**The Role of the Subthalamic Nucleus Zona Incerta in Distinct Behavioral**

**Phenotypes in a Rat Model of PTSD**

**SCIENTIFIC** **ABSTRACT**

This proposal investigates the role of the zona incerta (ZI), a subthalamic nucleus, in fear-related responses and behaviors associated with post-traumatic stress disorder (PTSD), expanding current neurobiological models of fear mechanisms. While the prefrontal cortex-amygdala-hippocampus circuit has been extensively studied for its role in exaggerated threat responses in PTSD, we hypothesize that the ZI, in conjunction with this circuit, plays a key role in processing interoceptive and exteroceptive signals related to PTSD.

Using chemogenetic tools such as DREADDs (designer receptors exclusively activated by designer drugs), we aim to uncover the molecular and anatomical foundations of ZI circuits influencing behavior. Our central hypothesis is that predator scent stress (PSS)-induced behavioral impairments—modeling avoidant and hyperarousal PTSD symptoms—are mediated by changes in ZI function, glucocorticoid receptor activity, and/or ZI projections affecting plasticity gene expression. We will test this through post-exposure behavioral assessments, chemogenetic manipulation of ZI activity in a region-, cell-, and projection-specific manner, and analysis of HPA-axis mediators and plasticity-related genes.

We further propose that ZI activation in response to stress initiates and maintains behavioral responses by engaging arousal, sensory, motor, visceromotor, and HPA-axis systems, and enhances neural plasticity to help the organism cope with acute threats and restore homeostasis. Different ZI neuronal subpopulations may stimulate circuits involved in adaptive stress responses.

Understanding the neural circuits involved in the generalization and extinction of traumatic fear memories is crucial, as PTSD affects millions globally, with current treatments often inadequate. Identifying pathways that drive the persistence or resolution of traumatic memories could lead to therapies targeting the root causes of PTSD, rather than just managing symptoms. A deeper understanding of the neurobiological mechanisms underlying adaptive and maladaptive fear processing will inform personalized interventions based on individual factors such as sex and developmental history.

By expanding knowledge of the ZI's role in fear modulation, this research may reveal novel therapeutic targets, leading to innovative treatments for PTSD and related anxiety disorders, and improving the quality of life for many affected individuals.

**A) SCIENTIFIC BACKGROUND**[[1]](#footnote-2)\*

**Post-traumatic stress disorder** (PTSD) is an incapacitating chronic syndrome affecting cognitive, emotional, and physiological processing and recovery from exposure to a potentially traumatic experience (1). Most individuals recover within one to four weeks after trauma, but a small proportion develop long-term psychopathology. The development of PTSD is often an evolving process and extends over time through a series of stages ranging from relatively contained distress to severe disability. PTSD affects multiple biological systems, including brain circuitry and neurochemistry, and cellular, immune, endocrine, and metabolic functions. Given the high personal, social, and economic burden of PTSD and the partial effectiveness of current therapies, there is an urgent need to understand the cellular and molecular changes underlying the disorder to develop more effective treatments.

A common approach to studying the effects of stress-related disorders involves identifying the risk factors that make certain individuals especially vulnerable to stress-related disorders. Although this approach is useful in many respects, little is learned about subjects who either do not demonstrate similar stress responses (i.e., stress-resistant) or who, despite being subjected to similar stress challenges, exhibit reactions of shorter duration that do not lead to stress responses (i.e., stress resilient). Therefore, understanding the neurobiological processes that lead to the development of specific PTSD symptoms, on the one hand, and identifying mechanisms that make individuals less vulnerable to stressful stimuli, on the other hand, are both critical for the development of effective treatments. PTSD-like animal models are most effective for investigating the entire spectrum of responses to stressful stimuli. An animal model can give a good approximation of certain aspects of the complex clinical disorder, enabling the study of questions raised in clinical research in a prospective study design and under much better controlled and reproducible conditions.

**Developing an animal model of PTSD:** We have developed a PTSD animal model that recapitulates two elements of clinical PTSD: (1) Symptom clusters are analyzed—similar to PTSD clinical diagnosis that requires specific symptoms from three well-defined symptom clusters to develop over time, and (2) Individual variability—from a group of animals exposed to the same stressful event, only a subgroup develops PTSD. Our model is original and innovative, as previous animal models of PTSD treated exposed populations as homogeneous groups. Recognizing individual variability in stress responses among animals supports the model's validity. Our Cut-off Behavioral Criteria model aligns animal behaviors with the DSM criteria for PTSD, and the clearly defined classification and reproducibility of the model yield results that are comparable to clinical findings.

In our model, adult rats are exposed to a cat urine predator scent stressor (PSS) for 10 minutes, as previously described (2-11). This model of predator exposure represents a life-threatening situation (criteria A) and provides a more natural stressor than electrical tail shocks or restraint, which may be analogous to extreme conditions such as torture. Seven days after the PSS exposure, animal behaviors are assessed using the elevated plus maze (EPM) and the acoustic startle response (ASR) test. Based on these assessments, rats are classified as exhibiting an ‘extreme behavioral response’ (EBR), ‘minimal behavioral response’ (MBR), or a ‘partial behavioral response’ (PBR), which falls between EBR and MBR (Fig. 1).

Classification into clearly defined groups allows for statistical analysis of the prevalence rate of specific response patterns. The proportion of rats showing extreme behavioral responses (EBR) (25%) aligns with epidemiological data on PTSD in trauma-exposed human populations, where 15–35% meet PTSD criteria and 20–30% exhibit partial or sub-symptomatic presentations. This alignment strengthens the model's face validity. We previously reported that the prevalence of the PTSD phenotype in wild rodent species ranged from 21.7% to 26.7%, similar to rates observed in laboratory rodents and trauma-exposed humans (12). This supports our translational PTSD model's ecological validity. The PSS paradigm has proven effective in inducing a range of expected behavioral and physiological responses, demonstrating strong face, construct, and predictive validity (13-22).

