Akt and its phosphorylation in nucleus accumbens mediate heroin-seeking behavior induced by cues in rats

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Abstract
Akt is initially identified as one of the downstream targets of phosphatidylinositol-3 kinase (PI3K) and is involved in morphine reward and tolerance. However, whether phosphorylation of Akt (p-Akt) mediates heroin relapse remains unclear. Here, we aimed to explore the role of p-Akt in the nucleus accumbens (NAc) in cue-induced heroin-seeking behaviors after withdrawal. First, rats were trained to self-administer heroin for 14 days, after which we assessed heroin-seeking behaviors induced by a context cue (CC) or by discrete conditioned cues (CS) after 1 day or 14 days of withdrawal. We found that the active responses induced by CC or CS after 14 days of withdrawal were higher than those after 1 day of withdrawal. Meanwhile, the expression of p-Akt in the NAc was also greatest when rats were exposed to the CS after 14 days of withdrawal. Additionally, a microinjection of LY294002, an inhibitor of PI3K, into the NAc inhibited the CS-induced heroin-seeking behaviors after 14 days of withdrawal. Finally, Akt1 or β-arrestin 2 was downregulated via a lentiviral injection to assess the effect on heroin seeking after 14 days of withdrawal. CS-induced heroin-seeking behavior was inhibited by downregulation of Akt1, but not β-arrestin 2, in the NAc. These data demonstrate that Akt phosphorylation in the NAc may play an important role in the incubation of heroin-seeking behavior, suggesting that the PI3K/Akt pathways may be involved in the process of heroin relapse and addiction.

KEYWORDS
drug seeking, heroin, incubation, nucleus accumbens, phosphatidylinositol-3 kinase

INTRODUCTION
Relapse into heroin use after prolonged abstinence is usually induced by exposure to drug-related cues that are associated with previous heroin reward. In rodent models, heroin-seeking behavior persists for over 2 months³ and progressively increases during the first 2 weeks after withdrawal,² also known as the heroin incubation of craving. This incubation of heroin-seeking behavior is critically associated with neuroadaptations in the mesocorticolimbic dopamine system after chronic heroin use and subsequent withdrawal.⁴ However, the molecular mechanisms underlying the incubation of heroin seeking remain largely unknown.
The serine/threonine protein kinase Akt, also termed protein kinase B, is an intracellular signal molecule initially identified as one of the downstream targets of phosphatidylinositol-3 kinase (PI3K). Phosphorylation of Akt (p-Akt) by PI3K can subsequently interact with downstream effectors and modulate various cellular processes, including growth, survival, differentiation, and glucose metabolism. There are two phosphorylation sites of Akt: serine 473 residue is activated in a PI3K-dependent pattern, while threonine 308 residue can also be activated by other signaling pathways, such as cAMP/PKA way. Akt has also been found to be regulated by addictive drugs within the mesocorticolic dopaminergic system that consists of cell bodies in the ventral tegmental area (VTA) which project to many forebrain areas, including the medial prefrontal cortex (mPFC), nucleus accumbens (NAC), and amygdala. The p-Akt-473 levels in the NAC of rats increased by acute morphine administration but significantly decreased after chronic morphine administration. It has been found that the decreased size of VTA dopamine neurons and diminished morphine reward after chronic morphine administration is mediated by the downregulation of brain-derived neurotrophic factor (BDNF)/the insulin receptor substrate 2 (IRS2)/p-Akt signaling pathway in the VTA. Recent evidence has revealed that the Akt pathway is involved in synaptic and structural neuroadaptations induced by addictive drugs. For example, cocaine self-administration increased the tropomyosin-related kinase B (TrkB)-dependent p-Akt protein levels in the NAC. Furthermore, the Akt/mammalian target of rapamycin (mTOR) signaling pathway in the NAC is critically involved in the bidirectional synaptic plasticity after acute or chronic cocaine administration. Methamphetamine-induced behavioral sensitization required increased levels of the NMDA Receptor 2B (GluN2B)-PP2A-Akt cascade in the dorsal striatum. Nicotine exposure during adolescence induces the profound upregulation of the Akt- glycogen synthase kinase 3(GSK-3) signaling pathways directly within the NAC. Furthermore, the p-Akt level in the NAC is reduced when rats are re-exposed to the cues associated cocaine memories in conditioned place preference model. However, the mechanisms underlying the regulation of p-Akt in the incubation of heroin seeking after withdrawal or cues-induced heroin-seeking behavior remains largely unknown.

