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NADPH-oxidase 4 promotes autophagy in spinal neurons through activating ER stress during the development of morphine tolerance

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Abstract

Morphine tolerance is one of the current challenging issues in the treatment of chronic pain. Recent studies have shown that ROS derived by NADPH oxidase (NOX) and endoplasmic reticulum (ER) stress is participated in the development of morphine tolerance. However, which NOX subtype initiates the ER stress during the development of morphine tolerance is not fully clear. NOX4 mainly expressed at intracellular membranes, such as ER and mitochondria, which sole function is to produce ROS as the major product. At present, whether NOX4 is activated and the mechanisms between NOX4 and ER stress during the development of morphine tolerance still need to be confirmed. Here, our research, for the first time, demonstrated that chronic administration of morphine up-regulated the expression of NOX4 at spinal cord through activating the three ER stress sensors (PERK, IRE1, ATF6), and subsequently leading to the activation of LC3B and P62 (a well-known autophagy marker) in GABAergic neurons. Therefore, our results may suggest that regulating NOX4 and the key factor of ER stress or autophagy may be a promising strategy to treat and prevent the development of morphine tolerance.

Introduction

Opioids, such as morphine, are extensively used in the treatment of postoperative pain and chronic pain as its powerful analgesic effects[1]. However, repeated administration of morphine may lead to analgesic tolerance[2], and need higher dose of drugs to achieve equivalent analgesic effect than usually, which significantly limit its clinical application. Over the past decades, some evidence has suggested multiple mechanisms of morphine tolerance, including internalization and desensitization of opioid receptor[3], upregulation of cAMP pathway[4], and autophagy in spinal neurons[5]. However, the exact mechanisms responsible for morphine tolerance are incompletely investigated.

Autophagy is an important cellular degradative mechanism by which cells use lysosomes to degrade misfolded proteins and damaged organelles and the molecular components are recycled and reintroduced for cellular metabolism[6]. It is showed that basal level of autophagy is critical for maintaining homeostasis during cellular stress. Furthermore, the abnormal of autophagy plays a key role in the development of Alzheimer's disease and Parkinson's disease[7]. Thus, a balanced level of autophagy is contributs to maintaining of normal cellular functions.

The functions of endoplasmic reticulum (ER) include protein synthetic and quality control, lipid metabolism and Ca^{2+} buffering[8]. Generally, ER stress is defined as the accumulation of misfolded or unfolded proteins in the ER lumen[9]. Then, the three unfolded protein response (UPR) sensors: protein kinase R like ER kinase (PERK), inositol-requiring protein 1 α (IRE−1 α) and activating transcription factor 6 (ATF6) are activated to restore the ER homeostasis. These sensors are usually rendered inactive and capped by the ER chaperone BIP under normal conditions[10]. However, excessive or prolonged ER stress inevitably initiates autophagy. Autophagy can reduce the unfolded and misfolded proteins and restore the ER homeostasis via negative feedback regulation.

The NADPH (NOX) oxidase family contains NOX1-5, Duox1 and Duox2, which sole function is to produce ROS as the major product[11]. On the one hand, high concentrations of ROS affect the transduction of REDOX signals[12]; and on the other hand, it would also inevitably affect post-translational modifications, which subsequently leading to ER stress to reestablish homeostasis[13]. Recent studies have shown that NOX oxidase is closely related to ER Stress[14]. However, which subtype initiates the ER stress during the development of morphine tolerance is still unclear. NOX4 is expressed in ER and mitochondria, which sole function is to produce ROS as a major product at intracellular membranes[15]. Recent study found that NOX4 participated in the development of neuropathic pain and cancer-induced bone pain[16]. However, the mechanisms between NOX4 and ER stress during the development of morphine tolerance still need to be confirmed.

Thus, we hypothesize that NOX4 is a critical factor-initiated ER stress and participated in the development of morphine tolerance by regulating autophagy in GABAergic neurons at the spinal cord. NOX4 may a new regulatory target for administration of morphine tolerance.

Materials And Methods

Materials and Methods

Animals

The male Sprague-Dawley rats (200–250g) were purchased from the Animals Experiment Center of Zhengzhou University (Zhengzhou, Henan, China). All rats were individually housed in clear plastic cages (water and food ad libitium,21 ± 1°C, humidity 45%–55%, 12–12h light/dark cycle). All experimental procedures were approved and reviewed by the Experimental Animals Care and Use Committee of Zhengzhou University, and consistent with the guidelines provided by the National Institutes of Health for the Care and Use of Laboratory Animals.

Intrathecal Catheterization

Intrathecal catheters were conducted following a previous study[17]. In brief, rats were anesthetized with phenobarbital (60 mg/kg,ip.), and then a sterile PE–10 catheter (inner diameter 0.3 mm,outer diameter 0.5 mm,Anilab Software & Instruments, Ningbo, Zhejiang,China) was insert into the subarachnoid cavity between L_4 and L_5 vertebral level, the catheter was fixed and tunneled to the back of neck. To confirm the placement of catheter, 10μl of 2% lidocaine was injected into subarachnoid through the catheter on the third day. Showing an immediate temporary motor block of both hind limbs considered to have proper placement. Then, rats were allowed to recover 7 days for the behavioral experiments.

Drug Administration

The morphine hydrochloride (2 μg/μl, Shenyang First Pharmaceutical Factory, Shenyang,China) was diluted in saline. The GLX351322 (Med Chem Express, MCE, China) was dissolved in 10% (v/v) DMSO

(Solarbio® Life Sciences, Beijing, China) to 100 μM and stored at−80°C until use. The 4-PBA (Selleck Chemical, Houston, USA) was diluted in 30% (v/v) DMSO and stored at−80°C until use. GLX351322(20 μM,15 μl)[18], 4-PBA(100 μg)[19] or vehicle were administrated through the PE−10 catheter 30 min before morphine injection and followed by 10 μl saline to flush the PE−10 catheter.