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|  | **Figure 1 The Cut-off Behavioral Criteria Model**. Behavioral models can be more closely matched with contemporary clinical conceptions of PTSD by using an approach that enables the classification of study animals into groups according to the degree of response to a stressor, i.e., the degree to which individual behavior is altered or disrupted. A) The data must demonstrate that the stressor had a significant effect on the overall behavior of exposed versus non-exposed populations at the time of assessment. B) To maximize the resolution and minimize false positives, extreme responses to both EPM and ASR performed in sequence are required for inclusion in the EBR group, whereas a negligible degree of response to both is required for inclusion in the MBR group. |

**Brain regions that may be involved in PTSD:** Most models addressing anxiety and PTSD have traditionally focused on amygdalocentric circuitry, given its key role in fear response, conditioning, and extinction processes (23). The prefrontal cortex-amygdala–hippocampus loop has received significant attention because of its association with prolonged and exaggerated threat responses in PTSD. This is attributed to heightened amygdala activity, reduced prefrontal top-down control, and diminished hippocampal function, all of which contribute to difficulties in suppressing conditioned fear during memory consolidation (24). In addition to its subcortical connections, the amygdala is crucial for processing emotionally driven reasoning across the prefrontal, cingulate, and insular cortices, which helps explain the maladaptive behaviors seen in PTSD. However, these structural findings remain inconsistent and may represent a risk factor rather than a definitive pathophysiological difference (25).

The thalamic and subthalamic regions, traditionally viewed as sensory and motor relays, have also been implicated in fear-related behaviors and PTSD (26). As hubs coordinating activity across cortical and subcortical networks, these regions are sensitive to physiological and psychological disturbances. Emerging evidence indicates that thalamic and subthalamic dysfunctions are associated with psychiatric disorders, including PTSD (27, 28). Functional imaging studies, such as fMRI, have demonstrated thalamic hypoactivity in people with PTSD exposed to trauma-related and trauma-unrelated emotional stimuli compared to non-PTSD controls (29, 30). This hypoactivity may result from altered arousal states, as the thalamus integrates input from arousal systems and sensory signals. SPECT studies also show reduced right thalamic blood flow in people with PTSD correlating with re-experience symptoms (31). Abnormal connectivity in thalamus-related pathways involved in emotional and cognitive processing has been observed in people with PTSD (32). Meta-analysis further suggests that the thalamus mediates cortical communication during traumatic memory recall (24, 33). Recent findings from resting-state functional connectivity studies revealed significant differences in thalamocortical connectivity between PTSD, trauma-exposed controls, and healthy groups, with these differences linked to clinical features (34). The reduced thalamic activation in PTSD may reflect a withdrawal of attention from external sensory stimuli. These findings suggest that alterations in thalamic and subthalamic regions play a crucial role in the pathophysiology of PTSD. However, whether subthalamic abnormalities directly underlie PTSD-related behaviors remains unclear.

The **Zona Incerta (ZI),** first described by Auguste Forel over a century ago as a “zone of uncertainty,” is a subthalamic structure with largely unclear functions. Characterized by chemical heterogeneity and extensive brain-wide connections, the ZI has recently gained attention for its role in processing sensory information (35, 36), neuronal development (37), hormonal regulation (38), sleep (39), pain (40), modulating defensive responses (41-44), and retrieval of fear-related memories (44-47).

As a key site for sensorimotor integration, the ZI processes sensory information and coordinates behavioral responses through its efferent projections (26, 48). In rodents, ZI activation has been shown to suppress generalization and enhance fear extinction (45, 47), while in humans, it aids in discriminating fearful from non-fearful stimuli (49). Given its role in processing somatosensory information and detecting threats, it remains unclear whether ZI dysfunction is directly involved in PTSD-related behaviors or if its abnormalities contribute to these behaviors. The ZI's role in establishing adaptive or vulnerable stress responses is also not well understood.

**B) RESEARCH OBJECTIVE AND EXPECTED SIGNIFICANCE**

Thalamic and subthalamic regions, especially the ZI, synchronize neural activity across cortical and subcortical networks, modulate information flow according to situational demands, and maintain adaptive fear associations. **We hypothesize that ZI alterations play a critical role in the pathophysiology of fear, anxiety-related behaviors, and PTSD.** We propose that the ZI modulates fear, anxiety, and PTSD-like responses by operating in parallel with the prefrontal cortex-amygdala-hippocampus circuit to dynamically process interoceptive and exteroceptive signals. Furthermore, we suggest that ZI stimulation may offer therapeutic potential by reducing fear responses and alleviating PTSD symptoms. This proposal expands the neurobiological framework of fear-related responses, highlighting the ZI's contribution to both adaptive and maladaptive fear processing.

**The overall aim** of the project is to identify the molecular mechanisms and anatomical regions in the ZI that mediate the PSS-induced PTSD-like behavioral impairments and to analyze the crosstalk between the ZI circuit and the HPA-axis.

The specific aims are:

**Aim 1** To determine the role of ZI in PSS-induced PTSD-like behavior and investigate the crosstalk between this neuronal pathway and the HPA-axis/glucocorticoid receptor.

**Aim 2** To analyze the role of specific neuronal subpopulations in the ZI—serotonergic, dopaminergic, GABAergic, and parvalbumin-expressing neurons—in PSS-induced PTSD-like behavior.

**Aim 3** To determine whether PSS-induced PTSD-like behavior and the HPA-axis response are mediated via the ZI→basomedial amygdala pathway.

Our animal model enables an in-depth investigation of the role of the ZI in shaping adaptive or vulnerability-based behavioral responses in rats exposed to PSS. It also allows us to explore the neurobiology of PTSD by linking the ZI with molecular parameters and individual behavioral outcomes. By integrating this research with established PTSD markers, such as HPA-axis dysfunction and altered brain circuitry, we aim to uncover mechanisms underlying individual differences in vulnerability to stress-related psychopathology, which is of critical importance.

