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The zona incerta regulates burying behavior and normalizes anxiety-like behavior in inescapable stressful male mice by object cue

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ABSTRACT

Inescapable stressful events often precipitate long-term alterations in emotion-related behaviors and poor sleep quality, with anxiety being a prevalent associated disorder. The defensive burying behavior of rodents is a response to imminent threats that becomes markedly pronounced in response to anxiety. However, the neural foundations of defensive burying behavior and etiology of anxiety remain largely unknown. In this study, we established a model employing object binding to elicit increased burying behavior in mice, thereby enhancing fear resolution and subsequently reducing anxious behaviors. Notably, the mice that associated shock with an object exhibited less object exploration and the zona incerta (ZI) neurons showed higher calcium activity during object exploration as compared to the Shock only mice. Although the calcium activity in ZI neurons of the Object mice was identical to the Shock only mice, the Object mice exhibited more burying behavior. Furthermore, the time spent in the center of the open-field test was directly proportional to the duration of burying behavior. Chemogenetic activation of ZI neurons extended the burying time and concomitantly ameliorated anxiety-like behavior. Importantly, chemogenetic enhancement of projection from ZI neurons to the ventral periaqueductal gray (vPAG), a brain region that plays a critical role in autonomic function, normalizes anxious behavior without influencing burying behavior. Collectively, these findings systematically reveal the functions and underlying mechanisms of the ZI-vPAG circuit in controlling behaviors akin to anxiety, offering significant insights into ZI's role in the pathophysiology of anxiety disorders.

1. Introduction

Anxiety disorder is a prevalent mental health condition that has been linked to genetic predisposition, illness, and environmental factors. Traumatic events have been proposed to play a pivotal role in the development and maintenance of anxiety disorders. Notably, inescapable stressful experiences can give rise to diverse pathologies, including psychological dysfunctions, such as anxiety (Marqueses et al., 2023; Seo, 2018). Among rodents, burying behavior is significantly pronounced in response to anxiety, as determined with the marble burying test, a common method to assess anxiety-like behaviors of laboratory rodents (Dixit et al., 2020). Defensive burying behavior, a phenomenon observed in rodents, involves meticulous rearrangement of bedding material through alternating forward paw movements and head shoveling motions to primarily conceal localized sources of threats or aversive stimuli (De Boer and Koolhaas, 2003). Rodents effectively mitigate potential life-threatening hazards by burying unfamiliar or harmful objects (Njung'e and Handley, 1991; Park et al., 2022). Interestingly, chronic stress diminishes burying behavior and increases immobility of rats, indicative of a shift from active to passive coping strategies. Intriguingly, burying a harmful probe has been linked to lower stress hormone levels (Fucich and Morilak, 2018). Furthermore, animals

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exhibiting heightened defensive burying have relatively lower mRNA levels of arginine vasopressin and oxytocin in the supraoptic and paraventricular nuclei of the hypothalamus (Linfoot et al., 2009). These findings suggest that burying behavior can be a positive strategy to mitigate stressful events. Despite these intriguing findings, the precise neural mechanisms underlying defensive burying behavior and potential associations with anxiety development remain unclear.

Various brain regions, including the hippocampus, prefrontal cortex, amygdala, bed nucleus of the stria terminalis, paraventricular thalamus, and insular cortex, have been implicated in the generalization of anxiety (Liu et al., 2020; Wang et al., 2019; Zhao et al., 2022). Recently, the zona incerta (ZI), a GABAergic subthalamic region, has been implicated in modulation of anxiety disorders, as chemogenetic inhibition of ZI neuron activity results in fear generalization, whereas increased ZI neuron activity can prevent conditioned fear responses (Venkataraman et al., 2019). The central amygdala forms monosynaptic connections with parvalbumin-positive neurons in the ZI, which are involved in both the acquisition of fear memories and recall of remote fear memories (Zhou et al., 2018). Stimulation in or near the ZI has been associated with reduced self-reported depression and anxiety, as well as improved ability to recognize fear in patients with Parkinson's disease (Burrows et al., 2012). Inactivation of the ZI significantly increased anxiety levels induced by repeated social defeat stress and contributed to the development of post-traumatic stress disorder (Zhou et al., 2021). Chemogenetic activation of neurons expressing glutamate decarboxylase in the ZI has been shown to significantly alleviate anxiety-like behaviors in mice at 1-day post-injection of complete Freud's adjuvant (Farzinpour et al., 2022). Optogenetic activation of neurons expressing somatostatin in the ZI triggers anxiety behavior, whereas inactivation of neurons expressing vesicular glutamate transporter 2 in the ZI and activation of calretinin-positive neurons reduce anxiety behavior (Li et al., 2021). GABAergic neurons in the ZI directly innervate the dorsolateral and ventrolateral excitatory neurons in the periaqueductal gray (PAG), driving both flight and freezing behaviors (Chou et al., 2018). The vPAG of the midbrain is considered a crucial component of the neural circuitry that triggers freezing and flight responses to threatening events (Tovote et al., 2016). However, the mechanism employed by the ZI to instruct the neurons of the vPAG to modulate anxiety disorders remains unclear.

In this study, a modified mouse model of object cue in response to stress was utilized to enhance the burying behavior and facilitate fear extinction by object exposure. Behavioral testing revealed that the mice exhibited reduced anxiety. The duration of time that the object mice spent in the center of the open field was found to be positively correlated with the time spent burying during the burying behavior test. Staining of the c-Fos protein and *in vivo* fiber photometry identified the ZI as a key brain region involved in burying behavior. Chemogenetic activation of ZI neurons increased the burying behavior and decreased anxiety-like behaviors in mice following electric shock treatment. Moreover, viral tracing demonstrated that vPAG was downstream of the ZI and plays a crucial role in the development of anxiety disorders. Activation of the ZI-vPAG pathway reduced anxious behavior in mice without influencing burying behavior. Taken together, these findings underscore the role of hyperactivity of the ZI in defensive burying behavior and highlight the potential for targeted manipulation for treatment of anxiety disorders based on a circuit-level understanding of behavioral abnormalities.

2. Materials and methods

2.1. Mice

Male C57BL/6J mice were purchased from the Model Animal Research Center (Nanjing, China). Five mice were housed in a standard cage under a 12-h reversed light-dark cycle with *ad libitum* access to food and water. The study protocol was approved by the Institutional Animal Care and Use Committee of Nanjing University and conducted in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals".

2.2. Inescapable foot shock (IFS)

Each mouse was placed in a fear behavior box and allowed to move freely for 5 min. Subsequently, an object was either introduced or not, and the mice were subjected to 10 IFSs (duration, 1 s; intensity, 1.50 mA) over the following 5 min, delivered uniformly throughout the session. The control group did not receive objects or IFSs.

2.3. Burying behavior test

The burying behavior of mice while caged was recorded for 5 min. An extra 20 s was used to place objects. Then, the burying behavior was recorded over the next 5 min. Burying behavior here refers to mice turning the bedding with their forelegs, hindlegs and tip of the nose, but not burying themselves.

2.4. The extinction phase

In the same fear behavior box, the mice were allowed to move freely for 5 min. Then, an object was placed in the box and the freezing time was recorded over the next 5 min.

