Inhibition of autophagy in the amygdala ameliorates anxiety-like behaviors induced by morphine-protracted withdrawal in male mice

Shuang Han[a,b,c,*], Chenchen Zhu[a,b,*], Dengjun Min[a,b,*] and Zicheng Li[a,b]

Objective **Morphine withdrawal triggers a range of negative affective states, wherein anxiety is typically common, significantly contributing to the morphine relapse. To date, the exact mechanism underlying morphine withdrawal-induced anxiety has remained unclear. Previous studies have proposed that autophagy is involved in the pathogenesis of morphine addiction and anxiety; however, the possible relationship between autophagy and morphine withdrawal-induced anxiety has not been explored before. In this study, we aimed to reveal the potential role of autophagy in anxiety-like behaviors elicited by protracted morphine withdrawal, and which brain region is involved.**

Methods **We established the model mice of anxiety by chronic intermittent escalating-dose morphine administration for 7 days and then withdrawing for 4 days. Anxious behaviors were detected using the Open field test and the Elevated plus maze test. Western blot was performed to measure the change of autophagy-associated proteins (ATG5, Beclin-1, LC3) in different brain regions.**

Results **Our results showed that intraperitoneal injection of an autophagy inhibitor 3-Methyladenine attenuated protracted morphine withdrawal-induced anxiety-like behaviors in male mice. Moreover, protracted morphine withdrawal predominantly promoted autophagy in the amygdala, rather than other related brain regions, suggesting the crucial involvement of amygdala in autophagy-mediated anxiety after morphine withdrawal. We further validated that 3-Methyladenine can effectively reduce autophagy-associated protein levels in the relevant brain region.**

Conclusion **These findings indicated that protracted morphine withdrawal-elicited autophagy in the amygdala contributes to the anxiety-like behaviors and may have implications for the future treatment of this disorder.**

NeuroReport 2025, **36**:487–496

Keywords: amygdala, anxiety, autophagy, morphine withdrawal

[a]College of Basic Medical Sciences, China Three Gorges University, [b]Hubei Key Laboratory of Tumor Microenvironment and Immunotherapy, China Three Gorges University and [c]The First College of Clinical Medical Science, China Three Gorges University, Yichang Central People's Hospital, Yichang, China

*Correspondence to Zicheng Li, PhD, College of Basic Medical Sciences, China Three Gorges University, Daxue Road 8, Hubei province, Yichang 443000, China
Tel: +86 0717 6393136; e-mail: zichengli@ctgu.edu.cn

*Shuang Han, Chenchen Zhu, and Dengjun Min contributed equally to the writing of this article.

Received 25 January 2025 **Accepted** 4 April 2025.

Introduction

Drug abuse disorder is a chronic and relapsing disease characterized by uncontrolled behaviors in craving and consuming drugs [1–3]. The 2022 National Survey on Drug Use and Health reported that approximately 48.7 million people were afflicted with substance use disorders in 2022. Morphine is one of the most commonly used substances for potent analgesia; however, it has a substantial propensity for addiction [4]. Upon cessation of morphine, the addict is confronted with withdrawal symptoms, wherein anxiety stands as a prominent negative emotion, significantly contributing to the morphine relapse [5,6]. Nevertheless, the biological mechanism responsible for anxiety symptoms triggered by morphine withdrawal remains elusive.

Autophagy represents a self-destructive mechanism that eliminates aberrant cytoplasmic cargoes through lysosomal degradation [7]. Critical steps involved include autophagy induction, nucleation, phagophore formation and elongation, autophagosome formation and fusion, as well as cytoplasmic components degradation [8,9]. The above process requires the orchestrated participation of multiple autophagy-related proteins (e.g. Beclin-1, LC3, and ATG5) to ensure molecular fidelity and functional regulation [10–12]. Mounting evidence demonstrates that autophagy is extensively engaged in the pathogenesis of various cerebral diseases, including morphine addiction [13], acute brain injuries [14], Parkinson's disease [15], Alzheimer's disease [16], and so on. It was reported that morphine-induced autophagy was decreased by ATG5 or ATG7 conditional knockout in dopaminergic neurons, which

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's website, www.neuroreport.com.

consequently protected mice from developing morphine addiction, thereby suggesting a link between autophagy and addiction [17]. Moreover, recent studies have revealed that autophagy is implicated in the development of negative emotions, such as anxiety disorder and depression [18–20]; however, the role of autophagy in morphine withdrawal–triggered anxiety has rarely been reported and the mechanism underlying it still remains unclear.

A myriad of experimental studies have demonstrated that multiple brain regions play essential roles in morphine addiction, such as the amygdala (Amy), medial prefrontal cortex, lateral habenula (LHb), nucleus accumbens (NAc), and ventral tegmental area (VTA) [21–23], wherein the Amy, which is comprised of the basolateral (BLA), central (CeA) nuclei, and the intercalated cells exerts a crucial function in processing emotions and reacting to external reward incentives [24,25]. For example, neurons expressing somatostatin within the CeA regulate anxiety by modulating the stria terminalis [26]. The lesions in the CeA increased anxiety-like behaviors induced by chronic stress [27]. Enhanced autophagy in the BLA causes mitochondrial loss, which disrupts synaptic transmission in the BLA–the bed nucleus of the stria terminalis anxiety pathway, ultimately resulting in anxiety-related behaviors [28]; but how Amy contributes to the development of anxiety-like behaviors following morphine withdrawal has not been answered yet.

Here, we revealed whether inhibition of autophagy in Amy could prevent the morphine protracted withdrawal mice from producing anxiety-like behaviors. We first examined the effect of an autophagy inhibitor on anxiety-like behaviors triggered by protracted morphine withdrawal. Second, we explored the consequences of morphine withdrawal on autophagy-associated proteins in different brain regions. Finally, we verified whether inhibiting autophagy could effectively reduce autophagy-associated proteins in the Amy. Our results suggest that inhibition of autophagy in Amy can blunt anxiety-like behaviors induced by protracted morphine withdrawal in male mice. This finding improves knowledge about morphine withdrawal-triggered anxiety and may provide new insights for pharmacotherapies.

Materials and methods

Animals

The male C57BL/6J mice (6–8 weeks old at arrival) weighing 18–23 g were purchased from the Experimental Animal Center of China Three Gorges University [CTGU, Certificate No. SCXK (E) 2017-0012; Yichang, China]. These specific pathogen-free animals were housed in a well-controlled environment (temperature 20–24 °C, relative humidity (61 ± 4) %, a standard 12 h light/dark cycle) with free access to food and water. A total of 108 mice were used for experiments, and each group consisted of 18 male mice. All animal experiments complied strictly with the Guidelines for the Care and Use of Laboratory Animals of CTGU under approval by the Ethics Committee (number of approval: No. 42010200008635).

