RESEARCH ARTICLE

A novel role of NK3 receptor signaling in bipolar disorder

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Abstract: Objective: Bipolar disorder (BD) affects more than 1% of the global population with limited therapeutic options. The neurokinin B (NKB)-neurokinin B receptor (NK3R) is involved in a variety of emotional activities. This study explored the role of NK3 receptor signaling in bipolar disorder. **Materials and methods**: In this study, a model of intracerebroventricular (ICV) administration of OUA-induced BD was used to investigate the possible role of NK3R signaling in BD. The involvement of NK3R in the expression of OUA-induced BD was assessed by genetically knocking down the NK3R-encoding *TACR3* gene with shRNA approach in the hippocampus and systemic administration of a NK3R antagonist ESN364,. Biochemical techniques were used to examine the NK3R-associated signaling changes and the oxidative stress parameters in the hippocampus of BD rats. **Results**: The NK3R expression level was elevated in the hippocampus BD rats. Both *TACR3* knockdown in the hippocampus and ESN364 treatment reversed the manic-like and depression-like behaviors in BD rats Inhibition of the NK3R signaling reversed oxidative stress-induced damage via upregulating the BDNF signaling pathway in the hippocampus. **Conclusion**: These results demonstrated that NK3R signaling plays a key role in the pathogenesis of BD and that pharmacological antagonist of NK3R such as ESN364 could represent a novel therapeutic strategy for the management of BD.

Keywords: bipolar disorder, hippocampus, NK3R, ESN364, oxidative stress

Abbreviations

BD:	bipolar disorder
NK3R:	tachykinin receptor 3
NKB:	neurokinin B
OUA:	ouabain
aCSF:	artificial cerebrospinal fluid
OFT:	open field test
FST:	forced swimming test
MDA:	malondialdehyde
GPx:	Glutathione peroxidase
shRNA:	short hairpin RNA
BDNF:	brain-derived neurotrophic factor

1 Introduction

Bipolar disorder (BD) is one of the most debilitating psychiatric disorders, which is characterized by its cyclical disruptive depressive and manic or hypomanic episodes. According to data from the latest World Mental Health Surveys conducted between 2001 and 2022, involving 156,331 respondents across 29 countries, the lifetime prevalence of 2.5% in men and 2.3% in women [1]. BD is broadly categorized into the two main categories: type I (BD-I) and type II (BD-II) [2, 3], with a global prevalence of 1.06% and 1.57%, respectively [4]. Although most patients require lifelong treatment, current strategies for managing bipolar disorder are limited. The standard approach involves the use of a mood stabilizer, such as lithium or valproate, in combination with atypical antipsychotic agents [5–7]. However, the current treatment of BD faces numerous challenges, including poor compliance, a lack of effective medications for BD ll type depression, and the occurrence of extrapyramidal reactions [7]. These challenges underscore the pressing need for a better understanding of the neurobiological mechanisms underlying bipolar disorder.

Oxidative stress (OS), which damages biomolecules and causes mitochondrial and dopamine system dysfunctions, is a persistent finding in patients with BD [8]. Two new classes of antioxidants have potential as a treatment for BD [9, 10].

The *TACR3* gene encodes tachykinin receptor 3 (NK3R), expressing in multiple mammalian brain regions, such as the hippocampus, the frontal, parietal and cingulate cortex. These brain regions are vital for emotion regulation, cognition, and behavior, and in patients with BD, these functions are often impaired. NK3R functions by binding to its high-affinity ligand, neurokinin B (NKB), leading to increased levels of dopamine, 5-HT and noradrenaline, and such changes are subsequently involved in a variety of neural activities. NK3R antagonists demonstrated anti-schizophrenia effect in human clinical trials [11–15] and antidepressant-like effect in animals [16, 17]. However, it is unclear whether NK3R signaling plays any role, if at all, in the pathophysiology of BD.

ESN364 is a small molecule NK3R antagonist with the pIC_{50} = 7.7 [18]. ESN364 was well-tolerated and rapidly bioavailable with linear pharmacokinetics and no drug accumulation with repeated, daily oral administration [19]. In the present study, we examined the role of NK3R in the hippocampus of ouabain(OUA)-induced BD rats using genetic (shRNA knockdown) and pharmacological (NK3R antagonist ESN364) approaches and further investigated the signaling pathways associated with NK3R in BD rats.

2 Material and methods

2.1 Animals

Adult male Wistar rats (Rattus norvegicus; 250–350 g) were purchased from a commercial vendor (Beijing Vital River Laboratory Animal Technology Co, Ltd, Beijing, China) and housed in a certified animal facility with a 12 h light/dark cycle, constant temperature and humidity (21-23°C and 40-60%) and free access to food and water. All animals were acclimated to the test environment for at least 2 days before the behavioral testing. All behavioral tests were performed by experimenters blind to treatment conditions. Behavioral tests were carried out between 8:00 and 15:00 h. All experimental procedures in this study were conducted in accordance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson & Altman, 2010) and all animal protocols were approved by the Laboratory Animals Care and Use Committee of Yantai University (registration number is YTU20181119) (Yantai, China).

2.2 Drugs and chemicals

ESN364 (molecular formula: C16H15FN6OS; molecular weight: 358.39) was purchased from MedChemExpress (United States) with the purity of $> 95\%$. Valproate was purchased from Sigma (United States). OUA was purchased from MedChemExpress (United States). Artificial cerebrospinal fluid (aCSF) was purchased from Shanghai Xin Yu Biotech Co., Ltd (Shanghai, China). For in vivo studies, ESN-364 and Sodium Valproate were prepared as a suspension by dissolving the compounds with 0.5% sodium carboxymethylcellulose(CMC-Na). OUA was dissolved in aCSF. Unless otherwise noted, all drugs were administered via oral gavage.

