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A stress-activated neuronal ensemble in the supramammillary nucleus produces anxiety-like behavior in male mice

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eLife Assessment

This manuscript provides a **valuable** contribution by identifying a stress-responsive circuit and its regulation of anxiety-related behaviors. The evidence is **convincing** that the supramammillary nucleus contains stress-responsive neurons that increase anxiety-like behaviors when activated, and that ventral subiculum projections to the supramammillary are also activated by stress and their inhibition alleviates some effects of stress. Evidence that this pathway encodes and is functionally specific to anxiety is, at present, not sufficiently support and will require future studies. This work offers new insights into how distinct circuits are activated by stress and can regulate emotional behaviors and will be of interest to those interested in brain systems of aversive emotional and behavioral states.

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Abstract

Anxiety is a prevalent negative emotional state induced by stress; however, the neural mechanism underlying anxiety is still largely unknown. We used acute and chronic stress to induce anxiety and test anxiety-like behavior; immunostaining, multichannel extracellular electrophysiological recording and Ca^{2+} imaging to evaluate neuronal activity; and virus-based neuronal tracing to label circuits and manipulate circuitry activity. Here, we identified a hypothalamic region, the supramammillary nucleus (SuM), plays important role in anxiety-like behavior. We then characterized a small ensemble of stress-activated neurons (SANs) that are recruited by stress. These SANs respond specifically to stress, and their activation robustly increases anxiety-like behavior in male mice. We also found that ventral subiculum (vSub)-SuM projection but not dorsal subiculum (dSub)-SuM projections encode anxiety-like behavior and that inhibition of these vSub-SuM projections has an antianxiety effect. These results indicate that the reactivation of stress-activated supramammillary cells and relevant neural circuits are important neural processes underlying anxiety-like behavior.

Introduction

Anxiety is a fundamental negative emotion observed in almost all mammal species. Long-lasting and uncontrollable anxiety often leads to several mental disorders, anxiety disorders, and even depression[5]. Recent studies have shown that the supramammillary nucleus (SuM), a part of the hypothalamus, regulates sleep[6], memory[7, 8], novelty exploration[7, 9], social memory[10, 11], neurogenesis[12], consciousness[13], locomotor activity[14], and theta oscillations in the

hippocampus[15]. Projections from the SuM to the hippocampus have been largely studied and were found to modulate either episodic memory[7] or social memory[10, 11] in depending on the subregion of the hippocampus targeted. Although the SuM is located near the mammillary nucleus, a key region implicated in emotion regulation via the Papez circuit, its role in regulating emotion has been explored only superficially, without in-depth investigation. Despite some discussions of this role of the SuM, no consistent conclusion has been reached thus far[12, 16, 17].

Activity-dependent activation of cells have been studied in many brain areas[18, 19]. Tagged cells react to specific stimuli, such as conditional stimuli[20, 21], pain[22], food[23], and even peripheral inflammation[24], and mediate the storage and retrieval of relevant memories. The manipulation of those believed memory-associated cells can alleviate neurodegenerative diseases[25] or inflammation[24]. Naturally, this has led us to consider whether there is special neuronal ensemble that play roles in regulating anxiety or anxiety-like behaviors.Click or tap here to enter text. Recent studies have focused on the role of the hippocampus and related neuronal afferents and efferents[27–29]. The dorsal part of the hippocampus mainly contributes to cognition, whereas the ventral hippocampus is often associated with emotion[30, 31]. Although the ventral hippocampus-hypothalamus circuit was reported to modulate anxiety[27, 28], it is still unknown whether the SuM is part of this regulatory circuit. Although the SuM sends and receives dense neuronal projections, few studies have focused on its afferents or its ability to modulate behavior and emotion[32].

In this study, we hypothesize that stress can recruit a special neuronal ensemble that exclusively encodes anxiety. To test this hypothesis, we first used multiple methods to assess whether the SuM responds to acute or chronic stress. The activity of the SuM was chemogenetically manipulated, and anxiety-like behavior in rodents was tested. We subsequently employed the targeted recombination in active populations (TRAP) strategy to label and manipulate stress-activated neurons in the SuM in terms of anxiety-like behavior. After demonstrating how the SuM modulates anxiety, we sought to identify upstream brain areas that may contribute to supramammillary function. We also examined the functions of neuronal projections from the ventral subiculum (vSub) to the SuM using fiber photometry to measure calcium dynamics, as well as chemogenetic manipulation. These results allowed us to characterize a previously unreported role of SuM in regulating anxiety-like behavior. In addition, we showed that projections from the vSub but not the dorsal subiculum (dSub) to the SuM govern chronic stress-induced anxiety-like behaviors.

Results

1. Stress increases neuronal activity in the SuM

c-Fos protein expression was assessed after acute stress exposure to test whether the SuM was activated (Figure 1 A). The number of c-Fos$^+$ cells was significantly increased by foot shock exposure (Figure 1 B-C). To investigate if SuM would be responsive to diverse stressors, we next examined whether chronic stress, which different mechanism underlying, affects neuronal activity in the SuM (Figure 1 D-E). We chose in vivo electrophysiological extracellular recordings to reveal the neuronal activity before and after chronic social defeat stress, the data shows that the firing rate of regular-spiking neurons (RNs) (Figure 1 F) but not fast-spiking neurons (FNs) (Supplemental Figure 1 A-B) increased after CSDS. Regarding local field potentials, there were no noticeable difference between the naïve and CSDS groups according to power spectrum analysis (Supplemental Figure 1 C-D). These results indicate that acute and chronic stress can strongly activate the supramammillary nucleus.

2. Activation of SuM produces anxiety-like behavior

After confirming the activation of SuM caused different types of stress, we then further investigate whether the SuM regulates anxiety behavior in mice. Chemogenetic manipulations were conducted to activate SuM neurons. The experiments were performed as shown in the workflow (Figure 2 A-B). The mice were subjected to the open field (OF) and EZM tests at least 2 weeks

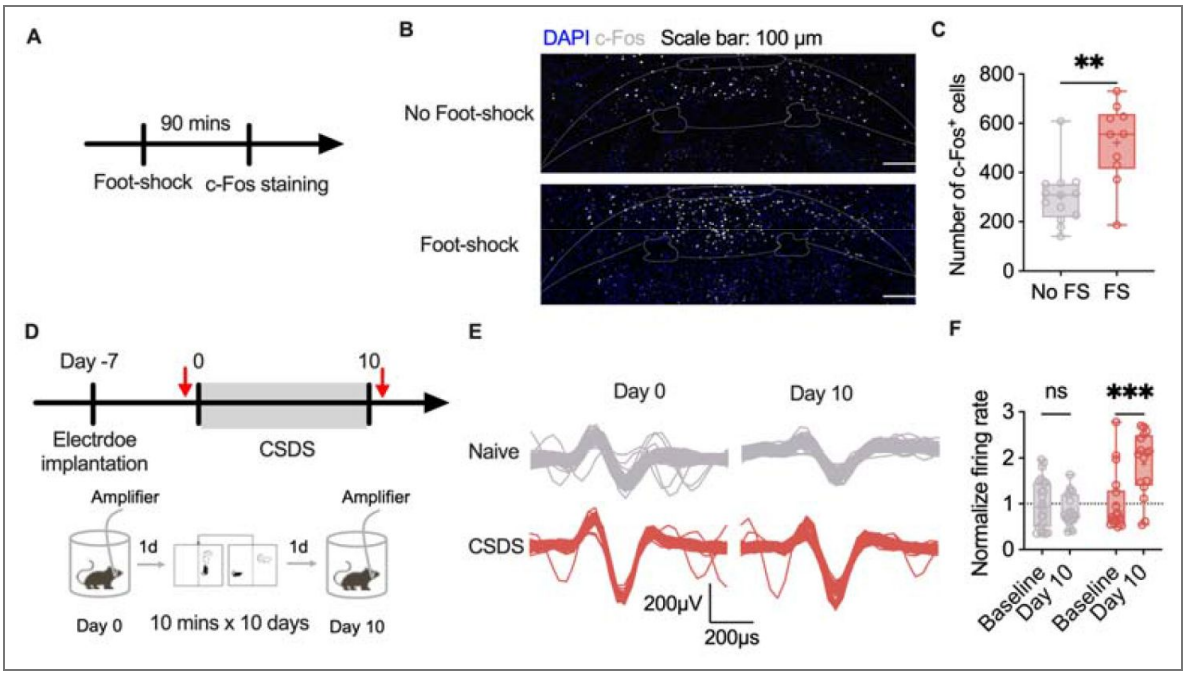

Figure 1. **Stress activates the SuM**

(A) Workflow of the c-Fos staining. **(B)** Representative images of c-Fos staining (DAPI: blue; c-Fos: white; scale bar: 100 μm). **(C)** Statistical analysis of the number of c-Fos-positive cells displayed in panel B. n = 10–13 per group; unpaired t test. **(D)** Workflow of CSDS exposure and in vivo recording. **(E)** Representative spikes acquired by multichannel recording. **(F)** Statistical analysis of the firing rates of RNs at baseline and after CSDS exposure. n = 16–23 per group; two-way ANOVA followed by Sidak's post hoc test. The bars in C and F indicate the Min to Max of all data point, and the "+" indicates mean value of all data point. "**", p <0.01; "***", p < 0.001. CSDS: chronic social stress; FS: foot shock. Also see Supplemental Figure 1.

after virus injection, followed by a reward-seeking test (Figure 2 E-H, Supplemental Figure 2 A). CNO was administered intraperitoneally 30 minutes before the test. Chemogenetic activation of the SuM did not affect the performance of mice in the OF test (Figure 2 E-F, Supplemental Figure 2 B). Compared with control mice, mice in which the SuM was activated explored the open arms of the EZM less (Figure 2 G), despite no change in distance traveled (Supplemental Figure 2 C). Moreover, mice in which the SuM was activated consumed less food than control mice did (Figure 2 F). These data suggest that there are neuronal ensembles that control the expression of anxiety behavior.

3. Identification of stress-activated neurons in the SuM

We next investigated whether an ensemble that encodes stress and controls the expression of anxiety exists. By crossbreeding Fos 2A-iCreERT2(TRAP2) and Rosa26-LSL-tdTomato (Ai14) mice, we generated TRAP2;Ai14 mice in which activated cells were genetically tagged for visualization (Figure 3 A). Foot shock exposure strongly activated neurons in the SuM but not adjacent areas (Figure 3 B-C). We hypothesized that these stress-activated neurons (SANs) respond exclusively to stress, but not to other stimuli such as reward. To validate the activity-dependent labeling in TRAP2;Ai14 mice, we labeled SANs in two cohorts of mice exposed either to the home cage condition or to foot shock. Several days after labeling, the mice were exposed to either sucrose pellets or social stress to induce reward-related or stress-related c-Fos expression, respectively. The reactivation of SANs under reward stimulation and stress was then compared (Figure 3 D-H). Foot shocks dramatically activated and labeled neurons in the SuM (Figure 3 F). Social stress but not reward (presentation of sucrose pellets) induced neuronal activation (Figure 3 G) and led to a much greater chance of reactivation of SANs (Figure 3 H). These data suggest the specific regulatory effect of the SuM on the effects of stress but not reward.

