Cellular/Molecular

The Epac-Phospholipase Cε Pathway Regulates Endocannabinoid Signaling and Cocaine-Induced Disinhibition of Ventral Tegmental Area Dopamine Neurons

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Exchange protein directly activated by cAMP (Epac) is a direct effector for the ubiquitous second messenger cAMP. Epac activates the phospholipase Cε (PLCε) pathway. PLCβ has been linked to the synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG). Here, we report that Epac facilitates endocannabinoid-mediated retrograde synaptic depression through activation of PLCε. Intracellular loading of a selective Epac agonist 8-CPT-2Me-cAMP into ventral tegmental area (VTA) dopamine neurons enabled previously ineffective stimuli to induce depolarization-induced suppression of inhibition (DSI) and long-term depression of IPSCs (I-LTD) in the VTA. DSI and I-LTD are mediated by 2-AG since they were blocked by a diacylglycerol lipase inhibitor. The effects of 8-CPT-2Me-cAMP on DSI and I-LTD were absent in Epac2 and PLCε knock-out mice, but remained intact in Epac1 knock-out mice. These results identify a novel mechanism for on-demand synthesis of retrograde signaling 2-AG by the Epac2-PLCε pathway. We investigated the functional significance of Epac2-PLCε-2-AG signaling in regulating inhibitory synaptic plasticity in VTA dopamine neurons induced by *in vivo* cocaine exposure. We showed that cocaine place conditioning led to a decrease in the frequency and amplitude of spontaneous IPSCs and an increase in action potential firing in wild-type mice, but not in Epac2 or PLCε knock-out mice. Together, these results indicate that the Epac2-PLCε-2-AG signaling cascade contributes to cocaine-induced disinhibition of VTA dopamine neurons.

Key words: endocannabinoid 2-arachidonoylglycerol; Epac; GABAergic disinhibition; phospholipase Cε; retrograde synaptic depression

Significance Statement

2-arachidonoylglycerol (2-AG) is an endogenous cannabinoid that depresses synaptic transmission through stimulation of CB_1 receptors. Among the six isoforms of phospholipase C (PLC; PLCβ, PLCγ, PLCδ, PLCε, PLCζ, PLCη), only PLCβ has been linked to 2-AG synthesis. Here we demonstrate that 8-CPT-2Me-cAMP, a selective agonist of the cAMP sensor protein Epac, enhances 2-AG-mediated synaptic depression in ventral tegmental area (VTA) dopamine neurons via activation of PLCε. These results identify a novel mechanism for 2-AG synthesis via activation of the Epac-PLCε pathway. Furthermore, we show that cocaine-induced conditioned place preference and disinhibition of VTA dopamine neurons were impaired in mice lacking Epac or PLCε. Thus, the Epac-PLCε signaling pathway contributes to cocaine-induced disinhibition of VTA dopamine neurons and formation of drug-associated memories.

Introduction

Exchange protein directly activated by cAMP (Epac) is a direct intracellular effector of cAMP (Kawasaki et al., 1998; de Rooij et al., 1998). Epac mediates diverse functions of cAMP by acting as a guanine nucleotide exchange factor for Rap, a Ras-like small GTPase (Cheng et al., 2008; Gloerich and Bos, 2010; Schmidt et

Received Sept. 3, 2016; revised Jan. 26, 2017; accepted Feb. 6, 2017.

Author contributions: J.T., X.L., C.V., and Q.-S.L. designed research; J.T., X.L., C.V., Y. Li, and L.Y. performed research; Y. Lu and A.V.S. contributed unpublished reagents/analytic tools; J.T., X.L., C.V., Y. Li, and L.Y. analyzed data; J.T., X.L., C.V., and Q.-S.L. wrote the paper.

This work was supported by National Institutes of Health Grants DA035217 and MH101146 (to Q.S.L.) and GM053536 (to A.V.S.). It was also partially funded through the Research and Education Initiative Fund, a component of the Advancing a Healthier Wisconsin endowment at the Medical College of Wisconsin. C.V. is a member of the Medical Scientist Training Program at MCW, which is partially supported by a training grant from NIGMS T32-GM080202.

The authors declare no competing financial interests.

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DOI:10.1523/JNEUROSCI.2810-16.2017

 0270-6474/17/373030-15$15.00/0

al., 2013). Epac-Rap activates phospholipase Cε (PLCε; Oestreich et al., 2007), a PLC isoform expressed in the heart (Oestreich et al., 2007) and brain (Wu et al., 2003). The Epac-Rap-PLCε signaling pathway regulates intracellular Ca^{2+} release in cardiac myocytes and cardiac contractility (Smrcka et al., 2012), but its role in neuronal signaling remains unknown.

Stimulation of $G_{q/11}$-coupled receptors, such as group-I mGluRs, leads to the activation of PLCβ (Hashimotodani et al., 2005), which hydrolyzes membrane phospholipid to produce a pair of second messengers, inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). DAG is subsequently converted into the endocannabinoid 2-arachidonoylglycerol (2-AG) by DAG lipase (DAGL; Di Marzo et al., 1998; Piomelli, 2003). The mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG) induces endocannabinoid-mediated retrograde synaptic depression (Maejima et al., 2001; Varma et al., 2001; Hashimotodani et al., 2005) and enhances depolarization-induced suppression of inhibition (DSI; Varma et al., 2001; Edwards et al., 2006). Synaptic stimulation of mGluRs induces endocannabinoid-mediated long-term depression (LTD) at excitatory and inhibitory synapses (I-LTD; Gerdeman et al., 2002; Robbe et al., 2002; Chevaleyre and Castillo, 2003, 2004). There are at least six isoforms of PLC (PLCβ, PLCγ, PLCδ, PLCε, PLCζ, PLCη; Rhee and Bae, 1997; Hwang et al., 2005). Among them, only PLCβ has been linked to 2-AG production via mGluRs (Hashimotodani et al., 2005). Based on the finding that Epac activates PLCε (Oestreich et al., 2007), we hypothesized that Epac-PLCε activation enhances 2-AG-mediated retrograde synaptic depression. To test this possibility, we examined the effects of the selective Epac agonist 8-CPT-2Me-cAMP (8-CPT) on DSI and I-LTD in dopamine neurons in the ventral tegmental area (VTA), and the involvement of Epac and PLCε was determined by using Epac (Yang et al., 2012) and PLCε knock-out mice (Oestreich et al., 2007).

Two genes, *Epac1* and *Epac2*, encode Epac proteins. Epac2 is abundantly expressed in the brain, whereas Epac1 expression in the brain is very low (Kawasaki et al., 1998; de Rooij et al., 1998). $Epac2^{-/-}$ mice exhibit deficits in social interaction and communication, but normal working and reference memory (Srivastava et al., 2012). Epac1 and Epac2 may be functionally redundant, as only Epac double-knock-out mice ($Epac^{-/-}$) exhibit deficits in spatial learning and memory (Yang et al., 2012). Hippocampal Epac signaling is required for memory retrieval (Ouyang et al., 2008; Ostroveanu et al., 2010). We have shown that cocaine conditioned place preference (CPP) was impaired in $Epac2^{-/-}$ mice but was not significantly altered in $Epac1^{-/-}$ mice (Liu et al., 2016). Thus, Epac may contribute to memory formation and/or retrieval by spatial and drug-associated cues. Additionally, Epac2 is required for the cocaine-induced insertion of surface GluA2-lacking AMPA receptors (AMPARs) in VTA dopamine neurons (Liu et al., 2016). Epac also regulates presynaptic glutamate release (Gekel and Neher, 2008; Zhao et al., 2013; Fernandes et al., 2015), LTP induction (Gelinas et al., 2008; Yang et al., 2012), and AMPAR trafficking (Ster et al., 2009; Woolfrey et al., 2009; Liu et al., 2016). However, the extent to which Epac regulates inhibitory transmission and plasticity remains essentially unknown.

Repeated cocaine exposure *in vivo* reduces GABAergic inhibition to VTA dopamine neurons *ex vivo* (Liu et al., 2005; Bocklisch et al., 2013). Endocannabinoid-mediated I-LTD provides a putative mechanism for cocaine-induced reduction of GABAergic inhibition (Pan et al., 2008a). We examined whether Epac-PLCε is required for the reduction of GABAergic inhibition to VTA dopamine neurons induced by cocaine exposure *in vivo*. Our studies reveal a novel mechanism for Epac-PLCε in regulating endocannabinoid-mediated retrograde synaptic depression and cocaine-induced inhibitory synaptic plasticity in VTA dopamine neurons.

Materials and Methods

Animals. Animal maintenance and use were in accordance with protocols approved by the Institutional Animal Care and Use Committee of Medical College of Wisconsin. Epac1 knock-out mice ($Epac1^{-/-}$), Epac2 knock-out mice ($Epac2^{-/-}$), and Epac1 and Epac2 double-knock-out mice ($Epac^{-/-}$) were generated and maintained on a 129Sv background in the laboratory of Youming Lu as detailed previously (Yang et al., 2012). The $Epac1^{-/-}$ or $Epac2^{-/-}$ mice were bred to 129Sv wild-type mice at Medical College of Wisconsin for ≥2 generations to generate heterozygous $Epac1^{+/-}$ or $Epac2^{+/-}$ breeders. Wild-type ($Epac^{+/+}$), $Epac1^{-/-}$, or $Epac2^{-/-}$ mice were generated by heterozygous × heterozygous breeding and all experiments were performed in age-matched littermates of either sex. Epac double knock-out ($Epac^{-/-}$) mice were generated by crossing $Epac1^{-/-}$ with $Epac2^{-/-}$ mice. $PLC\varepsilon^{-/-}$ mice were generated in the laboratory of Alan Smrcka and maintained on C57BL/6 background as described previously (Wang et al., 2005). Genotyping analysis was performed by using standard PCR technique on tail biopsies.

Brain-slice preparation. $Epac1^{-/-}$, $Epac2^{-/-}$, $Epac^{-/-}$ mice, their wild-type littermates, $PLC\varepsilon^{-/-}$ mice, and wild-type mice (P20–P30) of either sex were used for slice electrophysiology. In experiments described in Figures 6 and 8, adult wild-type, $Epac2^{-/-}$, and $PLC\varepsilon^{-/-}$ mice of either sex (8–9 weeks old at the beginning of the experiments) underwent place conditioning and behavioral tests (see below, CPP). Slices were prepared 24 h following behavioral tests. Mice were anesthetized by isoflurane inhalation and decapitated. The brain was embedded in low-melting-point agarose, and horizontal midbrain slices (200 μm thick) were cut using a vibrating slicer (Leica VT1200s), as described in our recent study (Chen et al., 2016). Slices were prepared in a choline-based solution containing the following (in mM): 110 choline chloride, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 7 $MgSO_4$, 26 $NaHCO_3$, 25 glucose, 11.6 sodium ascorbate, and 3.1 sodium pyruvate at room temperature. The slices were incubated at room temperature for 30–40 min in sucrose-based solution containing the following (in mM): 78 NaCl, 68 sucrose, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$, and 25 glucose. Then, the slices were allowed to recover for ≥30 min in the artificial CSF (ACSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose.