2.3 Surgical procedure

Animals were submitted to anesthesia by intramuscular injection of ketamine and xylazine (80 mg/kg and 10 mg/kg body weight, respectively). Then, after the rats were fixed at a stereotaxic apparatus, the skull was exposed using standard aseptic surgical procedure and a 27-gauge 9 mm guide cannula was placed at 0.9 mm posterior to bregma, 1.5 mm right from the midline and 1.0 mm above the lateral brain ventricle. Through a 2-mm hole made at the cranial bone, a cannula was implanted 2.6 mm ventral to the superior surface of the skull and fixed with dental acrylic cement. On the fourth day after the surgery, animals received an ICV injection of artificial cerebrospinal fluid (aCSF) 5 μL alone or in combination with OUA (10^{-3} M) [20, 21]. A cannula (30-gauge) was inserted inside the guide cannula and linked to a microsyringe through a polyethylene tube. The end of the infusion cannula extended 1.0 mm beyond the guide cannula toward the right lateral brain ventricle.

2.4 Behavioral tests

The experimenters were blind to the group assignments and drug treatments during all the behavioral tests.

2.4.1 Open field test

In rodents, increased locomotor activity is commonly considered as modeling the manic-like behavior in the OUA model [22]. The apparatus comprises a $60\times60\times50$ cm (length$\times$width $\times$height) box, with the floor divided into nine equal squares (20×20 cm). During the test, animals were placed in the box to explore the area for 5 min. Locomotor activity was recorded and analyzed using the TopScan monitoring system (CleverSys Inc. Reston, VA, United States) for the entire 5-min duration. The parameters measured in the test included the following: crossings (the total number of times rats crossed the squares throughout the entire test period), rearings (the total number of instances where rats exhibited erect postures during the entire test period) and total activity (the overall distance the rat moves throughout the test period) [23, 24].

2.4.2 Forced swimming test

The forced swimming test (FST) is a procedure that involves two individual exposures to a cylinder filled with warm water. The cylinder was designed with a height that prevents the animal from touching the floor of the apparatus or escaping. Composed of transparent acrylic and measuring 80 cm height and 30 cm diameter, the cylinder was filled with water (22–26°C) to a depth of 40 cm. On the 13th day following OUA treatment, the rats were individually placed into the water-filled cylinder for 15 min (training session). On the 14th day, soon after the OFT, rats were again submitted to FST for 5 min (test session). A trained observer recorded the rats' immobility and climbing in seconds for further analyses.

2.5 Brain samples

After behavioral analysis, rats were euthanized by decapitation. The skull was opened, and the cerebral content was excised and rapidly dissected on a chilled dish Petri. The prefrontal cortex and hippocampus were isolated and cleaned from the subcortical structures and white matter. All samples were kept frozen at -150 °C until the analyses were performed.

2.6 Viral-mediated gene knockdown

Short hairpin RNAs (shRNAs) were purchased from Shenggong Bioengineering (Shanghai) Co., LTD (Shanghai , China). AAV serotype 9 was used for the *TACR3* shRNA experiment. Rat shRNA sequences of *TACR3* were as follows: *TACR3-1* shRNA 5'-GCATGAGAACTGTCACCA ACTCTCGAGAGTTGGTGACAGTTCTCATGC-3'; *TACR3-2* shRNA 5'-GCAGGCTTCAAG AGAGCATTTCTCGAGAAATGCTCTCTTGAAGCCTGC-3'; *TACR3-3* shRNA 5'-GCCTCCA CCACATCAAGTTTCCTCGAGGAAACTTGATGTGGTGGAGGC-3'; *TACR3* shRNA con-taining EGFP-tagged AAV (AAV-U6-TACR3-shRNA) was constructed with a titer of 10^{12} viral particles/ml. AAV without shRNA of *TACR3* was used as control.

For virus injection, animals were submitted to anesthesia by intramuscular injection of ketamine and xylazine (80 mg/kg and 10 mg/kg body weight, respectively) 2 weeks before OUA injection and received stereotaxic surgery under aseptic condition. Bilateral injections of AAV particles (2 μl at each site) were slowly administered into the rats' hippocampus CA1 area (3.5 mm caudal to bregma, 2 mm lateral, and 3.5 mm depth) at a speed of 0.5 μl/min. After injection, the needle was left at the injection site for another 10 min to minimize the leakage of the viral suspension during the needle withdrawal.

2.7 Western blot

The samples were rapidly dissected and homogenized in radioimmunoprecipitation assay (RIPA) lysis buffer. Lysates were incubated on ice for 30 min, and samples were spun at 12000 r/min for 20 min. Then, the supernatants were collected and transferred to fresh tubes, and the protein concentrations were analyzed using the bicinchoninic acid (BCA) Protein Assay kit (BioTek SYNERGY neo2, United States). A total of 30 μg of protein samples were resolved on a 4-20% SDS-PAGE gel and transferred to polyvinylidene difluoride membranes for immunoblotting. After blocking for 2 h in 5% nonfat milk (m/v), the membranes were co-incubated at 4°C overnight with the following primary antibodies: rabbit anti-NK3R (0.5 – 1 μg/ml, Abcam, Cambridge, United Kingdom, Cat#ab124025), and rabbit anti-BDNF (1:1000, Abcam, Cambridge, United Kingdom, Cat#ab108319). After overnight incubation with primary antibodies, the membranes were then washed three times in TBST-Tween 20 and incubated for 1 h with the corresponding secondary antibodies: goat anti-mouse HRP (1:2000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#A0216) and goat anti-rabbit HRP (1:2000, Beyotime Institute of Biotechnology, Shanghai, China, Cat#A0208), and exposed onto ChampChemi 610 (Sagecreation, Bejjing, China) with Sage CapturePro software (Sagecreation,

Beijing, China). Signal intensities were quantified using Image J software and normalized to the corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band.

