the National Institutes of Health for the care of laboratory animals and by the Medical University of South Carolina Institutional Animal Care and Use Committee.

Surgery
Surgical procedures were performed on rats to implant catheters for receiving intravenous cocaine or saline infusions, or intracranial cannulae for the injection of pharmacological compounds directly to the prelimbic cortex. Rats were anesthetized using a ketamine HCl/xylazine mixture (0.57/0.87 mg/kg, respectively, i.p.) followed by ketorolac (2.0 mg/kg, i.p.) and Cefazolin (40 mg, i.p., or 10 mg/0.1 ml, i.v.). Subsequently, intrajugular catheters were implanted as described previously (Spencer et al., 2017). Catheter patency was maintained by daily flushing with cefazolin (1 g/5 ml during surgery and 1 g/10 ml daily for 1 week) and then with heparin for the remainder of the behavioral paradigms. Some rats received bilateral intracranial microinjections of either retigabine or vehicle [dimethylsulfoxide (DMSO)] via cannulae (double 28 ga barrel; 1.2–1.5 mm C-C; Plastics One) that were stereotactically implanted into the brain and reached 1 mm above PL-PFC using the following coordinates relative to bregma (in mm; anteroposterior, +3.0–3.5; dorsoventral, −3.0; mediolateral, ±0.75).

Behavioral procedures
The rationale underlying the self-administration, extinction, and cued reinstatement testing has been described in detail previously (Steketee and Kalivas, 2011; Bossert et al., 2013). The general timeline outlining the different behavioral phases is shown in Figure 1A. For Experiments 1, 2, 4, and 5, rats were trained to self-administer cocaine (i.e., the cocaine-SA group) on a fixed ratio 1 (FR1) schedule of reinforcement (2 h/session). Intravenous injection of cocaine (~0.2 mg/kg/infusion) was delivered contingent to pressing one (active) of two levers in response to a compound light (5 s) and tone (4 kHz, 78 dB, 5 s) cue that was followed by a 20 s time-out period. Each training session lasted 2 h, after which animals were returned to their home cages. A parallel set of time-matched control rats were "yoked" to the cocaine-SA group and received either saline (saline-yoked group) or noncontingent infusions of cocaine (cocaine-yoked group) every time the paired cocaine-SA rat pressed the active lever during the self-administration phase (Fig. 1A).

Experiment 1. Following the maintenance of stable self-administration behavior for 14 consecutive days (>10 infusions/session), the saline-yoked, cocaine-yoked, and cocaine-SA rats were killed 24 h after the last training session to harvest brain tissue for electrophysiological recordings (see Fig. 3).

Experiment 2. Twenty-four hours after the last cocaine self-administration (or yoked) training session, animals entered into extinction training to extinguish lever-pressing behavior (see Fig. 4). During the extinction period (14–19 d), the pressing of the active lever yielded neither cocaine nor the light/tone cue. Once the self-administration behavior was stably extinguished in cocaine-SA rats (<25 active lever presses for 3 consecutive days), saline-yoked and cocaine-SA rats were killed 15 min after
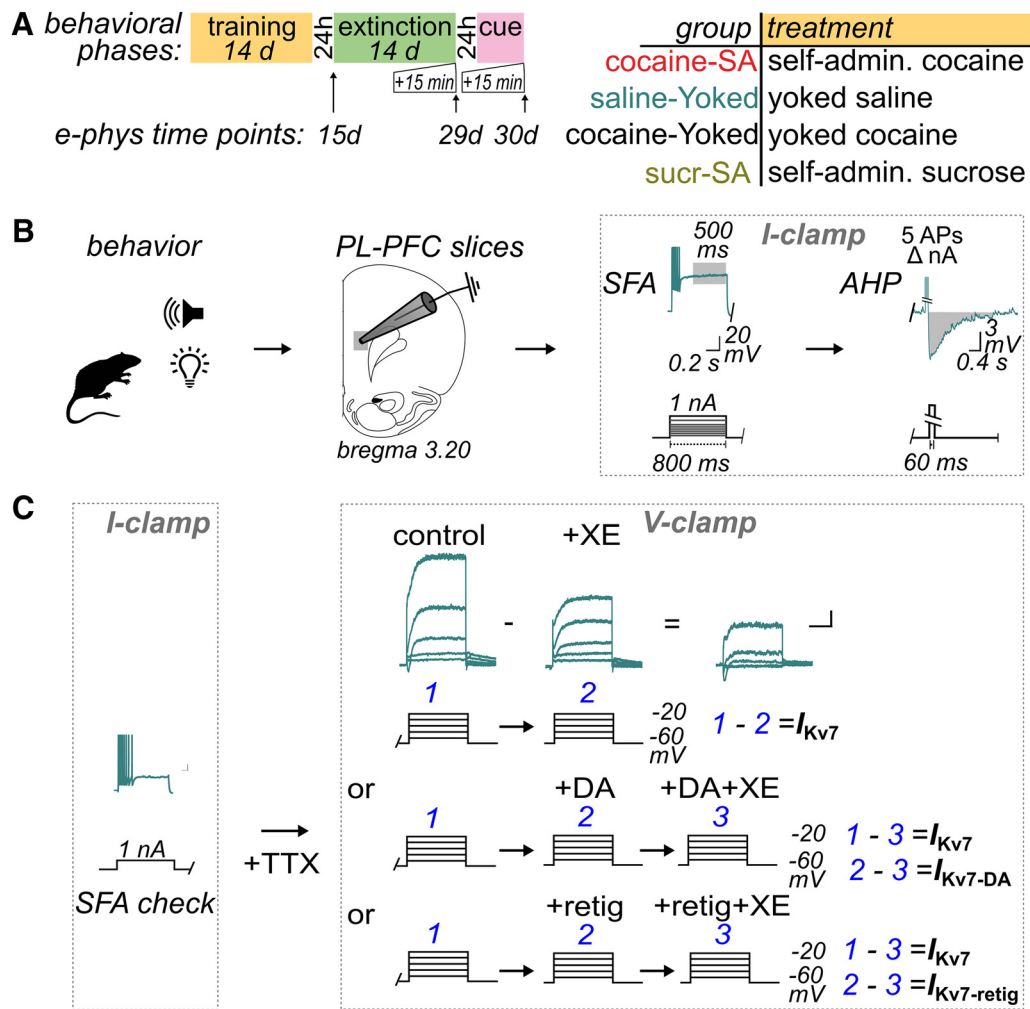
Figure 1. Experimental outline for behavioral training and physiological measurements. ***A***, Timeline shows the different behavioral phases, time points of electrophysiological measurements (e-phys), and the color-coded treatment groups. ***B***, After behavioral end points, brain slices containing PL-PFC were made for current-clamp measurements of SFA (measured during the final 500 ms of the step) and AHPs generated in response to five APs (expressed as the area under the curve). ***C***, In some experiments, after measuring firing to check for SFA with a 1 nA step, the I_{Kv7} was determined in voltage clamp. Currents were evoked with voltage steps (-60 to -20 mV; 800 ms; in 200 nM TTX). Currents generated after 8 min of incubation of the Kv7 channel antagonist, XE-991 (XE; 20 μM, 8 min) was subtracted from control (predrug) currents. To determine the effect of dopamine (DA; 10 μM) or the Kv7 channel stabilizer retigabine (retig; 20 μM) on I_{Kv7}, the currents generated after bath application of DA + XE-991 (20 μM, 8 min) or retigabine + XE-991 (20 μM, 8 min) were subtracted from currents after DA or retigabine alone, respectively. sucr, Sucrose.

completion of their last extinction session to harvest brain tissue for electrophysiological recordings.

Experiment 3. After the last cocaine self-administration session (see Fig. 5), six cocaine-SA rats were returned and remained in their home cage for the remainder of a 14 d withdrawal period (see Fig. 5A). Rats were killed on the last day of withdrawal for electrophysiological recordings. Another group of five unhandled and behaviorally naive rats were also killed for brain slices (see Fig. 5B). A third group of 12 rats was trained to self-administration sucrose (sucrose-SA; see Fig. 5C) using a procedure similar to those used in previously published studies (McGlinchey et al., 2016), where rats were handled daily for at least 1 week before sucrose self-administration training. Rats were trained to self-administer sucrose on an FR1 schedule of reinforcement (2 h/session) in operant chambers within sound-attenuating boxes, controlled by Med-PC IV software (Med Associates). Sucrose pellets (45 mg, Test Diet) were delivered contingent to pressing one (active) of two levers in response to a compound light (5 s) and tone cue (4 kHz, 78 dB, 5 s) followed by a 20 s time-out period, where additional presses had no programmed consequences. Presses on the inactive lever were recorded but had no programmed consequences. After 10–14 d of criterion sucrose-SA performance ($\geq$30 pellets/session), one group of six rats was killed 24 h after the last training session to harvest brain tissue for electrophysiological recordings. The remaining six rats underwent extinc-

tion training, where responding on either lever had no consequence. Rats received extinction training for a minimum of 10 d and until they met the criteria of <25 active lever presses for $\geq$3 consecutive days. Rats were killed 15 min after the last extinction session to harvest brain tissue for electrophysiological recordings.

Experiment 4. Once cocaine self-administration was completed and behavioral responding was stably extinguished (<25 active lever presses for 3 consecutive days), saline-yoked and cocaine-SA rats returned to the home cage for 24 h (see Fig. 6). The next day, they were tested for cue-induced reinstatement of lever pressing (Epstein et al., 2006). During the 2 h test, rats were re-exposed to the cocaine-paired cues (but, not cocaine) with a 20 s time-out period after each press of the active lever. Rats were killed immediately at the end of the 2 h test for electrophysiological recordings. In a separate set of cocaine-SA rats ($n = 27$ rats), retigabine (0.0015/0.015/0.15 nmol/0.5 μl prepared in saline with 0.1% DMSO) or vehicle (0.1% DMSO in 0.5 μl of saline) was microinjected into the PL-PFC 10 min before 2 h cued-induced reinstatement sessions. A randomized counterbalanced design with two to three cue reinstatement tests was used, with at least 2 d of extinction ($\leq$25 active lever presses) between tests (Smith et al., 2014). Following the completion of reinstatement testing, locomotor testing was conducted in novel open-field chambers (AccuScan Instruments). PL-PFC infusions (identical to those described above) of either retigabine or vehicle were made 10 min before

placement in the open-field chambers. The total distance traveled in the following 2 h was quantified using Versamax software. Data were included for analysis only if the histology identifying the center of injection sites confirmed PL-PFC cannulas placements, which ranged from ~3.2–4.2 mm anteroposterior.

Experiment 5. Transgenic cFos-GFP rats were trained to self-administer cocaine (i.e., the cocaine-SA group) using the same procedure as described above (see Fig. 7). A parallel set of time-matched control rats were "yoked" to the cocaine-SA group, but received saline (saline-yoked group) every time the paired cocaine-SA rat pressed the active lever during the self-administration phase. Following the same procedure as in Experiment 1, four saline-yoked and five cocaine-SA rats were killed immediately after the last 90 min extinction session to harvest brain tissue for electrophysiological recordings. The remaining four saline-yoked and 11 cocaine-SA rats underwent extinction training as described in Experiment 4 and then tested for cued reinstatement of lever pressing as described in Experiment 4, except that the session lasted 90 min. Rats were killed immediately at the end of the 90 min cue test for electrophysiological recordings.

Pharmacological compounds

Cocaine hydrochloride was obtained through the Drug Supply Program of the National Institute on Drug Abuse. DMSO and D-glucose were purchased from Sigma-Aldrich. All salts used in the preparation of physiological buffers were purchased from Fisher Chemicals. Dopamine, XE-991, kynurenic acid, and MK-801 maleate (dizocilpine) were purchased from Abcam. Retigabine was purchased from Axon Medchem.

PFC slice preparation

Rats were killed by rapid decapitation, the brain was removed, and a tissue block containing the PL-PFC was placed in ice-cold and oxygenated artificial CSF (aCSF), as follows (in mM[SCAP]): 126 NaCl, 2.5 KCl, 1.2 MgCl$_2$, 1.4 NaH$_2$PO$_4$, 25 NaHCO$_3$, 11 D-glucose, 0.4 ascorbate supplemented with MK-801 maleate (10 μM) and kynurenic acid (2.6 mM; Riegel and Williams, 2008; Williams et al., 2014). Thick sections (~200 μM) containing the PL-PFC region (Fig. 1B) were prepared using a vibrating microtome (Leica) and maintained in a vial containing MK-801 maleate (10 μM) dissolved in oxygenated aCSF in a 32°C water bath until recording. During recording, sections were perfused at a flow-rate of 2 ml/min with oxygenated aCSF at 33°C. The prelimbic layer V/VI was identified initially using a 5× objective, and later was magnified for patching with a 63× (0.9 numerical aperture) water-immersion lens fitted to an upright microscope (model BX 51WI, Olympus) equipped with gradient contrast infrared optics. GFP was visualized by fluorescence using a purpose-built LED system composed of parts from Thorlabs.