Slice electrophysiology. Whole-cell patch-clamp recordings were made using patch-clamp amplifiers (Multiclamp 700B) under infrared-differential interference contrast microscopy. Data acquisition and analysis were performed using DigiData 1440A and 1550B digitizers and analysis software pClamp 10 (Molecular Devices). Signals were filtered at 2 kHz and sampled at 10 kHz. Dopamine neurons in the VTA (medial to the medial terminal nucleus of the accessory optic tract) were identified by long duration (>1.5 ms) of spontaneous action potentials in cell-attached configuration (Chieng et al., 2011) and the presence of large I_h currents (>100 pA), rhythmic firing at low frequency (<5 Hz), and prominent afterhyperpolarization in whole-cell mode (Johnson and North, 1992; Jones and Kauer, 1999; Liu et al., 2005; Melis et al., 2008, 2013a). Neurons were voltage-clamped at −70 mV unless stated otherwise. Glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM) and D-2-amino-5-phosphonovaleric acid (D-AP-5; 50 μM) were present in the ACSF throughout the experiments. For recording of IPSCs, electrical stimulation was delivered by a bipolar tungsten stimulation electrode (WPI) placed ~150 μm rostral to the recorded dopamine neuron. Glass pipettes (3–5 MΩ) were filled with an internal solution containing the following (in mM): 90 K-gluconate, 50 KCl, 10 HEPES, 0.2 EGTA, 2 $MgCl_2$, 4 Mg-ATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine, pH 7.2 with KOH. Intracellular perfusion of 8-CPT via whole-cell pipette was performed based on published procedure (Lapointe and Szabo, 1987; Tang et al., 1992; Maathuis et al., 1997). Whole-cell recordings were performed with an Axopatch holder with suction and perfusion ports (catalog #660015, A-M Systems). 8-CPT-

containing internal solution (300 μM) was exchanged into the tip of the pipette via quartz tubing (Polymicro Technologies) connected to a PE-10 tube. Series resistance (15–30 MΩ) was monitored throughout all recordings, and data were discarded if the resistance changed by >20%. All recordings were performed at 32 ± 1°C by using an automatic temperature controller (Warner Instruments).

In vivo *electrophysiology.* Mice were anesthetized with intraperitoneal injection of urethane (1.5 mg/kg). Mice were positioned in a stereotaxic frame (David Kopf Instruments) and their body temperature was maintained at 37°C using a heating pad. Craniotomies were performed to allow single-unit recordings of VTA dopamine neurons. The areas for electrode insertion were moisturized with saline. Single-unit recording electrodes were pulled from micropipettes (outer diameter, 1 mm; inner diameter, 0.5 mm) to a resistance of 10–15 MΩ when filled with 0.5 M NaCl containing 1.5% neurobiotin. The electrode was lowered into the VTA [coordinates from bregma: anteroposterior −2.9 to −3.3 mm, mediolateral 0.6–1.1 mm, dorsoventral −3.9 to −4.5 mm] by a micromanipulator. A reference electrode was placed in the subcutaneous tissue. Single-unit activity was acquired with Multiclamp 700B amplifier and DigiData 1440A digitizer and analyzed by pClamp 10 (Molecular Devices). Signals were filtered at 2 kHz and sampled at 10 kHz. The bandpass filter was set between 0.3 and 5 kHz (Brischoux et al., 2009). Dopamine neurons were identified by a broad triphasic extracellular action potential with a width of >2 ms and a relatively slow firing rate (<10 Hz; Ungless et al., 2004). The burst firing is defined as the occurrence of two consecutive spikes in an interval of <80 ms and the termination of two consecutive spikes with an interval of >160 ms (Grace and Bunney, 1984; Bishop et al., 2010; Schiemann et al., 2012; Chen and Lodge, 2013). To confirm cell type and electrode placement, neurons were juxtacellularly labeled with neurobiotin via iontophoresis (Pinault, 1996; Bocklisch et al., 2013). Briefly, following electrophysiological recordings, positive current pulses (7 s on/off cycles) were applied through the recording electrode to the neuron for 10 min. The neurobiotin was allowed to transport within the neuron for another 1–2 h before the animals were killed for immunofluorescence staining (Pinault, 1996; Bocklisch et al., 2013).

CPP. Cocaine CPP was based on published procedures (Vialou et al., 2012). *Epac2*$^{-/-}$, *PLCε*$^{-/-}$, and wild-type control mice (8–9 weeks old) of either sex were placed into the middle chamber of the three-chamber conditioning apparatus (Med Associates) and allowed to explore three chambers freely for 20 min. Time spent in every chamber was recorded. Mice showing unconditioned side preference (≥180 s disparity) were excluded. On the second and third days, mice were injected with saline (0.9% NaCl, 2 ml/kg, i.p.) in the morning (between 8:00 and 10:00 A.M.) and confined to one chamber for 30 min, and then the mice were injected with cocaine (15 mg/kg, i.p.) in the afternoon (between 3:00 and 5:00 P.M.) and confined for 30 min to the other chamber. On the fourth day, mice were tested for side preference without treatment for 20 min (between 12:00 and 2:00 P.M.).

Immunofluorescence staining. Following *in vivo* single-unit recording of VTA dopamine neurons, mice were perfused transcardially with 4% paraformaldehyde in 4% sucrose-PBS, pH 7.4. Coronal VTA sections (30 μm) were cut with a Leica cryostat. VTA sections were first incubated with fluorescein streptavidin (1:200; Vector Laboratories) to retrieve the neurobiotin-labeled cell bodies. Then the selected tissue sections were incubated with primary antibody against tyrosine hydroxylase (TH; 1:300; Santa Cruz Biotechnology; 48 h) and anti-rabbit IgG Alexa Fluor 555 conjugate (1:500; Cell Signaling Technology) secondary antibody. Confocal imaging was performed using a Nikon TE2000-U inverted microscope equipped with the C1 Plus confocal system (laser light source for EGFP excitation, 488 nm, C-FL B-2E/C FITC filter cube; laser light source for Texas Red excitation, 561 nm, C-FL Y-2E/C Texas filter cube). The images were acquired using a 10× CFI Plan 10× apochromat objective (numerical aperture, 0.45) or a CFI Plan Fluor 40× oil objective (numerical aperture, 1.4) Nikon D-Eclipse C1 camera and EZ-C1 software.

Chemicals. 8-CPT sodium salt, D-(-)-2-amino-5-phosphonopentanoic acid, DHPG, 6-Bnz-cAMP sodium salt, and H-89 dihydrochloride were obtained from Tocris Bioscience. 7,8-dihydroxyflavone, CNQX, and all other common chemicals were obtained from Sigma-Aldrich. DO34 and DO53 were kindly provided by Benjamin Cravatt at Scripps Research Institute. Cocaine HCl was kindly provided by the National Institute on Drug Abuse Drug Supply Program. Drugs were prepared as concentrated stock solutions and stored at −20 or −80°C before use.

Statistics. Data are presented as the mean ± SEM. The magnitude of DSI and I-LTD was calculated as we have described previously (Pan et al., 2008a, 2009). sIPSCs were analyzed using Mini-analysis (Synaptosft). CPP scores were calculated as the time spent in the cocaine-conditioned chamber minus that in saline-conditioned chamber (Zhong et al., 2012). *In vivo* dopamine neuron firing was analyzed using Clampfit 10.6. Datasets were compared with either Student's *t* test or two-way ANOVA followed by Tukey's *post hoc* analysis. Results were considered significant at $p < 0.05$.

Results

Epac agonist 8-CPT facilitated DSI through activation of Epac2

We investigated whether the selective Epac agonist 8-CPT altered DSI in midbrain slices prepared from wild-type (*Epac*$^{+/+}$) and Epac1/2 (*Epac*$^{-/-}$) double-knock-out mice. Whole-cell voltage-clamp recordings were made from VTA dopamine neurons with control internal solution or internal solution containing the selective Epac agonist 8-CPT (100 μM). IPSCs were evoked by electrical stimulation of synaptic afferents at 4 s intervals in the presence of glutamate receptor antagonists CNQX (20 μM) and D-AP-5 (50 μM). We have shown that postsynaptic depolarization induced minimal or no DSI in VTA dopamine neurons in rats or wild-type mice (Pan et al., 2008a; Zhong et al., 2015). Consistent with these studies, we found that in wild-type slices, a brief depolarization of VTA dopamine neurons from −70 to 0 mV for 5 s did not induce significant depression of IPSCs with control internal solution (7.3 ± 4.0%, $n = 8$) but induced DSI with internal solution containing 8-CPT (23.4 ± 5.7%, $n = 9$; $t_{(15)} = 2.4$, $p = 0.031$ vs control; Fig. 1*A*). DSI induced with 8-CPT-containing internal solution was blocked by bath application of the CB_1 receptor antagonist AM251 (2 μM; 6.2 ± 5.3%, $n = 7$; $t_{(14)} = 2.3$, $p = 0.040$ vs 8-CPT; Fig. 1*A*). DSI was not induced in slices from Epac1 and Epac2 double-knock-out (*Epac*$^{-/-}$) mice with control or 8-CPT-containing internal solution (control, 4.2 ± 4.3%, $n = 10$; 8-CPT, 3.1 ± 5.8%, $n = 8$; $t_{(16)} = 1.0$, $p = 0.323$; Fig. 1*B*).

Two genes, *Epac1* and *Epac2*, encode Epac proteins (Kawasaki et al., 1998; de Rooij et al., 1998). We next determined whether Epac1 or Epac2 mediated the effects of 8-CPT on DSI. In *Epac1*$^{-/-}$ slices, DSI was induced with 8-CPT-containing internal solution but not with 8-CPT-free control internal solution (control, 3.7 ± 3.9%, $n = 8$; 8-CPT, 29.2 ± 3.7%, $n = 8$; $t_{(14)} = 4.7$, $p < 0.001$; Fig. 1*C*). In *Epac2*$^{-/-}$ slices, DSI was not induced with either internal solution (control, 1.9 ± 2.9%, $n = 8$; 8-CPT, 4.8 ± 2.6%, $n = 9$; $t_{(15)} = 1.8$, $p = 0.101$; Fig. 1*D*). Thus, Epac2, but not Epac1, mediates 8-CPT-induced facilitation of DSI in VTA dopamine neurons. Epac1 is ubiquitously expressed in all tissues and its expression in the brain is low. Meanwhile, Epac2 is richly expressed in the brain (Kawasaki et al., 1998; de Rooij et al., 1998), which may explain the lack of effect of 8-CPT on DSI in *Epac2*$^{-/-}$ slices.