2.8   qRT-PCR

Total RNA was extracted using an RNA extraction kit and then reverse-transcribed into cDNA using a reverse transcription kit. The resultant cDNA was quantified via qPCR using a SYBR Green method. The primer OligodT was used to complete qPCR according to the manufacturer's instructions (Sangong, 7 Shanghai, China). The primers used were as follows: *TACR3* forward, 5'-TACTGTTTCCCACTGCTC-3'; *TACR3* reverse, 5'-TGTTGGTAGATCGCAGTC-3'; GAPDH forward, 5'-GAAGGTCGGTGTGAACG-3'; GAPDH reverse, 5'-CCCATTTGATGTT AGCGG-3'.

The 10 μL reaction pool of RT-PCR included 5 μL of 2 × SYBR Green qPCR Mix, 0.4 μL of forward and reverse primer (10 nM), 0.5 μL of cDNA template, 0.2 μL of ROX Reference Dye II and 3.9 μL of RNase Free H2O. For qPCR analysis, the levels of mRNA were quantified by Power SYBR Green PCR Master Mix (Applied Biosystems, California, United States) in Fast 7500 Real-Time PCR System (Applied Biosystems, California, United States). Amplification reactions were conducted in triplicate, the procedure was set as one cycle of 95°C for 10 min, 40 cycles at 95°C for 15 s and 60°C for 60 s, followed by the melting stage of 95°C for 15 s, 60°C for 60 s and 95°C for 30 s, then the cooling stage of 37°C for 30 s. Gene expression levels for each sample were normalized to the expression level of the housekeeping gene encoding GAPDH within a given sample (ΔCt), and the difference between each group was used to determine the $\Delta\Delta$Ct. 2-$\Delta\Delta$Ct gave the relative fold changes of the gene expression levels in the hippocampus of different groups of rats.

2.9   Evaluation of oxidative stress in BD rats

2.9.1   Measurement of lipid peroxidation

The levels of the thiobarbituric acid-reactive species (TBARS), specifically malondialdehyde (MDA), were measured in samples of the cerebral structures using RIPA Lysis Buffer. The Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China, Cat#S0131S) was used for the direct quantitative measurement of the MDA level in brain tissue samples.

2.9.2   Determination of antioxidant enzyme activity

Glutathione peroxidase (GPx) catalyzes the reduction of peroxides coupled to glutathione oxidation. The Total Glutathione Peroxidase Assay Kit with NADPH (Beyotime Institute of Biotechnology, Shanghai, China, Cat#S0058) was used for the direct quantitative measurement of GPx activity levels.

2.9.3   Determination of protein carbonyl content

Carbonyl group content was determined using a Protein Carbonyl assay kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing Jiancheng Bioengineering Institute, Nanjing, China, Cat#A087-1-2).

2.10   Statistical analysis

All the data were presented as mean ± SEM. Data were analyzed using IBM SPSS Statistics version 21.0 (Chicago, IL, United States) and plotted using Graph Pad Prism software version 8.0 (San Diego, CA, United States). Data were analyzed by unpaired Student's t tests, or one-way analysis of variance (ANOVA) and followed by Dunnett's post-hoc test. Statistical significance was accepted at the level of $P < 0.05$. For detailed information, see the figure legends of the respective figures.

3   Results

3.1   Increased expression of NK3R in the hippocampus of OUA-induced BD rats

As a first attempt to examine the assocaition between NK3R and BD, we examined the protein expression levels in the prefrontal cortex and hippocampus of the model rats. The timeline for the establishment of BD models, behavioral testing, and brain tissue collection is

depicted in Figure 1A. Following 7 days of ICV administration of OUA, the open field test revealed increased numbers of crossings, rearings, and total activity in rats, signs of mania-like behaviors in this model (Figures 1B, 1C and 1D). After 14 days of ICV administration of OUA, depressive-like behaviors were present, as evidenced by an increase in the time of immobility (Figure 1E). Western blotting was performed to examine the protein expression levels of NK3R in the prefrontal cortex and hippocampus during the manic and depressive phases respectively. The results indicated that the expression of NK3R was significantly elevated in the hippocampus but not the prefrontal cortex of BD-treated rats (Figures 1F and 1G)
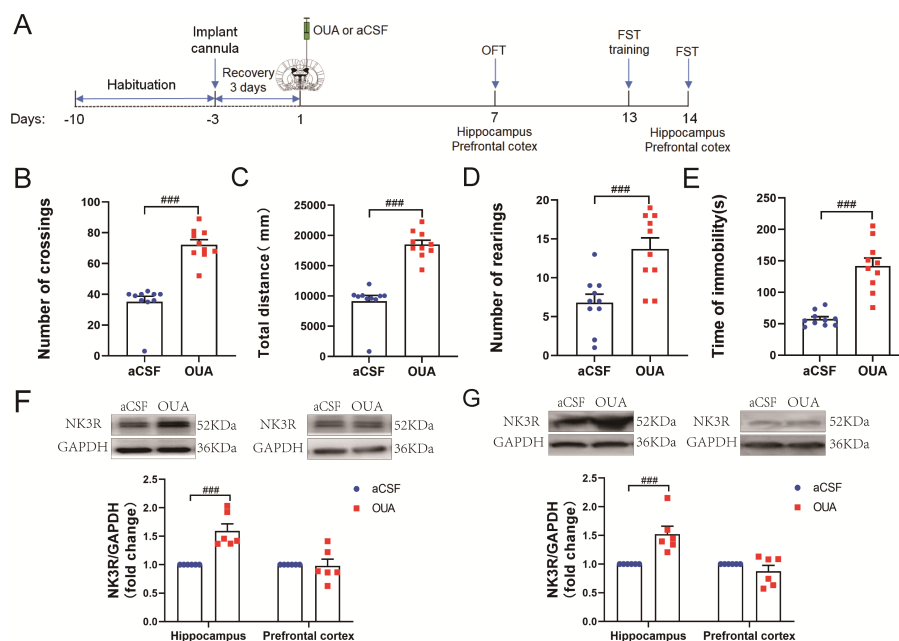

Figure 1   Timeline of this study (A). Expression of NK3R in the prefrontal cortex and hippocampus of OUA-induced BD rats. Representative Western blot bands and quantitative analysis of NK3R in the prefrontal cortex and hippocampus of rats 7 days (F) and 14 days (G) after OUA injection. Data were expressed as percentage of value of aCSF group. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values are expressed as mean $\pm$ SEM, n = 6 per group. $\#\#\# P < 0.001$ compared to aCSF group.

