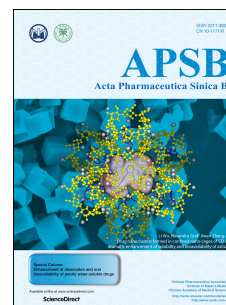


# Journal Pre-proof

Exendin-4 alleviates anxiety-like behaviors in chronic restraint stress mice *via* regulating BLA GLP-1R/BDNF signaling

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Original article

**Exendin-4 alleviates anxiety-like behaviors in chronic restraint stress mice *via* regulating BLA GLP-1R/BDNF signaling**

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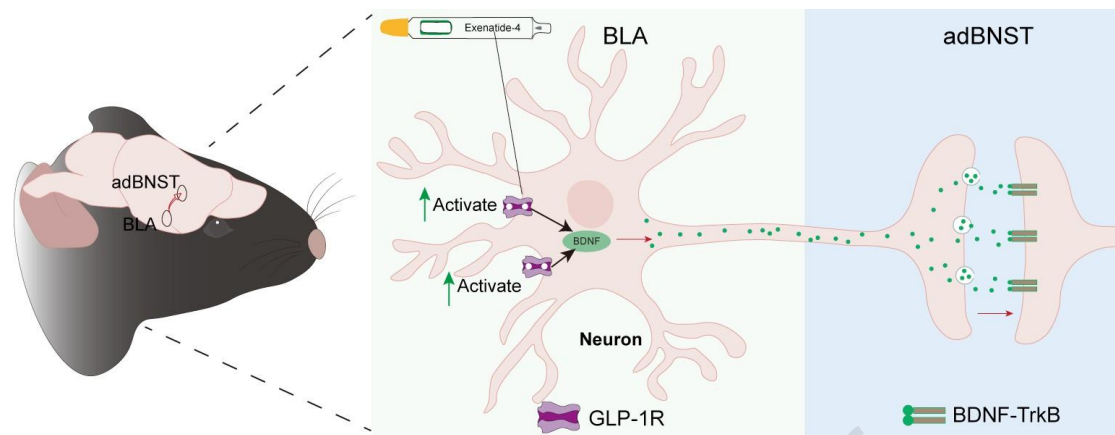
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**Running title:** Ex-4 attenuates anxiety-like behaviors by activating BLA GLP-1R/BDNF signaling.

## Graphical abstract



Activation of glucagon-like peptide-1 receptor (GLP-1R) in the BLA promotes BDNF release into the adBNST, thereby enhancing synaptic plasticity and alleviating anxiety-like behaviors in mice.

## Original article

# Exendin-4 alleviates anxiety-like behaviors in chronic restraint stress mice *via* regulating BLA GLP-1R/BDNF signaling

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**Running title:** Ex-4 attenuates anxiety-like behaviors by activating BLA GLP-1R/BDNF signaling.

**Abstract** Clinical studies have suggested that exendin-4 (Ex-4) exhibits therapeutic potential for anxiety disorders, yet the molecular mechanisms underlying its efficacy remain largely unclear. Our study demonstrates that Ex-4 at specific doses ameliorates anxiety-like behaviors in mice. Mechanistically, Ex-4 could cross the blood-brain barrier and enter the central nervous system, where it exerts anxiolytic effects by activating glucagon-like peptide-1 receptor (GLP-1R) in the basolateral amygdala (BLA). Anterior dorsal bed nucleus of the stria terminalis (adBNST) as a downstream projection target of BLA neurons. Chemogenomic inhibition of the BLA-adBNST circuit induced anxiety-like phenotypes, which were rescued by Ex-4 treatment. Brain-derived neurotrophic factor (BDNF), a critical neurotrophin implicated in neuropsychiatric disorders including anxiety and depression, was functionally interrogated in this pathway. Furthermore, conditional knockdown of *Bdnf* in the BLA-adBNST circuit abolished the anxiolytic effects of Ex-4, indicating that the activation of GLP-1R in BLA neurons drives BDNF release into adBNST to mitigate anxiety.

Taken together, these studies identify a central mechanism whereby Ex-4 attenuates anxiety-like behaviors by promoting BDNF release in the BLA-adBNST circuit.

**KEY WORDS** Anxiety; Exendin-4; GLP-1R; BLA; adBNST; BDNF; Anxiolytic effects; Molecular mechanisms

## 1. Introduction

Anxiety is characterized as a highly aroused, negatively valenced state, accompanied by heightened vigilance toward threatening cues that are uncertain, temporally or spatially distant, or internally generated through imagination and reflection<sup>1-3</sup>. Anxiety disorders impact an estimated 14%–18% of the global population at any time, constituting one of the most prevalent psychosomatic conditions<sup>4</sup>. Common treatments such as selective serotonin reuptake inhibitors or benzodiazepines are ineffective in subsets of patients or have abuse potential<sup>5,6</sup>. Therefore, it is crucial to elucidate the molecular mechanisms of anxiety disorders and to develop novel and effective therapeutic strategies.

Ex-4, a glucagon-like peptide-1 receptor agonist, modulates the rewarding value of food and drugs of abuse by activating GLP-1R within the mesolimbic reward system<sup>7,8</sup>. Recently published findings have also demonstrated its therapeutic potential for depression and anxiety<sup>9-11</sup>. For example, Ex-4 treatment has been shown to ameliorate anxiety-like behaviors in high-fat diet-fed mice<sup>12</sup>. GLP-1 drugs may improve anxiety-like behaviors by regulating the hypothalamic–pituitary–adrenal axis and enhancing the release of neurotransmitters, such as serotonin<sup>13</sup>. These observations raise the following question: how does Ex-4 produce anxiolytic effects? Does Ex-4 exert its effects by inhibiting anxiogenic circuits or by activating anxiolytic circuits?

GLP-1R is widely expressed in the central nervous system, such as the paraventricular nucleus (PVN), dorsolateral striatum (dLS), and arcuate nucleus (ARC). Furthermore, considerable numbers of GLP-1R neurons were observed in the periaqueductal grey (PAG), BLA, and the zona incerta (ZI)<sup>14</sup>. GLP-1R in different brain regions serves distinct functions. For example, GLP-1R expressed in the dLS has been implicated in modulating cocaine-seeking behaviors<sup>15</sup>. GLP-1R-positive neurons in the

lateral septum (LS) mediate anorectic and weight-reducing effects<sup>16</sup>. GLP-1R is also abundantly expressed in the BLA, which is primarily glutamatergic (~90%) and considered a major component of the amygdala<sup>17</sup>. Principal glutamatergic neurons in the BLA exhibit diverse and sometimes opposing roles depending on their projection targets throughout the brain<sup>18</sup>. Specifically, activation of BLA projections to the central amygdala (CeA) alleviates anxiety-like behaviors, whereas activation of BLA projections to the medial prefrontal cortex (mPFC) promotes anxiety<sup>19</sup>. Additionally, the bed nucleus of the stria terminalis (BNST), a downstream target of the BLA, can be subdivided into the anterodorsal BNST (adBNST) and oval BNST (ovBNST). Activation of the BLA–adBNST circuit reduces anxiety-like behaviors in mice, while its inhibition produces the opposite effect<sup>20</sup>. Nevertheless, the molecular mechanisms underlying these phenomena remain unclear.

To investigate these questions, we employed Western blotting, immunofluorescence, and cannula administration, which revealed that the GLP-1 receptor agonist Ex-4 primarily exerts anxiolytic effects *via* activation of GLP-1R localized on CaMKII $\alpha$ -positive neurons in the BLA. Combining chemogenetic approaches with pharmacological profiling, we further revealed that Ex-4 mediates its anxiolytic effects predominantly through the BLA–adBNST circuit. Mechanistically, Ex-4 enhances brain-derived neurotrophic factor (BDNF) expression in the BLA, which is subsequently transported to the adBNST, thereby facilitating synaptic plasticity and resilience within this pathway.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6 mice (23–25 g) were obtained from the Laboratory Animal Center of Nanjing Medical University. Animals were housed in a temperature-controlled environment (22–26 °C) under a 12 h light/dark cycle (lights on at 08:00 and off at 20:00), with food and water available *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Nanjing Medical

University (approval number: IACUC-2205069) and were conducted in accordance with national ethical guidelines.

## 2.2. Virus vectors

All viral vectors utilized, including rAAV-CaMKII $\alpha$ -Cre, AAV-DIO-hM4Di (Gi)-mCherry, AAV-DIO-mCherry and AAV<sub>retro</sub>-CaMKII $\alpha$ -Cre were sourced from BrainVTA (Wuhan, China). To achieve targeted silencing of *Glp1r*, a short-hairpin RNA (shRNA) carried by a recombinant AAV construct rAAV-DIO-(EGFP-U6)-shRNA (*Glp1r*)-WPRES was employed, which has been shown to reduce GLP-1R expression by over 80% *in vitro* and significantly suppresses *Glp1r* mRNA in the murine brain. The shRNA antisense sequence utilized was 5'-GATCGGGTTGCTGGTGGAAGGCGTGATCTGTACTCAAGAGGTACAGATACACGCCTTCCACCAGCAACCTTTTTT-3'<sup>21,22</sup>. The titer of the virus is  $5.17 \times 10^{12}$  vector genomes/mL. Its control is rAAV-DIO-(EGFP-U6)-shRNA (Scramble)-WPRES, at a titer of  $5.08 \times 10^{12}$  vector genomes/mL. The viral particles were purchased from BrainVTA. For *Bdnf* knockdown, we used a short-hairpin (shRNA) construct that knocks down *Bdnf* >70% in cultured cells and efficiently reduces BDNF transcripts in the mouse brain (rAAV-DIO-(EGFP-U6)-shRNA (*Bdnf*)-WPRES). The sequence of the shRNA was: 5'-GGTGATGCTCAGCAGTCAAGT-3'<sup>23</sup>. The titer of the virus is  $1.02 \times 10^{13}$  vector genomes/mL. Its control is rAAV-DIO-(EGFP-U6)-shRNA (Scramble)-WPRES, at a titer of  $1.32 \times 10^{13}$  vector genomes/mL. For *Bdnf* overexpression, we used AAV-DIO-*Bdnf*-EGFP-WPRES at a titer of  $1.15 \times 10^{13}$  vector genomes/mL. The control virus was AAV-DIO-EGFP-WPRES at a titer of  $1.35 \times 10^{13}$  vector genomes/mL. Viral particles were obtained from Obio Technology (Shanghai, China).