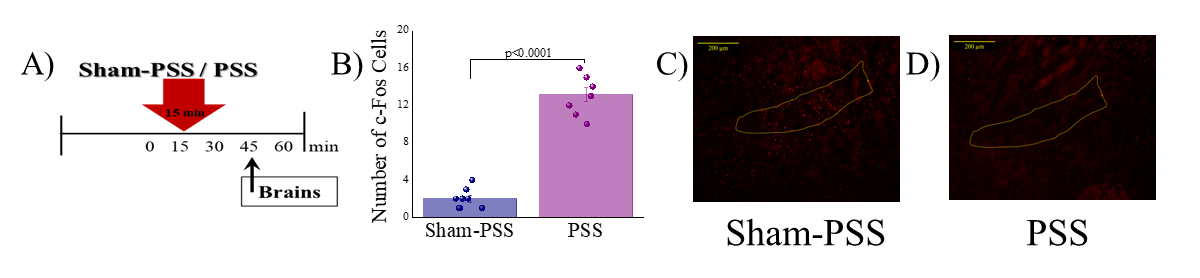
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**Aim 1 To determine the role of ZI in PSS-induced PTSD-like behavior and investigate the crosstalk between this neuronal pathway and the HPA-axis/glucocorticoid receptor.**

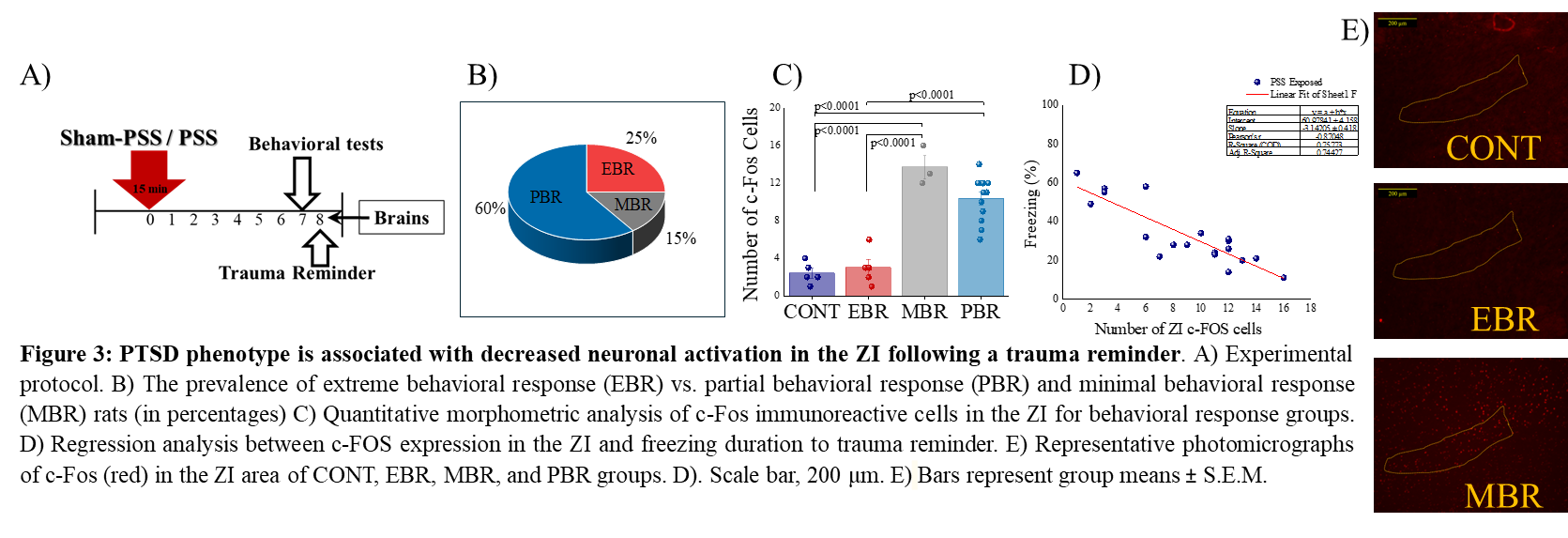
***Specific Aim 1a: To explore the sensitivity and response of the ZI to threatening experiences.***

**Preliminary Results** (Figure 2)**:** We assessed the expression of c-FOS, a marker of neuronal activation, after exposure to PSS or sham PSS, and following traumatic memory reactivation. We hypothesized that if the ZI encodes anxiety, c-FOS expression would increase in response to PSS or traumatic memory reactivation. To test this, 16 rats were exposed to PSS or sham PSS for 15 minutes. Thirty minutes later, brains were collected for c-FOS immunofluorescence staining. We found a significant increase in c-FOS-positive neurons in the medial ZI of PSS-exposed rats compared to sham PSS (F(1,14)=187, p<0.0001), confirming enhanced neuronal activity in the ZI following PSS exposure.



**Figure 2: PSS exposure enhances ZI neuronal activation. A**) Experimental protocol. **B**) The quantitative morphometric analysis of c-Fos immunoreactivity cells in the ZI for sham-PSS (n=8) and PSS-exposed rats (n = 8). C) Representative photomicrographs of c-FOS (red) in the ZI area in sham-PSS or (D) PSS-exposed rats. Scale bar, 200 μm. Bars represent group means ± S.E.M