3.2   *TACR3* knockdown sequence selection

Given that the NK3R expression was increased in the hippocampus of OUA-induced BD rats, we next examined whether genetic knockdown of this molecule had any functional consequence in OUA-induced BD rats. The timeline of AAV-shRNA injection, the establishment of BD model, behavioral testing, and tissue sampling was depicted in Figure 2A. Three short hairpin RNAs (shRNAs) were designed to interfere with NK3R expression, respectively. AAV 9 viruses containing shRNA *TACR3* (AAV-*TACR3*-1 shRNA, AAV- *TACR3*-2 shRNA or AAV- *TACR3*-3 shRNA), or negative control (AAV-NC) were microinjected into the rats' hippocampus CA1 area 15 days before cannulization surgery, and green fluorescence level was then examined 14 days after viral injections. Immunostaining showed that approximately 90% of the neurons were successfully infected (labeled by neuron specific marker EGFP) (Figure 2B). Except *TACR3*-1 shRNA, both the mRNA (Figure 2G) and protein (Figure 2F) levels of endogenous *TACR3* were substantially lower in neurons expressing shRNA *TACR3* than those of the neurons expressing control shRNA. *TACR3*-2 shRNA was chosen for subsequent studies due to its highest interference efficiency against *TACR3* and ability to attenuate manic-like behavior in BD rats (Figures 2C-2E).

3.3   Effects of *TACR3* knockdown on manic-like and depressive-like behaviors in OUA-induced BD rats

In order to examine the effects of *TACR3* knockdown on manic-like and depressive-like behaviors in OUA-induced BD rats, we further constructed *TACR3*-2 shRNA (AAV-*TACR3*-2 shRNA) to interfere with the expression of NK3R. The timeline of AAV-shRNA injection, the establishment of BD model, behavioral testing, and tissue sampling was depicted in Figure 3A.
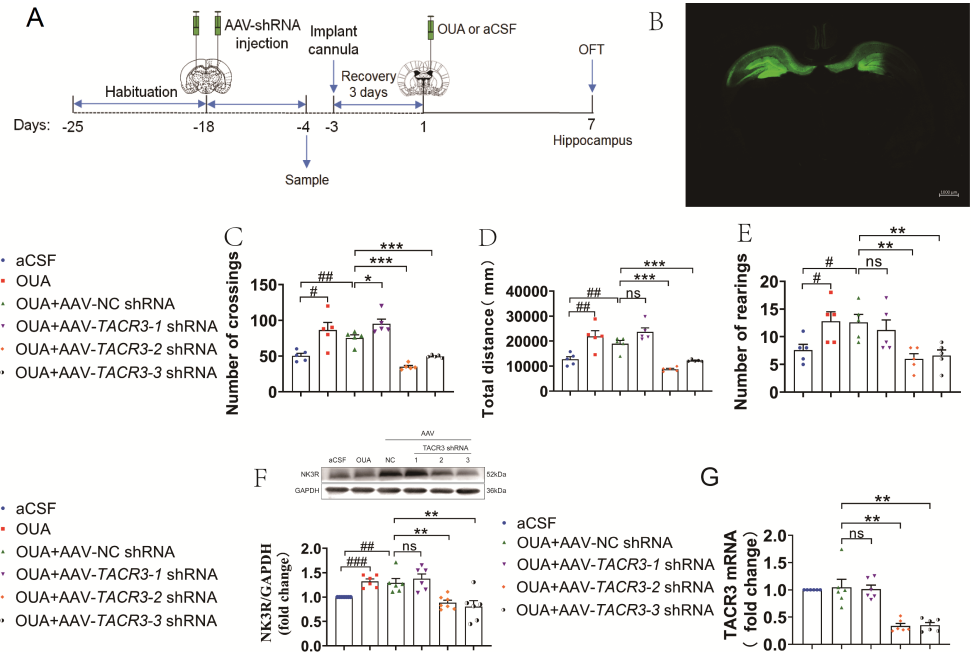
Figure 2 *TACR3* knockdown sequence selection. The timeline of AAV-shRNA injection, sampling, OUA ICV injection and behavioral testing (A). The expression of AAV shRNA in the rat's hippocampus (B). Effects of NK3R knockdown in the hippocampus on the 7th day of OUA injection on the number of crossings (C), total distance (D) and rearings (E) in animals (n = 5 per group). The Expression levels of NK3R in the hippocampus(F) (n = 6 per group). The expression of *TACR3* mRNA in the rat's hippocampus(G) (n = 6 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to aCSF group; $^{*}P < 0.01$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to AAV-NC shRNA group.
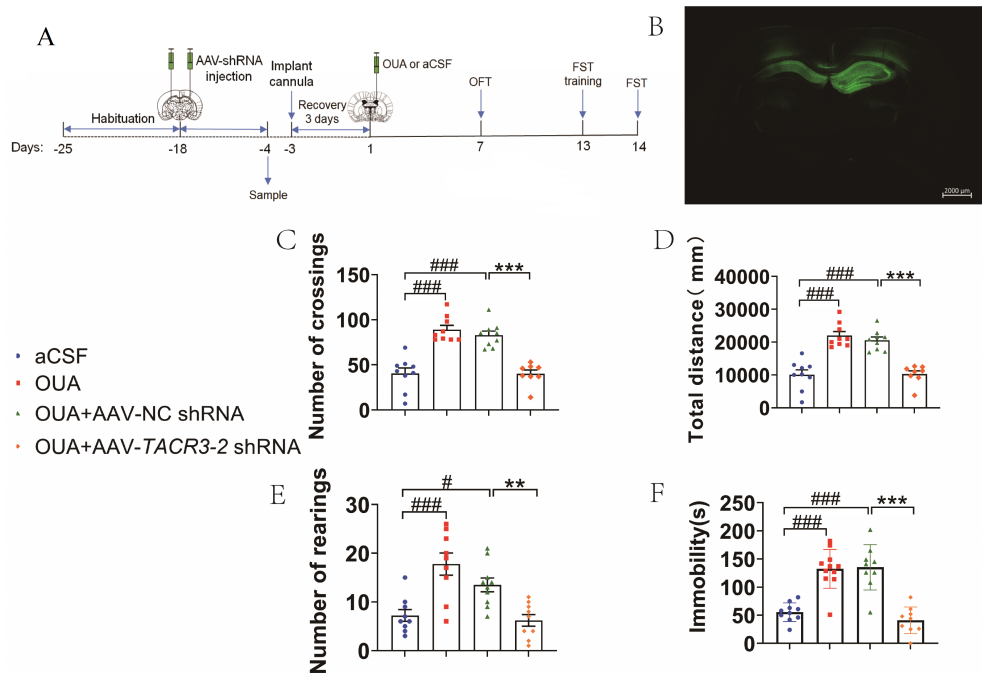