Morphine Tolerance and Behavioral Assessment

Morphine (10 μg, twice daily) was intrathecally injected for 9 consecutive days to induce morphine tolerance. An equal volume of saline was received in control group at the same time points. The antinociceptive effect of morphine was assessed 30 min after morphine intrathecally injected by the hot water tail-flick assay from day 1 to day 9, and the tail-flick test was performed in the morning. Briefly, the animals were placed in a plastic box, and the distal one-third of the tail was immersed into the water at 50 ± 0.2°C. The latency response was defined as the rapid removal of the tail remaining in the hot water. A 15 s cutoff time was used to minimize the tail injury. The analgesic effect of morphine was used to evaluate by the percentage of maximal possible antinociceptive effect (%MPE), %MPE=[(test latency-baseline latency)]/(15s - baseline latency).

Western Blots

Western Blots were conducted by a previous research[20]. Briefly, after 9 days of behavioral tests, rats were anesthetized with pentobarbital sodium (i.p. 60mg/kg), the segments of L_3-L_5 spinal cord were rapidly removed and homogenized using radio immunoprecipitation assay lysis buffer combined with phenylmethylsulfonyl fluoride (PMSF) and phosphatase inhibitor. Proteins concentration of the supernatants were gauged by using bicinchonininc acid (BCA) protein quantitation kit. Twenty-five μg proteins were separated and loaded on 10% or 12% SDS-PAGE, and transferred onto polyvinylidene fluoride membrane (Millipore, USA). The membrane was blocked by 5% bovine serum albumin (BSA) for 1.5h at room temperature and incubated overnight at 4°C with the primary antibodies (Table 1). Then, the membrane was washed and incubated with HRP-conjugated second antibodies. Finally, the membrane was detected with ECL reagent (K22030, Abbkine, Wuhan, China), and the results were measured and normalized to GAPDH using the Image Lab software (Bio-Rad,USA).

Table 1
The primary and secondary antibodies for WB and IF.

Antibody	Provider	Host	Catalog number	Dilution for WB	Dilution for IF
NOX4	Proteintech	Rabbit	11587-AP	1:1000	1: 40
BIP	Proteintech	Rabbit	14347-AP	1:1000	1: 35
PERK	Affinity	Rabbit	AF5034	1:1000	
P-PERK	Affinity	Rabbit	DF7576	1:1000	1: 35
IRE1	Affinity	Rabbit	DF7709	1:1000	
P-IRE1	Affinity	Rabbit	AF7150	1:1000	1: 40
ATF6	Affinity	Rabbit	DF6009	1:1000	1: 35
LC3B	Abmart	Rabbit	T55992	1:1000	1: 35
P62	Abmart	Rabbit	T55546	1:1000	
GAPDH	Proteintech	Rabbit	10494–1-AP	1:5000	
NeuN	Proteintech	Mouse	66836–1-Ig		1: 100
GFAP	CST	Mouse	#3670		1: 200
Iba–1	Abcam	Goat	Ab5076		1: 100
GAD67	Abmart	Mouse	MG510328		1: 40
Anti-Rabbit IgG HRP	Aspen	Goat	AS1107	1:5000	
CoraLite594 – conjugated Goat Anti-Rabbit IgG(H + L)	Proteintech	Goat	SA00013–4		1:100
CoraLite488-conjugated Goat Anti-Mouse IgG(H + L)	Proteintech	Goat	SA00013–1		1:100
CoraLite594 – conjugated Donkey Anti-Rabbit IgG(H + L)	Proteintech	Donkey	SA00013–8		1:100
Fluorescein (FITC)–conjugated Affinipure Donkey Anti-Goat IgG(H + L)	Proteintech	Donkey	SA00003–3		1:100

Immunofluorescence Staining

Rats were anesthetized with pentobarbital 60 mg/kg,i.p, and perfused with ice-cold 0.01M PBS and followed by 4%(v/v) paraformaldehyde (PFA) in 0.01M PBS. The L_3-L_5 spinal cord lumbar were immediately dissected and fixed in 4%(m/v) PFA at 4°C overnight, dehydrated in 20% and 30% sucrose solution for 48h at 4°C, and embedded in paraffin. The spinal tissue was sliced in 3 μm by a cryostat

(RM2245, Leica, Germany). Next, the section was dried at 65°C for 1h, and rinsed with PBS, Tris-EDTA buffer (PH9.0) was used to perform antigen repair. The sections were blocked with 10% donkey serum for 40 min at 37°C, and then incubated with the primary antibody (Table 1) overnight at 4°C, washed with 0.01M PBS and incubated with the followed secondary antibody (Table 1). The images were captured by a fluorescence microscope (ZIESS, Germany), and were analyzed by Image-Pro Plus 4 software(Media Cybernetics, Maryland, USA).

For double immunofluorescence, the sections were incubated with each primary antibody (Table 1) and NeuN/GFAP/Iba−1 overnight at 4°C, and then washed with 0.01M PBS and mixed with the secondary antibody (Table 1) for 2 h at room temperature. The images were captured by a fluorescence microscope (ZIESS, Germany), and were analyzed by Image-Pro Plus 4 software (Media Cybernetics, Maryland, USA).

Transmission Electron Microscopic Examination

Rats were anesthetized with pentobarbital 60 mg/kg, i.p, and transcardially perfused with ice-cold 0.01 M PBS and 4%(m/v) PFA. The L_3-L_5 spinal cord were quickly removed and fixed in 2.5% (v/v) glutaraldehyde for 4h at 4°C, post-fixed with 1% osmium tetroxide in 0.01 M PBS for 2 h at 20°C, dehydrated and embedded in epoxy resin, the spinal segments were sliced in 60 nm thickness ultramicrotome and stained with 2%(v/v) uranyl acetate and lead citrate. All images were captured by a Hitachi 7700 TEM system.

Statistical Analyses

All the data were presented as mean ± SEM and analyzed using Graphpad Prism 9 (Graphpad Software,San Diego,USA). The data of behavioral tests were analyzed by two-way ANOVA followed by Bonferroni Post hoc tests to assess the changes between each group after drug injection each time. The results of western blot were analyzed by one-way ANOVA followed by Post hoc Tukey's tests. All the statistical significance were considered at $P < 0.05$.

