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The hypothalamic orexin–medial prefrontal cortex pathway modulates susceptibility to depression in the learned helplessness paradigm in male mice

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Title: The hypothalamic orexin–medial prefrontal cortex pathway modulates susceptibility to depression in the learned helplessness paradigm in male mice

Abbreviated title: Orexin projection to mPFC modulates depression

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Conflict of Interest

The authors declare no competing financial interests.

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Abstract

Depression is a prevalent psychiatric disorder primarily characterized by behavioral despair and helplessness. The orexinergic system plays a crucial role in stress-induced depression; however, the precise mechanisms remain unclear. The medial prefrontal cortex (mPFC), a major target of orexinergic projections, is essential for emotional regulation. This study suggests that impaired orexinergic input to the mPFC underlies depression-like behaviors in murine models. In the learned helplessness (LH) paradigm, the majority of male mice subjected to inescapable foot shocks developed depression-like behaviors (classified as LH-susceptible), whereas a subset displayed resilience (classified as LH-resilient). LH-susceptible mice exhibited a reduced number of orexin⁺ neuronal somata in the hypothalamus, along with decreased expression of *prepro-orexin mRNA*. Correspondingly, the mPFC of these mice displayed decreased density of orexinergic terminals and lower Orexin A levels. In contrast, LH-resilient mice maintained stable hypothalamic orexin neuron counts, exhibited increased *prepro-orexin mRNA* expression, and preserved orexinergic terminal density in the mPFC. Chemogenetic activation of the hypothalamic orexin–mPFC pathway alleviated depression-like behaviors in LH-susceptible mice through Orexin A signaling. Conversely, inhibition of this pathway induced depression-like behaviors in LH-resilient mice. Additionally, LH-susceptible mice showed decreased excitatory synaptic transmission in layer V pyramidal neurons of the mPFC, which was improved by activation of hypothalamic orexinergic inputs; this improvement was attenuated by administration of an orexin receptor 1 (OX1R) antagonist. Collectively, these findings implicate the hypothalamic orexin–mPFC pathway as a pivotal regulator of excitatory synaptic transmission and suggest its involvement in modulating individual variability in susceptibility and resilience to depression.

Significance statement

Depression is a highly prevalent emotional disorder characterized by behavioral despair and helplessness under stress. This study demonstrates that the orexinergic system is critical for modulating resilience to aversive stimuli. Using the learned helplessness (LH) mouse model, we found that LH-susceptible mice, but not LH-resilient mice, show compromised hypothalamic orexinergic projections to the medial prefrontal cortex (mPFC), together with decreased excitatory synaptic transmission in layer V pyramidal neurons. Activation of the hypothalamic orexin–mPFC pathway alleviates depression-like behaviors in susceptible mice through Orexin A, while inhibition of this pathway induces depression-like phenotypes in resilient mice. These findings identify orexinergic signaling as a promising therapeutic target for depression.

Introduction

Depression is one of the most prevalent psychiatric disorders, often characterized by persistent low mood, sadness, hopelessness, and diminished interest or pleasure (Sahani and Singh, 2025). The pathogenesis of depression involves specific neuronal cell types distributed across multiple brain regions and their constituent neural circuits (Lv et al., 2022). The medial prefrontal cortex (mPFC) is a critical brain region involved in motor control, decision-making, and emotional regulation, and it is among the most structurally altered areas in the brains of individuals with depression (Price and Duman, 2020). Upon repeated exposure to foot shocks, mice utilize top-down regulation by the mPFC to decide the coping strategies against subsequent aversive stimuli, actively escaping or passively enduring (Wang et al., 2021; Abdulla et al., 2024). Activation of the mPFC promotes resilience to stress and supports active coping strategies. Conversely, functional impairments in the mPFC increase vulnerability to passive helplessness (Amat et al., 2005; Wu et al., 2021; Cheng et al., 2025). Acute stress induces the activation of NMDA and AMPA receptors in the mPFC of rats, leading to increased excitatory synaptic currents and enhanced synaptic excitability in this region (Yuen et al., 2011). In contrast, chronic stress reduces dendritic complexity in pyramidal neurons of the mPFC and decreases the excitability of glutamatergic pyramidal neurons (Luo et al., 2020), resulting in an imbalance in excitatory-inhibitory (E/I) signaling in mPFC neurons (Carcea and Froemke, 2013). Clinical studies have also shown that synapse-related gene expression in the mPFC is downregulated in patients with major depressive disorder (MDD) (Fries et al., 2023).

Orexin, an excitatory neuropeptide, was discovered in the hypothalamus of rats in 1998 (de Lecea et al., 1998; Sakurai et al., 1998). It consists of two isoforms, Orexin A and Orexin B, both derived from a common 130-amino-acid precursor peptide, prepro-orexin (PPO), through proteolytic processing (Kukkonen, 2017). These peptides are synthesized exclusively in the cell bodies of orexin-producing neurons located in the lateral hypothalamus, dorsomedial hypothalamus, and perifornical area (Harris and Aston-Jones, 2006; Jiao et al., 2025). Orexin binds to two G protein-coupled receptors, OX1R and OX2R, activating downstream signaling pathways that regulate various physiological functions (Sun et al., 2021). Dysregulation of the orexin system causes sleep disturbances, appetite issues, lack of motivation, and emotional instability, which are symptoms commonly observed in MDD (Wang et al., 2023). These observations suggest a significant association between the orexin system and depressive symptomatology; however, the precise mechanistic role of orexin in the pathophysiology of depression remains a subject of ongoing debate (Lutter et al., 2008; Nocjar et al., 2012; Jalewa et al., 2014). Orexin neurons project to multiple brain regions implicated in stress regulation, including the mPFC, where orexin receptors are densely expressed on pyramidal neurons (Sargin, 2019; Han et al., 2022). Orexin A exerts a direct excitatory effect on mPFC pyramidal neurons in a dose-dependent manner, which can be blocked by the OX1R antagonist SB-334867 (Yan et al., 2012).

Drawing on the research findings discussed above, we propose that the orexinergic neural pathway connecting the hypothalamus to the mPFC plays a critical regulatory role in the pathophysiology of depressive disorders. In the present study, a chronic foot shock paradigm was utilized to classify mice into learned helplessness (LH)-susceptible and LH-resilient groups based on their escape responses. By integrating behavioral assays, morphological and molecular analyses, as well as patch-clamp electrophysiology, alterations in orexinergic projections to the mPFC were examined. Furthermore, chemogenetic and pharmacological modulation of the

116 hypothalamic orexin–mPFC pathway was conducted to evaluate its impact on behavioral
117 outcomes, thereby elucidating the pathway’s potential role in the underlying mechanisms of
118 depression.

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Materials and Methods

Animals

Male orexin-Cre and C57BL/6J wild-type (WT) mice, aged 6–8 weeks, were used in this study. The orexin-Cre mice were generated by Shanghai Model Organisms Center, Inc. via CRISPR/Cas9-mediated insertion of the IRES-Cre-2A-EGFP-WPRE-pA cassette downstream of the Hcrt-201 transcript stop codon. WT mice were obtained from the Laboratory Animal Center of Ningxia Medical University. All animals were housed in an environment with controlled temperature ($20 \pm 2^\circ\text{C}$) and humidity (45%–70%), under a 12-h light/dark cycle, with free access to food and water. All efforts were made to minimize the use of animals and alleviate their suffering. The experimental procedures received approval from the Ethics and Animal Welfare Committee of Ningxia Medical University and were conducted in accordance with applicable governmental regulations.

Preparation procedure for the LH animal model

The LH paradigm was used to induce depression-like behavior in mice (Hammack et al., 2012; Brachman et al., 2016). The establishment and screening of the LH model consisted of two phases: (1) Training phase: Mice were placed in partitioned shuttle boxes (Med Associates, USA) and subjected to 360 inescapable foot shocks per day for six consecutive days. The shock intensity was set at 0.6 mA, with an average duration of 3 s. The inter-shock intervals were randomized and unpredictable, ranging from 5 to 8 s. (2) Testing phase: On day 7, following the final training session, classical shuttle box testing was performed to evaluate the effectiveness of LH induction. Prior to the formal test, mice were allowed to freely explore the shuttle box for 2 min. The test consisted of 30 trials. Each trial began with a 5-s cue light, followed by 25 s of light paired with a foot shock. If the mouse crossed to the other compartment within the 30-s window, the trial was recorded as a successful escape, and the latency to cross was measured. If the mouse failed to cross, the trial was recorded as an escape failure. In both cases, the shock was terminated after 30 s, and the next trial began. Mice with 15 or fewer crossings were classified as LH-susceptible, while those with more than 15 crossings were classified as LH-resilient.

Behavioral tests

The tail suspension test (TST) is widely used to assess antidepressant effects in rodents (Can et al., 2012). Mice were acclimated to the testing environment for 2 h prior to the test. Each mouse was suspended by the tail from a hook ~50 cm above the ground in a soundproof chamber. Immobility time was recorded using Smart 3.0 software over 7 min. The first 2 min were excluded from analysis; data from the final 5 min were used for statistical evaluation.

The open field test (OFT) was used to assess anxiety- and depression-related behaviors in mice (Meng et al., 2024). The test was conducted in a $50 \times 50 \times 40$ cm plastic box with white walls and a white bottom. The center 15×15 cm area was designated as the center zone, with the rest as the peripheral zone. Mice were first allowed to acclimatize to the environment for 2 h. Then each mouse was placed in the box facing a corner, and its activity was recorded for 10 min. The number of entries into the center zone and the duration in the center zone over the 10 min period were analyzed using the Smart 3.0 software. After each test, the box was cleaned with 75% ethanol, and testing resumed once the ethanol evaporated.

The sucrose preference test (SPT) was used to assess anhedonia in mice (Primo et al., 2023). Mice were singly housed prior to testing. On day 1, two bottles of water were provided for adaptation. On day 2, one bottle was replaced with 1% sucrose solution for 12 h. On day 3, mice underwent 24 h of food and water deprivation. On day 4, bottles containing water and sucrose solution were returned, with positions switched after 6 h. Sucrose preference was calculated as follows: Sucrose preference (%) = [sucrose intake / (sucrose intake + water intake)] × 100.

Surgery

All surgeries were performed under isoflurane anesthesia (3% induction, 1.5% maintenance) using a stereotaxic frame (RWD Life Science, Shenzhen, China). Mice were placed on a heating pad and secured in the frame. After shaving the scalp, a midline incision was made to expose the skull. Erythromycin ophthalmic ointment was applied to the eyes to prevent corneal drying.

Stereotaxic injections of retrograde tracer CTB. Using bregma as the reference point, a 1–2 mm cranial hole was drilled, and a microsyringe loaded with CTB (0.2%, Cat# 10433A1, List Labs, USA) was positioned into the left mPFC (AP = +1.98 mm, ML = +0.3 mm, DV = –1.64 mm). 50 nL of CTB solution was slowly injected. After the injection, the incision was sutured and the mice were placed on a 37°C warming pad for recovery. When fully awake, the mice were returned to individually ventilated cages (IVCs). One week later, immunofluorescence staining was performed to verify the injection site and identify retrogradely labeled cells.

Stereotaxic injections of viral vector. Surgical procedures were performed as described above. rAAV2/9-Ef1a-DIO-hM3D(Gq)-mCherry-WPREs, rAAV2/9-Ef1a-DIO-hM4D(Gi)-mCherry-WPREs or the control virus rAAV2/9-Ef1a-DIO-mCherry-WPREs (all $>2.00 \times 10^{12}$ vg/mL; provided by BrainVTA, Wuhan, China) was injected into the bilateral hypothalamus of orexin-Cre mice (200 nL on each side, bregma: AP = –1.5 mm, ML = ±0.7 mm, DV = –5.3 mm). Twenty-one days later, immunofluorescence staining was performed to verify the injection site and viral expression.

Cannula implantation. Surgical procedures were performed as described above. Stainless steel guide cannulae (Cat# 62102, RWD Life Science, Shenzhen, China) were implanted with the tip positioned 1 mm above the mPFC (bregma: AP = +1.98 mm, ML = ±0.3 mm, DV = –1.64 mm) unilaterally or bilaterally. Cannulae were secured to the skull with three stainless steel screws and dental acrylic. Orexin A/Orexin B/Dora were microinjected via the unilateral guide cannula (left or right side), while CNO was administered via the guide cannulae implanted into both sides of the mPFC. After drug administration, Nissl staining was performed to confirm cannula placement.