8-CPT is a cAMP analog that selectively activates Epac but not protein kinase A (PKA; Enserink et al., 2002), whereas 6-Bnz-cAMP is a cAMP analog that selectively activates PKA but does not affect Epac (Hewer et al., 2011). Nevertheless, they may have off-target effects at higher concentrations. As a control experiment, we examined whether 6-Bnz-cAMP altered DSI in VTA

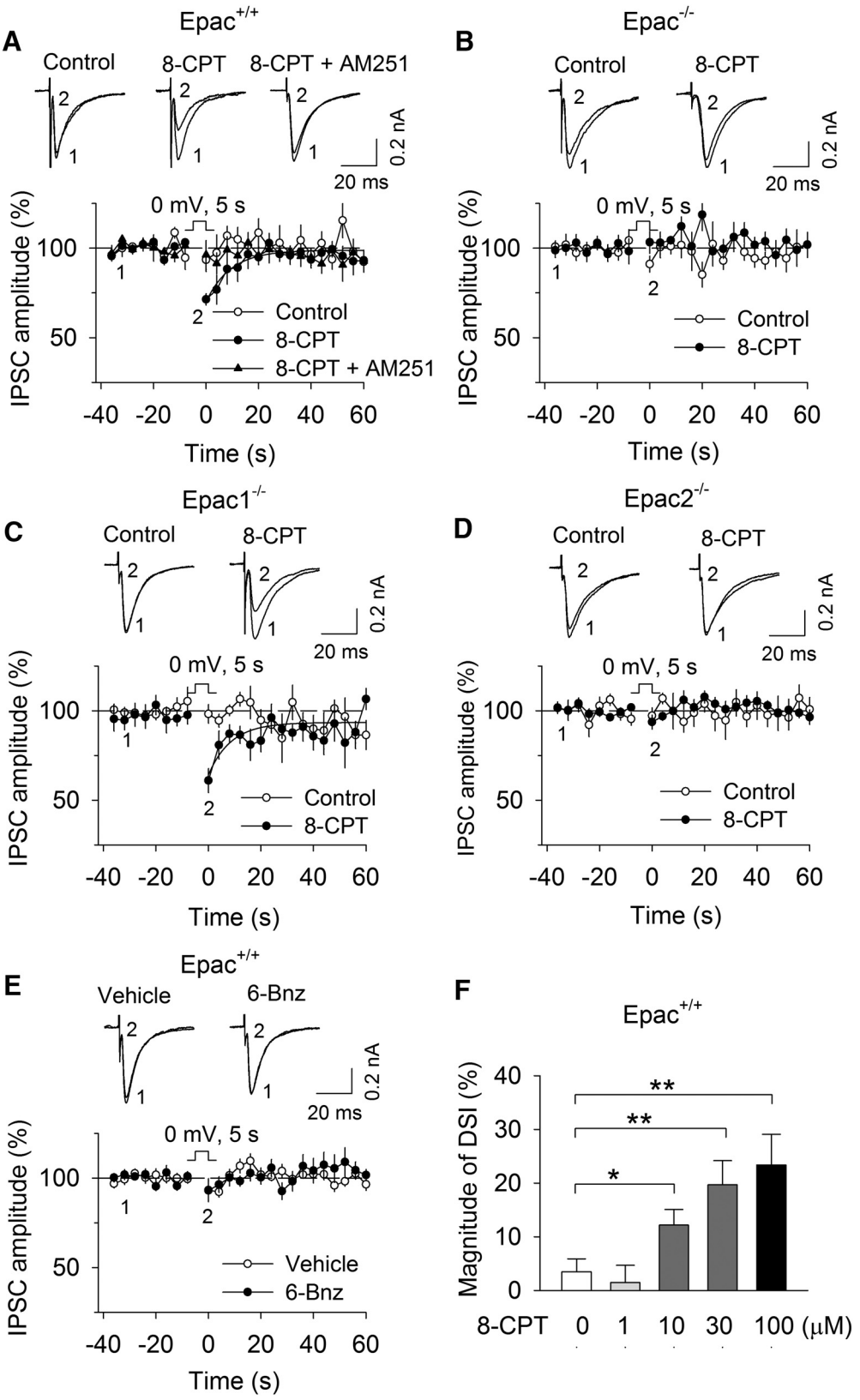

Figure 1. 8-CPT facilitated DSI by activating Epac2 in VTA dopamine neurons. ***A***, In $Epac^{+/+}$ slices, depolarization (from −70 to 0 mV, 5 s) did not induce DSI with control internal solution. Intracellular loading of 8-CPT (100 μM) enabled DSI ($n = 8–9$, $p = 0.031$). The 8-CPT-enabled DSI was blocked by the CB_1 receptor antagonist AM251 ($n = 7$, $p = 0.040$). Sample traces of evoked IPSCs were shown on the top and averaged DSI on the bottom. The solid lines are single exponential fitting curves of the decay of DSI. ***B***, DSI was not induced in $Epac^{-/-}$ slices with control internal solution or 8-CPT-containing internal solution ($n = 8–10$, $p = 0.323$). ***C***, In $Epac1^{-/-}$ slices, 8-CPT enabled DSI (both $n = 8$, $p < 0.001$). ***D***, DSI was not induced in $Epac2^{-/-}$ slices with control or 8-CPT-containing internal solution ($n = 8–9$, $p = 0.760$). ***E***, DSI was not induced in $Epac^{+/+}$ slices with control or 6-Bnz-cAMP-containing internal solution ($n = 8–9$, $p = 0.101$). ***F***, Dose-dependent effects of intracellular loading of different concentrations of 8-CPT ($n = 7–10$, $^{*}p < 0.05$, $^{**}p < 0.01$).

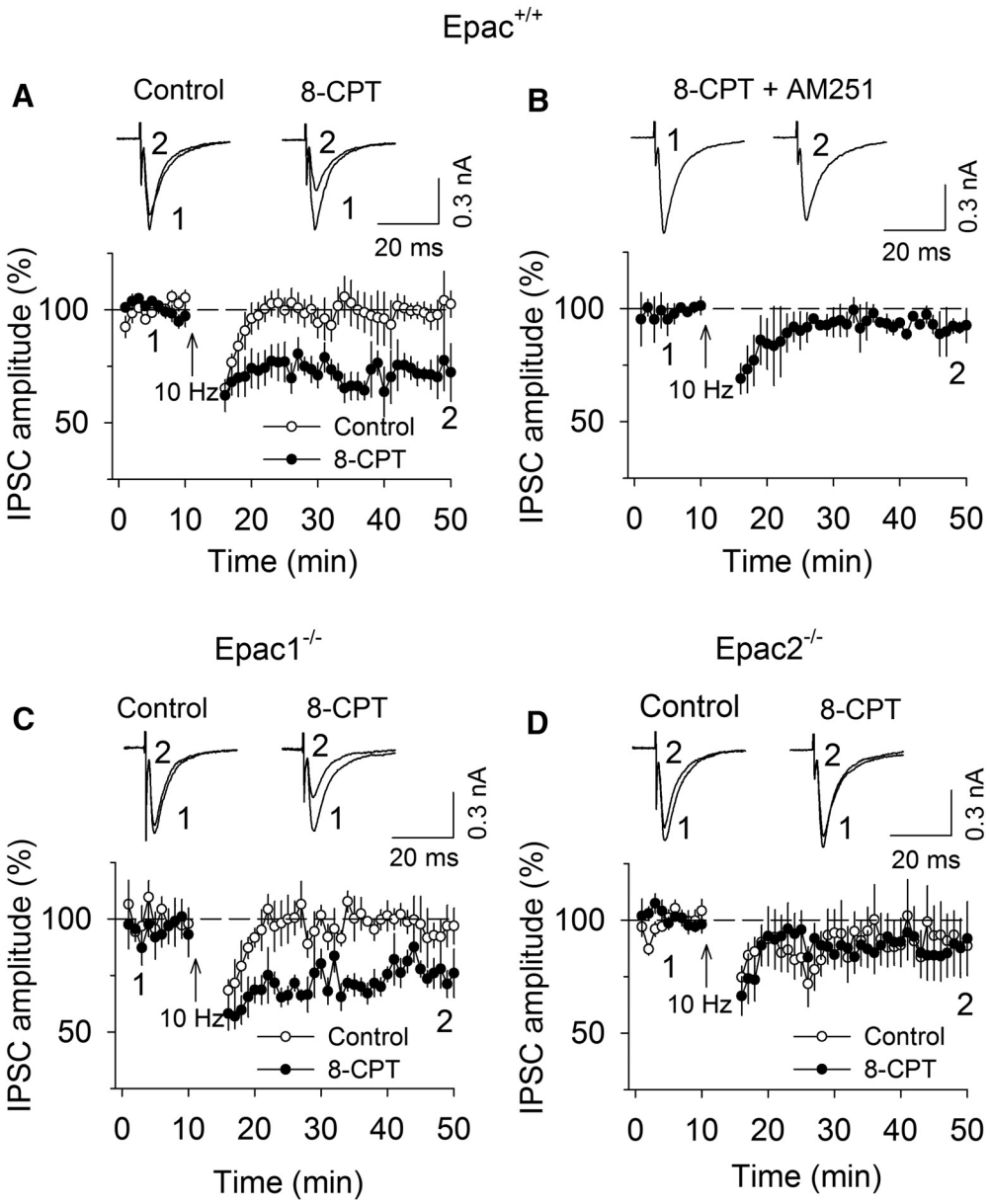

Figure 2. 8-CPT facilitated CB_1 receptor-mediated I-LTD through the activation of Epac2. ***A***, The 10 Hz, 5 min stimulation (arrow) did not induce I-LTD with control internal solution ($n = 6, p = 0.914$), while intracellular application of 8-CPT facilitated I-LTD ($n = 8, p = 0.022$). ***B***, 8-CPT-enabled I-LTD was blocked by AM251 ($n = 6, p = 0.355$). ***C***, In *Epac1*$^{-/-}$ mice, I-LTD was not induced with control internal solution ($n = 8, p = 0.789$), whereas 8-CPT enabled I-LTD ($n = 9, p = 0.030$). ***D***, I-LTD was not induced with control or 8-CPT-containing internal solution in *Epac2*$^{-/-}$ slices ($n = 6–7, p > 0.05$).

dopamine neurons in *Epac*$^{+/+}$ slices. 6-Bnz-cAMP (100 μM) was loaded into VTA dopamine neurons the same way as that of 8-CPT. However, 6-Bnz-cAMP did not enable DSI in VTA dopamine neurons (5.3 ± 4.9%, $n = 9$; $t_{(15)} = 0.3$ vs control, $p = 0.760$; Fig. 1*E*). Thus, PKA is not required for 8-CPT-induced facilitation of DSI in VTA dopamine neurons. We also examined whether intracellular loading of low concentrations of 8-CPT altered DSI in *Epac*$^{+/+}$ slices. We found that 8-CPT-induced facilitation of DSI was concentration-dependent at 1–100 μM (control, 3.5 ± 2.4%, $n = 8$; 1 μM, 1.5 ± 3.2%, $n = 7$, $t_{(13)} = 0.5$ vs control, $p = 0.620$; 10 μM, 12.2 ± 2.9%, $n = 10$, $t_{(16)} = 2.2$ vs control, $p = 0.040$; 30 μM, 19.7 ± 4.5%, $n = 8$; $t_{(14)} = 3.2$ vs control, $p = 0.007$; 100 μM, 23.4 ± 5.7%, $n = 9$; $t_{(15)} = 3.1$, $p = 0.008$ vs control; Fig. 1*F*).

