

## REGULAR RESEARCH ARTICLE

# Basolateral Amygdala SIRT1/PGC-1 $\alpha$ Mitochondrial Biogenesis Pathway Mediates Morphine Withdrawal-Associated Anxiety in Mice

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## Abstract

**Background:** Anxiety is a negative emotion that contributes to craving and relapse during drug withdrawal. Sirtuins 1 (SIRT1) has been reported to be critical in both negative emotions and drug addiction. However, it remains incompletely elucidated whether SIRT1 is involved in morphine withdrawal-associated anxiety.

**Methods:** We established a mouse model of anxiety-like behaviors induced by morphine withdrawal and then detected neuronal activity with immunofluorescence and mitochondrial morphology with electron microscopy, mitochondrial DNA contents with quantitative real-time PCR, and mitochondrial function with the ATP content detection kit and the Mitochondrial Complex IV Activity Kit in the basolateral amygdala (BLA). The mitochondrial molecules were detected by western blot. Then we used virus-mediated downregulation and overexpression of SIRT1 in BLA to investigate the effect of SIRT1 on anxiety and mitochondrial function. Finally, we examined the effects of pharmacological inhibition of SIRT1 on anxiety and mitochondrial function.

**Results:** We found that BLA neuronal activity, mitochondrial function, and mtDNA content were significantly higher in morphine withdrawal mice. Furthermore, the expression levels of mitochondrial molecules increased in BLA cells. Virus-mediated downregulation of SIRT1 in BLA prevented anxiety-like behaviors in morphine withdrawal mice, whereas overexpression of SIRT1 in BLA facilitated anxiety-like behaviors in untreated mice through the SIRT1/ peroxisome proliferator activated receptor gamma coactivator 1-alpha pathway. Intra-BLA infusion of selective SIRT1 antagonist EX527 effectively ameliorated anxiety-like behaviors and mitochondrial dysfunction in mice with morphine withdrawal.

**Conclusion:** Our results implicate a causal role for SIRT1 in the regulation of anxiety through actions on mitochondrial biogenesis. Inhibitors targeting SIRT1 may have therapeutic potential for the treatment of opioid withdrawal-associated anxiety.

**Keywords:** Anxiety, morphine withdrawal, basolateral amygdala, mitochondria, SIRT1

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## Significance Statement

Exposure to drug of abuse results in changes of mitochondrial activity in the BLA and consequently alters synaptic and behavioral plasticity. This study investigated the protective effects of the SIRT1 inhibitor on morphine-induced anxiety and mitochondrial dysfunction. BLA neurons were activated in morphine withdrawal-induced anxious mice. Mitochondria number and function increased in BLA of anxious mice. The SIRT1/PGC1 $\alpha$  pathway was upregulated in BLA of anxious mice. Our study suggests that SIRT1 is a key regulator of morphine withdrawal-associated anxiety by virus-mediated downregulation and overexpression of SIRT1 in BLA. Pharmacological inhibition of SIRT1 ameliorates withdrawal-associated anxiety in mice and restored dysregulation of mitochondrial biogenesis in BLA. Our data may have important therapeutic implications in opiate-induced anxiety.

## Introduction

Drug addiction is a chronic relapsing disorder. Withdrawal from addictive drugs is characterized by debilitating physical symptoms, refractory pathological memory, and aversive emotion in addicts (Ma et al., 2018; Wang et al., 2019). Maintaining prolonged abstinence from opioids is a major challenge in the treatment of drug addiction. After the somatic signs of withdrawal are no longer detectable, negative emotional responses gradually emerge and exacerbate overtime (Goeldner et al., 2011; Lee et al., 2014). Epidemiological studies revealed that negative symptoms (including anxiety and depression) in opioid addicts were positively associated with their craving score (Goeldner et al., 2011). Anxiety is an important negative emotion, which leads to craving and relapse during drug abstinence (Ma et al., 2018). Clinical studies show that opioid addicts still show obvious anxiety symptoms after 3 weeks of detoxification (Gossop et al., 1987; Herman et al., 2005). Furthermore, anxiety has an important potential risk of relapse after long-term abstinence (Willinger et al., 2002; LaRowe et al., 2017). A recent study found that withdrawal from repeated morphine exposure induced strong anxiety-like behaviors in mice (Qiao et al., 2021). However, the exact mechanism underlying the anxiety induced by opiate withdrawal is still unclear.

The basolateral amygdala (BLA) is a brain region that plays important roles in emotional response and sensory perception (Babaev et al., 2018). BLA integrates sensory inputs from the thalamus and cortex and sends projections to the central nuclei of the amygdala and eventually the brainstem and hypothalamus (Lu et al., 2014). Combining optogenetics and behavioral assays, our previous study demonstrated that BLA activation increased anxiety-like behaviors in mice (Yin et al., 2019). Nevertheless, chronic opioid exposure alters glutamate release, receptor function, and glutamate-linked synaptic plasticity in the amygdala (Yang et al., 2014; Song et al., 2018). Considering the critical role of BLA in both fear-learning and innate anxiety-like behaviors in many species, it is likely that synaptic plasticity in the principal BLA neurons was altered by chronic opioid exposure and further contributes to the expression of withdrawal-associated anxiety.

Drug addiction leads to numerous changes in the brain's metabolic profile, including changes in systemic energy balance, neural function, and metabolic pathways (Zaitso et al., 2014). Mitochondria are central for numerous metabolic pathways, including the production of adenosine triphosphate (ATP), intracellular Ca<sup>2+</sup> signaling, and the generation of reactive oxygen species (Kann and Kovacs, 2007). By generating energy and regulating subcellular homeostasis, mitochondria control fundamental processes in neuroplasticity, including neural differentiation, neurite outgrowth, neurotransmitter release, and dendritic remodeling (Cheng et al., 2010). Importantly, changes in the mitochondrial activity alter the synaptic plasticity and

participate in addiction processes (Sadakierska-Chudy et al., 2014). Mitochondrial biogenesis, which maintains mitochondrial homeostasis and coordinates nuclear and mitochondrial genomes, is regulated by a series of signaling factors (Shen et al., 2021). Several reports indicate that the NAD<sup>+</sup>-dependent deacetylase sirtuins 1 (SIRT1) and peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) serve as master regulators of mitochondrial biogenesis and function by controlling gene expression (Fanibunda et al., 2019). SIRT1 participates in maintaining the acquisition and consolidation of hippocampus-dependent short- and long-term memory (Heyward et al., 2012; Tang et al., 2018; El Hayek et al., 2019) and regulates neuronal differentiation and prevents neurodegeneration in several mouse models (Gomes et al., 2018). Previous study indicates that SIRT1 was upregulated in nucleus accumbens (NAc) in mice treated with chronic morphine and cocaine (Ferguson et al., 2013). SIRT1 in the principal hippocampus neuron also mediates anxiety- and depressive-like behaviors in mice (Libert et al., 2011; Abe-Higuchi et al., 2016). Therefore, we hypothesize that SIRT1 may be responsible for withdrawal-induced anxiety-like behaviors by acting as an upstream regulator of mitochondrial biogenesis in BLA.

In the present study, we investigated the neuronal activity of BLA in morphine withdrawal mice. By electron microscopy, qPCR, and immunoblotting, we determined the mitochondrial density, relative mtDNA contents, and expression of mitochondrial biogenesis pathway in the BLA of these mice. Combined with virus-mediated knockdown/overexpression (OE) and pharmacological inhibition of SIRT1 in the BLA, we found that the SIRT1/PGC-1 $\alpha$  signaling pathway positively regulated mitochondrial biogenesis and was positively correlated with the neural activity of BLA as well as anxiety-like behaviors in morphine withdrawal mice.

## MATERIALS AND METHODS

Detailed descriptions of all materials and methods can be found in the [supplementary materials](#). Experiments were performed at Xi'an Jiaotong University and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of Xi'an Jiaotong University. The experimental design included 4 experiments.

### Experiment 1: Spontaneous Withdrawal From Morphine-Induced Anxiety-Like Behaviors in Mice

A cohort of mice received repeated morphine administration (10 mg/kg, i.p.) or equal volume of saline once per day for 14 consecutive days. The mice were then subjected to a 2-week spontaneous withdrawal. The anxiety-like behaviors were

then examined with open-field test (OFT) and elevated plus maze (EPM). After the behavioral test, mice were perfused intracardially for immunofluorescent staining of cFos and electron microscopy. The rest of the mice were killed by decapitation for western blotting, real-time qPCR, and mitochondrial function detection.