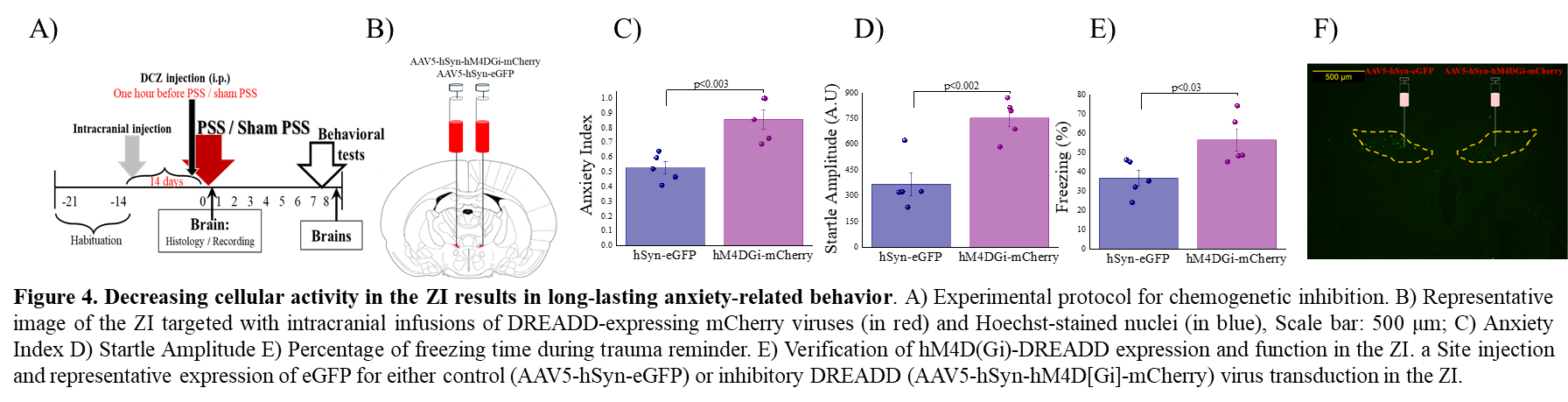
Next, we investigated the ZI response to traumatic memory reactivation (Figure 3). Twenty-five rats were exposed to PSS, and five served as sham controls. The elevated plus maze (EPM) and acoustic startle response (ASR) tests were conducted on Day 7 to classify behavioral responses. On Day 8, freezing behavior to a trauma reminder was assessed, and brains were collected 30 minutes afterward. Significant differences in ZI activity were observed between groups following the trauma reminder (F(3,21)=34.1, p<0.0001). Post hoc Bonferroni tests confirmed that the trauma reminder increased c-FOS expression in the ZI of PSS behavioral responders, both partially (PBR) and minimally (MBR), compared to the behaviorally resistant (EBR) and sham-PSS groups (p < 0.0001). c-FOS-positive cells were scarce in the ZI of the EBR and sham-PSS groups, with no significant differences between them.



These findings suggest that the PTSD phenotype is linked to reduced ZI neuronal activation following trauma reminder. **Our results indicate that the ZI encodes stress, anxiety-related, and PTSD-like behaviors, highlighting the value of investigating how reduced ZI activation during stress exposure may contribute to PTSD**.

***Specific Aim 1b: To determine whether manipulating ZI activity affects behavioral responses in control rats.*** We will use designer receptors exclusively activated by designer drugs (DREADDs) to modulate (decrease or increase) cellular activity in the ZI. Adeno-associated viruses (AAVs) containing the human synapsin (hSyn) promoter-driven transgenes will be injected into the ZI. Two weeks post-injection, deschloroclozapine (DCZ) will be administered intraperitoneally (i.p.) as a DREADD agonist (50). Behavioral assessments will follow one hour and seven days later, according to our animal model. We expect that ZI silencing will impair behavioral responses while increased activation will promote resilience.

**Preliminary Results** (Figure 4)**:**Twelve male rats underwent stereotaxic surgery under anesthesia (Ketamine 70 mg/kg and Xylazine 6 mg/kg). They were bilaterally microinfused with AAV5-hSyn-hM4D(Gi)-mCherry (#50475-AAV5, Addgene) (to reduce ZI activity) or AAV5-hSyn-EGFP (control) (#114472-AAV5, Addgene) into the ZI (AP: -2.12 mm, ML: ±1.2 mm, DV: -7.7 mm relative to bregma). Viral injections (50 nl) were delivered at 1 nl/sec. Two weeks later, DCZ (0.1 mg/kg, i.p.) was administered one hour before sham-PSS. Five rats were sacrificed 120 minutes post-DCZ injection for c-FOS immunohistochemistry (n=3) and patch-clamp recording (n=2) to validate DREADD effectiveness. Patch-clamp recordings from hSyn-hM4D(Gi)-mCherry–expressing ZI cells showed membrane hyperpolarization in response to DCZ. Behavioral tests were conducted 7 days later (N=8) (Figure 4). Chemogenetic inhibition of the ZI (AAV5-hSyn-hM4D(Gi) + DCZ), before sham-PSS significantly increased the anxiety index (F(1,8)=17.8, p<0.003), startle amplitude (F(1,8)=21.0, p<0.002), and freezing response to reminders (F(1,8)=7.8, p<0.03) compared to controls (AAV5-hSyn-EGFP + DCZ). These results demonstrate that ZI inhibition is sufficient to induce fear and anxiety-related behavior.



***Specific Aim 1c To examine whether selectively manipulating ZI cellular activity induces changes in behavioral responses, possibly mediated by HPA-axis/glucocorticoid receptors function changes.*** We will investigate the correlation between behavior and HPA-axis parameters in control rats. We predict that silencing the ZI will lead to impaired HPA-axis/glucocorticoid parameters akin to those observed in EBR animals, i.e., blunted HPA-stress response. Increasing cellular activity will promote faster and higher corticosterone levels.

***Specific Aim 1d: To determine whether manipulating ZI cellular activity immediately before PSS exposure correlates with behavioral stress responses.*** AAVs will be microinfused into the ZI. Two weeks post-injection, DCZ will be administered i.p. one hour before PSS exposure. Behavioral assessments will be performed 7 days later. We expect that silencing the ZI will disrupt fear learning and recall, resulting in elevated EBR-phenotype responses. Conversely, activating the ZI may reduce anxiety and fear responses, increasing resiliency.