## 2.3. Drugs

Clozapine-*N*-oxide (CNO; Sigma, St. Louis, MO, USA) was diluted in 0.9% saline and administered intraperitoneally (i.p.) at a dose of 1 mg/kg. Exendin-4 (Ex-4; MCE, Shanghai, China) was delivered *via* the same route at doses of 0.04, 0.2, 1, or 5  $\mu$ g/kg.

For local brain administration, Ex-4 was freshly dissolved in sterile saline and slowly microinjected at a total volume of 0.5  $\mu$ L over 5 min. The injector was left in place for an additional 5 min to facilitate diffusion and minimize reflux.

#### *2.4. Stereotaxic injections*

Under anesthesia with pentobarbital sodium (50 mg/kg, i.p.), mice were immobilized in a stereotaxic apparatus (RWD Life Science, Shenzhen, China). Targeted delivery was performed using a glass micropipette connected to an automatic microinjection system (RWD Life Science). Injections into the BLA were made at the following stereotaxic coordinates (relative to bregma): anteroposterior (AP)  $-1.5$  mm, mediolateral (ML)  $\pm 3.0$  mm, dorsoventral (DV)  $-4.5$  mm. The adBNST was targeted at AP  $+0.35$  mm, ML  $\pm 0.6$  mm, DV  $-4.2$  mm. Each hemisphere received 300 nL of viral suspension at a rate of 40 nL/min. To minimize reflux, the injection needle was left in place for 10 min post-infusion before being slowly withdrawn. Following the procedure, the incision was sutured, and the animals were allowed to recover in a temperature-controlled environment.

#### *2.5. Cannula implantation*

For selective region-specific drug microinfusion, a stainless steel guide cannula (RWD Life Science) was surgically implanted immediately above the BLA (AP:  $-1.5$  mm; ML:  $\pm 3$  mm; DV:  $-4.3$  mm; relative to bregma) and anchored with dental adhesive. Upon completion of surgery, a protective dummy cannula was placed to maintain patency. Animals were given at least one week for post-operative recovery before any intervention or testing. For each experimental session, either vehicle or active drug (0.5  $\mu$ L per animal) was slowly administered over a 5 min interval through the injection cannula, which was left in place for a further 5 min to minimize dispersion. Behavioral tests were initiated approximately 10 min following drug delivery. Upon completion of testing, brains were harvested to verify accurate cannula placement, and data from animals with misplaced implants were omitted from further analyses.

### 2.6. Chronic restraint stress (CRS)

Mice assigned to the chronic restraint group were individually placed in ventilated 50 mL conical tubes for 2 h daily (10:00–12:00) over a 10-day period. The tube dimensions closely matched each animal's body size, effectively limiting spontaneous movement. Control mice were gently transferred from their home cages to the experimental room, handled for 5 min, and then returned to the housing area after a comparable 2 h interval.

### 2.7. Open field test (OFT)

On the day of assessment, animals were habituated within an open-field arena (50×50×50 cm) for 6 h before testing. Each subject was then positioned in the center of the apparatus and granted free exploration for 10 min. Locomotor activity, total time spent, and entries into the central zone (defined as 50% of total area) were video recorded and quantified using TopScan (v2.00, Clever Sys Inc., Reston, VA, USA).

### 2.8. Elevated plus maze test (EPMT)

The elevated plus maze consisted of a central platform (6 × 6 cm) connected to 2 opposing open arms (35 × 6 cm each) and 2 opposing closed arms (35 × 6 × 19 cm), elevated 73 cm above the floor. At the start of each trial, mice were placed on the central platform facing an open arm and allowed to explore for 6 min. Time spent and entries into the open arm were recorded and analyzed using TopScan software (v2.00, Clever Sys Inc.). Animals that fell from the apparatus were excluded from further analysis.

### 2.9. Novelty-suppressed feeding test (NSFT)

Following 24 h of food and water deprivation, mice were placed individually in a novel cage (50 × 50 × 50 cm) containing a single food pellet positioned at the center on a white sheet. The latency to initiate feeding was recorded. Pre-test food weight (M1) was measured before the session, and post-test food weight (M2) was determined after

animals were allowed to feed for 2 h in their home cage. Food intake during the recovery period was calculated as M1–M2.

### 2.11. Tail suspension test (TST)

Mice were secured by adhesive tape roughly 1 cm from the distal tail and suspended 25 cm above the ground for 6 min. Immobility, defined as the complete absence of limb movement, was quantified during the final 4 min using the video tracking Forced Swim Scan system (Clever Sys Inc.).

### 2.12. Fiber photometry recordings

For fiber photometry recordings, a commercially available system (Thinker Tech, Nanjing, China) was employed. Two weeks after viral expression of the calcium indicator AAV-hSyn-GCaMP6s (BrainVTA) in the adBNST, an optical fiber (200  $\mu$ m core diameter, 5.0 mm length, NA 0.37) housed in a ceramic ferrule was implanted 0.2 mm above the adBNST. During the EPMT, GCaMP6s fluorescence was recorded as mice explored the open arms. Neurons expressing GCaMP6s were excited using a 470 nm LED (25  $\mu$ W at the fiber tip), while calcium-independent signals were simultaneously obtained using a 405 nm LED (20  $\mu$ W at the fiber tip). Mice were allowed to acclimate to the recording environment for at least 3 h before data acquisition.

The calcium signals and behavior recordings were both saved for synchronization purposes. Fluorescence signals were acquired and analyzed with MATLAB software. After smoothing the data with a moving average filter (20 ms span), the data were segmented based on behavioral events occurring within individual trials or bouts. The values of fluorescence change ( $\Delta F/F$ ) were calculated as Eq. (1):

$$\Delta F/F = (F - F_0)/F_0 \quad (1)$$

Where  $F_0$  is the baseline fluorescence signal averaged over a 2-s-long control time window.  $\Delta F/F$  values were presented with average plots with a shaded area indicating SEM.

### 2.13. Western blotting

Collected tissue samples were homogenized in RIPA buffer containing a cocktail of protease inhibitors (Invitrogen, Carlsbad, CA, USA). Samples underwent 2 cycles of freeze-grinding at 60 Hz for 30 s each, followed by centrifugation at 12,000 rpm (Thermo Fisher Scientific, Waltham, MA, USA) for 20 min at 4 °C. Protein levels were estimated by the bicinchoninic acid (BCA) method. Equal amounts (20 µg) were subjected to 10% SDS-PAGE and electrotransferred onto PVDF membranes (Millipore). After blocking with 5% non-fat dry milk for 1 h, membranes were probed overnight at 4 °C with primary antibodies anti-GLP-1R (1:3000, Immunoway, San Jose, CA, USA), anti-BDNF (1:1000, Abcam, Cambridge, UK), and anti- $\beta$ -Actin (1:3000, Sigma). Incubation with suitable HRP-conjugated secondary antibodies was performed for 1.5 h at room temperature. After washing with Tris-buffered saline containing 0.05% (v/v) Tween-20 (TBST), the immunoreactive bands were visualized using enhanced chemiluminescence (ECL) detection reagents and an imaging system (GE Healthcare, Pittsburgh, PA, USA), and band intensities were normalized to  $\beta$ -Actin using ImageJ software.

### 2.14. Immunofluorescence

For immunolabeling, deeply anesthetized mice were perfused initially with cold 0.9% saline, then with 4% paraformaldehyde (PFA). Isolated brains were post-fixed overnight at 4 °C in PFA, cryoprotected in increasing sucrose solutions (20% followed by 30% in PBS) until tissues settled. Coronal brain slices (30 µm) were prepared using a Leica CM1860 cryostat (Wetzlar, Germany). Sections were incubated in blocking buffer (0.3% Triton X-100 and 5% goat serum in PBS) for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies diluted in the same buffer. The primary antibodies used were anti-GLP-1R (1:200, Immunoway), anti-CaMKII $\alpha$  (1:200, Sigma), anti-GABA (1:500, Sigma), anti-GFAP (1:500, Bioss, Beijing, China), anti-Iba-1 (1:500, Wako, Saitama, Japan), and anti-BDNF (1:200,

Abcam). Following PBS washes, slices were then incubated with fluorescently labeled secondary antibodies for 1.5 h at room temperature. After three washes in PBS (5 min each), the sections were counterstained with DAPI (1:1000, Sigma) to confirm nuclear morphology. Subsequently, the slices were mounted using fluorescent mounting medium and stored at 4 °C until imaging. Immunofluorescence was assessed using an automated fluorescence imaging system (Leica Thunder). When primary antibodies originated from the same host species, multiplex TSA staining was applied. For sequential antibody labeling, brain slices were permeabilized and blocked at 37 °C for 1 h, followed by overnight incubation at 4 °C with the respective primary antibody. After being brought to room temperature for 15 min, the sections were washed 3 times in PBS (5 min each), incubated with an HRP-conjugated secondary antibody at 37 °C for 1.5 h, and then briefly exposed (5 min) to TSA-dendron-fluorophores (1:200, AiFang Biological, Changsha, China). After the chromogenic reaction, a stripping solution (AiFang Biological) was applied at 37 °C until complete elution was achieved. This cycle was repeated for each primary antibody, thereby labeling distinct antigens with different fluorophores. Following the final washing step, the sections were coverslipped with an anti-fade mounting medium containing DAPI and stored at 4 °C until analysis.