To investigate whether anxious effects and changes in molecules were induced by chronic morphine administration or subsequent morphine withdrawal symptoms, we recorded and analyzed withdrawal symptoms at 5 hours, 24 hours, 48 hours, and 14 days after the last injection of morphine with another cohort of mice. The morphine administration procedure was the same as in the above.

### Experiment 2: Effects of Viral-Mediated Knockdown of SIRT1 in the BLA on Withdrawal-Associated Anxiety and Mitochondrial Biogenesis

To address whether the SIRT1/PGC-1 $\alpha$  pathway is essential for the morphine withdrawal-associated anxiety, we knocked down the SIRT1 expression by stereotaxically microinjecting adeno-associated virus (AAV)-human synapsin (hSyn)-shRNA SIRT1-enhanced green fluorescent protein (eGFP) or AAV-hSyn-negative control (NC)-eGFP into the BLA of mice. The mice then received morphine administration for 14 consecutive days, and anxiety-like behaviors were tested 14 days after the last morphine injection. We investigated the mitochondrial function and expression levels of the hSIRT1/PGC-1 $\alpha$  pathway by western blotting. Three weeks after virus injection, the location of virus-mediated expression of eGFP was confirmed with a different group of mice by fluorescence microscopy.

To investigate the dynamic temporal patterns of SIRT1 expression in the BLA, we also investigated the expression of the SIRT1 gene at 1, 5, 10, and 14 days after 2 weeks of morphine administration (10 mg/kg, i.p., once per day).

### Experiment 3: Viral-Mediated OE of SIRT1 Promotes PGC-1 $\alpha$ Signaling in the BLA and Withdrawal Related Anxiety-Like Behaviors

To explore whether increasing SIRT1 in BLA induces anxiety-like behaviors, we overexpressed SIRT1 by stereotaxically injecting AAV-hSyn-SIRT1-eGFP into BLA in mice. After virus injection, these mice were kept in the home cage for 28 days and did not undergo any drug administration. Then anxiety-like behaviors were tested. After behavioral testing, mice were killed and BLA was separated for western blot and mitochondrial function detection.

### Experiment 4: Pharmacological Inhibition of SIRT1 Ameliorated Withdrawal-Associated Anxiety-Like Behaviors in Mice

Given that SIRT1 exerted robust effects on anxiety-like behaviors associated with morphine withdrawal in mice, we next sought to investigate whether pharmacological inhibition of SIRT1 could ameliorate anxiety-like behaviors in mice and restore dysregulation of mitochondrial biogenesis in BLA neurons. The morphine administration and withdrawal procedure were the same as in experiment 1. Three days before behavioral assessments, EX527 (a selective SIRT1 antagonist) or 2% dimethyl sulfoxide (DMSO) was microinjected bilaterally into BLA in mice with morphine withdrawal (0.1  $\mu$ M/d for 3 consecutive

days). After behavioral testing, mice were perfused for electron microscopy or killed by decapitation for real-time qPCR and mitochondrial function detection.

## RESULTS

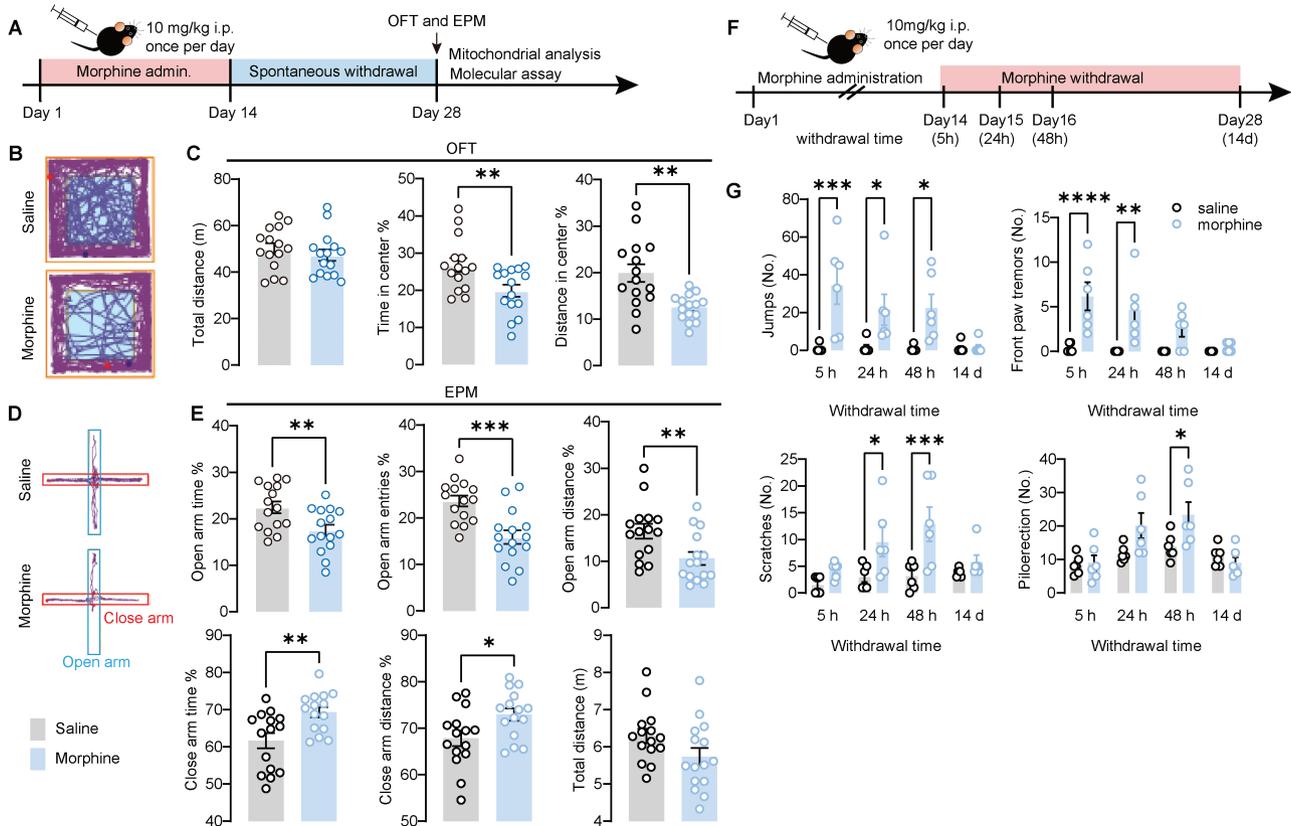
### Spontaneous Withdrawal From Morphine-Induced Anxiety-Like Behaviors in Mice

The timeline of the experiment is shown in Figure 1A. Mice received repeated morphine administration (10 mg/kg, i.p.) or saline once per day for 14 consecutive days ( $n=15$ /group). The mice were then subjected to a spontaneous withdrawal for 2 weeks. Anxiety-like behaviors were examined with OFT and EPM. The representative traces in OFT for the morphine and saline group are shown in Figure 1B. As shown in Figure 1C, we found that 2 weeks of spontaneous withdrawal did not affect the total distance ( $t_{(28)}=0.81$ ,  $P=.42$ ), while significantly decreasing the percentage of time spent in the center zone ( $t_{(28)}=2.82$ ,  $P<.01$ ) and the percentage of distance in the center zone ( $t_{(28)}=3.65$ ,  $P<.01$ ). The representative traces in EPM for the morphine and saline group are shown in Figure 1D. Morphine withdrawal mice showed significantly reduced open arm time percentage ( $t_{(28)}=2.81$ ,  $P<.01$ ), open arm entries percentage ( $t_{(28)}=4.10$ ,  $P<.001$ ), and open arm distance percentage ( $t_{(28)}=2.78$ ,  $P<.01$ ) compared with saline mice (Figure 1E). However, the closed arm time percentage ( $t_{(28)}=3.13$ ,  $P<.01$ ) and the closed arm distance percentage ( $t_{(28)}=2.43$ ,  $P<.05$ ) significantly increased (Figure 1E). There were no significant differences between the groups on the total distance in EPM (Figure 1E). These results indicate that 2 weeks of spontaneous withdrawal from morphine caused significant anxiety-like behaviors in mice.

