

NEUROSCIENCE

β -Arrestin–biased β -adrenergic signaling promotes extinction learning of cocaine reward memory

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Extinction learning of cocaine-associated contextual cues can help prevent cocaine addicts from relapsing. Pharmacological manipulation of β -adrenergic receptor (β -AR) during extinction learning is being developed as a potential strategy to treat drug addiction. We demonstrated that the extinction learning of cocaine-associated memory was mediated by β -arrestin2–biased but not heterotrimeric guanine nucleotide–binding protein (G protein)–dependent β -adrenergic signaling. We found that administration of the nonbiased β -AR antagonist propranolol, but not the G protein–biased β -AR antagonist carvedilol, blocked extinction learning of cocaine-conditioned place preference and the associated ERK activation in the infralimbic prefrontal cortex. Overexpression of β -arrestin2 in the infralimbic prefrontal cortex promoted extinction learning, which was blocked by propranolol. Knockout of β -arrestin2 in the infralimbic prefrontal cortex, specifically in excitatory neurons, impaired extinction learning of cocaine-conditioned place preference, which was not rescued by carvedilol. β -Arrestin2 signaling in infralimbic excitatory neurons was also required for the extinction learning in the cocaine self-administration model. Our results suggest that β -arrestin–biased β -adrenergic signaling in the infralimbic prefrontal cortex regulates extinction learning of cocaine-associated memories and could be therapeutically targeted to treat addiction.

INTRODUCTION

β -Adrenergic receptors (β -ARs), members of the large superfamily of heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors (GPCRs), are critically involved in central nervous system functions such as arousal, cognition, and stress-related behaviors. According to the classical model, catecholamine binding induces conformational changes in β -ARs that recruit and activate heterotrimeric G proteins, leading to the generation of cyclic adenosine monophosphate (cAMP). Consequently, GPCR kinases recognize and phosphorylate the activated receptors, triggering recruitment of cytosolic β -arrestin, which blocks β -AR coupling to Gs and terminates G protein–mediated signaling (1–3). However, it has become evident that the biological functions of β -arrestin can be extended beyond this single role. For instance, β -arrestin also acts as a multifunctional scaffold protein that interacts with several protein partners (4) and kinases, leading to the phosphorylation of various intracellular targets (5–7).

The functions of β -ARs have been primarily ascribed to their classical roles in stimulating Gs protein. The development of biased ligands for β -ARs, which selectively activate β -arrestin–mediated signaling pathway, has enabled research into the functions and potential therapeutic implications of β -arrestin–biased signaling pathway (8, 9). For example, carvedilol, which antagonizes β -AR/Gs signaling while mildly activating β -arrestin–mediated pathway, stimulates transactivation of the epidermal growth factor receptor through β 1-AR in a β -arrestin–dependent manner (10). We have found that β 1-AR/ β -arrestin/extracellular signal–regulated kinase (ERK) signaling, but not the classical G protein pathway in the entorhinal cortex, plays an important role in the reconsolidation of object recognition memory (11). Besides, β -arrestin–biased signaling of dopamine D2 receptor in medium spiny neurons of the striatum induces a significant potentiation of amphetamine-induced locomotion (12). Group I metabotropic glutamate receptors use a β -arrestin2–mediated signaling in pyramidal

hippocampal neurons to regulate synaptic strength (13). However, the roles of β -arrestin–biased signaling pathway in brain functions and behaviors remain largely unknown.

Drug addiction is an associative learning process, during which neutral stimuli acquire incentive motivational value when repeatedly paired with drug use (14). Exposure to the conditioned stimuli triggers the individual's craving for addictive drugs. Drug-associated memories can be attenuated by extinction-like cognitive therapy, resulting in attenuation of craving for drug (15). The infralimbic prefrontal cortex (IL-PFC) is believed to be critical for extinction learning and to play a major role in attenuating the original associative memory, reducing drug-seeking behavior and preventing relapse (16–20). Therefore, enhancement of IL-PFC functions by pharmacological cognitive enhancers is being considered as a strategy to augment the efficacy of extinction-like cognitive therapy. Better understanding of the intrinsic mechanisms underlying extinction learning within IL-PFC would therefore promote the development of new and more effective therapeutic agents that can enhance the efficacy of cognitive therapy and prevent relapse.

Here, we examined the role of G protein– and β -arrestin–biased β -adrenergic signaling pathways in the extinction of cocaine addiction using two behavioral paradigms: cocaine-conditioned place preference (CPP) and cocaine self-administration. We found that β -arrestin2–biased, but not G protein–dependent, β -adrenergic signaling in the excitatory neurons of IL-PFC regulated extinction learning of cocaine reward memory.

RESULTS**Extinction learning of cocaine-CPP is mediated by G protein–independent β -AR signaling pathway in the IL-PFC**

The CPP paradigm directly tests the association between the environment and the drug. After conditioning, animals show a preference for the compartment where the drug was received. Subsequent exposure to the chambers that have been previously paired with cocaine or saline without receiving the drug triggers extinction processes and activates

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brain regions mediating such processes (21, 22). To confirm that IL-PFC is engaged in extinction learning of cocaine-CPP, we quantified the numbers of c-Fos-positive neurons in the IL-PFC after extinction. After acquisition of the cocaine-CPP, mice were divided into three groups: extinction group that was subjected to a daily extinction trial from days 5 to 8, retrieval group that was subjected to a single extinction trial on day 8, and control group that was not exposed to extinction procedure (fig. S1A). On day 8, 1 hour after the last extinction trial, mice were sacrificed for immunohistochemical analysis. Quantitative analysis showed a significant increase in c-Fos-positive neurons in the IL-PFC of the extinction group compared with those of control and retrieval groups (fig. S1, B and C, and table S1), confirming that the IL-PFC could be involved in the extinction learning of cocaine-CPP.

To examine which β -AR signaling pathway, namely, β -arrestin or G protein signaling, was recruited during extinction learning of cocaine-CPP, we evaluated extinction learning in mice after bilateral infusions of unbiased or biased β -AR antagonists in the IL-PFC. The unbiased antagonist propranolol inhibits both β -arrestin and G protein β -adrenergic signaling pathways, whereas the biased antagonist carvedilol inhibits the β -AR/G protein signaling but partially stimulates β -AR/ β -arrestin signaling downstream of β -AR (23). Bilateral infusion of propranolol into the IL-PFC immediately after each extinction trial impaired extinction learning of cocaine-CPP (Fig. 1, A and B, and table S2), suggesting that activation of β -ARs was required for extinction learning of cocaine-CPP. In contrast to the inhibitory effect of propranolol, carvedilol did not attenuate extinction learning (Fig. 1C and table S2). These data suggest that β -arrestin-dependent signaling pathway, but not β -AR/G protein-dependent signaling, may be required in the IL-PFC for extinction learning of cocaine-CPP.

To support this notion, we examined the activation of ERK signaling pathway after extinction learning of cocaine-CPP. Upon β -adrenergic stimulation, β -arrestin mediates the activation of the ERK cascade in parallel with G protein/cAMP-dependent protein kinase/cAMP signaling. ERK signaling regulates synaptic plasticity and memory by stimulating transcription factors such as CREB (cAMP response element-binding protein) and Elk1 (ETS domain-containing protein) (24, 25). In line with our results on the effects of propranolol and carvedilol, we found that the phosphorylation of ERK in the IL-PFC was significantly increased after extinction learning (Fig. 1, D and E). This extinction-induced increase in phosphorylated ERK (pERK) was blocked by propranolol treatment (Fig. 1D and table S2). Moreover, carvedilol did not inhibit but rather increased ERK phosphorylation in the IL-PFC

to levels that were significantly higher than those induced by extinction learning alone (Fig. 1E and table S2).