For image analysis, all image acquisition and cell quantification were performed by investigators blinded to the experimental group assignments. To quantify fluorescent protein-positive neurons, automated cell counting was performed using ImageJ Fiji with relevant plugins and analysis tools. To ensure consistency across samples, staining, image acquisition (including exposure time and gain), and image analysis were conducted simultaneously for all specimens.

### 2.15. Statistical analysis

Data from two groups conforming to normal distribution were analyzed with unpaired two-tailed Student's *t*-test or paired *t*-test. One-way ANOVA followed by Tukey's *post hoc* test was used for multiple group comparisons involving a single factor, whereas

two-way ANOVA with Bonferroni post-tests was applied to dual-factor designs. All statistical evaluations were done in a blinded fashion using GraphPad Prism version 8. Results are displayed as mean  $\pm$  standard error (SEM); thresholds for significance were set at  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ , and  $^{****}P < 0.0001$ . Additional statistical information is available in Supporting Information Table S1.

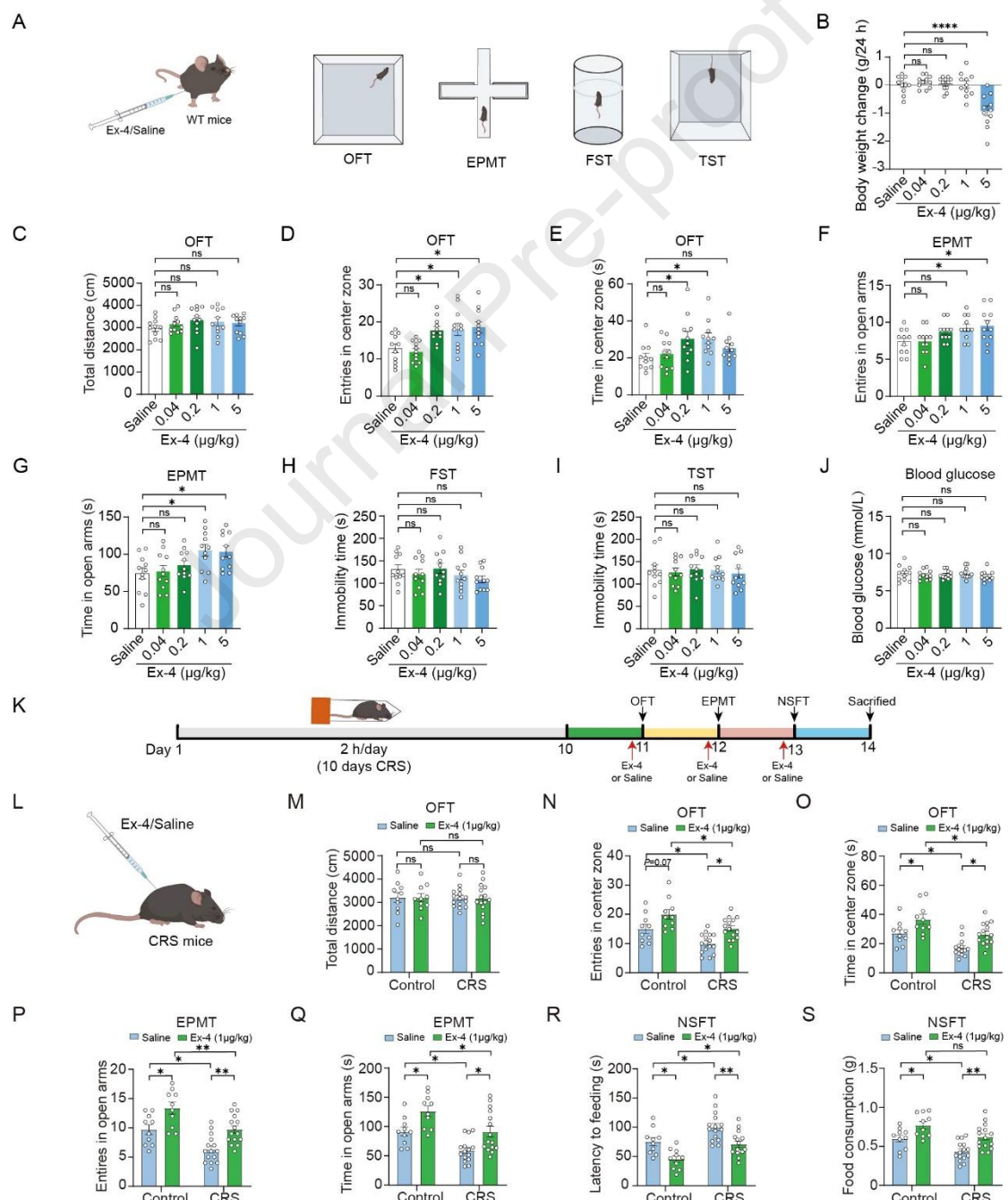
### 3. Results

#### *3.1. 1 $\mu\text{g/kg}$ Ex-4 ameliorates anxiety-like behaviors without affecting depression-like behaviors, body weight, or blood glucose in wild-type and CRS mice*

We performed behavioral tests to evaluate whether Ex-4 could ameliorate anxiety-like behaviors and depression-like behaviors. Mice received intraperitoneal injections of Ex-4 at doses of 0.04, 0.2, 1, and 5  $\mu\text{g/kg}$  (Fig. 1A). To assess locomotor activity following Ex-4 administration, mice were subjected to the OFT. No significant differences were observed in total distance traveled among the groups (Fig. 1C). However, the entries in the center zone and time spent in the center zone are increased at 0.2 and 1  $\mu\text{g/kg}$  (Fig. 1D and E). These findings suggest a potential anxiolytic effect of Ex-4. To further evaluate its impact on anxiety-like behaviors, separate groups of mice were tested in the EPMT. Administration of 1 and 5  $\mu\text{g/kg}$  Ex-4 significantly increased entries into and time spent in the open arms (Fig. 1F and G). No significant differences were observed in immobility time during the FST or TST among the groups (Fig. 1H and I). Furthermore, only 5  $\mu\text{g/kg}$  Ex-4 led to a reduction in body weight (Fig. 1B), whereas blood glucose levels remained unchanged across all groups (Fig. 1J). Collectively, these results indicate that intraperitoneal administration of 1  $\mu\text{g/kg}$  Ex-4 ameliorates anxiety-like behaviors without affecting depression-like behaviors, body weight, or blood glucose in wild-type mice.

Stress plays a causal role in anxiety, and CRS has been used as a method to induce anxiety-like behaviors in animal<sup>24,25</sup>. To understand the underlying mechanism of how Ex-4 ameliorates anxiety-like behaviors, we employ a rodent anxiety model induced by CRS exposure<sup>24</sup>, then intraperitoneal injection of 1  $\mu\text{g/kg}$  Ex-4 1 h before behavioral

tests (Fig. 1K and L). We found that a single dose of Ex-4 (1  $\mu\text{g/kg}$ ) intraperitoneally significantly increased the entries in the center zone and time spent in the center zone without affecting locomotor activity compared with CRS mice (Fig. 1M and O). Additionally, it could also increase the entries in open arms and time spent in open arms in EPMT (Fig. 1P and Q). In NSFT, 1  $\mu\text{g/kg}$  Ex-4 significantly increased food consumption and decreased latency to feed compared with the CRS group (Fig. 1R and S). These results suggest that 1  $\mu\text{g/kg}$  Ex-4 intraperitoneally could ameliorate anxiety-like behaviors in CRS mice.