Next, we evaluated morphine-induced somatic withdrawal symptoms in a new cohort of mice ( $n=6$ /group; Figure 1F). The number of jumps in the morphine group increased significantly at 5 hours ( $P<.001$ ), 24 hours ( $P<.05$ ), and 48 hours ( $P<.05$ ) following the last injection (Figure 1G). Furthermore, front paw tremors in the morphine group increased significantly at 5 hours ( $P<.0001$ ) and 24 hours ( $P<.01$ ) following the last injection (Figure 1G). The number of scratches in the morphine group increased significantly at 24 hours ( $P<.05$ ) and 48 hours ( $P<.001$ ) (Figure 1G). The piloerections in the morphine group increased significantly at 48 hours ( $P<.05$ ) following the last injection (Figure 1G). However, there was no significant difference between the groups on the number of jumps, front paw tremors, scratches, and piloerections 14 days after withdrawal.

### BLA Mitochondria Biogenesis Pathway Was Enhanced in Withdrawal-Associated Anxiety

To verify neuronal activation in BLA in morphine withdrawal mice, we examined the expression of the cFos protein in BLA by immunofluorescence ( $n=6$ /group). The timeline of the experiment is shown in Figure 2A. Compared with saline mice, we found massive expression of cFos in BLA in the morphine withdrawal mice ( $t_{(10)}=2.24$ ,  $P<.05$ ; Figure 2B–C). This result clearly indicated a significant activation of BLA cells. We used electron microscopy to analyze the mitochondrial ultrastructure (Figure 2D–E). Compared with saline mice, the total number of mitochondria ( $t_{(10)}=3.97$ ,  $P<.01$ ) was significantly increased in morphine withdrawal mice (Figure 2E). However, there were no differences in mitochondrial area between the groups (Figure 2E). Next, we classified mitochondria on the basis of pre- and postsynaptic, which revealed that the number of mitochondria



**Figure 1.** Spontaneous withdrawal from morphine-induced anxiety-like behaviors in mice. (A) Experimental procedures. (B) Representative traces show the movement of the mice in the open-field test (OFT). (C) The histogram illustrates the total distance, time in center percentage, and distance in center percentage in OFT. (D) Representative traces of the movement of the mice in the elevated plus-maze (EPM). (E) The histogram illustrates the open arm time percentage, open arm distance percentage, open arm distance percentage, close arm time percentage, close arm distance percentage, and total distance in EPM. Unpaired *t* test,  $n=15/\text{group}$ . (F) Experimental procedure of morphine-induced somatic withdrawal symptoms. (G) Histogram illustrates the numbers of jumps, front paw tremors, scratches, and piloerections.  $n=6/\text{group}$ . Repeated measure (RM) 2-way ANOVA. Data represent means  $\pm$  standard error of the means (SEM). \* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$ , compared with the saline-treated group.

in the postsynaptic increased selectively in morphine withdrawal mice ( $t_{(10)}=3.46$ ,  $P<.01$ ; Figure 2E).

As with mtDNA levels, we found that the transcription of *Cox1* ( $t_{(10)}=3.45$ ,  $P<.001$ ) and *Cyt b* ( $t_{(10)}=7.01$ ,  $P<.0001$ ; Figure 2F) increased significantly in mice with morphine withdrawal. Finally, to evaluate the functional changes of mitochondria, we also detect the production of production of ATP and the activity of the mitochondrial complex IV in the BLA with a multimode microplate detection system (Figure 2G). It is obvious that morphine induced an increase in ATP production ( $t_{(10)}=3.07$ ,  $P<.05$ ) and mitochondrial complex IV activity ( $t_{(10)}=2.91$ ,  $P<.05$ ). These data revealed that morphine withdrawal enhanced the process of mitochondrial biogenesis.

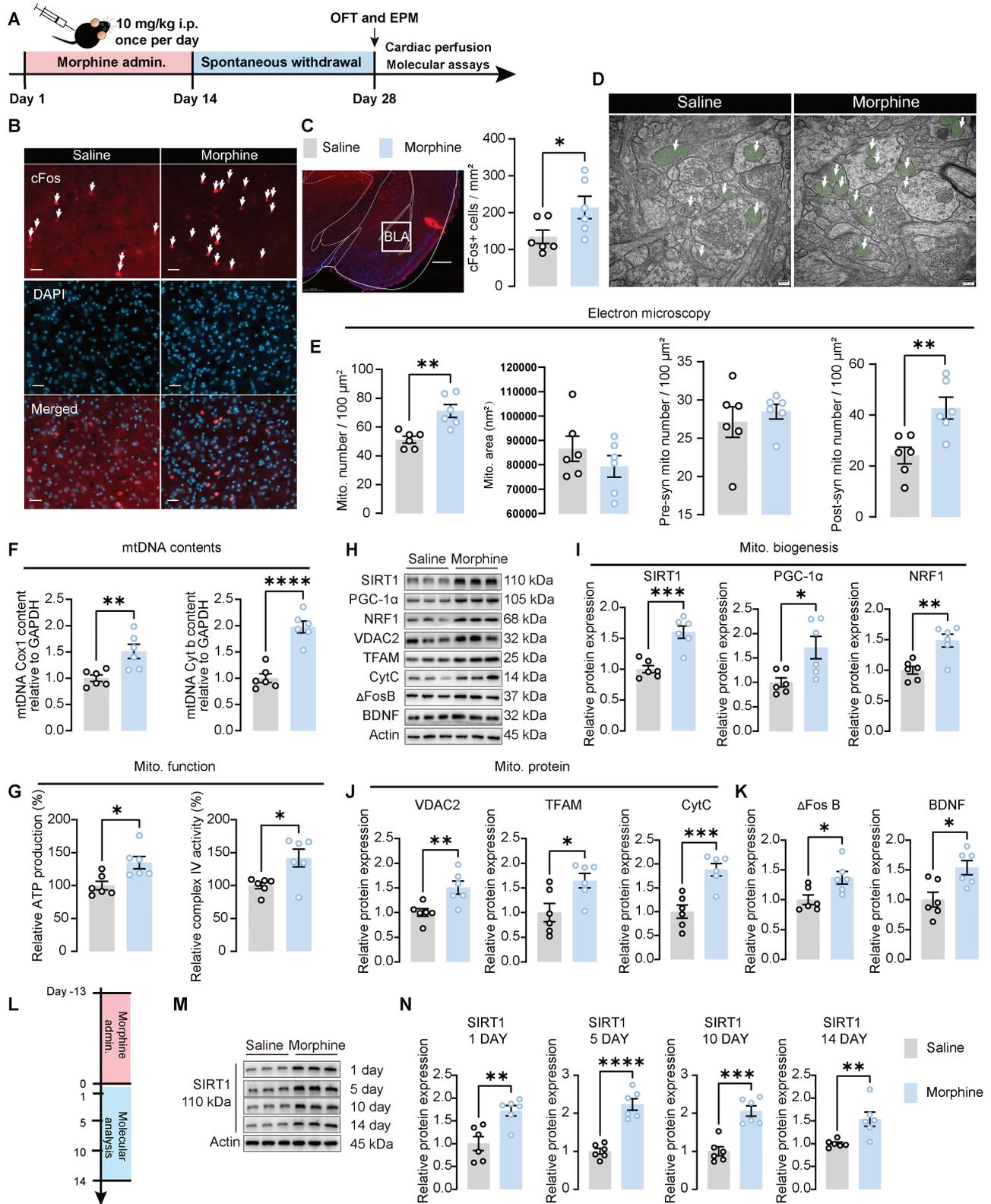
SIRT1 is a NAD<sup>+</sup>-dependent deacetylase, which is well established to induce mitochondrial biogenesis by its ability to regulate transcription involving PGC-1 $\alpha$  (Fanibunda et al., 2019). Representative immunoblot images were shown in Figure 2H ( $n=6/\text{group}$ ). We observed a significant upregulation of both SIRT1 ( $t_{(10)}=5.25$ ,  $P<.001$ ) and PGC-1 $\alpha$  ( $t_{(10)}=2.87$ ,  $P<.05$ ) in the BLA of mice with morphine withdrawal (Figure 2I;  $n=6/\text{group}$ ). As shown in Figure 2I–J, protein markers involved in mitochondrial biogenesis such as nuclear respiratory factor 1 (NRF1) ( $t_{(10)}=3.94$ ,  $P<.01$ ; Figure 2I), voltage dependent anion channel 2 (VDAC2) ( $t_{(10)}=3.30$ ,  $P<.01$ ; Figure 2J), transcription factor A (TFAM) ( $t_{(10)}=2.73$ ,  $P<.05$ ; Figure 2J), and cytochrome C (CytC) ( $t_{(10)}=4.66$ ,  $P<.001$ ; Figure 2J) also increased significantly. These results suggested that the SIRT1/PGC-1 $\alpha$  mitochondrial biogenesis pathway was activated in the BLA and may contribute to withdrawal-associated anxiety