4. Reactivation of SuM^{SANs} promotes anxiety-like behavior

To make sure mice are on similar basal condition while applying chemo-genetic manipulation, we subjected mice to an acute stress protocol involving foot shocks and then performed the elevated plus maze (EPM) and elevated zero maze (EZM) tests to evaluate anxiety on days 2 and 7 (Figure 4 A). The mice that experienced foot shocks showed decreases in the exploration time in the open arms on day 2. However, acute stress-induced anxiety was not detected on day 7 (Figure 4 B), which allow us to compare the reactivation of SANs produced anxiety-like behavior between groups at the same baseline. Seven days after SANs tagging, specific activation of SANs significantly increased the concentration of corticosterone (a peripheral indicator of stress) in the mouse serum (Figure 4 C-D). Experiments involving chemogenetic manipulation also revealed the sound-selective activation of SANs in the SuM (Figure 4 E-F). We then tested whether manipulating SANs in the SuM influences the anxiety-like behavior of the mice. The mice were subjected to the OF and EZM tests at least 1 week after SANs were tagged, followed by reward-seeking tests (Figure 4 G-H). CNO was administered intraperitoneally 30 minutes before the test. Chemogenetic activation of SANs decreased the total distance traveled by the mice in the OF and EZM tests (Supplemental Figure 2 F-G). The mice also presented decreases in the distance traveled in the central area (Figure 4 I) and time spent in the central area in the OF test (Figure 4 J), time spent in the open arms in the EZM test (Figure 4 K) and food consumption (Figure 4 L). These data suggest that SANs in the SuM encode anxiety-like behavior.

5. vSub-SuM projections encode anxiety-like behavior

The SuM receives afferents from various brain areas, and we identified projections to the SuM by using a nonvirus- and virus-based retrograde tracing strategy (Figure 5 A, Supplemental Figure 3 A-C). Afferents from the dSub and vSub were identified using CTB-647 and AAV (Supplemental Figure 4 A-B). These projection neurons expressed Vglut1 RNA but not Vgat RNA (Figure 5 B), suggesting that Sub-SuM projections are excitatory neuronal projections, as Vglut1 is a crucial marker of glutamatergic neurons. We then performed an electrophysiological experiment. Optogenetics-evoked postsynaptic currents in SuM neurons were blocked by perfusion with DNQX,

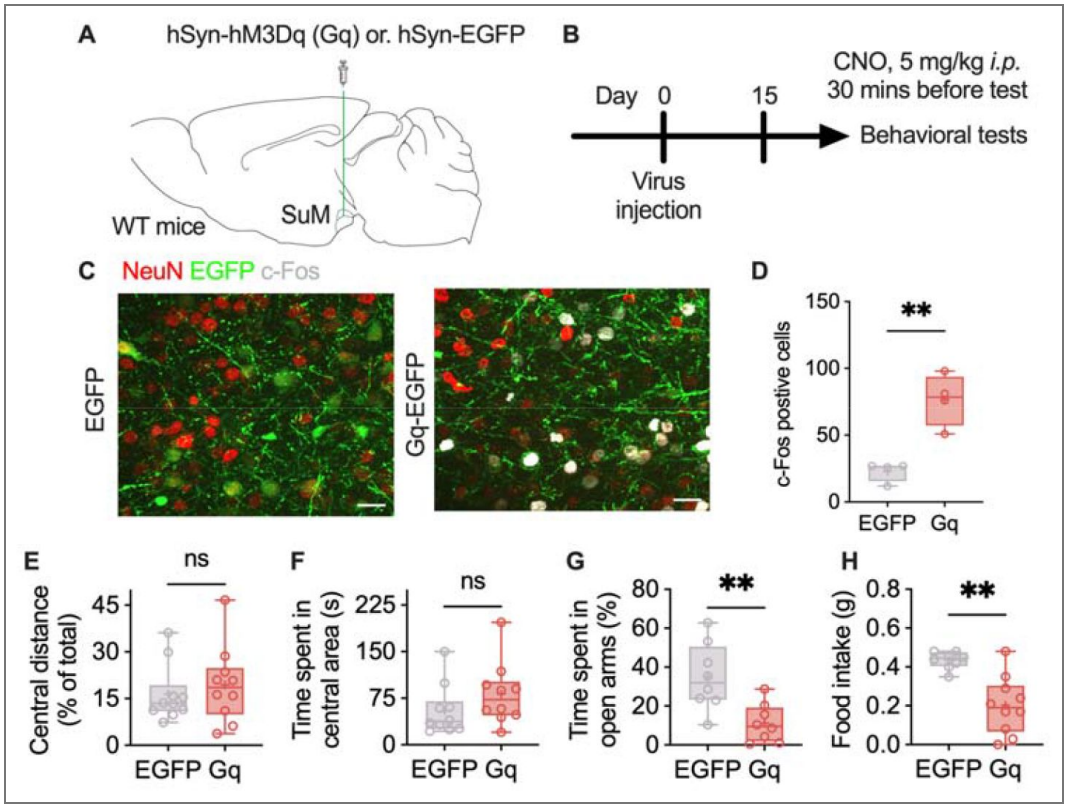

Figure 2. Activation of SuM produces anxiety-like behavior

(A-B) Virus injection information (A) and workflow for chemogenetic manipulation (B). **(C)** Representative c-Fos images. **(D)** Statistical analysis of the number of c-Fos-positive cells displayed in panel C. n = 4 per group; unpaired t test. **(E-F)** Statistical analysis of the distance traveled in the central area (E) and the time that the mice spent in the central area (F) in the OF test. n = 10 per group; unpaired t test for the data in (E) and the Mann-Whitney test for the data in (F). **(G)** Statistical analysis of the time that the mice spent in the open arms of the EZM. n = 8 per group; unpaired t test. **(H)** Statistical analysis of sucrose pellets consumed. n = 8–10 per group; Mann-Whitney test. The bars in D-H indicate the Min to Max of all data point, and the "+" indicates mean value of all data point. "ns", p >0.05; "**", p <0.01. Also see Supplemental Figure 2.

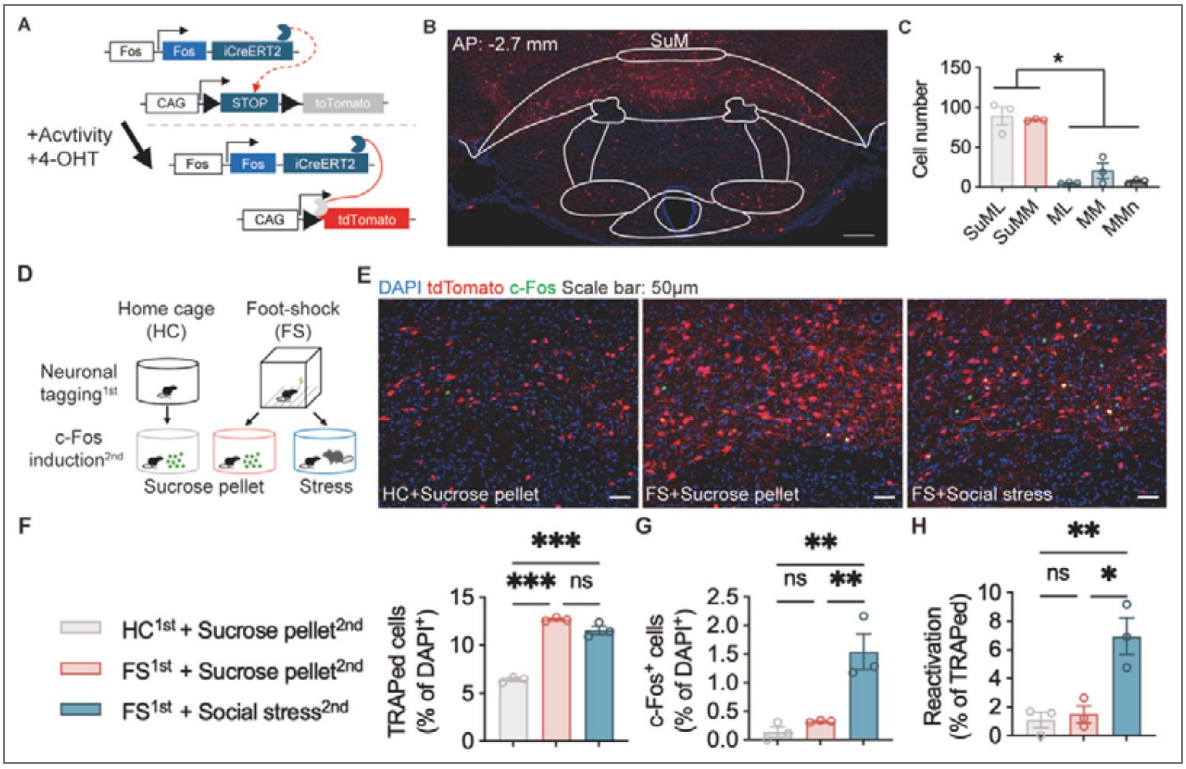

Figure 3. SANs in the SuM selectively respond to social stress but not reward

(A) Workflow of neuronal tagging. **(B)** Representative image of SANs in the SuM (DAPI: blue, tagged cells: red). **(C)** Quantitative statistics of stress-tagged cells in several brain areas. n = 3 per area; one-way ANOVA followed by Tukey'4s post hoc test. **(D)** Workflow of neuronal tagging and c-Fos staining. **(E)** Representative images of stress-tagged cells and c-Fos expression induced by sucrose and social stress (DAPI: blue, tdTomato: red, c-Fos: green). **(F)** Statistical analysis of the number of stress-tagged cells in the SuM. n = 3 per group; one-way ANOVA followed by Tukey's post hoc test. **(G)** Statistical analysis of the number of c-Fos$^+$ cells after sucrose or social stress exposure. n = 3 per group; one-way ANOVA followed by Tukey's post hoc test. **(H)** Statistical analysis of the reactivation of stress-tagged cells. n = 3 per group; one-way ANOVA followed by Tukey's post hoc test. The data in C and F-H are presented as the means ± SEMs. "ns", p >0.05; "*", p <0.05; "**", p <0.01; "***", p < 0.001.

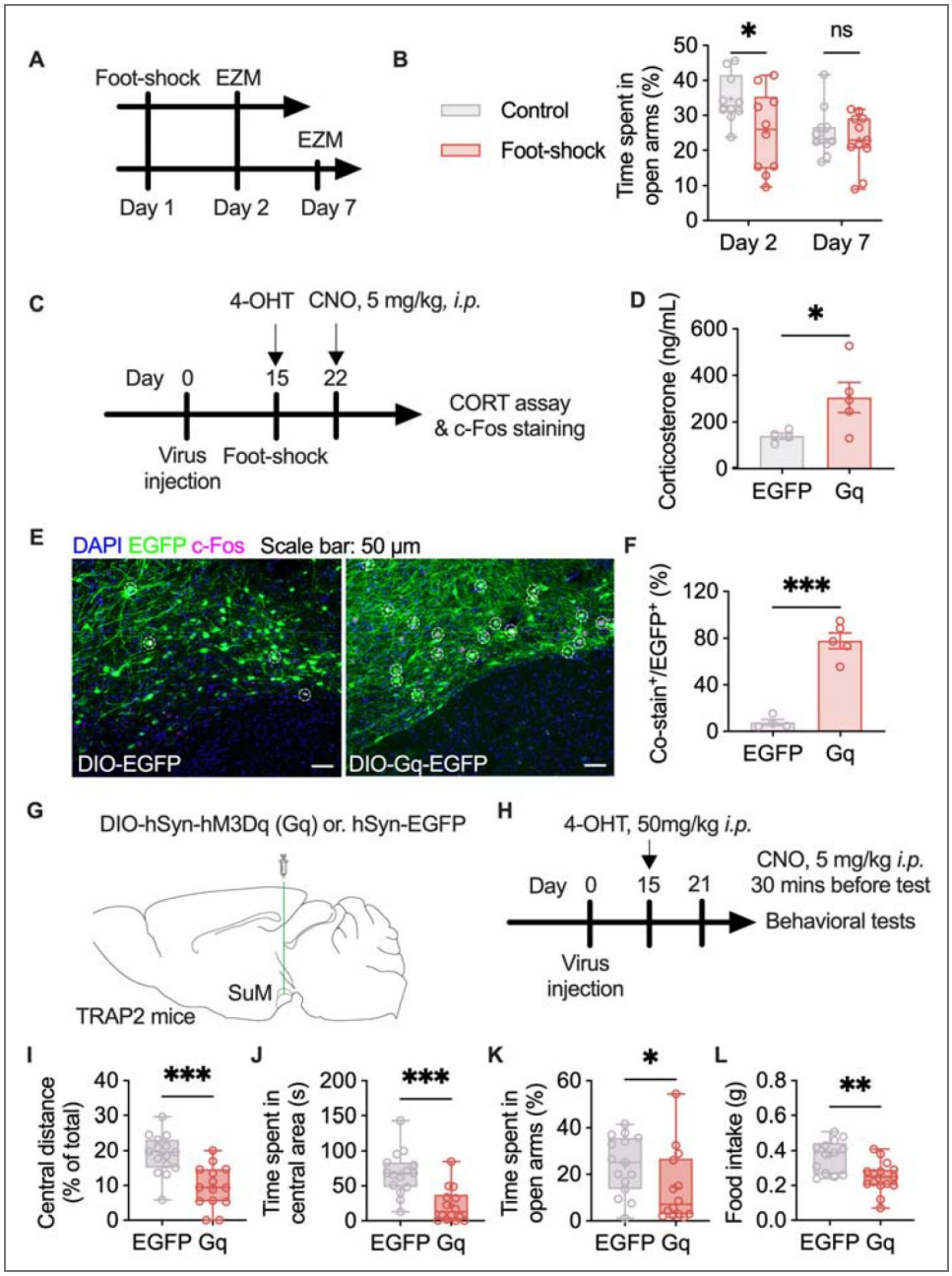

Figure 4. Selective chemogenetic activation of SANs elevates corticosterone level and produces anxiety-like behavior