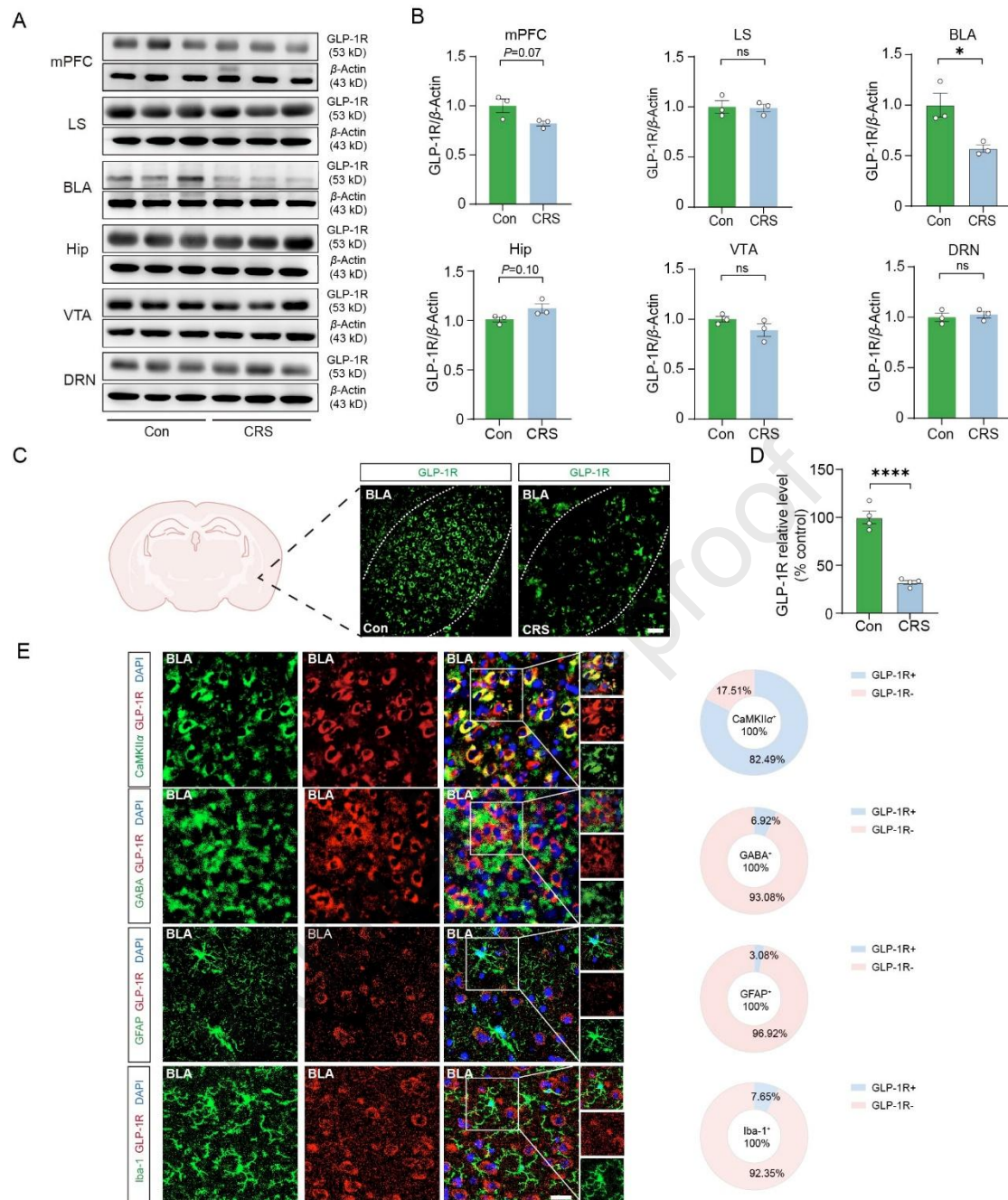
**Figure 1** 1 µg/kg Ex-4 ameliorates anxiety-like behaviors without affecting depression-like behaviors, body weight, or blood glucose in wild-type and CRS mice. (A) An experimental schematic of Ex-4 intraperitoneally and a series of behavior tests were used to assess general locomotion, anxiety-like behaviors, and depression-like behaviors ( $n=11$  for each group). (B) 24-h body weight in mice pretreated with Ex-4 (0, 0.04, 0.2, 1 or 5 µg/kg) prior to subsequent behavior tests. (C–E) The total distance, entries, and time spent in the center zone of different groups in OFT to assess general locomotion and anxiety-like behaviors. (F, G) The entries and time spent in open arms of different groups in EPMT to assess anxiety-like behaviors. (H, I) The immobility time in the FST and TST of different groups to assess depression-like behaviors. (J) The blood glucose changed after 24 h Ex-4 intraperitoneally in different groups. (K) Timeline of experiments. (L) Experimental schematic of Ex-4 intraperitoneal injection in CRS mice. For groups: Control+Saline or Ex-4,  $n=10$ ; CRS+Saline or Ex-4,  $n=15$ . (M, O) The total distance, entries, and time spent in the center zone of the OFT in different groups after Ex-4 intraperitoneal injection. (P, Q) The entries and time spent in the open arms of the EPMT in different groups after Ex-4 intraperitoneal injection. (R, S) The latency to feeding and food consumption of the NSFT in different groups after Ex-4 intraperitoneal injection. Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.0001$ ; ns, not significant. One-way ANOVA followed by Tukey's *post hoc* test analysis for (B–J). Two-way ANOVA followed by Bonferroni's *post hoc* analysis for (M–S).

### 3.2. CRS reduces GLP-1R expression in BLA CaMKII $\alpha$ neurons

Following CRS, mice exhibited pronounced anxiety-like behaviors in the OFT, EPMT, and NSFT. We then examined GLP-1R expression in the mPFC, LS, BLA, hippocampus (Hip), ventral tegmental area (VTA), and dorsal raphe nucleus (DRN) after chronic stress exposure. Notably, GLP-1R expression was significantly reduced in the BLA, whereas no significant changes were detected in the mPFC, LS, Hip, VTA,

or DRN (Fig. 2A and B). Immunostaining further confirmed that GLP-1R expression in the BLA was decreased following CRS (Fig. 2C and D).

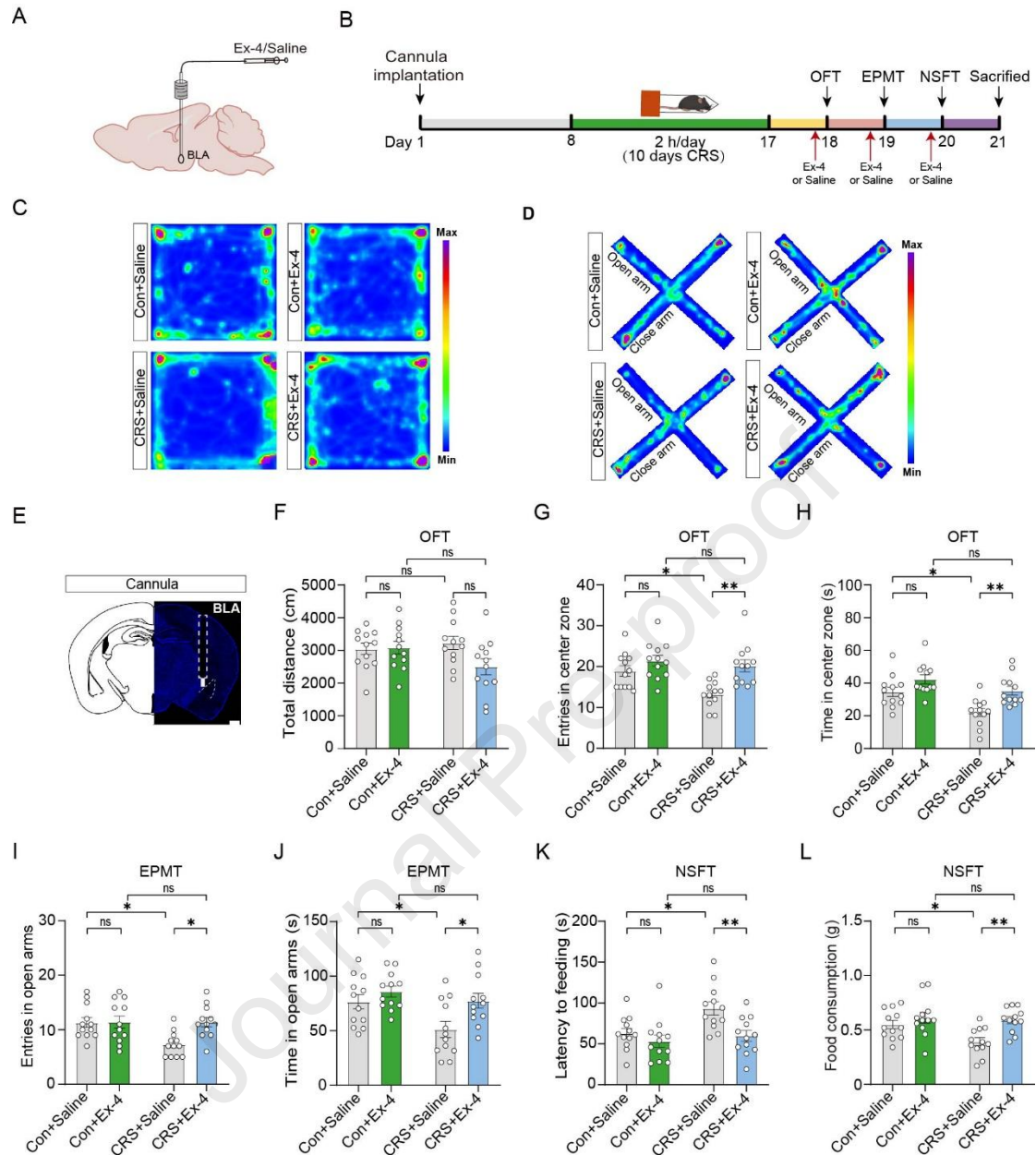
To determine whether GLP-1R expression was limited to a specific cell type, we further evaluated coronal sections from the BLA of wild-type mice. We found that most (82.49%) GLP-1R-positive cells co-expressed the PyN marker calcium/calmodulin-dependent protein kinase II $\alpha$  (CaMKII $\alpha$ ), with only a small fraction (6.92%) expressing gamma-aminobutyric acidergic marker GABA. Furthermore, GLP-1R-positive cells expressed little in the astrocyte marker GFAP (3.08%) and the microglia marker Iba1 (7.65%) (Fig. 2E), suggesting that most GLP-1R-positive cells in the BLA are CaMKII $\alpha$ . Taken together, these findings indicate that GLP-1R expression in CaMKII $\alpha$ -positive neurons of the BLA is decreased under CRS.