β -Arrestin2 in the IL-PFC is required for extinction learning of cocaine-CPP

Our results above suggested that the β -arrestin-biased β -AR signaling pathway was recruited during extinction learning in the IL-PFC. If so, then knockout of β -arrestin should impair extinction learning in the CPP task. To test this hypothesis, we selectively knocked out β -arrestin2 in the IL-PFC by bilateral infection of AAV-EF1 α :eGFP-T2A-Cre (AAV-eGFP-Cre) in *Arrb2*^{fllox/fllox} mice (fig. S3). *Arrb2*^{fllox/fllox} mice infected with AAV-EF1 α :eGFP (AAV-eGFP) served as the control

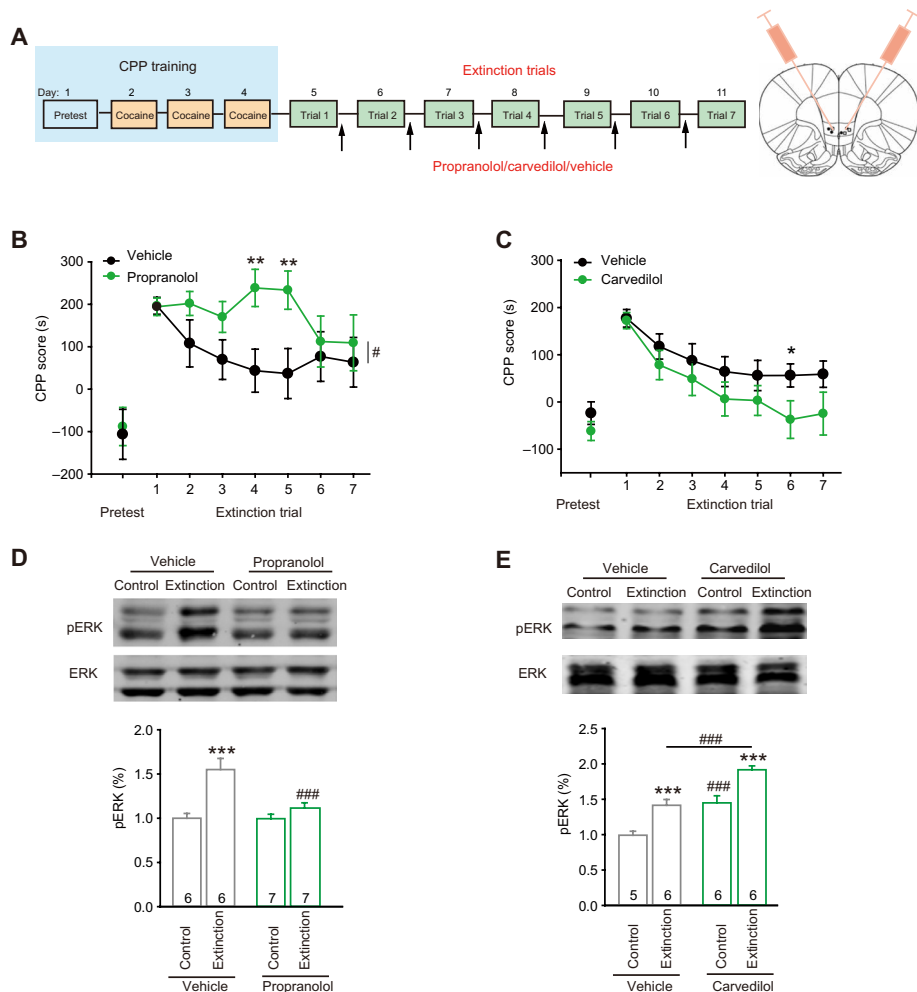


Fig. 1. The β -adrenergic receptor antagonist propranolol, but not carvedilol, impaired extinction learning of cocaine-CPP and extinction learning-induced ERK activation. (A) Experimental scheme. After cocaine-cocaine-conditioned place preference (CPP) training, mice were subjected to a daily extinction session for 7 days, and propranolol, carvedilol, or vehicle was infused in the infralimbic prefrontal cortex (IL-PFC) within 10 min after each extinction trial. For Western blotting, the IL-PFC was collected 15 min after the fourth extinction trial. (B) Effect of propranolol on extinction learning of cocaine-CPP (propranolol, 14 mice; vehicle, 19 mice). ** $P < 0.01$ compared to vehicle; # $P < 0.05$ as indicated. (C) Effect of carvedilol on extinction learning of cocaine-CPP (carvedilol, 27 mice; vehicle, 30 mice). * $P < 0.05$ compared to vehicle. (D) Effect of propranolol on extinction-induced extracellular signal-regulated kinase (ERK) activation in the IL-PFC. p, phosphorylated. *** $P < 0.005$ compared to control/vehicle; ### $P < 0.005$ compared to extinction/vehicle. (E) Effect of carvedilol on extinction-induced ERK activation in the IL-PFC. For (D) and (E), the number of mice per group is indicated in the bars. *** $P < 0.005$ compared to control/vehicle; ### $P < 0.005$ compared to vehicle. Error bars denote \pm SEM. See table S2 for additional statistical test details and results.