2.5. Anxiety behavioral tests

Open-field test (OFT). The OFT was conducted using a square Plexiglas chamber (50 × 50 × 38 cm). The central squares were defined as the center area. Under bright lighting, each mouse was placed in the center of the chamber and allowed to freely explore the new environment for 5 min. Movements were recorded with a video camera for subsequent behavioral analysis.

Elevated plus maze (EPM). The EPM apparatus, set 50 cm above the floor, features two open arms and two enclosed arms that intersect at right angles. Mice were positioned at the central intersection, facing an open arm, and permitted to explore for 5 min. Behavior was monitored and recorded with a video camera.

2.6. Virus injection

Before surgery, mice were anesthetized with 5% isoflurane and fixed on a stereotaxic instrument. Isoflurane anesthesia was maintained at 0.5%–1.0%. For manipulation of neurons in the ZI, 100 nL of adeno-associated virus (AAV), as rAAV-hSyn-hM3D(Gq)-EGFP-WPRE-pA, rAAV-hSyn-hM4D(Gi)-EGFP-WPRE-hGH pA, PT-1990 rAAV-hSyn-EGFP-WPRE-hGH pA, rAAV-hSyn-GCaMp6s-WPRE-hGH polyA, rAAV-DIO-hM3Dq-EYFP, or rAAV-DIO-EYFP (Wuhan Shumi Brain Science and Technology Co., Ltd, Wuhan, China) were delivered into the ZI (−1.2 mm from the bregma; ±1.6 mm lateral from the midline; 4.4 mm vertical from the cortical surface) and AAV2/2Retro-hSyn-Cre (Shanghai Taitool Bioscience Co., Ltd, Shanghai, China) into VPAG (−4.52 mm from bregma; ±0.42 mm lateral from the midline; 2.78 mm vertical from the cortical surface) of C57BL/6J mice in 10 min through a 1 μl microsampler. After finishing each injection, the needle was stayed for 5 min to spread the virus completely. After removing the needle from the injected point, scalp was sutured with a sterile suture needle. Subsequent experiments were conducted after about 4 weeks of viral expression. Data from mice that exhibited no or minimal viral infection bilaterally were excluded from the analysis.

2.7. Optical fiber embedding

At 2 weeks after virus injection, the mice were anesthetized with 5% isoflurane and fixed on a stereotaxic instrument. Isoflurane anesthesia was maintained at 0.5%–1.0%. An optical fiber (outer diameter, 125 μm; QAXK; ThinkerTech Nanjing BioScience Inc., Nanjing, China) was

inserted using a fiber clamp at a depth generally about 0.10–0.20 mm less than the depth of the virus injection and secured with dental cement. The mice were allowed to recover for 1–2 weeks before the experiment.

2.8. Fiber photometry recording

A fiber photometry system (DualColorMultichannel_TP_470_580; ThinkerTech Nanjing BioScience Inc.) was used for recording. The laser power at the fiber tip was adjusted from 20 to 30 μW. The relative change in fluorescence ($\Delta F/F$) was calculated as = (F - $F0$)/$F0$, where $F0$ is the baseline fluorescence signal averaged over 2.5 s before the events. All experimental data were processed and analyzed using MATLAB software (MathWorks, Inc., Natick, MA, USA).

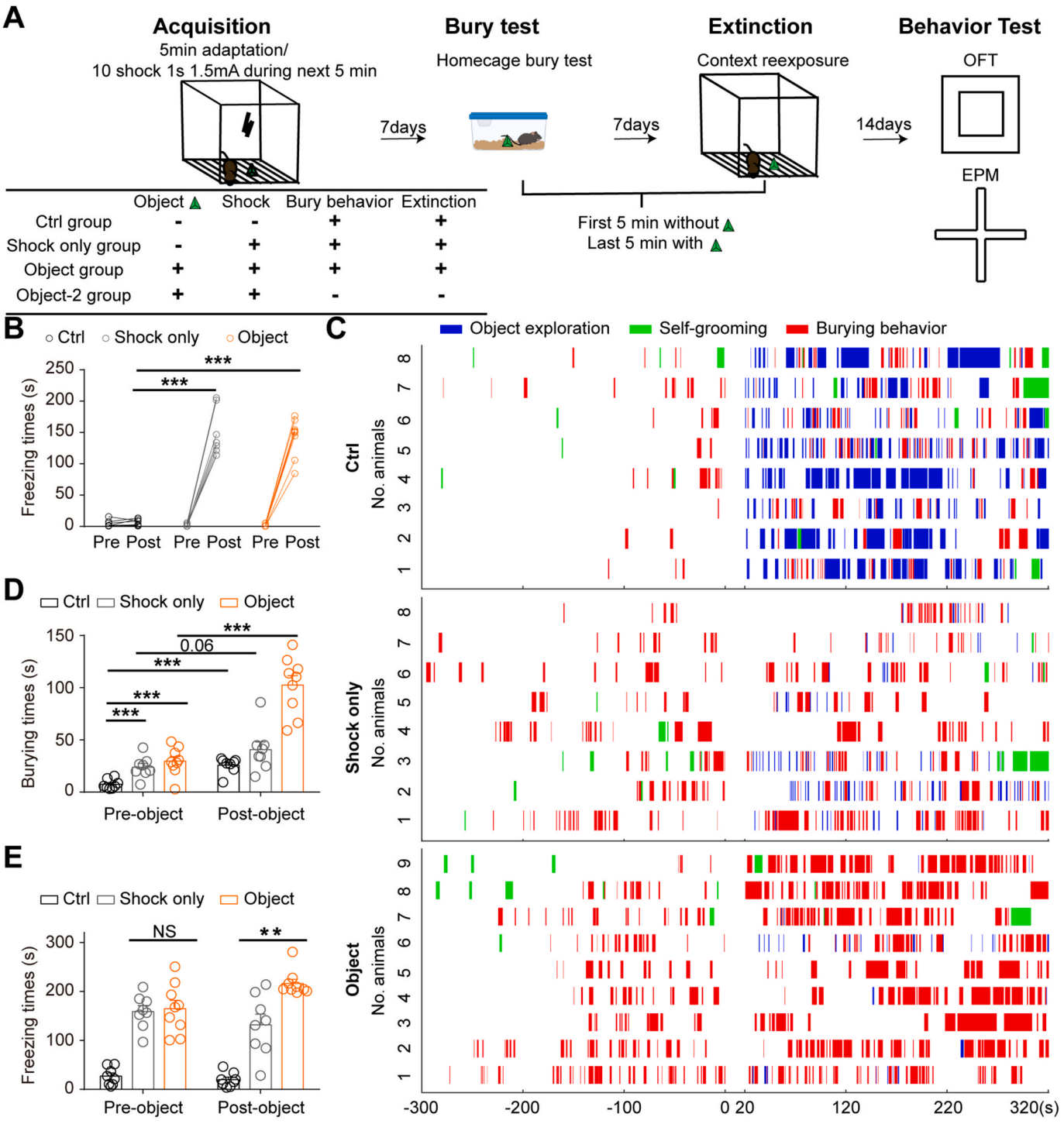

Fig. 1. Object mice paid more time on burying behavior. A. Schematic of stress model establishment and the experimental design. B. Freezing time before and after inescapable foot electric shock of Ctrl (n = 8 mice), Shock only (n = 8 mice) and Object group (n = 9 mice). C. Representative behavioral events assessed for the three group mice before and after object exposure. Blue represents exploring object, green represents self-grooming and red represents burying. D. Burying time before and after object exposure during the burying test. E. Freezing time before and after object exposure during context reexposure test.
Error bars, Data represent mean ± SEM $^{**}p < 0.01$, $^{***}p < 0.001$, Two-way ANOVA.