**Figure 2** CRS reduces GLP-1R expression in BLA CaMKII $\alpha$  neurons. (A, B) Protein expression and analysis of GLP-1R in the mPFC, LS, BLA, Hip, VTA, and DR after CRS ( $n=3$ ). (C, D) Representative images and quantification of GLP-1R in BLA from control and CRS mice ( $n=4$ ). Scale bar = 100  $\mu$ m. (E) The expression of GLP-1R colabeled with CaMKII $\alpha$ , GABA, GFAP, and Iba1 in BLA from wild-type mice. Right: the percentage of GLP-1R neurons that colocalized with markers was quantified ( $n=3$ ). Scale bar=50  $\mu$ m. Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ ; ns, not significant. Unpaired two-tailed Student's  $t$ -tests for (B) and (D).

### 3.3. Intra-BLA Ex-4 alleviates anxiety-like behaviors in CRS mice

The preliminary results suggest that systemic administration of the GLP-1R agonist Ex-4 (1  $\mu\text{g/kg}$ ) crosses the blood–brain barrier and selectively reduces anxiety-like behaviors without inducing malaise-like side effects. To determine whether activation of BLA GLP-1R is sufficient to reduce anxiety-like behaviors, mice were implanted with a cannula in the BLA before CRS, and Ex-4 or saline (0.025  $\mu\text{g/side}$ ) was administered 10 min before behavioral tests<sup>27,28</sup> (Fig. 3A, B, and E). We discovered intra-BLA Ex-4 significantly increased the entries in center zone and time spent in center zone without affecting locomotor activity in OFT in CRS mice (Fig. 3C, F–H). Additionally, it also increased the entries and time spent in the open arms in EPMT (Fig. 3D, I, and J), and significantly increased food consumption and decreased latency to feed compared with CRS group (Fig. 3K and L). These results indicate that intra-BLA Ex-4 effectively alleviates anxiety-like behaviors in CRS mice.

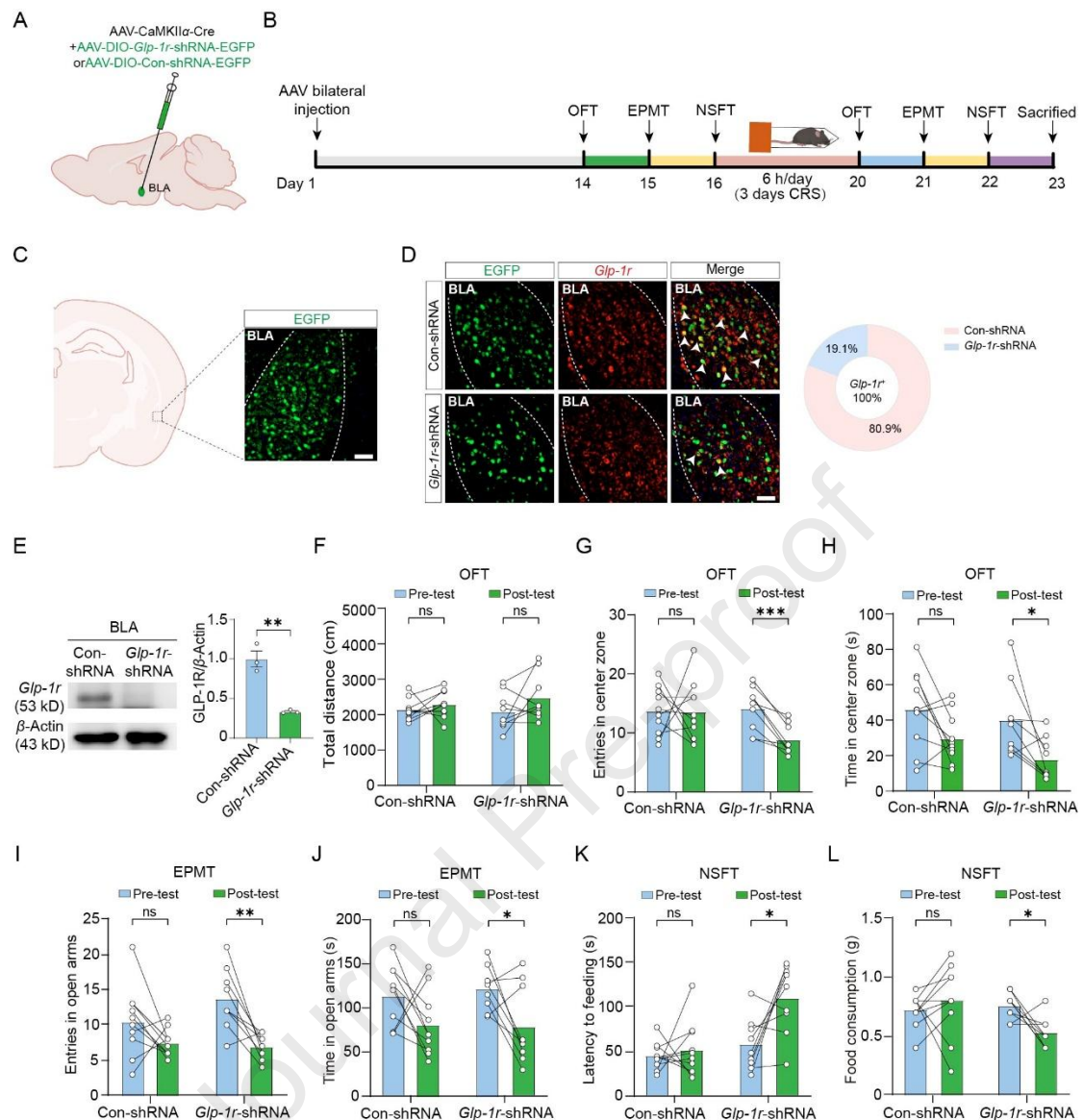


**Figure 3** Intra-BLA Ex-4 ameliorates anxiety-like behaviors in CRS mice. (A) Schematic representation of the cannula implantation in mice. (B) Timeline of experiments. (C, D) Representative heatmaps of different groups in the OFT and EPMT. Warmer colors indicate more time spent in that area;  $n=12$  for each group. (E) Representative image of cannula implantation in BLA. Scale bar = 500  $\mu$ m. (F–H) The total distance, entries, and time spent in the center zone of the OFT in different groups after saline or Ex-4 infusion in BLA. (I, J) The entries and time spent in the open arms of the EPMT in different groups after saline or Ex-4 infusion in BLA. (K, L) The latency to feeding and food consumption of the NSFT in different groups after saline or Ex-4

infusion in BLA. Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ ; ns, not significant. Two-way ANOVA followed by Bonferroni's *post hoc* test for (F–L).

#### 3.4. Knockdown of *Glp1r* in BLA *CaMKII $\alpha$* neurons facilitate stress-induced anxiety-like behaviors

To determine the role of BLA *CaMKII $\alpha$*  GLP-1R in anxiety-like behaviors, we bilaterally injected AAV-DIO-shRNA (*Glp1r*)-EGFP (*Glp1r*-shRNA) or AAV-DIO-shRNA (Scramble)-EGFP (Con-shRNA) together with AAV-*CaMKII $\alpha$* -Cre into the BLA of wild-type mice to achieve *Glp1r* knockdown in BLA *CaMKII $\alpha$*  neurons (Fig. 4A and C). Immunofluorescence and Western blot demonstrated the efficient knockdown of *Glp1r* in BLA (Fig. 4D and E). After 14 days, all mice were subjected to restraint stress for 6 h per day over 3 consecutive days to induce subthreshold CRS (SRS)<sup>29-31</sup>, and anxiety-like behaviors were assessed before and after stress exposure (Fig. 4B). At baseline, anxiety-like behaviors were comparable between *Glp1r*-shRNA mice and their controls. However, after repeated restraint stress, *Glp1r*-shRNA mice exhibited a marked decrease in both entries into and time spent in the center zone, while locomotor activity remained unchanged (Fig. 4F–H). Notably, these reductions were evident when each group was compared with its own performance before stress exposure. In addition, both the entries into and time spent in the open arms of the EPM were reduced after stress exposure (Fig. 4I and J). Moreover, in the NSFT, stress exposure increased the latency to feed and decreased food consumption (Fig. 4K and L). Behavioral tests demonstrated that knockdown of BLA *CaMKII $\alpha$*  GLP-1R, combined with exposure to a 3-day SRS, induced significant anxiety-like behaviors. Taken together, these findings highlight the critical role of BLA *CaMKII $\alpha$*  GLP-1R in mediating anxiety-like responses to chronic stress.