8-CPT facilitated CB_1 receptor-mediated I-LTD

The CB_1 receptor not only mediates short-term synaptic depression, such as depolarization-induced suppression of excitation (DSE) and DSI (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001), but also LTD and I-LTD (Gerdeman et al., 2002; Chevaleyre and Castillo, 2003; Safo and Regehr, 2005; Wang et al., 2010). We examined whether 8-CPT altered I-LTD induction in VTA dopamine neurons. Whole-cell recordings were made in wild-type slices with control internal solution or internal solution containing 8-CPT (100 μM). After a stable baseline recording of IPSCs for 10 min, repetitive synaptic stimulation (10 Hz, 5 min) was applied to induce I-LTD. Consistent with our previous studies (Pan et al., 2008a), we found that with control internal solution, the 10 Hz stimulation did not

induce significant long-lasting depression of IPSCs (96.7 ± 11.1% of baseline, $n = 6$; $t_{(10)} = 0.1$, $p = 0.914$; Fig. 2*A*), suggesting that this stimulation is subthreshold for I-LTD induction. However, the 10 Hz stimulation for 5 min induced I-LTD when 8-CPT was included in the internal solution (72.5 ± 10.0% of baseline, $n = 8$; $t_{(14)} = 2.6$, $p = 0.022$; Fig. 2*A*). This I-LTD was blocked by the continuous presence of AM251 (2 μM; 91.7 ± 5.0% of baseline, $n = 7$; $t_{(12)} = 1.0$, $p = 0.355$; Fig. 2*B*). These results indicate that 8-CPT enabled a subthreshold stimulus to induce CB_1 receptor-mediated I-LTD in VTA dopamine neurons.

We next examined whether 8-CPT altered I-LTD in VTA dopamine neurons in slices prepared from *Epac1*$^{-/-}$ and *Epac2*$^{-/-}$ mice. In *Epac1*$^{-/-}$ slices, application of the 10 Hz stimulation for 5 min did not induce significant I-LTD with control internal solution (96.8 ± 8.1% of baseline, $n = 8$; $t_{(14)} = 0.3$, $p = 0.789$; Fig. 2*C*), while the same stimulation induced robust I-LTD with 8-CPT-containing internal solution (74.9 ± 7.1% of baseline, $n = 9$; $t_{(16)} = 2.4$, $p = 0.030$; Fig. 2*C*). In *Epac2*$^{-/-}$ slices, the 10 Hz stimulation did not induce I-LTD when the recording pipette was filled with either control internal solution (91.7 ± 5.4% of baseline, $n = 7$; $t_{(12)} = 1.1$, $p = 0.293$) or internal solution containing 8-CPT (93.0 ± 3.6% of baseline, $n = 6$; $t_{(10)} = 1.6$, $p = 0.145$; Fig. 2*D*). Thus, 8-CPT facilitates I-LTD through activation of Epac2 but not Epac1.

Signaling mechanisms for 8-CPT-induced facilitation of DSI and I-LTD

Epac is known to activate PLCε (Oestreich et al., 2007). PLCε has a conserved phosphoinositide-specific PLC (phosphoinositidase) catalytic core that hydrolyzes phosphatidylinositol 1,4,5-bisphosphate (PIP_2) to IP_3 and DAG (Smrcka et al., 2012). DAG is a precursor for the endocannabinoid 2-AG and is converted into 2-AG by DAGL (Di Marzo et al., 1998; Piomelli, 2003). One possibility is that 8-CPT enhances DSI and I-LTD via the PLCε → DAG → 2-AG pathway. There are at least six isoforms of PLC (PLCβ, PLCγ, PLCδ, PLCε, PLCζ, PLCη; Rhee and Bae, 1997; Hwang et al., 2005). Currently available PLC inhibitors, such as U73122, cannot discriminate different PLC subtypes and have off-target effects (Walker et al., 1998; Hoover et al., 2008). We therefore tested whether 8-CPT altered DSI and I-LTD in VTA dopamine neurons in *PLCε*$^{-/-}$ mice (Wang et al., 2005). We found that DSI was induced in 8-CPT-filled VTA dopamine neurons in wild-type slices but not in *PLCε*$^{-/-}$ slices (wild type, 30.4 ± 1.3%, $n = 8$; *PLCε*$^{-/-}$, 7.7 ± 3.6%, $n = 9$; $t_{(15)} = 5.7$, $p < 0.001$; Fig. 3*A*). There is a possibility that genetic deletion of *PLCε* causes general deficits in 2-AG synthesis, which may account for the lack of effect of 8-CPT on DSI. We performed the following control experiments to test such a possibility.

Group-I mGluRs are coupled to the PLCβ pathway (Hashimotodani et al., 2005), while the tyrosine kinase receptor B (TrkB) is coupled to the PLCγ pathway (Reichardt, 2006). The mGluR agonist DHPG and the TrkB agonist 7,8-dihydroxyflavone (DHF) enhance DSI (Varma et al., 2001; Edwards et al., 2006; Zhong et al., 2015) and induced CB_1 receptor-dependent synaptic depression (Yu et al., 2013). We examined whether DHPG-induced and DHF-induced facilitation of DSI in VTA dopamine neurons was altered in *PLCε*$^{-/-}$ mice. In the presence of DHPG (2 μM) in the ACSF, a brief depolarization (−70 to 0 mV for 5 s) induced DSI with comparable magnitude in wild-type and *PLCε*$^{-/-}$ slices (wild type, 30.0 ± 7.6%, $n = 7$; *PLCε*$^{-/-}$, 29.9 ± 2.1%, $n = 8$; $t_{(13)} = 0.02$, $p = 0.985$; Fig. 3*B*). Similarly, in the presence of DHF (10 μM), there was no significant difference of DSI in wild-type and *PLCε*$^{-/-}$ slices (wild type, 33.6 ± 5.1%, $n = 9$; *PLCε*$^{-/-}$, 30.8 ± 7.3%, $n = 7$; $t_{(14)} = 0.3$, $p = 0.750$; Fig. 3*C*). Thus, genetic deletion of *PLCε* did not affect 2-AG synthesis through PLCβ and PLCγ pathways. These results indicate that 8-CPT facilitated DSI in VTA dopamine neurons via activation of PLCε.

We have shown earlier that 8-CPT enabled a subthreshold stimulus to induce I-LTD in VTA dopamine neurons (Fig. 2*A*). To determine whether PLCε mediates this effect of 8-CPT, we examined I-LTD in slices prepared from wild-type and *PLCε*$^{-/-}$ mice. Whole-cell recordings were made with internal solution containing 8-CPT (100 μM). We found that the 10 Hz stimulation induced I-LTD in wild-type slices (70.4 ± 7.4% of baseline, $n = 6$; $t_{(10)} = 3.3$, $p = 0.009$), but I-LTD was blocked in *PLCε*$^{-/-}$ slices (94.4 ± 11.4% of baseline, $n = 6$; $t_{(10)} = 0.4$, $p = 0.697$; Fig. 3*D*). Thus, the selective Epac agonist 8-CPT facilitated I-LTD via activation of PLCε.

As mentioned earlier, PLCε hydrolyzes PIP_2 to DAG (Smrcka et al., 2012), and DAG is hydrolyzed into 2-AG by DAGL (Di Marzo et al., 1998; Piomelli, 2003). If the Epac agonist 8-CPT enabled DSI through activation of the PLCε → DAG → 2-AG pathway, then DAGL inhibitors should block 8-CPT-induced facilitation of DSI and I-LTD. To test this possibility, we examined the effects of DO34, a recently developed, highly selective and potent DAG lipase inhibitor (Ogasawara et al., 2016), on DSI and I-LTD in wild-type slices. DO53, an inactive analog of DO34, was used as a negative control (Ogasawara et al., 2016). Slices were pretreated with DO34 (1 μM) or DO53 (10 μM) for 30 min and these compounds were present in the ACSF throughout the experiments. 8-CPT was included in the internal solution used for whole-cell recordings. We found that DO53 did not significantly alter DSI (31.7 ± 10.2%, $n = 8$; Fig. 4*A*) and I-LTD (68.5 ± 9.5% of baseline, $n = 6$; $t_{(10)} = 3.2$, $p = 0.010$; Fig. 4*B*), whereas DO34 blocked DSI (2.4 ± 2.6%, $n = 10$; $t_{(16)} = 3.1$, $p = 0.007$; Fig. 4*A*) and I-LTD (90.4 ± 8.4% of baseline, $n = 7$; $t_{(12)} = 1.1$, $p = 0.275$; Fig. 4*B*). These results suggest that 8-CPT enabled I-LTD via the PLCε → DAG → 2-AG pathway.

We examined whether intracellular perfusion of a higher concentration of 8-CPT (300 μM) via the patch pipette during whole-cell recordings affected basal IPSCs in VTA dopamine neurons. Recordings were initially made with 8-CPT-free internal solution. After stable baseline recordings of IPSCs for 5 min, 8-CPT was perfused to the tip of the patch pipette (see Materials and Methods). We found that 8-CPT perfusion caused gradual depression of IPSCs in *Epac*$^{+/+}$ slices (72.3 ± 6.8% of baseline, $n = 7$; $t_{(12)} = 3.0$, $p = 0.011$) but not in *Epac2*$^{-/-}$ slices (93.6 ± 7.7% of baseline, $n = 7$; $t_{(12)} = 0.4$, $p = 0.695$; Fig. 5*A*). 8-CPT-induced depression in *Epac*$^{+/+}$ slices was blocked by the DAGL inhibitor DO34 (1 μM; 96.8 ± 8.2% of baseline, $n = 6$; $t_{(11)} = 2.3$, $p = 0.043$ vs control; Fig. 5*B*) but was not significantly affected by the PKA inhibitor H89 (70.4 ± 6.3% of baseline, $n = 7$; $t_{(13)} = 0.2$, $p = 0.809$ vs control; Fig. 5*B*). In the latter experiment, slices were incubated (≥1 h) and continuously superfused with H89 (10 μM). We and others have shown that under this condition H89 was effective in blocking PKA signaling (Chevaleyre et al., 2007; Pan et al., 2008b). Together, these results indicate that 8-CPT depresses basal IPSCs through the activation of the Epac → PLCε → DAG → 2-AG pathway.