in mice. Given that SIRT1 is critical for the expression of brain derived neurotrophic factor (BDNF) and is regulated by transcription factor  $\Delta$ FosB (Ferguson et al., 2013), we examined the protein expression of  $\Delta$  FosB and BDNF. The results demonstrated that both  $\Delta$  FosB ( $t_{(10)}=2.81$ ,  $P<.05$ ) and BDNF ( $t_{(10)}=3.15$ ,  $P<.05$ ) were increased in the morphine group (Figure 2K).

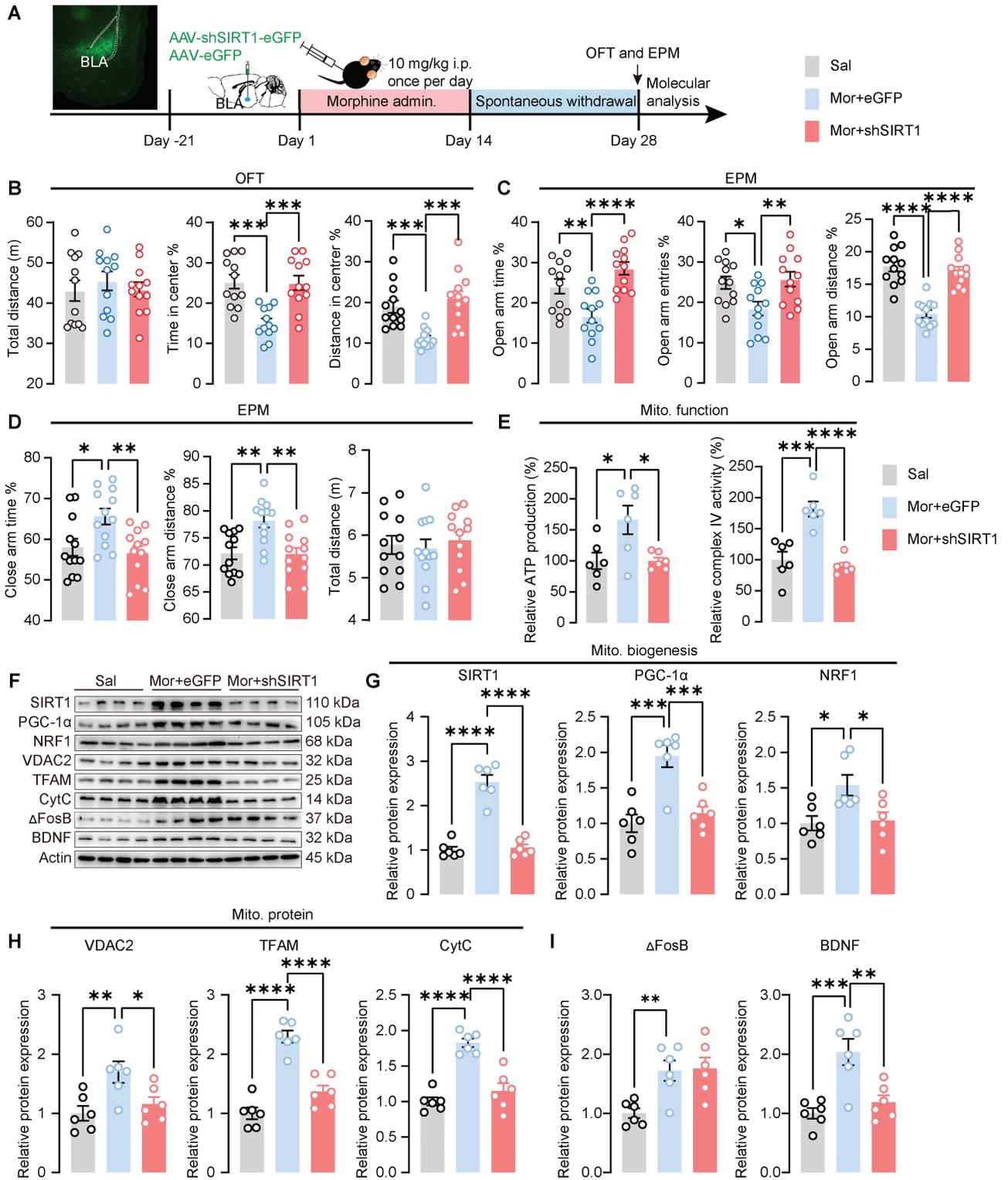
To test whether chronic morphine administration and spontaneous withdrawal induce dynamic expression of SIRT1 in the BLA, western blot was conducted to investigate the expression level of SIRT1 (Figure 2L). Representative immunoblot images are shown in Figure 2M ( $n=6/\text{group}$ ). The results show that SIRT1 expression was elevated on 1 day ( $t_{(10)}=3.79$ ,  $P<.01$ ), 5 days ( $t_{(10)}=7.39$ ,  $P<.0001$ ), 10 days ( $t_{(10)}=5.88$ ,  $P<.001$ ), and 14 days ( $t_{(10)}=3.39$ ,  $P<.01$ ) after morphine administration (Figure 2N), indicating that 2 weeks of morphine induced a long-lasting upregulation of SIRT1 expression in the BLA for at least 2 weeks.

### SIRT1 Is Necessary for Withdrawal-Associated Anxiety and Mitochondrial Biogenesis in BLA

To address whether the SIRT1/PGC-1 $\alpha$  pathway is essential for morphine withdrawal-associated anxiety, we knocked down the expression of SIRT1 by stereotactically microinjecting AAV-hSyn-shSIRT1-eGFP or AAV-hSyn-NC-eGFP into the BLA of mice. A different group of mice was used to confirm the location of virus-mediated eGFP expression. Three weeks after virus injection, fluorescence microscopy revealed that virus-mediated expression of eGFP was largely restricted in the BLA and along



**Figure 2.** Mitochondria biogenesis pathway was enhanced in basolateral amygdala (BLA) in morphine withdrawal mice. (A) Experimental procedures. (B) cFos immunostaining in BLA. Scale bar = 20  $\mu$ m. The arrows point to the cFos-positive cells. (C) Left: representative images showing the location of BLA. Scale bar = 500  $\mu$ m. Right: quantification of cFos+ cells in BLA. n = 6/group. (D) Representative images of mitochondria (shaded) in BLA neurons. Scale bar = 200 nm. The arrows point to the mitochondria. (E) Histogram illustrates the total number of mitochondrial numbers, mitochondrial area, presynaptic mitochondrial number, and postsynaptic mitochondrial number. n = 6/group. (F) Relative mitochondrial DNA (mtDNA) Cox1 and Cyt b content in BLA. n = 6/group. (G) The histogram illustrates the levels of ATP production and mitochondrial complex IV activity. (H) Representative immunoblots. (I-K) Histogram illustrates the protein expression of sirtuins 1 (SIRT1), PPAR-Gamma Coactivator 1-Alpha (PGC-1 $\alpha$ ), nuclear respiratory factor 1 (NRF1), voltage dependent anion channel 2 (VDAC2), transcription factor A, mitochondrial (TFAM), cytochrome C (CytC), delta FOSB ( $\Delta$ FosB), brain derived neurotrophic factor (BDNF). n = 6/group. (L) Experimental procedures. (M) Representative immunoblots. (N) Histogram illustrates the protein expression of SIRT1 on 1, 5, 10, and 14 days after the last injection of morphine. Unpaired t test. Data represent means  $\pm$  SEM. \* $P$  < .05, \*\* $P$  < .01, \*\*\* $P$  < .001, \*\*\*\* $P$  < .0001, compared with the saline group.