Electrophysiological procedures

Whole-cell recordings were performed using multiclamp 700B amplifiers (Molecular Devices). Neurons were voltage clamped at −70 mV using 1.5–2.5 MΩ glass microelectrodes filled with the following internal solution (in mM[SCAP]): 115 K-methylsulfate, 20 NaCl, 1.5 MgCl$_2$, 2.5 HEPES, 2 ATP, 0.3 GTP, and 0.1 EGTA, pH 7.3 and 265–270 mOsm. Series resistance (<10 MΩ) was compensated at 80%, and only cells with <15% series resistance change after drug application were included in the analysis. Recordings were collected on-line using AxoGraph X (AxoGraph Scientific) and digitized at 2–5 kHz. Layer V pyramidal cells were identified by location, morphology, and electrophysiological criteria including their characteristic hyperpolarized resting membrane potential and low input resistance (Gulledge and Jaffe, 1998; Ceci et al., 1999; Rosenkranz and Grace, 2002; Song et al., 2015). Passive membrane properties in these cells were consistent with published results (Gulledge and Jaffe, 1998; Ceci et al., 1999; Rosenkranz and Grace, 2002; Song et al., 2015) and did not vary among any of the treatment groups at any phase of behavioral training (average all groups across treatments: resting membrane potential: −67.2 ± 0.7 mV, $F_{(11,610)} = 1.084$, $p = 0.3715$; input resistance: 58 ± 2.0 MΩ, $F_{(11,747)} = 1.306$, $p = 0.2160$; membrane capacitance: 30.8 ± 1.0 pF/cm^2, $F_{(11,537)} = 1.529$, $p = 0.1172$). Brain slices prepared at the culmination of the behavioral assay (Fig. 1B) were used for current-clamp (*I*-clamp) recordings in Experiment 3 (see Figs. 5, 7).

Experiments 1, 2, and 4 contain both *I*-clamp and voltage-clamp (*V*-clamp) recordings (see Figs. 3, 4, 6).

For *I*-clamp experiments, the bridge was balanced routinely. Using published criteria, input–output (I–O) curves of action potential (AP) firing were generated by injecting a series of eight 0.8 s current steps, 1 s interval (100, 200, 300, 400, 500, 600, 800, and 1000 pA) and counting the numbers of APs evoked during the last 500 ms in each step epoch (Fig. 1B; Madison and Nicoll, 1984; Storm, 1990; Faber et al., 2001; Buchta et al., 2017). All firing traces shown represent the response to 1000 pA current injections. SFA or spike frequency accommodation is defined as the slowing of neuronal firing during a depolarizing current step (Madison and Nicoll, 1982; Lancaster and Nicoll, 1987; Aiken et al., 1995; Shah et al., 2006). Based on published studies, we identified SFA-positive (SFA$^+$) neurons as those showing five or fewer spikes during the final 500 ms of the 1 nA current injection (Madison and Nicoll, 1982; Lancaster and Nicoll, 1987; Aiken et al., 1995; Shah et al., 2006). AHPs were measured following calibration of a 60 ms current injection step to yield a burst of five APs from a holding potential of −70 mV (Fig. 1B; Coulter et al., 1989; Moyer et al., 2000; Gu et al., 2005). Because there are no clear criteria for the separation of slow and medium AHPs, we calculated total AHPs as the area below baseline following the fifth AP and the return of the membrane potential to baseline values (Coulter et al., 1989; Moyer et al., 2000; Gu et al., 2005).

For voltage-clamp experiments (Fig. 1C), cells were first sorted as spike frequency-adapting SFA$^+$ or SFA$^-$ by a single 1000 pA/800 ms current injection in *I*-clamp. After switching to *V*-clamp, 200 nM tetrodotoxin (TTX) was continuously applied to the bath perfusate. After 5 min, outward currents were generated in response to a series of long (800 ms) depolarizing steps (−60 to −20 mV, 10 mV increment). Because Kv7 conductance is a component of this net outward current (and not the total current), the outward currents were measured again after perfusion of the Kv7 channel antagonist XE-991 (20 μM, for 10 min; Fig. 1C). The long, slow Kv7 channel current (I_{Kv7}) activation kinetics and sensitivity to XE-991 have been characterized in previous reports (Delmas and Brown, 2005; Lawrence et al., 2006; Brown and Passmore, 2009). Consistent with published results, I_{Kv7} was voltage dependent, outwardly rectifying, and actively positive to −50 mV (Delmas and Brown, 2005; Lawrence et al., 2006; Brown and Passmore, 2009). The I_{Kv7} was measured by subtracting the outward currents generated in the presence of XE-991 from the baseline (predrug; Fig. 1C; Wang et al., 1998; Huang and Trussell, 2011). When the effects of dopamine or retigabine on Kv7 channel function were measured, cells were first sorted as SFA$^{+/-}$, then voltage clamped and injected with a series of five long (1 s) depolarizing steps (−70 to −20 mV, 10 mV increment) in the presence of 200 nM TTX (Fig. 1C). The outward current was measured 5 min after perfusion with 10 μM dopamine or 20 μM retigabine, and again 10 min after perfusion with dopamine plus 20 μM XE-991 or retigabine plus 20 μM XE-991 (Fig. 1C). The amplitude of the Kv7 current before and after dopamine or retigabine treatment was computed by subtracting the final set of currents from the first and second sets of currents, respectively (Fig. 1C). Recovery from dopamine or retigabine was not regularly examined, as full washout can exceed 25 min, a duration of time that negatively impacts the quality of patch-clamp recordings in older animals that have undergone operant training (Yang and Seamans, 1996; Zheng et al., 1999; Henze et al., 2000; Gorelova et al., 2002). The 10 μM dopamine concentration for study was selected for its relevance to earlier studies using concentrations of 10–30 μM (Malenka and Nicoll, 1986).

cFos immunohistochemistry

Immunohistochemical methods for verification of the cFos-GFP rats were adapted from previous studies (Taniguchi et al., 2017). Fluorescence labeling was used to quantify the colocalization of the intrinsic GFP signal (c-fos) and the protein product of the immediate early gene (cFos) between different treatment groups of the self-administration paradigm in layer V of the PFC. Sections were washed 3× 7 min in PBS, pH 7.4, and incubated for 1 h in blocking solution (3% BSA, 0.3% Triton X-100, 0.2% Tween, 3% normal donkey serum in PBS). Sections were then incubated overnight at 4°C with anti-Fos goat primary antibody (catalog #sc-52-G, lot B2513, Santa Cruz Biotechnology) diluted 1:1000 in blocking solution

and anti-GFP chicken (catalog #GFP-1020, lot 0316FP11, Aves Labs). Sections were washed in PBS 3× 7 min and incubated for 1.5 h at room temperature with donkey anti-chicken 488 (catalog #703-545-155, lot 119192, Jackson ImmunoResearch) 1:200 and donkey anti-goat Cy3 (catalog #705-165-147, lot 132480, Jackson ImmunoResearch) 1:200. Sections were washed, dehydrated, and mounted using ProLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific).

c-Fos GFP and Fos colocalization quantification

Ninety minutes after the start of the final day of self-administration of cocaine or yoked-saline or the first day of extinction training, rats were deeply anesthetized with isoflurane, as described previously (Cifani et al., 2012), and transcardially perfused [150 ml of saline and 150 ml of 4% paraformaldehyde (PFA) in PBS]. Whole-brain specimens were post-fixed in 4% PFA for 24 h and transferred into PBS for 24 h until immunohistochemistry procedures were conducted to confirm the c-Fos GFP transgene, as described previously (Cifani et al., 2012). The total c-Fos GFP/anti-Fos cell counts, the Pearson's correlation coefficients, and the Mander's overlap coefficients describing the coexpression of antibody staining for Fos and c-Fos GFP were obtained to verify the efficacy of the c-Fos GFP transgene (see Fig. 7). A confocal microscope (Zeiss LSM 510) was used to image PFA fixed brain sections. Fluorescent images of layer V PFC were taken using both Leica and NIS Elements Software (Nikon). Quantification of the colocalization of the GFP expression (indicative of c-fos) and the fos immunofluorescence was conducted using the Nikon NIS Elements Software on 10× bilateral images of layer V PFC.

Western blotting

Samples of the PL-PFC were lysed by sonication in RIPA buffer [50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 2 mM MgCl$_2$, 1% (v/v) NP40, 0.5% (mass/vol) sodium deoxycholate, and 0.1% (mass/vol) SDS], protease (catalog #B14002, BioTool) and phosphatase inhibitors (catalog #B15002, BioTool), and BitNuclease (catalog #B16002, BioTool). After sonication, lysates were clarified by centrifugation (15,000 × g, 10 min, 4°C), then protein concentrations were determined using the BCA Protein Assay (catalog #PI23225, Thermo Fisher Scientific). Approximately 15 μg of total proteins were loaded onto an SDS-PAGE gel and then transferred to polyvinylidene difluoride membranes (0.45 μm) and blocked at room temperature for 1 h in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) and 5% nonfat dry milk. Primary antibodies used for probing KCNQ2 (catalog #APC-050, Alomone Labs), KCNQ3 (catalog #APC-051, Alomone Labs), KCNQ5 (catalog #APC-155, Alomone Labs), and actin (catalog #A2066, Sigma-Aldrich) were diluted in TBST with 5% BSA. Immunoblots were revealed by enhanced luminescence (WBKLS0500, Millipore) before exposure to a photographic film. Films were scanned, digitized, and quantified using Un-Scan-It gel version 6.1 scanning software (Silk Scientific).

Experimental design and statistical analysis

All data are reported as the mean ± SEM, and statistical analyses were performed using GraphPad Prism. Electrophysiological data were analyzed using Axograph X. We used a generalized linear mixed-model ANOVA accounting for the clustering of cells within rats, as well as accounting for rats being used more than once to evaluate cell responses before and after drug application. This was achieved using two random effects in the analysis, one for the rats and another for cells within rats. An interaction between current or membrane potential and drug treatment was also included in the model. The between-subject variable was treatment (cocaine-SA, cocaine-yoked, saline-yoked, sucrose-SA), and the repeated measure was current (for action potential firing) or membrane potential (for current amplitude). Using least-squares means from the model, *post hoc* comparisons of drug treatment groups before and after were made with a Tukey adjustment for data related to Figures 3D, 4D, and 7, F and G, or a Sidak multiple-comparisons test for all other analysis. Model assumptions were evaluated based on the residuals, and no violations were found. AHP differences expressed as the area under the curve were evaluated using one-way repeated-measures ANOVAs or unpaired *t* tests. Two-way mixed repeated-measures ANOVAs were used to evaluate the effects of treatment (cocaine, saline, and sucrose), excitability (firing or current amplitude), and time (repeated steps of current or

membrane potential). All statistical evaluations of Kv7 channel *I–V* curves were conducted at data points positive to −50 mV, when channels are open and not inactivated (Lawrence et al., 2006; Huang and Trussell, 2011; Battefeld et al., 2014). Current and action potential responses of control and treated rats were typically compared at the maximal difference (1 nA for action potential firing or −20 mV for membrane currents). For graphical purposes and clarity, data are often presented separately for the cocaine and saline treatment groups. Data represent the mean ± SEM, with a α set at $p < 0.05$.

Results

This study addressed three interrelated questions. (1) What are the long-lasting alterations in PL-PFC excitability that occur following cocaine self-administration (withdrawal and extinction)? (2) What are the acute alterations in excitability that occur immediately following cue-induced reinstatement of cocaine seeking? (3) Do the observed alterations contribute to the cue-induced reinstatement of cocaine seeking?

To address these questions, we used the self-administration model of relapse to drug seeking (Shaham et al., 2003). Rats were trained to self-administer cocaine (0.20 mg/kg/infusion) for 14 d in operant chambers where drug infusions were paired with light/tone cues (Fig. 1A). All rats showed stable cocaine-SA responding (Fig. 2A). Parallel groups of rats received yoked infusions of saline or yoked infusions of cocaine (Fig. 2A). No differences in the total number of drug or saline infusions were found among the three groups (one-way ANOVA, $F_{(2,70)} = 1.819$, $p = 0.1697$). As expected, lever pressing in the three treatment groups differed (Fig. 2A; two-way ANOVA with repeated measures over time: treatment, $F_{(2,609)} = 91.33$, $p < 0.0001$; time, $F_{(14,609)} = 0.3788$, $p = 0.9806$; interaction, $F_{(28,609)} = 0.8388$, $p = 0.7060$). Cocaine-SA rats pressed the active lever significantly more than saline-yoked or cocaine-yoked controls (Fig. 2A; cocaine-SA vs saline-Y, $t_{(609)} = 12.17$, $p < 0.0001$; cocaine-SA vs cocaine-Y, $t_{(609)} = 9.205$, $p < 0.0001$; saline-yoked vs cocaine-Y, $t_{(609)} = 0.4642$, $p = 0.9544$). Self-administration training was followed by 14–19 d of Ext where neither cues nor drug was available. As previously reported in cocaine-SA-treated rats, the first day of extinction (Fig. 2A, vertical dashed line) was associated with a burst in lever pressing (Lerman and Iwata, 1995). As expected, rats trained for cocaine-SA showed stable context extinction of responding on the active levers (Fig. 2A).