Figure 3 Effects of *TACR3* knockdown on manic-like and depressive-like behaviors in OUA-induced BD rats. The timeline of AAV-shRNA injection, sampling, OUA ICV injection and behavioral testing (A). The expression of AAV shRNA in the rat's hippocampus (B). Effects of *TACR3* knockdown in the hippocampus on the 7th day of OUA injection on the number of crossings (C), total distance (D) and rearings (E) in animals (n = 8-9 per group). Effects of *TACR3* knocksown in the hippocampus on the 14th day of OUA injection on the time of immobility (F) in animals (n = 9-10 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared to aCSF group; $^{**}P < 0.01$, $^{***}P < 0.001$ compared to AAV-NC shRNA group.

Immunostaining showed that approximately 90% of the neurons were successfully infected (labeled by neuron 2 specific marker EGFP) 14 days after virus injection (Figure 3B). 25 days after virus microinjection, open field test was conducted to examine the level of manic-like behavior in OUA-induced BD rats. Results showed that *TARC3* knockdown significantly reversed manic-like behavior in BD rats (Figures 2C-2E). 32 days after virus microinjection, forced swimming test was performed to examine the level of depressive-like behavior. Results showed that *TARC3* knockdown significantly reversed depressive-like behavior in BD rats (Figure 3F).

3.4   Effects of *TACR3* knockdown on oxidative stress and BDNF expression in the hippocampus of OUA-induced BD rats

To examine the oxidative stress levels in the hippocampus of BD rats, we assessed the levels of MDA, GPx and protein carbonyl content. In the hippocampus of OUA-induced BD rats, the MDA activity (Figure 4A), GPx activity (Figure 4B) and protein carbonyl content (Figure 4C) were all significantly increased as compared to the aCSF-treated rats and, importantly, these changes were prevented by *TACR3* knockdown. For the expression level BDNF in the hippocampus, BD-treated rats were significantly lower than aCSF-treated rats (Figure 4D) and such a reduction was largely prevented by *TACR3* knockdown.
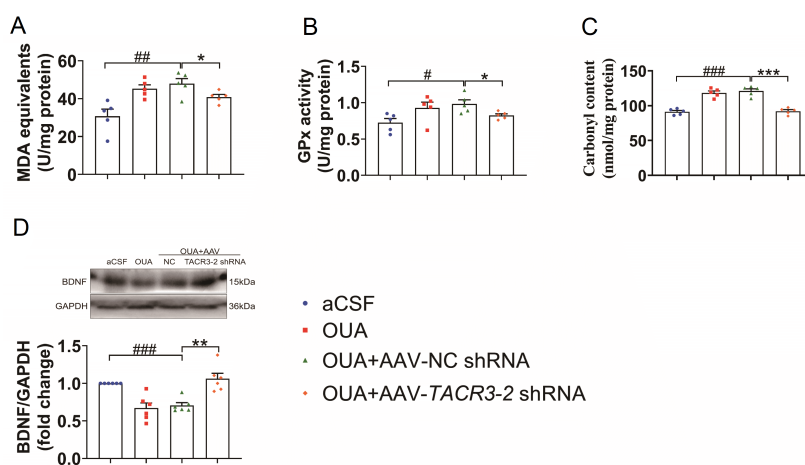

Figure 4   Effects of *TACR3* knockdown on the oxidative stress level and BDNF expression in the hippocampus of OUA-induced BD rats. Effects of *TACR3* knockdown on the levels of MDA (A), GPx activity (B) and protein carbonyl content (C) in the hippocampus of OUA-induced BD rats (n = 5 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values are expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to aCSF group. $^{*}P < 0.05$, $^{***}P < 0.001$ compared to OUA + AAV-NC shRNA group. Effects of *TACR3* knockdown on the BDNF in the hippocampus of OUA-induced BD rats (D). Data were quantified by densitometric analysis. Data were expressed as percentage of value of aCSF group. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 6 per group. $^{\#\#\#}P < 0.001$ compared to aCSF group; $^{**}P < 0.01$ compared to OUA + AAV-NC shRNA group.

3.5   Effects of ESN364 on locomotor activity in normal rats

The open field test was used to assess the impact of ESN364 on the locomotor activity within the dose range of 7.5 mg/kg and 30 mg/kg in normal rats. The results indicated that ESN364 at the doses tested had no significant effect on the spontaneous activity in rats. At higher doses than 30 mg/kg, ESN364 demonstrated sedative effects in rats (Figure 5).