Immunofluorescence staining

Mice were deeply anesthetized with isoflurane and transcardially perfused with 200 mL of 0.9% saline followed by 50 mL of 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 8 h and dehydrated in 30% sucrose for 48 h. Coronal sections (35 µm) were cut using a cryostat microtome (Leica, Germany). Brain sections were blocked with 10% normal goat serum and 1% Triton X-100 in 50 mM PBS for 2 h at room temperature, incubated with primary antibodies (rabbit anti-Orexin A, 1:1000, Cat# H-003-36, Phoenix, USA; goat anti-CTB, 1:1000, Cat# 7032A9, List Labs, USA; rabbit anti-Cre, 1:1000, Cat# ab216262, Abcam, UK) overnight at 4°C, and then incubated with secondary antibodies (Cy5 donkey anti-goat IgG, 1:500, Cat# 705-175-147, Jackson, USA; Alexa Fluor 488 donkey anti-rabbit IgG, 1:500, Cat# ab150073, Abcam, UK; Cy5 donkey anti-rabbit IgG, 1:500, Cat# 711-175-152, Jackson, USA) for 2 h at room

temperature. Sections were then mounted on glass slides, counterstained with 4',6-diamidino-2-phenylindole (DAPI, Cat# 0100-20, Southern Biotech, USA) and cover-slipped for imaging. Fluorescence images were acquired using a high-resolution fluorescence microscope (DM6 B, Leica, Germany). The specificity of the antibodies has been verified in our previous studies (He et al., 2022; Ma et al., 2023).

Nissl staining

Nissl staining was performed to confirm cannula placement. Brain sections were mounted on slides and air-dried, followed by staining in Nissl Staining Solution A (Cat# DK0022100, Solarbio, Beijing, China) at 56°C for 1 h. The sections were then placed in 1% hydrochloric acid in ethanol for 2 min, dehydrated through a graded ethanol series (75%, 85%, 95%, 100% I, 100% II), and cleared in xylene I and II (for 1 min each). Finally, coverslips were mounted using neutral balsam (Cat# 8590, Solarbio, Beijing, China).

Quantitative real-time PCR

Total RNA was extracted from fresh hypothalamic tissue using an RNA kit (Cat# DP304-02, TIANGEN, Beijing, China). The concentration and purity of the RNA were assessed with a NanoDrop 2000C (Thermo Fisher Scientific, USA). cDNA was synthesized using the PrimeScript™ RT Reagent Kit (Cat# RR037B, Takara, Japan) with gDNA Eraser (Cat# RR047A, Takara, Japan). qPCR was performed using the Premix Ex Taq™ II Kit (Cat# RR820A, Takara, Japan) on a CFX-96 system (Bio-Rad, USA). PCR conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Ct values were recorded, and relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method, normalized to the internal control. Primer sequences (5'–3') are as follows (S = sense; A = antisense):

Orexin.S.mus: CCTGCCGTCTCTACGAACTG

Orexin.A.mus: GGTGCTAAAGCGGTGGTAGT

Gapdh.S.mus: CCTCGTCCCGTAGACAAAATG

Gapdh.A.mus: TGAGGTCAATGAAGGGGTCGT

Western blotting

Mice were anesthetized with isoflurane, and mPFC tissues were rapidly dissected and homogenized in lysis buffer. Protein concentration was measured using a BCA kit (Cat# KGP250, KeyGEN Biotech, China). Equal protein amounts were separated by 12% SDS-PAGE and transferred to PVDF membranes. After blocking with a protein-free buffer, membranes were incubated overnight at 4°C with primary antibodies: rabbit anti-Orexin A (1:1000, Cat# orb158063, Biorbyt, UK), rabbit anti-Orexin B (1:1000, Cat# GTX129928, GeneTex, USA), and mouse anti-β-actin (1:3000, Cat# BS6007M, Bioworld, USA). After washing, membranes were incubated for 1.5 h at room temperature with HRP-conjugated donkey anti-rabbit (1:3000, Cat# A25222, Abbkine, China) or anti-mouse (1:3000, Cat# A21010, Abbkine, China) antibodies. Bands were visualized using ECL reagent (Cat# WP20005, Thermo Fisher Scientific, USA) mixed 1:1 (solutions A and B, 100 μL/lane) and imaged with the Amersham Imager 600. Band intensities were analyzed using ImageJ and normalized to β-actin.

Enzyme-linked immunosorbent assay (ELISA)

To assess the levels of Orexin A and B in the mPFC, tissue samples were obtained from the mPFC of mice. Quantification was conducted using commercial ELISA kits in accordance with the manufacturer's instructions: Mouse Orexin A ELISA Kit (Cat# QZ-14764, Jiubang Bio, China) and Mouse Orexin B ELISA Kit (Cat# QZ-14765, Jiubang Bio, China).

Transmission electron microscopy (TEM)

Mice were anesthetized with isoflurane, and brain tissues were promptly harvested. A small block (1 mm³) of the mPFC was meticulously dissected and immediately immersed in ice-cold saline to minimize cellular damage. Subsequently, the tissue was fixed in 2.5% glutaraldehyde at 4°C overnight. After embedding, ultrathin coronal sections (50–100 nm) were prepared and imaged using a transmission electron microscope.

Whole-cell patch-clamp electrophysiology

The mice were anesthetized with isoflurane and transcardially perfused with 20 mL of ice-cold slicing solution composed of the following (in mM): 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄·2H₂O, 10 glucose, 210 sucrose, 1.3 sodium ascorbate, 0.5 CaCl₂·2H₂O, and 7 MgSO₄, bubbled with 95% O₂/5% CO₂ (pH 7.4). Coronal brain slices (400 µm thick) containing the mPFC were prepared using a vibratome (Leica VT1000S). Slices were recovered at 32°C for 30 min in artificial cerebrospinal fluid (ACSF) consisting of (in mM): 2.5 KCl, 26.2 NaHCO₃, 1 NaH₂PO₄·2H₂O, 11 glucose, 119 NaCl, 2.5 CaCl₂·2H₂O, and 1.3 MgSO₄, continuously bubbled with 95% O₂/5% CO₂. Slices were then maintained at room temperature for ≥60 min before recording.

During recordings, brain slices were continuously perfused with ACSF (aerated with 95% O₂/5% CO₂) containing 50 µM picrotoxin (Cat# HY-101391, MCE, USA) at a flow rate of ~3 mL/min. Recording pipettes (3–5 MΩ) were pulled using a horizontal puller (Sutter P-1000) and filled with an internal solution composed of the following (in mM): 125 CsCl, 8 NaCl, 10 HEPES, 2 MgATP, 0.3 NaGTP, 0.2 EGTA, and 0.1% biocytin (pH 7.3, 290–300 mOsm). Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded from mPFC layer V pyramidal neurons voltage-clamped at -70 mV.

Signals were acquired using a patch-clamp amplifier (HEKA EPC10 Double USB, Germany), low-pass filtered at 2 kHz and digitized at 10 kHz. Data were recorded using PatchMaster software (HEKA, Germany). Recordings were excluded if the series resistance varied by more than 25% from the initial value. sEPSC waveforms were analyzed using Mini Analysis 6.0.1 (Synaptosoft).

Drugs

Orexin A (15.6–125 pmol/0.5 µL, Cat# T003-30, Phoenix Pharmaceuticals, USA) and Orexin B (15.6–125 pmol/0.5 µL, Cat# T003-31, Phoenix Pharmaceuticals, USA) were dissolved in ACSF. The dual orexin receptor antagonist (Dora, TCS-1102, 62.5–250 pmol/0.5 µL, Cat# 3818, TOCRIS Bio-Techne, UK) was also dissolved in ACSF. Clozapine-N-oxide (CNO, Cat# A3317, APExBIO, USA) was dissolved in DMSO as a stock solution and diluted in ACSF before use. For intra-mPFC microinjection, CNO was used at 1 mM (0.5 µL per side); for slice electrophysiology, CNO was bath-applied at 5 µM. The OX1R antagonist SB-334867 (Cat# HY-10895, MCE, USA)

was dissolved in DMSO as a 1 mM stock solution and was diluted to 10 μ M with ACSF before use.

Experimental Design and Statistical Analysis

To compare the number of orexin⁺ neurons in the lateral hypothalamus, serial sections of the hypothalamic region (7 sections/mouse, with a 140 μ m interval between adjacent sections) from control, LH-susceptible and LH-resilient mice ($n = 3$ each) were immunostained for orexin. Images were captured with identical exposure settings to ensure comparability between samples. All orexin-positive neurons exhibiting characteristic neuronal morphology and a visible nucleolus (used as a unique identifier for each neuron) were counted. Similarly, to assess the differences in orexin fiber density within the mPFC, serial brain sections containing the mPFC (4 sections/mouse) from control, LH-susceptible and LH-resilient mice ($n = 3$ each) were immunostained for orexin. Images were analyzed using the ImageJ software. The mPFC region was manually outlined in each section, and the area occupied by orexin-positive fibers was quantified using the “Threshold” function in ImageJ to separate specific orexin-positive fiber signals from background fluorescence. The percentage of orexin-positive fiber area relative to the total mPFC area was calculated.

For ELISA quantification of Orexin A and Orexin B peptide levels in the mPFC, two independent cohorts were analyzed. In the first cohort, WT mice (control and LH-susceptible groups, $n = 7$ mice per group) were used, with each sample measured in duplicate, and the average of the two technical replicates was used as a single value per mouse for statistical analysis. In the second cohort, following chemogenetic activation, orexin-Cre mice (control, LH-susceptible, and LH-susceptible+hM3D(Gq) groups, $n = 4$ mice per group) were analyzed, with each sample also measured in duplicate.

To assess potential impairments in excitatory synaptic transmission in the mPFC of LH-susceptible mice, transmission electron microscopy (TEM) was performed on ultrathin mPFC sections from control and LH-susceptible mice ($n = 3$ each). A total of 42 asymmetrical synapses per group were analyzed to compare postsynaptic density (PSD) length, PSD thickness, active zone (AZ) length, synaptic cleft width, and the number of synaptic vesicles. In addition, whole-cell patch-clamp recordings were conducted on layer V pyramidal neurons in the mPFC. In the comparison among control, LH-resilient, and LH-susceptible mice, 12 neurons from 4 control mice, 12 neurons from 4 LH-resilient mice and 14 neurons from 4 LH-susceptible mice were analyzed. In the chemogenetic activation experiments, sEPSCs were recorded from 6 neurons from 3 mice, with each neuron sequentially recorded under baseline, in the presence of CNO and in the presence of CNO+SB-334867.

GraphPad Prism 10.0 was used for statistical analyses. Figures were prepared with Adobe Illustrator 2024. Data are presented as the mean $\pm$ standard deviation (SD). Unless otherwise stated, n refers to the number of mice. For electrophysiological data, n refers to the number of neurons, and for TEM data, n refers to the number of synapses (from the indicated number of mice). All statistical tests were two-tailed. Comparisons between two groups were conducted using either paired or unpaired Student's t -tests. For multiple group comparisons, one-way or two-way analysis of variance (ANOVA) was used, followed by Tukey's post hoc tests to control for multiple comparisons, as specified in each figure legend. For comparisons across sequential recording conditions within the same neurons, repeated-measures one-way ANOVA was used. A

324 *P* value of less than 0.05 was considered statistically significant. Asterisks in figures indicate: * *P*
325 < 0.05, ** *P* < 0.01, *** *P* < 0.001, **** *P* < 0.0001.

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Results

Inescapable foot shocks induce LH-susceptible and LH-resilient phenotypes in mice

The study was conducted using the learned helplessness (LH) paradigm, with chronic inescapable foot shock stimulation administered to adult male C57BL/6J mice. Mice were subjected to daily foot shock training for 6 consecutive days, with a current intensity of 0.6 mA and an average duration of 3 s. The inter-shock intervals were randomized and unpredictable, ranging from 5 to 8 s. On day 7, mice underwent a 30-trial test in a shuttle box to assess helplessness-like behavior (Figure 1A). Results showed that among the 11 tested mice, 8 (72.7%) exhibited fewer than 15 crossings (range: 0–5) during the 30-trial test and were classified as LH-susceptible, while the remaining 3 mice (27.3%) exhibited more than 15 crossings (range: 16–20) and were classified as LH-resilient (Figure 1B, C). In contrast, all 8 mice in the control group, which did not undergo foot shock training, exhibited close to 30 crossings in the shuttle box test (Figure 1D). In addition, the average crossing latency, defined as the time required for mice to move from the shock compartment to the opposite compartment within 30 s, was also assessed. Shorter crossing latency is indicative of more efficient stress coping, whereas prolonged crossing latency reflects impaired coping and heightened stress vulnerability. Results showed that LH-susceptible mice exhibited significantly prolonged crossing latency compared with the control group, whereas no significant difference was observed between LH-resilient and control mice (Figure 1E). To further evaluate depression-like behaviors, the tail suspension test (TST), open field test (OFT), and sucrose preference test (SPT) were performed 24 h after the shuttle box test in all three groups. In the TST, LH-susceptible mice showed a significantly increased immobility time during the 5 min test period compared to the control group, indicating behavioral despair. No significant difference was observed between the LH-resilient and control groups (Figure 1F). In the OFT, LH-susceptible mice exhibited significantly fewer entries into the center zone and a reduced duration in the center compared to control mice, suggesting elevated anxiety-like behavior. LH-resilient mice showed no significant differences relative to controls (Figure 1H, I). Representative movement trajectories of the three groups in the OFT are shown in Figure 1G. In the SPT, LH-susceptible mice displayed a significantly reduced sucrose preference compared to controls, indicating anhedonia. No significant difference was observed between LH-resilient and control mice (Figure 1J).