Establishment of the anxiety model of morphine-protracted withdrawal

The administration of morphine to mice was implemented in accordance with our previous publication [29]. After a week-long acclimatization, mice were intraperitoneally (i.p.) injected with morphine (Northeast Pharmaceutical Group Shenyang No.1 Pharmaceutical Co., Ltd., Shenyang, China) given in escalating doses (20 mg/kg on day 1, 40 mg/kg on days 2 and 3, 80 mg/kg on days 4 and 5, and 100 mg/kg on days 6 and 7) or an equivalent volume of saline every 12 h (8 : 30 a.m. and 4 : 30 p.m.) for 7 consecutive days. Subsequently, all the mice underwent spontaneous withdrawal in their home cages without receiving injections. Steady and noticeable anxiety-like behaviors were displayed at days 3 and 4 following the final administration of morphine.

Behavioral test

As mentioned above, anxiety-like behaviors were evident on the third and fourth days after the last morphine injection. Consequently, the open-field test (OFT) was carried out on the third day after the last morphine injection meanwhile the elevated plus maze test (EPM) was carried out on the day afterward. In both behavioral tests, the automated video-tracking system was employed to observe and record the behavior of mice for a period of 8 min. The sampling frequency of this video-tracking system is 25 frames/s.

Open-field test

Each mouse was placed individually in the upper left corner of the open field (a 40 × 40 × 50 cm opaque plexiglass box without a top) exposed to 10 Lux illumination after a 30-min adaptation to the surroundings, and allowed to move freely. The time spent in the center (50% of the total area), the number of entries into the center, the total distance traveled and the amount of feces was measured by tracking and analysis software (TopScan Lite; CleverSys Inc, Reston, Virginia, USA). After each test, fields and equipments were cleaned and wiped using 75% alcohol to eliminate the impact of feces and urine odor on behavioral measurements.

Elevated plus maze test

The apparatus was placed on a fixed stand at a height of 50 cm above the floor and consisted of two open arms (65 cm length × 5 cm width each) and two closed arms (65 cm length × 5 cm width × 40 cm in height each) in a criss-cross pattern connected by a CeA platform (5 × 5 cm). The light intensity was 10 Lux. After 30 min of acclimatization, the mice were placed on the

CeA platform, with their heads facing the open arm in the same orientation, and allowed free movement. The number of entries into the open arms and the time spent there were measured by a tracking and analysis software (TopScan Lite; CleverSys Inc, Reston, Virginia, USA). After each test, the equipment was cleaned and wiped using 75% alcohol to eliminate the impact of feces and urine odor on behavioral measurements.

Protein sample preparation and western blot

Western blot was performed to detect the change of autophagy-associated proteins (ATG5, Beclin-1, and LC3) in different brain regions as previously reported.

The mice were anesthetized with 20% urethane after the EPM test and the brain tissue of the lateral hypothalamus, NAc, ventral pallidum, VTA, LHb, and Amy were quickly microdissected, then placed in –80 ℃ for storage until use. To extract the total protein from those brain regions, the brain samples were homogenized in radioimmunoprecipitation assay lysis and extraction buffer and subsequently centrifuged at 12 000 rpm at 4 ℃ for 10 min.

Protein concentrations were determined by the BCA assay kit (Applygen Technologies, Beijing, China) using the manufacturer protocol. A total of 30 μg protein of each sample was loaded onto 10% SDS-PAGE gel to separate. After electrophoresis, the proteins transferred on polyvinylidene difluoride membranes were incubated with the following primary antibodies for 15 h at 4 °C: ATG5 Rabbit mAb (1 : 1000, NBP178444; Cell Signaling Technology Inc., Danvers, Massachusetts, USA), Beclin-1Rabbit mAb (1 : 1000, #3495; Cell Signaling Technology Inc., Danvers, Massachusetts, USA), LC3 A/B antibody (1 : 1000, #4108; Cell Signaling Technology Inc., Danvers, Massachusetts, USA), and GAPDH polyclonal antibody (1 : 1000, A19056; ABclonal Biotech Co., Ltd., Woburn, Massachusetts, USA). Subsequent to washing, the blots were probed with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1 : 3000, AS014; ABclonal Biotech Co., Ltd., Woburn, Massachusetts, USA). Blot bands were then visualized with an ECL Super Kit (ABclonal Biotech Co., Ltd., Woburn, Massachusetts, USA), and densitometric data were analyzed by ImageJ software (ImageJ 1.5, NIH, Bethesda, Maryland, USA).

Reagent and solution

3-Methyladenine (3-MA) (HY-19312/CS-5207; MEDChemexpress llc) was diluted in saline and i.p. injected at a working concentration of 10 mM.

Statistical analysis

Statistical analysis of the data were performed with GraphPad Prism 8.0.2 and SPSS software. Two-way analysis of variance (ANOVA) followed by the least significant difference post-hoc test was utilized for comparing the four groups. Student's *t* test was used to compare the two groups. Data were presented in terms of mean ± SEM. Statistical significance was considered when *P* value was less than 0.05.