Epac2 is required for the reduction of GABAergic inhibition to dopamine neurons induced by cocaine CPP

We and others have shown that *in vivo* exposure to cocaine reduces GABAergic inhibition to VTA dopamine neurons in mid-

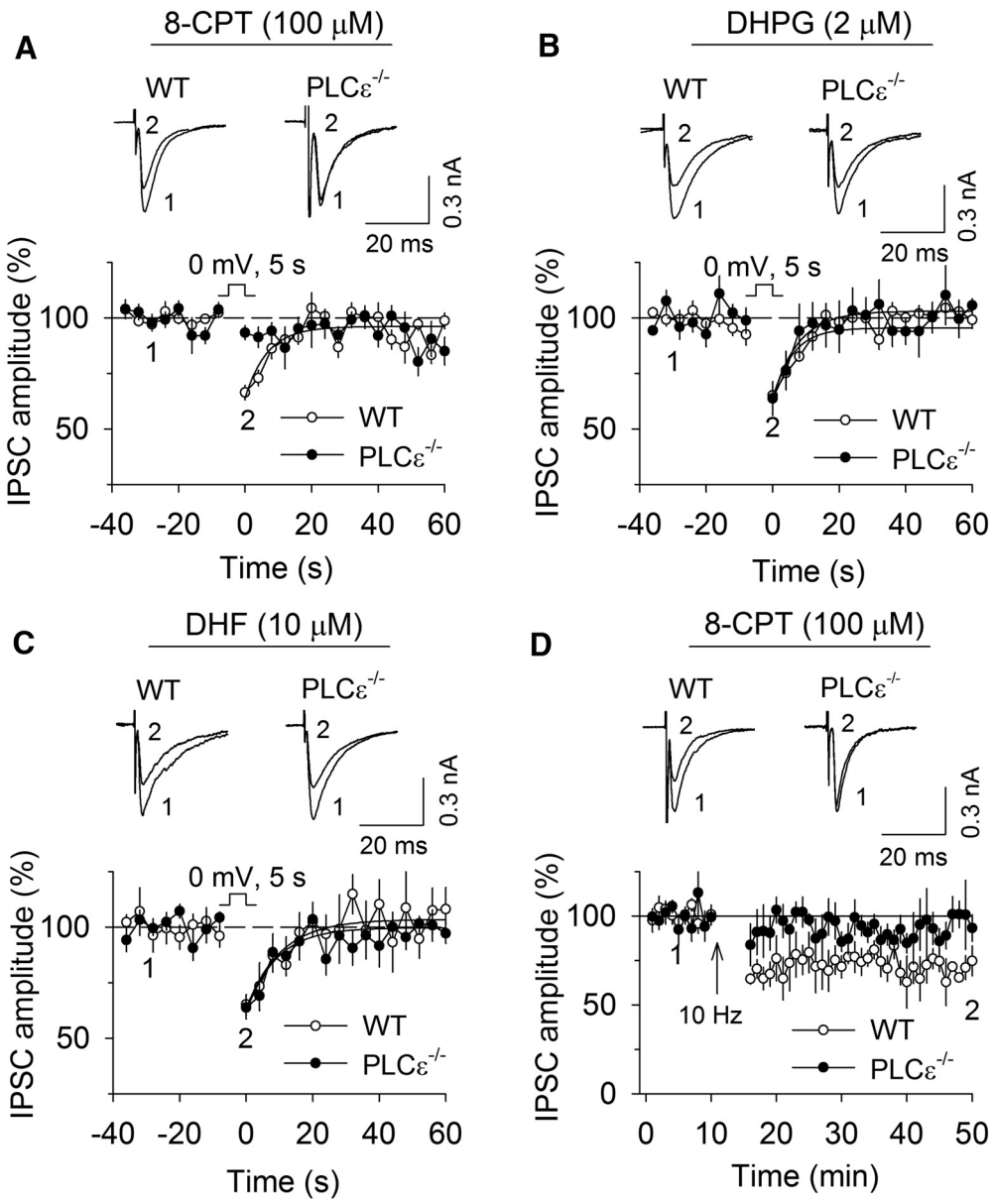

Figure 3. PLCε is required for 8-CPT-induced facilitation of DSI and I-LTD, but is not required for DHPG-induced or DHF-induced facilitation of DSI. ***A***, 8-CPT enabled DSI in wild-type (WT) slices, but not in *PLCε*$^{-/-}$ slices ($n = 8–9$, $p < 0.001$). ***B***, The mGluR1 agonist DHPG-enabled DSI was not altered in *PLCε*$^{-/-}$ slices ($n = 7–8$, $p = 0.985$). ***C***, TrkB agonist DHF-enabled DSI was not altered in *PLCε*$^{-/-}$ slices ($n = 7–9$, $p = 0.750$). ***D***, 8-CPT enabled I-LTD in WT slices ($n = 6$, $p = 0.009$). This I-LTD was absent in *PLCε*$^{-/-}$ slices ($n = 6$, $p = 0.697$).

brain slices *ex vivo* (Liu et al., 2005; Bocklisch et al., 2013). Our previous studies suggest that the endocannabinoid-mediated I-LTD provides a putative mechanism for cocaine-induced reduction of GABAergic inhibition (Pan et al., 2008a). Having shown that Epac2 is required for I-LTD induction in VTA dopamine neurons, we next determined whether cocaine CPP altered spontaneous IPSCs (sIPSCs) in the VTA and, if so, whether the alteration was dependent on Epac2. *Epac*$^{+/+}$ and *Epac2*$^{-/-}$ mice underwent saline and cocaine conditioning. Three mice that exhibited unconditioned place preference (≥180 s) during the pretest were excluded from further experiments. The remaining mice did not exhibit baseline bias ($p > 0.05$; Fig. 6*A*). Then, cocaine (15 mg/kg, i.p.) or saline place conditioning was conducted twice daily for 2 d. CPP was tested the next day without any drug or vehicle administration. Two-way ANOVA revealed that genotype ($F_{(1,36)} = 12.8$, $p = 0.001$) and cocaine place conditioning ($F_{(1,36)} = 64.1$, $p < 0.001$) had significant main effects on the preference score, and there was a significant interaction between genotype and cocaine conditioning ($F_{(1,36)} = 9.7$, $p = 0.004$; Fig. 6*B*). Tukey's *post hoc* tests showed that cocaine conditioning led to a significant increase in the preference score ($p < 0.001$) in *Epac*$^{+/+}$ mice and that cocaine CPP was attenuated in *Epac2*$^{-/-}$ mice ($p < 0.001$; Fig. 6*B*). These results are consistent with our recent studies showing that cocaine CPP is reduced in *Epac2*$^{-/-}$ mice (Liu et al., 2016).

One day after the CPP test, the mice were killed and midbrain slices were prepared. Spontaneous IPSCs were recorded from VTA dopamine neurons in these four groups of mice. We found that cocaine treatment and genotype had significant effects on the mean amplitude of sIPSCs (cocaine: $F_{(1,47)} = 5.9$, $p = 0.019$; genotype: $F_{(1,47)} = 5.9$, $p = 0.020$; cocaine × genotype interaction: $F_{(1,47)} = 15.8$, $p < 0.001$; Fig. 6*C*,*D*) and the mean frequency of sIPSCs (cocaine: $F_{(1,47)} = 4.5$, $p = 0.040$; genotype: $F_{(1,47)} = 9.2$, $p = 0.004$; cocaine × genotype interaction: $F_{(1,47)} = 9.5$, $p =$

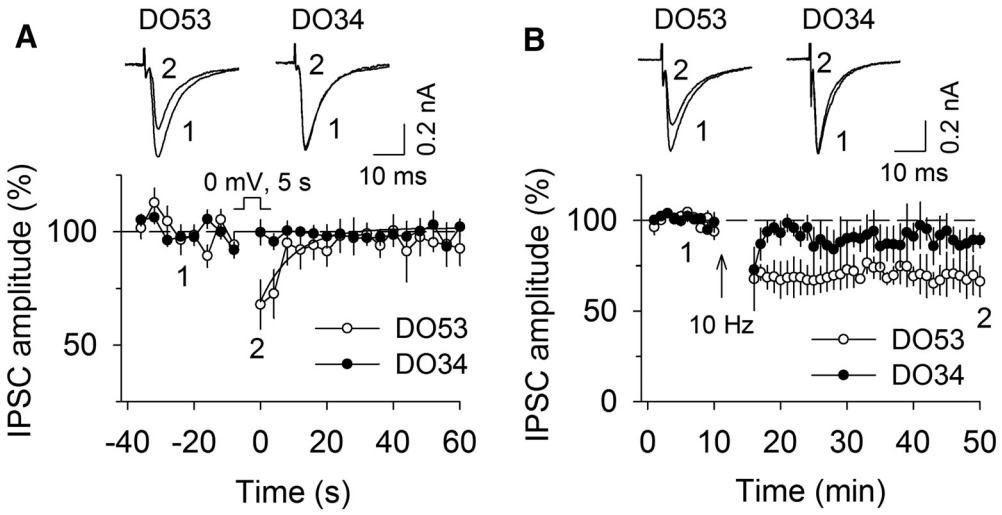

Figure 4. The DAGL inhibitor DO34 blocked DSI and I-LTD in wild-type slices. ***A***, ***B***, DAGL inhibitor DO34, but not the inactive analog DO53, blocked 8-CPT-enabled DSI ($n = 8–10$, $p = 0.007$; ***A***) and I-LTD ($n = 6–7$, $p = 0.010$; ***B***).

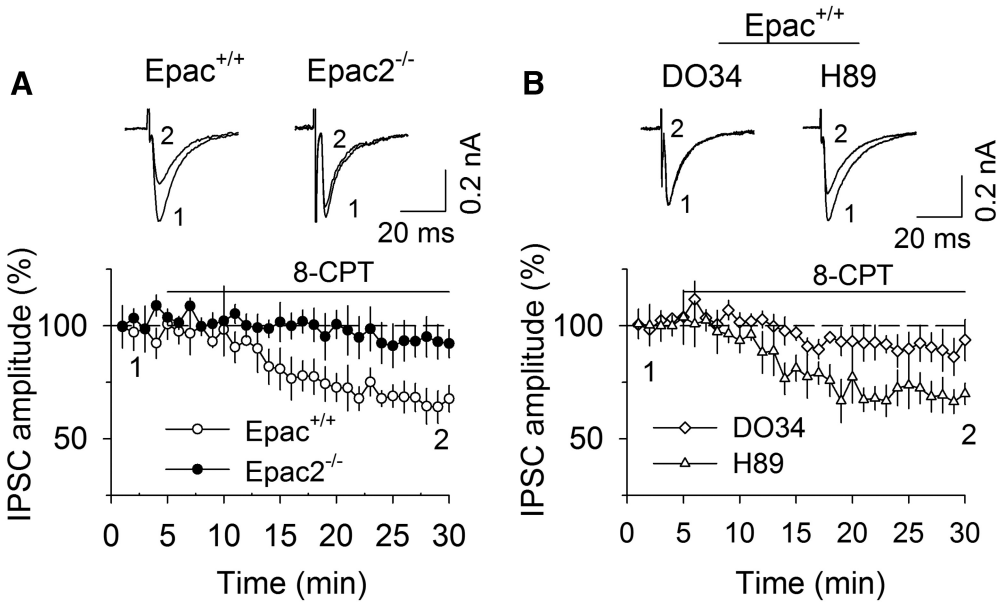

Figure 5. Intracellular perfusion of a higher concentration of 8-CPT (300 μM) depressed basal IPSCs. ***A***, 8-CPT depressed IPSCs in *Epac*$^{+/+}$ slices ($n = 7$, $p = 0.011$) but not in *Epac2*$^{-/-}$ slices ($n = 7$, $p = 0.695$). ***B***, 8-CPT-induced depression of IPSCs in the *Epac*$^{+/+}$ slices was blocked by the DAGL inhibitor DO34 ($n = 6–7$, $p = 0.043$) but was unaffected by the PKA inhibitor H89 ($n = 7–8$, $p = 0.809$).

0.004; Fig. 6*C*,*E*). Tukey's *post hoc* tests indicated that cocaine conditioning significantly decreased the mean amplitude ($p < 0.001$; Fig. 6*D*) and frequency of sIPSCs ($p < 0.001$; Fig. 6*E*) in *Epac*$^{+/+}$ mice. The cocaine-induced decreases in the amplitude and frequency of sIPSCs were absent in *Epac2*$^{-/-}$ mice ($p < 0.001$; Fig. 6*D*,*E*). The cumulative distribution for the amplitude of sIPSCs was shifted to the left (i.e., smaller value) in *Epac*$^{+/+}$ mice that received cocaine conditioning, and this shift was blocked in *Epac2*$^{-/-}$ mice (Fig. 6*F*). The cumulative distribution for interevent intervals of sIPSCs was shifted to the right (i.e., longer interval and less frequent) in *Epac*$^{+/+}$ mice that received cocaine conditioning, and this shift was blocked in *Epac2*$^{-/-}$ mice (Fig. 6*G*). Together, these results indicate that cocaine CPP led to the decrease in sIPSC amplitude and frequency in *Epac*$^{+/+}$ mice, and this decrease was blocked in *Epac2*$^{-/-}$ mice.