Excitatory synaptic transmission was impaired in layer V pyramidal neurons of the mPFC in LH-susceptible mice

Excitation/inhibition imbalance is considered one of the major neurobiological mechanisms underlying depression (Duman et al., 2016; Rahmani et al., 2022). We performed transmission electron microscopy (TEM), which revealed ultrastructural abnormalities in asymmetrical (predominantly excitatory) synapses in the mPFC of LH-susceptible mice. Specifically, compared with control mice ($n = 3$ mice, 42 synapses), LH-susceptible mice ($n = 3$ mice, 42 synapses) exhibited significant reductions in postsynaptic density (PSD) length and active zone (AZ) length, as well as a decreased number of synaptic vesicles (Figure 2A, C, D, F). No significant differences were observed in PSD thickness or synaptic cleft width between the two groups (Figure 2B, E).

We next recorded spontaneous excitatory postsynaptic currents (sEPSCs) from layer V pyramidal neurons in the mPFC of control ($n = 4$ mice, 12 neurons), LH-susceptible ($n = 4$ mice,

14 neurons), and LH-resilient ($n = 4$ mice, 12 neurons) groups. Compared with both control and LH-resilient mice, LH-susceptible mice exhibited a significantly lower mean sEPSC amplitude, together with a leftward shift in the cumulative amplitude distribution, indicating an increased proportion of low-amplitude events (Figure 2G, H). The mean sEPSC frequency was also significantly lower in LH-susceptible mice, accompanied by a rightward shift in the cumulative inter-event interval (IEI) distribution, indicating an increased proportion of long-interval events (Figure 2G, I). In contrast, sEPSC amplitude and frequency in LH-resilient mice were not significantly different from those of the control mice (Figure 2G–I). Together, these results demonstrate reduced spontaneous excitatory synaptic activity in layer V pyramidal neurons of the mPFC in LH-susceptible mice.

Hypothalamic orexin–mPFC projection was diminished in LH-susceptible mice

To explore the alterations in the orexin system in LH mice, we initially employed immunofluorescence staining to observe morphological and quantitative changes in orexinergic neuronal somata within the hypothalamus and orexinergic axonal projections in the mPFC. On coronal sections containing the lateral hypothalamic area, it was observed that the number of orexin immunoreactive somata was reduced in LH-susceptible mice, and some of the remaining orexin⁺ neuronal somata exhibited signs of atrophy compared to control mice. In contrast, no such changes were observed in LH-resilient mice (Figure 3A, panels a–c and a'–c'). Statistical analysis revealed that the total number of orexin⁺ neuronal somata in the hypothalamus of LH-susceptible mice was reduced by 39.54% compared to controls (Figure 3B). This reduction was observed across six serial coronal hypothalamic sections analyzed (Figure 3C). In addition, orexin⁺ axonal fibers in the mPFC were decreased in LH-susceptible mice, whereas no significant changes were observed in LH-resilient mice (Figure 3A, panels d–f and d'–f'). Statistical analysis showed that the density of orexin⁺ axonal fibers in the mPFC of LH-susceptible mice was reduced by 42.2%, and this reduction was observed in four serial sections of the mPFC (Figure 3D). We also examined the density of orexin⁺ fibers in other regions involved in mood regulation, including amygdalar subregions (LA, BA, CeA, and MeA) and the paraventricular thalamic nucleus (PVT). The results showed that, compared with the mPFC, the amygdala was relatively sparsely innervated by orexin⁺ fibers (Supplementary Figure 1A). Quantitative analysis of the amygdalar subregions revealed that LH-susceptible mice showed a significant reduction in orexin⁺ fiber density in the central amygdala (CeA) but not in LA, BA, or MeA (Supplementary Figure 1C–F). In addition, we observed that orexin⁺ signals were relatively abundant in the PVT (Supplementary Figure 1B). Further analysis showed that, compared with controls, the density of orexin⁺ fibers in the PVT was significantly reduced in LH-susceptible mice (Supplementary Figure 1G). These findings suggest that the reduction in orexin⁺ fibers in LH-susceptible mice is not restricted to the mPFC, but rather exhibits clear regional selectivity, with the most prominent reduction observed in the mPFC.

Subsequently, to determine whether the transcriptional level of orexin was altered in LH mice, we collected tissue from the lateral hypothalamus and performed qPCR to measure *prepro-orexin* mRNA expression. The results showed that *prepro-orexin* mRNA expression in the hypothalamus of LH-susceptible mice was significantly lower than that in control mice. In contrast, LH-resilient mice exhibited significantly higher levels of *prepro-orexin* mRNA expression compared to controls (Figure 3E). These findings indicate that the transcription of the orexin gene in the hypothalamus

is impaired in LH-susceptible mice, which may account for the reduction in the number of orexin-immunoreactive somata detected. In contrast, the increased expression of *prepro-orexin mRNA* in LH-resilient mice indicates an enhancement of the orexinergic system in these animals.

Furthermore, we investigated alterations in orexin neuropeptide concentrations within the mPFC. Fresh mPFC tissue samples were obtained from both control and LH-susceptible mice. The expression levels of Orexin A and Orexin B neuropeptides were quantified using Western blot (WB) and enzyme-linked immunosorbent assay (ELISA) techniques. WB analysis revealed a significant reduction in Orexin A protein expression in the mPFC of LH-susceptible mice, whereas Orexin B levels remained unaltered (Figure 3F, Supplementary Figure 2A, B). Correspondingly, ELISA results confirmed a significant decrease in Orexin A peptide levels in the mPFC of LH-susceptible mice, with no significant changes observed in Orexin B levels (Supplementary Figure 2C, D).

Chemogenetic activation of the hypothalamic orexin–mPFC neural pathway mediates antidepressant effects by enhancing excitatory transmission in LH-susceptible mice

The experimental results above suggest that the hypothalamic orexin–mPFC neural pathway may play a crucial role in depression induced by prolonged electric foot shock. Therefore, subsequent experiments focused on investigating this neural pathway. Initially, the retrograde tracer cholera toxin subunit B (CTB) was unilaterally injected into the mPFC of LH-susceptible mice to validate the hypothalamic orexin–mPFC pathway (Figure 4A). One week later, the mice were euthanized and perfused with paraformaldehyde for fixation, and coronal brain sections containing both the hypothalamus and mPFC were collected for double immunofluorescence staining of CTB and orexin. The results demonstrated a substantial population of neurons co-labeled with CTB⁺ and orexin⁺ in the hypothalamus, with a notably higher density of these co-labeled neurons observed in the supraforical region (Figure 4B). Quantitative analysis was conducted on the number of co-labeled neurons within seven coronal sections containing orexin neuronal somata in the hypothalamus of three LH-susceptible mice with accurate injection sites of CTB. The results showed that $46.29 \pm 2.81\%$ of orexin⁺ neurons were also CTB-labeled (Figure 4C), thereby confirming the projections of orexin neurons to the mPFC and establishing the hypothalamic orexin–mPFC neural pathway.

Subsequently, we specifically activated the hypothalamic orexin–mPFC neural pathway. The experimental procedure is illustrated in Figure 4D. A chemogenetic activating viral vector (rAAV2/9-Ef1a-DIO-hM3D(Gq)-mCherry-WPREs) was injected into the bilateral lateral hypothalamic area of orexin-Cre mice, and bilateral cannulae were implanted in the mPFC for subsequent local CNO infusion (Figure 4E, F). To control for non-specific effects of viral injection, a control group received injections of a control viral vector lacking the hM3D(Gq) gene (rAAV-Ef1a-DIO-mCherry-WPREs). Fluorescent microscopic examination of brain sections following completion of the behavioral experiments showed mCherry expression in orexin (*i.e.*, EGFP⁺) neurons in the lateral hypothalamus (Figure 4G). A few mCherry-labeled neurons did not show obvious EGFP fluorescence, which might be due to low EGFP expression in these neurons or bleaching of EGFP fluorescence during tissue processing procedures. In the orexin-Cre mice used in this study, orexin neurons were labeled by the EGFP reporter driven by the orexin promoter. Some orexin neurons might not be efficiently labeled with EGFP. To address this, we

performed anti-Cre immunostaining of brain sections from orexin-Cre mice. Quantitative analysis showed that $80.48 \pm 1.38\%$ of Cre⁺ neurons co-expressed EGFP, whereas the remaining Cre⁺ neurons were EGFP-negative (Supplementary Figure 3A, B).

Twenty-one days post-viral injection, a 7-day inescapable foot shock paradigm was administered to mice to identify individuals exhibiting susceptibility to learned helplessness. CNO was then bilaterally infused into the mPFC via the cannulae to activate orexinergic terminals in the mPFC, and behavioral testing was conducted 30 min later. In the TST, the LH-susceptible+hM3D(Gq)+CNO group exhibited significantly reduced immobility duration within 5 min compared to the LH-susceptible group, whereas no significant difference was observed between the LH-susceptible and LH-susceptible+mCherry+CNO groups (Figure 4H). In the OFT, the LH-susceptible+hM3D(Gq)+CNO group exhibited a significantly greater number of entries into the center zone and spent significantly more time there compared with the LH-susceptible group, with no significant difference between the LH-susceptible and LH-susceptible+mCherry+CNO groups (Figure 4I, J). In the SPT, the LH-susceptible+hM3D(Gq)+CNO group displayed significantly increased sucrose preference compared to the LH-susceptible group, whereas sucrose preference did not differ significantly between the LH-susceptible and LH-susceptible+mCherry+CNO groups (Figure 4K). In the shuttle box test, 6 out of 8 mice in the LH-susceptible+hM3D(Gq)+CNO group exhibited more than 15 crossings across 30 trials (Figure 4L). Following completion of the behavioral experiments, mPFC tissue was collected from each group, and the levels of Orexin A and Orexin B were measured by ELISA to assess changes in local orexin content after chemogenetic activation of the orexin–mPFC pathway. Compared with control mice, LH-susceptible mice exhibited significantly lower Orexin A levels in the mPFC. In contrast, local infusion of CNO into the mPFC to activate orexinergic terminals significantly increased Orexin A levels in LH-susceptible mice. No significant differences in Orexin B levels were observed among the groups (Figure 4M, N).

Orexin has been shown to exert a dose-dependent postsynaptic excitatory effect on layer V pyramidal neurons in the mPFC primarily through activating OX1R (Xia et al., 2005; Li et al., 2010; Yan et al., 2012). We observed that LH-susceptible mice exhibited impaired excitatory synaptic structure and transmission in the mPFC (Figure 2), whereas chemogenetic activation of the orexin–mPFC pathway alleviated depression-like behaviors. We therefore asked whether this behavioral improvement was mediated by the restoration of excitatory synaptic transmission in the mPFC. To address this question, we first bilaterally injected the Cre-dependent excitatory chemogenetic viral vector (rAAV2/9-Ef1a-DIO-hM3D(Gq)-mCherry-WPREs) into the lateral hypothalamic area of orexin-Cre mice. After 21 days of viral expression, the mice were subjected to a 7-day inescapable foot shock paradigm. Mice exhibiting the LH-susceptible phenotype were identified through the shuttle box test. Acute coronal brain slices containing the mPFC were then prepared from these LH-susceptible mice, and sEPSCs were recorded from layer V pyramidal neurons in the mPFC (Figure 5A). Following a stable baseline recording (Figure 5B, b1), CNO was bath-applied (Figure 5B, b2), followed by co-application of CNO and the OX1R antagonist SB-334867 in the same recorded neuron (Figure 5B, b3). Compared with baseline, CNO treatment significantly increased the mean amplitude and frequency of sEPSCs (Figure 5B–D). Upon addition of SB-334867, the mean amplitude of sEPSCs was markedly reduced, accompanied by a leftward shift in the cumulative amplitude distribution (Figure 5B–D). These results indicate that activation of the hypothalamic orexin–mPFC pathway may restore excitatory

synaptic transmission in layer V pyramidal neurons of the mPFC, predominantly via OX1R-mediated mechanisms.