Increased dopamine (DA) neurotransmission arising from the administration of amphetamine or from the lack of DA transporter results in the inactivation of Akt in the mouse striatum (including ventral NAC and dorsal caudate putamen). Another study has indicated that striatal dopamine D2 receptors exert their action in a cAMP-independent manner by promoting the formation of a signaling complex composed of Akt, protein phosphatase-2A (PP2A), and β-arrestin 2. The formation of this complex then leads to the inactivation of Akt after the dephosphorylation of its regulatory threonine 308 residue by PP2A. The inactivation of Akt, in turn, results in the activation of GSK-3, which eventually contributes to the activation of the transcription factor cAMP response element binding protein (CREB) and production of dopamine-associated behaviors.

The present study was first designed to characterize the expression of p-Akt in the NAC of rats during the incubation of heroin-seeking behavior induced by contextual cue (CC) or discrete conditioned cues (CS) after withdrawal. Furthermore, LY294002, an inhibitor of PI3K, was microinjected into the NAC to observe its effects on CS-induced heroin seeking after withdrawal. Finally, small interference RNA (siRNA) lentivirus of Akt1 or β-arrestin 2 was microinjected into the NAC to explore the possible interaction of Akt1 and β-arrestin 2 in the NAC on heroin-seeking behavior.

## EXPERIMENTAL PROCEDURES

### Subjects

Male Sprague–Dawley rats (250–300 g, Experimental Animal Center of Zhejiang Province, China) were housed in a temperature-controlled, ventilated colony room with a reversed 12-h light/dark cycle (lights onset 19:00 h, offset 07:00 h). *Ad libitum* food and water was provided in the home cage. Rats were acclimated to the environment for 1 week before experiments. The study design was approved by the Ethics Committee of Laboratory Animal Use and Care at Ningbo University, and all experiments were conducted during the dark period according to the specifications of the National Institute of Health Guide for the Care and Use of Laboratory Animals (Eighth edition).

### Drugs and construction of siRNA lentivirus

Heroin (diacetylmorphine HCl) was obtained from the National Institute of Forensic Science (Beijing, China). The Akt inhibitor LY294002 was purchased from Tocris, MO, USA, and dissolved in artificial cerebrospinal fluid (aCSF). Small hairpin RNA (shRNA) targeting Akt1 or β-arrestin 2 was constructed and synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). The rat Akt1 eukaryotic expression plasmid GP-Akt1 was constructed using the GP-GFP Vector containing a multiple cloning site for the insertion of shRNA constructs to be driven by an upstream H1 promoter and a downstream cytomegalovirus promoter-GFP (marker gene). The sequences of the shRNAs used in the present study were as follows: Akt1 (5′-GACCTTTATTGGCTACAAAGG-3′) and β-arrestin 2 (5′-GCAACTCAAGCACAGACAC-3′).

### Intravenous surgery

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and implanted with chronically indwelling intravenous catheters, as described previously. A silicon catheter (Silastic; length 3.5 cm, 0.5-mm inner diameter, 0.94-mm outer diameter) was inserted into the right external jugular, and the other end of the catheter (10 cm, PE20) was passed subcutaneously through an incision on the back of the body, where it then entered the custom-made fluid connector fixed to a jacket. The catheter was flushed daily with 0.3-ml saline containing penicillin B (20,000 units) and heparin (5 units) to prevent bacterial infection and maintain catheter patency. Following surgery, rats were allowed 7 days to recover prior to behavioral training.
2.4 | Heroin self-administration

After recovery from surgery, the animals were placed in operant chambers from Med Associates Inc. (ENV-114 M, Saint Albans, VT) for daily 4-h heroin self-administration training sessions. Operant chambers were equipped with two infrared beam nose-poke apertures on the same panel and located 5 cm above the floor of the box, those nose pokes had a green LED light inside each hole. Heroin was delivered through Tygon tubing attached to a syringe pump at a speed of 1.2 ml/min using a 5-ml syringe. A computer-assisted system was used to control experimental procedure using a MED Associates interface and running self-programmed software written in Borland Delphi 6.0. Every session was begun with a blue light inside the active nose-poke hole. The rat received a single heroin infusion (0.2 mg/ml, 0.05 mg/kg in each infusion) following the completion of an active nose poke under the FR1 schedule. Each infusion was paired with a 20-s illumination of the house light in combination with the noise of the infusion pump, which therefore served as the discrete conditioned cues (CS) paired with the drug infusion. A timeout period was imposed for 20 s, and the light inside the active nose-poke hole was turned off, during which a response had no programmed consequences but was still recorded. Illumination of the blue light in the active nose-poke hole signaled the end of the 20-s timeout period. The number of the inactive nose poke touched was recorded, but this had no programmed consequences. All rats were trained for 14 consecutive days to reach stable response activity levels.