(A) Workflow of the acute stress and anxiety tests. **(B)** Statistical analysis of the time that the mice spent in the open arms of the EZM. n = 10–11 per group; two-way ANOVA followed by Sidak post hoc test. **(C)** Workflow of the CORT assay and c-Fos staining. **(D)** Statistical analysis of the serum concentration of corticosterone after the application of CNO. n = 4-5 per group; unpaired t test. **(E)** Representative images of stress-tagged cells and c-Fos expression induced by chemogenetic manipulation (DAPI: blue, EGFP: green, c-Fos: violet). **(F)** Statistical analysis of the percentage of costained cells relative to $EGPF^+$ cells in the SuM. n = 4-5 per group; unpaired t test. **(G-H)** Virus injection information and workflow of chemogenetic manipulation. **(I-J)** Statistical analysis of the distance traveled in the central area (I) and the time that the mice spent in the central area (J) in the OF test. n = 14–15 per group; unpaired t test. **(K)** Statistical analysis of the time that the mice spent in the open arms of the EZM. n = 14–15 per group; Mann-Whitney test. **(L)** Statistical analysis of sucrose pellets consumed. n = 13–15 per group; unpaired t test. The data in D & F are presented as the means ± SEMs. The bars in B and I-L indicate the Min to Max of all data point, and the "+" indicates mean value of all data point. "*", p <0.05; "**", p <0.01; "***", p < 0.001. Also see Supplemental Figure 2.

which indicates the existence of glutamatergic projections from the Sub to the SuM (Figure 5 C-E). To investigate how Sub-SuM projections modulate stress and anxiety-like behavior, we then used fiber photometry to measure the calcium concentration to assess the activity patterns of the projection neurons (Figure 5 F-H). The projection neurons in the vSub but not those in the dSub were more strongly activated when the mice moved into the open arms from the closed arms of the EZM, (Figure 5 I-M). On the other hand, vSub but not dSub projection neurons show lower calcium activity while mice backing to the closed arms (Supplemental Figure 5 A-D). Following exposure to acute stress, both dSub-SuM and vSub-SuM projection neurons presented increased calcium activity (Figure 5 N-R). These data suggest that vSub-SuM projections, but not dSub-SuM projections, may participate in regulating anxiety-liker behavior.

6. Chronic inhibition of vSub-SuM projections alleviates anxiety-like behavior

After confirming the regulatory role of vSub-SuM projections in anxiety, we hypothesis that inhibition of this projection would alleviate chronic stress-induced anxiety. In the following experiments, the activity of these projections was chronically inhibited via a chemogenetic strategy. The mice were exposed to CSDS after the expression of the Gi protein was induced specifically on vSub-SuM projection neurons and their axons (Figure 6 A-B, D). The body weights of the mice were monitored throughout the entire procedure to assess their health (Figure 6 C). The mice showed no change in social interaction test scores after CSDS exposure (Figure 6 E). In the EZM test, the mice in which vSub-SuM projections were inhibited presented less anxiety-like behavior, as indicated by a longer time spent in the open arms of the EZM but no significant change in the distance traveled (Figure 6 F-G). Taken together, these data suggest that vSub-SuM projections are the essential neuronal projections for regulating chronic stress induced anxiety-like behavior.

Discussion

In this study, we combined multiple methods to determine whether the SuM is a brain region that involve in modulating anxiety. SuM neurons strongly respond to acute and chronic stress, and their activation results in robust increases in anxiety-like behavior in mice. We then defined a small ensemble of neurons that are activated by stress, called SANs. These SANs specifically respond to stressful stimuli but not reward. Selective activation of SANs in the SuM increases the serum concentration of corticosterone and anxiety-like behavior in mice. The neuronal circuits that may be underlie the regulation of anxiety were also determined in this study. The subiculum sends glutamatergic projections to the SuM and can be activated by stress, whereas only the vSub has a potential effect on the transition to anxiety. We finally determined that inhibition of SANs in the vSub project to the SuM is sufficient to alleviate anxiety in mice after CSDS exposure.

Regulation of anxiety avoidance by the SuM

The SuM has been demonstrated to respond to novel environments, social stimulation[7, 33], and stress[34, 35]. Given these findings, we assume that the SuM may be activated by foot shocks, a quantifiable acute stressor used in animal studies. Consistent with this hypothesis, we found that the SuM robustly positively responds to both acute and chronic stress through observable increases in c-Fos expression and increases in the neuronal firing rate. The activation of the SuM was also demonstrated to be essential for maintaining arousal[6, 13]. Sensitization to stressful events and high arousal are often associated with anxiety[36]. Thus, our data strongly suggest that the SuM potentially modulates anxiety. To further confirm whether the SuM participates in anxiety regulation, we recorded neuronal action potentials via multichannel extracellular recording while the mice were moving in the EPM, a traditional type of maze used to test anxiety in rodents. The change in the neuronal firing frequency when mice transitioned from the open arms to the closed arms supports the idea that the SuM may somewhat modulate anxiety. We then manipulated neuronal activity in the SuM via a chemogenetic method and subjected the mice to the EZM test, an improved test for assessing anxiety in rodents. The decrease in the exploration

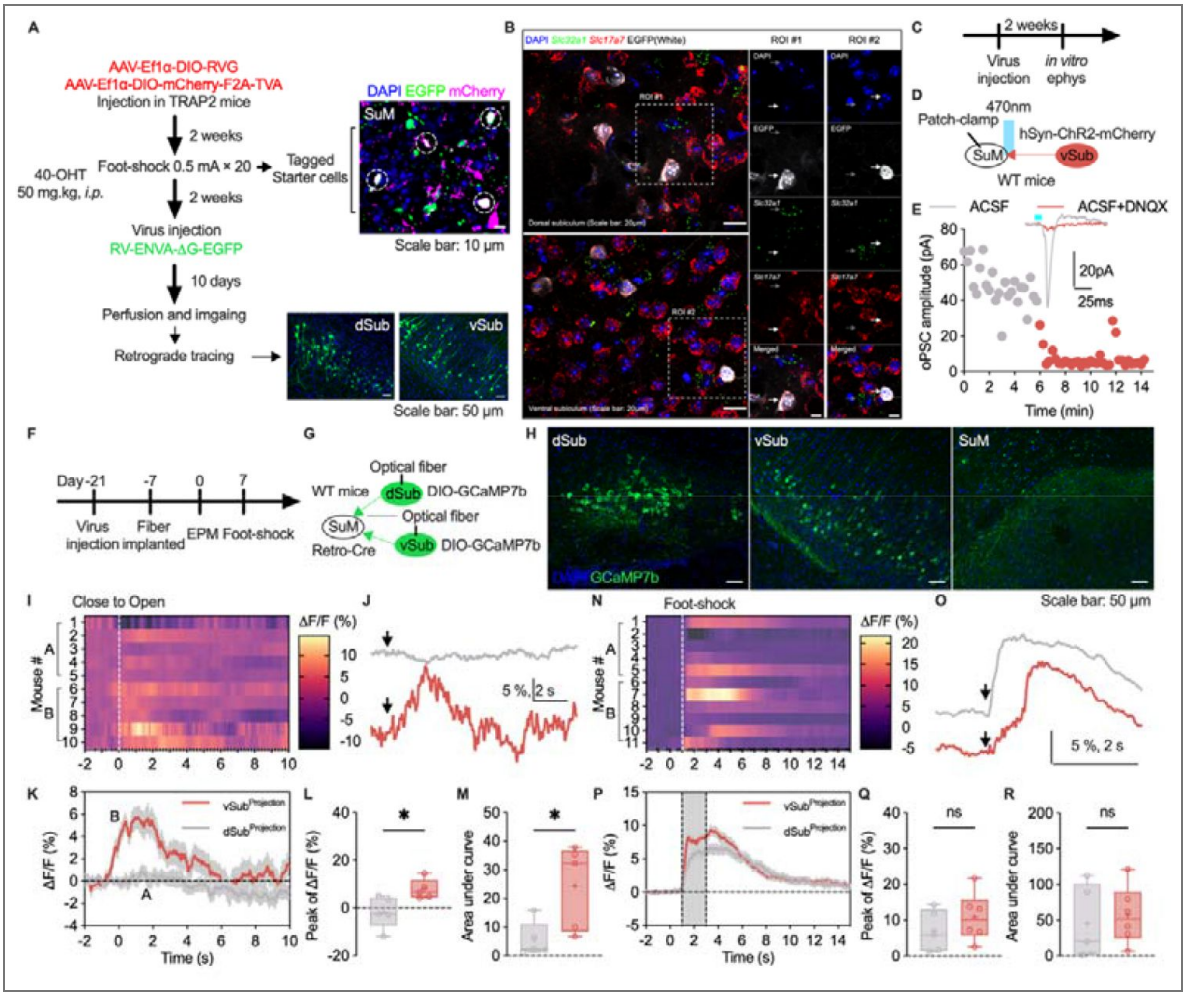

Figure 5. **vSub-SuM projections encoding anxiety-like behavior**

(A) Workflow of virus-based retrograde neuronal tracing. **(B)** Representative images of in situ RNA staining (DAPI: blue, Slc32a1: green, Slc17a7: red, EGFP: white). **(C)** Workflow of ex vivo electrophysiological recording. **(D)** Schematic of oPSCs in the SuM. **(E)** Representative traces of oPSCs. **(F)** Workflow of Ca^{2+} imaging. **(G)** Schematic of Ca^{2+} imaging of dSub and vSub projection neurons. **(H)** Representative images of GCaMP7b expression in the dSub, vSub and SuM (DAPI: blue, GCaMP7b: green). **(I)** Heatmap of the Ca^{2+} fluorescence intensity during the transition from the closed to the open arms. **(J)** Representative Ca^{2+} activity during the transition from the closed arms to the open arms. **(K)** Average ΔF/F of Ca^{2+} recorded in the dSub and vSub. **(L)** Statistical analysis of the peak Ca^{2+} activity. n = 5 per group; unpaired t test. **(M)** Statistical analysis of the area under the curve of Ca^{2+} activity. n = 5 per group; unpaired t test. **(N)** Heatmap of the Ca^{2+} fluorescence intensity during exposure to foot shocks. **(O)** Representative Ca^{2+} activity during exposure to foot shocks. **(P)** Average ΔF/F of Ca^{2+} recorded in the dSub and vSub. **(Q)** Statistical analysis of the peak Ca^{2+} activity. n = 5–6 per group; unpaired t test. **(R)** Statistical analysis of the area under the curve of Ca^{2+} activity. n = 5–6 per group; unpaired t test. The bars in L-M and Q-R indicate the Min to Max of all data point, and the "+" indicates mean value of all data point. "ns", p >0.05; "*", p <0.05. Also see Supplemental Figures 4 and 5.