Chemogenetic inhibition of the hypothalamic orexin–mPFC pathway induces depression-like behaviors in LH-resilient mice

Given that LH-resilient mice exposed to the same inescapable foot shock protocol did not exhibit depression-like behaviors, we next investigated the role of the hypothalamic orexin–mPFC neural pathway in mediating resilience. We specifically inhibited the hypothalamic orexin–mPFC pathway in LH-resilient mice and assessed the resulting effects on depression-related behaviors (Figure 6A). A chemogenetic inhibitory viral vector (rAAV2/9-Ef1a-DIO-hM4D(Gi)-mCherry-WPREs) was bilaterally injected into the hypothalamus of orexin-Cre mice. Simultaneously, cannulae were implanted bilaterally into the mPFC (Figure 6B, C). Immunofluorescence staining showed that the hM4D(Gi)-mCherry virus specifically transduced orexin neurons (*i.e.*, EGFP⁺) in the hypothalamus of orexin-Cre mice (Figure 6D). Twenty-one days post-viral injection, a 7-day inescapable foot shock paradigm was administered to mice to identify individuals exhibiting resilience to learned helplessness. CNO was then infused into the bilateral mPFC to inhibit orexinergic terminals in LH-resilient mice, followed by behavioral testing 30 minutes later. In the TST, the LH-resilient+hM4D(Gi)+CNO group exhibited significantly increased immobility duration within 5 min compared with the LH-resilient group, whereas no significant difference was observed between the LH-resilient and LH-resilient+mCherry+CNO groups (Figure 6E). In the OFT, the LH-resilient+hM4D(Gi)+CNO group exhibited a significantly reduced number of entries into the center zone and spent significantly less time there compared with the LH-resilient group, with no significant difference between the LH-resilient and LH-resilient+mCherry+CNO groups (Figure 6F, G). In the SPT, the LH-resilient+hM4D(Gi)+CNO group displayed significantly decreased sucrose preference compared with the LH-resilient group, whereas sucrose preference did not differ significantly between the LH-resilient and LH-resilient+mCherry+CNO groups (Figure 6H). In the shuttle box test, 7 out of 8 mice in the LH-resilient+hM4D(Gi)+CNO group exhibited fewer than 15 crossings across 30 trials (Figure 6I). These findings indicate that inhibition of the hypothalamic orexin–mPFC pathway induces depression-like behaviors in LH-resilient mice, manifested as helplessness, behavioral despair, and anhedonia.

Intra-mPFC administration of Orexin A alleviates depression-like behaviors in LH-susceptible mice

Orexin neurons exert their physiological effects through the release of two neuropeptides, Orexin A and Orexin B (Dale et al., 2022). To identify which orexin subtype is involved in mediating antidepressant-like effects, microinjection cannulae were stereotactically implanted into the mPFC to facilitate targeted delivery. Thirty minutes prior to each behavioral assessment, site-specific administration of Orexin A, Orexin B, or saline was conducted in LH-susceptible mice (Figure 7A, Part I). Nissl staining was performed to verify the accurate placement of the cannulae within the mPFC, and animals with incorrect placement were excluded from subsequent analyses (Figure 7B). The results demonstrated that intra-mPFC administration of Orexin A significantly alleviated depression-like behaviors in LH-susceptible mice. Specifically, in the TST, mice treated with Orexin A exhibited significantly reduced immobility time compared with the LH-susceptible group, whereas no significant difference was observed between the LH-susceptible and the LH-susceptible+saline groups (Figure 7C). In the OFT, the Orexin A group showed significantly more entries into the center zone and spent more time there, compared with the LH-susceptible group, with no significant difference between the LH-susceptible and the LH-susceptible+saline groups

(Figures 7D, E). In the SPT, Orexin A-treated mice displayed significantly increased sucrose preference compared with the LH-susceptible group, whereas sucrose preference did not differ significantly between the LH-susceptible and LH-susceptible+saline groups (Figure 7F). Moreover, in the 30-trial shuttle box test, 6 out of 8 mice receiving Orexin A achieved more than 15 successful crossings (Figure 7G). Collectively, these findings indicate that exogenous administration of Orexin A into the mPFC effectively alleviates depression-like behaviors in LH-susceptible mice. To verify whether Orexin A exerts its antidepressant effects via orexin receptors in the mPFC, we pretreated mice with a dual OX1R and OX2R antagonist (Dora) prior to intra-mPFC administration of Orexin A. The experimental procedure is illustrated in Figure 7A, Part II. The results showed that Dora pretreatment abolished the antidepressant effects of Orexin A. Specifically, in the TST, the LH-susceptible+Dora+Orexin A group exhibited significantly increased immobility time compared with the LH-susceptible+Orexin A group, whereas no significant difference was observed between the LH-susceptible+Dora+Orexin A and the LH-susceptible groups (Figure 7C). In the OFT, the LH-susceptible+Dora+Orexin A group showed significantly fewer entries into the center zone and spent significantly less time there compared with the LH-susceptible+Orexin A group, with no significant difference between the LH-susceptible+Dora+Orexin A and the LH-susceptible groups (Figures 7D, E). In the SPT, sucrose preference in the LH-susceptible+Dora+Orexin A group was significantly decreased compared with the LH-susceptible+Orexin A group and did not differ significantly from that of the LH-susceptible group (Figure 7F). Moreover, in the 30-trial shuttle box test, all 8 mice in the LH-susceptible+Dora+Orexin A group performed fewer than 15 crossings (Figure 7G). These findings indicate that Orexin A alleviates depression-like behaviors in LH-susceptible mice through the action of orexin receptors in the mPFC.

Subsequently, Orexin B was injected into the unilateral mPFC following the same protocol. In contrast to the effects observed with Orexin A, intra-mPFC administration of Orexin B did not result in a significant reduction in depression-like behaviors in LH-susceptible mice. Specifically, in the TST, no significant difference in immobility duration was detected between the Orexin B-treated group and the LH-susceptible group (Supplementary Figure 2E). Similarly, in the OFT, Orexin B treatment did not significantly affect either the number of center entries or the time spent in the center compared with the LH-susceptible group (Supplementary Figures 2F, G). Furthermore, the sucrose preference in the Orexin B group did not differ significantly from that of the LH-susceptible group (Supplementary Figure 2H). Additionally, during the 30-trial shuttle box test, the number of successful crossings remained low in Orexin B-treated mice, with all mice performing fewer than 15 crossings (Supplementary Figure 2I).

Discussion

In this study, mice were classified as either LH-susceptible or LH-resilient based on their escape responses to inescapable foot shocks. Compared with LH-resilient mice, LH-susceptible mice exhibited pronounced depression-like behaviors, including helplessness, despair, and anhedonia. Notably, LH-susceptible mice exhibited a significant reduction in the number of orexin neuronal somata within the hypothalamus, as well as decreased orexinergic fiber density in the mPFC. These observations prompted further investigation into the role of the hypothalamic orexin–mPFC neural pathway in regulating susceptibility to depression in the LH paradigm. Chemogenetic activation of this pathway in LH-susceptible mice elicited robust antidepressant-like effects, which were specifically mediated by Orexin A. In contrast, inhibition of this pathway in LH-resilient mice induced marked depression-like behaviors. Electrophysiological recordings further revealed impaired excitatory synaptic transmission in layer V pyramidal neurons of the

mPFC in LH-susceptible mice, which was improved by activation of orexinergic terminals, an effect that was attenuated by an OX1R antagonist.

The orexinergic system protects against learned helplessness in mice

Depression is considered a neural circuit disorder (Cui et al., 2024). Dysfunction of the orexin system correlates with depressive symptoms, but its exact role remains controversial (Arendt et al., 2014; Jalewa et al., 2014). In depression-related rodent models, orexin system changes vary. Orexin neuron size and number, as well as serum orexin levels, are reduced in Wistar-Kyoto rats (Taheri et al., 2001; Allard et al., 2004). Hypothalamic *prepro-orexin mRNA* and orexin neuron count decrease in the chronic social defeat stress (CSDS) model (Lutter et al., 2008), accompanied by lower orexin levels in the ventral tegmental area (VTA) and mPFC (Nocjar et al., 2012). In contrast, Flinders-sensitive line rats show increased hypothalamic orexin neuron numbers (Jalewa et al., 2014). In the chronic unpredictable mild stress (CUMS) model, the orexinergic system is hyperactive, and intracerebroventricular orexin antagonists alleviate anhedonia (Nollet et al., 2011; Nollet et al., 2012). In the LH paradigm, we found that the number of orexin⁺ neuronal somata in the hypothalamus was reduced in LH-susceptible mice, accompanied by decreased Orexin A levels in the mPFC. Infusion of Orexin A into the mPFC significantly ameliorated depression-like behaviors, suggesting that Orexin A exerts a predominant antidepressant effect. The mechanisms underlying the reduced number of orexin⁺ neuronal somata in LH-susceptible mice remain to be determined. Chronic stress might induce epigenetic suppression of the orexin gene (promoter hypermethylation), thereby reducing *prepro-orexin mRNA* transcription and leading to the loss of orexin neurons (Pu et al., 2022). Inhibition of nitric oxide synthase (NOS) has been shown to reduce NO signaling and alleviate depression-like behaviors in LH-susceptible mice (Stanquini et al., 2018). Excessive NO can cause S-nitrosylation of protein disulfide isomerase, leading to misfolded orexin protein and neuronal death (Uehara et al., 2006). Orexin A, but not Orexin B, contains two intramolecular disulfide bonds, making it susceptible to misfolding under nitrosative stress (Obukuro et al., 2013).

Recently, Liu et al. (2024) reported that inhibiting orexin terminals in the mPFC alleviates anhedonia in CUMS mice without affecting anxiety or despair behaviors (Liu et al., 2024). Our study shows that activating orexin terminals in the mPFC significantly alleviates despair, helplessness, and anhedonia in LH-susceptible mice, while inhibiting this pathway induces depression-like behaviors in LH-resilient mice. The discrepancy may arise from differences in animal models, stressor types, intensities, durations, or underlying mechanisms. The LH paradigm typically uses strong, inescapable shocks to induce helplessness. After inescapable foot shock, animals exhibit reduced activity and passive responses to negative stress, not due to motor impairment but to decreased response speed under intense stress, reflecting a motivational deficit (Vollmayr and Gass, 2013). By contrast, CUMS employs low-intensity, varied, unpredictable stressors (e.g., alternating cold/heat, cage tilt, noise) (Du Preez et al., 2021). The lower intensity and unpredictability in CUMS lead to sustained vigilance and gradual anhedonia with mild anxiety, rather than the extreme behavioral surrender of LH (Antoniuk et al., 2019). LH-susceptible mice represent a “yielding” helplessness phase, whereas high orexin levels promote active coping and motivation (Kuwaki, 2021). Under controllable or escapable stress situations, orexin neurons are strongly activated and project extensively to autonomic centers such as the nucleus raphe magnus (NRM), the rostral ventrolateral medullary area (RVLM), and the nucleus of the solitary tract (NTS), regulating heart rate, respiration, blood pressure, pain responses, and

alertness, supporting fight-or-flight behaviors (Young et al., 2005; Dutschmann et al., 2007; Shahid et al., 2011; Sugita et al., 2014; Carriave and Kuwaki, 2017; Kuwaki, 2021). Chemogenetic activation of hypothalamic orexin neurons increases active escape attempts in LH-susceptible mice (Kim et al., 2023). Conversely, orexin-deficient mice exhibit significant behavioral sluggishness and reduced autonomic responses to stress, such as insufficient heart rate increase, weakened escape responses, and loss of analgesic effects (Kayaba et al., 2003; Zhang et al., 2006). Thus, orexin is a critical hub linking arousal, emotion, and autonomic regulation and may act as an internal gauge of stress controllability, determining whether an individual adopts active (fight-or-flight) or passive (helpless) coping (Hsu and Wang, 2021; Kuwaki, 2021).

The neural mechanisms underlying helpless behavior also involve the dorsal raphe nucleus (DRN) and mPFC. Exposure to inescapable electric shocks activates aversive inputs transmitted to the DRN through the locus coeruleus (LC), bed nucleus of the stria terminalis (BNST), and lateral habenula (LHb), leading to hyperactivation of serotonergic (5-HT) neurons and the development of learned helplessness (Grahn et al., 2002). In contrast, resilient mice rely on excitatory glutamatergic projections from the mPFC to the DRN, which activate GABAergic neurons and suppress 5-HT activity, preventing learned helplessness (Celada et al., 2001; Jankowski and Sesack, 2004; Gabbott et al., 2005). Inhibition of the mPFC impairs controllability detection, rendering escape irrelevant, whereas its activation prevents helpless behavior induced by inescapable shocks (Amat et al., 2005; Amat et al., 2008). As a major source of upstream inputs, orexinergic neurons project directly to layer V pyramidal neurons in the mPFC and enhance their excitability. Our electrophysiological results showed that, compared with control and LH-resilient mice, LH-susceptible mice exhibited significantly reduced mean amplitude and frequency of sEPSCs in these neurons. Activation of the hypothalamic orexin–mPFC pathway restores synaptic activity and alleviates depression-like behaviors, whereas inhibition of this pathway induces similar behaviors in LH-resilient mice and reduces escape attempts to fewer than 15 during the 30-trial shuttle box test. These findings indicate that the orexin–mPFC circuit is essential for mediating antidepressant effects and promoting active coping strategies.