Rats from the cocaine-SA and saline-yoked groups also underwent testing for cued reinstatement (Fig. 2B). Relapse to cocaine seeking was modeled in a session where cocaine-associated cues were presented, but lever pressing did not result in drug delivery (Kalivas and McFarland, 2003; McLaughlin and See, 2003). Responses to reinstatement testing differed between the saline-yoked and cocaine-SA groups (two-way ANOVA: treatment, $F_{(1,33)} = 95.12$, $p < 0.0001$; time, $F_{(1,33)} = 105.6$, $p < 0.0001$; interaction, $F_{(1,33)} = 61.57$, $p < 0.0001$). Lever pressing in the cocaine-SA group increased above that observed during the last day of extinction training (Fig. 2B; $t_{(33)} = 13.8$, $p < 0.0001$). As predicted, rats in the saline-yoked group did not show increased lever pressing in response to light and tone cues (Fig. 2B; $t_{(33)} = 1.612$, $p = 0.2194$).

Active (but not passive) exposure to cocaine suppresses dopamine-sensitive inhibition: experiment 1

To determine whether cocaine self-administration causes acute changes in PL-PFC excitability, we killed animals 24 h after their last self-administration training session. In acute brain slices, we recorded layer V pyramidal neurons in the PFC in *I*-clamp to
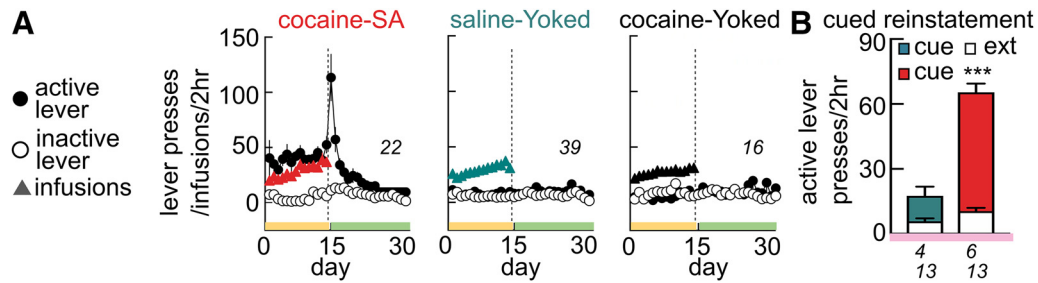
Figure 2. Behavioral responding in cocaine self-administration, yoked-cocaine, and yoked-saline rats. ***A***, Rats were presented with levers that resulted in the activation of a light and tone cue paired with the infusion of cocaine (cocaine-SA, n = 22; left), passive infusion of saline (saline-yoked, n = 39; middle), or passive infusion of cocaine (cocaine-yoked, n = 16; right). The vertical dashed line on time courses indicates the switch from self-administration to extinction. In extinction sessions, lever pressing produced no infusions or cue presentations. Behavioral responses for voluntary cocaine self-administration extinguished in 14 d. ***B***, Re-exposure to drug-conditioned cues (without cocaine) significantly increased lever pressing in rats with a history of cocaine-SA. Numbers in italics represent the total number of animals. For these and all other figures, error bars indicate the mean $\pm$ SEM. ***p $\leq$ 0.0001 compared with extinction using a Sidak test for multiple comparisons.

measure action potential firing to depolarizing current injections steps (0.8 s, 0.1–1 nA). We observed obvious differences in the firing responses among cocaine-SA, cocaine-yoked, and saline-yoked treatment groups during the depolarizing current steps (Fig. 3B1, inset; two-way ANOVA with repeated measures over current: treatment, $F_{(2,76)} = 34.89$, $p < 0.0001$; current, $F_{(7,532)} = 62.94$, $p < 0.0001$; interaction, $F_{(14,532)} = 19.90$, $p < 0.0001$). A *post hoc* analysis showed no significant firing differences between the two yoked-treatment groups ($p = 0.6400$), but a significant increase in cocaine-SA responses relative to the cocaine-yoked group ($p < 0.0001$), which was surprising given that the two groups received similar amounts of cocaine (Fig. 2A). As prior reports show heterogeneous subpopulations of cortical neurons (Cruz et al., 2013, 2015), we used scatter plots to compare the relative distribution of individual firing responses at the 1 nA current step. We categorized cell activity according to the degree of SFA, whereby SFA$^+$ neurons were classified as those showing five or fewer spikes during the final 500 ms of the 1 nA current injection (Fig. 3B1). In most cocaine-SA neurons (81%), training experience elevated firing (i.e., above the SFA threshold of five spikes; Fig. 3B1). By contrast, most firing responses in saline-yoked (91%) or noncontingent cocaine-yoked (79%) groups displayed robust SFA (i.e., SFA$^+$; Fig. 3B1). The SFA is modulated by the AHP that follows a depolarization step (Madison and Nicoll, 1986). AHPs were measured following calibration of a 60 ms current injection step to yield a burst of five APs from a holding potential of −70 mV (Fig. 1B) and measured the total AHP area (Coulter et al., 1989; Moyer et al., 2000; Gu et al., 2005). As expected, after training, the AHP size decreased in the cocaine-SA group, but not in the yoked groups (Fig. 3B2; one-way ANOVA: treatment, $F_{(2,41)} = 13.92$, $p < 0.0001$; *post hoc* test: cocaine-SA vs saline-yoked, $p < 0.0001$; cocaine-SA vs cocaine-yoked, $p = 0.0044$). These data suggest that learning to lever press for cocaine and not the mere exposure to cocaine itself increases neuronal excitability.

In other cortical regions, activation of Kv7 voltage-gated K$^+$ channels localized to the axon hillock suppresses neuronal output by enhancing SFA (Delmas and Brown, 2005; Lawrence et al., 2006; Brown and Passmore, 2009). To determine whether the cocaine-SA experience reduced this form of inhibition in PL-PFC cells, we sampled a range of membrane potentials in *V*-clamp (−60 to −20 mV) to measure I_{Kv7} values (Fig. 3C1, inset). We observed that voltage-dependent I_{Kv7} activated above −50 mV (Fig. 3C1). As with the previous firing measurements, the amplitude of I_{Kv7} also differed significantly between the cocaine-SA and saline-yoked treatments (Fig. 3C1; two-way ANOVA with

repeated measures of potential: treatment, $F_{(1,8)} = 5.973$, $p < 0.0403$; potential, $F_{(2,16)} = 26.67$, $p < 0.0001$; interaction, $F_{(4,32)} = 5.41$, $p < 0.0019$). When considering all cells sampled from the cocaine-SA and saline-yoked groups, the elevated basal level of firing at 1 nA correlated with the reduction in I_{Kv7} amplitudes (Fig. 3C2; $R^2 = 0.479$). Thus, at 24 h following the 2 weeks of cocaine self-administration, the loss of SFA may be due to functional suppression in Kv7 channel-mediated inhibition.

Our previous published work showed that bath application of dopamine or chemogenetic/optogenetic activation of VTA dopamine terminals suppressed SFA and AHPs in PL-PFC pyramidal cells (Buchta et al., 2017). Because repeated activation of cAMP-coupled GPCRs can sensitize adenylate cyclase to subsequently activate downstream signaling cascades (Johnston and Watts, 2003), we wondered whether dopamine suppresses SFA by closure of Kv7 channels and whether the repeated cocaine-SA impacted this function. To evaluate this, we sampled SFA, AHP, and I_{Kv7} before and during the application of 10 μM dopamine in the saline-yoked, cocaine-yoked, and cocaine-SA groups (Fig. 3D,E). A two-way ANOVA using cell type as the between-subjects factor and dopamine as a within-subject factor showed differences in both firing and AHPs (Fig. 3D1, firing: treatment, $F_{(2,15)} = 3.83$, $p = 0.0454$; dopamine, $F_{(1,10)} = 58.64$, $p < 0.0001$; interaction, $F_{(2,15)} = 11.58$, $p = 0.0009$; Fig. 3D2, AHP: treatment, $F_{(2,14)} = 1.02$, $p = 0.3860$; dopamine, $F_{(1,10)} = 29.56$, $p < 0.0003$; interaction, $F_{(2,14)} = 3.81$, $p = 0.0477$). At the 1 nA current injection step, all but one of the sampled PL-PFC cells in the cocaine-SA group lacked SFA, whereas all cells in the saline-yoked and cocaine-yoked groups displayed SFA (Fig. 3D1). In SFA$^+$ cells from both the saline-yoked and cocaine-yoked groups, dopamine increased firing responses and decreased AHPs relative to the predopamine levels (Tukey post-tests: Fig. 3D1, firing: saline-yoked/SFA$^+$, $t_{(15)} = -4.56$, $p = 0.0041$; cocaine-yoked/SFA$^+$, $t_{(15)} = -7.37$, $p < 0.0001$; Fig. 3D2, AHPs: saline-yoked, $t_{(14)} = 3.90$, $p < 0.0161$; cocaine-yoked, $t_{(14)} = 4.27$, $p = 0.0081$). By contrast, firing and AHP responses in the SFA$^-$ cells of the cocaine-SA group were not measurably changed by dopamine (Fig. 3D1; firing, $t_{(15)} = -1.03$, $p = 0.9023$; Fig. 3D2; AHP: $t_{(14)} = 1.05$, $p = 0.8920$). This indicates an alteration in the regulation of intrinsic inhibition by dopamine in the population of SFA$^-$ cells.

Given the diminished sensitivity to dopamine in the firing and AHP experiments in the cocaine-SA group, we examined the dopamine action on evoked I_{Kv7} currents in cells of the cocaine-SA group, all of which were SFA$^-$ (Fig. 3E). I_{Kv7} responses to dopamine in cocaine-SA and saline-yoked treatments
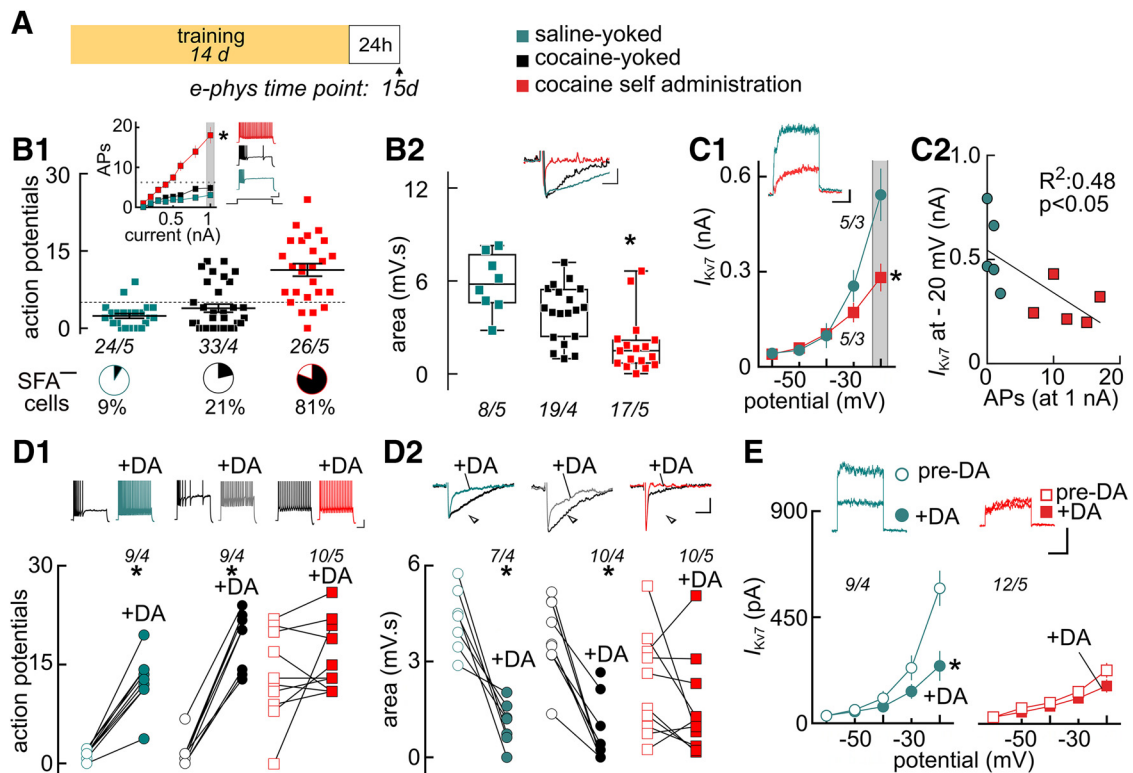
Figure 3. Experiment 1: training to self-administer cocaine (but not passive exposure to cocaine) suppressed intrinsic inhibition and inhibitory Kv7 channel currents. *A*, Timeline showing behavioral training ended 24 h before killing for electrophysiological recordings. *B1*, Scatter plots show firing responses of individual cells at 1 nA. Matching I–O curves (inset) show increased mean firing responses for the cocaine-SA relative to cocaine-yoked or saline-yoked treatment groups. Firing exceeded the upper limit for SFA (operationally defined as five or fewer spikes at 1 nA; dashed gray line) only in the cocaine-SA group. Black segments in the pie charts indicate the percentage of sampled cells that were SFA$^-$. *B2*, Scatter plots show decreased average AHPs in the cocaine-SA group only. *C1*, I–O curves show reduced I_{Kv7} currents in the cocaine-SA group. Before I_{Kv7} measurements, cells in the cocaine-SA and saline-yoked groups were confirmed to be SFA$^-$ and SFA$^+$, respectively. *C2*, Reductions in I_{Kv7} current amplitudes correlated with AP firing at the 1 nA step. *D1*, *D2*, Dot plot graphs show dopamine increased firing (*D1*) and decreased AHP (*D2*) in cocaine-SA/ SFA$^+$ and saline-yoked/SFA$^+$ cells. Dopamine produced no measurable change in cocaine-SA/SFA$^-$ cells. *E*, Dopamine reduced I_{Kv7} in the saline-yoked/SFA$^+$ but not in the cocaine-SA/SFA$^-$ cells. All insets show sample traces collected at 1 nA step for firing (I-clamp) or at −20 mV for Kv7 (V-clamp). Italicized numbers represent the number of cells/animals. Calibration: *B1*, 20 mV, 0.2 s; *B2*, 3 mV, 0.4 s; *C1*, 0.1 nA, 0.4 s; *D1*, 20 mV, 0.2 s; *D2*, 3 mV, 0.5 s; *E*, 0.1 nA, 0.4 s. *$p \leq 0.01$ compared with saline-yoked animals using a Sidak test (*B*, *C*, *E*) or a Tukey's test (*D1*, *D2*) for multiple comparisons. DA, Dopamine; e-phys, electrophysiological measurements.