2.9. Chemogenetic regulation

Clozapine N-oxide (MedChemExpress, Shanghai, China) was diluted with saline to 0.5 mg/mL and administered 30 min prior to each experimental session.

2.10. Immunohistochemical analysis

C57BL/6J mice were anesthetized with chloral hydrate (10%), transcardially perfused with ice-cold phosphate-buffered saline (PBS; 0.01 M), followed by 4% paraformaldehyde in PBS. Brains were resected and post-fixed overnight at 4 °C with 4% paraformaldehyde, cryoprotected with 30% sucrose in 0.1 M PBS for 2 days at 4 °C, and cut with a cryostat microtome (CM1950; Leica Microsystems GmbH, Wetzlar, Germany) into sections (thickness, 40 mm), which were permeabilized and blocked with 0.5% Triton X-100 and 5% bovine serum albumin for 1.5 h at room temperature, incubated for 24–48 h at 4 °C with an anti-rabbit antibody against c-Fos (dilution, 1:500; catalog no. 226008; Synaptic Systems GmbH, Göttingen, Germany), washed, and incubated at room temperature for 2 h with a goat anti-rabbit Alexa 488 secondary antibody against c-Fos (dilution, 1:500; Jackson ImmunoResearch Labs, West Grove, PA, USA). The nuclei were counterstained for 10 min with 4′,6-diamidino-2-phenylindole (2 mg/mL; catalog no. B2261; Sigma-Aldrich Corporation, St. Louis, MO, USA). Subsequently, the brain slices were mounted on glass slides with an anti-quenching agent (Vector Laboratories, Inc. Burlingame, CA, USA) and imaged under a confocal laser scanning microscope (TCS SP8; Leica Microsystems GmbH). The neurons were quantified using ImageJ software (https://imagej.net/ij/).

2.11. Statistical analysis

All data are presented as the mean ± standard error of the mean. The Student's *t*-test was used to compare data between two groups, One-way or Two-way ANOVA analysis of variance with Tukey's post hoc test for three or more. A probability (p) value < 0.05 was considered statistically significant.

3. Results

3.1. Object binding stressful events increased the buried behavior after re-exposure to the object and subsequently reduced anxious behaviors

The experimental design was adjusted from the original approach (see Fig. 1A). Following training involving IFS administered at 1.5 mA for 1 s, repeated 10 times, mice in both the Shock only group (n = 8) and the Object group (n = 9) exhibited increased freezing behavior during the subsequent 5 min as compared to the Control group (n = 8) (Fig. 1B, $p < 0.001$). After IFS exposure, spontaneous behavior in the homecage was recorded (Fig. 1C). The mice that experienced IFS displayed longer burying times than the Control mice (Fig. 1D, $p < 0.001$). After encountering a harmful stimulus, the Object mice exhibited a significantly longer duration of burying behavior as compared to the Shock only group (Fig. 1D, $p < 0.001$). Given that mice tend to bury unfavorable objects, this increased burying behavior, suggesting an association between the stressful event and the object. Interestingly, Control mice allocated more time to exploring the object than mice exposed to IFS (Fig. S1B, right, $p < 0.001$), while there was no significant difference in self-grooming time among the three groups (Fig. S1A, left). Subsequently, the mice were exposed to the conditioned context 7 days after the initial burying behavior, an event designated as the extinction phase. Remarkably, the mice that experienced IFS exhibited prolonged freezing behavior as compared to the Control mice, indicating a lasting fear effect resulting from the stressful experience (Fig. 1E, $p < 0.001$). Furthermore, after exposure to the object, the Object mice displayed increased freezing time relative to the Shock only group (Fig. 1E, $p < 0.01$), reinforcing the notion that these mice associated fear with the object.

Given that IFS can trigger anxiety-like behaviors in mice, we focused our attention on anxiety-related responses. Notably, the speed of movement of the three groups was unaffected by the stressful event, and the change to the total travel distance was not significant (Fig. 2A, middle and right, $p > 0.05$). However, the Shock only mice exhibited more immobility (Fig. 2A, left, $p < 0.05$) during the 5-min OFT as compared to the Control mice. Additionally, the Shock only group displayed fewer center entries (Fig. 2B, middle, $p < 0.01$), less time spent in the center (Fig. 2B, left, $p < 0.05$), and shorter center distances traveled (Fig. 2B, right, $p < 0.05$) than the Control group. Similarly, in the EPM, the Shock only mice had fewer open arms entries (Fig. 2C, middle, $p < 0.01$), less time spent in the open arms (Fig. 2C, left, $p < 0.05$), and shorter open arms distances traveled (Fig. 2C, right, $p < 0.05$) as compared to the Control group. These findings collectively indicate anxiety-like behavior in the Shock only mice. Surprisingly, the Object mice exhibited less anxiety-like behavior than the Shock only mice (Fig. 2A–C). To determine whether the reduction in anxiety of the Object mice was associated with burying behavior, we analyzed the linear relationship between burying behavior and the number of center entries in the OFT and the number of open arms entries in the EPM. Interestingly, neither object exploration nor self-grooming behavior showed a significant linear relationship with anxiety-like behaviors in mice (Fig. 2D and E, middle and right, $p > 0.05$). Furthermore, the Control mice, which had lower anxiety levels, spent more time on object exploring behavior (Fig. S1C, right, $p < 0.05$). However, the number of center entries (Fig. 2D, left, $p < 0.05$) and open arms entries (Fig. 2E, left, $p < 0.01$) demonstrated a significant linear relationship with the burying behavior of the Object mice. To determine whether burying behavior is key to improving anxious behavior, an Object-2 group of mice was established, consistent with the Object mice but without the burying behavior test and object exposure (Fig. 1A). After testing, the Object-2 mice had higher anxiety levels (Fig. 2A–C). These results suggest that Object mice exhibited lower anxiety levels, which correlated with increased burying behavior during object exposure.

3.2. The ZI neurons were involved in burying behavior

To examine the neuronal activity of the ZI and vPAG during anxiety generation, the number of cells expressing c-Fos in the ZI and vPAG were counted after exposing the mice to the three different phases (Fig. 3A). At stage 1, c-Fos expression in the ZI of the Object group was significantly higher, but lower in the subregion of vPAG of the Control group as compared to the IFS group (Fig. S2C). At stage 2, c-Fos expression in the ZI of the Object group was significantly higher than in the Control and Shock only groups (Fig. 3B and C). Meanwhile, c-Fos expression in the subregion of the vPAG remained consistent (Fig. 3C). At stage 3, c-Fos expression in vPAG was significantly higher in the Shock only group than the Control and Object groups, whereas expression levels in the ZI were similar among the three groups (Fig. S2D). To determine whether the ZI neurons are directly involved in burying behavior, rAAV-syn-gcamp6s was injected into ZI to monitor population calcium dynamics by *in vivo* fiber photometry (Fig. 3D and E). In the free-moving mice, there was no significant change to ZI neuron activity during object exploration in the Shock only group; while in the Object group this activity was increased (Fig. 3F top). In these two groups, Ca^{2+} transient analogously increased during burying behavior and decreased during self-grooming (Fig. 3F middle and bottom). These results demonstrate that the ZI neurons were involved in the burying behavior and can be activated by harmful objects.