Hypothalamic orexin neurons regulate antidepressant effects via OX1R-mediated synaptic transmission in the mPFC

Although characterized by orexin peptide release, orexin neurons also co-express and release other neuropeptides, including dynorphin (Chou et al., 2001; Muschamp et al., 2014), nociceptin/orphanin FQ (Maolood and Meister, 2010), and neurotensin (Furutani et al., 2013). They can also rapidly release glutamate via VGLUT2-positive terminals (Abrahamson et al., 2001; Torrealba et al., 2003; Henny et al., 2010; Warlow et al., 2024), indicating a capacity for fast excitatory neurotransmission. To determine whether orexin peptides within the mPFC mediate antidepressant-like effects, we infused Orexin A or B into the mPFC of LH-susceptible mice. Orexin A significantly reduced depression-like behaviors, an effect abolished by Dora, whereas Orexin B had no significant effect. In the mPFC, OX1R and OX2R are co-expressed predominantly in layers II/III and V on pyramidal neurons (Hervieu et al., 2001; Cluderay et al., 2002). Notably, the expression level of OX1R is higher than that of OX2R (Jin et al., 2016), and Orexin A has greater affinity for OX1R than for OX2R (Lang et al., 2004; Li et al., 2010). Consistent with this, Orexin A application depolarizes mPFC pyramidal neurons and elicits action potential firing (Yan et al., 2012). In our study, chemogenetic activation of orexinergic terminals within the mPFC markedly increased both the frequency and amplitude of sEPSCs in pyramidal neurons. The increased frequency likely results from CNO-induced activation of orexin terminals that

promotes orexin release. Application of the OX1R-specific antagonist SB-334867 markedly reduced sEPSC amplitude but had no significant effect on frequency. These findings indicate that the excitatory synaptic effects of orexin in the mPFC are predominantly mediated by OX1R on pyramidal neurons.

Despite these findings, several limitations should be acknowledged. First, this study lacked real-time in vivo monitoring of orexin neuropeptide dynamics, limiting direct assessment of activity-dependent release across behavioral states. Future studies using genetically encoded orexin sensors may provide more precise evaluation of orexin signaling within the mPFC. In addition, only male mice were used. Although this reduced endocrine variability, sex remains an important biological variable, and future studies should include female mice to determine sex-specific effects.

In summary, this study identifies the hypothalamic orexin–mPFC pathway as a crucial regulator of learned helplessness and antidepressant effects. Impairment of this pathway increases susceptibility to learned helplessness, whereas orexin appears to exert antidepressant effects primarily by enhancing excitatory synaptic transmission in mPFC pyramidal neurons through OX1R activation.

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Figure Legends

Figure 1. Inescapable foot shocks induce LH-susceptible and LH-resilient phenotypes in mice. **(A)** Schematic diagram of the LH paradigm protocol and screening procedure. **(B)** Number of crossings during the shuttle box test in 11 WT mice (green dots represent LH-susceptible mice; orange dots represent LH-resilient mice). **(C)** Proportion of LH-susceptible and LH-resilient mice among the 11 trained WT mice. **(D)** Number of crossings in the shuttle box test for control, LH-susceptible, and LH-resilient mice. **(E)** Latency to cross in the shuttle box test for control, LH-susceptible, and LH-resilient mice. $F_{(2, 16)} = 8.23$; $P = 0.0038$ for control mice versus LH-susceptible mice, $P = 0.96$ for control mice versus LH-resilient mice, $P = 0.0487$ for LH-susceptible mice versus LH-resilient mice. **(F)** Immobility time in the TST was significantly increased in LH-susceptible mice, $F_{(2, 16)} = 19.82$; $P = 0.0002$ for control mice versus LH-susceptible mice, $P = 0.51$

for control mice versus LH-resilient mice, $P = 0.0003$ for LH-susceptible mice versus LH-resilient mice. **(G)** Representative movement trajectories of control, LH-susceptible, and LH-resilient mice in the OFT. **(H)** In the OFT, LH-susceptible mice showed a decreased number of entries into the center zone, $F_{(2, 16)} = 19.92$; $P < 0.0001$ for control mice versus LH-susceptible mice, $P = 0.27$ for control mice versus LH-resilient mice, $P = 0.0215$ for LH-susceptible mice versus LH-resilient mice. **(I)** In the OFT, LH-susceptible mice exhibited a significant reduction in the time spent in the center zone, $F_{(2, 16)} = 13.74$; $P = 0.0003$ for control mice versus LH-susceptible mice, $P = 0.47$ for control mice versus LH-resilient mice, $P = 0.0443$ for LH-susceptible mice versus LH-resilient mice. **(J)** Sucrose preference was decreased in LH-susceptible mice in the SPT, $F_{(2, 16)} = 39.73$; $P < 0.0001$ for control mice versus LH-susceptible mice, $P = 0.48$ for control mice versus LH-resilient mice, $P = 0.0002$ for LH-susceptible mice versus LH-resilient mice. $n = 8$ mice for each of the control and LH-susceptible groups, $n = 3$ mice for the LH-resilient group. One-way ANOVA and Tukey's post hoc test were used to assess statistical differences. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns, not significant.

Figure 2. Excitatory synaptic deficits in the mPFC of LH-susceptible mice. **(A)** TEM images reveal ultrastructural abnormalities in excitatory synapses within the mPFC of LH-susceptible mice. a–c show representative images from the control group; d–f are from the LH-susceptible group, scale bar: 200 nm. **(B–F)** Quantitative analysis of excitatory synaptic ultrastructure. **(B)** No significant change in PSD thickness was observed in LH-susceptible mice, $t_{(82)} = 1.681$; $P = 0.097$ for control versus LH-susceptible mice; **(C)** PSD length was significantly reduced in LH-susceptible mice, $t_{(82)} = 10.65$; $P < 0.0001$ for control versus LH-susceptible mice; **(D)** AZ length was shortened in LH-susceptible mice, $t_{(82)} = 11.34$; $P < 0.0001$ for control versus LH-susceptible mice; **(E)** Synaptic cleft width remained unchanged in LH-susceptible mice, $t_{(82)} = 0.428$; $P = 0.67$ for control versus LH-susceptible mice; **(F)** The number of presynaptic vesicles was decreased in LH-susceptible mice, $t_{(82)} = 11.79$; $P < 0.0001$ for control versus LH-susceptible mice. For each group, 42 synapses were analyzed from 3 mice. Statistical comparisons were performed using unpaired Student's t-test. **(G)** Representative traces of sEPSCs recorded from layer V pyramidal neurons in the mPFC of control, LH-susceptible, and LH-resilient mice. **(H)** Quantification of mean sEPSC amplitude (right) and cumulative distribution (left) of amplitude, $F_{(2, 35)} = 43.11$; $P < 0.0001$ for control versus LH-susceptible mice, $P < 0.0001$ for LH-resilient versus LH-susceptible mice, $P = 0.1022$ for control versus LH-resilient mice. **(I)** Quantification of mean sEPSC frequency (right) and cumulative probability curves for inter-event interval (left), $F_{(2, 35)} = 104.9$; $P < 0.0001$ for control versus LH-susceptible mice, $P < 0.0001$ for LH-resilient versus LH-susceptible mice; $P = 0.39$ for control versus LH-resilient mice. $n = 12$ neurons from 4 mice (control), $n = 12$ neurons from 4 mice (LH-resilient), and $n = 14$ neurons from 4 mice (LH-susceptible). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine statistical significance. **** $P < 0.0001$, ns, not significant.

Figure 3. Downregulation of orexinergic inputs in the mPFC of LH-susceptible mice. **(A)** Immunofluorescence staining of orexin positive neuronal cell bodies (green) in the hypothalamus and orexinergic fiber terminals (green) in the mPFC of control, LH-susceptible, and LH-resilient mice. Panels a–c show the distribution of orexin neuronal cell bodies in the hypothalamus, scale bar: 500 μ m. Panels a'–c' are high-magnification images of the red boxes in a–c, scale bar: 100 μ m. Panels d–f show the distribution of orexinergic terminals in the mPFC, scale bar: 500 μ m. Panels d'–f' are high-magnification images of the red boxes in d–f, scale bar: 100 μ m. ic, internal capsule; opt, optic tract. **(B)** Total number of orexin positive neurons in the hypothalamus from

seven brain sections per mouse, $n = 3$ mice for each group. $F_{(2, 6)} = 255.5$; $P < 0.0001$ for control mice versus LH-susceptible mice, $P = 0.87$ for control mice versus LH-resilient mice. One-way ANOVA and Tukey's post hoc test were used to assess statistical differences. **(C)** Number of orexin neurons across seven hypothalamic sections based on the Allen Reference Atlas, $n = 3$ mice for each group. $F_{(2, 42)} = 316.1$; -1.34 mm, $P = 0.0022$ for control versus LH-susceptible, $P = 0.92$ for control versus LH-resilient; -1.46 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.57$ for control versus LH-resilient; -1.58 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.73$ for control versus LH-resilient; -1.70 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.92$ for control versus LH-resilient; -1.82 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.27$ for control versus LH-resilient; -1.94 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.30$ for control versus LH-resilient; -2.06 mm, $P = 0.47$ for control versus LH-susceptible, $P = 0.94$ for control versus LH-resilient. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test. **(D)** Percentage density of orexinergic fiber terminals across four mPFC sections based on the Allen Reference Atlas, $n = 3$ mice for each group. $F_{(2, 24)} = 99.1$; 1.98 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.51$ for control versus LH-resilient; 1.94 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.9$ for control versus LH-resilient; 1.78 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.57$ for control versus LH-resilient; 1.70 mm, $P < 0.0001$ for control versus LH-susceptible, $P = 0.72$ for control versus LH-resilient. Statistical analysis was performed using two-way ANOVA with Tukey's post hoc test. **(E)** Left: The red box indicates the tissue sampling region in the lateral hypothalamus. Right: qPCR results showing decreased *prepro-orexin mRNA* expression in LH-susceptible mice and increased expression in LH-resilient mice, $n = 8$ mice for each group. $F_{(2, 21)} = 54.65$; $P < 0.0001$ for control mice versus LH-susceptible mice, $P = 0.0021$ for control mice versus LH-resilient mice. Statistical analysis was conducted using one-way ANOVA followed by Tukey's post hoc test. **(F)** Left: The red box indicates the tissue sampling region in the mPFC. Right: WB results showing reduced Orexin A expression in the mPFC of LH-susceptible mice, $t_{(10)} = 4.55$; $P = 0.0011$ for control mice versus LH-susceptible mice, with no significant change in Orexin B, $t_{(10)} = 0.32$; $P = 0.754$ for control mice versus LH-susceptible mice, $n = 3$ mice for each group, with 2 repetitions per mouse. Statistical differences were analyzed using unpaired Student's t-test. **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, ns, not significant.

Figure 4. Chemogenetic activation of the hypothalamic orexin–mPFC pathway produces antidepressant effects. **(A)** Representative image showing the injection site of CTB in the unilateral mPFC, scale bar: 1000 μ m. PrL, prelimbic cortex; IL, infralimbic cortex; fmi, forceps minor of the corpus callosum. **(B)** Immunofluorescence double staining showing CTB⁺ neurons (green) and orexin⁺ neurons (red) in the hypothalamic region, scale bar: 500 μ m. b1–b3 present magnified images of the boxed area in B, scale bar: 100 μ m. f, fornix; 3V, third ventricle. **(C)** Quantification of the percentage of neurons double-labeled for CTB and orexin among total orexin⁺ neurons in the lateral hypothalamus, based on 7 brain sections per mouse from 3 LH-susceptible mice. **(D)** Schematic diagram of the experimental timeline for chemogenetic activation of the hypothalamic orexin–mPFC pathway. Clozapine-N-oxide (CNO, 1 mM, 0.5 μ L per side) was microinjected into the bilateral mPFC prior to behavioral testing. **(E)** Schematic showing injection of rAAV-DIO-hM3D(Gq)-mCherry into the bilateral lateral hypothalamic area of orexin-Cre mice, with cannula implantation in the bilateral mPFC. **(F)** Nissl staining confirming bilateral cannula placement in the mPFC, scale bar: 1000 μ m. **(G)** Representative fluorescence image

showing hM3D(Gq)-mCherry expression in the lateral hypothalamus, scale bar: 100 μ m. g shows a magnified view of the boxed area in G, scale bar: 40 μ m. **(H)** In the TST, chemogenetic activation of the orexin–mPFC pathway significantly reduced immobility time in LH-susceptible mice. $F_{(3, 28)} = 39.83$; $P < 0.0001$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.57$ for LH-susceptible mice versus LH-susceptible+mCherry+CNO mice. **(I)** In the OFT, activation of the orexin–mPFC pathway increased the number of entries into the center zone in LH-susceptible mice. $F_{(3, 28)} = 21.08$; $P = 0.0109$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.379$ for LH-susceptible mice versus LH-susceptible+mCherry+CNO mice. **(J)** In the OFT, activation of the orexin–mPFC pathway increased the duration spent in the center area in LH-susceptible mice, $F_{(3, 28)} = 55.10$; $P < 0.0001$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.672$ for LH-susceptible mice versus LH-susceptible+mCherry+CNO mice. **(K)** In the SPT, activation of the orexin–mPFC circuit enhanced sucrose preference in LH-susceptible mice, $F_{(3, 28)} = 25.02$; $P < 0.0001$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.9$ for LH-susceptible mice versus LH-susceptible+mCherry+CNO mice. **(L)** In the shuttle box test (30 trials), chemogenetic activation of the orexin–mPFC pathway increased the number of crossings in LH-susceptible mice. $n = 8$ mice for each group of behavioral tests. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine statistical significance. **(M, N)** ELISA showed that Orexin A levels in the mPFC were significantly reduced in LH-susceptible mice and restored by CNO, $F_{(2, 9)} = 18.35$; $P = 0.0005$ for control mice versus LH-susceptible mice, $P = 0.0127$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.1023$ for control mice versus LH-susceptible+hM3+CNO mice; whereas Orexin B levels were unchanged, $F_{(2, 9)} = 1.17$; $P = 0.65$ for control mice versus LH-susceptible mice, $P = 0.33$ for LH-susceptible mice versus LH-susceptible+hM3+CNO mice, $P = 0.812$ for control mice versus LH-susceptible+hM3+CNO mice. $n = 4$ mice per group, and each sample was measured in duplicate and averaged. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine statistical significance. **** $P < 0.0001$, *** $P < 0.001$, * $P < 0.05$, ns, not significant.