2.5 | Intracranial surgery and microinjection

After the last self-administration session, the rats were placed in the home chambers and underwent a spontaneous withdrawal session. In the withdrawal session, the rats did not have access to heroin, the heroin self-administration CC, or the heroin-associated CS. Next, all rats were divided into three groups in order to undergo different experiments. The first group of rats was tested for heroin-seeking behavior induced by CC or CS after different periods of withdrawal. The second group of rats was divided into three groups (eight rats per group) for intracranial surgery. These rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and positioned into a stereotaxic apparatus (Stoelting 51,950, USA). Guide cannulas, consisting of 24-gauge thin-walled stainless steel tubing (RWD Life Science Co., China), were implanted bilaterally 0.5 mm above the NAc (+1.4 mm anteroposterior, ±2.0 mm mediolateral, and −6.6 mm dorsoventral). Guide cannulas were secured with stainless steel screws and dental cement, and rats were allowed at least 7 days to recover before heroin-seeking behavioral testing. The last group of rats was divided into three groups (seven rats per group) for lentivirus vector microinjection. These rats were also anesthetized with sodium pentobarbital and positioned in a stereotaxic apparatus. Microinjection needles were bilaterally placed into NAc (+1.4 mm anteroposterior, ±2.0 mm mediolateral, and −7.1 mm dorsoventral), and 0.5 μl of virus per side was injected over 5 min; thus, the microinjection speed was 0.1 μl/min. The needles were left in place for an additional 5 min to allow for the diffusion of virus particles away from the injection site.

2.6 | Heroin-seeking behavior

After the completion of withdrawal for different days, all rats were replaced in the operant chambers for the assessment of their heroin-seeking behaviors induced by the CC or CS. In the CC test, there was no light and the infusion pump noise or heroin infusion. The CS test began with a 5-s light cue that previously predicted the drug’s availability and a house light that was previously associated with heroin infusion, after which each active nose-poke response resulted in another presentation of the CS but no heroin infusion. Active and inactive nose pokes during this testing phase of heroin-seeking behavior were recorded and added up.

2.6.1 | Effects of time duration on heroin-seeking behavior induced by a CC or CS

Four groups (eight per group) of rats were tested for heroin-seeking behavior induced by CC or CS after different durations of withdrawal. Following 1 day of withdrawal, two groups of rats were replaced in the operant chambers for 2 h to assess heroin-seeking behaviors induced by the CC or CS. The other two groups were tested after 14 days of withdrawal.

2.6.2 | Effects of LY294002 on CS-induced heroin-seeking behavior

Three groups of rats that had recovered from intracranial surgery were tested for heroin-seeking behaviors after 14 days of withdrawal. Prior to testing, plugs were removed, and bilateral infusion cannulas (31 gauge) were inserted, extending 0.5 mm beyond the tip of the guide cannulas. Rats were microinjected with LY294002 (2 or 5 mM) or vehicle (aCSF) into the NAc using a microinjection pump (MD-1001, Bioanalytical System Inc., IN) at a volume of 0.5 μl over 5 min. Fifteen minutes after completion of the microinjection, all rats were tested 2 h thereafter for heroin-seeking behaviors induced by the CS.

2.6.3 | Effects of β-arrestin 2 or Akt1 RNA interference on CS-induced heroin-seeking behaviors

Three groups of rats (n = 7) that had received a lentivirus microinjection were tested for heroin-seeking behaviors after 14 days of withdrawal. The negative control (NC), β-arrestin 2, or Akt1 lentivirus was expressed for 10–14 days. All rats were then tested heroin-seeking behaviors induced by the CS for 2 h. After testing, three rats
per group were used for western blot analysis, and the other four rats per group were used for the histology.