3.6   Effects of ESN364 on manic-like and depressive-like behaviors in OUA-induced BD rats

To determine the optimal dose of ESN364 for subsequent studies in rats with BD, we performed the open-field test on day 7 after the OUA injection. The timeline of the establishment of BD model and behavioral testing was depicted in Figure 6A. It was demonstrated that, within the dose range of 15 and 30 mg/kg, ESN364 dose-dependently amiliorated manic-like behaviors in BD rats (Figures 6B, 6C and 6D). Based on the results of open field test in normal rats, the dose of 15 mg/kg of ESN364 was selected for subsequent studies.

To test the effects of ESN364 on manic-like and depressive-like behaviors in rats with BD,
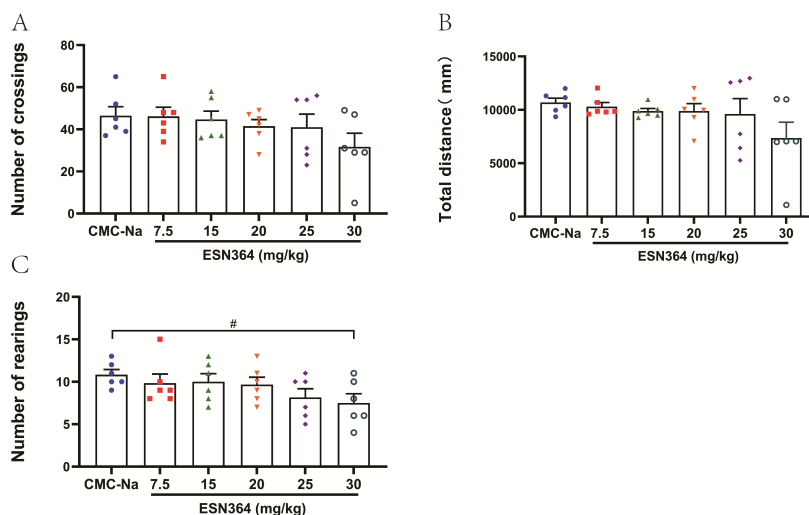
Figure 5   Effects of ESN364 on the locomotor activity in normal rats. Effects of ESN364 treatment on the number of crossings (A) total distance (B) and number of rearings (C) in normal animals. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean $\pm$ SEM, n = 6 per group. $^{\#}P < 0.05$ to CMC-Na group.
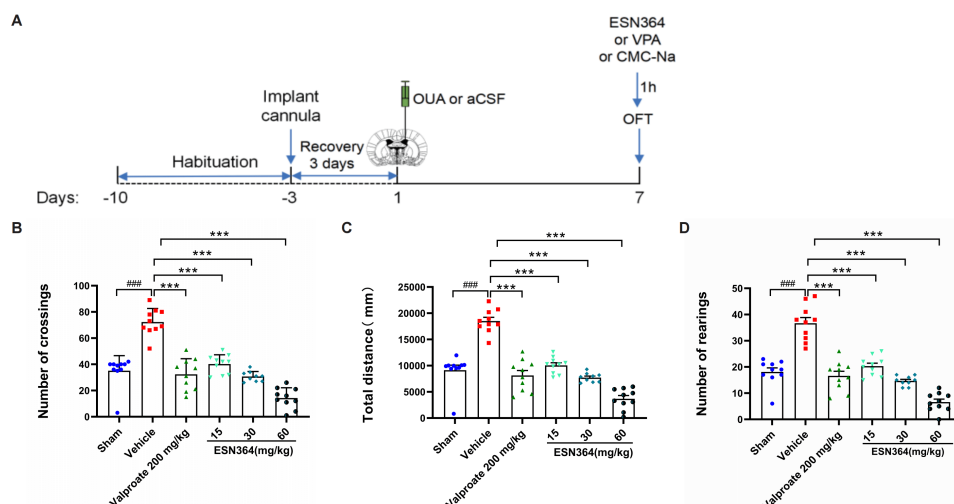

Figure 6   The dose-dependent effects of ESN364 on manic-like behaviors in OUA-induced BD rats. Scheme illustrating the experimental design used in this study (A). Effects of 200 mg/kg valproate or ESN364 (15 - 60 mg/kg) treatment on the 7th day of OUA injection on the number of crossings (B), total distance (C) and number of rearings (D) in animals. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean $\pm$ SEM, n = 10 per group. $^{\#\#\#}P < 0.001$ compared to Sham group; $^{***}P < 0.001$ compared to Vehicle group.

we performed the open-field test and forced swimming test. The timeline for the establishment of BD model and behavioral testing was depicted in Figure 7A. Compared to the artificial cerebrospinal fluid + sodium carboxymethylcellulose (aCSF + CMC-Na) group, both ESN364 (15 mg/kg) and valproate (200 mg/kg) demonstrated no significant effects on the locomotor activity in sham rats on day 7 and day 14 after the OUA injection, indicating that their effects on rats with BD were not associated with sedation. In contrast to the ouabain + carboxymethylcellulose (OUA + CMC-Na) group, both 1.5 mg/kg ESN364 and 200 mg/kg valproate significantly reduced the number of crossings, rearings, and the total travelled distance in OUA-induced BD rats in the open-field test on day 7 (Figure 7B, 7C and 7D) and decreased the immobility time in the forced swimming test on day 14 (Figure 7E).