differed significantly (two-way ANOVA with repeated measures of potential: treatment, $F_{(3,38)} = 5.902$, $p = 0.0021$; potential, $F_{(2,76)} = 65.03$, $p < 0.0001$; interaction, $F_{(6,76)} = 8.949$, $p < 0.0001$). To understand the nature of the interaction, we deconstructed individual treatment responses at −20 mV, a potential where I_{Kv7} was maximal. In support of our results above in Figure 3C1, follow-up comparisons between treatments showed significantly reduced I_{Kv7} amplitudes under baseline (predopamine) conditions in the cocaine-SA treatment group relative to saline-yoked controls ($p < 0.0001$). Furthermore, dopamine reduced the I_{Kv7} in the saline-yoked group ($p < 0.0001$), but not in the cocaine-SA group ($p = 0.6544$; Fig. 3E). In summary, the active self-administration (but not passive infusion) of cocaine was associated with a reduction in intrinsic inhibition, reduced sensitivity to dopamine, and suppression of inhibitory Kv7 ion channel currents.

Dopamine-sensitive inhibition is suppressed after 14 d extinction training: experiment 2

To determine whether the changes in PL-PFC excitability persisted after 14 d of extinction conditioning, rats were killed 15 min following the final extinction session (Fig. 4A). We excluded the cocaine-yoked group from further analysis since the previous experiment already showed that intrinsic inhibition was not altered by passive administration of cocaine, only by active

self-administration of cocaine. We found that after extinction training, average firing responses were different between the cocaine-SA and saline-yoked control treatments (Fig. 4B1; two-way ANOVA with repeated measures of current: treatment, $F_{(1,66)} = 5.309$, $p = 0.0244$; current, $F_{(7,462)} = 13.73$, $p < 0.0001$; interaction, $F_{(7,462)} = 4.694$, $p < 0.0001$). Firing responses at a 1 nA current step were elevated in the cocaine-SA group relative to those in saline-yoked controls ($t_{(528)} = 4.372$, $p = 0.0002$). Approximately half (57%) of cocaine-SA cells showed high firing (SFA$^-$ responses), whereas only a minority (11%) of saline-yoked control cells were SFA$^-$. Compared with training alone (without extinction; Fig. 3), there was a slight reduction in SFA$^-$ cells (from 81% to 57%) in the cocaine-SA group. Examination of individual cells showed evenly distributed responses in saline-yoked treatments, but heterogeneous responses in the cocaine-SA treatments (Fig. 4B2), suggesting that only a subset of cells is persistently hyperactive after extinction training. AHP measurements following extinction showed similar changes; in the cocaine-SA treatment group, the mean value declined, but individual cells tended to cluster at higher or lower responses (Fig. 4B3; unpaired t test: $t_{(80)} = 3.898$, $p = 0.0002$). Together, these data suggest that following extinction a subpopulation of SFA$^-$ cocaine-SA cells remain hypersensitive to depolarizing inputs.

To examine the involvement of Kv7 channels, first we measured the effects of the channel antagonist XE-991 (20 μM, 10 min)
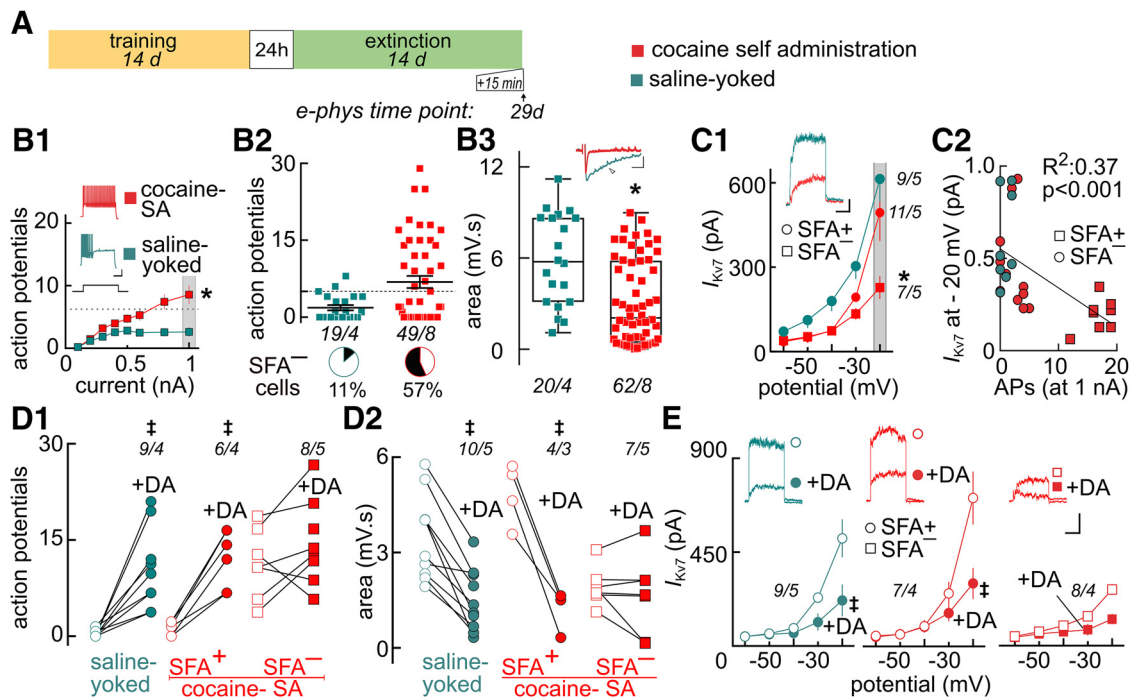
Figure 4. Extinguishing the behavioral responding for cocaine does not restore intrinsic inhibition or Kv7 channel currents. ***A***, Timeline showing cocaine-SA (14 d) and extinction (14 d) before killing for electrophysiological recordings at 15 min after the final extinction session. ***B1–B3***, Average firing (***B1***, I–O curves) and individual firing (***B2***, scatter plots at 1 nA) were increased in the cocaine-SA group, while AHP (***B3***, scatter plots) was decreased relative to saline-yoked controls. Black segments in the pie charts indicate the percentage of sampled cells that were SFA$^-$. ***C1***, I–O curves show reduced I_{Kv7} currents in the cocaine-SA/SFA$^-$ cells, but not the saline-yoked/SFA$^+$ or cocaine-SA/SFA$^+$ cells. ***C2***, Reductions in I_{Kv7} current amplitudes correlated with AP firing at the 1 nA step. ***D1, D2***, Dot plot graphs show that dopamine increased firing (***D1***) and decreased AHPs (***D2***) in SFA$^+$ cells from both saline-yoked and cocaine-SA groups. Dopamine did not measurably alter cocaine-SA/SFA$^-$ cells. ***E***, Dopamine reduced I_{Kv7} in SFA$^+$ cells of saline-yoked and cocaine-SA rats, but not in SFA$^-$ cells. All insets showing sample traces were collected at 1 nA step for firing (I-clamp) or at -20 mV for Kv7 (V-clamp). Italicized numbers represent the number of cells/animals. Calibration: ***B1***, 20 mV, 0.2 s; ***B3***, 3 mV, 0.4 s; ***C1***, 0.1 nA, 0.4 s; ***D1***, 20 mV, 0.2 s; ***D2***, 3 mV, 0.5 s; ***E***, 0.1 nA, 0.4 s. *$p \leq 0.001$ compared with saline-yoked animals using a Sidak test (***B, C, E***) or a Tukey test (‡; ***D***) for multiple comparisons. DA, Dopamine; e-phys, electrophysiological measurements.

on firing frequency and second we measured the I_{Kv7} value in voltage clamp in cells from the saline-yoked group and the population of SFA$^-$ cells from cocaine-SA rats immediately after the final extinction session. First, responses to XE-991 differed in saline-yoked SFA$^+$ cells and the subpopulation of SFA$^-$ cells from the cocaine-SA rats (two-way ANOVA with repeated measures of current: treatment, $F_{(3,30)} = 17.89$, $p < 0.0001$; current, $F_{(7,210)} = 94.48$, $p < 0.0001$; interaction, $F_{(21,210)} = 10.23$, $p < 0.0001$; data not shown). A subsequent analysis within the saline-yoked group showed XE-991 increased firing across the full range of current steps, culminating in a 10-fold potentiation at the 1 nA current step (two-way ANOVA with repeated measures of current: XE991, $F_{(1,112)} = 42.42$, $p = 0.0001$; current, $F_{(7,112)} = 10.06$, $p < 0.0001$; interaction, $F_{(7,112)} = 6.971$, $p < 0.0001$). By contrast, the application of XE-991 did not alter the input–output curve for SFA$^-$ cells within the cocaine-SA group (two-way ANOVA with repeated measures of current: XE-991, $F_{(1,16)} = 0.5168$, $p = 0.4826$; current, $F_{(7,112)} = 95.73$, $p < 0.0001$; interaction, $F_{(7,112)} = 0.8562$, $p = 0.5435$). A follow-up analysis of baseline (pre-XE-991) conditions comparing saline-yoked and cocaine-SA treatments at different current steps showed elevated action potential firing in the cocaine-SA (SFA$^-$ cells) group, indicating that firing may already be maximal and therefore less prone to further potentiation by blockade of Kv7 channels (two-way ANOVA with repeated measures of current: treatment, $F_{(1,15)} = 42.82$, $p = 0.0001$; current, $F_{(7,105)} = 30.83$, $p < 0.0001$; interaction, $F_{(7,105)} = 23.46$, $p < 0.0001$). Together, these data indicate that after extinction from cocaine self-administration, a population of PL-PFC cells show enhanced firing responses un-

der baseline conditions and a related diminished sensitivity to Kv7 channel block.

Second, to corroborate these results, we measured I_{Kv7} responses (Fig. 4C1, inset) in all cells across membrane potentials. We found significant differences among the three groups (saline-yoked/SFA$^+$, cocaine-SA/SFA$^+$, and cocaine-SA/SFA$^-$; Fig. 4C1; two-way ANOVA with repeated measures of potential: group/SFA, $F_{(2,29)} = 8.669$, $p = 0.0015$; potential, $F_{(2,48)} = 58.94$, $p < 0.0001$; interaction, $F_{(4,48)} = 4.344$, $p = 0.0044$). At -20 mV, I_{Kv7} responses in SFA$^-$ cells in the cocaine-SA group were reduced relative to the SFA$^+$ cells in the saline-yoked group (Fig. 4C1; cocaine-SA/SFA$^+$ vs cocaine-SA/SFA$^-$: $t_{(72)} = 3.817$, $p = 0.0008$; saline-Y/SFA$^+$ vs cocaine-SA/SFA$^-$: $t_{(72)} = 5.634$, $p < 0.0001$; saline-yoked vs cocaine-SA/SFA$^+$: $t_{(72)} = 2.211$, $p = 0.0878$). Similar to findings at 24 h after cocaine-SA, the basal level of firing at 1 nA correlated with I_{Kv7} amplitudes (Fig. 4C2; Ext, $R^2 = 0.370$). These results highlight an enduring functional shift in inhibition in a population of SFA$^-$ cells in the cocaine-SA group.

Given that dopamine regulates SFA in the PFC, we examined whether this regulation is altered following extinction (Fig. 4D1,D2) and again noted differences between the groups in both firing and AHPs (firing: treatment, $F_{(2,8)} = 4.78$, $p = 0.0432$; dopamine, $F_{(1,9)} = 26.69$, $p < 0.0006$; interaction, $F_{(2,8)} = 6.27$, $p = 0.0230$; AHP: treatment, $F_{(2,7)} = 3.08$, $p = 0.1097$; dopamine, $F_{(1,8)} = 57.20$, $p < 0.0001$; interaction, $F_{(2,7)} = 12.18$, $p = 0.0053$). In SFA$^+$ cells of either saline-yoked or cocaine-SA rats, bath application of dopamine (10 μM, 10 min) produced robust changes in SFA and AHPs (Tukey post-test; Fig. 4D1, saline-

yoked/SFA$^+$: $t_{(8)} = -4.59$, $p = 0.0151$; cocaine-SA/SFA$^+$: $t_{(8)} = -4.06$, $p = 0.0293$; Fig. 4D2, saline-yoked/SFA$^+$: $t_{(7)} = 6.25$, $p < 0.0036$; cocaine-SA/SFA$^+$: $t_{(7)} = 5.83$, $p = 0.0053$). In contrast, in SFA$^-$ cells (only observed in the cocaine-SA group) there was no measurable dopamine-induced change in firing or AHPs (Tukey post-test; Fig. 4D1, cocaine-SA/SFA$^+$: $t_{(8)} = -0.18$, $p = 1.0000$; Fig. 4D2, AHP: $t_{(7)} = 0.90$, $p = 0.9356$).