Results

1. Repeated administration of morphine promotes antinociceptive tolerance

Rats in MT group was intrathecally injected morphine (10 μg/5 μl, twice a day, 09:00, 16:00) for consecutive 9 days. An equal volume of saline was administrated at the same time in sham group rats. The behavior test was assessed 30 min after morphine or saline intrathecally injected by the hot water tail-flick assay on day 1, 3, 5, 7 and day 9. The baseline latency of hot water tail-flick was similar among all the rats. Figure 1B The %MPE of morphine were strongest on the day 1 and gradually decreased from day 3 to day 9. There was no difference in %MPE between intrathecally injected morphine and saline on day 7 and day 9. These demonstrated the model of morphine tolerance was successful establishment.

2. Activation of NOX4 and BIP in the spinal cord participated in the development of morphine tolerance
To clarify the role of NOX4 in the spinal cord during the repeated administration of morphine, we

analyzed the protein level of NOX4 and BIP using western blots. Figure 2A On day 9, the protein level of NOX4 and BIP at the spinal cord in morphine tolerant rats was significantly increased when compared with naïve group and sham group. Furthermore, we examined the cellular localization of NOX4 and BIP in the spinal dorsal horn using double immunofluorescence staining. Figure 2B,C showed that NOX4 and BIP was highly expressed in spinal dorsal horn. Both NOX4 and BIP were mainly coexpressed with NeuN, and a small part of coexpressed with Iba−1, but not coexpressed with GFAP. These data showed that activation of NOX4 and BIP in the spinal cord participated in the development of morphine tolerance.

3. NOX4 contributed to the development of morphine tolerance by initiating the ER stress response and autophagy in the spinal cord

To investigate the role of spinal NOX4 during the development of morphine tolerance, we adopt intrathecal injection the specific NOX4 inhibitor GLX351322(20 μM, 15 μl, once a day) 30 min before morphine administration. Figure 3A showed that GLX351322 did not affect on the %MPE from day 1 to day 9, while the level of %MPE by daily GLX351322 co-administration with morphine was significantly higher than morphine tolerance group rats. Figure 3B In morphine-tolerant rats, the endoplasmic reticulum at spinal cord was significantly swollen compared with sham group rats measured by transmission electron microscopy (TEM). GLX351322 pretreatment inhibited the activation of NOX4 and subsequently down-regulated the expression of ER stress sensors BIP, PERK, p-PERK, IRE1, p-IRE1and ATF6. These tests were performed by western blots Fig. 3C. Next, we investigate the cellular localization of BIP, p-PERK, p-IRE1, ATF6 using double immunofluorescence staining. Figure 3D, E showed that p-PERK and p-IRE1 were mainly coexpressed with NeuN, and a small part coexpressed with GFAP and Iba-1. Figure 3F ATF6 were coexpressed with NeuN, GFAP. These results showed that NOX4 contributed to the development of morphine tolerance by initiating the ER stress response.

To further examination the role of NOX4 and autophagy during morphine-induced autophagy, we adopted intrathecal injection the specific NOX4 inhibitor GLX351322(20 μM,15 μl, once a day) 30 min before morphine administration. Figure 3C The activation of NOX4, LC3B and P62 induced by repeated administration of morphine was significantly inhibited by GLX351322, which prevent the development of morphine tolerance. Next, we clarify the cellular localization of LC3B in spinal dorsal horn. Figure 3G showed that LC3B was mainly coexpressed with NeuN. These data indicate that NOX4 contributed to the development of morphine tolerance by initiating the ER stress response and autophagy in the spinal cord.
4. The role of ER stress during repeated administration of morphine-mediated autophagy

There were some ways to induce autophagy, in this work, ER stress pathway were focused. To confirm the role of ER stress in mediating autophagy during chronic administration of morphine, we used intrathecal injection an ER stress inhibitor 4-PBA (100 μg, once a day) 30 min before morphine administration.

Figure 4A showed that 4-PBA did not affect on the %MPE from day 1 to day 9, while the level of %MPE by daily 4-PBA co-administration with morphine was significantly higher than morphine tolerant rats. Statistical analysis showed that there was not significantly difference between sham group and 4-PBA group rats. Figure 4B,C The activation of NOX4, BIP, PERK, p-PERK, IRE1, p-IRE1, ATF6, LC3 and P62 induced by repeated administration of morphine was significantly inhibited by 4-PBA. These results demonstrate that ER stress mediates autophagy and plays a significant role during chronic administration of morphine. Figure 4D The number of autophagic vacuoles at spinal cord in morphine-tolerant rats were higher than sham group rats measured by TEM (indicated by arrows).

To examination autophagy induced by chronic administration of morphine in the spinal cord, we used LC3B to mark autophagosomes, and GAD67 to label GABAergic neurons at the spinal cords in morphine tolerance rats. Figure 4E showed that LC3B was mainly localized in GAD67 positive neurons. These results indicate that autophagy induced by chronic administration of morphine in the spinal cord is mainly localized in GABAergic neurons.

Discussion and Conclusions

In this work, we demonstrated that chronic administration of morphine upregulated the expression of NOX4 at spinal cord through activating ER stress response, and subsequently leading to the upregulation of autophagy in GABAergic neurons. Furthermore, blocking NOX4 activation could repress ER stress response and autophagy at spinal cord, which significantly prevented the development of morphine tolerance. Taken together, our study, for the first time, shows the importance of NOX4 and ER stress response and autophagy in GABAergic neurons at spinal cord during the development of morphine tolerance.

GABAergic neurons are the primary inhibitory neurons in the central nervous system[21] and play a critical role during the modulate nociception through inhibiting the release of glutamate[22]. Interestingly, A recent study reported that repeated administration of morphine up-regulation the expression of GFAP was involved the ER stress-autophagy axis in brain; and there was not autophagy in the midbrain GABAergic neurons during chronic morphine administration[23]. Moreover, another studies reported that there was not autophagy in microglia or neurons during repeated administration of morphine[24, 25]. Conversely, our work showed that ER stress mediated autophagy was mainly co-expressed with GABAergic neurons, and non-coexpressed with GFAP or Iba-1 at spinal cord during the development of morphine. This may be related with different animals and methods used in the research, and multiple mechanisms between in the brain and spinal cord during chronic administration of morphine.