Results

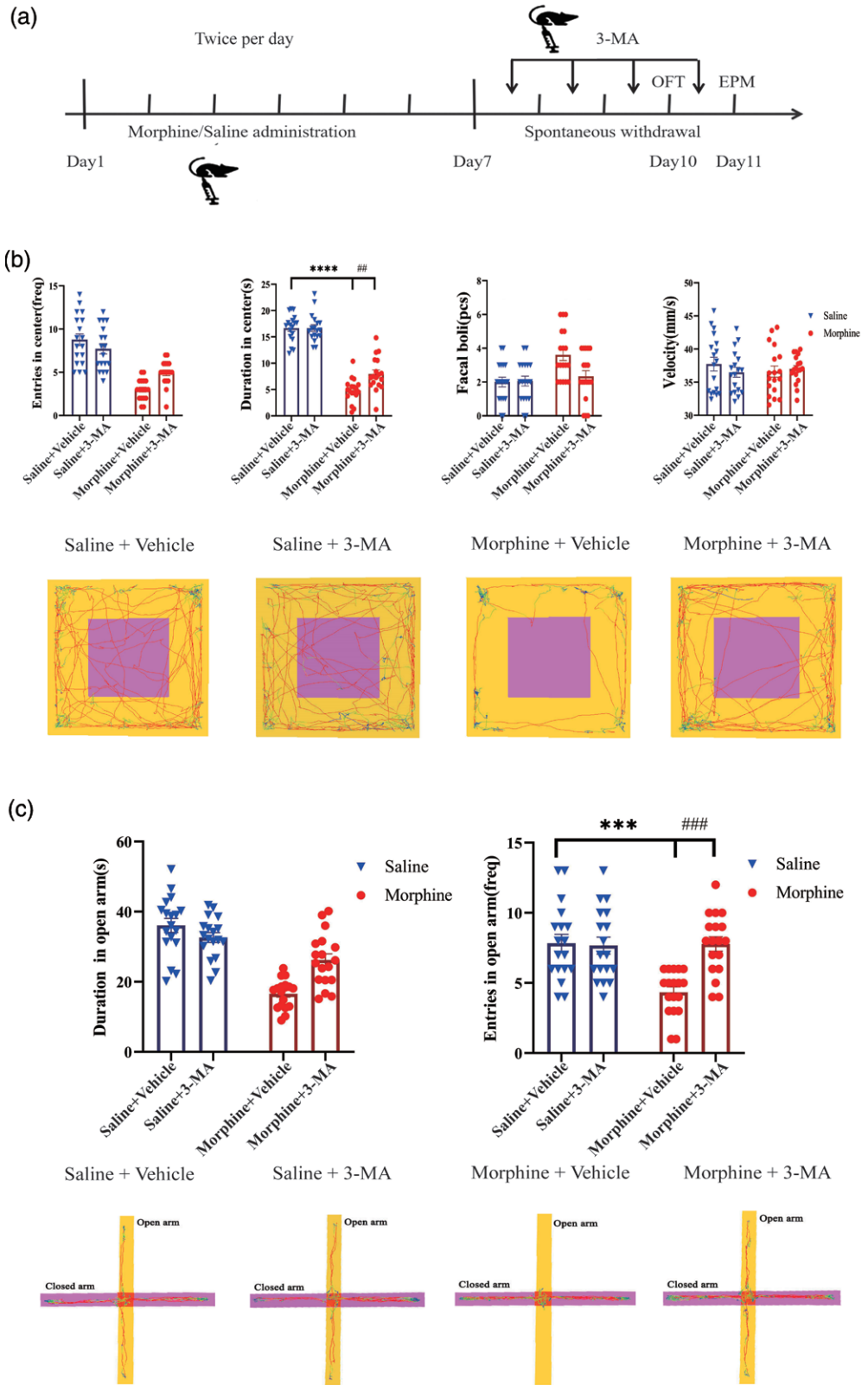
Intraperitoneal administration of autophagy inhibitor ameliorated anxiety-like behaviors following protracted morphine withdrawal in male mice

To assess the role of autophagy in anxiety-like behaviors induced by protracted morphine withdrawal, we treated the mice with 3-MA, a common autophagy inhibitor, during morphine withdrawal via i.p. injecting. The experimental procedure was depicted in Fig. 1a. Mice were stochastically allocated into four groups: saline withdrawal + vehicle (saline + vehicle), saline withdrawal + 3-MA (saline + 3-MA), morphine withdrawal + vehicle (morphine + vehicle), morphine withdrawal + 3-MA (morphine + 3-MA).

In our OFT, the two-way ANOVA showed significant differences for the morphine treatment on center durations: $F_{(1,68)} = 6.088$, $P = 0.0161$, the 3-MA treatment on [center entries: $F_{(1,68)} = 72.33$, $P < 0.0001$; center durations: $F_{(1,68)} = 263.6$, $P < 0.0001$; feces: $F_{(1,68)} = 9.158$, $P = 0.0035$] and morphine treatment × 3-MA treatment interaction on [center entries: $F_{(1,68)} = 8.893$, $P = 0.0040$; center durations: $F_{(1,68)} = 6.149$, $P = 0.0156$; feces: $F_{(1,68)} = 4.563$, $P = 0.0363$]. Post-hoc analysis revealed that the morphine + 3-MA group stayed in the center significantly more than the morphine + vehicle group ($P = 0.0050$). Meanwhile, the two-way ANOVA showed no differences for the morphine treatment on center entries [$F_{(1,68)} = 0.7808$, $P = 0.38$] and feces [$F_{(1,68)} = 3.834$, $P = 0.0543$]. The experimental data confirmed that 3-MA substantially mitigated anxiety-like behaviors resulting from morphine withdrawal in mice. In addition, the locomotor speed of the mice exhibited no significant difference across all groups, which suggested the locomotor function remained uncompromised in each group.