Correspondingly, responses of inhibitory Kv7 currents to dopamine also differed between treatment groups (Fig. 4E; two-way ANOVA with repeated measures of potential, treatment $F_{(5,42)} = 5.025$, $p = 0.0011$; potential $F_{(2,84)} = 75.74$, $p = 0.0001$; interaction: $F_{(10,84)} = 7.968$, $p < 0.0001$). This difference could be explained, in a subsequent analysis, by the finding that most cells in the saline-yoked group were SFA$^+$. However, in both the saline-yoked/SFA$^+$ and cocaine-SA/SFA$^+$ groups, dopamine reduced I_{Kv7} amplitudes (two-way ANOVA with repeated measures of potential: saline-yoked/SFA$^+$, dopamine $F_{(1,16)} = 6.534$, $p = 0.0201$; potential $F_{(2,32)} = 28.37$, $p = 0.0001$; interaction: $F_{(2,32)} = 6.185$, $p < 0.0054$; cocaine-SA/SFA$^+$, dopamine $F_{(1,12)} = 5.646$, $p = 0.0350$; potential $F_{(2,24)} = 31.5$, $p = 0.0001$; interaction: $F_{(2,24)} = 7.927$, $p = 0.0023$). In contrast, dopamine did not alter I_{Kv7} amplitudes in cocaine-SA/SFA$^-$ cells (Fig. 4E; two-way ANOVA with repeated measures of potential: dopamine, $F_{(1,14)} = 3.695$, $p = 0.0752$; potential, $F_{(2,28)} = 19.44$, $p < 0.0001$; interaction, $F_{(2,28)} = 9.973$, $p < 0.0005$). A final comparison of the baseline (pre-dopamine, -20 mV) conditions among the three groups showed significantly reduced I_{Kv7} amplitudes in the cocaine-SA/SFA$^-$ group relative to cocaine-SA/SFA$^+$ cells (Fig. 4E; one-way ANOVA, $F_{(2,21)} = 3.653$, $p = 0.0435$; post hoc saline-yoked/SFA$^+$ vs cocaine-SA/SFA$^+$: $t_{(21)} = 1.578$, $p = 0.3404$; post hoc saline-yoked/SFA$^+$ vs cocaine-SA/SFA$^-$: $t_{(21)} = 1.936$, $p = 0.1865$; cocaine-SA/SFA$^+$ vs cocaine-SA/SFA$^-$: $t_{(21)} = 3.354$, $p = 0.0090$). This suggests that the baseline amplitude of I_{Kv7} in cocaine-SA/SFA$^-$ cells was already reduced before bath application of dopamine. Together, these data indicate that after 2 weeks of cocaine self-administration a population of SFA$^-$ cells experience a reduction in intrinsic inhibition and therefore reduced sensitivity to dopamine regulation, and that this relationship persists even after the behavioral extinction of drug-paired associations.

Inhibition following withdrawal from cocaine-SA or sucrose-SA with extinction training: experiment 3

Typically, reductions in intrinsic inhibition are transient, learning specific, and behaviorally appropriate (Oh and Disterhoft, 2015). To determine whether extinction learning is required for the enduring reduction in intrinsic inhibition in the cocaine-SA/SFA$^-$ population, we sampled cells from cocaine-SA animals that did not undergo extinction training, but instead underwent 14 d of home-cage withdrawal (Fig. 5A). Like the firing responses in extinguished animals (Fig. 4B), average firing responses in approximately half of cells in the withdrawal group exceeded the maximal limit for SFA, and the individual responses for firing and AHPs clustered at high and low values (Fig. 5A). This indicates that intrinsic inhibition is also modified in cocaine-SA animals that did not experience extinction training. In addition, we killed a group of naive animals without any prior experience of handling and surgery. Cells from naive rats displayed firing and AHP responses (Fig. 5B) qualitatively identical to those observed after saline-yoked treatment (Fig. 4B). This indicates that the cocaine-SA-induced adaption in intrinsic inhibition in the PFC did not require extinction training or the handling associated with the surgical procedures.

Self-administration of sucrose can modify some of the same brain reward circuitry stimulated by drugs of abuse (McGlinchey et al., 2016). We next determined whether sucrose, a nondrug reinforcer, produced adaptations in intrinsic inhibition in the PFC similar to those observed after cocaine self-administration. Rats trained to self-administer sucrose pellets paired with light/tone cues (Fig. 5C1) for 10–14 d showed stable lever pressing (Fig. 5C2). During extinction conditions (Ext, 14–19 d) when neither cues nor sucrose was available, lever-press responding diminished as expected (Fig. 5C2). To evaluate firing and AHP responses, rats were killed either at 24 h after the final self-administration training session (training; day 15) or immediately after the final extinction (Ext; day 29). Firing responses in the sucrose-SA rats after training and extinction differed (Fig. 5C3; two-way ANOVA with repeated measures of current: treatment, $F_{(1,45)} = 5.525$, $p = 0.0232$; current, $F_{(7,315)} = 20.97$, $p < 0.0001$; interaction, $F_{(7,315)} = 4.921$, $p < 0.0001$). After training, approximately half of the sampled sucrose-SA cells showed elevated firing (SFA$^-$, 56%; Fig. 5C4), which is a much lower proportion than we observed after cocaine-SA training (Fig. 3B1, 81%). In contrast, following extinction, few cells in the sucrose-SA group showed elevated firing (Fig. 5C4, SFA$^-$, 14%). In fact, sucrose-SA responses after extinction more resembled those described previously in the saline-yoked with extinction rats (Fig. 4B, 11%) or in naive rats (Fig. 5B, 5%), and did not resemble responses in the cocaine-SA extinction group (Fig. 4B2, 57%). AHPs in the sucrose-SA rats after training and extinction also differed (Fig. 5C5; unpaired t test: $t_{(41)} = 6.876$, $p < 0.0001$). AHP responses in the sucrose-SA with extinction group resembled responses in the saline-yoked with extinction (Fig. 4B) or in naive rats (Fig. 5B, 5%), and diverged from AHP responses in the cocaine-SA extinction group (Fig. 4B3). Together, this indicates that training to self-administer drug (cocaine) and nondrug (sucrose) reinforcers produces qualitatively similar reductions in intrinsic inhibition early on, but for drug reinforcers these modifications are persistent even after withdrawal or extinction. These data are consistent with those of previous studies reporting that drug and nondrug reinforcers produce different cellular adaptations in PL-PFC neurons (Whitaker et al., 2017).

Stabilization of Kv7-mediated inhibition suppresses cued-reinstatement: experiment 4

To determine whether a loss of intrinsic inhibition contributes to relapse and to what extent the stabilization of inhibitory Kv7 channels would reduce relapse, we measured responses in rats 15 min after a 2 h cue-reinstatement session (Fig. 6A). Following cued reinstatement testing (Fig. 2B), the cocaine-SA group showed robust differences in firing from saline-yoked controls (Fig. 6B1; two-way ANOVA: treatment, $F_{(1,43)} = 34.22$, $p < 0.0001$; current, $F_{(7,301)} = 36.09$, $p < 0.0001$; interaction, $F_{(7,301)} = 23.16$, $p < 0.0001$). A post hoc analysis showed elevated firing (SFA$^-$ responses) in the cocaine-SA group (1 nA current step: $t_{(344)} = 10.68$, $p < 0.0001$). Although after cue testing most cocaine-SA cells (72%) showed this firing response, only a minority (11%) of saline-yoked controls were SFA$^-$ (Fig. 6B2). Although visual inspection of dot plots depicting individual cell AHP responses in cocaine-SA and saline-yoked groups showed an apparent divergent distribution of responses in the cocaine-SA group (Fig. 6B3), the difference in the average AHP between groups did not reach statistical significance (Fig. 6B3; t test: treatment, $t_{(41)} = 1.847$, $p = 0.0720$). Thus, cued reinstatement is associated with potentiated firing responses in SFA$^-$ cells from rats with extinguished behavioral responding for cocaine self-administration.
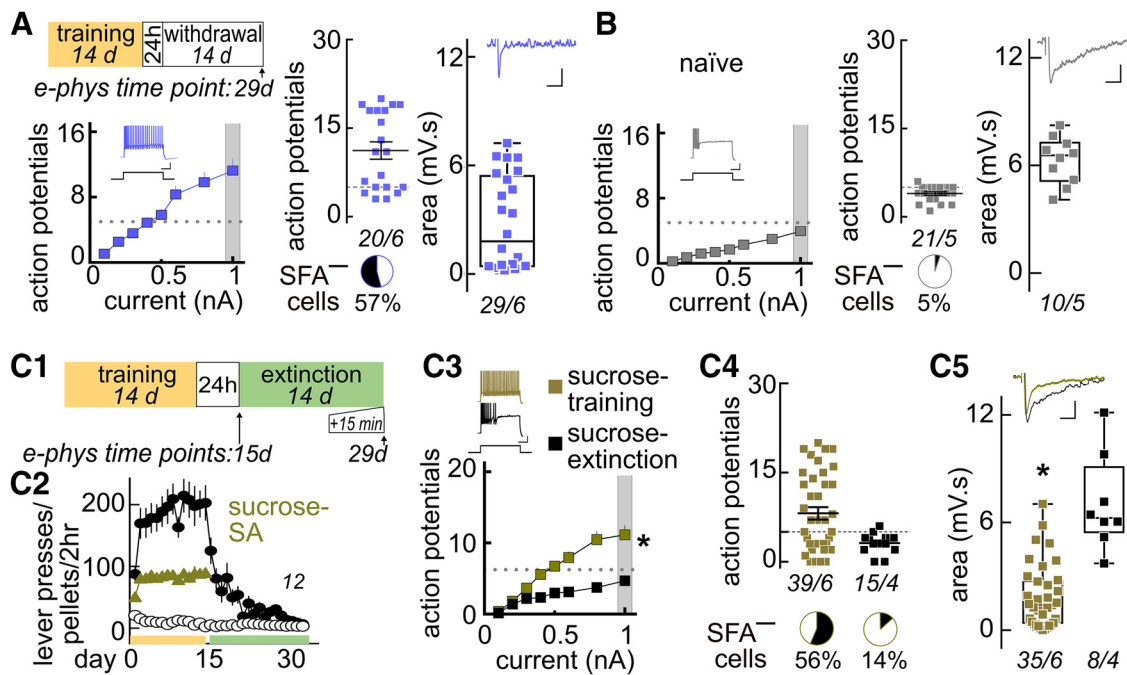
Figure 5. Differences in intrinsic inhibition following withdrawal from cocaine-SA and behavioral extinction from sucrose-SA. **A**, Top, Timeline showing cocaine-SA with 14 d of home-cage withdrawal, ending immediately before the preparation of brain slices for electrophysiological recordings (e-phys). Cells from rats withdrawn from cocaine-SA (without extinction training) showed elevated average firing beyond the maximal range for SFA (I–O curve with inset sample trace collected at 1 nA), heterogeneous individual firing responses (scatter plots at 1 nA), and reduced AHPs (inset, sample trace and scatter plots). **B**, Responses from unhandled, experimentally naive rats showed robust SFA across the I–O curve and homogeneous individual firing (scatter plots at 1 nA) and AHP (scatter plots) responses. **C1**, Timeline showing 14 d of sucrose-SA followed by extinction (14 d). Preparation of brain slices occurred at either 24 h after the last SA session (day 15) or 15 min after the final extinction session (day 29). **C2**, Rats were presented with levers that resulted in the activation of a light and tone cue paired with the delivery of a sucrose pellet. The vertical dashed line on time courses indicates the switch from self-administration to extinction. In extinction sessions, lever pressing had no consequence. Behavioral responses for sucrose extinguished in 14 d. **C3–C5**, Comparison of responses showing the increased excitability associated with sucrose-SA resolves with extinction, including average firing (**C3**, I–O curves), individual firing responses (**C4**, scatter plot, at 1 nA), and AHPs (**C5**, scatter plot). The black segments in the pie charts indicate the percentage of sampled cells that were SFA⁻. Italicized numbers indicate the number of cells/animal. Calibration: **A, B, C**, firing traces, 20 mV, 0.2 s; **A, B, C**, AHP traces, 3 mV, 0.4 s. *$p \leq 0.001$ compared with sucrose-extinction using a Sidak test for multiple comparisons or unpaired t test.