**Figure 3.** SIRT1 is necessary for withdrawal-associated anxiety and mitochondrial biogenesis in BLA. (A) Experimental procedures. The coronal brain slice showing the expression of eGFP after virus injection in BLA. (B) Histogram illustrates the total distance, time in center percentage, and distance in center percentage in OFT.  $n=12$ /group. (C) The histogram illustrates the open arm time percentage, open arm entries percentage, and open arm distance percentage in EPM. (D) Histogram illustrates the closed arm time percentage, the closed arm distance percentage, and the total distance in EPM.  $n=12$ /group. (E) Histogram illustrates the levels of ATP production and mitochondrial complex IV activity.  $n=6$ /group. (F) Representative immunoblots. (G–I) Histogram illustrates the protein expression of SIRT1, PGC-1 $\alpha$ , NRF1, VDAC2, TFAM, CytC,  $\Delta$ FosB, and BDNF.  $n=6$ /group. Ordinary 1-way ANOVA. Data represent means  $\pm$  SEM. \* $P<.05$ , \*\* $P<.01$ , \*\*\* $P<.001$ , \*\*\*\* $P<.0001$ , compared between the labeled groups.

the needle tract of the injected area (Figure 3A). We found that knockdown of SIRT1 in BLA had no effect on locomotor activity ( $F_{(2,33)}=0.34, P=.72$ ) in morphine withdrawal mice (Figure 3B;  $n=12/\text{group}$ ). However, knockdown of SIRT1 effectively prevented anxiety-like behaviors in withdrawal mice by reversing the decreased time in center percentage ( $P<.001$ ;  $F_{(2,33)}=13.55, P<.0001$ ) and distance in center zone percentage ( $P<.001$ ;  $F_{(2,33)}=12.14, P<.001$ ) in OFT (Figure 3B;  $n=12/\text{group}$ ). For EPM, knockdown of SIRT1 in BLA also reversed open arm time percentage ( $P<.0001$ ;  $F_{(2,33)}=12.28, P<.001$ ), open arm entries percentage ( $P<.01$ ;  $F_{(2,33)}=5.49, P<.01$ ), and open arm distance percentage ( $P<.0001$ ;  $F_{(2,33)}=32.40, P<.0001$ ) in morphine withdrawal mice (Figure 3C;  $n=12/\text{group}$ ). On the contrary, the closed arm time percentage ( $P<.01$ ;  $F_{(2,33)}=6.20, P<.01$ ) and closed arm distance percentage ( $P<.01$ ;  $F_{(2,33)}=9.03, P<.001$ ) were significantly decreased by suppression of SIRT1 in morphine withdrawal mice (Figure 3D). The total distance traveled in EPM did not differ among all 3 groups (Figure 3D). However, knockdown of SIRT1 in BLA did not affect anxiety-like behaviors in saline mice (supplementary Figure 1A–D). These results revealed that knockdown of SIRT1 in BLA neurons could prevent anxiety-like behaviors in morphine withdrawal mice.

Next, we investigated mitochondrial function and found that SIRT1 suppression decreased ATP production ( $P<.05$ ;  $F_{(2,15)}=5.80, P<.05$ ) and mitochondrial complex IV activity ( $P<.0001$ ;  $F_{(2,15)}=21.78, P<.0001$ ) in the BLA of morphine (Figure 3E;  $n=6/\text{group}$ ). The expression levels of the SIRT1/PGC-1 $\alpha$  pathway were also detected by western blot. Representative images of immunoblots are shown in Figure 3F ( $n=6/\text{group}$ ). A 1-way ANOVA showed that SIRT1 expression in the Mor+shSIRT1 group was decreased compared with the Mor+eGFP group ( $P<.0001$ ;  $F_{(2,15)}=59.31, P<.0001$ ). Compared with the Mor+eGFP group, the expression level of mitochondrial biogenesis-related proteins, such as PGC-1 $\alpha$  ( $P<.001$ ;  $F_{(2,15)}=16.01, P<.001$ ), NRF1 ( $P<.05$ ;  $F_{(2,15)}=5.77, P<.05$ ), VDAC2 ( $P<.05$ ;  $F_{(2,15)}=6.32, P<.05$ ), TFAM ( $P<.0001$ ;  $F_{(2,15)}=42.79, P<.0001$ ), and CytC ( $P<.0001$ ;  $F_{(2,15)}=30.81, P<.0001$ ), were also decreased in the Mor+shSIRT1 group (Figure 3G–H). These results indicated that the SIRT1/PGC-1 $\alpha$  signaling pathway is critical for anxiety-like behaviors induced by morphine withdrawal. Knockdown of SIRT1 did not affect the expression of  $\Delta\text{FosB}$  ( $P=.98$ ;  $F_{(2,15)}=7.71, P<.01$ ) in the Mor+shSIRT1 group but reversed the expression of BDNF ( $P<.01$ ;  $F_{(2,15)}=12.85, P<.001$ ) in the Mor+shSIRT1 group (Figure 3I). However, knockdown of SIRT1 did not affect the mtDNA transcription and mitochondrial function (supplementary Figure 1E–G).

### OE of SIRT1 in BLA Induced Anxiety-Like Behaviors Through PGC-1 $\alpha$ Signaling Pathway

To explore whether increasing SIRT1 in BLA is sufficient to induce anxiety-like behaviors, we overexpressed SIRT1 by stereotaxically injecting AAV-hSyn-SIRT1-eGFP or AAV-hSyn-NC-eGFP into the BLA in mice. After the virus injection, these mice were kept in the home cage for 28 days and did not undergo any drug administration (Figure 4A;  $n=12/\text{group}$ ). Compared with GFP controls, SIRT1 OE in BLA had no significant effect on locomotor activity in mice ( $t_{(22)}=0.21, P=.83$ ; Figure 4B). However, SIRT1 OE significantly decreased the time in center zone percentage ( $t_{(22)}=2.96, P<.01$ ) and distance in center zone percentage ( $t_{(22)}=5.27, P<.0001$ ) in the OFT (Figure 4B). Regarding the EPM test, the open arm time percentage ( $t_{(22)}=4.88, P<.0001$ ), open arm entries percentage ( $t_{(22)}=2.57, P<.05$ ), and open arm distance percentage ( $t_{(22)}=2.99, P<.01$ ) were significantly decreased in SIRT1 OE mice (Figure 4C). On the contrary, the closed arm time