3.3. Chemogenetic activation of ZI extended the burying time and concomitantly ameliorated anxiety-like behavior in the shock only group

Considering the involvement of ZI neurons in burying behavior, we investigated whether increasing ZI activity directly influences burying behavior. The ZI of mice was injected with rAAV-hsyn-EGFP, rAAV-

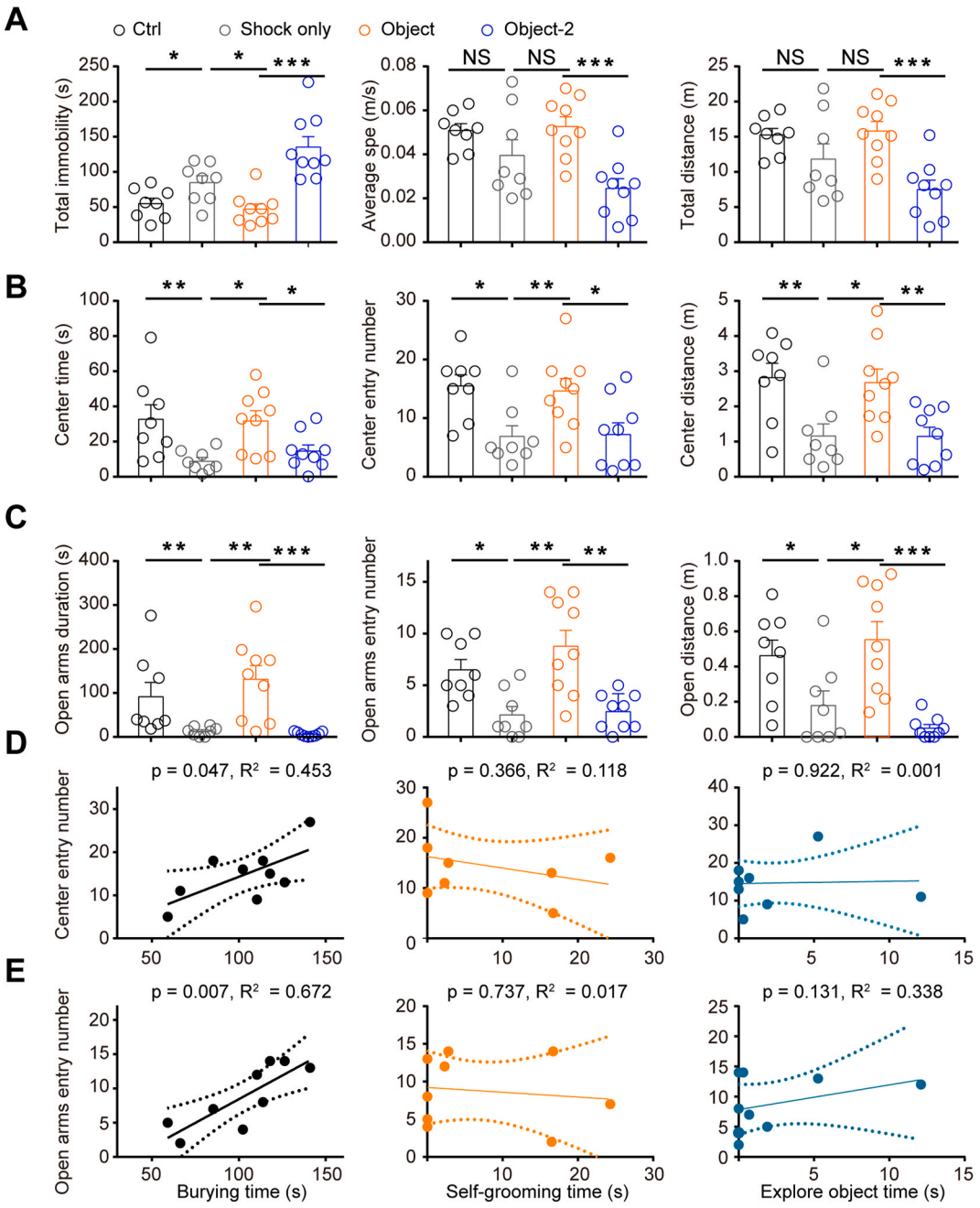

Fig. 2. The Object mice showed less anxiety behavior. A. The four groups of mice movement related to anxiety was assessed based on immobile time (left) and total travel distance (right); motor ability based on the average speed (middle).

B. The four groups of mice movement related to anxiety was assessed based on the time in the center zone (left), number of entries into the center zone (middle), and the center distance(right).

C. The three groups of mice movement related to anxiety was assessed based on the time in open arms (left), number of entries into the open arms (middle), and open arms travel distance (right).

D. The time of staying in the center of the open field was related to the burying time of the mice in burying behavior in the Object group, but not the time of self-grooming and exploring objects.

E. The time of staying in the open arms was related to the burying time of the mice in burying behavior in the Object group, but not the time of self-grooming and exploring objects.

Error bars, Data represent mean ± SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, One-way ANOVA.

hsyn-hM4Di-EGFP, or rAAV-hsyn-hM3Dq-EGFP to specifically regulate the ZI neurons (see Fig. 4A). After virus expression for over 14 days, the mice were subjected to IFS and allocated to the Shock only group or Object group (Fig. 4C). Following IFS, all mice exhibited high levels of freezing behavior (Fig. 4D). During the burying behavior test, specific activation of ZI neurons increased the burying time both before and after object exposure in the Shock only group (Fig. 4F, left). Conversely, in the Object group, specific inhibition of ZI neurons was found to reduce the burying time after exposure to the aversive object. However, increasing activity of the ZI neurons did not significantly impact the burying behavior either before or after exposure (Fig. 4F, right). Additionally, chemogenetic enhancement of ZI neuron activity slightly increased self-