Because changes in SFA responses following reinstatement testing exceeded those following extinction, we determined whether cued reinstatement exposure also suppressed the function of Kv7 channels (Fig. 6C). A comparison of I_{Kv7} function after cued reinstatement testing showed obvious differences between the SFA⁺ cells (from both the saline-yoked and cocaine-SA groups) and SFA⁻ cells in the cocaine-SA group (Fig. 6C; two-way ANOVA with repeated measures of potential: treatment, $F_{(2,29)} = 9.064$, $p = 0.0006$; potential, $F_{(2,58)} = 80.98$, $p < 0.0001$; interaction, $F_{(4,58)} = 7.272$, $p < 0.0001$). A *post hoc* analysis of responses at -20 mV indicated no difference in I_{Kv7} amplitude between SFA⁺ cells from saline-yoked and cocaine-SA rats (Fig. 6C; $t_{(87)} = 0.8783$, $p = 0.7673$), but a significant reduction of I_{Kv7} amplitudes in the SFA⁻ cell population (Fig. 6C; cocaine-SA/SFA⁻ vs cocaine-SA/SFA⁺: $t_{(87)} = 4.718$, $p < 0.0001$; cocaine-SA/SFA⁻ vs saline-yoked/SFA⁺: $t_{(87)} = 6.166$, $p < 0.0001$). Thus, the function of Kv7 channels is suppressed in SFA⁻ cells following re-exposure to drug paired cues that reinstate drug seeking.

To determine whether the stabilization of Kv7 channels with retigabine could restore SFA and I_{Kv7} in neurons from cocaine-SA-experienced animals, two types of experiments were performed. In the first experiment, current-clamp recordings were conducted in slices prepared at 15 min after a 2 h cue test to sample firing both before and after bath application of retigabine (20 μM, 10 min). Firing responses in the saline-yoked and cocaine-SA treatments differed considerably at this time point (Fig. 6D; two-way ANOVA: treatment, $F_{(3,34)} = 22.44$, $p = 0.0001$; current, $F_{(7,238)} = 9.626$, $p = 0.0001$; interaction, $F_{(21,238)} = 14.49$, $p < 0.0001$). A *post*

hoc analysis of the retigabine action measured at the 1 nA current step indicated no measurable retigabine effect on firing in SFA⁺ cells from saline-yoked rats ($t_{(272)} = 0.1590$, $p > 0.9999$), but a robust retigabine-mediated reduction of firing in SFA⁻ cells from cocaine-SA rats ($t_{(272)} = 14.2$, $p < 0.0001$). Presumably, the nominal response of controls to retigabine reflected strong pre-existing intrinsic inhibition, whereas the pronounced retigabine action across the full range of current injections in SFA⁻ cells in cocaine-SA rats reflected a pre-existing state of weakened Kv7 channel-mediated inhibition.

To test this notion, a second group of experiments determined whether exposure to drug-paired cues reduced Kv7 channel inhibition and to what extent the reduction was rescued by retigabine. Following the cued reinstatement test, I_{Kv7} currents were measured before and after bath application of retigabine (20 μM, 10 min). Retigabine responses in SFA⁺ cells from saline-yoked controls and SFA⁻ cells from cocaine-SA rats differed (Fig. 6E; two-way ANOVA; treatment, $F_{(3,32)} = 5.503$, $p = 0.0037$; potential, $F_{(2,64)} = 81.19$, $p < 0.0001$; interaction, $F_{(6,64)} = 3.559$, $p = 0.0042$). Subsequent analysis showed that retigabine increased the amplitude of I_{Kv7} in SFA⁻ cells from cocaine-SA rats ($t_{(96)} = 1.672$, $p = 0.0138$) without significantly altering the I_{Kv7} responses in SFA⁺ cells from saline-yoked rats ($t_{(96)} = 1.672$, $p = 0.4609$). This indicates that retigabine partially restored Kv7 channel inhibition to SFA⁻ cells from cocaine-SA rats, but, in contrast, produced little measurable change in neurons with normal pre-existing levels of intrinsic inhibition.

Cue-induced reinstatement of cocaine seeking is blocked by reversible pharmacological or chemogenetic/optogenetic inacti-
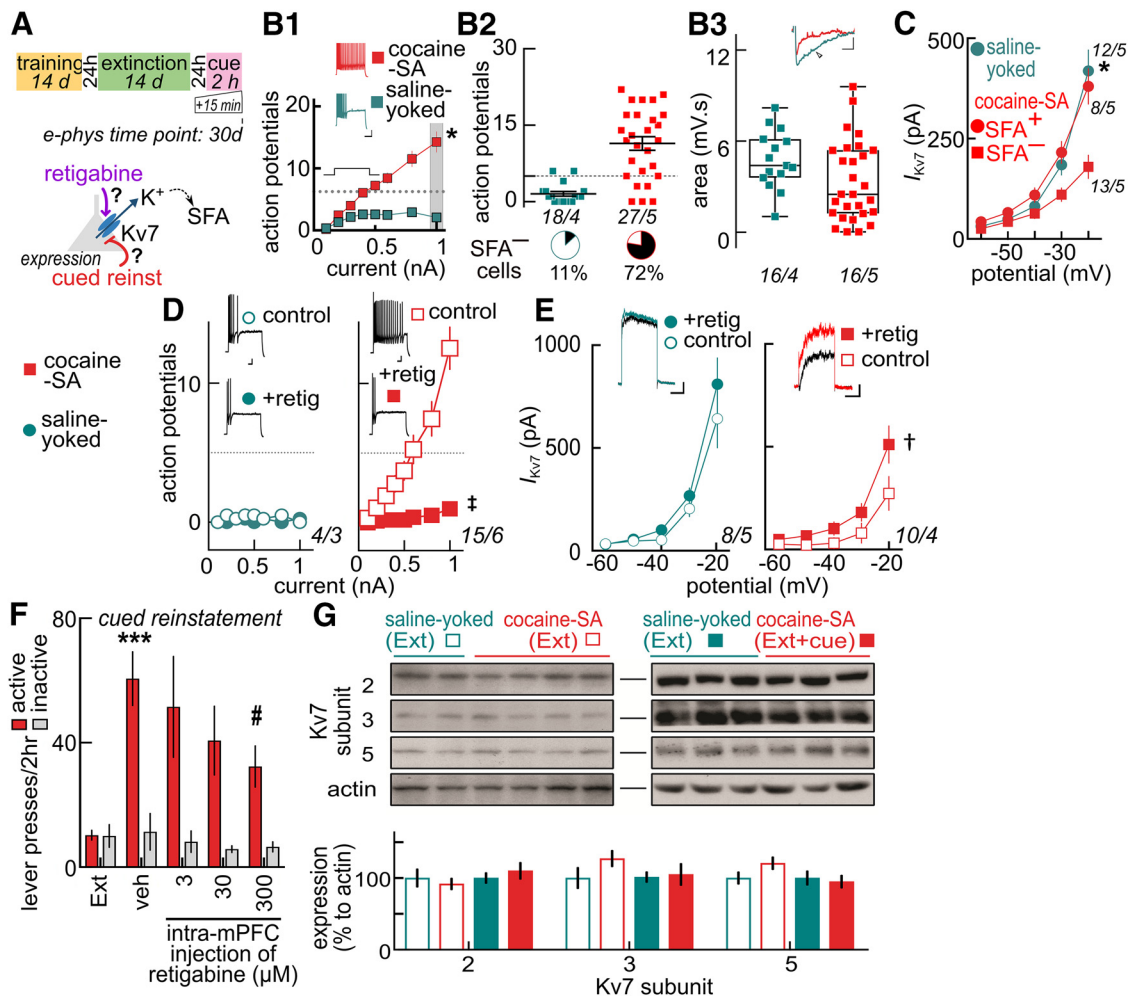
Figure 6. Cues that reinstate cocaine seeking rekindle the suppression of intrinsic inhibition: Restoration of inhibition by stabilization of Kv7 channels reduces cocaine seeking. ***A***, Timeline showing the preparation of brain slices, occurring 15 min after the 2 h cued reinstatement test. Diagram shows proposed retigabine (retig) action on I_{Kv7} in SFA$^-$ cells during reinstatement. ***B1–B3***, Comparison of responses showing cued reinstatement reduces intrinsic inhibition in cocaine-SA treatments relative to saline-yoked controls. Shown are average firing (***B1***, I–O curves), individual firing responses (***B2***, scatter plot, at 1 nA), and AHPs (***B3***, scatter plot). Black segments in pie charts below indicate the percentage of sampled cells that were SFA$^-$. ***C***, Relative to SFA$^+$ (cocaine-SA or saline-yoked) cells, I_{Kv7} currents are reduced in SFA$^-$ (cocaine-SA) cells. ***D, E***, Summary graphs showing retigabine (20 μM) reduced firing (***D***) and increased I_{Kv7} (***E***) only in SFA$^-$ (cocaine-SA) cells, and not in SFA$^+$ (saline-yoked and cocaine-SA) cells. ***F***, Bilateral microinjection of retigabine into PL-PFC 10 min before reinstatement testing resulted in a dose-dependent reduction of cue-induced reinstatement. ***G***, Western blots of PL-PFC punch lysates showing Kv7 subunit expression (7.2, 7.3, 7.5) as a percentage of actin control did not differ between saline-yoked and cocaine-SA, either after extinction or after cued reinstatement. Calibration: ***B1, D***, 20 mV, 0.2 s; ***B3***, 3 mV, 0.4 s; ***C1, E***, 0.1 nA, 0.4 s. All insets showing sample traces were collected at the 1 nA step for firing (*I*-clamp) or at −20 mV for Kv7 (*V*-clamp). Italicized numbers indicate the cells/animal. Sidak test for multiple comparisons: $p \leq 0.0001$ compared with saline-yoked (*; ***B1, C***) or preretigabine (‡; ***D***); $p \leq 0.01$ compared with preretigabine (†; ***E***). ***F***, ***p ≤ 0.0001, vs ext; #$p = 0.0299$, vehicle vs retig (300 μM). e-phys, Electrophysiological recordings; vehicle, veh.

vation of cells in the PL-PFC or their terminals in the nucleus accumbens (Kalivas and McFarland, 2003; Gipson et al., 2013, 2014; Stefanik et al., 2016). It is possible that the drug-paired cues activated PL-PFC cells with reduced intrinsic inhibition, thus causing enhanced firing that promotes cocaine seeking. To determine whether the stabilization of inhibitory PL-PFC Kv7 channels would reduce cued reinstatement of cocaine-seeking behavior, we used a counterbalanced design using cocaine-SA rats that were extinguished from cocaine seeking and microinjected retigabine or vehicle directly into PL-PFC region 10 min before reinstatement testing. Infusions altered the average lever-pressing behavior for drug-associated cues (Fig. 6F; two-way ANOVA: concentration, $F_{(4,110)} = 5.561$, $p = 0.0004$; lever, $F_{(1,110)} = 56.64$, $p < 0.0001$; interaction, $F_{(4,110)} = 5.504$, $p = 0.0004$). A *post hoc* comparison of pressing between the last extinction session and the cue session showed potentiated active lever pressing in vehicle-injected animals (Fig. 6F; $t_{(110)} = 6.233$, $p < 0.0001$). The magnitude of the reinstatement was compara-

ble to what we observed in our earlier experiments (Fig. 2B) and consistent with previous reports (Venniro et al., 2016). Relative to vehicle infusions, retigabine dose-dependently reduced the reinstatement of active lever pressing significantly at 300 μM (Fig. 6G; 3 μM, $t_{(110)} = 0.8641$, $p = 0.9928$; 30 μM, $t_{(110)} = 2.073$, $p = 0.3387$; 300 μM, $t_{(110)} = 3.036$, $p = 0.0295$). A comparison of the total distance traveled by the same rats in a novel chamber locomotion test did not show significant differences caused by retigabine in any of the treatment groups (one-way ANOVA comparing vehicle, retigabine (30 μM), and retigabine (300 μM): $F_{(2,33)} = 0.5745$, $p = 0.5685$), suggesting that the retigabine action on reinstatement was not due to a nonspecific reduction in motor behavior. The cannula placements for PL-PFC microinjections were verified histologically in all animals included in the analysis.

To determine whether these cocaine experiences altered the protein expression of Kv7 channel subunits, separate groups of saline-yoked and cocaine-SA rats were killed 15 min after the 2 h reinstatement test for a Western blot analysis (Fig. 6G). A com-

parison of the PL-PFC tissue homogenates showed no differences in the expression levels of the three principle subunits of the neuronal Kv7 family (Kv7.2, Kv7.3, and Kv7.5; Fig. 5G; two-way ANOVA: treatment, $F_{(3,84)} = 0.6641$, $p = 0.5764$; subunits, $F_{(2,84)} = 0.3579$, $p = 0.7002$; interaction, $F_{(6,84)} = 7699$, $p = 0.5957$). Together, these results suggest that rather than reducing Kv7 channel expression, the operant training for cocaine desensitizes the function of inhibitory Kv7 channels. Thus, by stabilizing the open state of Kv7 channels, retigabine reduces cue-induced reinstatement of cocaine seeking when injected into the PL-PFC.