2.7 | Western blot analysis

Rats were killed immediately after heroin-seeking testing. The rats were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and decapitated. The NAc tissues were isolated from the removed brain by gross dissection, and extracts were prepared from an individual rat. Western blots were carried out according to standard methods. Briefly, the NAc tissues were directly lysed in sodium dodecyl sulfate sample buffer and incubated at 95°C for 10 min before being loaded onto a 10% sodium dodecyl sulfate-polyacrylamide gel. The protein concentration was determined before being loaded onto gel and then 50-μg protein per sample was loaded onto each track. Proteins were separated by electrophoresis and then transferred to a nitrocellulose membrane (BioRad, Hercules, California, USA). The membrane was blocked in 5% milk-TBST and probed with Akt or p-Akt-308 or p-Akt-473 antibody (9272s or 9275s or 9271s, Cell Signaling Technology Inc., USA) and p-CREB antibody (sc-7978-R, Santa Cruz Biotechnology Inc., USA) (all at a 1:1000 dilution ratio), all in 3% milk-TBST, and reacted with the horseradish peroxidase-conjugated secondary antibody (1:2000) in 3% milk-TBST. Immunoreactive protein bands were detected by enhanced chemiluminescence reagents (ECL; Amersham Pharmacia Biotech, Piscataway, NJ) on an X-ray film or scanned by the Odyssey Imaging System (LI-COR Biosciences, Lincoln, USA), and immunoblots were evaluated by integrating densitometry using GeneSnap and GeneTools (Chemigenius Gel Documentation System, Syngene, Cambridge, UK). β-Actin (4967s, Cell Signaling Technology Inc., USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-365062, Santa Cruz Biotechnology Inc., USA) served as an internal protein control (1:2000).

2.8 | Histology

Once all testing was completed, the rats that had been microinjected with the lentivirus were anesthetized and transcardially perfused with saline, followed by a 4% polyformalin solution. The brains were sectioned on a Cryostat Microtome (Leica CM1850, Germany) in the coronal plane at a thickness of 30 μm. Lentiviral expression was observed by fluorescent microscopy (Olympus BX 53, Japan), and the localization of injection sites was mapped onto a schematic diagram of the rat brain.18

2.9 | Statistical analysis

Total nose-poke responses during behavioral testing were assessed using a two-way ANOVA followed by Bonferroni post-tests over the active and inactive nose pokes. The expressions of p-Akt and p-CREB protein were analyzed using a one-way ANOVA followed by Bonferroni post hoc comparisons between group means. Data are presented as means ± SEM. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). $P < 0.05$ or $P < 0.01$ was considered to indicate a statistically significant difference.

3 | RESULTS

3.1 | Establishment of the heroin self-administration model

Figure 1 revealed similar findings to those that we had previously described.19 The active pokes and heroin infusions of rats reached a stable high level within 14 days of heroin self-administration training. In contrast, the inactive pokes of rats were still at a low level after

**FIGURE 1** Effects of 14 days of training on the number of pokes and infusions during heroin self-administration in rats. (A) The active pokes but not the inactive pokes at days 7–14 increased significantly when compared to those at day 1, $**P < 0.01$, compared with the active pokes at day 1, $n = 24$. (B) The heroin infusions at days 7–14 increased significantly when compared to those at day 1, $***P < 0.01$, compared with the heroin infusions at day 1, $n = 24$.
14 days of training. Two-way repeated measures ANOVA for active pokes and inactive pokes during 14 days showed a significant main effect for day \((F_{13, 644} = 12.66, P < 0.001)\), pokes \((F_{1, 644} = 1.197, P < 0.001)\), and an interaction of day \(\times\) pokes \((F_{13, 644} = 14.07, P < 0.001)\), with significant increase in active pokes on days 7–14 when compared to those at day 1 \((P < 0.01, \text{respectively})\) but no significant differences in inactive pokes across 14 days (Figure 1A). One-way ANOVA revealed a significant main effect for day on the heroin infusions \((F_{13, 322} = 15.61, P < 0.001)\). Post-hoc Bonferroni comparisons revealed that the heroin infusions on days 7–14 were significantly increased when compared to those on day 1 \((P < 0.01, \text{respectively})\).