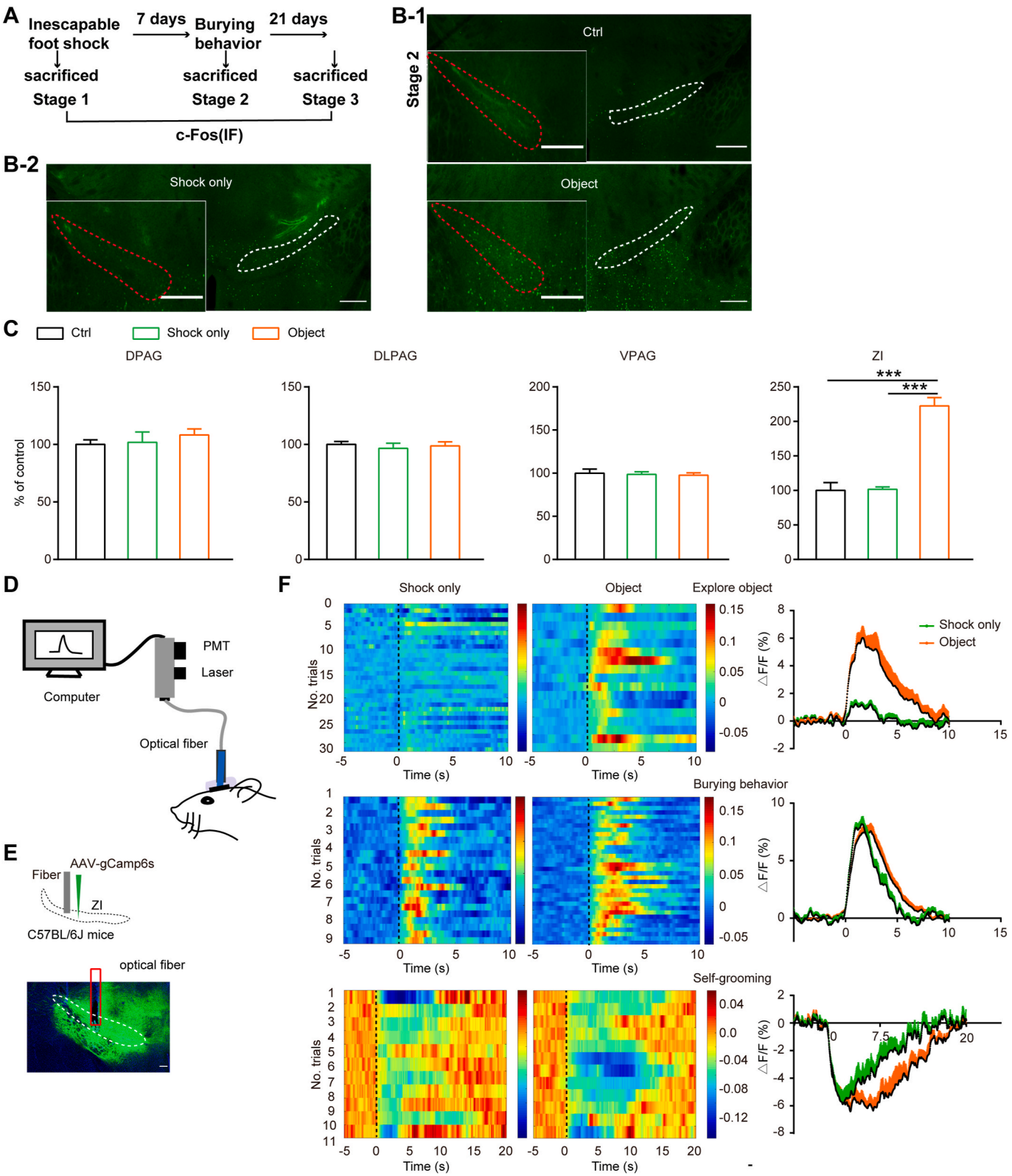

Fig. 3. ZI neurons were involved in burying behavior. A. Schematic diagram of the experimental design. B1-2. Illustration of c-Fos expression in ZI of three groups of mice after burying behavior. Scale bar = 300 μm. Enlarge view shown in inset, Scale bar = 400 μm. C. The percentage of c-Fos expression in DPAG, DLPAG, VPAG and ZI of three groups of mice. D Setup of the fiber photometry system for head-fixed mice. E Schematic diagram of virus injection and implanted fiber in ZI of C57BL/6J mouse. Scale bar = 150 μm.

F Left: Representative heatmap of Ca2+ signals aligned to the initiation of exploring object (top), burying (middle) and self-grooming (bottom) in the Shock only mice (left, n = 5 mice) and Object mice (right, n = 5 mice), Color scales on the right indicate ΔF/F. Right: Mean Ca2+ transients aligned to events starting with stimuli. Data are means ± SEM. ***$p < 0.001$, One-way ANOVA.

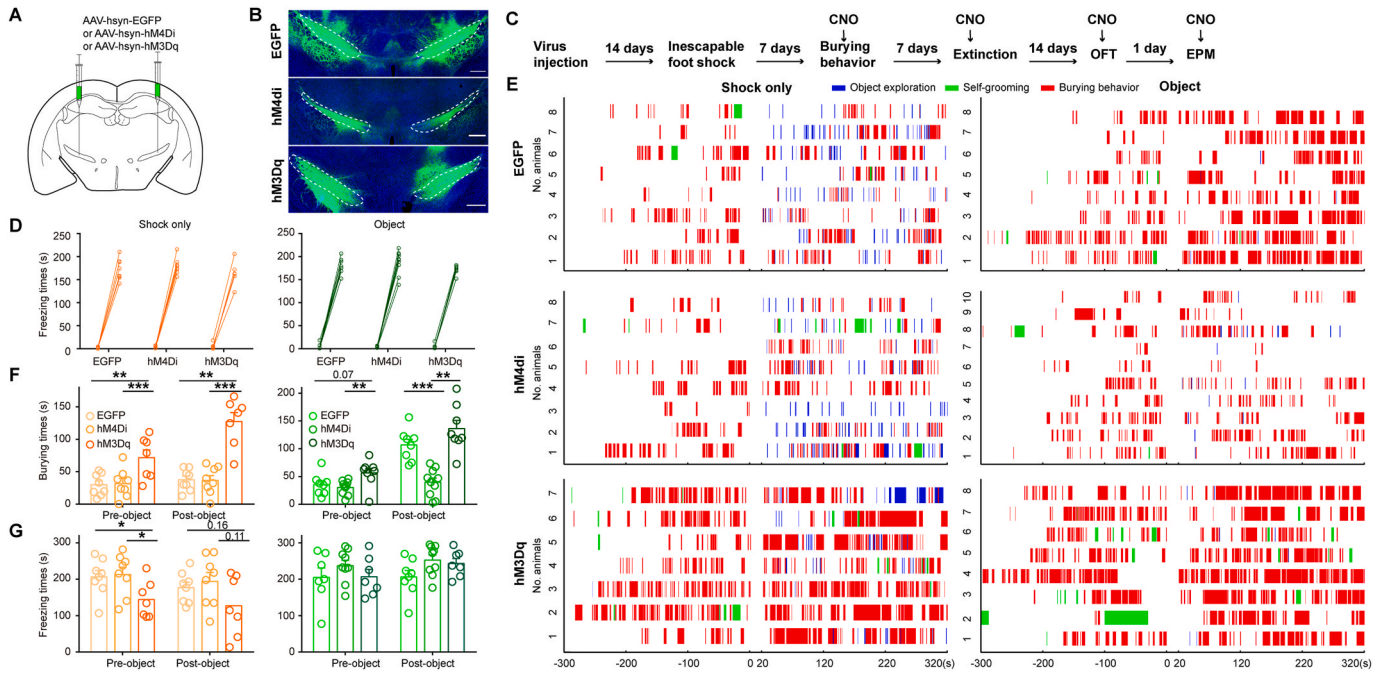

Fig. 4. Activation of ZI neurons by chemogenetics increased burying behavior in the Shock only group. A. Stereotaxic injection of AAVs into the ZI. B. Illustration of ZI infections with EGFP, hM4Di and hM3Dq in C57BL/6J mice. Scale bar = 300 μm. C. Schematic diagram of the experimental design. D. Freezing time before and after inescapable foot electric shock of Shock only (Left, EGFP, n = 8 mice, hM4Di, n = 8 mice, hM3Dq, n = 7 mice) and Object group (Right, EGFP, n = 8 mice, hM4Di, n = 10 mice, hM3Dq, n = 8 mice). E. Representative behavioral events were assessed for the six groups before and after object exposure. Blue represents exploring object, green represents self-grooming and red represents burying. F. Burying time before and after object exposure during the burying test. G. Freezing time before and after object exposure during context reexposure test.
Error bars, Data represent mean ± SEM * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, One-way ANOVA.