Reduced inhibition in cFos-GFP$^+$, but not cFos-GFP$^-$ cells: experiment 5

The experiments above used a generalized selection criterion of PL-PFC neurons based on location, morphology, and electrophysiological parameters to show that re-exposure to drug-conditioned cues to reinstate drug seeking also rekindled the reduction in intrinsic inhibition that emerges after cocaine self-administration. This occurred in the majority of SFA$^-$ cells, despite the extinction of behavioral responding for cocaine. Because published studies have shown a causal link between the persistent expression of cocaine-related behaviors and the activation of select ensembles of neurons following re-exposure to drug-associated cues and context (Koya et al., 2009; Bossert et al., 2011; Fanous et al., 2012; Bobadilla et al., 2017), we evaluated whether the PL-PFC neurons with reduced inhibition represented a similar engram. Behaviorally relevant ensembles of cells have been identified in behaviorally activated cFos-GFP transgenic rats, where activation of the c-fos promoter drives the expression of green fluorescent protein (cFos-GFP; Fig. 7A; Koya et al., 2012). Using the same line of transgenic animals, we determined whether the expression of Fos paralleled the cocaine-SA modification in intrinsic inhibition.

Transgenic rats underwent self-administration training for cocaine or yoked infusion of saline followed by extinction (Fig. 7B) and cued reinstatement (Fig. 7C). No differences in the total number of drug or saline infusions were found between the group of transgenic rats and the wild-type rats examined above in Figure 2A (one-way ANOVA: $F_{(3, 90)} = 0.3502$, $p = 0.7891$). Similar to the above responses in wild-type rats (Fig. 2A), transgenic rats trained to self-administer cocaine pressed the active lever significantly more than saline-yoked controls (Fig. 7B; two-way ANOVA with repeated measures over time: treatment, $F_{(1,16)} = 21.89$, $p = 0.0003$; time, $F_{(13,208)} = 1.221$, $p = 0.2661$; interaction, $F_{(13,208)} = 1.963$, $p = 0.0253$). Active lever pressing for drug was extinguished over 2 weeks of extinction training (Fig. 7B). The responses to reinstatement testing differed between the saline-yoked and cocaine-SA groups (two-way ANOVA: treatment, $F_{(1,25)} = 34.56$, $p < 0.0001$; time, $F_{(1,25)} = 35.38$, $p < 0.0001$; interaction, $F_{(1,25)} = 38.10$, $p < 0.0001$). Lever pressing in the transgenic cocaine-SA group increased above that observed during the last day of extinction training (Fig. 7C; $t_{(25)} = 8.649$, $p < 0.0001$). As predicted, rats in the transgenic saline-yoked group did not show increased lever pressing in response to light and tone cues (Fig. 7C; $t_{(25)} = 0.1576$, $p = 0.9846$).

To corroborate previous reports validating the transgene, one rat from each of the saline-yoked and the cocaine-SA training groups received cardiac perfusion 15 min following the first extinction session (day 15) for subsequent immunofluorescent evaluation. This time point is associated with a burst of lever-pressing activity in cocaine-SA rats (Fig. 7B). The GFP fluorescence levels in fixed sections visualized on a confocal microscope showed a high degree of overlap of cFos-GFP and endogenous

immunolabeled Fos (Fig. 7D1,D2), which is consistent with other published work (Koya et al., 2012). A similar group of animals killed immediately following the final extinction session (day 30) showed qualitatively similar GFP$^+$ cells in living brain slices when evaluated on a confocal microscope (Fig. 7E1). Cells from living brain slices collected at the same time point (day 30) were also suitable for high-magnification *ex vivo* electrophysiology experiments (Fig. 7E2).

Fluorescence-guided patch-clamp recordings of GFP$^{+/-}$ cells were performed in slices from the cFos-GFP transgenic rats (Fig. 7F; Whitaker et al., 2017). Where possible, we attempted to sample equal numbers of GFP$^{+/-}$ cells from each brain slice. In contrast to experiment 5, the final extinction or cue-test session was halted after 90 min to maximize the likelihood of a cFos-GFP signal. The firing responses of GFP$^+$ and GFP$^-$ cells in the saline-yoked and cocaine-SA groups after extinction differed (Fig. 7F; three-way ANOVA with repeated measures: treatment, $F_{(1,380)} = 1.02$, $p = 0.3140$; current, $F_{(1,380)} = 58.80$, $p < 0.0001$; GFP$^{+/-}$, $F_{(1,380)} = 0.99$, $p < 0.3194$; interaction, $F_{(1,380)} = 35.47$, $p < 0.0001$). In particular, changes in the current amplitude varied by both treatment (saline-yoked or cocaine-SA) and GFP expression (Fig. 7F; current $\times$ treatment, $F_{(1,380)} = 32.85$, $p < 0.0001$; current $\times$ GFP$^{+/-}$, $F_{(1,380)} = 70.37$, $p < 0.0001$). Visual inspection of only the saline-yoked treatments suggested that the number of action potentials increased with increasing current injection, without obvious differences between GFP$^+$ and GFP$^-$ cells (Fig. 7F). In contrast, with the cocaine-SA treatments, GFP$^+$ and GFP$^-$ cell responses to depolarizing current appeared noticeably different (Fig. 7F). Even in brain slices from the same cocaine-SA with extinction animal, GFP$^+$ cells from cocaine-SA with extinction rats showed on average a threefold to fourfold enhancement of firing relative to the GFP$^-$ cells in the same animals (1 nA step). The dot-plot summary for firing indicated that most cells (both GFP$^+$ and GFP$^-$) in the saline-yoked group displayed SFA after extinction (Fig. 7F). By contrast, in the cocaine-SA group, most GFP$^+$ cells lacked SFA, whereas the GFP$^-$ cells in the same group were qualitatively similar to saline-yoked responses after extinction (Fig. 7F).

Following extinction, AHPs in GFP$^{+/-}$ cells in the saline-yoked and cocaine-SA treatment groups also differed (two-way ANOVA: treatment, $F_{(1,51)} = 11.18$, $p = 0.0016$; GFP$^{+/-}$, $F_{(1,51)} = 3.74$, $p = 0.0588$; interaction, $F_{(1,51)} = 4.10$, $p = 0.0483$). A follow-up analysis showed that AHPs in GFP$^+$ cells from cocaine-SA rats were reduced relative to the saline-yoked rats (Fig. 7F; *post hoc* test: $t_{(51)} = 3.88$, $p = 0.0017$). In contrast, AHPs in GFP$^-$ cells in the saline-yoked and cocaine-SA groups were similar (Fig. 7F; *post hoc* test: $t_{(51)} = 0.91$, $p = 0.7975$). Comparing AHP responses within respective treatment groups, GFP$^+$ and GFP$^-$ cells were equivalent for the saline-yoked groups, but differed for the cocaine-SA groups (Fig. 7F; *post hoc* saline-yoked group, $t_{(51)} = -0.06$, $p = 0.7975$; cocaine-SA group, $t_{(51)} = 2.99$, $p = 0.0218$). These data indicate that in animals with a history of cocaine-SA treatment (but not saline-yoked treatment), the expression of GFP$^+$ overlaps with the persistent reduction in intrinsic inhibition after extinction training.

The effect of cocaine-predictive cues was similar to that observed above with extinction; however, GFP$^-$ cells were typically more difficult to find. After cue exposure, the firing responses of GFP$^{+/-}$ cells in the saline-yoked and cocaine-SA groups also differed (Fig. 7G; three-way ANOVA with repeated measures of treatment: $F_{(1,401)} = 2.26$, $p = 01331$; current: $F_{(1,401)} = 80.80$, $p < 0.0001$; GFP$^{+/-}$: $F_{(1,401)} = 3.06$, $p < 0.0812$; interaction: $F_{(1,401)} = 114.14$, $p < 0.0001$). As above, changes in the current
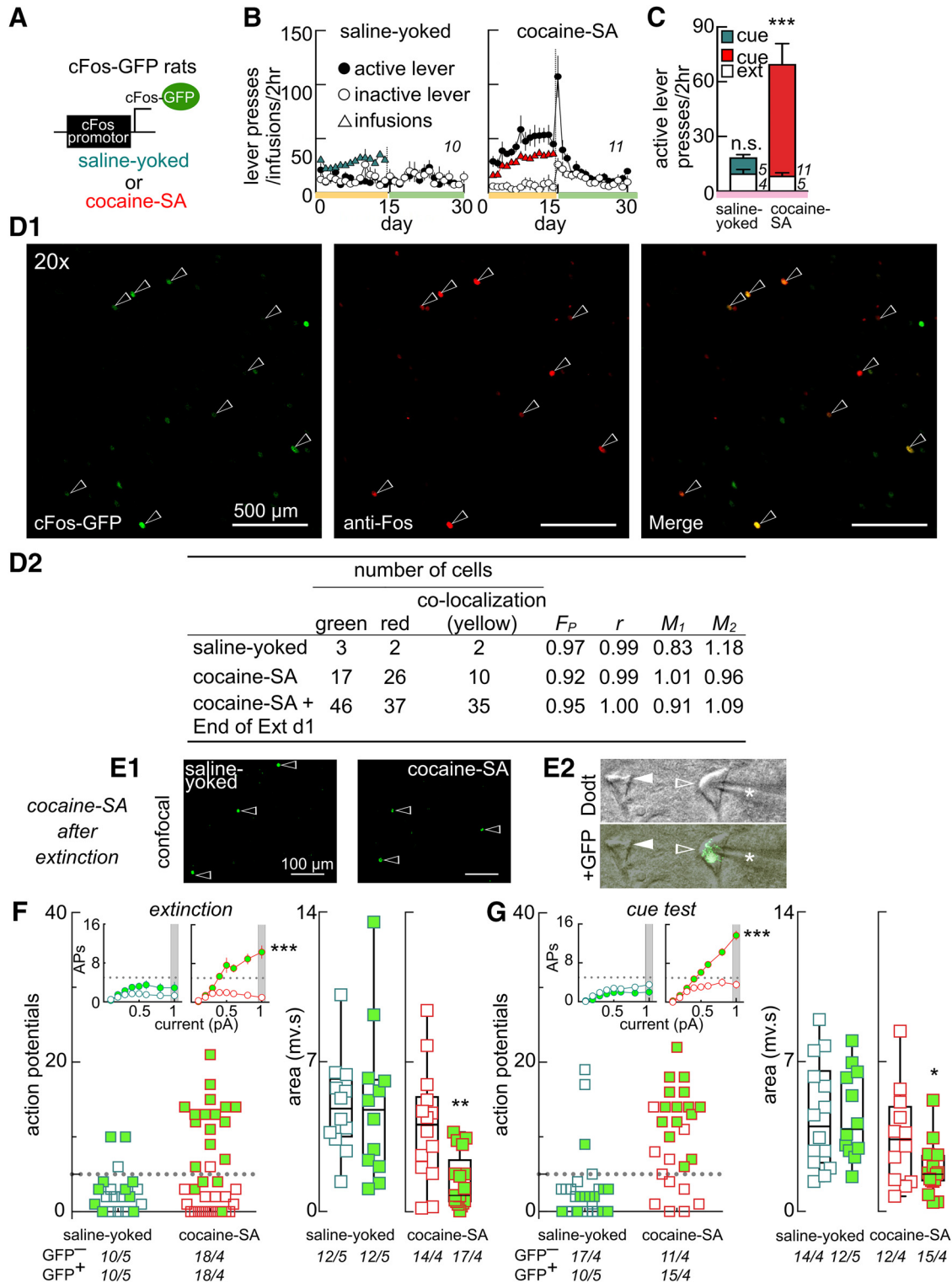
Figure 7. Characterization of intrinsic inhibition in cFos-GFP$^+$ and cFos-GFP$^-$ cells in the PL-PFC after extinction and cued reinstatement of cocaine seeking. **A**, Schematic of the transgene containing a c-fos promoter that induces GFP in strongly activated Fos-expressing neurons, which can be sampled in electrophysiological slice preparations. **B**, Lever-pressing behavior for Long–Evans rats providing the PL-PFC electrophysiology recordings and immunohistochemistry. Rats were presented with levers that resulted in the activation of a light and tone cue paired with the infusion of cocaine (cocaine-SA, right) or yoked passive infusion of saline (saline-yoked, left). The vertical dashed line on the time courses indicates the switch from SA to Ext. In Ext sessions, lever pressing had no consequence. **C**, Re-exposure to cues (without saline or rewards) significantly increased lever pressing in rats with a history of cocaine-SA (Sidak *post hoc*; ***$p \leq 0.0001$, cue vs extinction). **D1**, Confocal photomicrographs showing colabeling (white arrows) of GFP (green) and Fos (red) using immunofluorescence histochemistry. Micrographs are from a c-Fos-GFP rat that underwent cocaine-SA and a single Ext training session before perfusion (day 15). **D2**, Table summarizing GFP/Fos colabeling in transgenic rats with different treatments: Rat 1, saline-yoked + 14 d EXT(killing on day 28); Rat 2-cocaine-SA (killing on day 14); Rat 3 cocaine-SA + 1 d Ext (killing on day 15). A high degree of colocalization between fluorophores was evident from the Pearson's correlation coefficient (F_P; summarizing the ratio of cells that colocalize) and Manders' overlap coefficient (r; showing the overlap between channels). **E1**, Confocal photomicrographs show similarly low levels of GFP-IR (open arrowheads) in the PFC of a saline-yoked (left) and cocaine-SA (right) rat immediately after the final Ext session (day 28). **E2**, Photomicrographs of pyramidal neurons in a living brain slice from a cFos-GFP rat killed after the final Ext session (day 28). Cells were visualized in the electrophysiology recording chamber by contrast (*Figure legend continues.*)

amplitude varied by both treatment (saline-yoked or cocaine-SA) and GFP expression (Fig. 7G; current × treatment: $F_{(1,401)} = 140.37$, $p < 0.0001$; current × GFP$^{+/-}$: $F_{(1,401)} = 69.93$, $p < 0.0001$). In the saline-yoked treatments, action potential firing in GFP$^+$ and GFP$^-$ cells increased in a similar fashion (Fig. 7G). In contrast, in cocaine-SA treatments, the response of GFP$^+$ and GFP$^-$ cells to depolarizing current was noticeably different, and all GFP$^+$ cells lacked SFA (Fig. 7F). In the cocaine-SA group, GFP$^+$ cells showed a fourfold greater number of action potentials than in the GFP$^-$ cells (1 nA step). After cue exposure, AHPs in GFP$^{+/-}$ cells in the cocaine-SA and saline-yoked treatments differed as a function of treatment (Fig. 7G; two-way ANOVA: treatment, $F_{(1,49)} = 10.00$, $p = 0.0027$; GFP$^{+/-}$, $F_{(1,49)} = 1.73$, $p < 0.1944$; interaction, $F_{(1,49)} = 1.96$, $p < 0.1681$). Data from the extinction and cued reinstatement experiments shown above indicate that the SFA$^-$ cells from rats with a history of cocaine-SA and conditioned cues also express cFos-GFP. In other studies, in behaviorally extinguished animals, GFP$^+$ neurons comprising ensemble networks display strong activation in response to drug context environments (Koya et al., 2009, 2012). The nature of the Fos-generating stimulus in our behaviorally extinguished cocaine-SA animals remains to be determined.