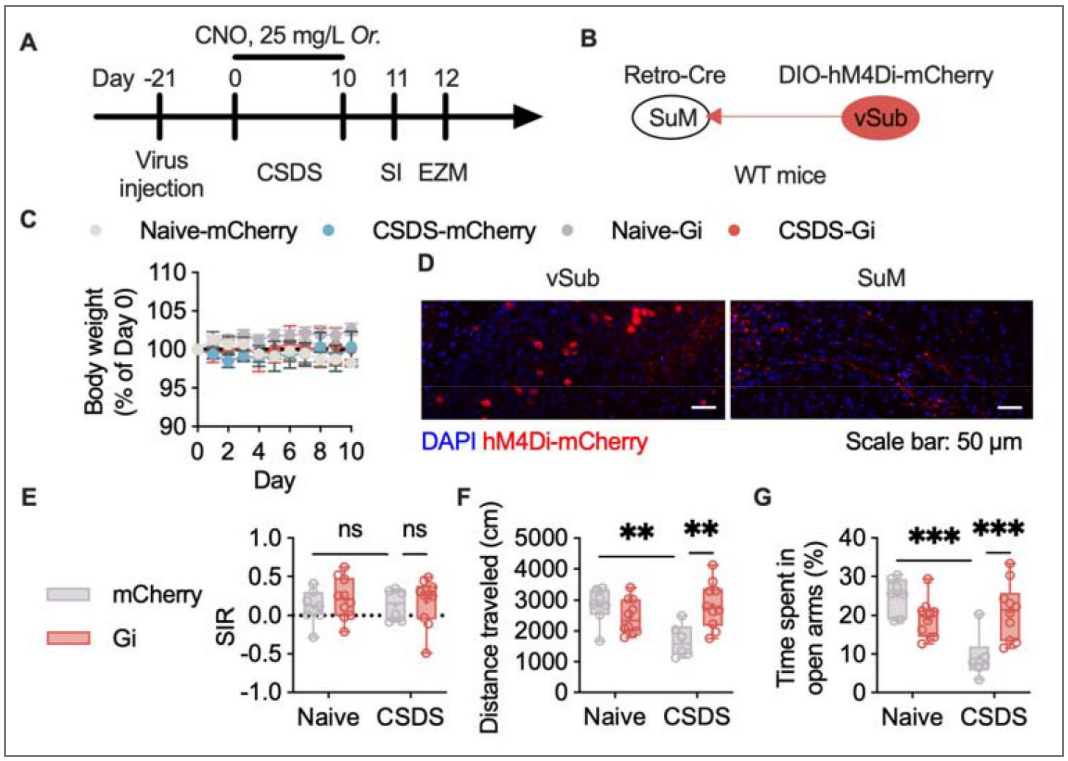

Figure 6. Selective inhibition of vSub-SuM projections alleviated CSDS-induced anxiety

(A) Workflow of CSDS and chemogenetic manipulation. **(B)** Schematic of chemogenetic manipulation of specific projections. **(C)** Body weight during CSDS exposure. **(D)** Representative images of virus expression. **(E)** Statistical analysis of the social interaction ratio after CSDS exposure. n = 6-10 per group; two-way ANOVA followed by Sidak's post hoc test. **(F)** Statistical analysis of the distance that the mice traveled in the EZM. n = 6-10 per group; two-way ANOVA followed by Sidak's post hoc test. **(G)** Statistical analysis of the time that the mice spent in the open arms of the EZM; two-way ANOVA followed by Sidak post hoc test. The bars in E-G indicate the Min to Max of all data point, and the "+" indicates mean value of all data point. "ns", p >0.05; "*", p <0.05; "**", p <0.01; "***", p < 0.001.

time in the open arms by mice in which the SuM was activated by hM3Dq indicated increased anxiety. We noted that these results are inconsistent with those of some previous reports. Some studies have reported that lesions in the SuM and adjacent areas decrease anxiogenic behavior in rats[15, 37, 38]. López-Ferreras et al. performed the OF test, a complicated test, and reported that the chemogenetic activation of neurons in the SuM results in increased anxiety-like behaviors in rats[16, 17]. However, further experiments involving specific test (e.g., the EZM test) are needed to confirm whether there is a potential difference across species.

The role of stress-activated neurons in regulating anxiety avoidance

Recent studies have highlighted the importance of activity-tagged neuronal ensembles in regulating various behaviors, particularly memory[20, 39–41], food consumption[22], the inflammatory response[24] and emotion[26, 42]. A negative experience-related neuronal ensemble in the hippocampus was found to increase susceptibility to chronic stress[26]. A recent study reported that the lateral habenula contains a small population of neurons that are recruited in response to stress and mediate the development of depression in mice[42]. These studies suggest that SANs may be important for emotional regulation. In this study, we found that the SuM was more strongly activated by acute stress than was adjacent areas. SANs were more likely to be reactivated by social stress than by sucrose reward, indicating their potential to specifically encode anxiety. The serum corticosterone concentration can be used as a marker of stress-induced change in the peripheral blood. Previous studies showed serum corticosterone can be increased by various stress stimulation[39–42]Click or tap here to enter text.; meanwhile, intentionally supplementing the diet with corticosterone can induce anxiety-like behaviors in rodents[43]. Our data showed that the chemogenetic activation of SANs in the SuM increased the serum corticosterone concentration, whereas the inactivation of SANs had no effect, suggesting the chemogenetic manipulation of SANs may cause similar anxiety effect like real stressors. These findings in combination with the results of the OF and EZM suggest that SANs in the SuM are more likely involved in modulating anxiety-like avoidance. However, the reactivation rate of SANs caused by different stressor was relatively lower than the initial activation rate caused by foot shock (Figure 3). This suggests that stress-activated neuronal clusters may have more flexible recruitment principles, with only a small number of neurons potentially encoding emotional information, while most other neurons remain involved in encoding other neural activities. Studies in other field, particularly studies of memory engram, has shown that the sets of neurons activated during learning are dynamic and exhibit high flexibility[44, 45]. While the activation of SANs produced anxiety-like behavior, the future study will examine whether silencing SuM SANs, either during stress exposure or during anxiety testing, can prevent or reduce stress-induced anxiety. We also found that both the nonselective activation of SuM neurons and the selective activation of SANs in the SuM significantly suppressed the consumption of sucrose pellets. This result may be attributed to the anxiety-induced suppression of reward seeking[43]. However, further experiments are still needed to confirm whether this effect is anxiety dependent and whether basal food consumption is affected.

A relevant neural circuit that regulates anxiety avoidance

The SuM recruits and is targeted by neuronal projections in the hippocampus, medial septum, and cortex[32]. To further understand the circuitry through which the SuM regulates anxiety, we identified projections from the dSub and the vSub to the SuM[46]. Fiber photometry was used to measure the Ca^{2+} concentration in projection neurons in the dSub and vSub, and the results revealed increased Ca^{2+} activity in vSub-SuM projection neurons but not dSub-SuM projection neurons when the mice transited from the closed arms to the open arms, indicating that vSub-SuM projections encode anxiety. To confirm the regulation of anxiety by vSub-SuM projections, we exposed mice to CSDS and found that constant inhibition of vSub-SuM activity significantly abolished CSDS-induced anxiety in mice. Unlike the dorsal hippocampus, which is involved in the regulation of cognition, the ventral hippocampus is often involved in regulating emotion[47]. The

ventral CA1 area and its projections to the lateral hypothalamic area were found to mediate innate anxiety, and its activation increases anxiety-like behavior in mice[28]. Although very close spatially, neurons in the subiculum are somewhat different from those in the CA1 region[48, 49]. The vSub and its downstream brain areas were found to regulate anxiety[50, 51]. Jing-Jing et al. reported that the vSub and its projections to the anterior hypothalamic nucleus are essential for anxiety because the inhibition of these projections decreases anxiety-like behavior[27]. Our data are consistent with these findings and suggest the mediating role of the vSub and its projections to different subareas in the hypothalamus.

In summary, the activation of SuM increases anxiety-like behavior. Stressful event recruits a neuronal ensemble in SuM. The activation of SANs also significantly increases anxiety-like behavior and suppresses reward seeking. SuM receives glutamatergic projections from the vSub, and inhibition of these projections can diminish CSDS-induced anxiety-like behavior. These results suggest that SuM plays important role in regulating anxiety-like behavior and furthermore studies are worthy performing.

Methods and Materials

Animals

Male C57BL6/J mice aged 12–20 weeks were used. $Fos^{2A\text{-}iCreERT2}$ (TRAP2) mice were a gift from Wenting Wang (JAX, cat. No. 030323). Rosa26-CAG-LSL-tdTomato (Ai14) mice were purchased from the Shanghai Model Organisms Center (cat. No. NM-KI-225042). Male CD-1 mice aged 8–10 months were purchased from Charles River (cat. No. 201). To construct TRAP2;Ai14 mice, homozygous male TRAP2 mice and homozygous female Ai14 mice were bred. Homozygous TRAP2;Ai14 mice were maintained and used in the experiments. We performed genotyping for TRAP2 and Ai14 via PCR with the following primers: TRAP2 (wild-type: 357 bp, mutant: 232 bp): wild-type forward: GT CCGGTTCCTTCTATGCAG, mutant forward: CCTTGCAAAAGTATTACATCACG, common: GAACCTTCGA GGGAAGACG; Ai14 (wild-type: 297 bp, mutant: 196 bp): wild-type forward: AAGGGAGCTGCAGTGG AGTA, wild-type reverse: CCGAAAATCTGTGGGAAGTC, mutant forward: GGCATTAAAGCAGCGTATC C, mutant reverse: CTGTTCCTGTACGGCATGG.

The mice were housed 4–5 per cage at a constant temperature and humidity (22 ± 1°C, 30–40% RH)on a day–night cycle (lights on from 08:00–20:00) with a fixed-intensity light source. Each mouse was acclimated to the testing environment for 1–2 min. Acclimation was performed for three days before the behavioral experiments. The mice were fed ad libitum and euthanized with CO_2 after all the tests were finished. The experimental protocols described here were approved by the Animal Ethics Committee of Shaanxi Normal University.

Behavioral procedure

Acute stress exposure

The mice were exposed to acute stress according to a previously reported procedure[52]; specifically, they were exposed to 20 foot shocks with an intensity of 0.5 mA that were randomly delivered across 10 minutes. The foot shocks were delivered in a fear conditioning box (Med-associates).

Chronic stress exposure

We used a CSDS protocol to induce anxiety and depression in the mice[53]. Each C57BL6/J mouse was housed with one CD-1 mouse, and the mice were separated by a transparent plexiglass board with several small holes. The mice were allowed to contact each other directly for 10 minutes every day for 10 days. Body weight was measured and recorded every day before contact.

OF test

The OF test was carried out in a 50 × 50 × 35 cm arena made of white plexiglass. The mice were allowed to move freely in the arena for 10 minutes, and the distance the mice traveled and the time the mice spent in the central area were recorded and analyzed.

EPM test

The EPM consisted of two open arms (30 × 7 cm), two closed arms (30 × 7 × 14 cm) and a central area (7 × 7 cm). The mice were allowed to move freely in the arena for 10 min, and the time the mice spent in the open arms was recorded and analyzed.