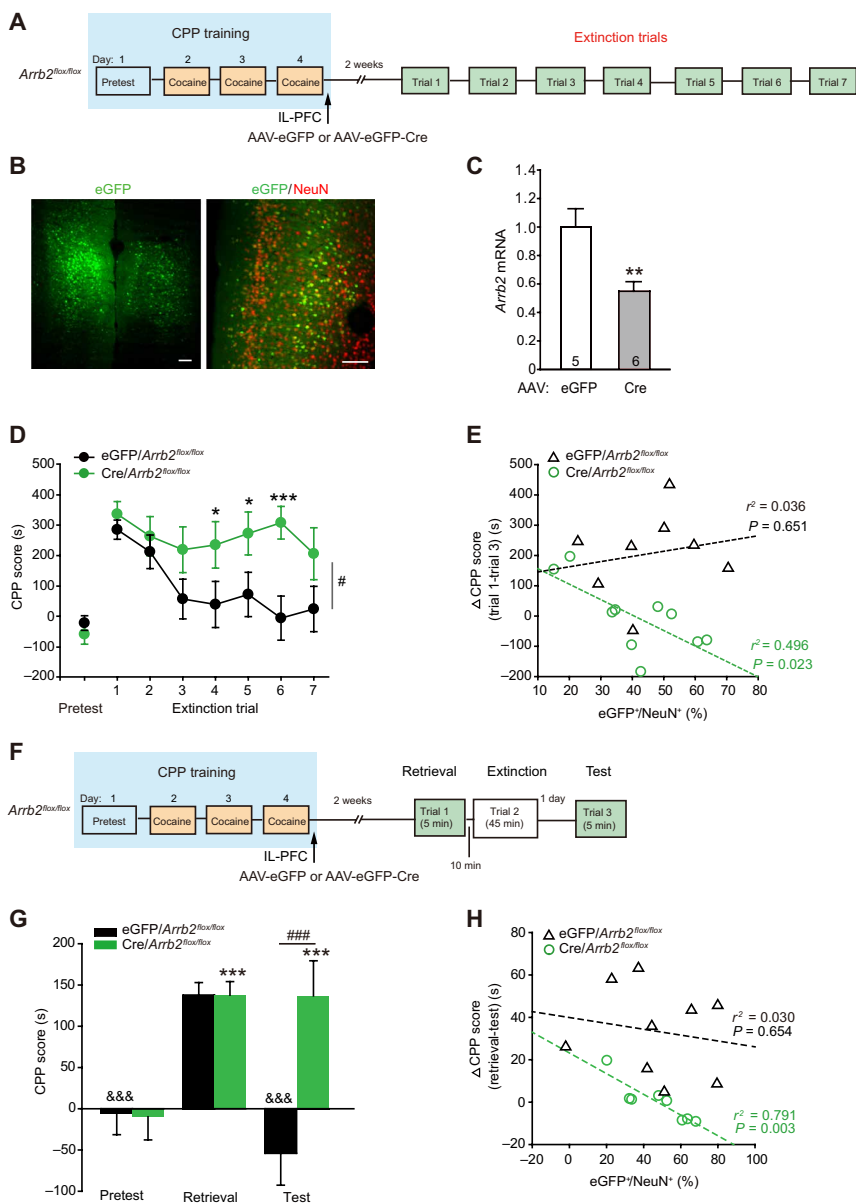


Fig. 2. β -Arrestin2 local knockout in the IL-PFC blocks extinction learning of cocaine-CPP. (A) Experimental scheme. AAV-EF1 α :eGFP-T2A-Cre or AAV-EF1 α :eGFP was infused into the IL-PFC of *Arrb2*^{fllox/fllox} mice after cocaine-CPP training. After a 2-week recovery, the mice were subjected to daily extinction sessions. (B) Infection of neurons in the IL-PFC by adeno-associated virus (AAV) and immunostaining for neuronal nuclei (NeuN) 2 weeks after bilateral AAV injection. Scale bars, 100 μ m. (C) *Arrb2* messenger RNA (mRNA) expression 2 weeks after injection of AAV-EF1 α :eGFP (eGFP) or AAV-EF1 α :eGFP-T2A-Cre (Cre) into the IL-PFC of *Arrb2*^{fllox/fllox} mice. Number of mice per group is indicated in the bars. ***P* < 0.01 compared to enhanced green fluorescent protein (eGFP). (D) Effect of β -arrestin2 deletion in the IL-PFC on extinction learning of cocaine-CPP (eGFP/*Arrb2*^{fllox/fllox}, 16 mice; Cre/*Arrb2*^{fllox/fllox}, 18 mice). **P* < 0.05, ****P* < 0.005 compared to eGFP/*Arrb2*^{fllox/fllox}; #*P* < 0.05 as indicated. (E) Correlation of the percentage of eGFP⁺ neurons in the IL-PFC of *Arrb2*^{fllox/fllox} mice with the extent of cocaine-CPP extinction (eGFP/*Arrb2*^{fllox/fllox}, 8 mice; Cre/*Arrb2*^{fllox/fllox}, 10 mice). (F) Experimental scheme. After cocaine-CPP training, AAVs were infused in the IL-PFC of *Arrb2*^{fllox/fllox} mice. Two weeks later, these mice were subjected to retrieval-extinction sessions, and the memory retention test was performed 1 day later. (G) Effect of β -arrestin2 ablation in the IL-PFC on the memory retention test after retrieval-extinction session of cocaine-CPP (eGFP/*Arrb2*^{fllox/fllox}, 17 mice; Cre/*Arrb2*^{fllox/fllox}, 16 mice). ****P* < 0.005 compared to Cre/*Arrb2*^{fllox/fllox} in pretest; ###*P* < 0.005 as indicated; &&&*P* < 0.005 compared to eGFP/*Arrb2*^{fllox/fllox} in retrieval. (H) Correlation of the percentage of eGFP⁺ neurons in the IL-PFC of *Arrb2*^{fllox/fllox} mice with the extent of the extinction of cocaine-CPP. Error bars denote \pm SEM (eGFP/*Arrb2*^{fllox/fllox}, nine mice; Cre/*Arrb2*^{fllox/fllox}, eight mice). See table S3 for additional statistical test details and results.

in these experiments. After CPP training, mice were infused with adeno-associated virus (AAV), and 2 weeks later, the daily extinction sessions were commenced for 7 days (one trial per day) (Fig. 2A). The expression of Cre in the IL-PFC (Fig. 2B) significantly decreased *Arrb2* messenger RNA (mRNA) abundance in the IL-PFC of *Arrb2*^{fllox/fllox} mice (Fig. 2C and table S3). β -Arrestin2 knockout in the IL-PFC of *Arrb2*^{fllox/fllox} mice through infusion of AAV-eGFP-Cre impaired CPP extinction learning (Fig. 2D and table S3). Furthermore, the extent of extinction learning impairment (calculated as the difference in CPP score between trial 1 and trial 3) correlated with the percentage of enhanced green fluorescent protein (eGFP)/Cre-positive neurons (in which β -arrestin2 had been ablated) in the IL-PFC (Fig. 2E and table S3).

We also investigated the role of β -arrestin2 in the IL-PFC in the retrieval-extinction paradigm, in which a 5-min brief retrieval trial is followed by prolonged extinction learning (Fig. 2F). This paradigm is considered to be a promising nonpharmacological therapeutic approach for enhancing extinction efficacy and reducing drug craving and relapse (26). β -Arrestin2 ablation in the IL-PFC significantly impaired extinction learning of cocaine-CPP induced by this retrieval-extinction session (Fig. 2G and table S3). Furthermore, the retention of extinction correlated to β -arrestin2 ablation (Fig. 2H and table S3). Thus, β -arrestin2 in the IL-PFC appeared to be indispensable for extinction learning of cocaine-CPP.

Next, we investigated whether overexpression of β -arrestin2 in the IL-PFC would enhance extinction learning of cocaine-CPP. After cocaine-CPP training, we infused AAV-hSyn:HA-Arrb2-T2A-eGFP (AAV-Arrb2-eGFP) in the IL-PFC of mice. AAV-hSyn:Gal-T2A-eGFP (AAV-Gal-eGFP)-infused mice served as the control group. Infusion of AAV-Arrb2-eGFP in the IL-PFC resulted in overexpression of β -arrestin2 mainly in this brain region (Fig. 3, A to D, and table S4). Overexpression of β -arrestin2 in the IL-PFC enhanced extinction learning of cocaine-CPP (Fig. 3E and table S4). Similar to our correlative analyses described above, the extent of CPP extinction levels correlated with the percentage of β -arrestin2/eGFP-positive neurons in the IL-PFC (Fig. 3F and table S4). Together, our results suggest that β -arrestin2 in the IL-PFC might regulate extinction learning of cocaine-CPP in a bidirectional manner.

β -Arrestin-biased β -adrenergic signaling pathway promotes extinction learning of cocaine-CPP

To further demonstrate whether β -ARs mediate cocaine-CPP extinction through the downstream β -arrestin2-biased signaling pathway, we infused AAV-Arrb2-eGFP in the IL-PFC of wild-type mice or AAV-eGFP-Cre in the IL-PFC of *Arrb2*^{fllox/fllox} mice after cocaine-CPP training. Two weeks later,

propranolol or carvedilol was infused into the IL-PFC after each CPP extinction trial (Fig. 4A). The enhanced extinction learning in mice overexpressing β -arrestin2 in the IL-PFC was impaired by propranolol (Fig. 4B and table S5). Furthermore, the impairment of extinction learning caused by β -arrestin2 deletion in the IL-PFC was not rescued by carvedilol (Fig. 4C and table S5). Together, our results suggest that the β -AR/ β -arrestin2-mediated signaling pathway in the IL-PFC regulated extinction learning of cocaine-CPP.