Collectively, the above studies demonstrate that the reduction of intrinsic inhibition in a subpopulation of PL-PFC neurons after repeated operant cocaine-SA coincides temporally with changes in both drug seeking and neuronal activation. In summary, these experiments indicate the following: (1) operant training for a drug (cocaine) or nondrug (sucrose) reinforcer is associated with neuromodification of two powerful types of intrinsic inhibition; (2) whereas the modification for the nondrug reinforcer resolves during extinction, it persists for the drug reinforcer cocaine in a subpopulation of cells throughout extinction and cued reinstatement of cocaine seeking; and (3) the persistent excitability is also associated with a Fos-GFP$^+$ signature. Whether the activation of Fos reflects a re-exposure to the same environment where the animal first learned to associate the light and tone cues with cocaine or, alternatively, reflects that competing activation of different ensembles (extinction vs cue) remains to be determined.

Discussion

In rats, we found that learning to self-administer a drug reinforcer (cocaine) reduced PL-PFC intrinsic inhibition (as measured by SFA and AHPs) mediated by Kv7 channels. This modification persisted in a subpopulation of PL-PFC neurons throughout extinction and was enhanced further by exposure to drug-predictive cues. Such modifications were absent after simi-

lar treatments with a nondrug reinforcer (sucrose) or the same drug reinforcer (cocaine) administered passively under a yoked schedule. Cellular plasticity that is resistant to behavioral extinction and unusually robust is considered important for drug-seeking behaviors. An existing prevailing theory is that drug seeking stems from repeated intense neuronal activity that causes downstream biochemical changes in signal transduction, and thereby alters the responsiveness of cortical neurons to neurotransmitters that encode and interpret cues. In line with this, we found that dopamine regulation of intrinsic inhibition was occluded only in this hyper-responsive neural subpopulation and coincided with the expression of the cFos-GFP transgene, suggesting prior robust dopamine transmission. Pharmacological stabilization of Kv7 channels with retigabine normalized SFA, potentiated I_{Kv7}, and reduced reinstatement behavior. By contrast, cells lacking this modification showed normal levels of I_{Kv7} inhibition and insensitivity to retigabine, suggesting that some pharmacological specificity for the activated subpopulation of cells mediating drug seeking. As Western blot analysis showed no overall changes in channel protein expression levels, it appears that repeated cocaine-SA causes a functional desensitization of inhibitory Kv7 channels. Given the role of SFA in the synchronization of neuronal assemblies, this pathological shift away from intrinsic inhibition in this subpopulation of neurons may facilitate drug-seeking behavior.

Role of intrinsic inhibition in cocaine self-administration and seeking

In primate PFC, learning to acquire a nondrug reward results in the formation of task-relevant neural ensembles (Buschman et al., 2012). The flexible nature of ensembles to form and reform underlies cognitive flexibility (Sejnowski and Paulsen, 2006; Womelsdorf and Fries, 2007). Chronic drug use impairs cognitive flexibility, rendering addicts unable to shift away from behavioral patterns learned during drug taking (Briand et al., 2008; Kalivas and O'Brien, 2008; Porter et al., 2011; Schultz, 2011). SFA and AHPs are candidate cellular mechanisms that promote learning and memory by facilitating neuronal synchronization (Crook et al., 1998; Prescott and Sejnowski, 2008; Prescott et al., 2008; Sehgal et al., 2014). Transient reductions in SFA/AHP mechanisms by neuromodulators like dopamine accelerate the transition of LTD into LTP (Zaitsev and Anwyl, 2012). In other brain regions, reductions in intrinsic inhibition bias the recruitment of neuronal ensembles toward excitation and facilitate memory storage (Yiu et al., 2014) and possibly behavioral responses to drug-associated cues.

Reductions in intrinsic inhibition are learning specific (Moyer et al., 1996; Thompson et al., 1996). Because such reductions are normally transient and normalize shortly after behavioral learning, the underlying mechanisms are considered essential for memory consolidation (Moyer et al., 1996). The restabilization or recovery of inhibition is equally important then to acquire new learning and to extinguish a behavior (Kufahl et al., 2009; Pentkowski et al., 2012, 2014). Thus, persistent drug memories and craving could be associated with deficits in this process. In this regard, our finding that reinforcement learning for a drug reinforcer (cocaine) and a nondrug reinforcer (sucrose) differentially altered intrinsic inhibition is important. Drug and nondrug reinforcers differ in the degree to which they facilitate PFC dopamine release and form cue-reinforcement associations (Ciccocioppo et al., 2004; Tunstall and Kearns, 2014; Tunstall et al., 2014). Following repeated cocaine-SA, the combination of robust release of

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(*Figure legend continued.*) (Dodt) optics, and overlaid with pseudocolor fluorescence to show a GFP$^+$ (open arrowhead) and a GFP$^-$ (closed arrowhead) nucleus. Star indicates the recording electrode. All subjects were killed immediately following their final 90 min behavioral session. *F*, *G*, Comparison of responses from GFP$^+$ and GFP$^-$ cells from cocaine-SA-treated (red) and saline-yoked-treated (blue) rats following Ext (*F*) or cue reinstatement (*G*). Closed and open symbols denote responses in GFP$^+$ and GFP$^-$ cells, respectively. Measurements included average firing (inset, I–O curves), individual firing (scatter plots of responses at 1 nA), and AHPs (scatter plots). Where possible, equal numbers of GFP$^+$/GFP$^-$ cells in animals were sampled. Note the elevated firing and reduced AHPs only in a subset of GFP$^+$ cells from cocaine-SA rats, but not from saline-yoked rats. Italicized numbers in *B* indicate the number of animals, whereas those in *C*, *F*, and *G* indicate the number of cells/animal. Tukey multiple comparison post-test: ***$p \leq 0.0001$ (current × treatment and current × GFP$^{+/-}$) comparing I–O (insets) after extinction or cue; **$p = 0.0218$ comparing AHPs from GFP$^+$ and GFP$^-$ cells from the cocaine-SA group after extinction; *$p \leq 0.05$ effect of treatment comparing AHPs after cue.

PFC dopamine and persistent unremitting reduction in intrinsic inhibition may bias cortical function toward future drug seeking.

Select adaptations in a subpopulation of PL-PFC neurons

Previous studies indicate that sparse ensembles of neurons can encode a "seek" rule learned by the repeated presentation of drug-predictive cues and that selective ablation or inactivation of these neurons reduces reinstatement for food and drug seeking (Bossert et al., 2011; Cruz et al., 2014; Warren et al., 2016; Whitaker et al., 2017) as well as incubation of drug craving (Fanous et al., 2012; Funk et al., 2016; Caprioli et al., 2017). These studies used cFos-LacZ transgenic rat strategies, whereby the LacZ signal serves as a proxy for the endogenous cFos gene product, and can also be targeted for selective inactivation by Duano2 (Cruz et al., 2013). The Fos gene product is a known neuronal marker of intense firing activity (Morgan and Curran, 1991) and is activated by cocaine self-administration and context or cue-induced reinstatement (for review, see Cruz et al., 2015). Fos expression requires robust elevations in calcium influx (McClung and Nestler, 2008) and is associated with downstream biochemical changes that regulate gene expression (Cruz et al., 2013).

Activity-induced increases in Fos are considered to represent the integration of neuronal activity driven by numerous synaptic alterations (Cifani et al., 2012; Koya et al., 2012; Whitaker et al., 2017; Ziminski et al., 2017, 2018). Using cFos-GFP transgenic rats, we observed that cocaine-SA training decreased intrinsic inhibition selectively in cFos/GFP$^+$ neurons. Although the increased firing and increased Fos is logical, it contrasts with other reports showing reductions in excitatory synaptic strength (AMPAR/NMDAR current ratios) and glutamate release in cFos/GFP$^+$ cells after stress reinstatement of food seeking (Cifani et al., 2012; Koya et al., 2012). In those instances, the disparity in the cFos and excitation relationship was explained to reflect indirect homeostatic counteradaptations. Differences in those studies and our results may reflect differing reinforcers, brain regions, or the use of drug-associated cues. Regardless, these studies and ours highlight the importance of studying functional changes, both synaptic and intrinsic, in behaviorally relevant neurons.

The influence of dopamine

Dopamine exerts a multifaceted role in learning (Hollerman and Schultz, 1998), network connectivity (Eytan et al., 2004), and neuronal synchronization (Puig et al., 2014), including a stabilizing influence on cellular activity within neural ensembles (O'Donnell, 2003; Puig and Miller, 2012; Miyawaki et al., 2014). The role of dopamine in drug reward processing appears to facilitate the encoding of reward prediction, imprinting incentive value to reinforcers (energizing approach behavior) and facilitating the learning of reward associations (conditioning) through its modulation of subcortical (including the nucleus accumbens) and cortical brain regions (Di Chiara, 1999; Schultz, 1999, 2002; Waelti et al., 2001; Redish, 2004; Steinberg et al., 2013). Cellular adaptations in dopaminergic signaling contribute to relapse to drug seeking (Hyman et al., 2006), in part by disrupting transmission in the prefrontal cortex (Anderson and Pierce, 2005; Dong et al., 2005; Huang et al., 2007; Sidiropoulou et al., 2009; Buchta and Riegel, 2015). In tissue from rats with a history of repeated cocaine-SA training, we observed a decreased ability of dopamine to alter firing and I_{Kv7} in a subpopulation of cells, which may reflect prior sensitization or overactivation in this neuronal population. Unexpectedly, the SFA$^+$ population of cells from both the saline-yoked and the cocaine-SA treatments showed normal responses to dopamine, indicating some neuro-

nal selectivity in the modification of intrinsic inhibition. The reason for this selectivity is unclear but may reflect differences in dopamine receptor expression. Chronic exposure to cocaine causes rapid, robust, and persistent increases in D1 receptor-mediated signaling that result in a prominent upregulation of Fos (Henry and White, 1995; McClung and Nestler, 2003). Functional D1 receptors are required for Fos induction by cocaine (Drago et al., 1996; Moratalla et al., 1996; Zhang et al., 2002, 2004), and cocaine-induced behaviors are greatly reduced in transgenic mice with inactivated Fos in D1 receptor-expressing cells throughout the brain (Zhang et al., 2006). In SFA$^-$ cells, we observed both occluded dopamine responses and a strong association with the cFos-GFP transgene (even under extinction-like conditions). Perhaps prior robust overactivation or sensitization of dopamine signaling functionally reduces intrinsic inhibition and further modulation by dopamine in these neurons.

Kv7 ion channels

Why Kv7 channel currents are reduced following cocaine-SA is unclear. Cocaine exposure is known to elevate levels of intracellular calcium, which reportedly can initiate a well described calmodulin-sensitive suppression of the Kv7 ion channel activity, resulting in hyperexcitability (Kirkwood et al., 1991; Selyanko and Brown, 1996; Cruzblanca et al., 1998; Gamper and Shapiro, 2003; Gamper et al., 2003). Other possible mechanisms regulating the channel will also need to be examined, including elevated cAMP and protein kinase A activity (Dong et al., 2005; Nasif et al., 2005; Ford et al., 2009) and/or reduced levels of PtdIns(4,5)P2 (Park et al., 2001; Zhang and Linden, 2003; Li et al., 2005). The adaptations outlined above all likely contribute to increased cue-induced drug-seeking behavior long after the cessation of drug use. Nevertheless, our results demonstrate a functional role for Kv7 channel-mediated inhibition in a behaviorally relevant subpopulation of PL-PFC neurons important for cue-induced reinstatement of drug seeking. Future studies combining pharmacological treatments like retigabine with newer strategies for time-sensitive labeling of behaviorally relevant neurons using cFos-TetTag in transgenic mice (Reijmers et al., 2007) may further refine precisely when these adaptations disrupt Kv7 channel function and how this contributes to cue-induced cocaine seeking.

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