EZM test

The EZM was used to test whether the mice were anxious. The EZM used in this study was made of organic glass (height of 60 cm), with an inner diameter of 51.8 cm and an outer diameter of 65 cm. The closed arms of the EZM were separated by two 15 cm-high pieces of organic glass, the outer one of which was opaque. After a 15-minute habituation period, the mice were placed into the EZM and allowed to move freely for 10 minutes. Videos were recorded and analyzed using EthoVisionXT software. The time that the mice spent in the open arms was compared between the groups to evaluate anxiety-like behavior.

Reward seeking

On the first day, sucrose pellets were provided for habituation. The mice were then deprived of food on the second day. On the third day, the mice were placed in a new home cage without bedding and allowed to eat freely for 2 hours. The pellets were weighed to evaluate whether the experimental manipulation influenced reward seeking by the mice.

Social interaction test (SIT)

The SIT was conducted as previously described[53]. The SIT involved two 2.5-minute phases. In the first phase (no-target phase), we placed each C57BL6/J mouse in the periphery of the arena opposite the social interaction area (SIA). We allowed the animal to explore the arena freely. In the second phase (with-target phase), each C57BL6/J mouse was placed in the arena again, with a new CD-1 mouse in the SIA. The social interaction ratio (SIR) was calculated using the following formula:

$$Social\ interaction\ ratio\ (SIR) = \frac{Time\ in\ SIA^{With-target} - Time\ in\ SIA^{No-target}}{Time\ in\ SIA^{With-target} + Time\ in\ SIA^{No-target}}$$

Neuronal tagging of stress-activated neurons

To specifically label SANs, TRAP2 or TRAP2;Ai14 mice were intraperitoneally (i.p.) injected with 4-hydroxytamoxifen (4-OHT, 50 mg/kg) immediately after acute stress exposure or the learning phase of the CFC test. The mice were subjected to the next experiment or test after 7 days to allow Cre-dependent recombination.

4-OHT (CAS No. 68392-35-8. Sigma, cat. No. H6278 or Bidepharm, cat. No. BD00958757) was dissolved in DMSO at a concentration of 62.5 mg/mL and diluted with vehicle (containing 10% TWEEN-80 and 80% saline) on the day of neuronal tagging. The final concentration of DMSO was kept below 10% to avoid toxicity.

Observation of the reactivation of SANs

One week after neuronal tagging, whether previously tagged SANs were reactivated in TRAP2;Ai14 mice when they were subjected to social stress was assessed. The mice in the first group were underwent neuronal tagging in their home cages, and c-Fos expression was induced by sucrose pellets. The mice in the second group were subjected to neuronal tagging in response to foot shock exposure, and c-Fos expression was induced by sucrose pellets. The mice in the third group were subjected to neuronal tagging in response to foot shock exposure, and c-Fos expression was induced by social stress (one CD-1 mouse was placed in the home cage). The mice were then sacrificed 90 minutes after sucrose pellet feeding or social stress exposure, and c-Fos immunofluorescence staining was performed. The number of c-Fos-positive neurons was counted to determine whether reward and cross-strain social stress could activate neurons in the SuM. The likelihood of SAN reactivation was calculated using the following formula:

$$Reactivation\ chance\ (\%) = (c - Fos^{+}/DAPI^{+}) \times (tdTomato^{+}/DAPI^{+}) \times 100$$

Viral vectors

An adeno-associated virus (AAV) vector was used to label and manipulate specific neurons or determine the calcium concentration. To manipulate the neuronal activity in the SuM, AAV2/9-hSyn-hM3Dq-EGFP (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-0891) or its control vector AAV2/9-hSyn-EGFP (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-1990) was injected into the SuM of mice.

To manipulate the activity of SANs in the SuM, AAV2/9-hSyn-DIO-hM3Dq -EGFP (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-0891) or its control vector AAV2/9-hSyn-DIO-EGFP (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-1103) was injected into the SuM of TRAP2 mice.

To chronically inhibit vSub-SuM circuitry activity, AAV2/Retro-hSyn-Cre (titer: 2.00E+12 GC/mL, Taitool, cat. No. S0278) was injected into the SuM, and AAV2/9-hSyn-DIO-hM4Di-mCherry (titer: 2.00E+12 GC/mL, Taitool, cat. No. S0193) or its control vector AAV2/9-hSyn-DIO-mCherry (titer: 2.00E+12 GC/mL, Braincase, cat. No. BC-0025) was injected into the vSub of wild-type mice.

To determine the calcium concentration in dSub/vSub-SuM projection neurons, AAV2/Retro-hSyn-Cre (titer: 2.00E+12 GC/mL, Taitool, cat. No. S0278) was injected into the SuM, and AAV2/9-hSyn-DIO-GCaMP7b (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-2892) was injected into the dSub/vSub of wild-type mice.

For the ex vivo electrophysiological experiment, AAV2/9-hSyn-ChR2-mCherry (titer: 5.00E+12 GC/mL, BrainVTA, cat. No. PT-0150) was injected into the vSub of wild-type mice.

Stereotaxic surgery

The mice were anesthetized using isoflurane at a concentration of 1.5~2.0%. Virus was injected into the SuM (AP: -2.8, ML: 0, DV: -4.5 mm), dSub (AP: -2.8, ML: ±0.7, DV: -1.7 mm) or vSub (AP: -3.5, ML: ±3.0, DV: -4.6 mm) according to the experimental design. If only one type of virus needed to be injected into a single brain area, the final volume was typically 150 nL. Otherwise, the final volume of the virus mixture was 200 nL. The viruses were injected at a rate of 50 nL/min. The syringe was held in place for at least 5 minutes and carefully removed from the brain. The mice were then returned to their home cages, and their health was monitored on the following days. All the mice that underwent surgery were subjected to the subsequent experiment after 2 weeks or more to allow virus expression.

For fiber photometry, ceramic ferrules (outer diameter: 2.5 mm, core diameter: 0.2 mm, NA: 0.50) were inserted into the dSub (AP: -2.8, ML: ±0.7, DV: -1.5 mm) or vSub (AP: -3.5, ML: ±3.0, DV: -4.4 mm) 2 weeks after virus injection under the guidance of a laser (wavelength: 470 nm). Calcium imaging was conducted at least 1 week after ferrule implementation.

Fiber photometry

Commercially available equipment (Thinker Tech) was used to determine the calcium concentration. The fluorescence signal was activated by a laser at 470 nm, and the signal was transmitted through a low-autofluorescence fiber-optic patch cord and rotary (doric lenses) and collected. The final activation intensity was set to ~40 μW. The sampling rate was 50 Hz for all the recordings. The mice were habituated to the fiber-optic patch cord for 3 consecutive days before recording. A TTL lasting 0.1 s was delivered by the software to mark the timepoint when the mouse moved from a closed arm to an open arms in the EPM (USB-IO box, Noldus). Continuous data were stored as *.tdms files and analyzed using custom-made software in MATLAB.

Chemogenetic manipulation

To manipulate neuronal activity in the SuM in wild-type and TRAP2 mice, clozapine N-oxide (CNO, 5 mg/kg; Cayman, cat. No. 25780) was injected i.p. 30 minutes before behavioral tests were performed. For chronic inhibition of circuit activity, CNO was administered orally (25 mg/L).

For acute and chronic experiments, CNO was dissolved in DMSO at a concentration of 10 mg/mL and stored at -20°C or in saline at a concentration of 1 mg/mL and stored at -80°C. The storage solution was diluted with saline to a concentration of 0.75 mg/mL to prepare a working solution

for acute manipulation or to a concentration of 25 mg/L to prepare a working solution for chronic inhibition on the day of the experiment.

Immunofluorescence

The mice were anesthetized with 20% urethane and perfused with PBS or saline. The mouse brain was dissected and immersed in 4% paraformaldehyde (PFA) at 4°C overnight. The PFA solution was then replaced with a 30% sucrose solution. After the brain sank to the bottom, it was embedded in optimal cutting temperature (OCT) compound and frozen in a cryostat (CM1950, Leica). Coronal slices (40 μm) were cut and collected in a 24-well plate. After the residual OCT was removed with PBS, the slices were blocked with 0.3% Triton X-100 and 10% normal donkey serum at room temperature (RT) for 2 hours. The slices were then incubated with diluted primary antibody (rabbit anti-c-Fos, 1:500, Cell Signaling Technology, cat. No. 2250) at 4°C overnight. The next day, the slices were washed and incubated with secondary antibody dilutions (donkey anti-rabbit conjugated to AF647, 1:500, Jackson ImmunoResearch, cat. No. 706-605-148) at RT for 2 hours. After washing, the slices were transferred to slides and mounted with an antifade reagent (Thermo Fisher, cat. No. P36981). Images of the slices were collected using a Zeiss M2 microscope and then analyzed.

RNA fluorescence in-situ hybridization

The samples were processed as described in the *Immunofluorescence* section. Slices (10 μm thick) were cut and dried at RT for ~15 minutes and then heated at 37°C for 30 minutes in a hybridization oven. The baked slides were then moved to precooled 4% PFA solution for fixation (~15 minutes). The slices were dehydrated in 100% ethanol at RT for 5 minutes. The dehydration step was then repeated. The following steps were performed as recommended by the manufacturer (ACDbio, cat. No. 323100). To label vglut1, vglut2 and vgat RNA, the slices were hybridized with Mm-*Slc17a7* (ACDbio, cat. No. 416631-C1), Mm-*Slc17a6* (ACDbio, cat. No. 319171-C1) and Mm-*Slc32a1* (ACDbio, cat. No. 319191-C3), respectively. The samples were then stained with Opal dye.

Costaining of protein and RNA

After confirming RNA staining, the slices were blocked in 10% normal goat serum for 1 hour. The blocking solution was removed, and the slices were incubated with diluted primary antibody (mouse anti-GFP, 1:500, Thermo Fisher, cat. No. MA5-16256; rabbit anti-tdTomato, 1:500, Oasis BioFarm, cat. No. OB-PRB013) at 4°C overnight. The slides were washed with PBS and incubated with secondary antibody solution (goat anti-rabbit/mouse conjugated to HRP, Proteintech, cat. No. PR30009) at RT for 1 hour (in the dark). After washing, the slices were stained with Opal dye at RT for 30 minutes. The slides were then mounted and imaged.

Corticosterone assay

Mouse whole blood was collected 90 min after CNO injection (5 mg/kg, i.p.). The samples were subsequently centrifuged at 2000×g for 10 minutes at 4°C after being left to stand at RT for 30–60 minutes. The supernatant was then carefully collected as the serum. Corticosterone levels were then measured using a commercial ELISA kit (Beyotime, cat. No. PC100) according to the manufacturer's instructions.

Ex vivo electrophysiology

The mice were anesthetized with urethane and then decapitated. The brain was quickly removed from the skull and immersed in precooled sucrose-based cutting solution (in mM, 225 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 11 D-glucose, 5 L-ascorbic acid, 3 sodium pyruvate, 7 $MgSO_4{\cdot}7H_2O$, 0.5 $CaCl_2$). After being fixed on a metal pallet, the brain was cut into 300-μm slices. The slices were then collected and incubated in artificial cerebrospinal fluid (ACSF) containing (in mM) 122 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 11 D-glucose, 2 $MgSO_4{\cdot}7H_2O$, and 2 $CaCl_2$ equilibrated with 95% O_2-5% CO_2 at 28°C for at least 1 hour before recording.

To evoke postsynaptic currents (PSCs) using light, whole-cell recording of global SuM neurons near axons illuminated by ChR2-mCherry injected into the vSub was performed. The final light intensity at the end of the optical fiber was set to ~5 mW/mm^2. Then, blue light (470 nm, width: 10 ms, frequency: 0.05 Hz) was used to evoke optically induced PSCs (oPSCs). DNQX (20 μM) was perfused into the ACSF to isolate AMPA-dependent currents from the oPSCs after establishing a 5-minute baseline.