**Figure 4** Knockdown of *Glp1r* in BLA CaMKIIα neurons facilitates stress-induced anxiety-like behaviors. (A) Schematic representation of virus injection. (B) Timeline of experiments. (C) Coronal brain slice includes a schematic (left) and the histology (right) of the virus injection site in the BLA. Scale bar = 100 μm. (D) Left: immunostaining of *Glp1r*-shRNA EGFP-labeled GLP-1R neurons. Arrowheads indicate neurons double-positive for both EGFP and GLP-1R. Right: quantification of co-labeled neurons ( $n=3$ ). Scale bar = 50 μm. (E) Representative Western blot images and quantification of *Glp1r* knockdown efficiency ( $n=3$ ). (F–H) The total distance, entries, and time spent in the center zone of the OFT in different groups before and after 3-day restraint stress. (I, J) The entries and time spent in the open arms of the EPMT in different groups before and after 3-day restraint stress. (K, L) The latency to feeding and food consumption of the

NSFT in different groups before and after 3-day restraint stress. Control-shRNA mice,  $n=10$ ; *Glp1r*-shRNA mice,  $n=9$ . Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, not significant. Unpaired two-tailed Student's  $t$ -tests for (E). Paired  $t$ -test used for (F–L).

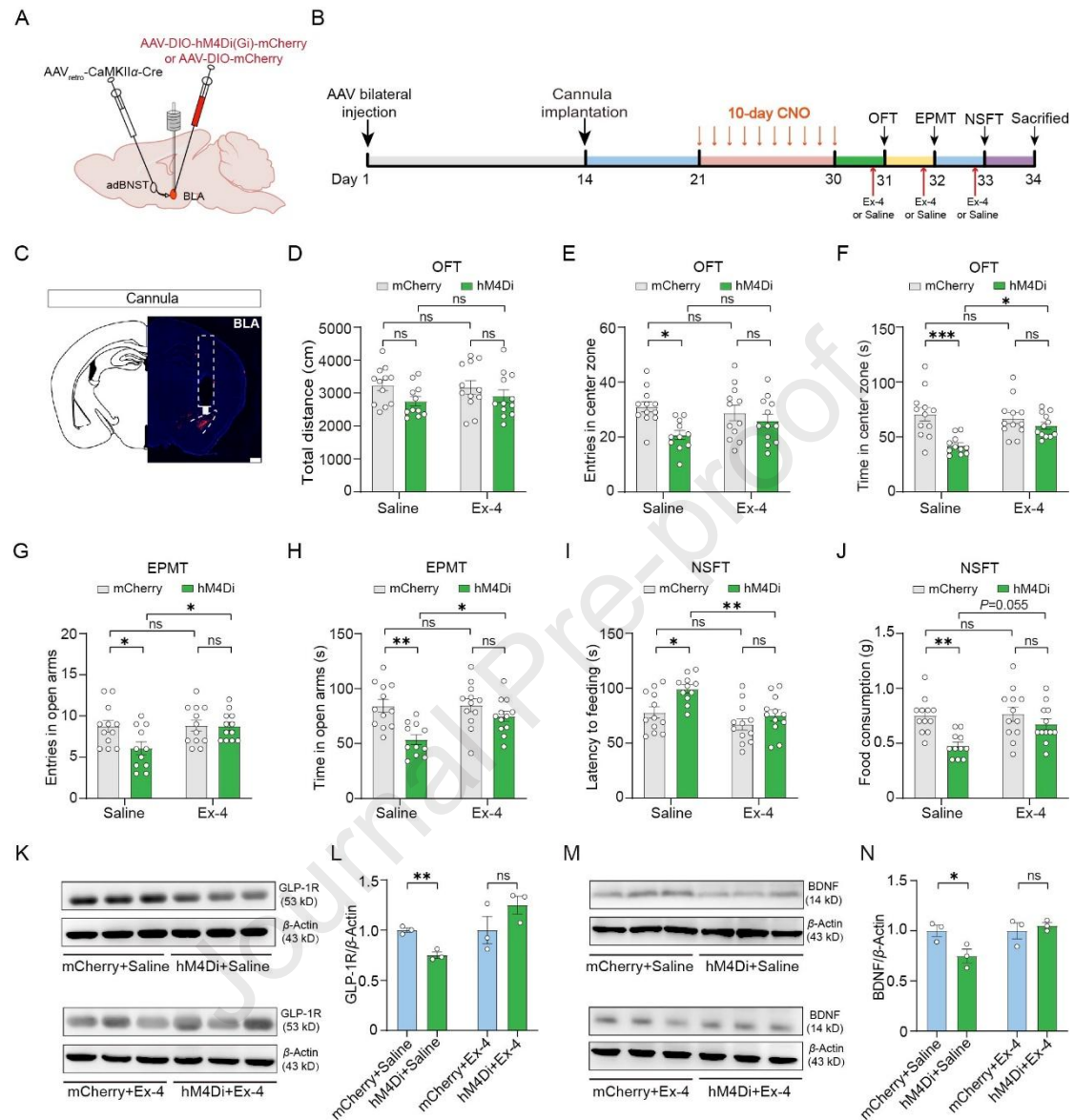
### 3.5. Ex-4 reduces anxiety-Like behaviors via the BLA–adBNST circuit in mice

Based on the above findings, we found that Ex-4 reduces anxiety-like behaviors by activating GLP-1R on BLA CaMKII $\alpha$  neurons. We then focused on the BLA–adBNST circuit, which is known to encode anxiety-like behaviors<sup>20,32</sup> and asked whether the observed effects of Ex-4 depended on this pathway. To address this question, wild-type mice were injected with AAV-DIO-hM4Di (Gi)-mCherry or AAV-DIO-mCherry into the BLA, and AAV<sub>retro</sub>-CaMKII $\alpha$ -Cre was injected into the adBNST (Fig. 5A). After the virus expression, cannula was implanted into BLA. After one week of recovery, mice received daily intraperitoneal injections of Clozapine-*N*-oxide or saline for 10 days. Meanwhile, Ex-4 or saline was administered into the BLA *via* cannula 10 min before behavioral tests (Fig. 5B and C). We found that the mice display anxiety-like behaviors after inhibiting BLA-adBNST circuit, this is consistent with the previous research results<sup>20</sup>. However, inhibiting BLA-adBNST circuit then infused Ex-4 could ameliorate anxiety-like behaviors, shows the entries in center zone and time spent in center zone significantly increased without affecting locomotor activity compared with control animals injected with AAV-DIO-hM4Di (Gi)-mCherry virus (Fig. 5D–F). In addition, both the entries into and time spent in the open arms of the EPMT were increased (Fig. 5G and H). Moreover, in the NSFT, stress exposure decreased the latency to feed and increased food consumption (Fig. 5I and J). These results indicate that the anxiolytic effects of Ex-4 depend on activation of the BLA–adBNST circuit. Notably, similar to the results shown in Fig. 3F–L, intra-BLA administration of Ex-4, unlike intraperitoneal injection (Fig. 1A–G), did not alleviate anxiety-like behaviors in wild-type mice. We speculate that, unlike in CRS-model mice, Ex-4 administration does not markedly alter adBNST neuronal activity in wild-type mice, and this lack of change

may be insufficient to drive anxiety-like behavioral differences. To investigate the underlying reason, we conducted both *in vivo* and *ex vivo* experiments to assess changes in adBNST neuronal activity following Ex-4 administration in the BLA (Supporting Information Fig. S1A and S1B). In the *ex vivo* c-Fos experiment, Ex-4 administration in the BLA in the control group did not produce a marked increase in adBNST neuronal activity compared with saline treatment (Fig. S1C and S1D), whereas CRS mice exhibited a significant increase in neuronal activity following Ex-4 administration relative to the saline group (Fig. S1E and S1F). To further validate our conclusion, we performed *in vivo* fiber photometry, which yielded results consistent with the *ex vivo* c-Fos findings. Specifically, Ex-4 administration did not markedly increase adBNST neuronal activity in control mice, but significantly enhanced neuronal activity in CRS mice (Supporting Information Fig. S2A–S2F). These results indicate that intra-BLA Ex-4 of wild-type mice does not alter the anxiety levels, but it could ameliorate anxiety-like behaviors in CRS mice.

To further investigate the underlying mechanism, we performed Western blot analysis to examine GLP-1R protein expression. The results showed that inhibition of the BLA–adBNST circuit led to a reduction in GLP-1R protein levels in the BLA compared with controls (Fig. 5K and L). These findings prompted us to investigate how GLP-1R modulate anxiety-like behaviors *via* the BLA–adBNST circuit in mice. BDNF is a well-known neuromodulator that plays a critical role in anxiety<sup>33-35</sup>, and previous studies have shown that BDNF in the BLA is also involved in regulating anxiety-like behaviors in mice<sup>36</sup>. Other studies have indicated that activation of GLP-1R leads to phosphorylation of the transcription factor CREB through the cAMP/PKA and PI3K/Akt pathways, thereby enhancing BDNF expression<sup>37-39</sup>. Based on these observations, we hypothesized that reduced GLP-1R activity decreases BDNF levels in the BLA, leading to reduced BDNF release to the adBNST and consequently promoting anxiety-like behaviors in mice. Consistent with this hypothesis, Western blot analysis showed that when GLP-1R protein levels were reduced, BDNF expression in the BLA was also decreased following inhibition of the BLA–adBNST circuit (Fig. 5M and N).

Collectively, these results indicate that Ex-4 alleviates anxiety-like behaviors *via* the BLA–adBNST circuit, potentially through a mechanism involving BDNF.