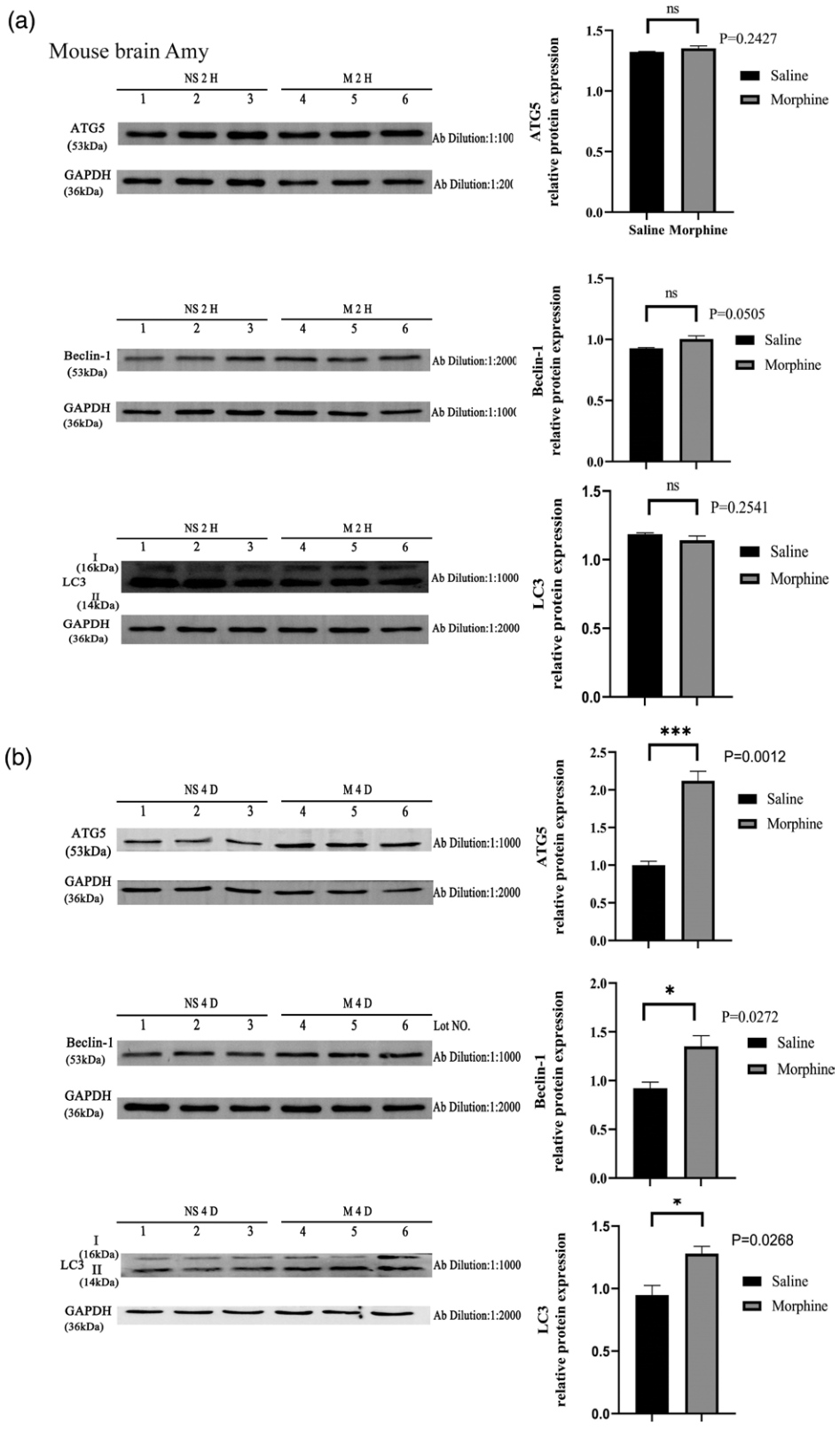
Consistent with the OFT, the EPM test showed similar results (Fig. 1c). The two-way ANOVA showed significant differences for the morphine treatment on [entries in open arms: $F_{(1,68)} = 9.092$, $P = 0.0036$], the 3-MA treatment on [entries in open arms: $F_{(1,68)} = 9.718$, $P = 0.0027$; duration in open arms: $F_{(1,68)} = 67.18$, $P < 0.0001$] and morphine treatment × 3-MA treatment interaction on [entries in open arms: $F_{(1,68)} = 11.03$, $P = 0.0014$; duration in open arms: $F_{(1,68)} = 17.07$, $P = 0.0001$]. Post-hoc analysis revealed that the 3-MA (i.p.) significantly rescued the decreased open arm entries induced by morphine withdrawal ($P = 0.0002$ and $P = 0.0003$, respectively). Together, these results demonstrated that inhibition of autophagy could ameliorate the severity of anxiety-like symptoms triggered by protracted morphine withdrawal.

Fig. 1



Autophagy inhibitor ameliorated anxiety-like behaviors triggered by protracted morphine withdrawal. (a) Experimental schedule. (b)The histograms of center entries, center durations, the quantity of feces, and locomotor speed in the OFT. The trajectories in the OFT. (c) The histograms of durations spent and entries into the open arms in the EPM. The trajectories in the EPM. Mean ± SEM, two-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus saline group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus morphine group. 3-MA, 3-methyladenine; ANOVA, analysis of variance; EPM, elevated plus maze test OFT, open-field test.

Fig. 2



Protracted morphine withdrawal–elevated autophagy in the Amy. (a) Representative western blot images and histograms of the expression of ATG5, Beclin-1, and LC3 in the Amy at 2 h after the final morphine or saline administration. (b) Representative WB images and histograms of the expression of ATG5, Beclin-1, and LC3 in the Amy at 4 days after the final morphine or saline administration. Mean ± SEM. Student's *t* test, $^{*}P < 0.05$, $^{***}P < 0.001$. Amy, amygdala; ns, no significant difference.

Protracted morphine withdrawal but not morphine itself upregulated autophagy in the amygdala

Amy, one of the brain regions involved in morphine addiction, has a substantial correlation with the development of anxiety [25]. To further investigate whether autophagy in the Amy is influenced by prolonged morphine withdrawal, we analyzed the expression levels of autophagy-related proteins at 2 h and 4 days following the final administration of morphine or saline. As shown in Fig. 2a, western blot analysis indicated that there were no significant differences in the total protein levels of ATG5, Beclin-1, and LC3 in the Amy between morphine- and saline-withdrawal group at 2-h time point ($P = 0.2427$, $P = 0.0505$, and $P = 0.2541$, respectively). These results suggested that morphine itself failed to cause alterations in autophagy within Amy. However, at the 4-day time point, a marked increase in the ATG5, Beclin-1, and LC3 protein levels was observed in the morphine-withdrawal group compared with the saline-withdrawal group($P = 0.0012$,$P = 0.0272$,and$P = 0.0268$,respectively). The data revealed that protracted morphine withdrawal (4 days) but not morphine itself (2 h after last injection), could induce elevated expression levels of autophagy-associated proteins in the Amy.