ER stress is defined as the accumulation of misfolded or unfolded proteins in the ER lumen. Under normal condition, BIP, a well-known ER stress sensor, was inactive and capped with three UPR sensors PERK, ATF6, IRE-1[26]. Under stress condition, BIP was activated and isolated from the three sensors, which initiated the UPR response to restore ER homeostasis and enhanced the adaptive capacity[27]. The main

effect of PERK was to attenuate the newly translation proteins of mRNA into the stressed ER lumen and increased the expression of autophagy genes to re-establish homeostasis[28]. Studies revealed that IRE-1 was able to promote the expression of chaperone regulate the degradation of damaged proteins[29] and ATF6 is participated in maintaining the ER homeostas by affect Golgi translocation and promoted the release of bZIP transcription factor[30].

Studies shown that the PERK pathway promote basolateral amygdala GABAergic neuron injury participated in the development of stress-induced mental disorders[31]; and the IRE1 signaling exacerbates Alzheimer's disease pathogenesis[32]; the ER stress-ATF6 axis mediates cellular senescence through p38 pathway[33]. Moreover, these pathways participated in the development of neurodegenerative disorder,such as Alzheimer’s disease and Parkinson’s disease[34]. In particular, whether ER stress response contributes to autophagy in GABAergic neurons at spinal cord during repeated administration of morphine is still unclear.

Our findings indicated that BIP and the three main ER stress sensors (PERK, ATF6 and IRE1) participated in the development of morphine tolerance. In spinal cord, BIP and were mainly co-expressed with NeuN; p-PERK, p-IRE1 and ATF6 were co-expressed with NeuN, GFAP, and Iba-1. These results shows that the interaction between neurons and glial cell during the development of morphine tolerance. Next, we found that 4-PBA (an ER stress inhibitor) significantly down-regulated the expression of BIP, p-PERK, IRE1 and ATF6, and subsequently leaded to the activation of LC3B and P62, a well-known marker of autophagy at spinal cord during the development of morphine tolerance. LC3B is mainly co-expressed with GAD67, a marker of GABAergic neurons. Taken together, our study for the first time demonstrates a direct link between ER stress and autophagy in GABAergic neurons at spinal cord during the development of morphine tolerance.

Furthermore, studies shown that elevated level of ROS contributes to the development of morphine tolerance[35], which would inevitably affect post-translational modifications, and subsequently leading to ER stress to reestablish homeostasis[36]. Moreover, NOX oxidase is the major source for superoxide generation during the repeated administration of morphine[37]. The NOX oxidase family contains NOX1-5, Duox1 and Duox2, which sole function is to produce ROS as the major product. However, the mechanisms of different NOX subtype in morphine tolerance remains known little.

NOX4 is the first discovered as a specific subtype in renal, which has been detected in nociceptive system and cardiovascular system in recent study[38]. It is mainly expressed at intracellular membranes, such as ER and mitochondria[39]. Recent studies found that NOX4 participated in the development of neuropathic pain[40] and cancer-induced bone pain[41]. However, whether NOX4 is activated and the exact mechanisms between NOX4 and ER stress during the development of morphine tolerance still need to be confirmed.

Study have indicated that NOX4 specifically activate IRE1α but no other ER stress sensors participated in the development of hypertension[42]. Our results shown that the three ER stress sensors were activated

and NOX4 was mainly coexpressed with NeuN, indicating that there may be different mechanisms between hypertension and morphine tolerance. Furthermore, muscosterone relieves inflammatory pain through downregulated the expression of NOX4 and NLRP3 inflammasome[43]. Similarly, downregulated the expression of NOX4 protect GABAergic neurons from cell death in the amelioration of neuropathic pain at injured spinal cord[44]. These data indicated that NOX4 is a key target for treatment of chronic pain. We for the first time found that GLX351322, a specific inhibitor of NOX4[45], significantly inhibit autophagy in GABAergic neurons through these three pathways of ER stress at spinal cord, which prevent the development of morphine tolerance.

In summary, our study, for the first time, demonstrated that chronic administration of morphine upregulated the expression of NOX4 at spinal cord, which activatited the three ER stress sensors (PERK, IRE1, ATF6), and subsequently lead to the activation of LC3B and P62, a well-known autophagy marker, in GABAergic neurons. Therefore, modulating the key factor of NOX4, ER stress, or autophagy may be a promising strategy to treat and prevent the development of morphine tolerance.

Abbreviations

ALS, amyotrophic lateral sclerosis

ANOVA, analysis of variance

ATF6, activating transcription factor6

BCA, bicinchonininc acid

BIP, binding immunoglobulin protein

BL, baseline latency

BSA, bovine serum albumin

cAMP, adenosine 3',5'monophosphate

DAPI, 4, 6-diamidino-2-phenylindole

ER, endoplasmic reticulum

GAD, glutamic acid decarboxylase

GAPDH, glyceraldehyde-3-phosphatedehydrogenase

GFAP, glial fibrillary acidic protein

HRP, horseradish peroxidase

Iba-1, ionized calcium-binding adapter molecule 1

IRE1, inositol-requiring enzyme 1

%MPE, maximal possible antinociceptive effect

NeuN, neuronal nuclei

NOX, NADPH-oxidases

PBS, phosphate buffer saline

PERK, protein kinase RNA-like ER kinase

PFA, paraformaldehyde

PVDF, polyvinylidene fluoride

ROS, reactive oxygen species

RT, room temperature

SCI, spinal cord injury

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM, mean of standard error

TBS, tris buffered saline

TEM, transmission electron microscopy

UPR, unfolded protein response

Declarations

Acknowledgements

None.