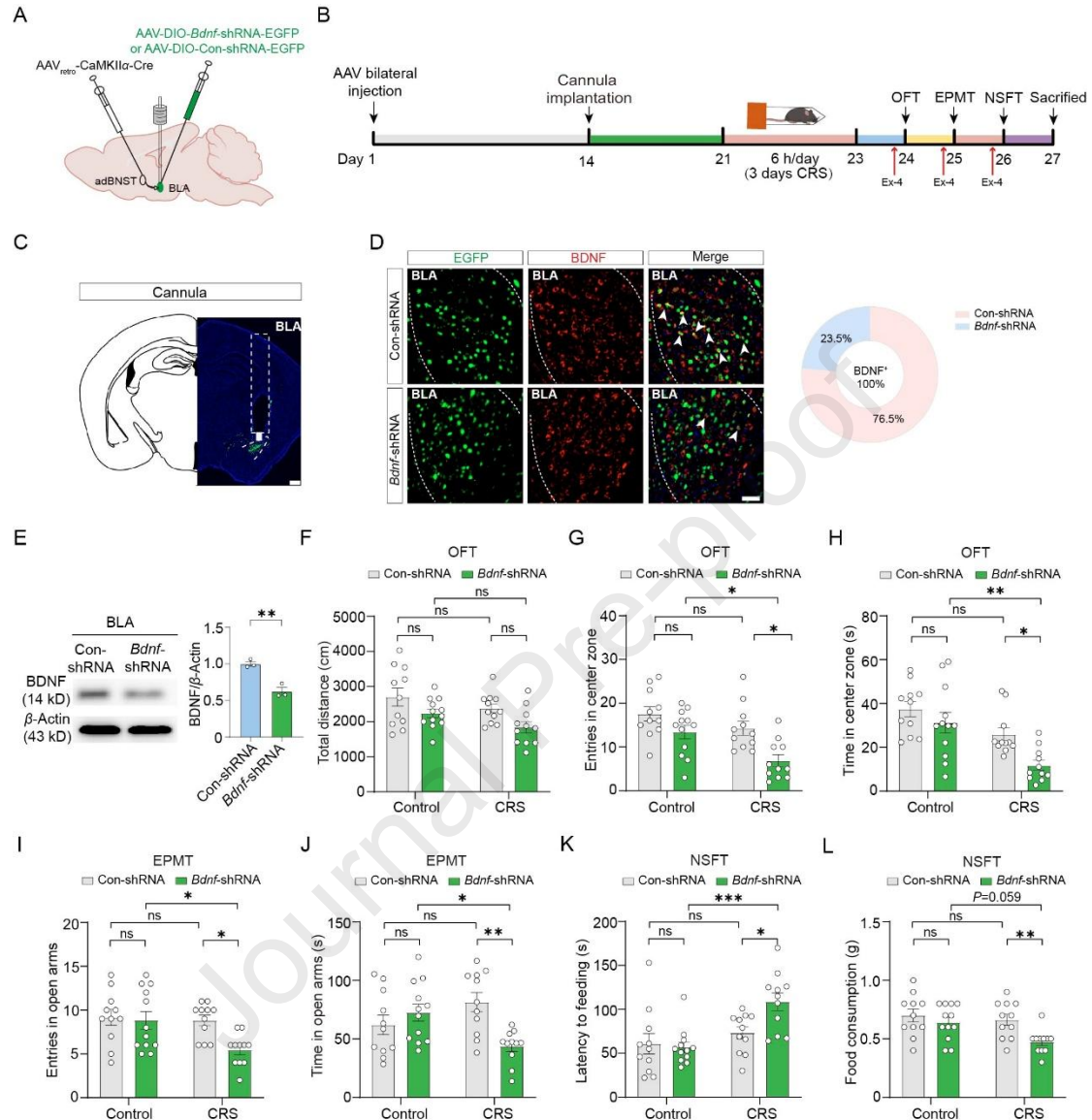


of GLP-1R in the mice inhibit the BLA-adBNST circuit and intra-BLA Ex-4 after inhibit BLA-adBNST circuit ( $n=3$ ). (M, N) The protein expression and analysis of BDNF in the mice inhibit BLA-adBNST circuit and intra-BLA Ex-4 after inhibiting the BLA-adBNST circuit ( $n=3$ ). Data represented as mean  $\pm$  SEM.  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$ ; ns, not significant. Unpaired two-tailed Student's *t*-tests for (L) and (N). Two-way ANOVA followed by Bonferroni's *post hoc* analysis for (D–J).

### 3.6. GLP-1R ameliorate anxiety-like behaviors through regulating BDNF signaling in the BLA-adBNST circuit

To further confirm this conclusion, Western blot and immunofluorescence were performed on mice with *Glp1r* knocked down in BLA CaMKII $\alpha$  neurons. The results showed that knockdown of *Glp1r* also led to a reduction of BDNF in the BLA (Supporting Information Fig. S3A–S3C). Building on these findings, we further investigated the role of BDNF in the BLA CaMKII $\alpha$ –adBNST circuit by deleting BDNF in this pathway through injection of AAV<sub>retro</sub>-CaMKII $\alpha$ -Cre into the adBNST and AAV-DIO-*Bdnf*-shRNA-EGFP or AAV-DIO-Con-shRNA-EGFP into the BLA (Fig. 6A). Mice received injections of viruses into the BLA, followed by cannula implantation in all animals (Fig. 6C). Half of the mice were subjected to SRS, while the other half served as non-stressed controls. Ex-4 was administered *via* the implanted cannula 10 min before behavioral testing in all mice (Fig. 6B). The efficiency of the *Bdnf* knockdown was confirmed by Western blot and immunofluorescence analysis (Fig. 6D and E). Behavioral tests indicated the *Bdnf*-knockdown mice, which suffer from SRS exhibited significantly reduced entries in the center zone and time spent in the center zone significantly reduce without affecting locomotor activity (Fig. 6F–H). And the entries in open arms and time spent in open arms in EPMT were also reduced (Fig. 6I and J). Moreover, the latency to feed was increased, and the food consumption was decreased in NSFT (Fig. 6K and L). Meanwhile, Ex-4 could not rescue the anxiety-like behaviors of the mice (Fig. 6F–L). These data suggest that when *Bdnf* was knocked down in the BLA-adBNST circuit, it facilitates stress-induced anxiety-like behaviors.

Collectively, these results demonstrate that GLP-1R regulates anxiety-like behaviors through BLA-adBNST BDNF signaling.