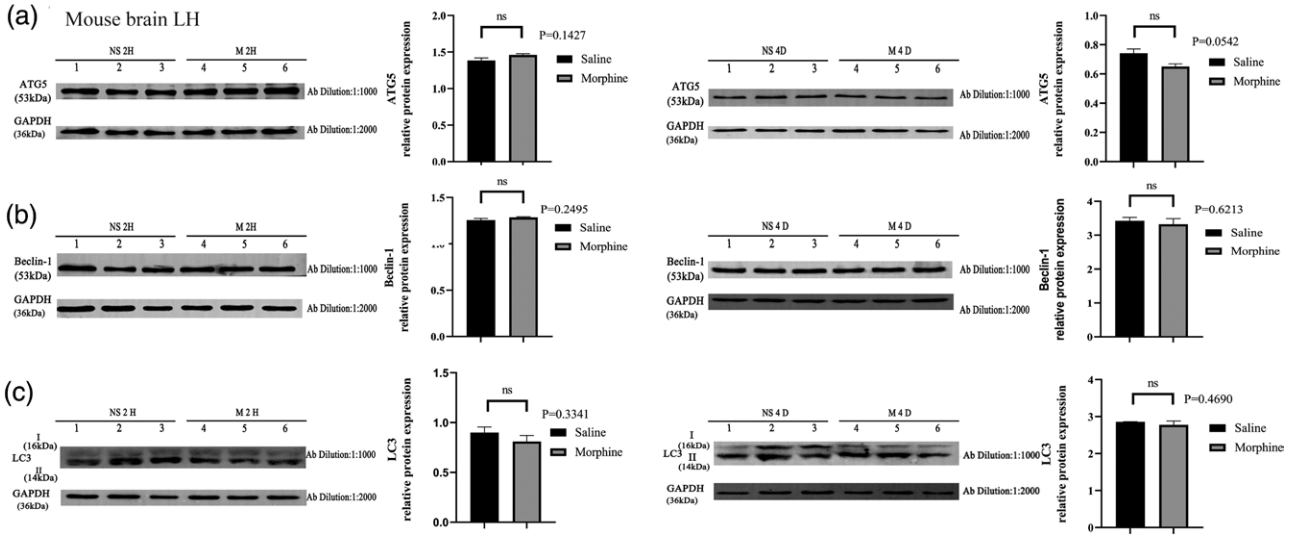
Protracted morphine withdrawal had no effect on autophagy-related signaling in the lateral hypothalamus, nucleus accumbens, ventral pallidum, ventral tegmental area, and lateral habenula

Considering that multiple brain regions are engaged in drug addiction or anxiety, we then detected the autophagy-associated proteins in the lateral hypothalamus, NAc, ventral pallidum, VTA, and LHb 2 h or 4 days following discontinuation of morphine or saline, to determine whether autophagy in these regions were also affected by morphine withdrawal. We found that at 2 h after morphine withdrawal, no difference was observed in expression levels of ATG5, Beclin-1, and LC3 measured in lateral hypothalamus between morphine- and saline-withdrawal mice group, indicating that expression levels of autophagy-associated proteins in the lateral hypothalamus were not altered in the mice subjected to 2-h morphine withdrawal. We further tested the alteration of ATG5, Beclin-1, and LC3 in the lateral hypothalamus by 4 days after morphine withdrawal, and also found that expression levels of autophagy-associated proteins remained unchanged (Fig. 3). Similar experimental results were observed in the other four brain regions mentioned above (Supplementary Figs. 1–4, Supplemental Digital Content 1, *http://links.lww.com/WNR/A822*). These results indicated that neither morphine itself (2 h) nor protracted morphine withdrawal (4 days) could induce an elevation of autophagy in lateral hypothalamus, NAc, ventral pallidum, VTA, and LHb, suggesting that these five brain regions might be not involved in the autophagy-mediated anxiety-like responses following protracted withdrawal from morphine.

Inhibition of autophagy reduced morphine-protracted withdrawal-induced upregulation of autophagy-associated proteins in the Amy

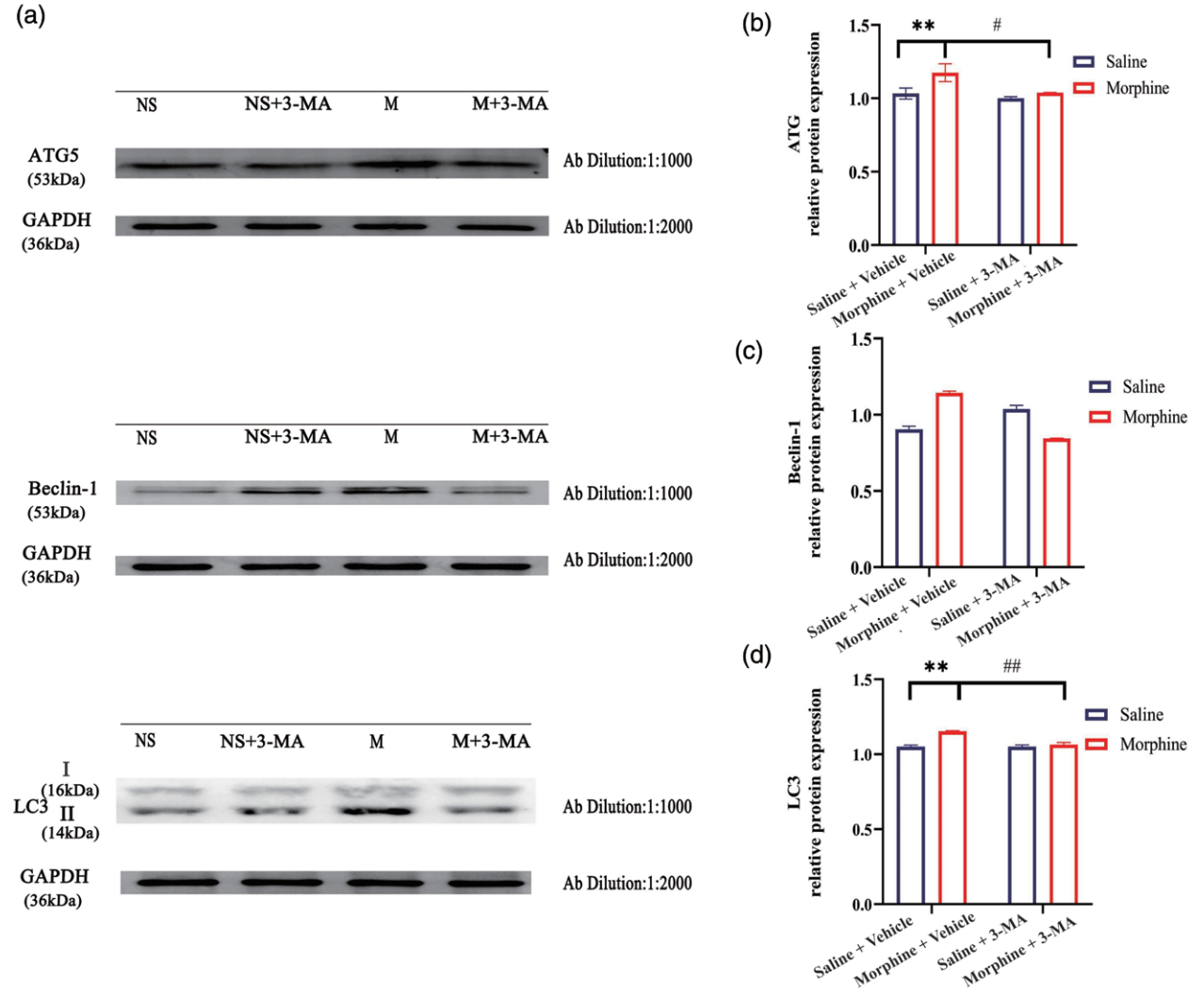
To determine whether autophagy inhibitor efficiently reduced expression levels of autophagy-associated

Fig. 3



Levels of autophagy in the LH after morphine withdrawal. (a–c) Representative western blot images and histograms of the expression of ATG5, Beclin-1, and LC3 in the LH at 2 h or 4-day time point after the final morphine or saline administration. Mean ± SEM. Student's *t* test. LH, lateral hypothalamus; ns, no significant difference.