β -Arrestin signaling in excitatory neurons in the IL-PFC mediates extinction learning of cocaine-CPP and cocaine self-administration memories

We examined the role of β -arrestin2 signaling in excitatory or inhibitory neurons in the IL-PFC in extinction learning of cocaine-CPP. First, we infused AAV-CaMKII α :GFP-T2A-Cre into the IL-PFC of

Arrb2^{fllox/fllox} mice after cocaine-CPP training. Two weeks later, the daily extinction session was performed (Fig. 5A). The expression of Cre in neurons was verified by fluorescence imaging of eGFP and neuronal nuclei (NeuN) (Fig. 5B). The selective deletion of β -arrestin2 in excitatory neurons in the IL-PFC significantly impaired extinction learning of cocaine-CPP (Fig. 5C and table S6). Next, we selectively knocked down β -arrestin2 in inhibitory neurons. We infused a lentivirus that expressed Cre-dependent β -arrestin2 short hairpin-mediated RNA (shRNA) (LV-DIO-*Arrb2*-shRNA) into the IL-PFC of *GAD2*:Cre mice. β -Arrestin2 shRNA decreased *Arrb2* mRNA by 50% in Neuro 2A (N2A) cells (fig. S3, A to C, and table S7). Three weeks after the lentivirus infusion into the IL-PFC of *GAD2*:Cre mice, extinction sessions were commenced (fig. S3D). β -Arrestin2 knockdown in inhibitory neurons in the IL-PFC did not attenuate extinction learning of cocaine-CPP (fig. S3E and table S7). These results suggest that β -arrestin signaling in the excitatory neurons of the IL-PFC might mediate extinction learning of cocaine-CPP.

Excitatory neurons in the IL-PFC send projections to the shell of nucleus accumbens (NAc). To examine whether β -arrestin2 expression in the IL-PFC neurons that project to NAc shell is critical for extinction learning of cocaine-CPP, we simultaneously infused a retrogradely transported canine adenovirus-2-expressing Cre (CAV2-Cre) (27, 28) into the NAc shell and a Cre-dependent AAV (AAV-hSyn:FLEX-tdTomato) into the IL-PFC of *Arrb2*^{fllox/fllox} mice after cocaine-CPP training. The expression of tdTomato was detected in the IL-PFC (Fig. 5D), indicating that Cre recombinase was functionally expressed in the IL-PFC neurons targeting the NAc shell. β -Arrestin2 conditional knockout in the IL-PFC neurons targeting the NAc shell impaired extinction learning of cocaine-CPP (Fig. 5E and table S6). Therefore, β -arrestin signaling in the IL-PFC glutamatergic neurons projecting to the NAc shell regulated extinction learning of cocaine-CPP.

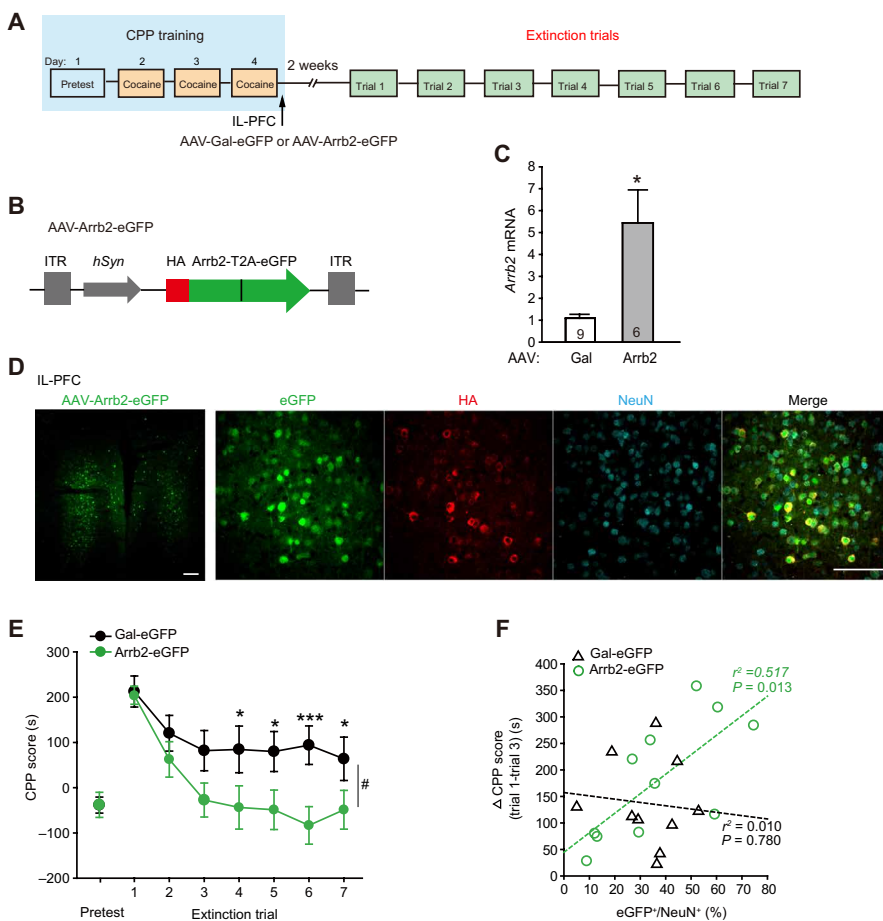


Fig. 3. Overexpression of β -arrestin2 in the IL-PFC promotes extinction learning of cocaine-CPP.

(A) Experimental scheme. After cocaine-CPP training, AAVs were infused into the IL-PFC. After a 2-week recovery, daily extinction sessions were carried out. (B) Construction of recombinant AAV encoding hemagglutinin (HA)-tagged β -arrestin2 under the control of a human synapsin promoter inserted between inverted terminal repeats (ITR) (AAV-hSyn:HA-Arrb2-T2A-eGFP). (C) Effect of injection of AAV-hSyn:HA-Arrb2-T2A-eGFP or AAV-hSyn:HA-galactosidase (Gal)-T2A-eGFP on *Arrb2* mRNA abundance. Number of mice per group is indicated in the bars. * $P < 0.05$ compared to Gal. (D) Left: The expression of eGFP in IL-PFC. Right: Co-immunostaining of HA and NeuN in the IL-PFC. Scale bars, 100 μ m. (E) Effect of β -arrestin2 overexpression on extinction learning of cocaine-CPP (Arrb2-eGFP, 31 mice; Gal-eGFP, 16 mice). * $P < 0.05$, *** $P < 0.005$, compared to AAV-Gal-eGFP; # $P < 0.05$ as indicated. (F) Correlation of the percentage of eGFP⁺ neurons in the IL-PFC with the extent of the extinction of cocaine-CPP (Arrb2-eGFP, 10 mice; Gal-eGFP, 11 mice). Error bars denote \pm SEM. See table S4 for additional statistical test details and results.