grooming behavior in the Object group, although not significantly (Fig. S3A, right). Interestingly, activation or inhibition of ZI neurons had no significant effect on the time spent exploring objects in the Shock only and Object groups (Fig. S3B). In the Shock only group, ZI neuron activation reduced freezing time during the extinction phase (Fig. 4G, left). However, activation or inhibition of ZI neurons did not significantly impact freezing time in the Object groups (Fig. 4G, right). These findings suggest that chemogenetic activation of ZI neurons is sufficient to induce burying behavior in mice.

To determine whether changes in ZI neuronal activity alter anxiety-like behavior, anxiety-like behavior was assessed 2 weeks after re-exposure. In the Shock only group, chemogenetic inhibition of ZI neurons did not alter anxiety-like behavior (Fig. 5A–C). However, chemogenetic activation of ZI neurons significantly reduced immobile time and increased the number of times entering the center and the duration of stay in the center of the open field (Fig. 5A and B). In the EPM test, chemogenetic activation of ZI neurons significantly increased the number of entries and the distance traveled in the open arms (Fig. 5C). In the Object group, chemogenetic inhibition of ZI neurons reduced the time spent in the center of the open field as compared to the Control and Activation groups, while the retention time in the open arms of the EPM was significantly reduced (Fig. 5B and C). These data indicate that reducing ZI neuron activity increased anxiety-like behavior in the Object group, and increasing ZI neuron activity by chemogenetics decreased the anxiety-like behavior of the Shock only group.

3.4. Chemogenetic enhancement of the projection from the ZI to the vPAG normalized anxious behavior

Since c-Fos expression in the vPAG subregion was similar in the Object and Control groups, the potential involvement of the vPAG in inducing burying behavior and improving anxiety-like behavior was investigated. Injection of the rAAV-hsyn-EYFP virus into the ZI revealed abundant fiber projections from the ZI to the vPAG, indicating functional connectivity between these two brain regions (Fig. 6A). To further validate the function of the ZI-vPAG pathway, the vPAG was injected with AAV-retro-cre and the ZI with rAAV-DIO-hM3Dq-EYFP (Fig. 6B). After exposure to IFS, both groups of mice exhibited high levels of freezing behavior (Fig. S4D, top). Following 21 days of virus expression, clozapine N-oxide (5 mg/kg, i.p.) was administered to specifically activate the vPAG-projecting ZI neurons (Fig. 6C). The results showed that c-Fos expression was significantly increased in the ZI and decreased in the vPAG (Figs. S4A–C). To remove the restriction of the object, the subsequent burying behavior and extinction event were assessed without exposure to the object. During the burying test, burying time and self-grooming time were recorded (Fig. 6D). Interestingly, neither burying behavior nor self-grooming behavior was affected by chemogenetic activation of the ZI-vPAG pathway (Fig. 6E, Fig. S4D, bottom). However, during the extinction event, the hM3Dq group exhibited significantly reduced freezing time (Fig. 6F). Additionally, activation of the ZI-vPAG pathway significantly improved anxiety-like behavior in mice. Specifically, the center entry times in the open field and the open arms of the EPM were significantly higher in the hM3Dq group than the EYFP group (Fig. 6G, right, Fig. 6H). These results suggest that the ZI-vPAG pathway does not directly regulate burying behavior but plays a crucial role in fear extinction and improved anxiety-like behavior in mice.

4. Discussion

The aim of this study was to provide experimental evidence supporting the role of the ZI in anxious behavior. This is in response to recent clinical reports that suggest deep brain stimulation (DBS) of the ZI alleviates anxiety-like behavior associated with Parkinson's disease (PD) (Burrows et al., 2012; Gourisankar et al., 2018). A modified stress model was adopted to enhance anxiety-like behavior in mice, which increased