Figure 5. Activation of the hypothalamic orexin–mPFC pathway enhances excitatory synaptic transmission in layer V pyramidal neurons of LH-susceptible mice via OX1R. **(A)** Schematic of the experimental timeline showing bilateral rAAV-DIO-hM3D(Gq)-mCherry injection into the lateral hypothalamic area of orexin-Cre mice, followed by screening for LH-susceptible mice and whole-cell patch-clamp recordings from layer V pyramidal neurons in the mPFC. (a1–a2) Representative images of whole-cell patch-clamp recording from a layer V pyramidal neuron in the mPFC. The white arrow indicates the recording pipette targeting the pyramidal neuron. **(B)** Representative continuous trace of sEPSCs recorded at baseline and during CNO, and CNO+SB-334867 treatment (top). Lower panels (b1–b3) show enlarged traces under baseline, CNO, and CNO+SB-334867 conditions. **(C)** Quantification of mean sEPSC amplitude (right) and cumulative distribution (left) of amplitude, $F_{(1.135, 5.676)} = 12.73$; $P = 0.0239$ for baseline versus hM3+CNO, $P = 0.0003$ for hM3+CNO versus hM3+CNO+SB-334867. **(D)** Quantification of mean sEPSC frequency (right) and cumulative probability curves for inter-event interval (left), $F_{(1.595, 7.975)} = 29.14$; $P = 0.0006$ for baseline versus hM3+CNO, $P = 0.315$ for hM3+CNO versus hM3+CNO+SB-334867. $n = 6$ neurons from 3 mice. Data were analyzed using repeated-measures one-way ANOVA with Geisser–Greenhouse correction, followed by Tukey's multiple comparisons test to determine statistical significance. *** $P < 0.001$, * $P < 0.05$, ns, not significant.

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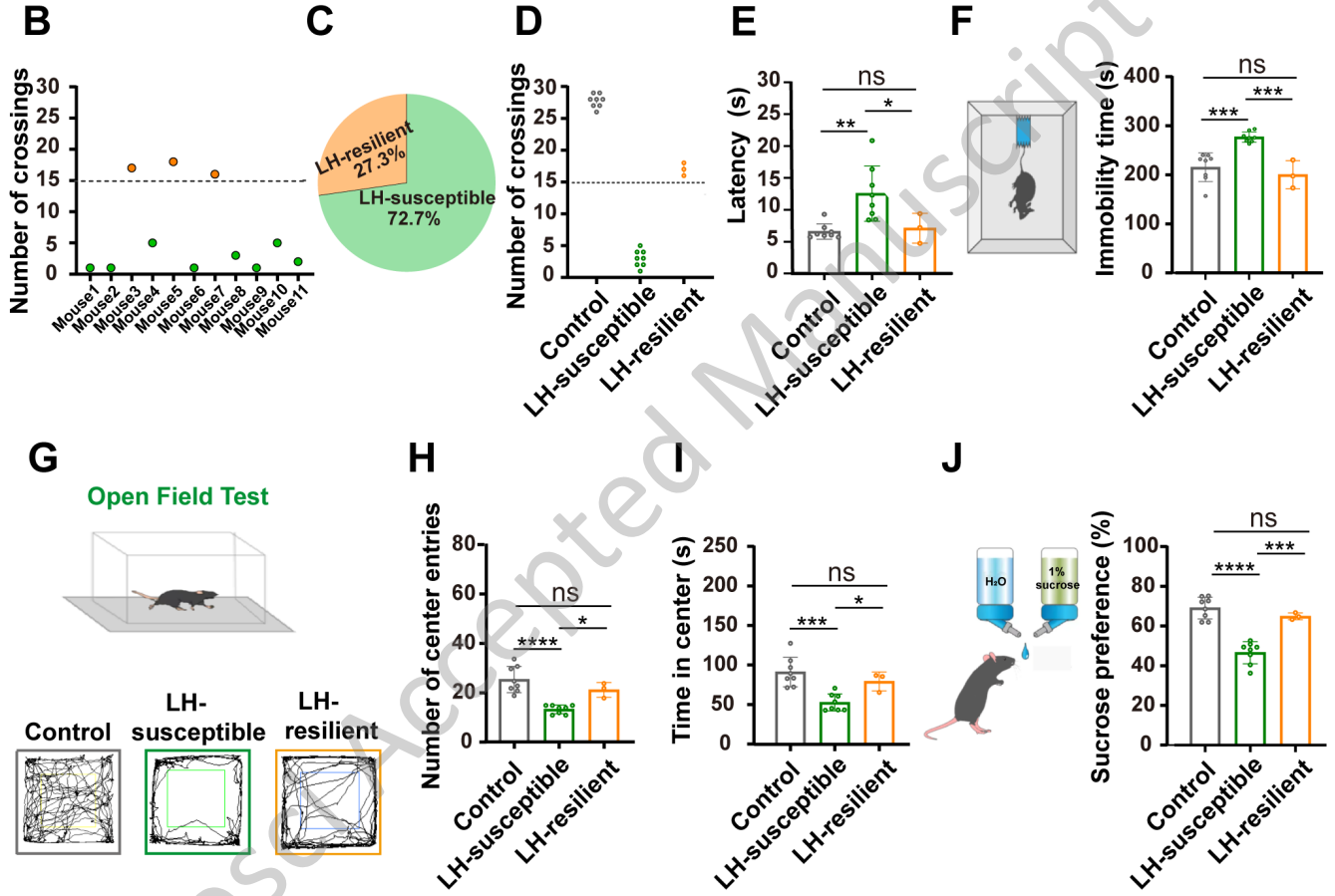
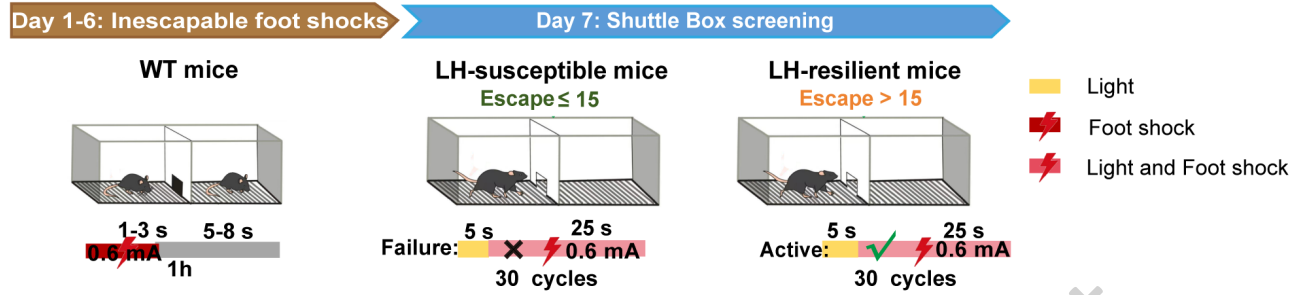
Figure 6. Chemogenetic inhibition of the hypothalamic orexin–mPFC pathway induces depression-like behaviors in LH-resilient mice. **(A)** Schematic diagram of the experimental timeline for chemogenetic inhibition of the hypothalamic orexin–mPFC pathway. CNO (1 mM, 0.5 μ L per side) was microinjected into the bilateral mPFC prior to behavioral testing. **(B)** Illustration showing rAAV-DIO-hM4D(Gi)-mCherry injection into the bilateral lateral hypothalamic area of orexin-Cre mice, with cannula implantation in the bilateral mPFC. **(C)** Nissl-stained image showing bilateral cannula placements in the mPFC, scale bar: 1000 μ m. **(D)** Representative fluorescence image showing rAAV-DIO-hM4D(Gi)-mCherry expression in the lateral hypothalamus, scale bar: 100 μ m. Panel d shows a magnified view of the boxed area, scale bar: 20 μ m. **(E)** In the TST, chemogenetic inhibition of the orexin–mPFC pathway significantly increased immobility time in LH-resilient mice. $F_{(4, 35)} = 21.16$; $P < 0.0001$ for LH-resilient mice versus LH-resilient+hM4+CNO mice, $P = 0.9$ for LH-resilient mice versus LH-resilient+mCherry+CNO mice. **(F)** In the OFT, inhibition of the orexin–mPFC pathway significantly reduced the number of entries into the center zone in LH-resilient mice, $F_{(4, 35)} = 13.25$; $P = 0.0003$ for LH-resilient mice versus LH-resilient+hM4+CNO mice, $P = 0.95$ for LH-resilient mice versus LH-resilient+mCherry+CNO mice. **(G)** In the OFT, inhibition of the orexin–mPFC pathway significantly decreased the duration spent in the center area of LH-resilient mice, $F_{(4, 35)} = 34.2$; $P < 0.0001$ for LH-resilient mice versus LH-resilient+hM4+CNO mice, $P = 0.91$ for LH-resilient mice versus LH-resilient+mCherry+CNO mice. **(H)** In the SPT, inhibition of the orexin–mPFC circuit led to decreased sucrose preference in LH-resilient mice, $F_{(4, 35)} = 29.68$; $P < 0.0001$ for LH-resilient mice versus LH-resilient+hM4+CNO mice, $P = 0.83$ for LH-resilient mice versus LH-resilient+mCherry+CNO mice. **(I)** In the shuttle box test (30 trials), chemogenetic inhibition of the hypothalamic orexin–mPFC pathway reduced the number of crossings in LH-resilient mice. $n = 8$ mice for each group of behavioral tests. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine statistical significance. **** $P < 0.0001$, *** $P < 0.001$, ns, not significant.