Cocaine-induced reduction of GABAergic inhibition was attenuated in *PLCε*$^{-/-}$ mice

Having shown that Epac2 is required for the cocaine-induced decrease in sIPSCs in VTA dopamine neurons (Fig. 6), we next determined whether PLCε is involved in this process. We examined whether cocaine-induced reduction of GABAergic inhibition was altered in *PLCε*$^{-/-}$ mice. Wild-type and *PLCε*$^{-/-}$ mice underwent saline and cocaine conditioning as described above. CPP was tested the next day without any drug or vehicle administration. Two-way ANOVA revealed that genotype ($F_{(1,36)} = 11.4$, $p = 0.002$) and cocaine place conditioning ($F_{(1,36)} = 127.2$, $p < 0.001$) had significant main effects on the preference score, and there was a significant interaction between genotype and cocaine conditioning ($F_{(1,36)} = 7.6$, $p = 0.009$; Fig. 7*B*). Tukey's *post hoc* tests showed that cocaine conditioning led to a significant

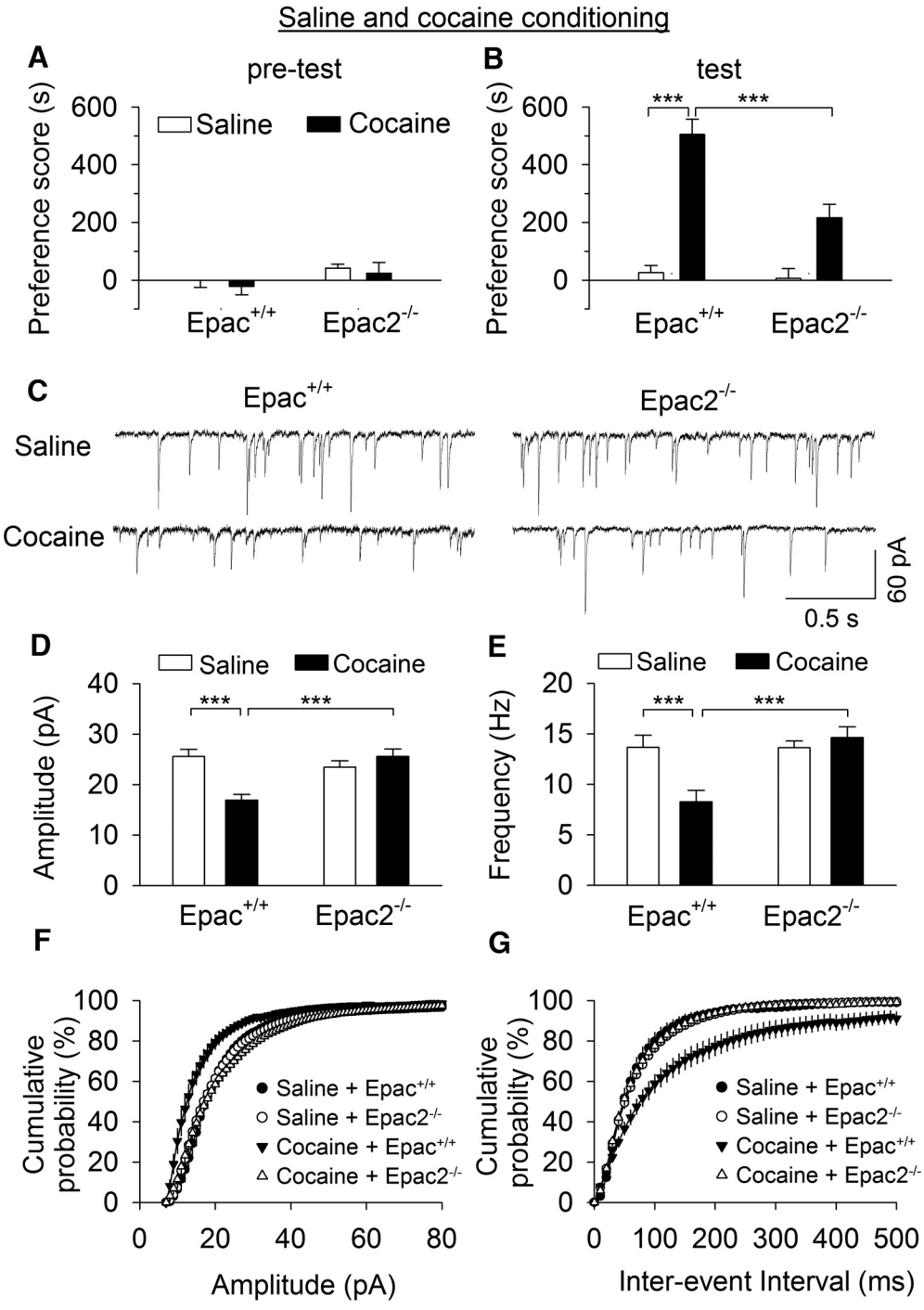

Figure 6. Epac2 is required for the reduction of GABAergic inhibition to dopamine neurons induced by cocaine conditioning. ***A***, *Epac*$^{+/+}$ and *Epac2*$^{-/-}$ mice exhibited no significant unconditioned preference in each chamber during pretest ($n = 9–10$, $p > 0.05$). ***B***, In *Epac*$^{+/+}$ mice, cocaine conditioning induced a significant increase in preference score compared with saline conditioning ($n = 9–10$, ***$p < 0.001$). *Epac2*$^{-/-}$ mice exhibited a significant decrease in the preference score compared with that of *Epac*$^{+/+}$ mice ($n = 9–10$, ***$p < 0.001$). ***C***, Representative sIPSCs recorded from VTA dopamine neurons in slices prepared from saline-conditioned or cocaine-conditioned *Epac*$^{+/+}$ or *Epac2*$^{-/-}$ mice. ***D***, ***E***, The averaged amplitude (***D***) and frequency (***E***) of sIPSCs in VTA dopamine neurons in these four groups of mice. The mean amplitude and frequency of sIPSCs were significantly decreased in cocaine-conditioned *Epac*$^{+/+}$ mice (both $n = 12$, ***$p < 0.001$), and this decrease was blocked in *Epac2*$^{-/-}$ mice (both $n = 12$, ***$p < 0.001$). ***F***, ***G***, The cumulative probability plots indicated that cocaine conditioning led to shifts in the distribution of the amplitude (***F***) and interevent intervals (***G***) in *Epac*$^{+/+}$ mice. These shifts were blocked in *Epac2*$^{-/-}$ mice ($n = 12$, $p < 0.01$).

increase in the preference score ($p < 0.001$) in wild-type mice, and cocaine-induced CPP was attenuated in *PLCε*$^{-/-}$ mice ($p < 0.001$; Fig. 7*B*).

One day after the CPP test, the mice were killed and midbrain slices were prepared. Spontaneous IPSCs were recorded from VTA dopamine neurons in these four groups of mice. We found that cocaine conditioning ($F_{(1,55)} = 16.6$, $p < 0.001$) and genotype ($F_{(1,55)} = 4.9$, $p = 0.032$) had significant effects on the mean amplitude of sIPSCs, and there was a significant interaction between cocaine conditioning and genotype ($F_{(1,55)} = 27.9$, $p < 0.001$; Fig. 7*C*,*D*). Tukey's *post hoc* tests indicated that cocaine conditioning significantly decreased the mean amplitude of