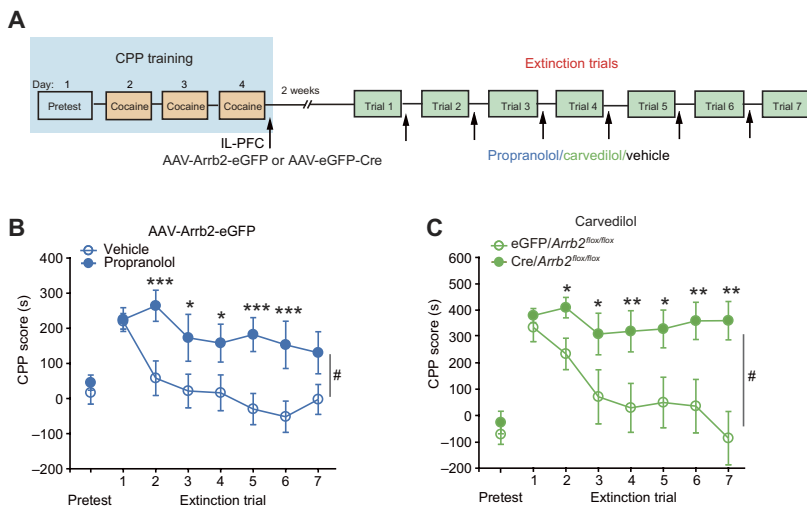


Fig. 4. Extinction learning of cocaine-CPP depends on β -arrestin2-biased β -adrenergic signaling. (A) Experimental scheme. AAV-hSyn:HA-Arrb2-T2A-eGFP or AAV-EF1 α :eGFP-T2A-Cre was infused bilaterally in the IL-PFC of C57 mice or *Arrb2^{lox/lox}* mice after cocaine-CPP training. Propranolol or carvedilol was infused in the IL-PFC within 10 min after each extinction trial. (B) Effect of propranolol on the enhanced extinction caused by β -arrestin2 overexpression in the IL-PFC (vehicle; 17 mice; propranolol, 15 mice). * $P < 0.05$, *** $P < 0.005$ compared to vehicle; # $P < 0.05$ as indicated. (C) Effect of carvedilol on the impaired CPP extinction caused by β -arrestin2 knockout in the IL-PFC (Cre/*Arrb2^{lox/lox}*, 16 mice; eGFP/*Arrb2^{lox/lox}*, 15 mice). * $P < 0.05$, *** $P < 0.01$ compared to eGFP/*Arrb2^{lox/lox}*; # $P < 0.05$ as indicated. Error bars denote \pm SEM. See table S5 for additional statistical test details and results.

and subjected the mice to self-administration training followed by nose poke extinction and cue extinction trials. β -Arrestin2-selective knockdown in the IL-PFC did not affect cocaine self-administration (Fig. 5G and table S6) but significantly impaired nose poke extinction learning during the early extinction sessions (Fig. 5H and table S6). Next, the mice received cue extinction training or did not receive this training. In the reinstatement test, the mice infused with LV-DIO-Arrb2-shRNA in the IL-PFC had more nose pokes for cocaine than those infused with LV-DIO-scramble shRNA (Fig. 5I and table S6). These data suggest that β -arrestin2 signaling in the IL-PFC excitatory neurons was critically involved in the extinction learning of discrete conditional cues, in addition to nose poke extinction learning in cocaine self-administration.

DISCUSSION

Here, we found that IL-PFC infusion of propranolol, but not of carvedilol, impaired extinction learning of cocaine-CPP memory and ERK activation induced by extinction learning. Furthermore, β -arrestin2 knockout in IL-PFC excitatory neurons disrupted extinction learning, whereas β -arrestin2 overexpression in IL-PFC promoted extinction learning of cocaine-CPP. In a cocaine self-administration mouse model, β -arrestin2-selective knockdown in IL-PFC excitatory neurons also impaired extinction learning of cocaine self-administration memory. Our study suggests that β -arrestin2-biased β -adrenergic signaling in the IL-PFC excitatory neurons regulated extinction learning of cocaine reward memory.

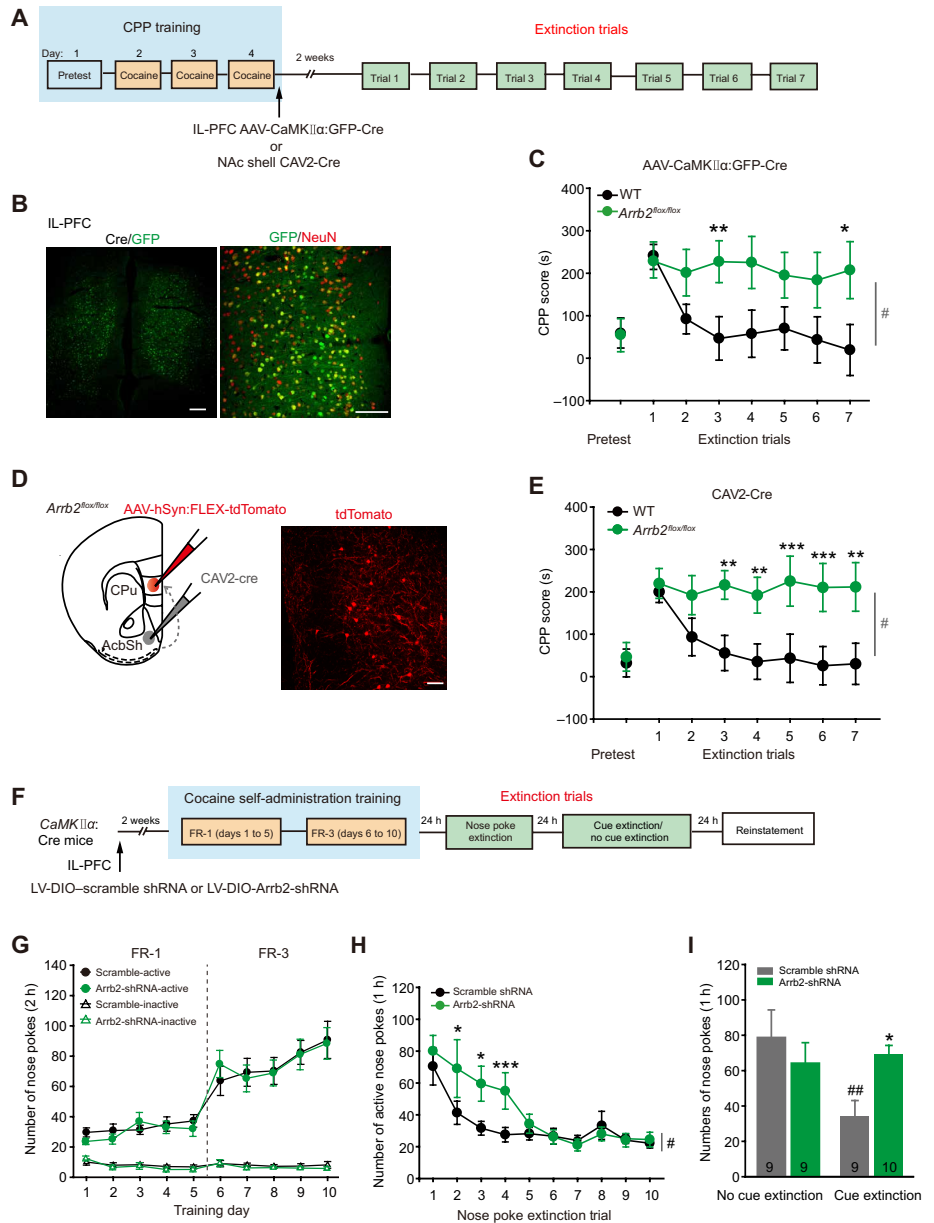
Various brain regions have been implicated in extinction of drug seeking, such as the IL-PFC (17, 18, 31) and NAc (32, 33). The neurons in the IL-PFC send projections to the NAc shell (34). Extinction of

cocaine self-administration increases c-Fos levels in the IL-PFC (35), indicating that extinction learning processes engage distinct neuronal populations in the IL-PFC. Inactivation of the IL-PFC after extinction impairs extinction of cocaine self-administration (31, 36). Consistent with these findings, we found that the IL-PFC was activated during extinction learning of cocaine-CPP as demonstrated by the increased levels of c-Fos and pERK in the IL-PFC induced by extinction learning. β -AR blockade by propranolol in the IL-PFC impaired extinction learning of cocaine reward memory. However, the IL-PFC may serve different roles in cocaine addiction compared to heroin addiction, because extinction of cocaine seeking, but not that of heroin seeking, depends on activation of the IL-PFC (37). Nonselective global inhibition of the IL-PFC decreased context-induced reinstatement of heroin seeking (38). Inhibition of glutamatergic projections from the IL-PFC to the NAc shell decreases context-induced reinstatement for heroin (39). In addition to cocaine and heroin, activation of metabotropic glutamate receptor 5 in the IL-PFC facilitates alcohol extinction by inhibiting K_{Ca2} channels (40). These studies suggest that the IL-PFC may have different roles in regulating extinction learning of different drugs. Because the IL-PFC represents a common node in the extinction circuits for addiction, treatments that target this region may help to alleviate the symptoms of addictive disorders (41).