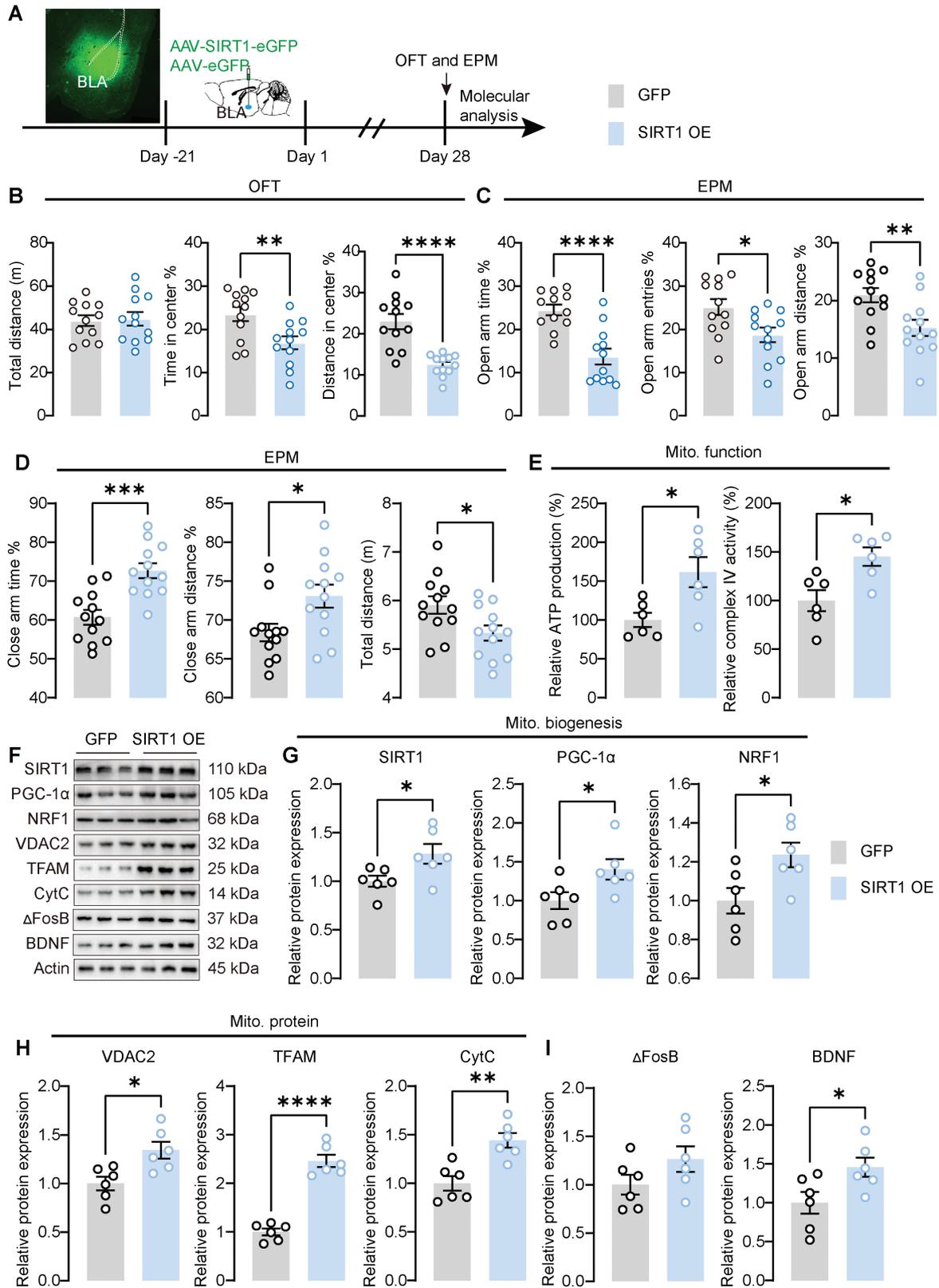
percentage ( $t_{(22)}=4.40, P<.001$ ) and the closed arm distance percentage ( $t_{(22)}=2.53, P<.05$ ) were significantly increased in SIRT1 OE mice (Figure 4D). Interestingly, the total distance in the EPM ( $t_{(22)}=2.43, P<.05$ ) was significantly decreased in SIRT1 OE mice (Figure 4D).

SIRT1 OE significantly increased ATP production ( $t_{(10)}=2.87, P<.05$ ) and mitochondrial complex IV activity ( $t_{(10)}=3.13, P<.05$ ) in the BLA (Figure 4E;  $n=6/\text{group}$ ). The expression of SIRT1, PGC-1 $\alpha$ , TFAM, and NRF1 was also examined (Figure 4F;  $n=6/\text{group}$ ). As expected, the expression level of SIRT1 in BLA was significantly increased by injection of AAV-hSyn-SIRT1-eGFP ( $t_{(10)}=2.42, P<.05$ ; Figure 4G). Furthermore, protein levels of PGC-1 $\alpha$  ( $t_{(10)}=2.38, P<.05$ ; Figure 4G), NRF1 ( $t_{(10)}=2.57, P<.05$ ; Figure 4G), VDAC2 ( $t_{(10)}=3.07, P<.05$ ; Figure 4H), TFAM ( $t_{(10)}=10.06, P<.0001$ ; Figure 4H), and CytC ( $t_{(10)}=4.18, P<.01$ ; Figure 4H) were significantly elevated in the SIRT1 OE group. These results indicated that OE of SIRT1 in BLA facilitates anxiety-like behaviors in mice through the SIRT1/PGC-1 $\alpha$  pathway. The expression of  $\Delta\text{FosB}$  did not change between the groups (Figure 4I). The expression of BDNF ( $t_{(10)}=2.45, P<.05$ ; Figure 4I) also increased in SIRT1 OE mice.

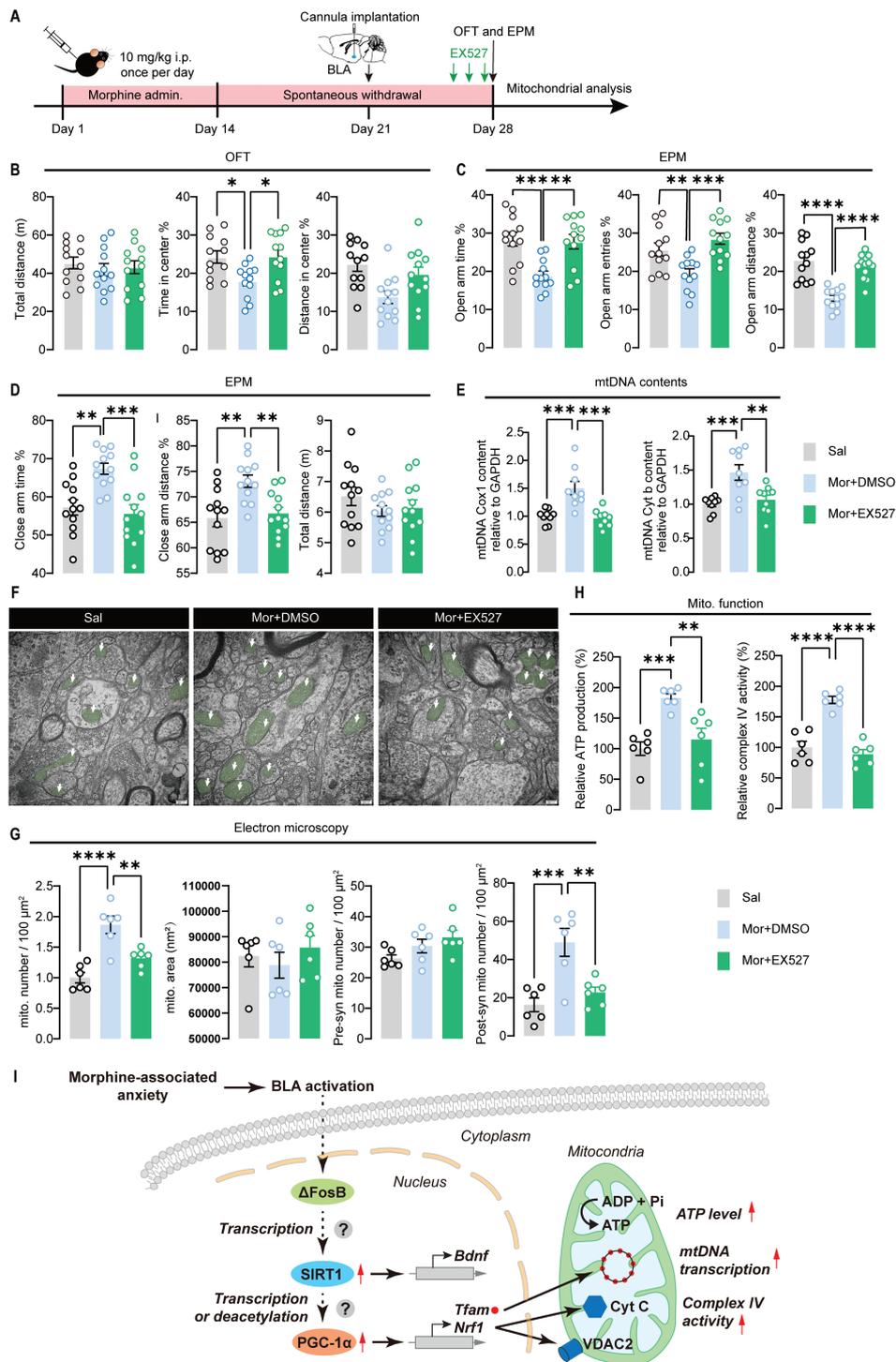
### Pharmacological Inhibition of SIRT1 Ameliorated Withdrawal-Associated Anxiety-Like Behaviors in Mice