***Specific Aim 1e: To examine whether manipulating ZI cellular activity immediately before PSS exposure induces changes in behavioral responses, potentially mediated by HPA-axis/glucocorticoid receptor function changes.*** We predict that silencing the ZI will correlate with extreme behavioral stress responses associated with PTSD vulnerabilitywhile increasing cellular activation will correlate with resilience***.*** This evaluation will aid in determining the neuroendocrine mechanisms involved in PSS-induced behavioral disruption associated with the ZI.

**Aim 2 To analyze the role of specific neuronal subpopulations in the ZI—serotonergic, dopaminergic, GABAergic, and parvalbumin-expressing neurons—in PSS-induced PTSD-like behavior.**

The ZI is a heterogeneous structure containing several distinct neuronal populations that vary in cytoarchitecture, connectivity, and immune marker distribution (45, 51). Key neuronal types include glutamatergic (52), GABAergic (53), serotoninergic (54), dopaminergic neurons (55), and others expressing parvalbumin, calbindin, and somatostatin. This part of the study aims to examine the contribution of specific ZI subpopulations to individual variability in stress and anxiety responses to PSS exposure.

Objective—***To determine the role and location of ZI subpopulations*** ***encoding fear, anxiety, and PTSD-related behaviors***.Immunofluorescence will be used to assess the biochemical diversity of ZI neurons in our animal model. We will determine the anatomical distribution and identify which of the four ZI sectors—rostral, dorsal, ventral, and caudal (56)—contribute to specific functions. Double-label immunohistochemistry for c-FOS and specific neuronal markers will determine the activation of these cell populations. In the second part of the study under Aim 2b, chemogenetic techniques will be employed to manipulate ZI subpopulations to determine their functional role in PTSD-related behaviors.

***2as*** compared to controls

Neuronal subpopulations of interest will be labeled with the following antibodies: serotonergic, tryptophan hydroxylase 2 (anti-TPH2); dopaminergic (DA), tyrosine hydroxylase (anti-TH); GABAergic, gamma-aminobutyric acid (anti-GABAergic); and parvalbumin PV (anti-PV). These four neuronal subpopulations play pivotal roles in stress response and PTSD-related behaviors (57, 58). Dopamine, GABA, and serotonin are key regulators of fear, stress, and PTSD vulnerability, and their dysfunctions are linked to PTSD symptoms (59, 60).

**Preliminary results** (Figure 5)**:** We examined the topographic distribution of the serotonin marker TPH2 in the ZI of rats subjected to the PTSD protocol. Twenty-five rats were exposed to either PSS or sham-PSS for 15 minutes. On day 7, we conducted the EPM and ASR tests to classify the animals into behavioral response groups, followed by freezing responses assessment to a trauma reminder on day 8. One hour after the test, brain tissues were collected. We used anti-TPH2 (ab-184505, Abcam) and c-FOS antibodies (ab-208942, Abcam) to label the subpopulations in the ZI. TPH2 immunoreactivity was observed in cells with large, rounded somata and finely labeled dendrites, particularly in the rostral ZI. Our findings suggest that plasticity within the TPH2-immunoreactive populations may contribute to differences in stress vulnerability between susceptible and resilient animals. Statistical analysis found significant differences in TPH2-immunoreactive cells among the groups (F(3,21)=18.4, p<0.0001), with a decreased density of TPH2-positive cells and fibers in the EBR and PBR groups compared to the Sham-PSS group (p < 0.001 and p < 0.0004, respectively). Additionally, the MBR group had significantly more activated serotonergic neurons than the EBR group (p < 0.0003), as indicated by co-expression of TPH2 and c-FOS (Figure 5E).

A screenshot of a video game

Description automatically generated

These findings suggest that PTSD-related vulnerability (EBR) induced by PSS is linked to fewer activated serotonergic neurons in the ZI, while resilience is associated with a higher number of activated serotonergic neurons. Our preliminary data indicate that the ZI's serotonergic subpopulation plays a role in encoding fear, anxiety-related behaviors, and PTSD-like responses.

***Specific Aim 2b:*** ***To determine which of the ZI neuronal subpopulations contribute to PSS-induced behavioral and HPA-axis impairments.*** Chemogenetic tools will be used to selectively manipulate ZI neuronal subpopulations associated with PTSD-like responses, which had been identified under Specific Aim 2a. We will administer AAV vectors into the ZI and use DCZ as chemogenetic actuators, followed by behavioral tests and freezing response assessments. We hypothesize that silencing ZI serotonergic, dopaminergic, or GABAergic neurons will impair behavioral resilience and increase PTSD vulnerability, while their activation will promote resilience; in contrast, silencing PV neurons will increase PTSD vulnerability, and their activation will promote resilience. We also expect a correlation between ZI subpopulation neuron density, corticosterone levels, and anxiety.

**Aim 3: To determine whether PSS-induced PTSD-like behavior and the HPA-axis response are mediated via the ZI→basomedial amygdala** **(BMA) pathway.**

Recent studies suggest this circuit modulates aversive expectations during stress (61). We will first investigate the anatomical properties of the ZI→BMA projection using anterograde and retrograde tracing. AAV encoding enhanced green fluorescence protein (AAV-CaMKIIa-EGFP) will be injected into the ZI for anterograde tracing of axonal terminals in the BMA. We predict dense EGFP-positive terminals in the BMA compared to the basolateral amygdala. To retrogradely trace BMA neurons projecting to the ZI, red fluorescent RetroBeads will be injected into the BMA. We expect a higher number of labeled ZI neurons projecting to the BMA than to the BLA, indicating preferential ZI innervation of the BMA. Next, we will examine c-FOS expression in ZI neurons projecting to the BMA following PSS (labeling them with RetroBeads). We predict a significant increase in c-FOS-positive BMA-projecting ZI neurons compared to controls. Chemogenetic inhibition of ZI→BMA neurons will then be used to assess their role in stress-related behavior and HPA-axis activity (measured via plasma corticosterone) following PSS. For further analysis, we will use an intersectional chemogenetic strategy to inhibit the ZI→BMA circuit. AAVretro-Syn-Cre will be injected into the BMA, and AAV-ef1a-DIO-hM4DmCherry into the ZI (creating ZI-BMA hM4D rats). We predict that inhibiting this pathway will increase anxiety-related behaviors and vulnerable phenotypes. Additionally, we will assess HPA-axis activity by measuring corticosterone levels in response to PSS after chemogenetic inhibition of ZI→BMA neurons. In a related experiment using PVT-CeA hM4D mice, inhibition of CeA-projecting PVT neurons reduced stress-induced plasma corticosterone levels, suggesting this approach may modulate hormonal stress responses. Finally, to confirm the ZI→BMA circuit’s role in stress responses, we will inhibit ZI axonal terminals in the BMA using chemogenetic. AAV-CaMKIIa-hM4D-mCherry will be injected into the ZI, and drug delivery cannulas implanted into the BMA for local inhibition of ZI neurons' axonal terminals.