An important area of research is the role of noradrenergic mechanisms in extinction learning of associative memories. Activation of β -ARs by norepinephrine in the IL-PFC facilitates extinction of contextual fear memory (42), and propranolol infusion into the IL-PFC impairs the extinction of fear memory (43). However, these studies did not clarify whether the G protein-dependent or the β -arrestin-dependent β -adrenergic signaling pathway is recruited during extinction learning.

Our study supports the hypothesis that β -ARs in IL-PFC could be critically involved in extinction learning of drug addiction associative memories. Furthermore, our results suggest that the β -arrestin-biased β -AR signaling is required for the extinction of reward memory induced by cocaine. We found that infusion of propranolol, a β -AR antagonist that blocks both G protein and β -arrestin signaling pathways, in the IL-PFC impaired extinction learning of cocaine-CPP. By contrast, carvedilol, which selectively blocks a β -AR/G protein signaling, did not have these effects, indicating that G protein signaling was not required for extinction learning (Fig. 1, A to C). In addition, injection of carvedilol into the IL-PFC did not promote cocaine extinction in mice with β -arrestin2 knockout in the IL-PFC. β -Arrestin2 deletion in the excitatory neurons in IL-PFC also inhibited extinction learning of cocaine reward memories. However, β -arrestin2 overexpression in IL-PFC accelerated extinction learning. These results support the notion that extinction learning of cocaine-seeking behavior is mediated by a G protein-independent, β -arrestin-biased signaling pathway. Here, we also used the cocaine self-administration mouse model. We performed a Pavlovian cue extinction procedure designed to mimic the human cue-based exposure therapy. We found that β -arrestin2 knockdown in IL-PFC excitatory neurons impaired discrete cue and context extinction, which further supported our main conclusion that β -arrestin-biased signaling in the IL-PFC could mediate the extinction of cocaine reward memories.

Fig. 5. β -arrestin2 deletion in excitatory neurons in the IL-PFC blocks extinction learning of cocaine reward memory. (A) Experimental scheme. AAV-*CaMKII α* :GFP-Cre was infused into the IL-PFC of *Arrb2^{lox/lox}* mice and their wild-type (WT) littermates after cocaine-CPP training. In another cohort of mice, *CAV2-Cre* was infused in the nucleus accumbens (NAc) shell to infect IL-PFC neurons projecting to the NAc shell in a retrograde fashion, and AAV-hSyn:FLEX-tdTomato was infused in the IL-PFC of *Arrb2^{lox/lox}* mice and their WT littermates. After a 2-week recovery, the mice were subjected to daily extinction sessions. (B) Immunostaining for NeuN 2 weeks after bilateral AAV injection. Scale bars, 100 μ m. (C) Effect of β -arrestin2 deletion in excitatory neurons in the IL-PFC on extinction learning of cocaine-CPP (WT, 16 mice; *Arrb2^{lox/lox}*, 15 mice). * P < 0.05, ** P < 0.01 compared to WT; # P < 0.05 as indicated. (D) AAV injection sites (left) and expression of tdTomato (right) in the IL-PFC. Scale bar, 100 μ m. CPU, caudate putamen; AcbSh, NAc shell. (E) Effect of β -arrestin2 deletion in IL-PFC neurons projecting to the NAc shell after the acquisition of cocaine-CPP on extinction learning (WT, 18 mice; *Arrb2^{lox/lox}*, 17 mice). ** P < 0.01, *** P < 0.005 compared to WT; # P < 0.05 as indicated. (F) Experimental scheme. LV-DIO-*Arrb2*-shRNA (short hairpin-mediated RNA) or LV-DIO-scramble shRNA was infused into the IL-PFC of *CaMKII α* :Cre mice before cocaine self-administration training. (G) Effect of LV-DIO-*Arrb2*-shRNA and LV-DIO-scramble shRNA injection on cocaine self-administration (0.5 mg/kg per infusion) training on fixed-ratio 1 (FR-1) and FR-3 schedule. Active, portal producing a cocaine injection; inactive, portal yielding no cocaine injection (scramble shRNA, 19 mice; *Arrb2*-shRNA, 19 mice). (H) Effect of β -arrestin2 knockdown in excitatory neurons in the IL-PFC on extinction learning of cocaine self-administration in nose poke extinction session (scramble shRNA, 19 mice; *Arrb2*-shRNA, 19 mice). * P < 0.05, *** P < 0.005 compared to scramble shRNA; # P < 0.05 as indicated. (I) Effect of β -arrestin2 knockdown in excitatory neurons in the IL-PFC on cue-induced reinstatement after cue extinction session. Number of mice per group is indicated in the bars. * P < 0.05 compared to scramble shRNA/cue extinction; ## P < 0.01 compared to scramble shRNA/no cue extinction. Error bars denote \pm SEM. See table S6 for additional statistical test details and results.



Studying neural mechanisms underlying extinction of drug memory could help in the development of new therapeutic approaches to prevent drug relapse. Treatment of drug addiction could be achieved nonpharmacologically by triggering the extinction of the association between drug use and conditional cues. Our results suggest that the β -arrestin-biased β -adrenergic ligands may help in treating drug dependence and addictions as an adjunct treatment with exposure therapy.

MATERIALS AND METHODS

Animals

Arrb2^{lox/lox} mice that had loxP site on either side of exon 2 of *Arrb2* (S2) were generated by laboratory of G. Pei (Shanghai Institutes for Bio-

logical Sciences, Chinese Academy of Sciences) and backcrossed onto a C57BL/6J background (>10 generations). *CaMKII α* :Cre [B6.Cg-Tg (Camk2a-Cre) T29-1Stl/J] and *GAD2*:Cre [B6N.Cg-Gad2tm2 (Cre) Zjh/J] mice were obtained from the Jackson Laboratory. Seven-week-old male C57BL/6J mice, weighing about 22 g, were purchased from Slaccas Lab Animal Ltd. The male mice were housed under standard conditions of a reversed 12-hour light/dark cycle with access to food and water ad libitum, and behavioral experiments were carried out during the light cycle. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Animal Care and Use Committee of Shanghai Medical College of Fudan University. Male *Arrb2^{lox/lox}* mice at 8 to 10 weeks of age were used for all behavioral tests. *Arrb2^{lox/lox}* mice and subsequent offsprings were genotyped using the following

primer sets: 5'-TTGCCGACGCTCTGAAGAAGC-3'; 5'-AGGAAGGA-TTGTCTCCCAGTATGAC-3'. The *CaMKII α* :Cre and *GAD*:Cre mice were genotyped with the following primers sets: 5'-GATCTCCGGTA-TTGAACCTCCAGC-3'; 5'-GCTAAACATGCTTCATCGTCGG-3'.

Reagents

Cocaine-hydrochloride (Qinghai Pharmaceutical Firm) and (+)-propranolol HCl (Sigma-Aldrich) were dissolved in saline. Carvedilol (Tocris Bioscience) was dissolved with vehicle of saline containing 1% dimethyl-sulfoxide. Mice were infused with (+)-propranolol HCl (1.0 μ g) or carvedilol (2.5 μ g) bilaterally into IL-PFC. Controls received an equivalent volume of vehicle.