### 3.2 | P-Akt expression in the NAc exposed to a CC or CS after 1 day or 14 days of withdrawal

Following the self-administration sessions, rats were assessed for the number of nose pokes induced by CC or CS after 1 day or 14 days of withdrawal. As illustrated in Figure 2A, a two-way ANOVA revealed a significant main effect for treatment \((F_{3, 56} = 158.9, P < 0.001)\), pokes \((F_{1, 56} = 628.6, P < 0.001)\), and an interaction of treatment \(\times\) pokes \((F_{3, 56} = 135.1, P < 0.001)\). Post-hoc Bonferroni comparisons revealed that the active pokes induced by either the CC or CS after 14 days of withdrawal were significantly increased when compared to those after 1 day of withdrawal \((P < 0.01, \text{respectively})\). Additionally, the active pokes induced by CS were significantly increased when compared to those induced by CC not only after 1 day but also after 14 days of withdrawal \((P < 0.01, \text{respectively})\). There was no difference in the inactive pokes of all groups.

**Figure 2** Effects of withdrawal period on the heroin-seeking behavior and expression of p-Akt-473 in the NAc. (A) Number of active or inactive nose-poke responses induced by a context cue (CC) or discrete conditioned cues (CS) after 1 day or 14 days of withdrawal. Data are expressed as mean ± SEM, \(n = 8\) per group. (B) The insert immune-blot bands show representative changes in the expression of p-Akt-473 and \(\beta\)-Actin in the same NAc tissue sample. Histogram figures show the relative level of p-Akt-473 protein by densitometric quantification. Data are expressed as mean ± SEM, \(n = 3\) per group. CC 1 day: exposure to CC following 1 day of withdrawal after heroin self-administration; CS 1 day: exposure to discrete CS following 1 day of withdrawal; CC 14 day: exposure to CC following 14 days of withdrawal; CS 14 day: exposure to discrete CS following 14 days of withdrawal. \(*P < 0.01, \text{CC 14 days compared with CC 1 day or CS 14 days compared with CS 1 day}\). \#P < 0.05, \##P < 0.01, CC 1 day compared with CC 14 days or CS 14 days compared with CS 1 day.

### 3.3 | Effects of LY294002 on heroin-seeking behavior and p-Akt levels in the NAc

Figure 3A revealed the effects of LY294002 on heroin-seeking behavior induced by a CC after 14 days of withdrawal. A two-way ANOVA revealed a significant main effect for dose of LY294002 \((F_{2, 42} = 35, P < 0.001)\), pokes \((F_{1, 42} = 321, P < 0.001)\), and a dose \(\times\) pokes interaction \((F_{2, 42} = 32, P < 0.001)\). Post-hoc Bonferroni comparisons revealed a significant inhibition in the active pokes of the 2-mM LY294002 group or 5-mM LY294002 group \((P < 0.01, \text{respectively})\), as compared with the vehicle control group. There were no differences in the inactive pokes across the three groups.

Figure 3B revealed the p-Akt-473 levels in the NAc immediately after the rats completed the CS-induced heroin-seeking testing. A one-way ANOVA revealed a significant inhibition on the p-Akt-473
level after treatment with LY294002 ($F_{(2,6)} = 19.73, P < 0.01$). Post hoc Bonferroni comparisons revealed that the p-Akt-473 levels in the NAc were significantly inhibited after injection with 2-mM LY294002 ($P < 0.05$) or 5-mM LY294002 ($P < 0.01$), as compared with those in the control group. There were no differences in the expression of total-Akt across groups.

### 3.4 | GFP protein expression after Akt1 lentivirus microinjection and cannula placement sites in the NAc

Following the heroin-seeking test, the rats that had been microinjected with a lentivirus were assessed for protein levels by western blot or histologically assessed for their levels of GFP protein expression. As illustrated in Figure 4A, a coronal section of the rat brain demonstrated the expression of GFP protein in the NAc. The overlay magnification of the NAc shows the neurons transfected with an Akt1 lentivirus. Scale bars = 50 μm. (C) Diagrams of coronal sections of the rat brain show the microinjection sites (NAc) of all rats.

### 3.5 | Effects of β-arrestin 2 or Akt1 RNA interference on heroin-seeking behavior or p-Akt and p-CREB expression in the NAc

The effects of β-arrestin 2 or Akt1 RNA interference on heroin-seeking behavior induced by a CS after 14 days of withdrawal were shown in Figure 5A. A two-way ANOVA revealed a
significant treatment effect for treatment \((F_{(2, 36)} = 6.126, P < 0.01)\), pokes \((F_{(1, 36)} = 497.5, P < 0.001)\), and a treatment \(\times\) pokes interaction \((F_{(2, 36)} = 5.514, P < 0.01)\). Post-hoc Bonferroni comparisons revealed a significant inhibition in the active pokes of the Akt1 group \((P < 0.05)\) compared to those of the NC group. However, \(\beta\)-arrestin 2 interference had no significant effect on the active pokes. There were no differences in the inactive pokes among groups.