**C.ii. RESEARCH DESIGN & METHODS:**

All procedures will strictly adhere to NIH guidelines for the Care and Use of Laboratory Animals.

**Animals**:Male and female Sprague-Dawley rats (Harlan Jerusalem) will be used in all experiments. Rats will arrive at 6 weeks of age and acclimate to the vivarium for at least one week. Rats will have ad-lib access to standard rat chow.

**Sample size:** Using a 95% confidence level (alpha=0.05) and 80% power, the sample size was estimated as N=(Zα/2+Zβ)²×σ²×(r+1)/d²×r, with σ=1.283, r=2:1, and d=1.528. This calculation indicated 20 rats per group. A total of 720 rats (360 male, 360 female) will be required.

**Exposure to stressor (PSS):** Test animals will be placed on used cat litter for 10 minutes (in use by the cat for 2 days, sifted for stools), while controls will be exposed to fresh litter for the same duration (7, 11, 16, 62-77).

**Behavioral tests:** The behavioral tests were chosen to cover a range of unconditioned and conditioned models of anxiety and fear associated with PTSD: anxiety and fearful behavior on the elevated plus maze (EPM) and non-habituated exaggerated startle reaction (ASR). Behavioral parameters will be assessed 7 days post-exposure, based on the consistent finding that extreme changes that remain constant at 7 days after exposure represent "chronic symptoms" (71) and persist in the long run (7, 11, 16, 62-71). All test sessions will be video-recorded and analyzed with ETHO-VISION (Noldus).

*Elevated plus maze:*The EPMis a plus-shaped platform with two opposing open arms and two opposing closed arms(78). Mice will be placed on the central platform facing an open arm and allowed to explore the maze for 5 min. The behaviors assessed will be time spent in the open and closed arms; open- and closed-arm entries; total exploration and anxiety index (AI). Total exploration will be calculated as the number of entries into any arm of the maze (total arm entries) to distinguish between impaired exploratory behavior, exploration limited to closed arms (avoidance), and free exploration. AI integrates the EPM behavioral measures as follows:

AI values range from 0 to 1, where an increase in the index expresses increased anxiety-like behavior.

*Acoustic startle response*will bemeasured using two ventilated startle chambers (San Diego Instruments). Each startle session will start with a 5-minute acclimatization period to a background of 68 dB white noise, following which 30 acoustic startle trial stimuli will be presented (110 dB white noise of 40 ms duration with inter-trial intervals of 30 s or 45 s). The following parameters will be assessed: (1) mean startle amplitude and (2) percent of startle response habituation to repeated presentation of the acoustic pulse (the change between the response to the first block of sound stimuli and the last).

*Exposure to trauma reminder*: A disproportionate psychophysiological response to trauma cues is integral to the clinical definition of PTSD. To model this component of PTSD, a stimulus that is not intrinsically threatening, yet acts as a clear-cut reminder of the traumatic stressor, will be employed. The behavioral outcome measure that we found reliable and valid is the freezing behavior (duration of immobility) of the rodent placed on the unused litter. As freezing behavior indicates a sense of immediate threat and intense fear, and as this behavior was engendered by a neutral reminder 7–8 days (or more) after stress exposure implies that a memory-related process and contextual association of the stimulus must have occurred.

We will use the "*Cut-off Behavioral Criteria*" (Figure 1) to classify individual animals according to their individual patterns of responses to various manipulations. Such a classification will enable us to correlate the behavioral effects on the whole (individual) organism with underlying biomolecular and physiological parameters.

**Stereotaxic surgeries:** Rats will be anesthetized with isoflurane (using an anesthesia machine) and placed in a stereotaxic apparatus. Erythromycin eye ointment will protect the eyes. The scalp will be cut along the midline to expose the skull. The rat skull will be adjusted under a microscope to ensure it is horizontal. Small holes will be drilled into the skull for virus injection. After carefully removing the dura, the specific viruses will be injected into the target brain regions (See each experiment).

Virus injection: Bilateral stereotaxic AAV injections into ZI will be performed using the following stereotaxic coordinates: rostral (rZI): AP: -1.8 mm, ML: ±1.0 mm and DV: -7.55 mm relative to Bregma. Medial (mZI): AP: -2.12 mm, ML: ±1.2 mm and DV:-7.7 mm relative to Bregma. Caudal (cZI): AP: -3.8 mm, ML: ±2.5 mm and DV: -7.5 mm relative to Bregma. Viruses will be injected at a final volume of 50 nl (experiment 1), or 100 nl (experiment 3), at the rate of 20 nl/sec using a Hamilton syringe equipped with a micropump controller (World Precisions Instruments, Micro4, UMC4). The syringes will be slowly withdrawn after a 10-minute diffusion period following injection. After recovery from anesthesia, rats will be housed in cages supplied with plenty of food and water. Rats that will be used for retrograde tracing will be perfused 3–5 days after injection of RetroBeads.