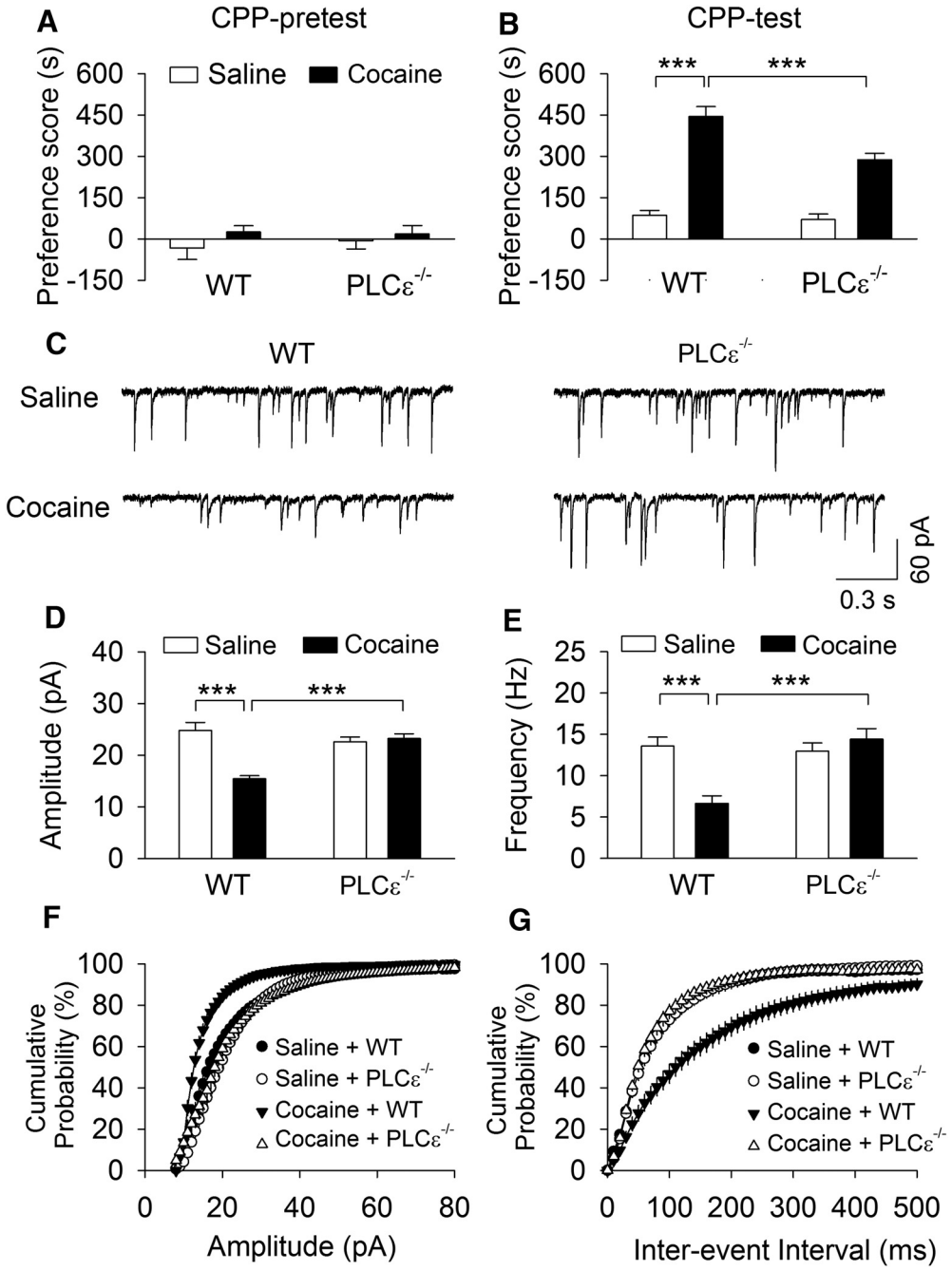

Figure 7. Cocaine conditioning-induced reduction of GABAergic inhibition was attenuated in $PLC\varepsilon^{-/-}$ mice. ***A***, Wild-type (WT) and $PLC\varepsilon^{-/-}$ mice exhibited no significant unconditioned preference (baseline bias) in each chamber during pretest ($n = 8–10$, $p > 0.05$). ***B***, Cocaine CPP was attenuated in $PLC\varepsilon^{-/-}$ mice compared with that of WT mice ($n = 9–10$, $^{***}p < 0.01$). ***C***, Representative sIPSCs recorded from VTA dopamine neurons in slices prepared from saline-conditioned or cocaine-conditioned WT or $PLC\varepsilon^{-/-}$ mice. ***D***, ***E***, The averaged amplitude (***D***) and frequency (***E***) of sIPSCs in VTA dopamine neurons in these four groups of mice. The mean amplitude and frequency of sIPSCs were significantly decreased in cocaine-conditioned WT mice ($n = 14–15$, $^{***}p < 0.001$), and this decrease was blocked in $PLC\varepsilon^{-/-}$ mice (both $n = 14$, $^{***}p < 0.001$). ***F***, ***G***, The cumulative probability plots indicated that cocaine conditioning led to shifts in the distribution of the amplitude (***F***) and interevent intervals (***G***) in WT mice. These shifts were blocked in $PLC\varepsilon^{-/-}$ mice ($n = 12$, $p < 0.01$).

sIPSCs ($p < 0.001$; Fig. 7*D*) in wild-type mice; this decrease was blocked in $PLC\varepsilon^{-/-}$ mice ($p < 0.001$; Fig. 7*D*). The cumulative distribution for the amplitude of sIPSCs was shifted to the left (i.e., smaller value) in wild-type mice that received cocaine conditioning, and this shift was blocked in $PLC\varepsilon^{-/-}$ mice (Fig. 7*F*).

Cocaine conditioning ($F_{(1,55)} = 6.5$, $p = 0.014$) and genotype ($F_{(1,55)} = 10.9$, $p = 0.002$) had significant effects on the mean frequency of sIPSCs, and there was a significant interaction between cocaine conditioning and genotype ($F_{(1,55)} = 15.7$, $p < 0.001$; Fig. 7*C*,*E*). Tukey's *post hoc* tests indicated that cocaine conditioning significantly decreased the frequency of sIPSCs ($p < 0.001$; Fig. 7*E*) in wild-type mice, and this decrease was blocked in $PLC\varepsilon^{-/-}$ mice ($p < 0.001$; Fig. 7*E*). The cumulative distribution for interevent intervals of sIPSCs was shifted to the right (i.e., longer interval and less frequent) in wild-type mice that received cocaine conditioning, and this shift was blocked in $PLC\varepsilon^{-/-}$ mice (Fig. 7*G*). Together, these results indicate that cocaine CPP led to the reduction of GABAergic inhibition to

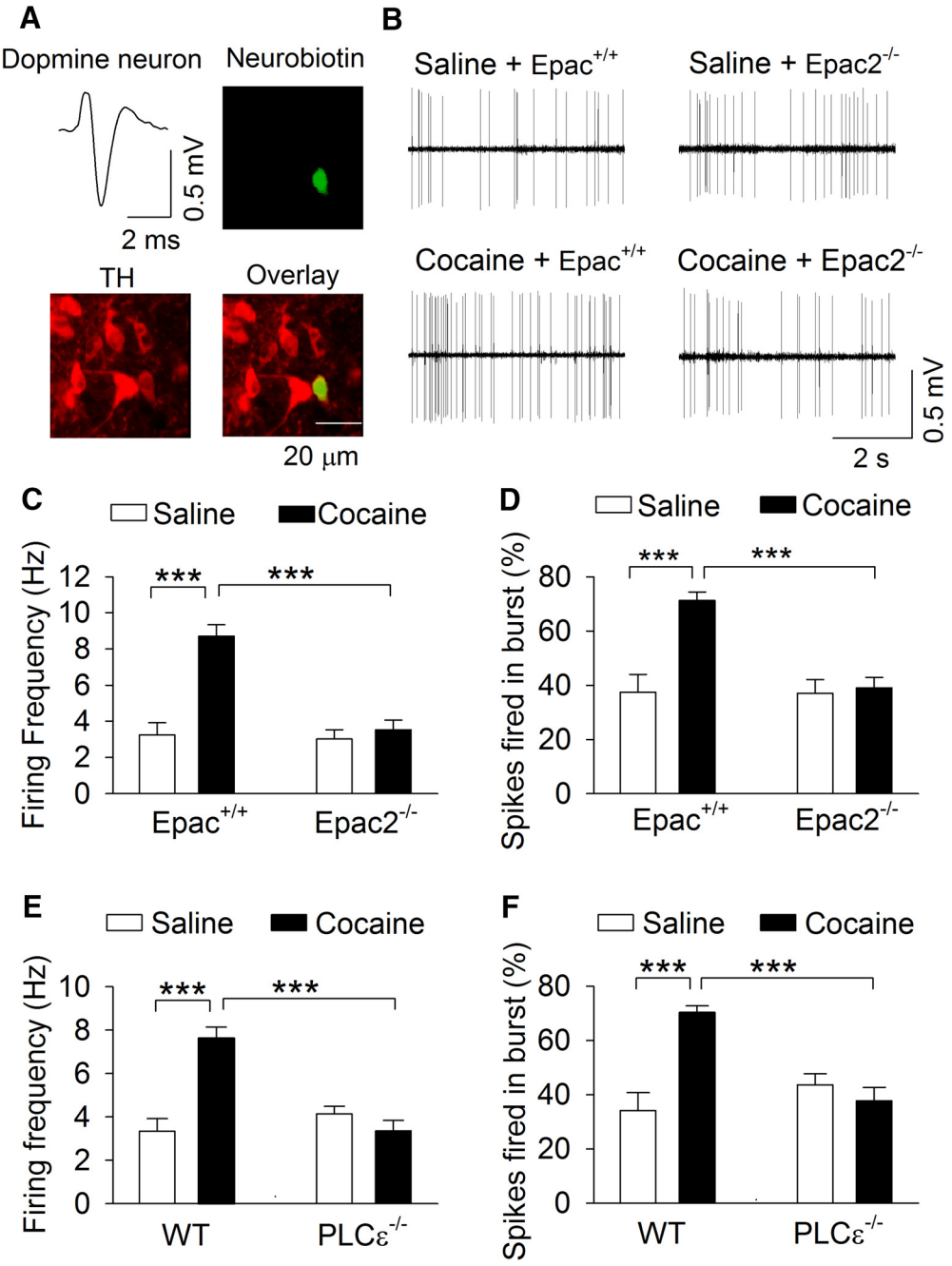

Figure 8. The Epac-PLCε pathway is required for cocaine conditioning-induced increase in action potential firing of VTA dopamine neurons *in vivo*. ***A***, Dopamine neurons were identified by a broad triphasic extracellular action potential of a width of >2 ms and juxtacellular labeling with neurobiotin. *Post hoc* immunostaining showed that the electrophysiologically identified dopamine neuron was colabeled with neurobiotin (green) and TH (red). ***B***, Examples of action potential firing recorded from saline-conditioned or cocaine-conditioned $Epac^{+/+}$ and $Epac2^{-/-}$ mice. ***C***, ***D***, Cocaine conditioning led to increases in the frequency of action potential firing (***C***) and the proportion of spikes that occurred in a burst (***D***) in $Epac^{+/+}$ mice (both $n = 12–13$, $^{***}p < 0.001$). These increases were blocked in $Epac2^{-/-}$ mice ($n = 13–14$, $^{***}p < 0.001$). ***E***, ***F***, Repeated cocaine exposure caused increases in the frequency of action potential firing (***E***) and the proportion of spikes that occurred in a burst (***F***; both $n = 11–12$, $^{***}p < 0.001$) in WT mice. These increases were blocked in $PLC\varepsilon^{-/-}$ mice (both $n = 12–15$, $^{***}p < 0.001$).

VTA dopamine neurons in wild-type mice, and this reduction was blocked in $PLC\varepsilon^{-/-}$ mice.

Cocaine CPP produced Epac2-PLCε-dependent increase in action potential firing of VTA dopamine neurons *in vivo*

Cocaine-induced reduction of the amplitude and frequency sIPSCs in VTA dopamine neurons may cause disinhibition and therefore increase the excitability of these neurons. To test this, we made *in vivo* single-unit extracellular recordings from VTA dopamine neurons in saline-conditioned and cocaine-conditioned wild-type, $Epac2^{-/-}$, and $PLC\varepsilon^{-/-}$ mice shown in Figures 6 and 7, respectively. One day after the CPP test, the mice were anesthetized with urethane and *in vivo* single-unit recordings were performed. Dopamine neurons were identified by a broad triphasic extracellular action potential with a width of >2 ms and a relatively slow firing rate (<10 Hz; Ungless et al., 2004; Fig. 8*A*,*B*). Dopamine neurons were validated postmortem via juxtacellular labeling with neurobiotin and TH immunostaining (Ungless et al., 2004; Chaudhury et al., 2013; Fig. 8*A*). In $Epac2^{-/-}$ mice, we found that cocaine place conditioning and

genotype had significant effects on the frequency of action potential firing (cocaine: $F_{(1,51)} = 21.1$, $p < 0.001$; $Epac2^{-/-}$: $F_{(1,51)} = 26.0$, $p < 0.001$; cocaine × $Epac2^{-/-}$ interaction $F_{(1,51)} = 17.9$, $p < 0.001$; Fig. 8*B*,*C*), and the percentage of spikes in bursts in VTA dopamine neurons (cocaine: $F_{(1,51)} = 14.4$, $p < 0.001$; $Epac2^{-/-}$: $F_{(1,51)} = 11.9$, $p = 0.001$; cocaine × $Epac2^{-/-}$ interaction: $F_{(1,51)} = 11.3$, $p = 0.002$; Fig. 8*B*,*D*). Tukey's *post hoc* tests indicated that cocaine place conditioning significantly increased the frequency of action potential firing and the percentage of spikes in bursts in $Epac^{+/+}$ mice (both p's < 0.001), but not in $Epac2^{-/-}$ mice (both p's > 0.05). There were no significant differences of the firing frequency and the percentage of spikes in bursts between $Epac^{+/+}$ and $Epac2^{-/-}$ mice that received saline conditioning (both p's > 0.05).