Figure 5B showed the effects of \(\beta\)-arrestin 2 or Akt1 RNA interference on the protein expression levels of p-Akt-308 in the NAc. A one-way ANOVA revealed a significant treatment effect of RNA interference on p-Akt-308 levels \((F_{(2, 6)} = 46.60, P < 0.001)\). Post-hoc Bonferroni comparisons revealed that the protein expression of p-Akt-308 was increased after \(\beta\)-arrestin 2 interference \((P < 0.05)\) but decreased after Akt1 interference compared to that of the NC group \((P < 0.01)\). There were no differences in the total-Akt protein expression levels among those groups.

Figure 5C showed the effects of \(\beta\)-arrestin 2 or Akt1 RNA interference on p-Akt-473 protein expression levels in the NAc. A one-way ANOVA revealed a significant treatment effect of RNA interference on p-Akt-473 levels \((F_{(2, 6)} = 254.2, P < 0.001)\). Post-hoc Bonferroni comparisons revealed that the protein expression of p-Akt-473 was decreased after Akt1 interference \((P < 0.01)\) but not after \(\beta\)-arrestin 2 interference when compared to those of the NC group. There were no differences in the protein expression levels of GAPDH among groups.

4 | DISCUSSION

In the present study, we found that heroin-seeking behaviors induced by CC or CS were enhanced following a prolonged withdrawal period, reflecting the elevated levels of p-Akt-473 in the NAc. Microinjection of LY294002 significantly decreased CS-induced heroin-seeking behaviors and the expression of p-Akt-473 protein in the NAc after 14 days of withdrawal. Lentivirus-mediated Akt1 downregulation in the NAc also attenuated the cue-induced heroin-seeking behavior after 14 days of withdrawal. Meanwhile, p-Akt-308 and p-Akt-473 expression levels were all significantly decreased, while p-CREB expression was increased after Akt1 downregulation in the NAc. Lentivirus-mediated \(\beta\)-arrestin 2 knockdown increased the expression of p-Akt-308 in the NAc, without affecting heroin-seeking behavior. These data demonstrated that Akt phosphorylation in the NAc may be involved in the incubation of heroin-seeking and cue-induced heroin-seeking behavior.

Heroin-seeking behavior induced by CC or CS was more strongly enhanced after 14 days of withdrawal than that after 1 day of withdrawal. This is consistent with other studies that demonstrated a time-dependent increase in heroin seeking not only in the extinction test but also in the CS test which may include several extinction sessions prior to the test.20,21 In our study, the CC reflects the extinction environment, while the CS-induced heroin-seeking test does not
Akt activity varies in the different phases of drug addiction and has brain region specificity. The expression of p-Akt in the NAc increases after a single morphine injection but significantly decreases after chronic morphine administration. In the striatum of amphetamine sensitization rats, the expression of p-Akt significantly increases 15 min after an amphetamine injection on the challenge day but decreases at 2 h after the amphetamine challenge. Because amphetamine can stimulate Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) activity and CaMKII subsequently regulates the complex of PP2A and Akt, reduced Akt activity in response to amphetamine can be blocked by the treatment by CaMKII inhibition in culture cells. Furthermore, reward memory formation in the basolateral amygdala-mPFC circuit involves a functional link between D1 receptors and extracellular signal-related kinase 1/2 (ERK1/2) signaling substrates in opiate-naïve state rats but switches to a D2R/Ca\(^{2+}\)/CaMKII-dependent memory substrate following chronic opiate exposure. The “rapid” Akt activation in vitro or vivo may be mediated by dopamine D1 receptors and subsequently cAMP/PKA pathway, whereas the “late” Akt deactivation may be considered a result of dopamine D2 receptors that stimulate an Akt-β-arrestin-PP2A inactivation complex and underlie the reward tolerance of drugs. Our study found that the expression of p-Akt-473 in the NAc increased when rats were exposed to either a CC or CS after prolonged withdrawal, indicating that the activation of Akt signaling also occurred after prolonged withdrawal. Moreover, the enhancement of p-Akt-473 is positively related to the increase in heroin-seeking behaviors. These results suggest that the activation of Akt signaling in the NAc may contribute to the incubation of the heroin-seeking behavior after prolonged withdrawal.