Given that SIRT1 exerted robust effects on anxiety-like behaviors in morphine withdrawal mice, we next sought to investigate whether pharmacological inhibition of SIRT1 could ameliorate anxiety-like behaviors in mice and restore dysregulation of mitochondrial biogenesis in BLA neurons. We implanted mice with bilateral cannulas aimed at the BLA and established a morphine withdrawal-induced anxiety model following the experiment procedure shown in Figure 5A. Three days before behavioral assessments, EX527 (a selective SIRT1 antagonist) or 2% DMSO was microinjected bilaterally into BLA in mice (0.1  $\mu\text{M}/\text{d}$  for 3 consecutive days) ( $n=12/\text{group}$ ). For OFT, 1-way ANOVA demonstrated that EX527 had no significant effect on locomotor activity ( $F_{(2,33)}=0.32, P=.73$ ; Figure 5B) in morphine withdrawal mice. However, compared with DMSO-treated mice, EX527 completely reversed the decreased time in center percentage ( $P<.05$ ;  $F_{(2,33)}=5.60, P<.01$ ) and distance in center percentage ( $P<.05$ ;  $F_{(2,33)}=5.98, P<.01$ ) in OFT (Figure 5B), as well as the reduced open arm time percentage ( $P<.01$ ;  $F_{(2,33)}=10.32, P<.001$ ), open arm entries percentage ( $P<.001$ ;  $F_{(2,33)}=10.05, P<.001$ ), and open arm distance percentage ( $P<.0001$ ;  $F_{(2,33)}=23.44, P<.0001$ ) in EPM (Figure 5C). Furthermore, the closed arm time ( $P<.001$ ;  $F_{(2,33)}=10.39, P<.001$ ) and closed arm distance ( $P<.01$ ;  $F_{(2,33)}=8.03, P<.01$ ) were also reversed by administering EX527 to mice with morphine withdrawal (Figure 5D). However, there was no total distance difference between these 3 groups (Figure 5D). Inhibition of SIRT1 with EX527 in BLA did not affect anxiety-like behaviors in saline mice (supplementary Figure 2A–D).

As shown in Figure 5E, we qualified the level of mtDNA after the behavior test and found that the relative content of mtDNA Cox1 ( $P<.001$ ;  $F_{(2,24)}=14.55, P<.0001$ ) and Cyt b ( $P<.01$ ;  $F_{(2,24)}=10.04, P<.001$ ) was significantly decreased by EX527 compared with the DMSO group ( $n=9/\text{group}$ ). The result of the electron microscopy showed that the number of mitochondria was also reversed by EX527 treatment in morphine withdrawal mice ( $P<.01$ ;  $F_{(2,15)}=18.00, P<.001$ ;  $n=6/\text{group}$ ; Figure 5F–G). Morphine withdrawal and EX527 treatment did not alter mitochondrial area (Figure 5G) and presynaptic mitochondrial number (Figure 5G). Consistently, EX527 also reversed postsynaptic mitochondrial number in



**Figure 4.** Overexpression of SIRT1 in BLA-induced anxiety-like behaviors through PGC-1 $\alpha$  pathway. (A) Experimental procedures. The coronal brain slice showing the expression of eGFP after virus injection in BLA. (B) Histogram illustrates the total distance, time in center percentage, and distance in center percentage in OFT. n=12/group. (C) The histogram illustrates the open arm time percentage, open arm entries percentage, and open arm distance percentage in EPM. (D) Histogram illustrate closed arm time percentage, closed arm distance percentage, and total distance in EPM. n=12/group. (E) Histogram illustrates the levels of ATP production and mitochondrial complex IV activity. n=6/group. (F) Representative immunoblots. (G-I) Histogram illustrates the protein expression of SIRT1, PGC-1 $\alpha$ , NRF1, VDAC2, TFAM, CytC,  $\Delta$ FosB, and BDNF. n=6/group. Unpaired t test. Data represent means  $\pm$  SEM. \* $P$ <.05, \*\* $P$ <.01, \*\*\* $P$ <.001, \*\*\*\* $P$ <.0001, compared with the GFP control group.



**Figure 5.** Pharmacological inhibition of SIRT1 ameliorated withdrawal-associated anxiety-like behaviors in mice. (A) Experimental procedures. (B) The histogram illustrates the total distance, time in center percentage, and distance in center percentage in OFT.  $n=12/\text{group}$ . (C) The histogram illustrates the open arm time percentage, open arm entries percentage, and open arm distance percentage in EPM. (D) Histogram illustrates closed arm time percentage, closed arm distance percentage, and total distance in EPM.  $n=12/\text{group}$ . (E) Relative mtDNA Cox1 and Cyt b content in BLA cells.  $n=9/\text{group}$ . (F) Representative images of mitochondria (shaded) in BLA neurons. Scale bar = 200 nm. Arrows point to the mitochondria. (G) Histogram illustrates the total number of mitochondrial numbers, mitochondrial area, presynaptic mitochondrial number, and postsynaptic mitochondrial number.  $n=6/\text{group}$ . (H) The histogram illustrates the levels of ATP production and mitochondrial complex IV activity.  $n=6/\text{group}$ . Ordinary 1-way ANOVA. Data represent means  $\pm$  SEM. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ , \*\*\*\* $P < .0001$ , comparison between labeled groups. (I) Diagram illustrating SIRT1/PGC-1 $\alpha$  pathway in anxiety-like behaviors associated with morphine withdrawal. Chronic administration of morphine and spontaneous withdrawal contributed to the activation of BLA neurons and induced anxiety-like behaviors in mice. Increased  $\Delta\text{FosB}$  promoted the expression of SIRT1. SIRT1 enhances PGC-1 $\alpha$  activity and, together, promotes the transcription of BDNF, TFAM, and NRF1. NRF1 promoted the expression of outer membrane proteins VDAC2, inner membrane proteins CytC, and matrix proteins TFAM. Moreover, the increased TFAM protein promotes the transcription and replication of mtDNA, including Cox1 and Cyt b. These adaptations ultimately cause an increase in mitochondrial complex IV activity and an increase in the level of ATP.

mice with withdrawal from morphine ( $P < .01$ ;  $F_{(2, 15)} = 12.12$ ,  $P < .001$ ; **Figure 5G**). Furthermore, EX527 decreased ATP production ( $P < .01$ ;  $F_{(2, 15)} = 11.65$ ,  $P < .001$ ) and mitochondrial complex IV activity ( $P < .0001$ ;  $F_{(2, 15)} = 37.11$ ,  $P < .0001$ ) in the BLA of morphine withdrawal mice (**Figure 5H**;  $n = 6$ /group). However, inhibition of SIRT1 with EX527 did not affect the transcription of mtDNA and mitochondrial function (**supplementary Figure 2E–F**). These results suggested that pharmacological inhibition of SIRT1 could ameliorate withdrawal-associated anxiety-like behaviors in mice by inhibiting mitochondrial biogenesis in BLA neurons.

## Discussion

The present study provides several important insights into the mechanism underlying morphine-associated anxiety. First, we confirmed that long-term spontaneous withdrawal from morphine induces anxiety-like behaviors in mice, during which physical withdrawal symptoms were no longer detectable. Second, neuronal activity and the total number of mitochondria increased significantly in BLA in mice with anxiety-like behaviors. Furthermore, the SIRT1-mediated mitochondrial biogenesis pathway was activated in BLA neurons. We also found that downregulation of SIRT1 in BLA prevented anxiety-like behaviors in morphine withdrawal mice, whereas OE of SIRT1 in BLA facilitated anxiety-like behaviors in untreated mice via the SIRT1/PGC-1 $\alpha$  pathway. Intra-BLA infusion with the selective SIRT1 antagonist EX527 ameliorated anxiety-like behaviors and mitochondrial dysfunction in morphine-withdrawal mice. These results suggest that SIRT1 in BLA regulates morphine withdrawal-associated anxiety in mice through the mitochondrial biogenesis pathway.