Fig. 4



3-MA administration counteracted the increased ATG5, Beclin-1, and LC3 expression levels in the Amy triggered by morphine-protracted withdrawal. (a) Representative western blot images. (b–d) The histograms of the expression of ATG5, Beclin-1, and LC3 in the Amy at 4 days after morphine withdrawal. Mean ± SEM, Two-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$ versus saline group, $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus morphine group. 3-MA, 3-methyladenine; ANOVA, analysis of variance.

proteins in the Amy after morphine protracted withdrawal, which further mediated the onset of anxiety, we examined autophagy levels in the Amy with or without the administration of 3-MA at 4 days after morphine withdrawal.

As shown in Fig. 4, the two-way ANOVA showed significant differences for the morphine treatment on [ATG5: $F_{(1,8)} = 16.41$, $P = 0.0037$; Beclin-1: $F_{(1,8)} = 22.72$, $P = 0.0014$; LC3: $F_{(1,8)} = 14.65$, $P = 0.0050$], the 3-MA treatment on [ATG5: $F_{(1,8)} = 18.58$, $P = 0.0026$; LC3: $F_{(1,8)} = 24.77$, $P = 0.0011$] and morphine treatment × 3-MA treatment interaction [ATG5: $F_{(1,8)} = 6.172$, $P = 0.0379$; Beclin-1: $F_{(1,8)} = 151.3$, $P < 0.0001$; LC3: $F_{(1,8)} = 14.35$, $P = 0.0053$]. Post-hoc analysis demonstrated that the increased ATG5 and LC3 expression levels in the Amy triggered by morphine protracted withdrawal were restored by repeated i.p. 3-MA treatment ($P = 0.0102$ and $P = 0.0039$, respectively). These results supported that the efficient reduction of autophagy-associated proteins in the Amy caused by the 3-MA.

Discussion

Morphine, a widely utilized opioid for severe pain management, has been shown to elicit detrimental side effects including addiction [4]. Withdrawal from morphine

usually manifests anxiety, which serves as a key factor in relapse to morphine seeking [6]. However, the biological mechanisms responsible for anxiety triggered by morphine withdrawal remain currently unknown.

Given the extensive research revealing autophagy's crucial role in morphine addiction pathogenesis [17], we speculate that autophagy may be actively involved in anxiety-like behaviors following morphine withdrawal. The present study is devoted to investigate the effects of autophagy in related brain regions on morphine withdrawal-triggered anxiety and possible underlying molecular basis.

Autophagy is a self-degradative and recyclable process of aberrant cytoplasmic contents [7]. While autophagy is essential for normal homeostasis, it is engaged in the development of many diseases. Most of the relevant studies focus on the relationship between autophagy and morphine addiction. It has been recently reported that morphine elicits autophagy in dopaminergic neurons by regulating Atg5 and Atg7, which contribute to addictive behaviors in mice [17]. Besides, when administrated with morphine, increased autophagy activation is observed in an HT22 cell line, a cellular model for addiction, immortalized neuronal cell lines, and C57BL/6 murine hippocampi [13,30,31]. In our present study, however, we found that autophagy levels remained unaltered in Amy, lateral hypothalamus, NAc, ventral pallidum, VTA, and LHb during morphine administration but weres elevated in Amy during morphine withdrawal. This inconsistency likely arises from different experimental models and methods. In addition, different brain regions tested in each experiment may also have an effect on the results.

Recently, several studies on the role of autophagy in anxiety have been reported. Of note, these results are conflicting. According to Li *et al*., [19] high-fat diet-caused obesity might trigger anxiety-like behaviors in mice by inhibiting autophagy. Concordantly, it was also reported that the ameliorative effects of resveratrol in Lipopolysaccharide-induced anxiety-like behavior was mediated by the promotion of autophagy levels in the hippocampus [20]. Taken together, these studies indicated the ameliorative role of autophagy on anxiety symptoms. However, in Parkinson's disease, the seemingly contradictory role of autophagy was revealed in the development of anxiety disorders. Yan *et al*. [18] found that atorvastatin, a protective agent against Parkinson's disease, alleviated anxiety and depression by reducing NADPH Oxidase 2-mediated autophagy in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned mice, suggesting that autophagy can induce anxiety. Whether autophagy promotes or alleviates anxiety is under debate and remains for further exploration. In particular, to date, there has been a paucity of research on the role of autophagy in morphine withdrawal–triggered anxiety-like behaviors. Thus, our present study primarily aimed to elucidate the potential involvement of autophagy in the etiology of anxiety-like behaviors triggered by protracted morphine abstinence in male mice and its involved mechanisms.

In this study, mice model of anxiety following morphine-protracted withdrawal was established by using protocols in accordance with our previous publication [29]. Anxiety-like behaviors was assessed by the OFT and EPM at days 3 and 4 after the final administration of morphine, when anxiety symptom was the most stable and pronounced. To figure out whether autophagy was involved in anxiety after morphine withdrawal and to delineate its specific role in this process, we used 3-MA to inhibit autophagy during morphine withdrawal. The results revealed that inhibition of autophagy rescues anxiety-like symptoms triggered by protracted morphine withdrawal in male mice. To our knowledge, this is the first study to report the relationship between autophagy and anxiety triggered by morphine withdrawal, which could be a potential target for therapeutics.

To further explore the underlying mechanisms implicated in autophagy-induced anxiety after morphine withdrawal, we proceeded to investigate the relevant brain regions and protein molecules involved. Because the Amy is a key brain region associated with morphine addiction and is closely linked to anxiety [22–24], we examined changes in autophagy levels within the Amy 4 days after the final morphine administration, a time point corresponding to peak anxiety symptoms. Western blot analysis was used to determine the total protein levels of ATG5, Beclin-1, and LC3, which are common markers for autophagy. The results demonstrated that autophagy-related protein levels in the Amy were upregulated following prolonged morphine withdrawal, suggesting that morphine withdrawal–induced autophagy activation in the Amy may contribute to the onset of anxiety-like behaviors.