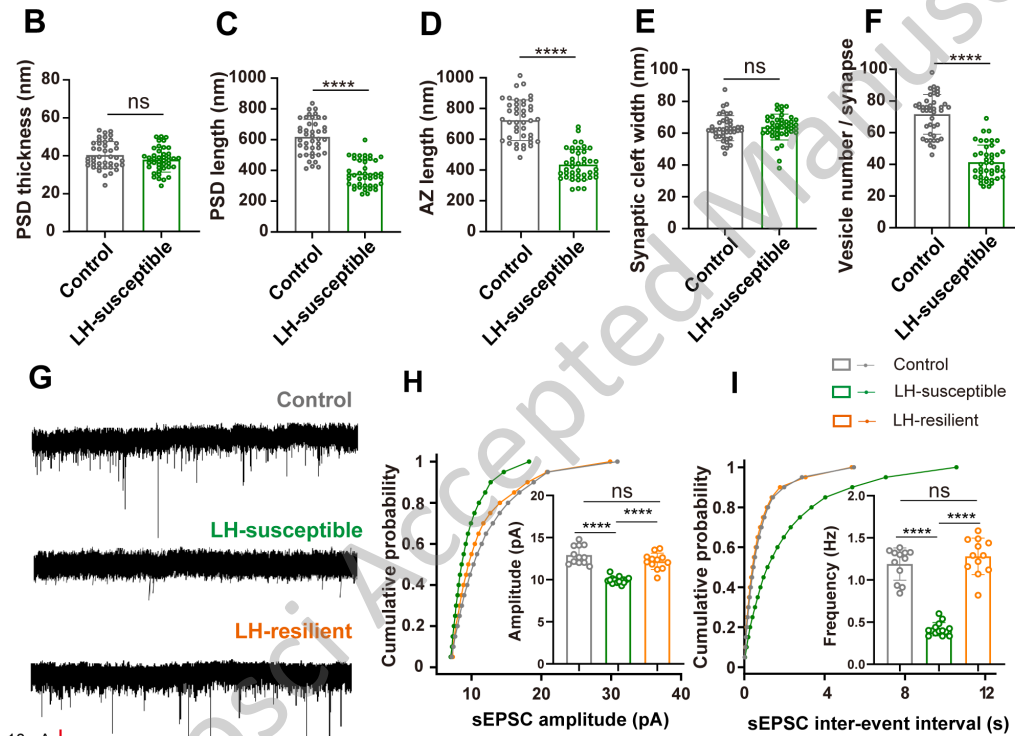
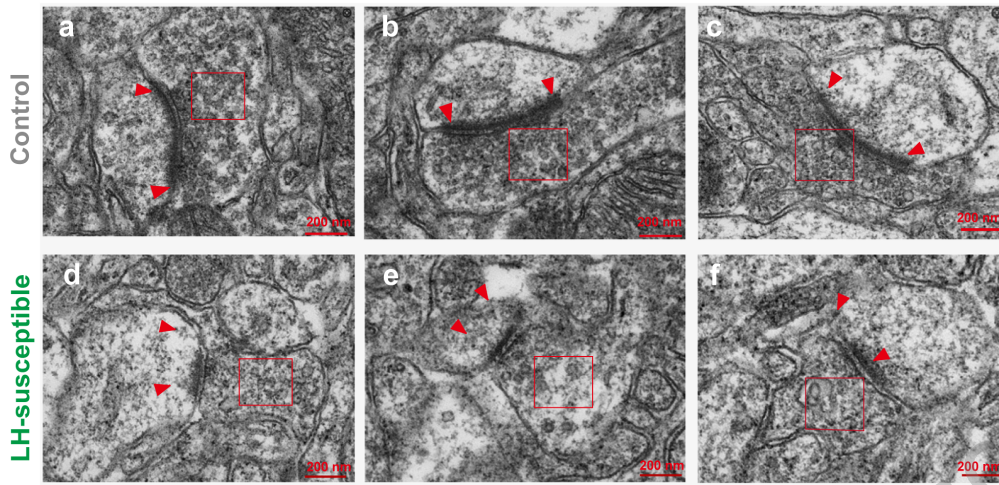
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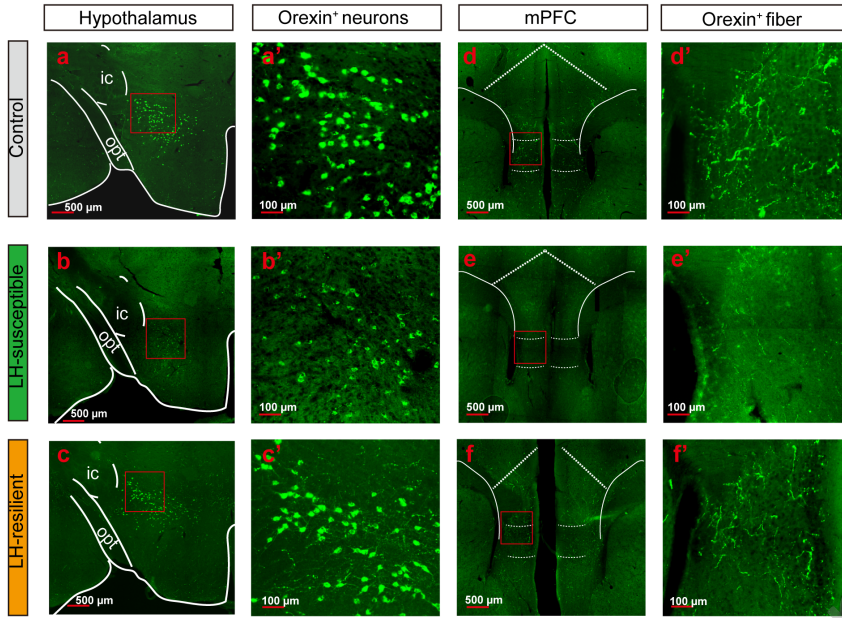
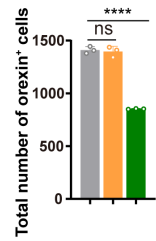
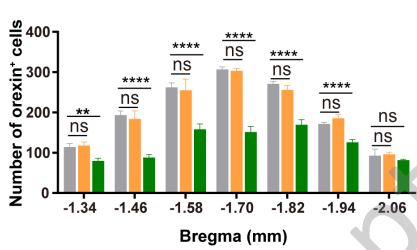
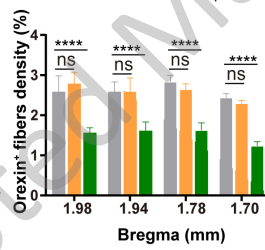
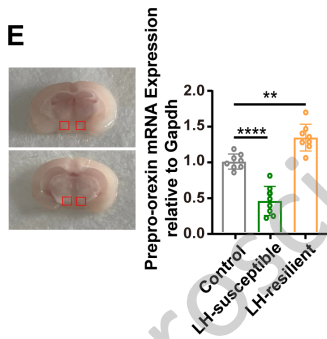
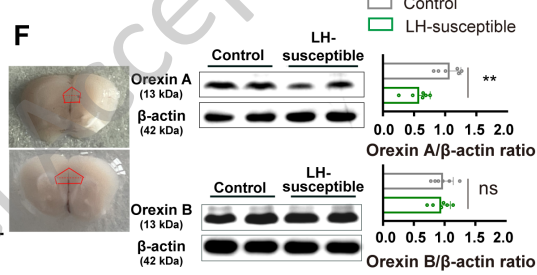
Figure 7. Intra-mPFC infusion of Orexin A alleviates depression-like behaviors in LH-susceptible mice. **(A)** Part I: Experimental design showing infusion of Orexin A, saline, or Orexin B into the unilateral mPFC; Part II: Experimental procedure for pre-infusion of Dora followed by Orexin A into the unilateral mPFC. **(B)** Schematic representation of cannula placements in the mPFC for Orexin A injection. Black circles indicate correct placements ($n = 8$ mice), while red squares denote misplacements excluded from analysis ($n = 4$ mice). **(C)** In the TST, unilateral infusion of Orexin A into the mPFC significantly reduced immobility time in LH-susceptible mice, an effect reversed by pre-treatment with Dora, $F_{(4, 35)} = 56.96$; $P < 0.0001$ for LH-susceptible+Orexin A mice versus LH-susceptible mice; $P = 0.96$ for LH-susceptible+saline mice versus LH-susceptible mice, $P < 0.0001$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible+Orexin A mice, $P = 0.9$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible mice. **(D)** In the OFT, Orexin A injection into the mPFC increased the number of entries into the center zone in LH-susceptible mice, an effect reversed by pre-treatment with Dora, $F_{(4, 35)} = 23.05$; $P < 0.0001$ for LH-susceptible+Orexin A mice versus LH-susceptible mice, $P = 0.550$ for LH-susceptible+saline mice versus LH-susceptible mice, $P < 0.0001$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible+Orexin A mice; $P = 0.9$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible mice. **(E)** In the OFT, Orexin A injection into the mPFC increased the duration spent in the center area in LH-susceptible mice, an effect reversed by pre-treatment with Dora, $F_{(4, 35)} =$

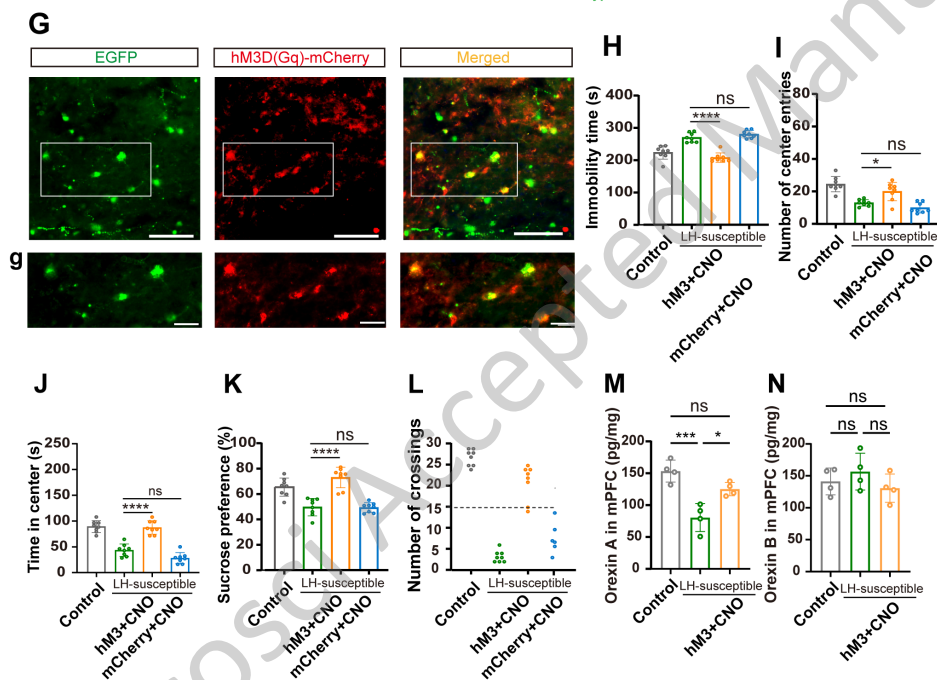
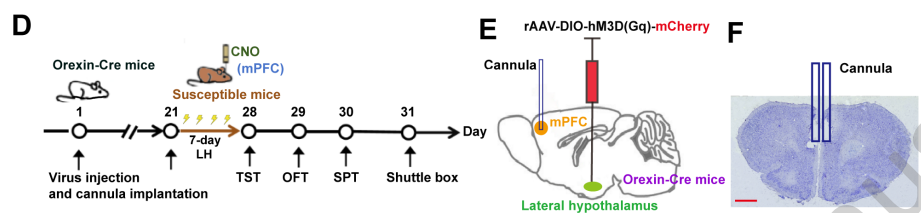
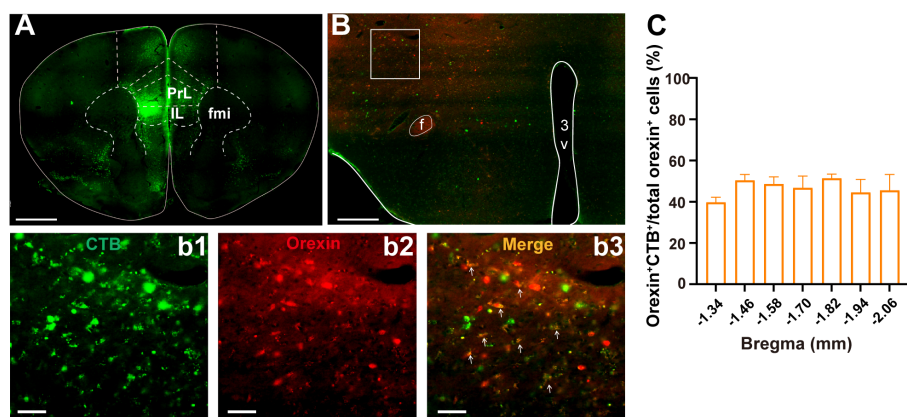
46.54; $P < 0.0001$ for LH-susceptible+Orexin A mice versus LH-susceptible mice; $P = 0.92$ for LH-susceptible+saline mice versus LH-susceptible mice, $P < 0.0001$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible+Orexin A mice, $P = 0.931$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible mice. **(F)** In the SPT, Orexin A administration into the mPFC enhanced sucrose preference in LH-susceptible mice, $F_{(4, 35)} = 63.56$; $P < 0.0001$ for LH-susceptible+Orexin A mice versus LH-susceptible mice, $P = 0.418$ for LH-susceptible+saline mice versus LH-susceptible mice, $P < 0.0001$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible+Orexin A mice, $P = 0.629$ for LH-susceptible+Dora+Orexin A mice versus LH-susceptible mice. **(G)** In the 30-trial shuttle box test, Orexin A injection into the mPFC increased the number of crossings in LH-susceptible mice, an effect reversed by pre-treatment with Dora. $n = 8$ mice for each group of behavioral tests. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine statistical significance. **** $P < 0.0001$, *ns*, not significant.