Chemogenetic Manipulation: Two weeks later (to allow for optimal viral expression), chemogenetic actuator DCZ (HelloBio, catalog #HB9126) will be dissolved in 0.9% saline and will be injected intraperitoneally at a dose of 0.1 mg/kg, one hour before PSS exposure. This new DREADD agonist DCZ represents an extremely potent, highly brain penetrant, and selective actuator for hM3Dq and hM4Di DREADDs (50).

Behavioral tests will be performed 7 days after PSS exposure, according to our animal model (8-10, 79, 80).

Collection of urine:To collect urine, the rats will be placed individually into plastic buckets (1.1-L volume) and will be provided with a plastic salad dish (250 cc). The rats that do not urinate spontaneously will be excluded from the study.Urine will be collected with a 1-ml syringe, centrifuged at 800×g for 10 min, diluted 1:10 in EIA diluent, and stored in polypropylene tubes at –20 °C.

Collection of blood: At the end of the study, the animals will be decapitated, and blood from the trunk will be collected into plastic tubes containing 100 μl of heparin (25,000 units/ml). Corticosterone (urine and plasma) levels will be measured with an AssayMax Corticosterone ELISA Kit.

Immunohistochemistry will be used to 1) Assess the role and location of ZI subpopulations in PTSD-related behaviors (Specific Aim B). 2) Validate the specificity and efficacy of AAV DREADD infection. 3) Validate the placement of intra-cranial virus injections. For aim 1, we will use the following antibodies: anti-TPH2 (ab-184505, Abcam), anti-c-FOS (ab-300443, Abcam), Anti-TH (ab-ab6211, Abcam), anti-GABAergic (ab-308437, Abcam), anti-PV (ab-181086, Abcam). For aim 2, from each study group, 2-3 rats will be sacrificed for approximately 90 minutes following DCZ injection via transcardial perfusion. For aim 3, rats will be sacrificed 1h after behavioral assessments. Rats will be anesthetized (Ketamine (70 mg/kg) and Xylazine (6 mg/kg)) and will be perfused transcardially with phosphate-buffered saline (PBS) followed by 100 mL 4% paraformaldehyde (PFA). The brains will be post-fixed in 4% PFA for 4 hours and then transferred to 20% and 30% sucrose until the brains sink completely. Brains will be coronally sectioned at 35 mm using a freezing microtome (CM 3050S, Leica).

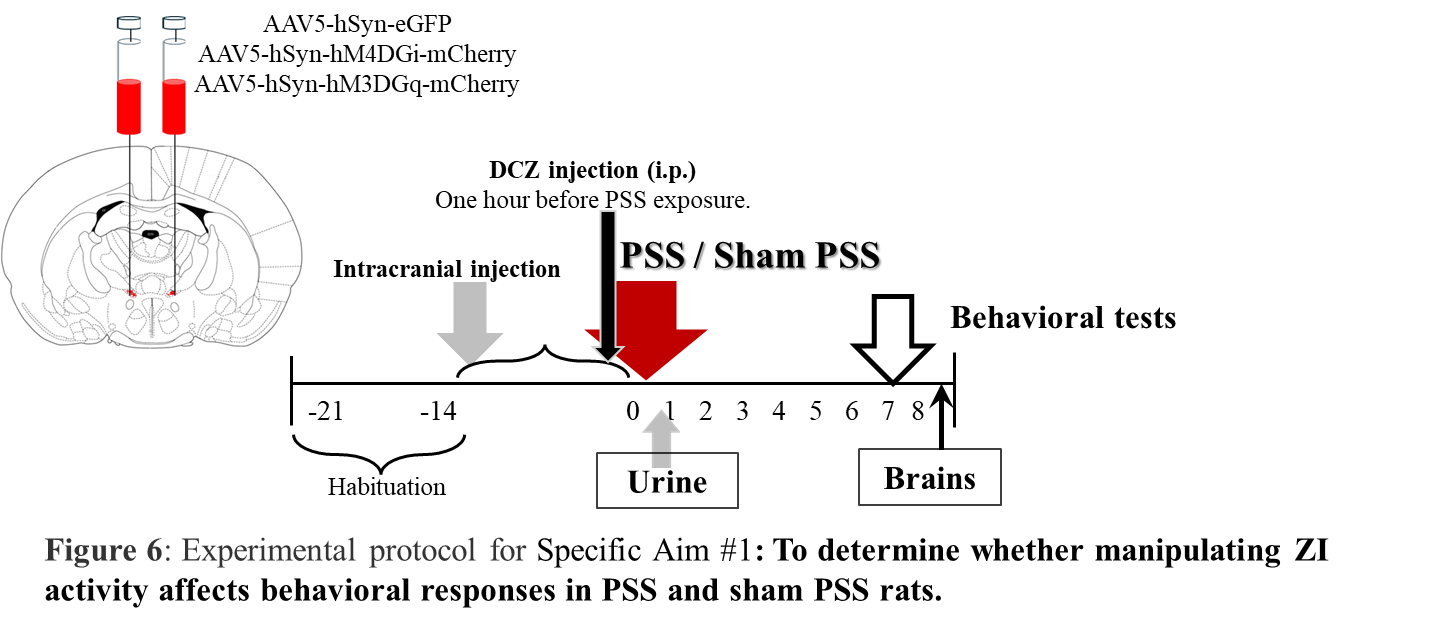
Rats will be anesthetized (Ketamine (70 mg/kg) and Xylazine (6 mg/kg)) and will be perfused transcardially with phosphate-buffered saline (PBS) followed by 100 mL 4% paraformaldehyde (PFA). The brains will be post-fixed in 4% PFA for 4 hours and then transferred to 20% and 30% sucrose until the brains sink completely. Brains will be coronally sectioned at 10μm (#1) or 35μm (#2 and #2) using a freezing microtome (CM 3050S, Leica).

To validate the specificity and efficacy of AAV DREADD infection, we will evaluate the induction of c-FOS expression. We chose c-FOS immunolabeling as the readout because induction of c-FOS protein is a widely used index of neuronal activity with a temporally defined expression window (81). From each study group, 3-4 rats will be sacrificed for approximately 90 minutes following DCZ injection via transcardial perfusion.