To ascertain whether morphine itself induces alterations of autophagy within Amy, we also determined the level of autophagy in Amy 2 h after last morphine administration. The results suggested that morphine itself fails to induce changes in autophagy within Amy. In summary, we found that protracted morphine withdrawal (4 days) but not morphine itself (2 h) induced increased expression levels of autophagy-associated proteins in Amy of male mice. Coupled with the evidence that 3-MA effectively diminishes autophagy levels in Amy, we can identify Amy as a crucial brain region involved in morphine withdrawal–induced anxiety.

Multiple brain regions are involved in drug addiction or anxiety. It has been reported that the medium spiny neurons in the NAc can project to the VTA and ventral pallidum, with those expressing dopamine receptor D2 inhibiting the activity of GABAergic neurons in ventral pallidum via projections, thereby promoting anxiety [31].

In our previous study, we demonstrated that HCN1 in the LHb plays a role in mediating anxiety-like behaviors induced by morphine abstinence [32]. It also has been revealed that $LS^{GABAergic}$-lateral hypothalamus circuit is critical to modulate pain and anxiety comorbidities [33]. So we next examined the role of lateral hypothalamus, NAc, ventral pallidum, VTA, and LHb in morphine withdrawal–caused anxiety. Autophagy-associated proteins were tested by using western blot and no significant change within these five brain regions were observed after protracted withdrawal from morphine. The results seemed to suggest that the lateral hypothalamus, NAc, ventral pallidum, VTA, and LHb are not associated with autophagy-mediated anxiety after morphine withdrawal; however, it does not imply that they are completely unrelated. Because our modeling method may generate an artifact that autophagy alterations are confined to a particular brain region. A similar result was observed in a study conducted by Pan *et al.*'s [34] team. Pan *et al.* used a different modeling method in their study and found that the administration of chronic morphine predominantly triggers autophagy in hippocampus, rather than other brain regions. Different protocols of morphine administration may specifically promote autophagy within certain brain regions by activating different neural circuits. In addition, these five brain regions can also participate in morphine withdrawal–induced anxiety via different pathways besides autophagy, which warrants further study.

Collectively, under our experimental condition, the Amy is a specific brain region involved in autophagy-mediated anxiety-like behavior during protracted morphine withdrawal. Regrettably, we did not perform further validation experiments to increase the reliability of our results. Virus-mediated knockdown and overexpression of autophagy in the Amy could be used in future studies to confirm the effect of autophagy in the Amy on anxiety behaviors in morphine-withdrawn mice. At the same time, we also plan to prioritize multiomics approaches (e.g. qRT-PCR and RNA sequencing) in future studies to dissect the temporal dynamics of the mRNA expression of autophagy-related genes.

Another limitation of this present study is that we measured the expression levels of autophagy only at two-time points: 2 h and 4 days after morphine withdrawal, thus failing to provide an ongoing monitoring of autophagic activity. We will monitor the autophagy levels for various times in subsequent experiments and plot a dynamic curve to better reveal the changes in autophagy during this process.

The third limitation is that only male mice were used. We focused exclusively on male mice for the reason that female rodents exhibit cyclic fluctuations in estrogen and progesterone levels during the estrous cycle, which are known to modulate anxiety-like behaviors and confound interpretation of withdrawal-related phenotypes. Thus, the findings demonstrate limited generalizability to female mice populations, and consequently, caution must be exercised when extrapolating these conclusions to human female subjects. Given these considerations, we plan to conduct future investigations that incorporate sex as a biologically relevant variable in experimental design.

In summary, our present study demonstrates that protracted morphine abstinence can promote autophagy in the Amy, which may further induce anxiety-like behaviors in male mice. It is the first time to reveal the critical role of autophagy in anxiety caused by protracted morphine withdrawal. These novel findings shed light on the functional linkage between morphine withdrawal, autophagy, and anxiety, thereby indicating that inhibiting autophagy could serve as a potential therapeutic approach for anxiety-like behaviors induced by morphine withdrawal.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (NSFC) (No. 81971248 and 31200814).

Z.L. conceptualized and designed the study. C.Z. wrote and edited the article. S.H. carried out behavioral tests and made analysis. D.M. and S.H. performed and analyzed western blot. Z.L. revised the article and approved the submitted version.

Data will be available on request.

Conflicts of interest

There are no conflicts of interest.

References

1 Rosenthal A, Ebrahimi C, Wedemeyer F, Romanczuk-Seiferth N, Beck A. The treatment of substance use disorders: recent developments and new perspectives. *Neuropsychobiology* 2022; **81**:451–472.
2 Kong D, Sun JX, Yang JQ, YS LI, Bi K, Zhang ZY, *et al*. Ketogenic diet: a potential adjunctive treatment for substance use disorders. *Front Nutr* 2023; **10**:1191903.
3 Severino AL, Evans CJ. Digging through the roots of addiction: understanding the causes that underlie the insidious problem of substance use disorders (SUDs). *Neurosci Biobehav Rev* 2019; **106**:3–4.
4 Srivastava AB, Mariani JJ, Levin FR. New directions in the treatment of opioid withdrawal. *Lancet* 2020; **395**:1938–1948.
5 Blum K, Han D, Bowirrat A, Downs BW, Bagchi D, Thanos PK, *et al*. Genetic addiction risk and psychological profiling analyses for 'preaddiction' severity index. *J Pers Med* 2022; **12**:1772.
6 Sutter MB, Watson H, Yonke N, Weitzen S, Leeman L. Morphine versus methadone for neonatal opioid withdrawal syndrome: a randomized controlled pilot study. *BMC Pediatr* 2022; **22**:345.
7 Fleming A, Bourdenx M, Fujimaki M, Karabiyik C, Krause GJ, Lopez A, *et al*. The different autophagy degradation pathways and neurodegeneration. *Neuron* 2022; **110**:935–966.
8 Ren H, Zhai W, Lu X, Wang G. The cross-links of endoplasmic reticulum stress, autophagy, and neurodegeneration in Parkinson's disease. *Front Aging Neurosci* 2021; **13**.
9 Chen Y, Zhou X, Chu B, Xie Q, Liu Z, Luo D, Zhang J. Restraint stress, foot shock and corticosterone differentially alter autophagy in the rat hippocampus, basolateral amygdala and prefrontal cortex. *Neurochem Res* 2023; **49**:492–506.
10 Chen Y, Zhang W, Guo X, Ren J, Gao A. The crosstalk between autophagy and apoptosis was mediated by phosphorylation of Bcl-2 and beclin1 in benzene-induced hematotoxicity. *Cell Death Dis* 2019; **10**.