In vivo electrophysiology

The mice were anesthetized with 2% isoflurane and fixed to a stereotaxic device. A sixteen-channel microwire electrode array (KD-MWA, KedouBC), a 4x4 array of 25 μm NiTi wires spaced 200 μm apart, was slowly inserted into the mouse brain. Four small nails were first inserted into the skull, with a ground wire presoldered onto one of them. The electrode array was left in the SuM (AP: -2.8, ML: 0, DV: -4.55 mm), and then dental cement was used to fix it onto the skull.

The mice were introduced to the recording area at least one week after surgery. During the day, the electrode array attached to the mouse skull was connected to the OpenEphys acquisition board through an Intan head stage. An OpenEphys GUI was used to visualize and save electrical signals. The mice were allowed to move freely inside a home cage-like arena for at least 20 minutes. Only data acquired during the last 5 min were saved and then analyzed via Python-based software and a customized Python script.

Spikes were detected and divided into single units using SpikeInterface (*https://github.com/SpikeInterface/spikeinterface*). Continuous binary raw data (sampling rate: 30 kHz) were imported and filtered using a bandpass butter filter at a cutoff value of 300 Hz. Movement artifacts were removed by subtracting medians across all channels. The templates were then extracted and fitted using spyking-circus2 inside the SpikeInterface frame. Neurons meeting the following criteria were excluded from the subsequent analysis: (1) spikes with refracting period violations smaller than 1 ms, accounting for more than 2% of total spikes, and (2) a total frequency lower than 0.2 Hz. Neurons with spike frequencies ≥ 10 Hz were considered RNs, whereas those with spike frequencies < 10 Hz were considered FNs, as reported in a previous study[7]. The local field potential was extracted and analyzed using the power spectrum analysis tool in MATLAB.

Statistical analysis

The data are presented as the means ± SEMs in all the figures in this manuscript. For normally distributed data with equal standard deviations, independent t tests for unpaired data and dependent t tests for paired data were performed in GraphPad software to compare mean values between two groups. Otherwise, the Mann-Whitney test for unpaired data and the Wilcoxon test for paired data were performed instead. One-way ANOVA followed by Tukey's post hoc test and two-way ANOVA followed by Sidak's post hoc test were performed to compare mean values among more than three groups. A p value less than 0.05 was considered to indicate a statistically significant difference between groups. "*" represents $p < 0.05$, "**" represents $p < 0.01$, and "***" represents $p < 0.001$. Detailed statistical information referring to specific figure can be found in Table 1.

Data availability

Data reported in this paper will be shared by the correspondence author upon request.

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Supplemental information

Additional information

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Peer reviews

Reviewer #1 (Public review):

In the revised manuscript, the authors refine their conclusions, narrow their interpretation, and add limited new analyses but have not added additional new data or made fundamental changes in the analyses of their data.

The central findings are that the SuM contains neurons that are activated by stressors (foot shock and social defeat). Chemogenetic activation of SuM and the neurons genetically tagged as active during foot shocks, which the authors define as Stress Activated Neurons, increases classic anxiety-like behaviors. The subiculum projects to the SuM, and terminals in the SuM from the ventral versus dorsal subiculum are differentially active during elevated plus-maze transitions. Chronic inhibition of vSub neurons that project to SuM mitigates CSDS-induced anxiety-like behaviors.

Due to limitations in the data and experimental design the findings are felt to remain incomplete. A central limitation is the discordance between the temporal resolution of the behavioral assays and the neural interventions used. This weakens support for the conclusions drawn about the causal roles the SuM and specific vSub projections to SuM (vSub→SuM) may play in anxiety and anxiety-like behaviors. The authors acknowledge this limitation but do not address it experimentally in the revised manuscript. Furthermore, the connection between chronic inhibition of vSub→SuM neurons for 10 days and the alleviation of CSDS-induced anxiety is incomplete. Separately, the use of foot shock and social defeat stressors in connection with SuM neurons, with limited exploration of the potential (or lack thereof) relation between the two groups, further limits the ability to draw conclusions from the data.

Although a number of interesting points are raised through the experiments the weakness noted will reduce the impact of the work in the field.

https://doi.org/10.7554/eLife.108593.2.sa3

Reviewer #2 (Public review):

This manuscript investigates the neural mechanisms of anxiety and identifies the supramammillary nucleus (SuM) as a critical hub in mediating anxiety-related behaviors. The authors describe a population of neurons in the SuM that are activated by acute and chronic stress. While their activity is not required for fear memory recall, reactivation of these neurons after chronic stress robustly increases anxiety-like behaviors as well as physiological stress markers. Circuit analysis further shows that these stress-activated neurons are driven by inputs from the ventral, but not dorsal, subiculum, and inhibition of this pathway exerts an anxiolytic effect.

The study provides an elegant integration of techniques linking stress, neuronal ensembles, and circuit function, advancing our understanding of the neural substrates of anxiety. A particularly notable point is the selective role of these stress-activated neurons in anxiety, but not in associative fear memory, highlighting functional distinctions between neural circuits underlying anxiety and fear.

The recruited neuronal population is activated by acute and chronic stress, though the overlap across stress exposures is partial, suggesting that further studies will be important to define how these neurons respond under other stressors and conditions.

Overall, this work identifies SuM stress-activated neurons and their ventral subiculum inputs as central elements of anxiety circuitry, providing a valuable framework for future studies and potential targeted interventions for stress-related disorders.

https://doi.org/10.7554/eLife.108593.2.sa2

Reviewer #3 (Public review):

Summary:

The authors aim to investigate the mechanisms of anxiety. The paper focuses on supramammillary nucleus (SuM) based on a fos screen and recordings showing that footshock and social defeat stress increases activity in this region. Using activity-dependent tagging, they show that reactivation of stress-activated neurons in SuM has an anxiety-like effect, reducing open-arm exploration in the elevated zero task. They then investigate the ventral subiculum as a potential source of anxiety-related information for SuM. They show that ventral subiculum (vSub) inputs to SuM are more strongly activated than dSub when mice explore open arms of the elevated zero. Finally, they show that DREADD-mediated inhibition of vSub-SuM projections alleviates stress-enhanced anxiety. Overall the results provide good evidence that SuM contains a stress-activated neuronal population whose later activity increases anxiety-like behavior. It further provides evidence that vSub projects to SuM are activated by stress and their inhibition alleviates some effects of stress.

Strengths:

Strengths of this paper include the use of convergent methods (e.g., fos plus electrode recordings, footshock and social defeat) to demonstrate that the SuM is activated by different forms of stress. The activity-dependent tagging experiment shows that footshock-activated SuM neurons are reactivated by social defeat but not sucrose is also compelling because it

provides evidence that SuM neurons are driven by some integrative aspect of stress rather than by a simple sensory stimulus.

Weaknesses:

The strength of some evidence is judged to be incomplete. The paper provides good evidence that SuM contains stress-responsive neurons, and the activity of these neurons increases some measure of anxiety-like behavior. However, the evidence that the vSub-SuM projection "encodes anxiety" and that the SuM is a key regulator of anxiety is judged to be incomplete. I am not convinced that the identified SuM cells have a specific anxiety function. As the authors mention in the introduction, SuM regulates exploration and theta activity. Since theta potently regulates hippocampal function, there is the concern that SuM manipulations could have broad effects beyond anxiety-like behavior.

https://doi.org/10.7554/eLife.108593.2.sa1

Author Response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Weaknesses*
>
> *As presented, the manuscript has limitations that weaken support for the central conclusions drawn by the authors. Many of the findings align with prior work on this topic, but do not extend those findings substantially.*
>
> *An overarching limitation is the lack of temporal resolution in the manipulations relative to the behavioral assays. This is particularly important for anxiety-like behaviors, as antecedent exposures can alter performance. In the open field and elevated zero maze assays, testing occurred 30 minutes after CNO injection. During much of this interval, the targeted neurons were likely active, making it difficult to determine whether observed behavioral changes were primary - resulting directly from SuM neuronal activity - or secondary, reflecting a stress-like state induced by prolonged activation of SuM and related circuits. This concern also applies to the chronic inhibition of ventral subiculum (vSub) neurons during 10 days of CSDS.*

We appreciate the reviewer's concern regarding the timing of CNO administration relative to behavioral testing. The 30-minute interval was selected according to some previous studies[1, 2]. This window ensures stable and specific neuronal manipulation while minimizing off-target effects and was strictly performed through all experiments. We acknowledge that shorter interval (~15 mins) can be efficient to produce biological effect in vivo[3, 4]. We repeated chemogenetic tests 2-3 times to make sure to get reliable data for statistical analysis. However, we cannot exclude potential side-effects caused by chemogenetically prolonged activation of SuM because of its poor temporal resolution compared to optogenetic manipulation. We agree that employing techniques with higher temporal resolution, such as optogenetics, in future studies would provide an excellent complement to these findings.

> *The combination of stressors (foot shock and CSDS) and behavioral assays further complicates interpretation. The precise role of SuM neurons, including SANs, remains unclear. Both vSub and dSub neurons responded to foot shock, but only vSub neurons showed activity differences associated with open-arm transitions in the EZM.*

We agree that the use of multiple stressors (foot shock and CSDS) adds complexity to the interpretation. Our rationale was to test the generality of the SuM response and the role of SANs across different stress modalities (acute vs. chronic). The key finding is that while both vSub and dSub projections to the SuM were activated by the acute stressor of foot shock (Figure 5N-R), only the vSub-SuM pathway showed a significant increase in calcium activity specifically during the anxiety-provoking transition from the closed to the open arms of the EZM (Figure 5I-M). This dissociation suggests a selective role for the vSub-SuM circuit in encoding anxiety-related information, beyond a general response to stress.

> *In light of prior studies linking SuM to locomotion (Farrell et al., Science 2021; Escobedo et al., eLife 2024), the absence of analyses connecting subpopulations to locomotor changes weakens the claim that vSub neurons selectively encode anxiety. Because open- and closed-arm transitions are inherently tied to locomotor activity, locomotion must be carefully controlled to avoid confounding interpretations.*

We thank the reviewer for highlighting the important studies linking the SuM to locomotion. We acknowledge this known function and carefully considered it in our analyses. Non-selective activation of the entire SuM didn't affect total distance traveled in open field and elevated zero maze (Supplemental Figure 2 B-C). Although the locomotion of mice in OF and EZM was affected while targeting SANs, we also compared the travel distance in the central area of OF, to some extent, to minimize the influence of locomotion on the estimation of anxiety produced avoidance to the central area (Figure 4 I). We agree that future work delineating the specific subpopulations within the SuM that regulate locomotion versus anxiety would be highly valuable.

> *Another limitation is the narrow behavioral scope. Beyond open field and EZM, no additional assays were used to assess how SAN reactivation affects other behaviors. Without richer behavioral analyses, interpretations about fear engrams, freezing, or broader stress-related functions of SuM remain incomplete.*
>
> *In addition, small n values across several datasets reduce confidence in the strength of the conclusions.*

We acknowledge that the primary focus on OF and EZM tests is a limitation in fully characterizing the behavioral profile of SAN manipulation. These tests were selected as they are well-validated, standard assays for anxiety-like behavior in rodents[5–10]. However, we also included the reward-seeking test, where activation of SANs significantly suppressed sucrose consumption (Figure 4L), suggesting a broader impact on motivational state that is often linked to anxiety. We fully agree with the reviewer that employing a richer behavioral battery—such as tests for social avoidance, conditioned place aversion, or Pavlovian fear conditioning—in future studies will be essential to comprehensively define the functional scope of SuM SANs and to conclusively dissect their role from fear memory engrams.