Ethical Approval

All experimental procedures were approved and reviewed by the Experimental Animals Care and Use Committee of Zhengzhou University, and consistent with the guidelines provided by the National Institutes of Health for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Xuyang Xiao, Zhisong Li, and Huilian Bu Conceived and designed the project; Xuyang Xiao, Jingjie Yang, Yue Si, and Yaowei Xu performed the experiments; Xuyang Xiao, Qian Bai, Zhitao Wang and Yan Chen interpreted and analyzed the experiments data Xuyang Xiao drafted the paper, Huilian Bu and Zhisong Li revised the manuscript. All authors read and approved the final version of the research for publication.

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Availability of data and materials

The raw data supporting the results of this study are available from the corresponding author upon reasonable request.

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Figures

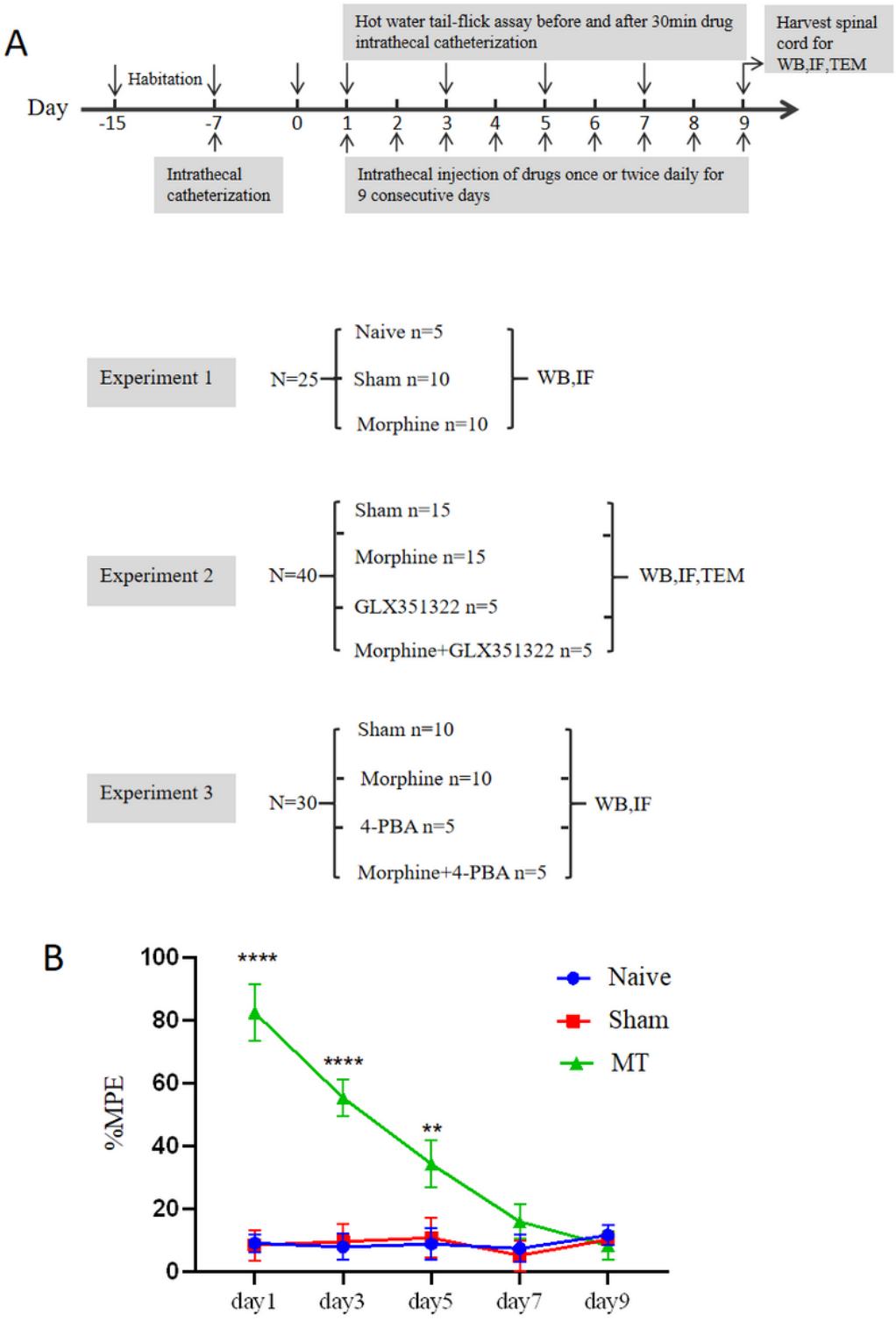

Figure 1

Repeated administration of morphine promotes antinociceptive tolerance

(A) Experiments scheme, sample size and animal groups. There were 25 rats employed in experiments 1 (15 rats were employed in WB, and 10 rats were employed in IF, n=5 each group). In experiments 2 and experiments 3, 20 rats were employed in WB, 10 rats were employed in IF, and 10 rats were employed in TEM, n=5 each group.

(B) The %MPE of repeated injection intrathecally morphine(10 μg/5 μl, twice daily) on day 7 and day 9 were significantly decreased compared with the BL on day1 (**P<0.01, ****P<0.001, compare with naïve group, n=5 in each group). %MPE, maximal possible antinociceptive effect; BL, baseline latency; WB, western blots; IF, immunofluorescence staining; TEM, transmission electron microscopy.