3.7   Effects of ESN364 on oxidative stress and BDNF expression in the hippocampus of OUA-induced BD rats

To examine the oxidative stress levels in the hippocampus of BD rats, we assessed the levels of MDA, GPx and protein carbonyl content. During the manic and depressive phases, the MDA

activity (Figures 8A and 8D), GPx activity (Figures 8B and 8E) and protein carbonyl content (Figures 8C and 8F) in the hippocampus were all significantly increased in BD rats, as compared to aCSF + vehicle-treated rats, and such change was largely prevented by 15 mg/kg ESN364 treatment.
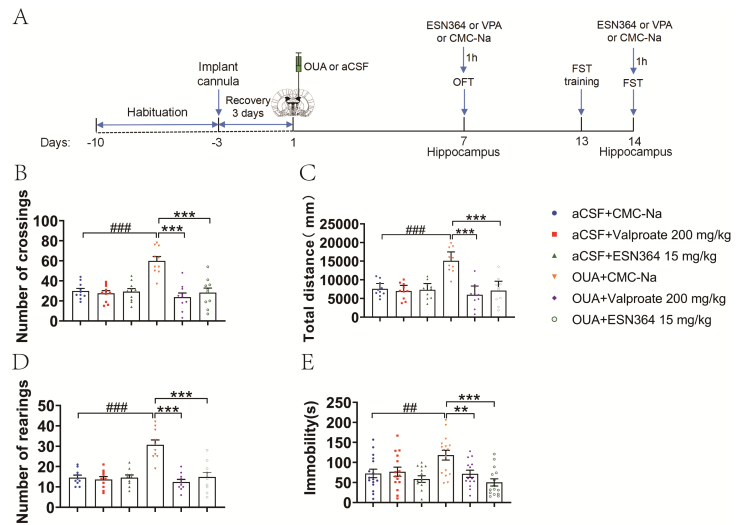

Figure 7   Effects of ESN364 on manic-like and depressive-like behaviors in OUA-induced BD rats. Timeline of the study (A). Effects of 200 mg/kg valproate or 15 mg/kg ESN364 on the 7th day of OUA injection on the number of crossings (B), total distance (C) and number of rearings (D) in rats (n = 10 per group). Effects of 200 mg/kg valproate or 15 mg/kg ESN364 on the 14th day of OUA injection on the duration of immobility (E) in rats (n = 14-16 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to aCSF + CMC-Na group; $^{**}P < 0.01$, $^{***}P < 0.001$ compared to OUA + CMC-Na group.
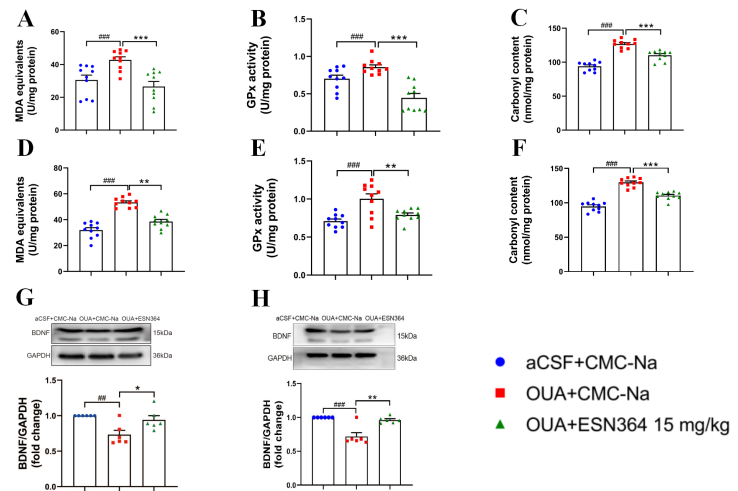

Figure 8   Effects of ESN364 on the oxidative stress level and BDNF expression in the hippocampus of OUA-induced BD rats. Effects of 15 mg/kg ESN364 treatment on the levels of MDA (A), GPx activity (B) and protein carbonyl content (C) in the hippocampus of rats 7 days after OUA injection (n = 10 per group). Effects of 15 mg/kg ESN364 treatment on the levels of MDA (D), GPx activity (E) and protein carbonyl content (F) in the hippocampus of rats 14 days after OUA injection (n = 10 per group). Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM. $^{\#\#\#}P < 0.001$ compared to aCSF+ CMC-Na group; $^{**}P < 0.01$, $^{***}P < 0.001$ compared to OUA+ CMC-Na group. Effects of ESN364 or NK3R knockdown on the BDNF in the hippocampus of OUA-induced BD rats. The representative western blot level of BDNF (G) in the hippocampus of rats 7 days after OUA injection. The representative western blot level of BDNF (H) in the hippocampus of rats 14 days after OUA injection. Data were quantified by densitometric analysis. Data were expressed as percentage of value of aCSF+ CMC-Na group. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values were expressed as mean ± SEM, n = 6 per group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to aCSF + CMC-Na group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to OUA + CMC-Na group.

We also examined the effects of ESN364 on the expression level of BDNF, a downstream signaling molecule. During the manic and depressive phases, the expression levels of BDNF in the hippocampus in BD rats were all significantly reduced as compared to aCSF + vehicle-treated rats (Figures 8G and 8H, which were significantly prevented by 15 mg/kg ESN364 treatment.

4 Discussion

Using the OUA-induced BD model in rats, the current study for the first time revealed a direct involvement of NK3R signaling in BD. Specifically, we found that OUA treatment led to upregulation of NK3R expression level in the hippocampus and manic-like and depressive-like symptoms in rats, both of which were prevented by genetic knockdown of NK3R protein and a NK3R antagonist ESN364 treatment. Mechanistically, OUA treatment led to the activation of NK3R/BDNF signaling and increased oxidative stress, both of which were prevented by NK3R knockdown or ESN364 treatment. These results revealed a novel and crucial role of NK3R signaling in the pathophysiology of BD and suggested that NK3R inhibitors may have the potential as novel pharmacotherapies for BD. A schematic figure depicting the potential mechanisms is shown in Figure 8.
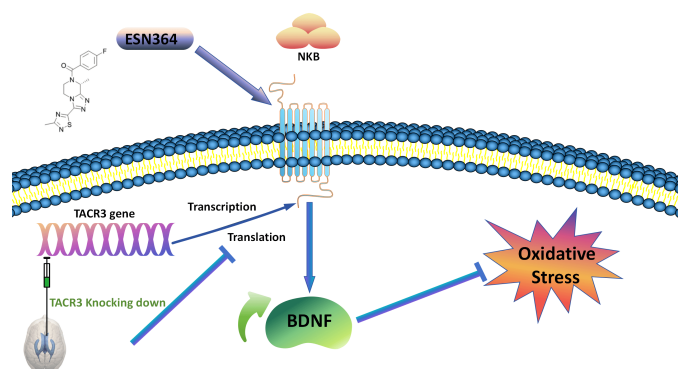