> *Figure level concerns:*
>
> *(1) Figure 1: In Figure 1, the acute recruitment of SuM neurons by for shock is paired with changes in neural activity induced by social defeat stress. Although interesting, the connections of changes induced by a chronic stressor to Fos induction following acute foot shock are unclear and do not establish a baseline for the studies in Figure 3 on activation of SANs by social stressors.*

Thank you for this important comment. We agree that directly linking acute foot shock-induced cFos expression with chronic social defeat stress (CSDS) electrophysiological changes may create an interpretive gap. In Figure 1, we aimed to demonstrate that both acute (foot shock) and chronic (CSDS) stressors can activate SuM neurons, using complementary

methods (cFos for acute, in vivo recording for chronic). We did not intend to imply that the same neuronal population responds identically to both stressors.

To address this, we have clarified in the text that the purpose of Figure 1 is to show that SuM is responsive to diverse stressors, rather than to establish a direct mechanistic link between acute and chronic activation patterns. The baseline for SAN studies in Figure 3 is established through the TRAP2 tagging protocol following foot shock, independent of the CSDS model. We acknowledge that future studies should compare SAN recruitment across acute vs. chronic stressors to better define their functional overlap.

> *(2) Figure 2: The chemogenetic experiments using AAV-hSyn-Gq-DREADDs lack data or images, or hit maps showing viral spread across animals. This omission is critical given the small size of SuM, where viral spread directly determines which neurons are manipulated. Without this, it is difficult to interpret findings in the context of prior studies on SuM circuits involved in threats and rewards.*

Please see Supplemental Figure 2 for the infection area of AAV.

> *(3) Figure 3: The TRAP experiments show that the number of labeled neurons following foot shock (Figure 3F) is approximately double that of baseline home-cage animals, though y-axis scaling complicates interpretation. It is unclear whether this reflects true Fos induction, low TRAP efficiency, or baseline recombination.*

We thank the reviewer for pointing out the axis scaling issue. We have modified the y-axis to start from 0. The SuM nucleus has been reported to play role in the awake of rodents, it's reasonable to have some basal neuronal activation after 4-OHT i.p. injection.

> *Overlap analyses are also limited. For example, it is not shown what proportion of foot shock SANs are reactivated by subsequent foot shock. Comparisons of Fos induction after sucrose reward are also weakened by the very low Fos signal observed. If sucrose reward does not robustly induce Fos in SuM, its utility in distinguishing reward- versus stress-activated neurons is questionable. Thus, conclusions about overlap between SANs and socially stressed neurons remain uncertain due to the missing quantification of Fos+ populations.*

Thank you for the question. We have replaced the reactivation chance graph with a new reactivation percent analysis graph to show the proportion of SANs that reactivated by subsequent sucrose reward or stress. The rationale we use social stress other than foot shock is to show the potential generality of foot-shock tagged neurons. The lower expression of cFos after sucrose exposure suggest first, the SuM may not involve in reward regulation, which we agree with you; second, those SANs are more likely to modulate anxiety-like behavior but not reward.

> *(4) Supplemental Figure 3: The claim that "SANs in the SuM encode anxiety but not fear memory" is not well supported. Inhibition of SANs (Gi-DREADDs) did not alter freezing behavior, but the absence of change could reflect technical issues (e.g., insufficient TRAP efficiency, low expression of Gi-DREADDs). Moreover, the manuscript does not provide a positive control showing that SuM SANs inhibition alters anxiety-like behavior, making it difficult to interpret the negative result. Prior work (Escobedo et al., eLife 2024) suggests SuM neurons drive active responses, not freezing, raising further interpretive questions.*

We agree that here we didn't provide enough data to confirm there is no regulation effect of SuM-SANs on fear memory. Relevant statement has been removed to avoid any further misunderstanding.

> *(5) Figure 4: The statement that corticosterone concentration is "usually used to estimate whether an individual is anxious" (line 236) is an overstatement. Corticosterone*

> *fluctuates dynamically across the day and responds to a broad range of stimuli beyond anxiety.*

Thank you for your kind reminder. Corticosterone/cortisol, the primary stress hormone, is a well-established biomarker whose levels are elevated in response to stress and in anxiety states.[11, 12]. Some studies also reported that supplying corticosterone can produce anxiety-like behaviors in rodents[13–16]. We collect the blood sample at the same timepoint in Figure 4 C-D. We agree that line 236 is a kind of overstatement and has modified.

> *(6) Figures 5-6: The conclusion that vSub neurons encode anxiety-like behavior is not firmly supported. Data from photo-activating terminals in SuM is shown for ex vivo recording, but not in vivo behavior, which would strengthen support for this conclusion. Both vSub and dSub neurons responded to foot shock. The key evidence comes from apparent differential recruitment during open-arm exploration. However, the timing appears to lag arm entry, no data are provided for closed-arm entry, and there is heterogeneity across animals. These limitations reduce confidence in the authors' central claim regarding vSub-specific encoding of anxiety.*

We thank the reviewer for this important point. To address the concern regarding the in vivo behavioral encoding specificity of the vSub-SuM pathway, we further analyzed the in vivo fiber photometry data. The new analysis revealed that calcium activity in vSub-SuM projection neurons exhibited bidirectional, instantaneous, and specific changes during transitions between the open and closed arms of the elevated plus maze: their activity significantly and immediately decreased when mice moved from the open arm to the closed arm (new results shown in Supplemental Figure 5), and conversely, significantly and immediately increased upon transitioning from the closed to the open arm. However, under the same behavioral events, dSub-SuM projection neurons showed no significant change in activity. We hope this finding could strengthens the role of the vSub-SuM pathway in encoding anxiety-like behavior.

> *An appraisal of whether the authors achieved their aims, and whether the results support their conclusions:*
>
> *(1) From the data presented, the authors conclude that "the SuM is the critical brain region that regulates anxiety" (line 190). This interpretation appears overstated, as it downplays well-established contributions of other brain regions and does not place SuM's role within a broader network context. The data support that SuM neurons are recruited by foot shock and, to a lesser extent, by acute social stress. However, the alterations in activity of SuM subpopulations following chronic stress reported in Figure 1 remain largely unexplored, limiting insight into their functional relevance.*

Thank you for the suggestion. We have modified the line 190 with cautious “In this study, we combined multiple methods to determine whether the SuM is a brain region that involve in modulating anxiety.”

> *(2) The limited temporal resolution of DREADD-based manipulations leaves alternative explanations untested. For example, if SANs encode signals of threat, generalized stress, or nociception, then prolonged activation could indirectly alter behavior in the open field and EZM assays, rather than reflecting direct anxiety regulation.*

We discussed the DREADD method in the first part in our response.

> *(3) The conclusion that "SuM store information about stress but not memory" (line 240) is not fully supported, particularly with respect to possible roles in memory. The lack of a role in memory of events, as opposed to the output of threat or stress memory, may be true, but is functionally untested in presented experiments. The data do indicate activation of the SuM neuron by foot shock, which has been previously reported*

> *(Escobedo et al eLife 2024). The changes in SuM activity following chronic stress (Figure 1) are intriguing, but their relationship to "stress information storage" is not clearly established.*

Thank you for your valuable comments. Foot-shock-activated neurons may play role in modulate any of the following anxiety-like behaviors and emotional memory (fear memory). We realized that we didn't fully test all aspects of anxiety and memory, thus resulting in some overstatements in the manuscript. It is more proper to focus on "anxiety avoidance" according to the reduced open-arm exploration in EZM/EPM.

> ***Reviewer #2 (Public review):***
>
> *This manuscript investigates the neural mechanisms of anxiety and identifies the supramammillary nucleus (SuM) as a critical hub in mediating anxiety-related behaviors. The authors describe a population of neurons in the SuM that are activated by acute and chronic stress. While their activity is not required for fear memory recall, reactivation of these neurons after chronic stress robustly increases anxiety-like behaviors as well as physiological stress markers. Circuit analysis further shows that these stress-activated neurons are driven by inputs from the ventral, but not dorsal, subiculum, and inhibition of this pathway exerts an anxiolytic effect.*
>
> *The study provides an elegant integration of techniques to link stress, neuronal ensembles, and circuit function, thereby advancing our understanding of the neural substrates of anxiety. A particularly notable point is the selective role of these stress-activated neurons in anxiety, but not in associative fear memory, which highlights functional distinctions between neural circuits underlying anxiety and fear.*
>
> *Some aspects would benefit from clarification. For example, how selective is the recruitment of this population to stress compared with other aversive states, and how should one best interpret their definition as "stress-activated neurons" given the relatively modest overlap across stress exposures? In addition, the use of the term "engram" in this context raises conceptual questions. Is it appropriate to describe a neuronal ensemble encoding an emotional state as an engram, a term usually tied to specific memory recall?*
>
> *Overall, this work makes a valuable contribution by identifying SuM stress-activated neurons and their ventral subiculum inputs as central elements of the circuitry underlying anxiety. These findings provide a valuable framework for future studies investigating anxiety circuitry and may inform the development of targeted interventions for stress-related disorders.*

We thank the reviewer for raising these important points. We agree that further clarification is warranted. In our study, we compared SAN reactivation across different stimuli: foot shock (acute physical stress), social stress (chronic psychosocial stress), and sucrose reward (non-aversive positive stimulus). As shown in Figure 3, SANs in the supramammillary nucleus (SuM) were significantly reactivated by social stress but not by sucrose reward. Moreover, the c-Fos response in SuM was markedly higher after foot shock compared to home cage controls (Figure 1). While we did not test all possible aversive states (e.g., pain, sickness), our data support that SuM SANs are preferentially recruited by stressors rather than by reward or neutral conditions. We acknowledge that the overlap across stress modalities is not complete, which may reflect differences in stress intensity, duration, or circuit engagement. Future work will systematically compare SAN recruitment across diverse aversive and non-aversive states to further define their selectivity.

The term "stress-activated neurons" (SANs) here refers to neurons that are reliably activated by at least one type of stressor and can be reactivated by subsequent stress exposure. The

partial overlap across stressors likely reflects the diversity of stress responses and the possibility that distinct subpopulations within SuM may encode different aspects of aversive experience. Importantly, chemogenetic activation of SANs was sufficient to induce anxiety-like behavior and elevate corticosterone (Figure 4), supporting their functional role in stress-related behavioral and physiological outputs. We have revised the manuscript to clarify that SANs represent a stress-responsive ensemble rather than a uniform population activated identically by all stressors.

We appreciate the reviewer's conceptual caution. In the revised manuscript, we intentionally avoided using the term "engram" to describe SANs. Our focus is on a stress-activated neuronal ensemble that drives anxiety-like behavior, not on memory recall per se. We refer to SANs as an "ensemble" or "population" rather than an engram, consistent with the TRAP-based labeling approach used to capture neurons activated during a specific experience. We agree that "engram" is best reserved for memory-encoding cells and will ensure this distinction remains clear throughout the text.