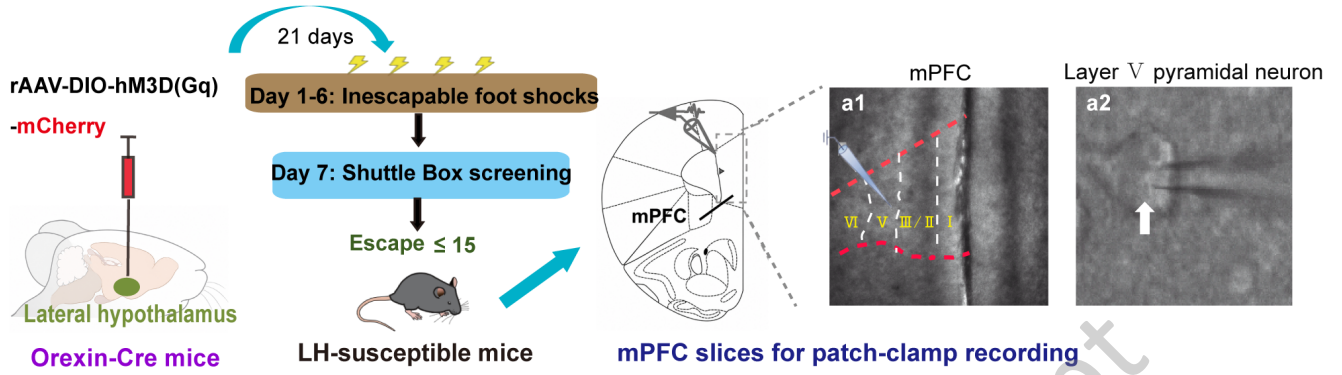
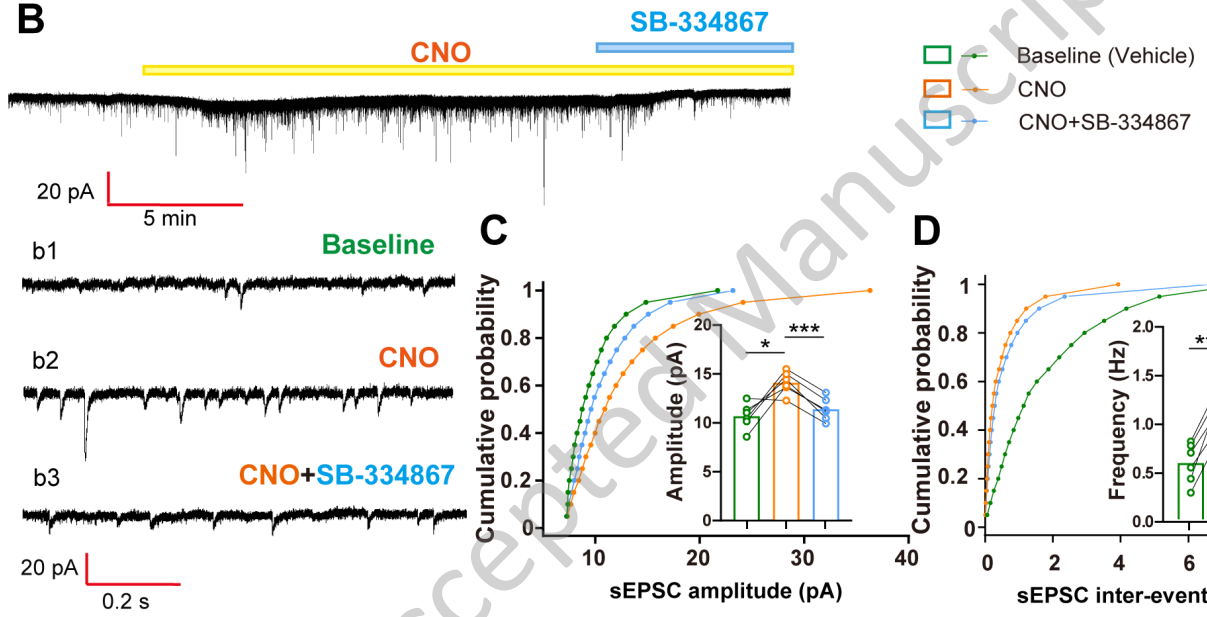
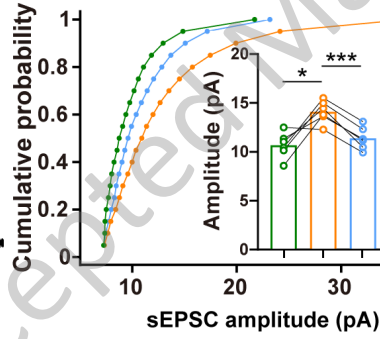
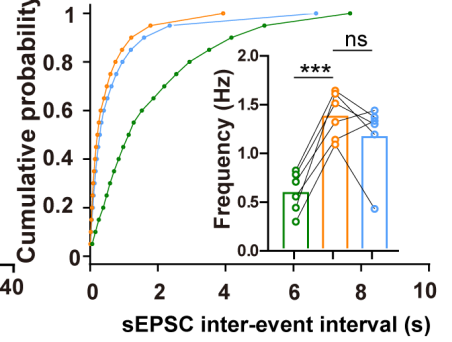
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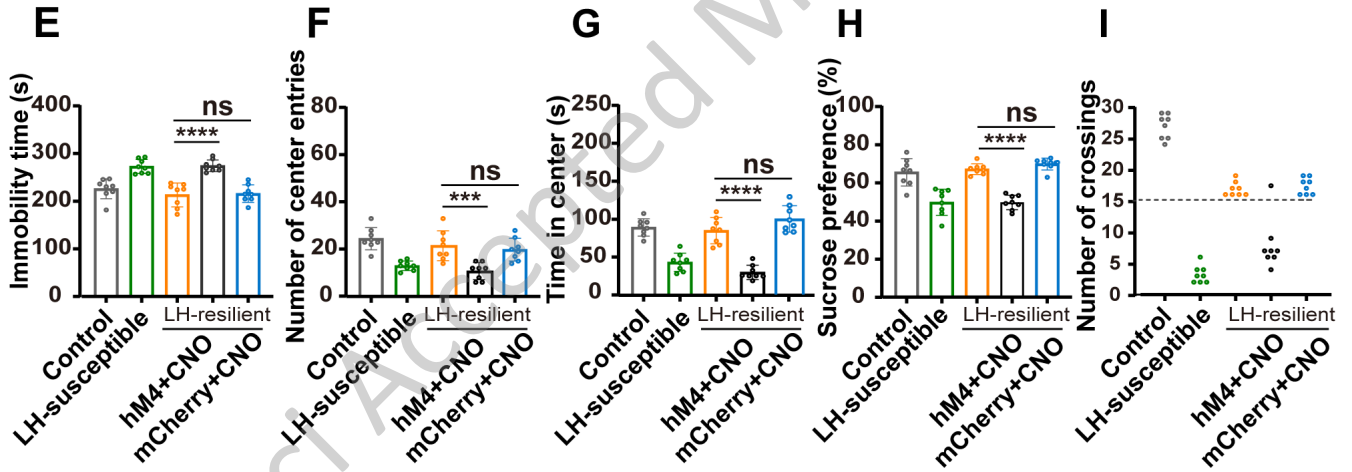
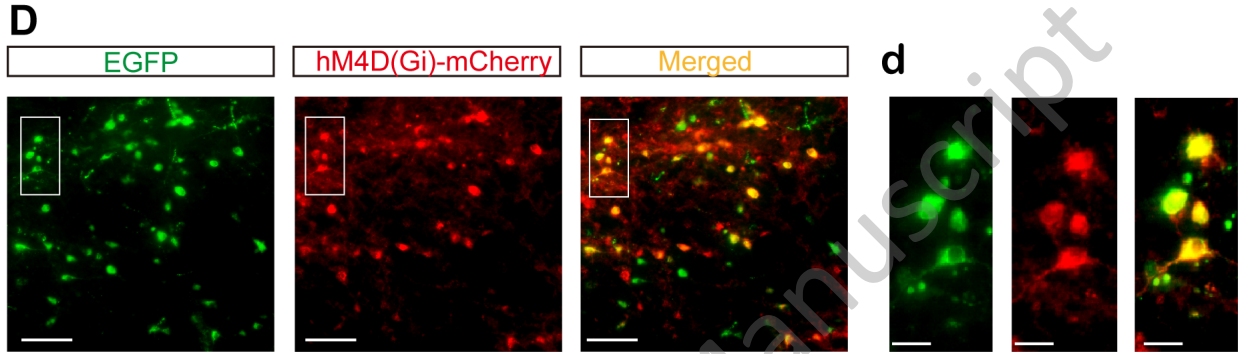
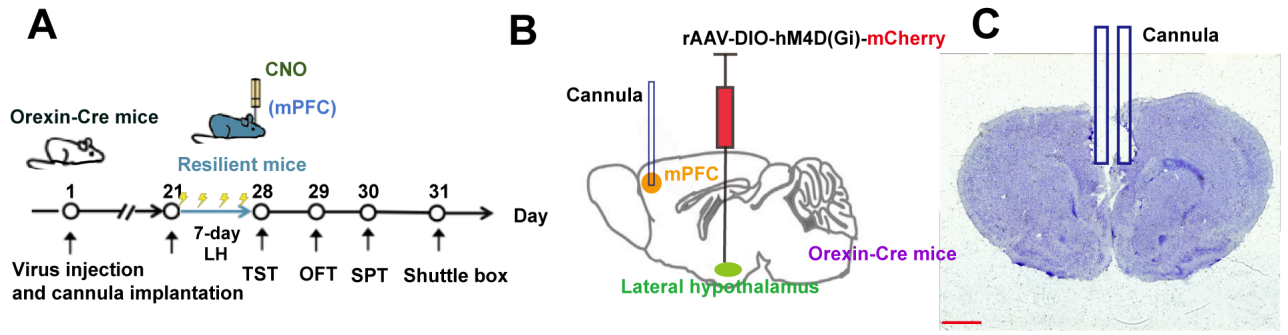


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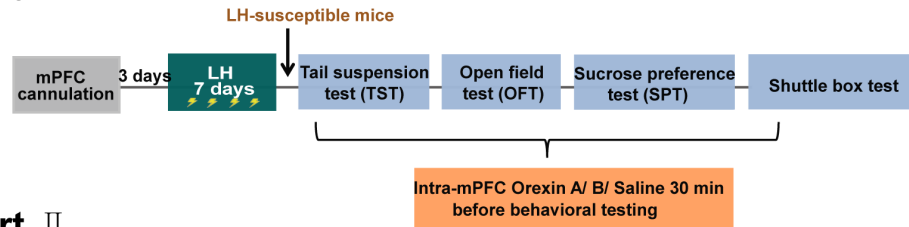


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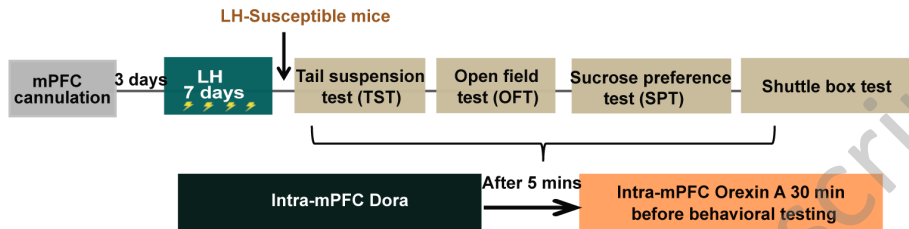


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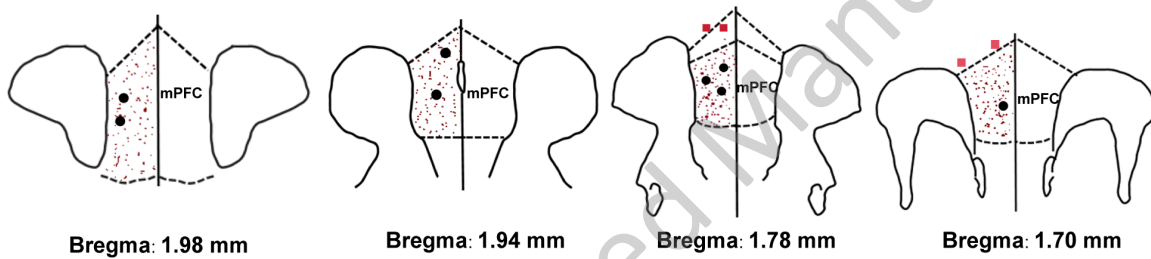
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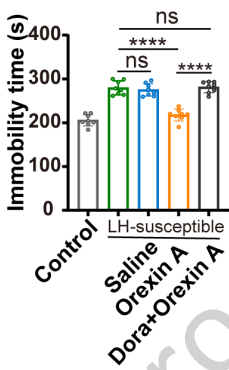
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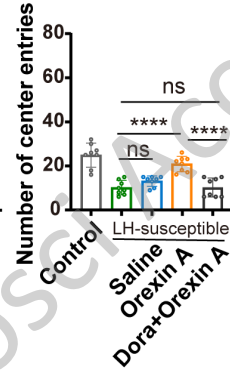
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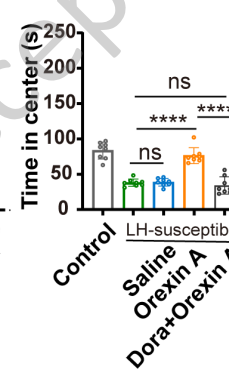
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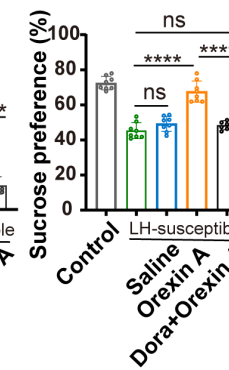
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