In $PLC\varepsilon^{-/-}$ mice, cocaine place conditioning and genotype had significant effects on the frequency of action potential firing (cocaine: $F_{(1,50)} = 14.4$, $p < 0.001$; $PLC\varepsilon^{-/-}$: $F_{(1,50)} = 14.1$, $p < 0.001$; cocaine × $PLC\varepsilon^{-/-}$ interaction $F_{(1,50)} = 29.9$, $p < 0.001$; Fig. 8*E*), and the percentage of spikes in bursts in VTA dopamine neurons (cocaine: $F_{(1,50)} = 11.1$, $p = 0.001$; $PLC\varepsilon^{-/-}$: $F_{(1,50)} = 6.4$, $p = 0.015$; cocaine × $PLC\varepsilon^{-/-}$ interaction: $F_{(1,50)} = 21.4$, $p < 0.001$; Fig. 8*F*). Tukey's *post hoc* tests indicated that cocaine place conditioning significantly increased the frequency of action potential firing and the percentage of spikes in bursts in wild-type mice (both p's < 0.001), but not in $PLC\varepsilon^{-/-}$ mice (both p's > 0.05). There were no significant differences of the firing frequency and the percentage of spikes in bursts between wild-type and $PLC\varepsilon^{-/-}$ mice that received saline conditioning (both p's > 0.05). Thus, cocaine place conditioning caused an increase in the excitability of VTA dopamine neurons, and this increase was blocked in *Epac2* and *PLCε* knock-out mice.

Discussion

The present study has shown that the selective Epac agonist 8-CPT enabled CB_1 receptor-mediated DSI and I-LTD in VTA dopamine neurons in wild-type mice, and the effects of 8-CPT were blocked in Epac2-deficient and PLCε-deficient mice. These results uncovered a novel mechanism for on-demand synthesis of retrograde signaling 2-AG by the Epac2-PLCε pathway. In addition, we provide evidence that Epac2-PLCε is required for the reduction of GABAergic inhibition to VTA dopamine neurons induced by cocaine place conditioning.

Facilitation of DSI and I-LTD by 8-CPT

Consistent with previous studies (Pan et al., 2008a), we found that depolarization of VTA dopamine neurons was not sufficient to induce DSI in VTA dopamine neurons, and repetitive synaptic stimulation at 10 Hz for 5 min was subthreshold for I-LTD induction. The present study showed that 8-CPT enabled DSI and I-LTD, and the effects of 8-CPT were blocked in $Epac2^{-/-}$ mice, but not in $Epac1^{-/-}$ mice. Thus, 8-CPT facilitated DSI and I-LTD by activating Epac2. The lack of effects in $Epac1^{-/-}$ mice may be explained by low expression of Epac1 in the brain (Kawasaki et al., 1998; de Rooij et al., 1998; Ostroveanu et al., 2010). Previous studies have shown that robust DSI was induced by depolarization alone in rat VTA dopamine neurons (Melis et al., 2009, 2013b, 2014). In these studies, IPSCs were evoked by selectively stimulating rostromedial tegmental nucleus (RMTg) afferents, which highly express CB_1 receptors (Melis et al., 2014). In support of this premise, the CB_1 receptor agonist WIN55212-2 produced robust depression of IPSCs evoked by stimulating the RMTg and blocked RMTg-evoked suppression of VTA dopamine neuron firing (Melis et al., 2009; Lecca et al., 2012). In the present study, IPSCs were evoked by nonselectively stimulating inhibitory synaptic inputs, which may potentially account for the absence of DSI from depolarization alone in our study.

DSI and I-LTD are mediated by the activation of CB_1 receptors by 2-AG, as they were blocked by pharmacological inhibition or genetic knock-out of DAGL (Chevaleyre and Castillo, 2003; Pan et al., 2009; Gao et al., 2010; Tanimura et al., 2010) and prolonged by monoacylglycerol lipase inhibitors (Pan et al., 2009; Patel et al., 2009; Straiker et al., 2009). Indeed, we found that 8-CPT-enabled DSI and I-LTD were abolished by a recently developed, highly selective, and potent DAGL inhibitor DO34 (Ogasawara et al., 2016), but not by the inactive analog DO53. Thus, an increase in 2-AG production is likely responsible for 8-CPT-induced facilitation of DSI and I-LTD. In support of this idea, we found that intracellular perfusion of a high concentration of 8-CPT (300 μM) induced depression of IPSCs in $Epac^{+/+}$ slices, and the 8-CPT-induced depression was blocked by DO34 and was absent in $Epac2^{-/-}$ slices. These results provide evidence that 8-CPT facilitated DSI and I-LTD by increasing 2-AG production.

The group-I mGluR agonist DHPG induces 2-AG-mediated retrograde synaptic depression (Maejima et al., 2001; Varma et al., 2001) and facilitates DSI (Varma et al., 2001; Edwards et al., 2006). The effects of DHPG are likely mediated by increasing 2-AG production. Group-I mGluRs are coupled to PLCβ (Hashimotodani et al., 2005), which cleaves PIP_2 into IP_3 and DAG, and the latter is subsequently converted into 2-AG by DAGL (Di Marzo et al., 1998; Piomelli, 2003). There are at least six isoforms of PLC (PLCβ, PLCγ, PLCδ, PLCε, PLCζ, PLCη; Rhee and Bae, 1997; Hwang et al., 2005), and among them, PLCβ is required for 2-AG synthesis induced by group-I mGluR activation and depolarization-induced Ca^{2+} influx (Hashimotodani et al., 2005). TrkB receptor agonists BDNF and DHF enhance DSI and I-LTD in VTA dopamine neurons (Zhong et al., 2015). Given that TrkB is coupled to PLCγ (Reichardt, 2006), a role for PLCγ in 2-AG synthesis has been speculated but has not been examined experimentally (Zhong et al., 2015). Epac activates PLCε via its direct effector, the small GTPase Rap (Schmidt et al., 2001; Oestreich et al., 2007). We tested the possibility that 8-CPT enabled DSI and I-LTD through activation of PLCε and found that the effects of 8-CPT on DSI and I-LTD were absent in $PLC\varepsilon^{-/-}$ slices. The lack of effects of 8-CPT cannot be attributed to gross disruption of 2-AG production due to permanent loss of PLCε from early development, since the mGluR agonist DHPG and the TrkB agonist DHF enabled DSI in wild-type and $PLC\varepsilon^{-/-}$ slices. These results suggest that activation of the Epac2-PLCε pathway induces synthesis of retrograde signaling 2-AG. Although all PLC isoforms are capable of producing DAG (Rhee and Bae, 1997; Hwang et al., 2005), 2-AG production via mGluRs and depolarization-induced Ca^{2+} influx was previously only linked to PLCβ (Hashimotodani et al., 2005). The present results provide evidence that PLCε is involved in synthesizing retrograde signaling 2-AG.

Regulation of cocaine-induced reduction of GABAergic inhibition by the Epac2-PLCε pathway

We have shown that repeated cocaine exposure *in vivo* reduces the strength of GABAergic inhibition to VTA dopamine neurons (Liu et al., 2005; Pan et al., 2008a), which primes excitatory synapses for LTP induction (Liu et al., 2005; Pan et al., 2011). Endocannabinoid-mediated I-LTD may constitute a mechanism for cocaine-induced reduction of GABAergic inhibition (Pan et al., 2008a). Having shown that 8-CPT enabled DSI and I-LTD via

activating Epac2 and PLCε, we examined whether the Epac-PLCε pathway is required for the reduction of GABAergic inhibition to VTA dopamine neurons induced by cocaine exposure *in vivo*. We have shown recently that cocaine CPP was impaired in *Epac2*$^{-/-}$ mice but was not altered in *Epac1*$^{-/-}$ mice (Liu et al., 2016). We examined whether cocaine CPP was accompanied by a change in GABAergic inhibition to VTA dopamine neurons. The results showed that cocaine place conditioning caused decreases in the frequency and amplitude of sIPSCs in wild-type mice, but not in *Epac2*$^{-/-}$ mice or in *PLCε*$^{-/-}$ mice. Thus, the Epac-PLCε pathway is required for the cocaine-induced reduction of GABAergic inhibition to VTA dopamine neurons, and the disruption of Epac signaling attenuates the behavioral reinforcement induced by cocaine. Although cocaine-induced decreases in amplitude and frequency of sIPSCs were blocked in the *Epac2*$^{-/-}$ mice, CPP was only attenuated in these mice. CPP is a complex behavior that is likely involved in multiple signaling pathways and multiple brain regions (Bardo and Bevins, 2000).

What might be the mechanism for the involvement of Epac-PLCε in cocaine-induced reduction of GABAergic inhibition? A common cocaine-induced neuroadaptation is an upregulation of cAMP signaling in the mesolimbic dopamine system (Nestler, 2001; Anderson and Pierce, 2005). Repeated cocaine exposure *in vivo* leads to reduction of $G\alpha_{i/o}$-protein levels (Nestler et al., 1990; Striplin and Kalivas, 1992) and enhancement of adenylate cyclase activity and cAMP accumulation (Watts and Neve, 2005). Cocaine-induced upregulation of cAMP signaling may activate the Epac-PLCε pathway, leading to increased 2-AG production. The repeated activation of this signaling cascade may induce an I-LTD-like synaptic modulation via cAMP-induced Epac2 activation in VTA dopamine neurons, leading to 2-AG synthesis and a reduction in the strength of GABAergic inhibition to VTA dopamine neurons. By activating PLCε, Epac links the cAMP-adenylate cyclase pathway and the DAG/IP_3-PLC pathway and provides a novel mechanism for the crosstalk between these two common G-protein signaling pathways.

Epac2-PLCε is required for cocaine-induced increase in dopamine neuron excitability

Cocaine-induced reduction of GABAergic inhibition causes disinhibition of VTA dopamine neurons and increases their excitability (Liu et al., 2005; Bocklisch et al., 2013). We found that cocaine conditioning increased *in vivo* action potential firing in VTA dopamine neurons, and this increase was blocked in *Epac2*$^{-/-}$ and *PLCε*$^{-/-}$ mice. Thus, the Epac2-PLCε pathway is involved in the cocaine-induced increase in excitability in VTA dopamine neurons. We have shown that intraperitoneal injection of AM251 blocked the decreases in IPSCs in VTA dopamine neurons induced by repeated cocaine exposure *in vivo* (Pan et al., 2008a). It is thus likely that endocannabinoid signaling contributes to cocaine-induced reduction of GABAergic inhibition and the increase in action potential firing. Cocaine exposure *in vivo* in rats or mice led to increases in insertion of GluA2-lacking AMPARs (Bellone and Lüscher, 2006; Good and Lupica, 2010; Liu et al., 2016) and in AMPAR/NMDAR ratio (Ungless et al., 2001; Borgland et al., 2004; Liu et al., 2005; Bellone and Lüscher, 2006; Mameli et al., 2007; 2009; Argilli et al., 2008). We have shown that Epac2 is required for both effects of cocaine (Liu et al., 2016). Thus, it is likely that both the cocaine-induced increase in the strength of excitatory synapses and decrease in the strength of inhibitory synapses contribute to the increase in excitability and action potential firing in VTA dopamine neurons.

In summary, we have shown that 8-CPT facilitated DSI and I-LTD in VTA dopamine neurons, and these effects were mediated via the activation of the Epac-PLCε pathway. In addition, this signaling pathway is also involved in cocaine conditioning-induced reduction of GABAergic inhibition and an increase in dopamine neuron excitability. Together, our studies suggest that Epac-PLCε plays a critical role in mediating cocaine-induced long-term synaptic plasticity at both excitatory and inhibitory synapses.

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