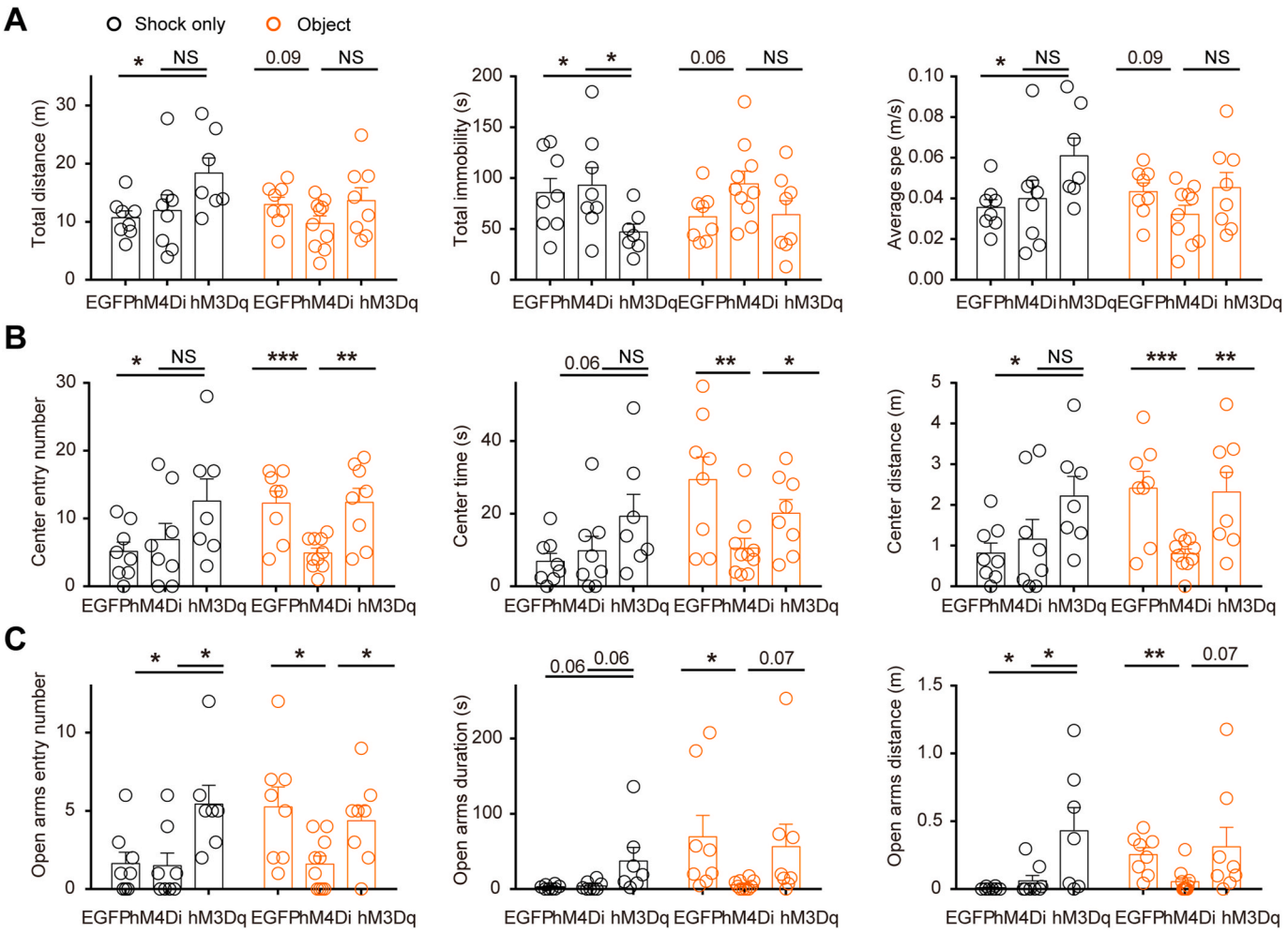

Fig. 5. Inhibition of ZI neuron activity by chemogenetics increased anxiety-like behavior in the Object mice. A. The six groups of mice movement related to anxiety was assessed based on total travel distance (left) and immobile time (middle) and motor ability based on the average speed (right).
B. The six groups of mice movements related to anxiety were assessed based on the number of entries into the center zone (left), time in the center zone (middle), and center distance(right).
C. The six groups of mice movement related to anxiety was assessed based on the number of entries into the open arms (left), time in the open arms (middle), and open arms travel distance (right).
Error bars, Data represent mean ± SEM, $*p < 0.05$, $**p < 0.01$ $***p < 0.001$, Two-way ANOVA.

defensive burying behavior towards object binding and promoted fear extinction. These findings further illustrate that the ZI plays a crucial role in regulating the burying behavior of mice. Chemogenetic activation of ZI neurons increased burying behavior and decreased anxiety-like behavior. We provide evidence that the regulatory effect of ZI is achieved through projections to the vPAG. Collectively, these results clarify a novel function of the ZI in regulation of anxiety disorders.

Burying behavior is a classic rodent behavior, commonly referred to as defensive burying with the marble burying test, where small objects, such as glass marbles or unfamiliar objects, are placed in a test cage to measure the number of marbles buried by the mice (Carter et al., 2020; Partridge et al., 2023). This method is generally used to measure anxiety levels, with a higher number of buried marbles indicating higher anxiety levels (Parveen et al., 2023). Later, marble burying was used as a model to mimic the repetitive and persistent behavior observed in obsessive-compulsive disorder (de Brouwer et al., 2019). Defensive burying refers to the act of burying unfamiliar or harmful objects to remove or avoid potential life-threatening dangers (De Boer and Koolhaas, 2003). The specificity of burying behavior is controversial. Burying behavior has been linked to lower stress hormone levels and is considered a positive coping strategy. However, the burying behavior of chronically stressed rats decreased and immobile time increased significantly (Fucich and Morilak, 2018). After chronic restraint stress, self-grooming behavior increased, whereas chemogenetic activation of the olfactory tubercle D3 neurons increased grooming to normalize depression-like behaviors induced by chronic restraint stress (Zhang et al., 2023). Consistent with previous results, spontaneous burying behavior was significantly higher after IFS than in the Control group (Fig. 1D). Object exposure was used to increase the induced burying behavior to normalize anxious behavior (Figs. 1D and 2A–C). Based on these findings, we propose that the effect of burying behavior is positive, similar to self-grooming behavior.

Chronic restraint stress can increase self-grooming behavior, but did not significantly change the spontaneous self-grooming behavior of mice exposed to IFS (Fig. S1A). This may be influenced by the modified mode and the detection time interval. Additionally, levels of self-grooming are not necessarily positively correlated with levels of anxiety. The burying behavior and anxiety levels in mice exhibiting high levels of self-grooming on the EPM were consistent with those exhibiting low levels of self-grooming (Reimer et al., 2015). PlexinA1 and Itgb3 knockout mice showed altered levels of self-grooming without affecting anxiety levels (Jahan et al., 2020; Lopuch et al., 2022). Our results demonstrated that the level of anxiety in the Object group was closely related to induced burying behavior (Fig. 2D and E left). Although both