11 Stempels FC, Janssens MH, ter Beest M, Mesman RJ, Revelo NH, Ioannidis M, van den Bogaart G. Novel and conventional inhibitors of canonical autophagy differently affect LC3-associated phagocytosis. *FEBS Lett* 2022; **596**:491–509.
12 El-Hage N, Rodriguez M, Dever SM, Masvekar RR, Gewirtz DA, Shacka JJ. HIV-1 and morphine regulation of autophagy in microglia: limited interactions in the context of HIV-1 infection and opioid abuse. *J Virol* 2015; **89**:1024–1035.
13 Gao S, Li E, Gao H. Long non-coding RNA MEG3 attends to morphine-mediated autophagy of HT22 cells through modulating ERK pathway. *Pharm Biol* 2019; **57**:536–542.
14 He C, Xu Y, Sun J, Li L, Zhang JH, Wang Y. Autophagy and apoptosis in acute brain injuries: from mechanism to treatment. *Antioxid Redox Signal* 2023; **38**:234–257.
15 Minchev D, Kazakova M, Sarafian V. Neuroinflammation and autophagy in parkinson's disease – novel perspectives. *Int J Mol Sci* 2022; **23**:14997.
16 Eshraghi M, Adlimoghaddam A, Mahmoodzadeh A, Sharifzad F, Yasavoli-Sharahi H, Lorzadeh S, *et al*. Alzheimer's disease pathogenesis: role of autophagy and mitophagy focusing in microglia. *Int J Mol Sci* 2021; **22**:3330.
17 Su L-Y, Luo R, Liu Q, Su J-R, Yang L-X, Ding Y-Q, *et al*. Atg5- and Atg7-dependent autophagy in dopaminergic neurons regulates cellular and behavioral responses to morphine. *Autophagy* 2017; **13**:1496–1511.
18 Yan J, Huang J, Liu A, Wu J, Fan H, Shen M, *et al*. Atorvastatin improves motor function, anxiety and depression by NOX2-mediated autophagy and oxidative stress in MPTP-lesioned mice. *Aging (Milano)* 2021; **13**:831–845.
19 Li Y, Cheng Y, Zhou Y, Du H, Zhang C, Zhao Z, *et al*. High fat diet-induced obesity leads to depressive and anxiety-like behaviors in mice via AMPK/mTOR-mediated autophagy. *Exp Neurol* 2022; **348**:113949.
20 Tian Q, Fan X, Ma J, Han Y, Li D, Jiang S, *et al*. Resveratrol ameliorates lipopolysaccharide-induced anxiety-like behavior by attenuating YAP-mediated neuro-inflammation and promoting hippocampal autophagy in mice. *Toxicol Appl Pharmacol* 2020; **408**:115261.
21 Jacobsen JHW, Hutchinson MR, Mustafa S. Drug addiction: targeting dynamic neuroimmune receptor interactions as a potential therapeutic strategy. *Curr Opin Pharmacol* 2016; **26**:131–137.
22 Liu JF, Li JX. Drug addiction: a curable mental disorder? *Acta Pharmacol Sin* 2018; **39**:1823–1829.
23 Volkow ND, Morales M. The brain on drugs: from reward to addiction. *Cell* 2015; **162**:712–725.
24 Meisner OC, Nair A, Chang SWC. Amygdala connectivity and implications for social cognition and disorders. In: Aminofe MJ, Boller F, Swab DE, editors. *The temporal lobe*. Handbook of Clinical Neurology Series; 2022. pp. 381–403.
25 Šimić G, Tkalčić M, Vukić V, Mulc D, Španić E, Šagud M, *et al*. Understanding emotions: origins and roles of the amygdala. *Biomolecules* 2021; **11**:823.
26 Ahrens S, Wu MV, Furlan A, Hwang G-R, Paik R, Li H, *et al*. A central extended amygdala circuit that modulates anxiety. *J Neurosci* 2018; **38**:5567–5583.
27 Ventura-Silva AP, Melo A, Ferreira AC, Carvalho MM, Campos FL, Sousa N, Pêgo JM. Excitotoxic lesions in the central nucleus of the amygdala attenuate stress-induced anxiety behavior. *Front Behav Neurosci* 2013; **7**:32.
28 Johnson J, Li Z. Fuel for emotion: how mitophagy in the BLA can mediate increased anxiety in stressful social situations. *Autophagy* 2021; **18**:465–466.
29 Ye H, Cao T, Shu Q, Chen Y, Lu Y, He Z, Li Z. Blockade of orexin receptor 1 attenuates morphine protracted abstinence-induced anxiety-like behaviors in male mice. *Psychoneuroendocrinology* 2023; **152**:106108.
30 Liu LT, Song YQ, Chen XS, Liu Y, Zhu JJ, Zhou LM, *et al*. Morphine-induced RACK1-dependent autophagy in immortalized neuronal cell lines. *Br J Pharmacol* 2020; **177**:1609–1621.
31 Correia R, Coimbra B, Domingues AV, Wezik M, Vieitas-Gaspar N, Gaspar R, *et al*. Involvement of nucleus accumbens D2-medium spiny neurons projecting to the ventral pallidum in anxiety-like behaviour. *J Psychiatry Neurosci* 2023; **48**:E267–E284.
32 Li Z, Shu Q, Chen Q, Yang H, Liu L, He Z, *et al*. HCN1 in the lateral habenula contributes to morphine abstinence-induced anxiety-like behaviors in male mice. *J Psychiatr Res* 2024; **171**:185–196.
33 Wang D, Pan X, Zhou Y, Wu Z, Ren K, Liu H, *et al*. Lateral septum-lateral hypothalamus circuit dysfunction in comorbid pain and anxiety. *Mol Psychiatry* 2023; **28**:1090–1100.
34 Pan J, He L, Li X, Li M, Zhang X, Venesky J, *et al*. Activating autophagy in hippocampal cells alleviates the morphine-induced memory impairment. *Mol Neurobiol* 2016; **54**:1710–1724.