Figure 9 A schematic figure depicting the potential mechanisms

There are few well-validated animal models of BD due to its complicated clinical demonstrations [25]. The OUA model of BD was developed based on the "Na^+/K^+ ATPase hypothesis", which proposed that decreased activity of the enzyme is a key factor contributing to the occurrence of manic and depressive mood episodes in patients [21,26]. In addition, ICV administration of OUA in rats induces molecular changes similar to those detected in BD patients, including neurotrophic factor alterations and oxidative stress [27–30]. To test our hypothesis that NK3R signaling is involved in the pathophysiology of BD, we selected OUA-induced BD model in this study due to its high validity. OUA treatment led to manic-like symptoms on day 7 and depressive-like symptoms on day 14 in rats, consistent with the literatures [31, 32]. Western blotting results revealed that the NK3R expression level was up-regulated in the hippocampus at both the manic and the depressive phases. These results suggest that the NK3R in the hippocampus might be involved in the pathophysiological development of BD.

To further elucidate the role of NK3R in BD, two approaches were employed : genetic knockdown of NK3R expression and pharmacological antagonism of NK3R. Remarkably, we found that both the knockdown of the TACR3 in hippocampus CA1 and a NK3R antagonist ESN364 treatment significantly reduced both manic-like behaviors and depressive-like behaviors in BD rats, which provided direct functional evidence for the direct involvement of NK3R in BD pathophysiology.

A small molecule NK3R inhibitor can serve two purposes: (1) it tests whether pharmacological inhibition of NK3R can recapitulate the observed reduced manic-like and depressive-like behavioral phenotypes as seen in the TACR3 knockdown study, thus further supporting the novel role of NK3R in the modulation of BD; (2) it paves the way for validating NK3R as a potential drug target for pharmacotherapy of BD. Indeed, rats treated with ESN364 showed very similar behavioral phenotypes to rats with TACR3 knockdown. In this regard, ESN364 demonstrated dose-dependent and significant in vivo anti-manic-like and anti-depressant-like efficacy. Importantly, the effects of ESN364 are not attributable to and unrelated to its sedation effects as in the open field test in normal rats 15 mg/kg of ESN364 did not affect the spontaneous activity. Because this dose is efficacious in BD model rats, this suggests that NK3R antagonists can produce specific anti-BD effects and have the potential as novel pharmacotherapies for BD.

Oxidative stress is known to be involved in the pathophysiology of BD as changes of oxidative stress index and activity of antioxidant enzymes are observed in BD patients [33]. The central nervous system is especially vulnerable to oxidative stress because the brain uses large amounts of oxygen, which increases the production of free radicals and ROS [34, 35]. Failure to eliminate ROS efficiently and timely can lead to oxidative injury (for example, the peroxidation of lipids, DNA, and proteins) to the neurons [36–38]. Carbonyl proteins and MDA have been used as markers of Oxidative stress. GPx converts H_2O_2 to water and oxygen [39–44], which is also used as a measure of oxidative stress. Previous preclinical studies demonstrated that the manic-like behaviors and depressive-like behaviors induced by OUA treatment was accompanied by increased level of oxidative stress [32, 45]. Here, both TACR3 knockdown and ESN364 treatment were able to decrease the elevation in MDA, GPx activity and protein carbonyl content caused by OUA. Thus, the antimanic and antidepressant-like effects of TACR3 knockdown and ESN364 treatment might be partially medited by the attenuation of oxidative stress.

The human TACR3 gene is located on chromosome 4q24 and encodes the NK3R protein, which binds to NKB. Early studies revealed that NK3R interacts with dopamine pathways, stimulating dopamine release and evoking dopamine-mediated behaviors [46–48]. Clinical and preclinical studies demonstrated that NK3R antagonists improved positive symptoms in schizophrenia [11–15], and these antagonists also exhibited antidepressant activity [16, 17]. BDNF plays a crucial role in the development, plasticity, and survival of neurons [34, 49, 50]. Six meta-analyses have reported decreased BDNF levels in BD patients compared to both healthy individuals and those with unipolar depression. Lowered BDNF levels were observed during both manic and depressive phases in BD [51–55]. The established link between higher oxidative stress levels and decreased BDNF levels in BD has been well-demonstrated [43, 56]. Decreased BDNF can lead to oxidative stress, providing another pathway through which it may contribute to changes associated with BD [57, 58]. In this study, we found that the expression of NK3R protein in the hippocampus of BD rats was up-regulated during both manic and depressive phases, correlating with the upregulation of BDNF signaling pathways and oxidative stress. This phenomenon could be reversed by TACR3 knockdown or ESN364 treatment, suggesting that inhibition of NK3R signaling might reverse oxidative stress-induced cellular daamage by upregulating the BDNF signaling pathway in the hippocampus.

5 Conclusion

In summary, this study is the first to reveal a novel role of TACR3 in the development and maintenance of BD. It also provided direct evidence that NK3R is a viable pharmacological target to treat the manic-like and depressive-like behaviors in BD. Given the promising in vivo efficacy of ESN364 for inhibiting NK3R and improving BD-related behavioral phenotypes, it seems a viable strategy to develop small molecule NK3R antagonists as novel pharmacotherapies for BD and warrants further investigation.

Ethical approval

All experimental procedures in this study were conducted in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals and all animal protocols were approved by the Laboratory Animals Care and Use Committee of Yantai University (registration number is YTU20220329) (Yantai, China). Efforts were made to minimize the number of animals used and to limit their suffering.

Conflicts of interest

The authors have no conflicts of interest to declare in relation to this work.

Funding support

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Acknowledgements

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