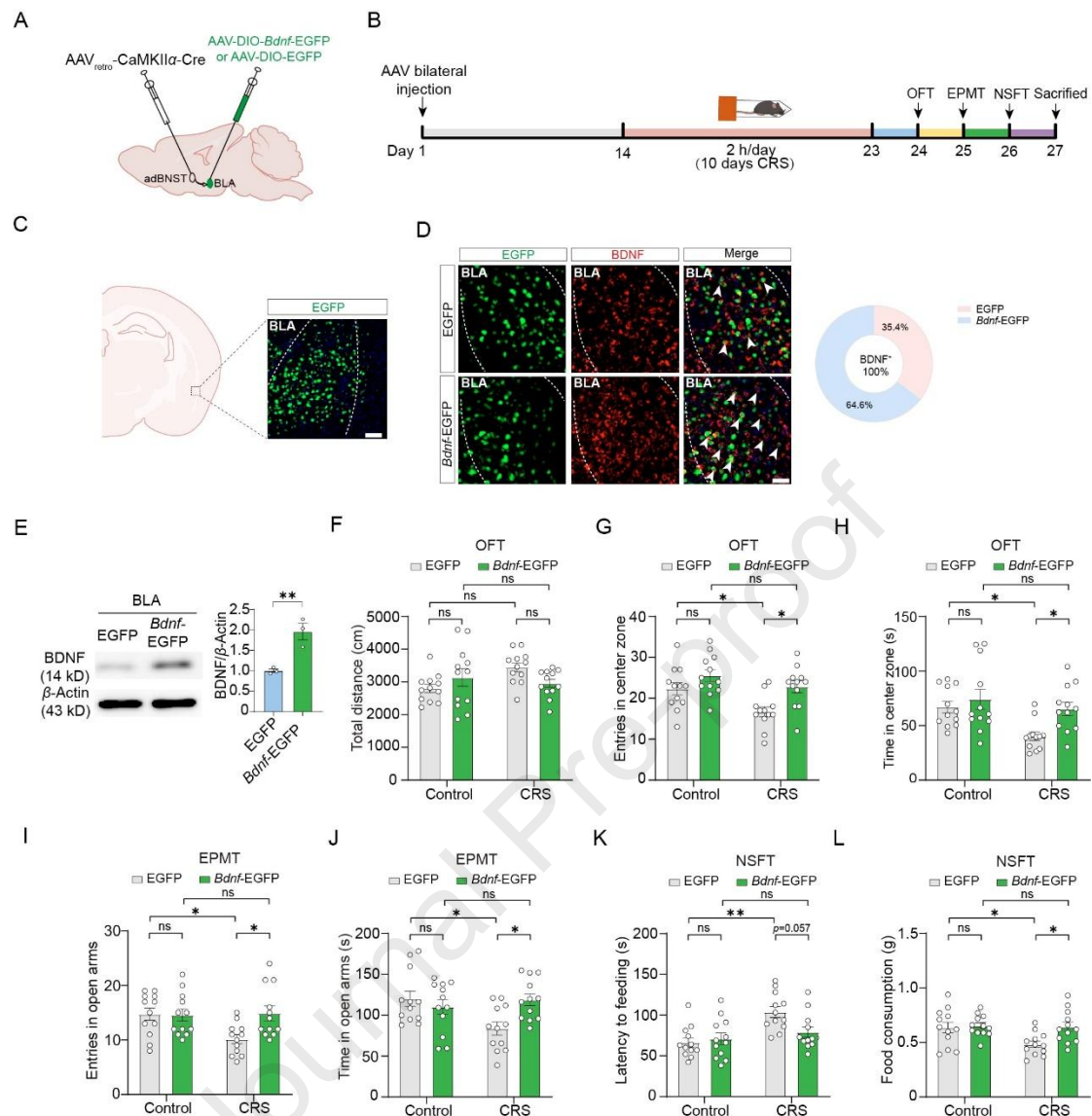


**Figure 6** GLP-1R ameliorate anxiety-like behaviors through regulating BDNF signaling in the BLA-adBNST circuit. (A) Schematic representation of virus injection. (B) Timeline of experiments. (C) Representative image of virus injection and cannula implantation in BLA. Scale bar = 500  $\mu$ m. (D) Left: immunostaining of Bdnf-shRNA EGFP-labeled BDNF neurons. Arrowheads indicate neurons double-positive for both EGFP and BDNF. Right: quantification of co-labeled neurons ( $n=3$ ). Scale bar = 50  $\mu$ m. (E) Representative Western blot images and quantification of Bdnf knockdown efficiency ( $n=3$ ). (F-H) The total distance, entries, and time spent in the center zone of the OFT in different groups. (I, J) The entries and time spent in the open arms of the

EPMT in different groups. (K, L) The latency to feeding and food consumption of the NSFT in different groups. Control+Con-shRNA,  $n=11$ ; Control+*Bdnf*-shRNA,  $n=12$ ; CRS+Con-shRNA,  $n=11$ ; CRS+*Bdnf*-shRNA,  $n=11$ . Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ns, not significant. Unpaired two-tailed Student's *t*-tests for (E). Two-way ANOVA followed by Bonferroni's *post hoc* analysis for (F–L).

### 3.7. Overexpression of *Bdnf* in the BLA-adBNST circuit ameliorates anxiety-like behaviors

To further substantiate our findings, *Bdnf* was overexpressed specifically in the BLA-adBNST pathway to assess its impact on anxiety-like behaviors (Fig. 7A). Next, all the mice were injected with AAV<sub>retro</sub>-CaMKII $\alpha$ -Cre into the adBNST and AAV-DIO-*Bdnf*-EGFP or AAV-DIO-EGFP into the BLA, then half of them subjected to CRS. Subsequently, a series of tests was conducted to assess anxiety-like behaviors (Fig. 7B). The efficiency of the *Bdnf* overexpression was confirmed by Western blot and immunofluorescence analysis (Fig. 7C–E). Consistent with our expectations, *Bdnf* overexpression in the BLA–adBNST circuit significantly rescued anxiety-like behaviors, including increased time spent and entries into the center of the OFT and the open arms of the EPMT, reduced latency to feed, and increased food intake in the NSFT, without affecting locomotor activity (Fig. 7F–L). Taken together, these results demonstrate that GLP-1R regulates anxiety-like behaviors *via* BDNF signaling in the BLA–adBNST circuit.



CRS+*Bdnf*-EGFP,  $n=12$ . Data represented as mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ ; ns, not significant. Unpaired two-tailed Student's  $t$ -tests for (E). Two-way ANOVA followed by Bonferroni's *post hoc* analysis for (F–L).

#### 4. Discussion

In this study, we identify a critical role of BLA GLP-1R in modulating anxiety-like behaviors *via* the BLA–adBNST circuit. Mechanistically, this regulation is mediated by GLP-1R in BLA neurons that increase BDNF expression, which subsequently drives anterograde BDNF signaling along the BLA–adBNST projection. Together with the well-established role of the BLA–adBNST circuit in anxiety disorders, our findings provide novel insights into the underlying mechanisms and identify BLA GLP-1R as a key molecular substrate that regulates anxiety through BDNF signaling within this circuit. These results further suggest that targeting GLP-1R and the associated BDNF pathway may represent a promising therapeutic strategy for anxiety disorders.

Our current study offers considerable advancement in the knowledge of the function and regulation of anxiety disorders. First, we identified a region-specific role of GLP-1R in the BLA, demonstrating its critical involvement in the behavioral regulation of anxiety-like behaviors. This finding aligns with the notion that GLP-1-based drugs can alleviate mood disorders by modulating the release of neurotransmitters such as dopamine and serotonin<sup>13</sup>. For example, the GLP-1R agonist liraglutide has been reported to attenuate depressive- and anxiety-like behaviors by enhancing Hip plasticity<sup>40</sup>. Consistently, our results show that systemic administration of the GLP-1R agonist Ex-4 (1  $\mu\text{g/kg}$ ) ameliorates anxiety-like behaviors without affecting depression-like behaviors, body weight, or blood glucose levels. Furthermore, Western blot and immunofluorescence results show that GLP-1R expression in BLA was decreased after CRS, and the GLP-1R mainly co-localized with CaMKII $\alpha$  neurons. Pharmacological activation of BLA GLP-1R after CRS effectively rescued anxiety-like behaviors. However, previous studies have identified a distinct role for GLP-1R in the dLS, where they modulate behavioral responses to cocaine without affecting anxiety<sup>15</sup>.

GLP-1R in the LS is also involved in weight regulation<sup>16</sup>. Our study demonstrates that GLP-1R in the BLA specifically contributes to anxiety-like behaviors. The apparent discrepancies across studies may reflect the region-specific functions of GLP-1R in different brain areas.

Second, we demonstrated an anxiety-alleviating-associated increase in BDNF expression, which relies on the BLA GLP-1R activity. BDNF has been well recognized as a key mediator of anxiety<sup>40</sup> and has a close connection with GLP-1R<sup>37-39,42</sup>, and many anxiolytics may exert their effects *via* interactions with BDNF signaling<sup>43-45</sup>. We focused on BDNF as a potential downstream key molecule regulated by GLP-1R in BLA for anxiety. In the past study, mRNA sequencing analysis has revealed an extinction-associated expression of BDNF<sup>23</sup>. From a mechanistic perspective, we hypothesize that GLP-1R activation may lead to phosphorylation of the transcription factor CREB *via* the cAMP/PKA, PI3K/Akt, and MAPK/ERK pathways, subsequently increasing BDNF levels in glutamatergic neurons of the BLA. However, this proposed mechanism remains to be experimentally tested and warrants further investigation<sup>37-39</sup>. Studies have shown that *Bdnf* deletion in the BLA and surrounding area increases anxiety levels in mice<sup>36</sup>, which is different from us. In our study, knockdown of *Bdnf* in the BLA was not sufficient to induce anxiety-like behaviors but facilitated stress-induced anxiety-like behaviors. We attribute this discrepancy to methodological differences: Xie et al.<sup>36</sup> used a broad BLA *Bdnf* knockout, while our shRNA approach targeted only CaMKII $\alpha$  neurons. This suggests BDNF from non-neuronal sources (*e.g.*, glia, astrocytes) or adjacent regions (central nucleus of the amygdala) may compensate under basal conditions.

Third, research has revealed that the synaptic weakening of BLA<sup>CaMKII $\alpha$</sup> -adBNST<sup>GABA</sup> circuit is the cause of anxiety occurrence, and reversing this change would improve anxiety<sup>46</sup>. Our study proved most of the changes were dependent on GLP-1R in BLA, highlighting the importance of BLA GLP-1R neurons in anxiety disorders. In order to prove GLP-1R-dependent BLA-adBNST BDNF signaling is associated with anxiety disorder, the *Bdnf* was knocked down in the BLA-adBNST

circuit, then Ex-4 was infused into BLA. It was found that Ex-4 could not successfully rescue the anxiety disorder in these mice. These results indicate that Ex-4 could rescue anxiety disorder through activation of GLP-1R-dependent BLA-adBNST BDNF signaling.

## 5. Conclusions

In summary, we propose a working model in which BLA GLP-1R regulates anxiety-like behaviors by enhancing BDNF expression in the BLA. This is followed by anterograde BDNF signaling from the BLA to the adBNST, which modulates NMDAR function at BLA–adBNST synapses, thereby promoting synaptic plasticity and resilience. Our findings highlight a critical role for BLA GLP-1R–BDNF signaling in the regulation of anxiety-like behaviors. This work provides significant insights into the neural mechanisms through which GLP-1R influences neuronal circuit function, as well as the contribution of adBNST activity to anxiety. Collectively, these results may inform the development of novel therapeutic strategies targeting anxiety-related symptoms across a range of neuropsychiatric disorders.

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## Author contributions

Gang Hu, Qian Zhang: designed the experiments and supervised the project. Qian Zhang, Ke Wei: investigation, formal analysis, writing original draft preparation. Ke Wei, Meng Yu, Lei Gao, Qiaoyue Zhang: conducting all the experiments. Jianhua Ding:

experimental guide. Qian Zhang, Ke Wei: made critical revision for the manuscript.

### Conflicts of interest

The authors declare no conflicts of interest.

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