Cannula implantation and microinjection

Mice were anesthetized with 10% chloral hydrate, placed in a stereotaxic apparatus, and implanted with 26-gauge pedestal guide cannulas (Plastics One Inc.) targeting the IL-PFC according to the following coordinates: anteroposterior (AP), +1.8 mm; mediolateral (ML), \pm 1.4 mm; and dorsoventral (DV), -2.7 mm with an angle of 20° and fixed with dental acrylic cement. On the day of injection, a surgical grade, 33-gauge steel needle with a 0.5-mm projection was connected to tubing attached to a 1-ml infusion pump (BAS Bioanalytical Systems Inc.). A volume of 0.5 μ l was infused bilaterally over a period of 5 min immediately after each extinction trial.

Vector construction

pSico vector (Addgene plasmid no. 11578) allows for Cre-dependent and stable expression of shRNAs in cells and transgenic mice (44). The shRNA coding oligos (5'-GCGACTTTGTAGATCACCTGG-3') targeting *Arrb2* and a scramble oligos (5'-TCGAAGTATTCCGCG-TACGTT-3') as a control were subcloned into pSico vector using XhoI and HpaI restriction sites.

Cell culture and transfection

N2A cells were grown to confluence in 24-well plates. Two hours before transfection, the Dulbecco's modified Eagle's medium (DMEM)/F12 containing fetal serum was replaced with serum-free DMEM. The pSico vector containing the mouse *Arrb2*-shRNA was transfected into N2A cells by using Lipofectamine 2000 Transfection Reagent (Invitrogen, Thermo Fisher Scientific).

Viral constructs and microinjection

AAV2/9 with titer exceeding 2×10^{12} vector genome (vg) ml⁻¹ (Neuron Biotech Co. Ltd. and Shanghai Taitool Bioscience Co. Ltd.) and lentivirus with titer exceeding 1×10^9 vg ml⁻¹ (GeneChem Technology Co. Ltd.) were used. To conditionally knockout β -arrestin2 in the IL-PFC, we infused the packaged AAV that encodes Cre recombinase under the control of an *EF1 α* promoter (AAV-EF1 α :eGFP-T2A-Cre) or *CaMKII α* promoter (AAV-CaMKII α :GFP-T2A-Cre) into the IL-PFC of *Arrb2*^{flox/flox} mice. AAV-EF1 α :eGFP was used as a control for AAV-EF1 α :eGFP-T2A-Cre. To selectively knock down β -arrestin2 in excitatory or inhibitory neurons, we infused the Cre-dependent lentivirus (LV-DIO-*Arrb2*-shRNA or LV-DIO-Scramble-shRNA) into the IL-PFC of *CaMKII α* :Cre or *GAD2*:Cre mice. Cre was also delivered retrogradely by CAV2 that has the ability to enter axon terminals and thereby retrogradely infects projection neurons (CAV2-Cre, Shanghai Taitool Bioscience Co. Ltd.). To overexpress β -arrestin2 in the IL-PFC, we infused AAV expressing N-terminal hemagglutinin (HA)-tagged wild-type β -arrestin2 and an eGFP reporter under the control of a human syn-

apsin promoter (AAV-hSyn:HA-Arrb2-T2A-eGFP) into the IL-PFC and used AAV-hSyn:Gal-T2A-eGFP as a control. To microinject AAV (45), we positioned an anesthetized mouse in a stereotaxic apparatus with the injection syringe of 36-gauge tips (World Precision Instruments Inc.) aimed at the IL-PFC or NAc shell. The intended stereotaxic coordinates were as follows: AP, +1.80 mm; ML, \pm 0.30 mm; and DV, -3.00 mm for the IL-PFC and AP, +1.60 mm; ML, \pm 0.50 mm; and DV, -4.80 mm for the NAc shell. AAV (0.2 μ l) or lentivirus (0.3 μ l) was infused at a rate of 0.05 μ l/min.

Cocaine-CPP training and extinction learning

The CPP apparatus consists of two equal-sized chambers (15 cm \times 15 cm \times 15 cm) with distinct tactile and visual cues, separated by a removable divider. On day 1 (pretest session), naive mice were allowed free access and exploration to both chambers for 15 min, and the time spent in each chamber was recorded by video. Mice that spent >65% (>585 s) or <35% (<315 s) of the total time (900 s) in one side were eliminated from the subsequent CPP experiments. On days 2 to 4 (training session), mice were injected with saline (4 ml/kg, intraperitoneally) daily in the morning. Immediately after the saline injection, mice were placed in one chamber (designated as saline-paired) and allowed to explore for 30 min. Five hours later, mice were injected with cocaine (10 mg/kg, intraperitoneally) and placed into the other chamber (designated as cocaine-paired) for 30 min.

In daily extinction training session, after cocaine-CPP training, the mice were moved to the experiment room and given an injection of saline (4 ml/kg, intraperitoneally), and then, the mice were immediately placed in CPP apparatus to allow free access to both chambers for 10 min. The time spent in each chamber was recorded, and a CPP score was determined by subtracting time spent on the drug-paired chamber from that on the saline-paired chamber for each daily extinction trial. For immunohistochemistry experiments, mice in the no extinction control group were removed to the experiment room and injected with saline similarly to mice in the extinction group except that they were not exposed to the CPP apparatus. Every second and third batch of β -arrestin2 conditional knockout mice or β -arrestin2 overexpression mice was used to test the correlation of efficiency of AAV infection with CPP score.

In retrieval-extinction session, the mice experienced a 5-min retrieval and a 45-min extinction learning with a 10-min interval in between. During the retrieval phase, mice were injected with saline and allowed free access to two chambers of the CPP apparatus for 5 min, after which mice were returned to home cage and stayed there for 10 min. During the extinction phase, mice went through the same procedure as the retrieval phase mentioned above except that the duration was 45 min. One day later, mice were tested for preference for the cocaine-paired side by reexposure to the same apparatus for 5 min.

Cocaine self-administration training and extinction learning

All behavioral experiments were performed during the light phase and took place in mouse operant chambers (ENV-307W-CT, Med Associates) situated in a sound-attenuating cubicle (Med Associates). Surgery was performed in accordance with a method that our laboratory previously used (46). Before cocaine self-administration training session, animals were initially maintained at 85% of original body weight and trained under a fixed-ratio 1 (FR-1) schedule of food pellets for nose poke responses in the operant chambers. After stable nose poke for food pellets was achieved, animals were anaesthetized with 10% chloral hydrate, implanted with a single silastic catheter in

the right jugular, and bilaterally injected with lentivirus into the IL-PFC, as previously mentioned. Mice were housed in pairs before surgery and singly after surgery. Catheters were flushed every day with 0.1-ml saline solution containing gentamycin (0.33 mg/ml) and heparin (30 U/ml). They were allowed to recover for 2 weeks before the start of behavioral experiments.

In the cocaine self-administration session, animals were trained to self-administer intravenous injections of cocaine (0.5 mg/kg per infusion delivered in 2 s) during a 2-hour session daily over 10 days under the FR-1 (5 days) and FR-3 (5 days) schedule of reinforcement with a 10-s time out. Responding on the active portal produced a cocaine injection accompanied with the conditional stimuli, which was stimulus light illumination and an audible tone for 10 s. Inactive portal yielded no consequence. Any animal that failed to self-administer at least 10 infusions of cocaine per session averaged across the last 3 days of self-administration was removed from the study.

The day after the final self-administration session, animals received daily 1-hour nose poke extinction session for 10 days. The active portal had no programmed consequences. The day after the final nose poke extinction session, half of the mice received a cue extinction session (cue extinction). After a 2-min baseline period, the 10-s tone-light cue was presented every 30 s for 60 times. The remaining mice (no cue extinction group) were handled on this day but were not placed into the operant chamber (47–49). The day after a cue extinction session, animals were tested for cue-induced reinstatement. During a 1-hour session, the previously active portal produced the cocaine-associated cues on an FR-1 schedule without infusing cocaine.