In the present study, the inhibition of Akt activity by LY294002 decreased the CS-induced heroin-seeking behavior after prolonged withdrawal in a dose-dependent manner, alongside a decrease of p-Akt-473 in the NAc. Since previous other study has shown that intracerebroventricular microinjection with LY294002 did not affect mice locomotion activity. The effect of LY294002 on heroin-seeking behavior and expression of p-Akt in the NAc did not account for the inhibitory action of locomotion. Thus, the results suggested that the PI3K/Akt signaling pathway may underlie the heroin-seeking behavior induced by CS. PI3K can be regulated by neurotrophic factors, one of the most important of them being BDNF. BDNF colocalizes with tyrosine hydroxylase in midbrain DA neurons and therefore can play a role in the synaptic plasticity of these neurons. The downregulation of Akt/mTORC2 signaling pathway mediates the decrease in the size of VTA dopamine neurons and reward tolerance after chronic morphine. This study shows that BDNF and TrkB in the VTA are downregulated in morphine-induced CPP. The expression levels of BDNF in the NAc of rats significantly decrease after chronic heroin treatment, whereas BDNF expression is significantly increased in these rats when they undergo either naloxone-induced withdrawal or spontaneous withdrawal 1 day after the last heroin treatment. However, this explanation is in conflict with that of another study that suggests that BDNF protein in the NAc is not involved in heroin incubation. Several methodological issues should be considered when these discrepant results are interpreted. With regard to BDNF, one possibility is the difference in the molecular assay. Another result indicates that the BDNF serum levels in heroin-dependent patients are lower than those of healthy controls at baseline and increased after 26 weeks of abstinence. Additionally, the intravenous injection of the inhibitor of TrkB, the receptor of BDNF, dose-dependently reduced the reinstatement of cocaine consumption in rats that were allowed either short or long access times to cocaine self-administration. Increased Akt phosphorylation is observed in rat ventromedial PFC after 3 or 30 days of withdrawal from cocaine treatment; an intra-PFC infusion of the PI3K inhibitor wortmannin could reduce cocaine-seeking behaviors elicited by a cue. Therefore, the upregulation of PI3K/Akt signaling pathway in our study may be mediated by the increased BDNF expression in the NAc, and the PI3K/Akt signaling pathway is particularly involved in CS-induced heroin seeking after prolonged withdrawal.

Akt1 is one of Akt isoforms and is ubiquitously expressed in tissues. The downregulation of Akt1 by a lentivirus decreased p-Akt-308 and p-Akt-473 expression levels and increased p-CREB expression in the NAc. This is consistent with our previous results that demonstrated that the elevated expression of p-CREB in the NAc by an inhibitor of phosphodiesterase 4 could reduce CS-induced heroin-seeking behaviors after prolonged withdrawal. The downregulation of β-arrestin 2 increased p-Akt-308 expression in the NAc but not p-Akt-473 or p-CREB. The ineffectiveness of β-arrestin 2 on heroin seeking was complex and may be due to a number of factors. First, tolerance to the behavioral effects of morphine is prevented by the downregulation of β-arrestin-2 in mice, but opioid withdrawal at the cellular level in periaqueductal gray neurons is unaffected by β-arrestin 2 deletion. Second, β-arrestin 2 can also interact with other signaling molecules, such as ERK. In addition, the dopamine D1 receptor-dependent β-arrestin 2/p-ERK signaling complex in the NAc mediates morphine-induced locomotor activity but not conditioned place preference. Taken together, CS-induced heroin-seeking behavior after prolonged withdrawal might not be directly affected by the β-arrestin 2/Akt/PP2A signaling pathway.

In conclusion, the present results demonstrated that Akt and its phosphorylation in the NAc mediate the incubation of heroin-seeking behaviors after prolonged withdrawal, suggesting that Akt may thus serve as a potential target of therapies for the treatment of heroin relapse and addiction.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHORS CONTRIBUTION
HZ performed the experiments, analyzed the data, and wrote the manuscript. DZ, ZL, ML, FD, and QH performed the experiments. HL was responsible for the study concept and supervised the experiments. WZ was responsible for study design and critically revised the manuscript. All authors critically reviewed content and approved final version for publication.

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