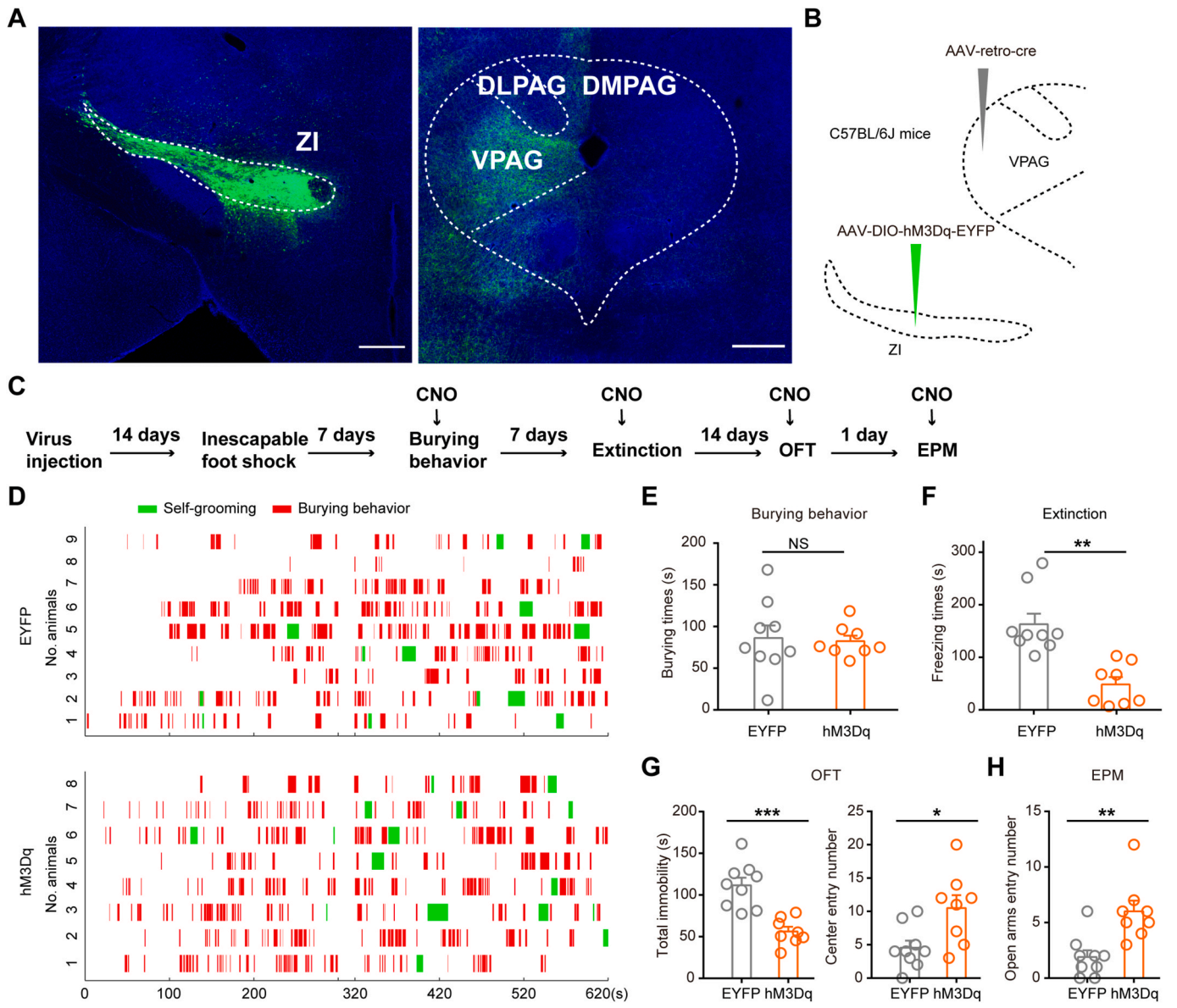

Fig. 6. Activation of the ZI-VPAG pathway normalized IFS-induced anxiety behavior. A. ZI sends projections to VPAG. Scale bar = 300 μm (left), scale bar = 200 μm (right).

B. Stereotaxic injection of AAVs into the ZI and VPAG.

C. Schematic diagram of the experimental design.

D. Representative behavioral events were assessed for the two groups during the burying test. Green represents self-grooming and red represents burying.

E. Total burying time during burying test.

F. Total freezing time during context reexposure test.

G. The two groups of mice movements related to anxiety were assessed based on immobile time (left) and the number of entries into the center zone (right).

H. The six groups of mice movements related to anxiety were assessed based on the number of entries into the open arms.

Error bars, Data represent mean ± SEM, $*p < 0.05$, $**p < 0.01$ $***p < 0.001$, T-test.

self-grooming and burying behavior are repetitive physical behaviors that are critical to emotional disorders in mice, burying behavior has been far less studied than self-grooming. Calcium signals of medial paralemniscal nucleus (MPL^{SST} neurons) are significantly increased during spontaneous and stress-induced self-grooming, and MPL^{sst} neurons project to the various regions of the brain (vPAG and the ventral tegmental, lateral hypothalamic, periventricular, and medial preoptic areas) to regulate self-grooming (Sun et al., 2022). The activity of glutamate-decarboxylase 2+ neurons in the interpeduncular nucleus decreased, while that of dopamine transporter + neurons in the ventral tegmental area were increased in stress-induced self-grooming behavior (Klenowski et al., 2023). Our results showed that ZI neuronal activity increased calcium signals during self-grooming and burying behavior (Fig. 3F bottom and 3F middle), while chemogenetic activation of ZI neurons improved spontaneous burying behavior (Fig. 4F), but did not change self-grooming behavior (Fig. S3A). Collectively, these findings suggest that burying behavior is key to normalizing IFS-induced anxiety-like behavior.

The marble burying test has been utilized to measure compulsive behavior in mice (Lazic, 2015), and furthermore, mice exhibiting Big nest-building (burying behavior), serve as a spontaneous animal model for obsessive-compulsive disorder (OCD) (Mitra et al., 2017). Surprisingly, compared to the SMALL strains, the BIG strains exhibit less anxiety-like behavior in the EPM test, which is analogous to the positive

correlation between the number of entries into the open arms and the time dedicated to burying behavior in the Object group (2E left). The highly GABAergic structure within the ZI is important to alleviating essential tremor (Lee et al., 2019), and ZI may also serve as a potential target area for deep brain stimulation in OCD (Haber et al., 2023). In two patients with PD who also have a long history of OCD, the implantation of DBS electrodes in the anteromedial subthalamic nucleus (STN) or between the anteromedial STN and ZI, and/or the anterior part of the ZI, not only improved Parkinsonian disability but also ameliorated obsessive-compulsive symptoms (Ricci et al., 2024). Our findings demonstrate that specifically suppressing the activity of ZI neurons in Object mice leads to a reduction in burying behavior (Fig. 4F right) and a concurrent increase in anxiety-like behaviors (Fig. 5). In summary, the ZI is a crucial brain region in the regulation of anxiety disorders and OCD.

The ZI contains a large number of inhibitory neurons. Stimulation of GABAergic cells in the ZI has been shown to inhibit fear generalization and enhance extinction learning (Chou et al., 2018). The ZI neurons of Object mice showed a significant increase in calcium release during object exploration (Fig. 3F), suggesting that these neurons may be responsive to the presence of harmful objects. This also confirmed again that ZI neurons participate in fear generalization. Additionally, targeted photogenetic stimulation of the ZI dopaminergic projection in the nucleus reuniens of the thalamus enhances extinction recall (Venkataraman et al., 2021). The central amygdala-ZI ^{parvalbumin} is involved in the acquisition of fear memories and the recall of remote fear memories (Zhou et al., 2018). ZI neurons play a key role in the regulation of burying behavior, but more precise specific subtypes of neurons need further exploration. Optogenetic activation and inhibition of the ZIr-vPAG pathway, respectively, reduce and enhance the sound-induced innate flight response and conditioned freezing response. We found that the ZI brain region mainly projects to the vPAG (Fig. 6A). However, there was no significant difference in burying behavior between chemogenetically activated ZI-vPAG mice and the Control group (Fig. 6E), suggesting that other brain regions downstream of the ZI regulate burying behavior. Notably, activation of the ZI brain region reduced c-Fos expression in the vPAG (Figs. S4A–C). Chemogenetic activation of the ZI-vPAG pathway significantly reduced freezing time during extinction events (Fig. 6F) and normalized anxiety-like behavior induced by IFS (Fig. 6G and H; Figs. S4E and F). Inducing burying behavior significantly increased c-Fos expression in the ZI brain region of mice (Fig. 3B). Overall, the results indicated that induced burying behavior improved anxiety-like behavior by increasing the activity of the ZI brain region and the ZI-vPAG pathway.

In conclusion, our research offers new insights into the pivotal roles and neural circuitry of ZI neurons in modulating burying behavior and alleviating anxiety induced by IFS. Increased activity in the ZI further regulates downstream activity in the vPAG, thereby normalizing IFS-induced anxiety-like behavior. This insight broadens our comprehension of the connectivity and influence of burying behavior-related neurons on those involved in emotional regulation within the mammalian brain, suggesting that repetitive physical behavior may provide humans with satisfaction and relief.

CRediT authorship contribution statement

Yueqin Liu: Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Lianli Qiu:** Writing – original draft, Methodology, Data curation, Conceptualization. **Jiahui Qian:** Writing – original draft, Visualization, Methodology, Data curation. **Qiang Xu:** Visualization, Methodology. **Rongfeng Qi:** Visualization, Investigation. **Yifeng Luo:** Supervision. **Zhihong Cao:** Supervision. **Zhiqiang Zhang:** Supervision. **Wei Wu:** Writing – review & editing, Visualization, Methodology, Conceptualization. **Longjiang Zhang:** Writing – review & editing, Supervision, Investigation. **Guangming Lu:** Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization.

Declaration of competing interest

There is no conflict of interest between the authors.

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Appendix A. Supplementary data

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