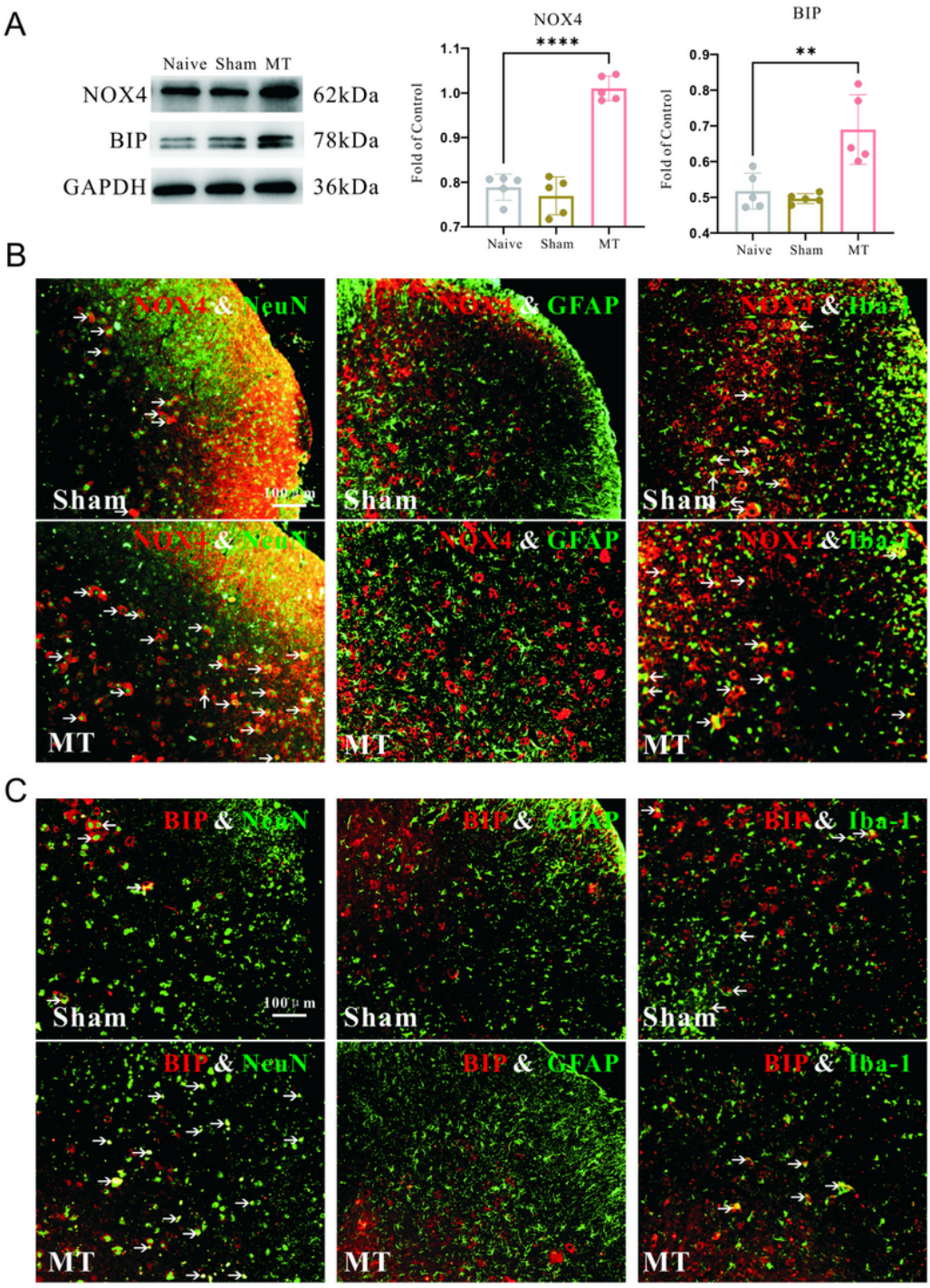

Figure 2

The expression of NOX4 and BIP at spinal cord in morphine-tolerance rats

(A) The expression of NOX4 and BIP was significantly increased in morphine-tolerant rats as measured by WB (**P <0.01, ****P <0.0001).

(B) The double immunostaining results showed that NOX4 was mainly coexpressed with NeuN, and a small part of NOX4 was coexpressed with Iba-1 (indicated by arrows).

(C) The double immunostaining results showed that BIP was mainly coexpressed with NeuN, and a small part of BIP was coexpressed with Iba-1 (indicated by arrows). NOX4, NADPH-oxidase 4; BIP, binding immunoglobulin protein; Iba-1, ionized calcium-binding adapter molecule 1; NeuN, neuronal nuclei.