> ***Reviewer #3 (Public review):***
>
> *Weaknesses:*
>
> *The strength of some of the evidence is judged to be incomplete. The paper provides good evidence that SuM contains stress-responsive neurons, and the activity of these neurons increases some measure of anxiety-like behavior. However, the evidence that the vSub-SuM projection "encodes anxiety" and that the SuM is a key regulator of anxiety is judged to be incomplete. The claim that SuM generates an "anxiety engram" is also judged to be incompletely supported by the evidence. Namely, what is unclear is whether these cells/regions encode anxiety per se versus modulate behaviors (like exploration) that tend to correlate with anxiety. Since many brain regions respond to footshock and other stressors, the response of SuM to these stimuli is not strong evidence for a role in anxiety. I am not convinced that the identified SuM cells have a specific anxiety function. As the authors mention in the introduction, SuM regulates exploration and theta activity. Since theta potently regulates hippocampal function, there is the concern that SuM manipulations could have broad effects. As shown in Supplementary Figure 2, stimulating stress-responsive cells in SuM potently reduces general locomotor exploration. This raises concerns that the manipulation could have broader effects that go beyond just changes in anxiety-like behavior. Furthermore, the meaning of an "anxiety engram" is unclear. Would this engram encode stress, the sense of a potential threat, or the behavioral response? A more developed analysis of the behavioral correlates of SuM activity and the behavioral effects of SuM manipulations could give insight into these questions.*

We appreciate the reviewer's thoughtful critique regarding the specificity of SuM's role in anxiety and the interpretation of our findings. We acknowledge that SuM has broad functions, including regulating exploration and hippocampal theta. However, our data show that general SuM activation increases anxiety-like measures (reduced open-arm time in EZM, decreased center exploration in OF) without altering total locomotion (Fig. 2, Suppl. Fig. 2). The locomotor reduction in SAN activation experiments (Suppl. Fig. 2F–G) was observed alongside clear anxiety-like behavioral changes (e.g. suppressed reward seeking), suggesting that the effects are not solely due to motor suppression. We agree that the methods we used to estimate anxiety-like behaviors base on mice movement when testing, and this could be a shortage of this research when trying to link the data to anxiety. Therefore it will be more proper to interpret the results as modulation of anxiety-like behavior (anxiety related avoidance) but not anxiety itself. We have modified the manuscript to describe more precise to avoid overstatement.

Our fiber photometry data (Fig. 5) show that vSub–SuM projection neurons increase activity specifically when mice enter open arms of the EZM—a behavioral transition associated with anxiety—whereas dSub–SuM projections do not. This activity correlates with anxiety-related behavior, not merely with movement or stress per se.

We also agree that the term “engram” may be misleading in this context. In the manuscript, we refer to SANs as a “stress-activated neuronal ensemble” rather than an anxiety engram. Our data indicate that these neurons are recruited by stress and their reactivation produces more anxiety related avoidance to open arms. We have revised the text to avoid conceptual overreach and to clarify that SuM SANs likely contribute to a state of sustained anxiety/avoidance.

> ***Recommendations for the authors:***
>
> ***Reviewing Editor Comments:***
>
> *Should you choose to revise your manuscript, if you have not already done so, please include full statistical reporting, including exact p-values wherever possible alongside the summary statistics (test statistic and df) and, where appropriate, 95% confidence intervals. These should be reported for all key questions and not only when the p-value is less than 0.05 in the main manuscript.*
>
> *Readers would also benefit from noting that the subjects were male in the abstract and discussion of the limitations of the exclusion of females.*

Thank you for the suggestion. We have included the full statistical detail in a separate sheet as Table 1. Also, we have modified the title of the manuscript to reflect the sex of the mice.

> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) In line 211, the authors state, "we recorded neuronal action potentials via multichannel extracellular recording while the mice were moving in the EPM, a traditional type of maze used to test anxiety in rodents,". However, it is unclear what data is presented in the paper, that is, extracellular recordings from SuM in mice on the elevated plus maze.*

We have deleted the description of multichannel recording data in EPM as the data was removed earlier.

> *Minor corrections to the text and figures.*
>
> *(2) For bar plots, perhaps clarify how the data is presented. For example, in Figure 4, "The data in B, D, E and I-L are presented as the means {plus minus} SEMs," but this does not appear to be plotted as a mean with SEM error bars because the error bars cover all the values.*

Corrected.

> *(3) In Figure 5, the white text for EGFP in panel B is very difficult to see.*

Corrected.

> *(4) For Figure 5D, it would be helpful to more clearly specify which neurons in SuM were recorded from. Was it SANs or all SuM neurons?*

We did whole-cell recording on all SuM neurons.

> *(5) Fos2A-iCreERT2 is mislabeled as "Fos2A-iCreERT" in the methods.*

Corrected.

> *(6) The sentence at line 139 "To make sure foot shock induced anxiety won't last until manipulation, we subjected139mice to an acute stress protocol involving foot shocks and then performed the elevated plus140maze (EPM) and elevated zero maze (EZM) tests to evaluate anxiety on days 2 and 7," is unclear as written.*

Thank you for pointing this. We have modified the sentence to make it more clear. “To make sure mice are on similar basal condition while applying chemo-genetic manipulation, we subjected mice to an acute stress protocol involving foot shocks and then performed the elevated plus maze (EPM) and elevated zero maze (EZM) tests to evaluate anxiety on days 2 and 7 (Figure 4 A). The mice that experienced foot shocks showed decreases in the exploration time in the open arms on day 2. However, acute stress-induced anxiety was not detected on day 7 (Figure 4 B), which allow us to compare the reactivation of SANs produced anxiety-like behavior between groups at the same baseline.”

> *(7) The details of the viral injections used for ex vivo electrophysiology are not sufficient to understand the experiment and the implications of the data. Which neurons (SANs?) are recorded from, what percent of those had inputs, were the sub-neurons globally labeled or just SANs?*

We performed whole-cell recording on global SuM neurons to show if the projection is innervated by glutamergic neurons in Sub as shown in Figure 5-B that the projection neurons in Sub are exclusively vglut1 expressed. Based on this aim of the experiment, we didn’t keep any neurons that were not response to the light stimulation, therefore can’t calculate the input percent in this case. We have added words to clearly show that we did global SuM neurons in Methods.

> *(8) The scale used in Figure 6C renders that data unreadable. 120 to 40% changes in body weight are well beyond the variability in the data.*

We have modified the axis (90 to 110%) to show the body weight change clearer.

> *(9) The dose of CNO used, 5 mg/kg, is high, and using lower doses or other DREADD ligands is worth considering.*

Thank you for your valuable comment. We have noticed that people are using relatively lower dose of CNO or other DREADD ligands that are reported much higher affinity and less side-effect. The dose of 5mg/kg was adapted from earlier papers that using DREADD and show no obvious side-effect in mice[17], e.g locomotion (S Figure 2B), in our experiments, so we keep using this dose in this project to make it consistent across different cohorts of experiments. We are switching to DCZ to avoid any potential side-effect of CNO in the following experiments based on this project.

> ***Reviewer #2 (Recommendations for the authors):***
>
> *This is a strong manuscript that provides important insights into the role of the supramammillary nucleus (SuM) and its inputs from the ventral subiculum in regulating anxiety. The combination of behavioral, imaging, electrophysiological, and circuit manipulation approaches is impressive, and the distinction the authors propose between anxiety-related and fear-related circuits is conceptually important.*

> *There are, however, some points that I think need clarification. The authors emphasize that the hippocampus is essential for fear memory recall, yet they do not directly evaluate whether the SuM-hippocampal pathway might contribute differentially to anxiety versus fear memory. Addressing this would help to explain where the dissociation between the two processes arises.*

Thank you for the suggestion. We realized that we didn't collect enough data to exclude the role of those SANs on memory, especially fear memory, a memory formation bases on strong emotional training as aforementioned. The data and relevant discussion have been removed to avoid misunderstanding and overstatement.

> *I am also not fully convinced about the definition of the "stress-activated neurons" (SANs). The overlap across repeated stress exposures is quite modest (around 20%), which suggests that this population may not be strictly stress-specific but rather a dynamic subset that is preferentially, though not exclusively, engaged by stress. Related to this, the use of the term "engram" raises conceptual questions. Since the classic engram refers to an ensemble encoding and recalling a specific memory, it is not obvious whether it is appropriate to apply the term to a neuronal population that appears to represent a persistent emotional state. The authors should consider justifying this choice of terminology more carefully or adopting a different term.*

Thank you for your important comments. Yes we agree that the SANs in this manuscript are more likely dynamic subset other than exclusive foot-stress engaged "engram". That's why we use "stress-activated neurons" but not "engram" to describe this neuronal ensemble. To avoid further misleading, we have made some modification to reduce the use of "engram" across the manuscript.

> *Some parts of the text also need more precision. For example, the statement in lines 63-65 that "few studies have explored emotion-related engram cells" is potentially misleading, as most engram studies focus on memories with a strong emotional component. The rationale for this claim should be clarified.*

This sentence has been deleted since it is not necessary to link the text and misleading.

> *In Figure 1, the choice of methods is also puzzling: cFos immunostaining is used after shock delivery, while electrophysiology is used for the CSDS paradigm. It would be helpful to explain why different readouts were chosen for different stress models, and whether this may affect the comparability of the results.*

Thank you for this important comment. In Figure 1, we aimed to demonstrate that both acute (foot shock) and chronic (CSDS) stressors can activate SuM neurons, using complementary methods (cFos for acute, in vivo recording for chronic). The reason we chose different method is that acute stress produces transit effect while chronic stress produces long-lasting effect. To our knowledge, cFos is a well-established marker for strong neuronal activation, but with short lifespan (~4-6 hours) and suits acute paradigm better. In vivo recording allows us to compare the neuronal activity before and after chronic experiments within subjects and has ability to reveal cumulative effect which cFos cannot. To address this, we have clarified in the text that the purpose of Figure 1 in Line 112-113: "To investigate if SuM would be responsive to diverse stressors, we next examined whether chronic stress, which different mechanism underlying…"

> *Finally, some additional details would strengthen the presentation. The discussion of corticosterone and other physiological markers could be expanded to indicate whether these effects were robust across stress paradigms. Similarly, the relatively modest overlap between SANs activated by different stressors could be framed more explicitly as part of a broader principle of flexible ensemble recruitment in anxiety-related circuits.*

Thank you for your suggestion. We have added more discussion about the corticosterone and the flexibility of SANs in the manuscript. See Line 267-270: “The serum corticosterone concentration can be used as a marker of stress-induced change in the peripheral blood. Previous studies showed serum corticosterone can be increased by various stress stimulation [39–42]; meanwhile, intentionally supplementing the diet with corticosterone can induce anxiety-like behaviors in rodents[43].” and Line 275-281: “However, the reactivation rate of SANs caused by different stressor was relatively lower than the initial activation rate caused by foot shock (Figure 3). This suggests that stress-activated neuronal clusters may have more flexible recruitment principles, with only a small number of neurons potentially encoding emotional information, while most other neurons remain involved in encoding other neural activities. Studies in other field, particularly studies of memory engram, has shown that the sets of neurons activated during learning are dynamic and exhibit high flexibility [44, 45].”

> *Overall, the work is of high quality and provides a valuable contribution to the field, but addressing these points would help sharpen the mechanistic claims and ensure that the conceptual framework is as clear and precise as the experimental data.*
>
> ***Reviewer #3 (Recommendations for the authors):***
>
> *(1) Since increased SuM activity is hypothesized to mediate the effects of stress on anxiety-like behavior, a logical step would be to test for necessity by silencing the stress-activated SuM cells.*

We agree this is a logical and valuable experiment. While our current study focused primarily on the sufficiency of SuM/SAN activation to induce anxiety-like behavior, we acknowledge that inhibition experiments would provide critical complementary evidence for necessity. We have added a statement in the Discussion noting that “future studies should examine whether silencing SuM SANs, either during stress exposure or during anxiety testing, can prevent or reduce stress-induced anxiety”. This will help establish a more complete causal role.

> *(2) Discuss what is meant by "anxiety engram" and what features of anxiety the labeled cells might encode.*

We concur that “stress-activated neuron (SAN)” is a more precise descriptor than “engram” in this context. We have revised the text to avoid the potentially misleading term “engram” and instead refer to a “stress-activated neuron”. The labeled cells are preferentially reactivated by stress (not reward), and their activation promotes both behavioral avoidance and physiological stress markers (corticosterone). They likely contribute to the maintenance of an anxious state under perceived threat, rather than encoding discrete threat cues or memories.

> *(3) A more nuanced analysis of behavioral correlates of SuM activity and/or the behavioral effects of SuM manipulations would strengthen this paper.*

To provide a more nuanced understanding of the behavioral correlates, we have performed additional analyses on our fiber photometry data (now presented in Supplemental Figure 6). and have also planned additional experiments for the future study to deepen our understanding.

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