It is well established that the amygdala generates anxiety-related behavioral outputs by integrating information from cortical and thalamic sensory inputs (**Babaev et al., 2018**). Our cFos immunostaining result suggests that BLA neurons were activated after 2 weeks of withdrawal from morphine. Substantial observations indicate alterations in mitochondria and metabolism in highly anxious individuals and, conversely, anxiety symptoms in humans suffering from mitochondrial disorders (**Filiou and Sandi, 2019**). Carmen Sandi's group demonstrated that mitochondrial function in the NAc is a critical mediating factor in the subordinate status displayed by highly anxious rats (**Hollis et al., 2015**). They also showed alterations in mitochondria (i.e., respiration, and volume) and medium spiny neurons (i.e., dendritic complexity and spine density) in NAc in highly anxious animals. Viral overexpression of MFN2 in NAc reversed all these behavioral, mitochondrial, and neuronal phenotypes (**Gebara et al., 2021**). Interestingly, our electron microscope results revealed that both mitochondria number and mtDNA content increased significantly in the BLA of morphine-related anxious mice. Drug-evoked synaptic plasticity would require large amounts of energy, hence increasing the cellular energetic demand for ATP. It is interesting to speculate that this heightened energetic demand following morphine dependence may be causal to an increase in mitochondrial biogenesis and function in the BLA.

Consistent with the electron microscope results, we found increased expression levels of mitochondrial biogenesis markers, such as PGC-1 $\alpha$ , TFAM, and NRF1, in the BLA of morphine withdrawal mice. PGC-1 $\alpha$  is a key regulator of mitochondrial biogenesis. TFAM and NRF1 are the downstream targets of PGC-1 $\alpha$ , which co-regulate mitochondrial function with PGC-1 $\alpha$  (**Tian et al., 2019**). Once activated by phosphorylation or deacetylation, PGC-1 $\alpha$  can enhance the expression of TFAM and NRF1, which generates new mitochondria by promoting mitochondrial DNA

and proteins synthesis (**Li et al., 2017**). Nevertheless, we found increased mitochondrial function (such as increased ATP production and ETC complex IV activity) in the BLA of morphine-related anxious mice. Given the prominent role of mitochondrial function in mood, it is reasonable to speculate that withdrawal from morphine drives an increase in mitochondrial biogenesis in the BLA. Excess mitochondrial biogenesis beyond what is physiologically necessary may lead to an increase in reactive oxygen species levels or alter mitostasis that may influence anxiety-like behavior.

SIRT1 is widely expressed in the brain. Our results suggest that SIRT1 expression in BLA was increased after 2 weeks of withdrawal from chronic morphine. We have recently studied the role of SIRT1 in BLA in depression-like behaviors in mice, using the chronic unpredictable mild stress model. Knockdown of SIRT1 in BLA glutamatergic neurons reversed depression-like behaviors in chronic unpredictable mild stress mice, whereas overexpression of SIRT1 in BLA glutamatergic neurons induced depression-like behaviors in nonstressed control mice (**Guo et al., 2021**). Consistent with our results, a previous study suggested that SIRT1 overexpression in NAc increased depressive- and anxiety-like behaviors in mice (**Kim et al., 2016**). On the contrary, brain-specific SIRT1 knockout achieved an anxiolytic effect in mice (**Libert et al., 2011**). Interestingly, we also found that  $\Delta$ FosB and BDNF were increased in BLA in anxious mice.  $\Delta$ FosB has been reported in NAc to in part mediate an increase in SIRT1 levels following chronic morphine treatment (**Ferguson et al., 2013**). Therefore, it is possible that morphine-induced increase in  $\Delta$ FosB drives an increase in SIRT1 through the transcriptional mechanism. BDNF is well known for its role in neuronal development and synaptic function (**An et al., 2020**). In agreement with previous studies, we found that knockdown or pharmacological inhibition of SIRT1 reversed BDNF expression in morphine withdrawal mice. These results implied that BDNF could be a downstream target of SIRT1.

SIRT1 deacetylates a variety of substrates, including PGC-1 $\alpha$  (**Li et al., 2011**). It should be mentioned that acetylation is one of the most important regulatory mechanisms for the activity of PGC-1 $\alpha$ . Decreased acetylation promotes the activation of PGC-1 $\alpha$ . The virus-mediated overexpression of SIRT1 in NAc has been reported to enhance the rewarding effect of morphine in mice, whereas local suppression of SIRT1 from NAc decreased the rewarding effect of morphine (**Ferguson et al., 2013**). We hypothesized that the observed increase in SIRT1 upregulates the expression of TFAM and NRF1, possibly by promoting the deacetylation of PGC-1 $\alpha$ , in the BLA of morphine withdrawal mice. To verify this hypothesis, we microinjected viruses into bilateral BLA to overexpress or knockdown SIRT1. We found that knockdown of SIRT1 in BLA prevented anxiety-like behaviors in morphine withdrawal mice. Moreover, knockdown of SIRT1 also inhibited the expression of TFAM and NRF1. Interestingly, knockdown of SIRT1 decreased the expression level of PGC-1 $\alpha$ . It is possible that SIRT1 also promotes PGC-1 $\alpha$  transcription (**Yuan et al., 2016**). In contrast, SIRT1 overexpression in BLA not only induced obvious anxiety-like behaviors in untreated mice but also increased the expression of PGC-1 $\alpha$ , TFAM, and NRF1 in BLA neurons. These results suggest that long-term withdrawal from morphine activates the SIRT1/PGC-1 $\alpha$  pathway in BLA neurons, which enhances the transcription of NRF1 and TFAM that drives mitochondrial biogenesis.

Since we demonstrated the pivotal role of SIRT1 in mitochondrial biogenesis and morphine-associated anxiety, we proposed that the SIRT1 antagonist, such as EX527, could rescue anxiety-like behaviors and mitochondrial dysfunction from

withdrawal of morphine. We showed that microinjection of EX527 into BLA effectively ameliorated withdrawal-associated anxiety-like behaviors in mice. This finding was supported by another preclinical study that microinjection of EX527 into NAC decreased anxiety-like behaviors induced by chronic social defeat in mice (Kim et al., 2016). Another important key result of our study is that the excess mitochondrial biogenesis in BLA neurons in anxious mice was abolished by EX527 administration. However, it should be noted that the experimental design of SIRT1 knockdown (using shRNA) and SIRT1 inhibition (using EX527) differed in the timing of SIRT1 inhibition and therefore addressed slightly different questions. For the knockdown experiment, SIRT1-shRNA was expressed before starting morphine treatment in the addiction phase as well as during the withdrawal phase. We do not know if SIRT1 is essential to morphine addiction during chronic morphine treatment and if this may affect withdrawal-phase anxiety. For the pharmacological inhibition experiment, EX527 was administered only during the last 3 days of withdrawal. This result demonstrated that after spontaneous withdrawal with SIRT1 on board, a short-duration (3-day) acute inhibition of SIRT1 at the end is sufficient to reverse withdrawal-associated anxiety.

The present study has several limitations. Despite the evidence suggesting that SIRT1 can activate PGC-1 $\alpha$  through deacetylation or transcriptionally increase the expression of PGC-1 $\alpha$  (Li et al., 2011; Yuan et al., 2016), our findings did not directly prove these mechanisms in the mouse model of withdrawal-associated anxiety. Furthermore, we only studied the role of the SIRT1/PGC-1 $\alpha$  signaling pathway, and it is not the sole mechanism underlying anxiety-like behaviors in morphine withdrawal mice, so many more studies are needed.

## CONCLUSION

Our current study demonstrated for the first time, to our knowledge, that the SIRT1/PGC-1 $\alpha$  pathway is activated in BLA in morphine withdrawal-associated anxious mice (Figure 5I). We speculate that withdrawal from morphine caused abnormal activation of BLA neurons and contributes to anxiety-like behaviors. The increased firing rate of BLA neurons requires higher amounts of energy, which can lead to neurons upregulating mitochondrial biogenesis through the SIRT1/PGC-1 $\alpha$  pathway. The specific antagonist EX527 ameliorates withdrawal-associated anxiety-like behaviors in mice and restores dysregulation of mitochondrial biogenesis in BLA neurons. Thus, pharmacological inhibition of SIRT1 may have therapeutic potential for the treatment of anxiety associated with opioid withdrawal.

## Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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## Author Contributions

F.Y. and Y.W. designed the study; J.Z., Y.L., and Y.Z. carried out behavioral experiments; F.Y., J.Z., and Y.L. analyzed behavior

experimental results; D.L., X.Y., and Y.F. carried out and analyzed molecular experiments; J.L. and H.Z. carried out and analyzed electron microscopy results; F.Y. and Y.W. wrote the manuscript; and Y.W. and S.W. approved the final version to be published.

## Interest Statement

The authors have no conflicts of interest to declare.

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