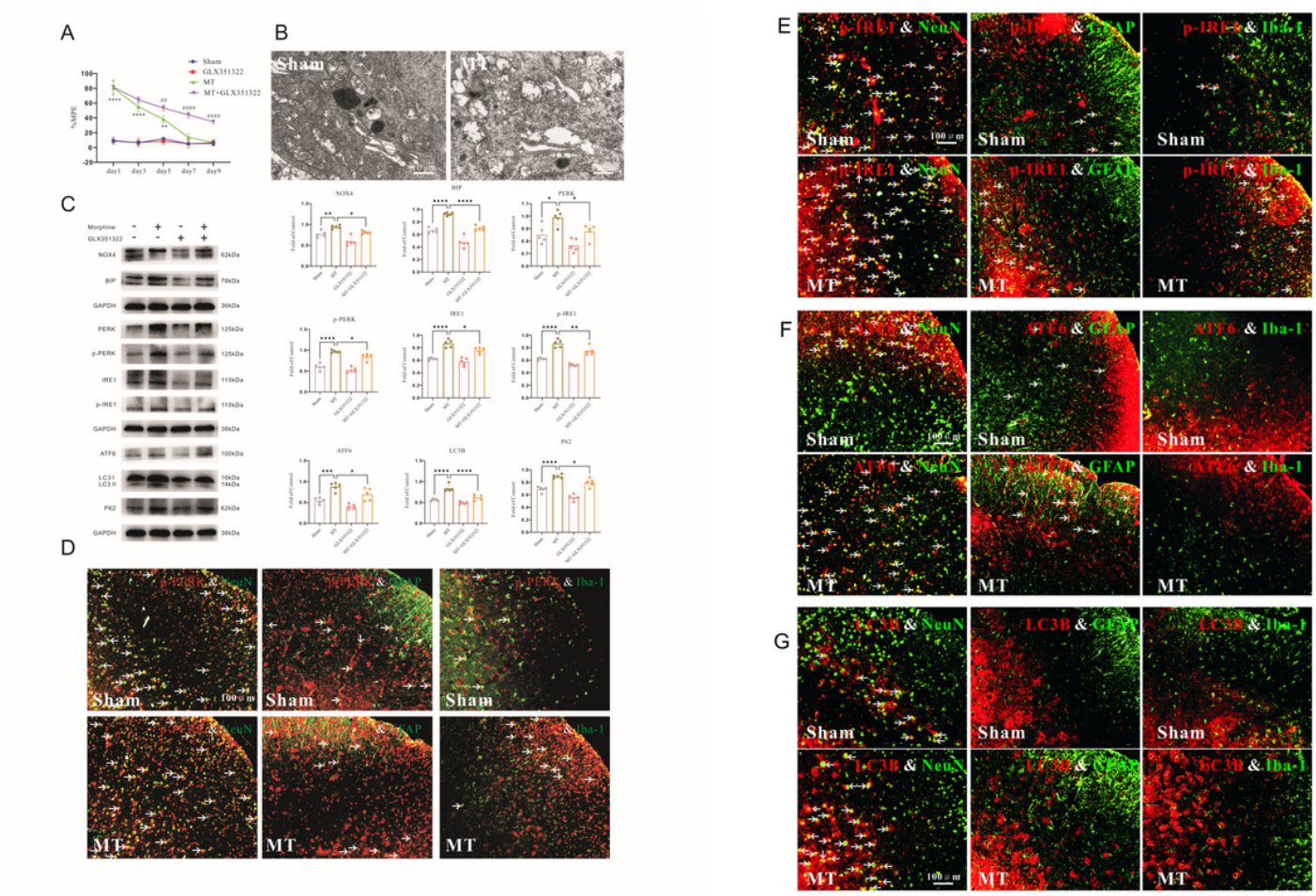

Figure 3

Effect of NOX4 specific inhibitor GLX351322 during the development of morphine tolerance

(A) Rats were intrathecally injected GLX351322 (20 μM,15 μl, once daily) for consecutive 9 days. The %MPE in rats pretreatment with GLX351322 prevent the development of morphine tolerance (**P<0.01, ****P<0.0001, compared with the baseline; $^{\#\#}P$<0.01,$^{\#\#\#\#}P$<0.0001, compared with the MT group).

(B) The results of TEM in spinal cord. The normal ER in sham group and the swollen ER in MT group (indicated by arrows)

(C) The expression of NOX4, the ER stress marker (BIP, PERK, p-PERK, IRE1, p-IRE1, ATF6) and the autophagy marker (LC3B and P62) were significantly increased in morphine-tolerant rats (*P<0.05,**P<0.01,***P<0.001,****P<0.0001). Pretreatment with GLX351322 before intrathecal injection of morphine

significantly downregulated the expression of NOX4, the ER stress marker (BIP, PERK, p-PERK, IRE1, p-IRE1, ATF6) and the autophagy marker (LC3B and P62) compared with morphine-tolerant rats. (*P <0.05,**P <0.01,****P <0.0001).

(D) The double immunostaining results showed that p-PERK was mainly coexpressed with NeuN, and a small part of p-PERK was coexpressed with GFAP and Iba-1 (indicated by arrows).

(E) The double immunostaining results showed that p-IRE1 was mainly coexpressed with NeuN, and a small part of p-PERK was coexpressed with GFAP and Iba-1 (indicated by arrows).

(F) The double immunostaining results showed that ATF6 was mainly coexpressed with NeuN, and a small part of ATF6 was coexpressed with GFAP (indicated by arrows).

(G) The double immunostaining results showed that LC3B was mainly coexpressed with NeuN. %MPE, maximal possible antinociceptive effect; TEM, transmission electron microscopy; ER, endoplasmic reticulum; MT, morphine tolerance; PERK, protein kinase RNA-like ER kinase; IRE1, inositol-requiring enzyme 1; ATF6, activating transcription factor6; GFAP, glial fibrillary acidic protein.