Brain lysate preparation and Western blotting

Mice were decapitated, and the brains were removed immediately. The IL-PFC was dissected within 5 min on ice, according to stereotaxis coordinates with matrix for mouse (Plastic One Inc.). Tissue samples were homogenized with lysis buffer [10 mM tris-HCl (pH 7.4), 1% Triton X-100, 50 mM NaCl, 1.0 mM EGTA, 1.0 mM EDTA, 50 mM NaF, 100 μ M Na₃VO₄, 1.0 μ M phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, and protease inhibitors (Roche cocktail tablets)], incubated on ice for 10 min, and centrifuged at 1000g for 30 min at 4°C. Supernatants of samples were assayed for protein content and then diluted to equal protein concentration of 1.5 μ g/ μ l. Samples were further diluted 1:5 in SDS-polyacrylamide gel electrophoresis loading buffer [0.3 mM tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 6% β -mercaptoethanol, and 0.012% w/v bromophenol blue] and heated at 95°C for 5 min. Protein (30 μ g per aliquot) was loaded onto 10% SDS polyacrylamide gel. Proteins were then transferred onto nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in tris-buffered saline (TBS) for 1 hour at room temperature and probed with primary antibody [1:1000, anti-pERK (Cell Signaling Technology); 1:2000, anti-ERK (Cell Signaling Technology); 1:2000, anti- β -tubulin (Sigma)] at 4°C overnight and then incubated with IRDye 700DX- or 800DX-conjugated anti-rabbit immunoglobulin G (IgG) (1:50000; Rockland Immunochemicals Inc.) for 1 hour at room temperature. The membrane was rinsed in TBS with Tween 20 (0.1%) and scanned with the appropriate channels (Odyssey, LI-COR Biosciences). The immunoblots were analyzed with Image-Pro Plus to measure the optical density of the bands of pERK or ERK. ERK activation (relative pERK/ERK levels) was calculated by normalizing the intensity of pERK to the total ERK expression. Data of pERK/ERK levels for each group were expressed as percentage of the averaged values in the control group.

Immunohistochemistry

Mice were anesthetized with 10% chloral hydrate and sacrificed with transcardial perfusion with 0.9% saline followed by 4.0% paraformaldehyde in 0.1 M Na₂HPO₄/NaH₂PO₄ buffer (pH 7.4). The entire brain was quickly removed, processed for post-fixing in 4.0% paraformaldehyde for 4 hours, and cryoprotected with 30% sucrose/phosphate-buffered saline (PBS) for 3 days. The brain was sectioned into 30- μ m slices by a vibratome (Leica). After blocking in 5.0% normal goat serum in PBS, floating sections were incubated in primary antibody (1:2000; anti-c-Fos, Santa Cruz) at 4°C overnight. After rinsing in PBS, sections were incubated for 2 hours with the biotinylated anti-rabbit IgG (1:300). Sections were rinsed and incubated at room temperature for 40 min in avidin-biotin complex solution (Vector Laboratories) in PBS. The peroxidase reaction was visualized in 0.01 M PBS containing 0.025% diaminobenzidine tetrahydrochloride and 0.03% H₂O₂. The sections were then washed and visualized by an image system (Spot Advanced 4.1.2). The quantification of c-Fos-positive cells in IL-PFC was counted with three serial sections by Image-Pro Plus. Labeled cells above the same threshold determined from control animals were counted (50). The brain slices were also analyzed with double immunofluorescence by incubating with primary antibodies [1:500, anti-HA (Sigma); 1:1000, anti-NeuN (Millipore)] overnight at 4°C. After extensive washing with PBS, the slices were incubated with two fluorescence secondary antibody (Jackson ImmunoResearch) overnight at 4°C or at room temperature for 1 hour. The sections were then washed and visualized under a LSM 510 laser confocal fluorescence microscope (Carl Zeiss). Data in the graphs are means \pm SEM.

Real-time quantitative polymerase chain reaction

Mice were decapitated, and the IL-PFC was dissected on ice immediately with Matrix for Mouse (Plastic one Inc.), according to stereotaxis coordinates. Total RNA was extracted from tissues using the TRIzol Reagent (Thermo Fisher Scientific Inc.). For N2A cells, DMEM media was removed and replaced with TRIzol 48 hours after transfection. The reverse transcription with random primers was conducted according to the Superscript First-Strand Synthesis System (TAKARA). Quantitative reverse transcription polymerase chain reaction (PCR) amplification of the complementary DNA was performed on samples in triplicate with Power SYBR Green PCR Master Mix (TAKARA). The primers for examining *Arrb2* conditional knockout were designed to flank exon 2 (5'-AAGTCGAGCCCTAACTGCAA-3' and 5'-CAGGATCCACAAGCACCAC-3'), and the primers for verifying *Arrb2* mRNA expression were 5'-TGCTCCGTCACACTGCAGCCA-3' and 5'-CATGAGGAAGTGGCGTGTGGTT-3'. Primers for verifying the knockdown efficacy of pSico-DIO-*Arrb2*-shRNA plasmid in N2A cells were 5'-CACGCCACTTCTCATGTCT-3' and 5'-GCAAATGTCGGCGTACTGTC-3'. *Arrb2* mRNA expression was normalized to the internal control glyceraldehyde-3-phosphate dehydrogenase (5'-GTGGAGTCATACTGGAACATGTAG-3' and 5'-AATGGTAGAGGTCGGTGTG-3').

Statistical analysis

All the data were recorded and analyzed by two students who were blinded to treatment or genotype. Data are means \pm SEM and analyzed using Stata 14. Data were analyzed with Kolmogorov-Smirnov test for distribution. The data for *Arrb2* mRNA abundance were tested with two-tailed Student's *t* test or Mann-Whitney rank sum test with unequal variances. Quantification of immunostaining or Western blotting was assessed using one-way or two-way analysis of variance

(ANOVA) with Bonferroni post hoc test where appropriate. For behavioral comparison, data were analyzed with two-way repeated measures ANOVA, followed by the Bonferroni post hoc test. Two-way ANOVA by ranks or Friedman's two-way repeated measures ANOVA by ranks was used for nonnormally distributed data in memory retention test. $P < 0.05$ was considered statistically significant. Data are means \pm SEM.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/11/512/eaam5402/DC1

Fig. S1. The IL-PFC was activated during extinction learning of cocaine-induced CPP.

Fig. S2. Generation of β -arrestin2 conditional knockout mice.

Fig. S3. Conditional knockout of β -arrestin2 in IL-PFC inhibitory neurons did not impair extinction learning of cocaine-CPP.

Table S1. Summary of statistics for fig. S1.

Table S2. Summary of statistics for Fig. 1.

Table S3. Summary of statistics for Fig. 2.

Table S4. Summary of statistics for Fig. 3.

Table S5. Summary of statistics for Fig. 4.

Table S6. Summary of statistics for Fig. 5.

Table S7. Summary of statistics for fig. S3.

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β -Arrestin–biased β -adrenergic signaling promotes extinction learning of cocaine reward memory

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Extinguishing memories to fight addiction

Relapse in cocaine addicts can be prevented by training addicts not to respond to drug-associated cues. This process is called extinction learning in animal models of drug addiction. Activation of the β -adrenergic receptor, which can preferentially stimulate signaling mediated by β -arrestins or G proteins, is implicated in extinction learning. Huang *et al.* determined that the β -arrestin–dependent pathway was required downstream of β -adrenergic receptor activation to promote extinction learning in two different models, including a self-administration paradigm. Specifically, β -arrestin signaling was required in excitatory neurons in the infralimbic prefrontal cortex, a region of the brain involved in attenuating the association between drugs and cues. Thus, enhancing β -arrestin signaling in the infralimbic prefrontal cortex may augment the effect of cognitive therapy in preventing drug-seeking behavior and relapse in cocaine addicts.

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