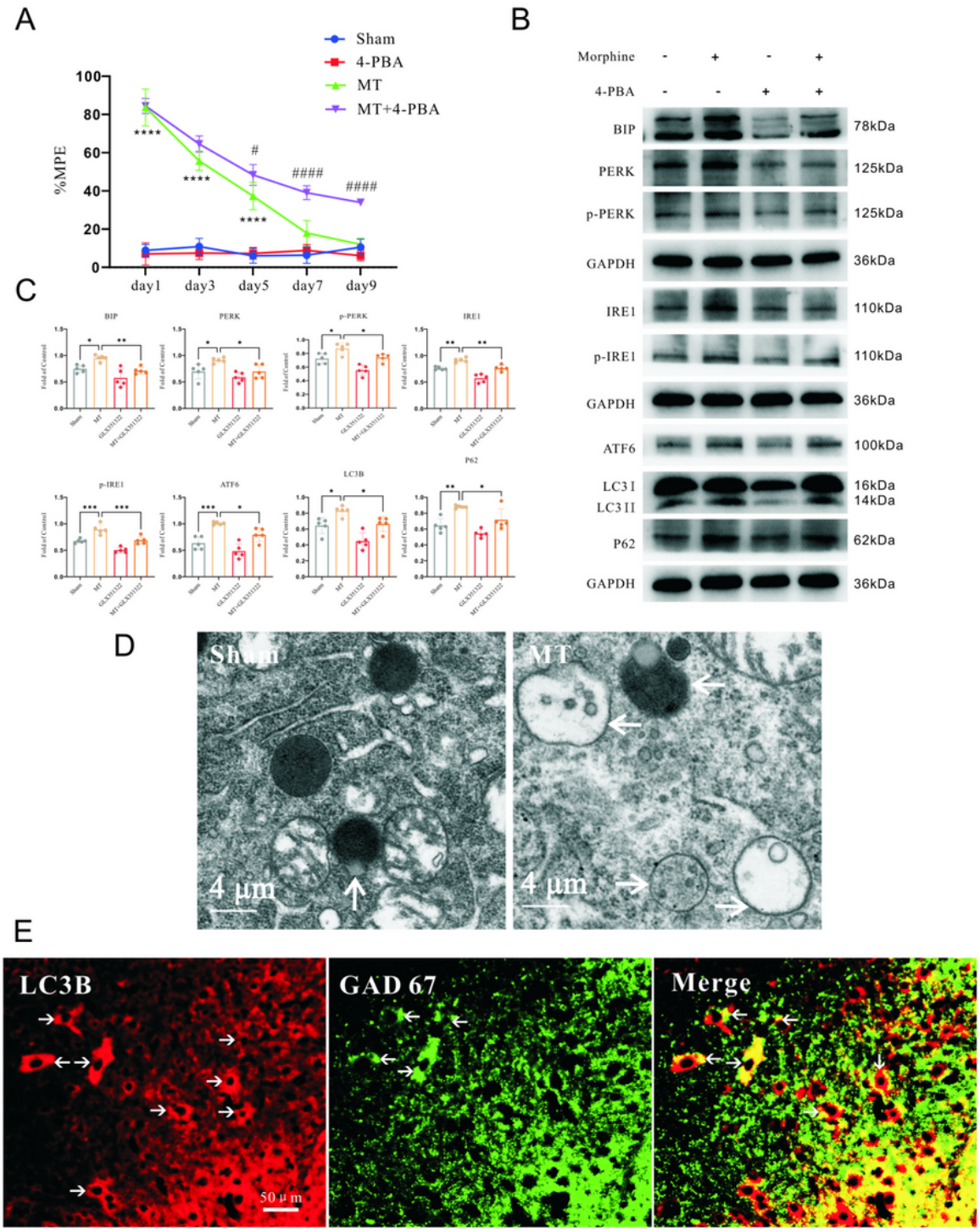

Figure 4

Effect of ER stress inhibitor 4-PBA during the development of morphine tolerance

(A) Rats were intrathecally injected 4-PBA (100 μg, once daily) for consecutive 9 days. The %MPE in rats pretreatment with 4-PBA prevent the development of morphine tolerance (****P <0.0001, compared with the baseline; #P <0.05 ####P <0.0001, compared with the MT group).

(B, C) Pretreatment with 4-PBA before intrathecal injection of morphine significantly downregulated the expression of the ER stress marker (BIP, PERK, p-PERK, IRE1, p-IRE1, ATF6) and the autophagy marker (LC3B and P62) compared with morphine-tolerant rats. (*P<0.05,**P<0.01,***P<0.001).

(A) The results of TEM in spinal cord. The number of autophagic vacuoles in MT group was higher than sham group (indicated by arrows).

(B) The double immunostaining results showed that LC3B was mainly localized in GAD67 positive neurons (indicated by arrows). GAD67, glutamic acid decarboxylase 67.

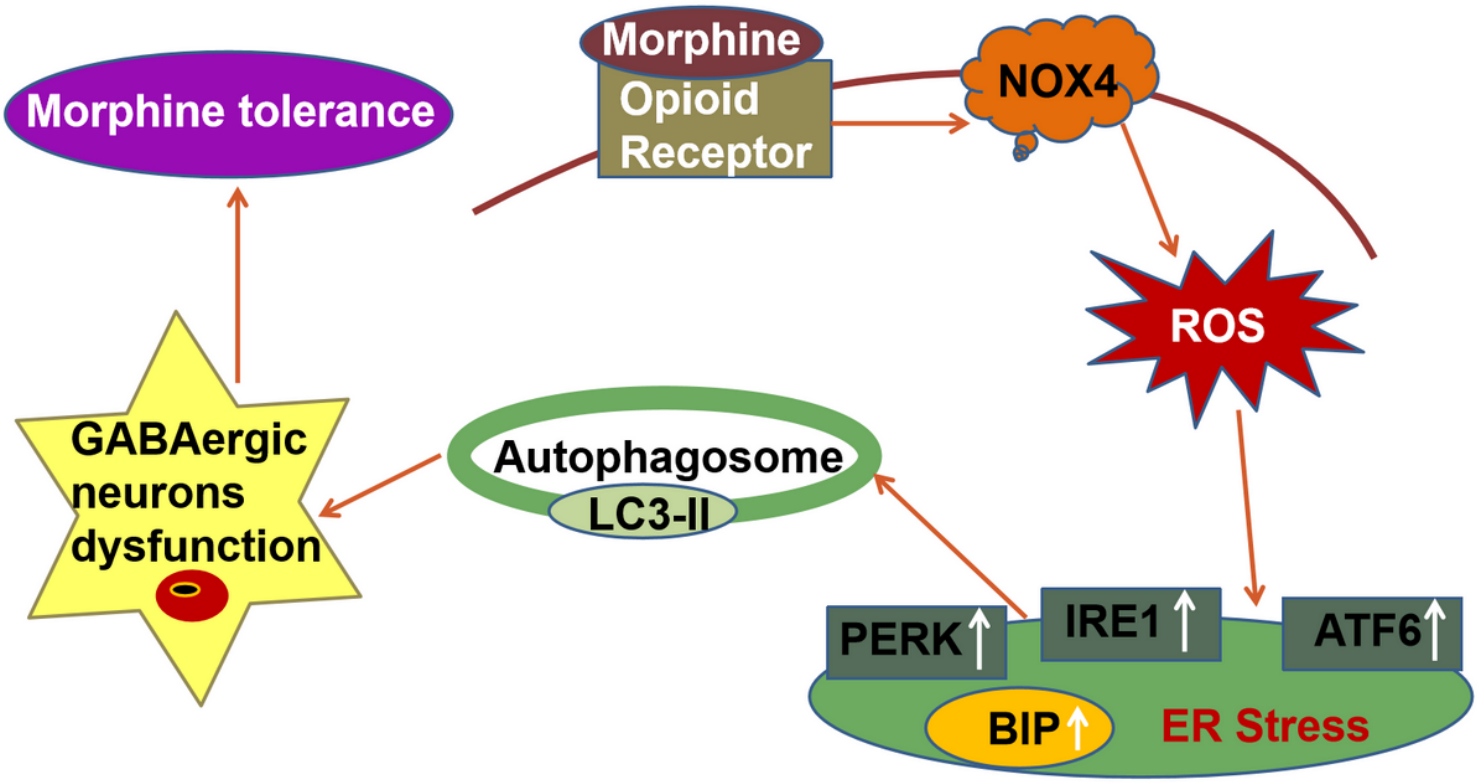

Figure 5

The schematic illustration of NOX4 participated in the development of morphine tolerance.