To validate the placement of intra-cranial virus injections: Brain sections will be stained with Hoechst nuclear stain (1:1000). The position of GFP or mCherry-positive cells will be assessed using Leica fluorescent microscope. Rats with incorrect targeting viruses or tracers will be excluded from data analysis.

Image acquisition and quantification**:** Fluorescence images of the ZI will be captured using a Leica 3050S microscope, and c-FOS expression will be quantified using MCID Core Imaging software. c-FOS immunoreactivity will be quantified across three consecutive sections per animal in both the left and right hemispheres.

**Experiment for *Specific Aim 1***: Rats will be randomly assigned to one of six groups (n=20 per sex group, 120 males, 120 females): 1) Unexposed +AAV5-hSyn-eGFP (control), 2) Unexposed + AAV5-hSyn-hM4DGi-mCherry (inhibitory), 3) Unexposed + AAV5-hSyn-hM3DGq-mCherry (excitatory), 4) PSS + AAV5-hSyn-eGFP, 5) PSS + AAV5-hSyn-hM4DGi-mCherry, or 6) PSS + AAV5-hSyn-hM3DGq-mCherry. Under anesthesia, rats will undergo stereotaxic surgery for bilateral injection of the respective DREADD virus (excitatory: AAV5-hSyn-hM3Dq.mCherry, inhibitory: AAV5-hSyn-hM4Di.mCherry, or control: AAV5-hSyn.mCherry) into the medial ZI (AP: -2.12 mm, ML: ±1.2 mm, DV: -7.7 mm relative to Bregma). Two weeks later, DCZ (0.1 mg/kg, #HB9126 HelloBio) will be dissolved in 0.9% saline and injected i.p. one hour before PSS or sham-PSS exposure to assess the role of ZI manipulation in stress-induced behavioral disruption. In each group, 2-3 rats will be sacrificed 90 minutes post-DCZ injection via transcardial perfusion. Seven days post-exposure, behavioral tests will be conducted, followed by deep anesthesia and transcardial perfusion 24 hours later. Brains will be removed, post-fixed, cryoprotected (30% sucrose in PBS), and frozen at −80°C. Immunohistochemistry will confirm viral vector infection and target location.



**Experiment for *Specific Aim 2a***: Forty rats (20 male, 20 female) will be exposed to either PSS or sham-PSS for 15 minutes. On day 7, EPM and ASR tests will be conducted. On day 8, freezing responses to a trauma reminder will be assessed, followed by brain tissue collection one hour later. Immunohistochemistry (details provided) will be used to examine the role and location of ZI subpopulations in PTSD-related behaviors. If ZI subpopulations are implicated in PTSD-like responses, we will explore their role in PSS-induced behavioral and HPA-axis impairments.

***Specific Aim 2b:*** Rats will be randomly assigned to one of three groups (n=20 per sex group, 60 males, 60 females): 1) excitatory, 2) inhibitory, or 3) mCherry control viruses- for each ZI subpopulation (4 subpopulation). Each group will undergo stereotaxic surgery for bilateral injection of the respective virus into the ZI (specific sites based on Stage 1 results). Two weeks later, DCZ will be injected intraperitoneally one hour before PSS or sham-PSS. Urine will be collected during the first hour post-PSS. In each group, 2-3 rats will be sacrificed 90 minutes after DCZ injection via transcardial perfusion. Seven days post-exposure, rats will undergo behavioral tests, and, 24 hours later, they will be deeply anesthetized and transcardially perfused and their brains removed for immunohistochemistry to confirm viral vector infection and location. The following virus will be used:

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | To reduce activity | To enhance activity | Control virus |  |
| ***ZI-serotonergic neurons*** | AAV5-hSyn-DIO-hM4Di-mCherry | AAV5-hSyn-DIO-hM3Dq-mCherry | AAVDJ-Syn1-DIO-eGFP | Addgene |
| ***ZI-DAergic neurons****:* | AAV-TH-hM4Dq-mCherry | AAV-TH-hM3Dq-mCherry | AAV-TH-mCherry | Addgene |
| ***ZI-GABAergic neurons*** | pAAV-GAD67-hM4D(Gi)-mCherry-WPRE | pAAV-GAD67-hM3D(Gq)-mCherry-WPRE | pAAV-GAD67-MCS-mCherry-3Flag | Addgene |
| ***ZI-PV neurons*** | rAAV-EF1a-DIO-hM4D(Gi)-mCherry-WPREs | rAAV-EF1a-DIO-hM3D(Gq)-mCherry-WPREs | rAAV-hSyn-DIOEGFP-WPRE-hGHpA | BrainVTA |

Addressing potential issues: If manipulating the ZI subpopulation does not lead to behavioral changes or altered c-FOS expression across groups, we will adjust the experiment to target a different ZI subdivision. Alternatively, if no changes are observed, we will modify the timing of the manipulation relative to stress exposure. Given our preliminary results, which suggest reduced ZI activation following a trauma reminder in the PTSD phenotype, we plan to shift the ZI manipulation to an earlier time point, either before or during the trauma reminder (via DCZ injection).

**Experiment for *Specific Aim 3***: The protocol is identical to Experiments #A1 and #A2.

To anterogradely trace the ZI projections: AAV-CaMKIIa-EGFP will be injected into the ZI. To retrogradely trace ZI neurons projecting to the BMA: RetroBeads (R180-1000, Lumaflouor) will be injected into the BMA (bregma, AP = -1.8 mm; ML = +3.9 mm; DV = -9.0 mm). To chemogenetic inhibition of ZI neuronal axonal terminals in the BMA: AAV-CaMKIIa-hM4D-mCherry (Obio Technology, China) will be injected into the ZI. To chemogenetic inhibition of BMA-projecting ZI neurons: AAVretro-Syn-Cre will be bilaterally injected into the BMA and AAV-EF1a-DIO-hM4D-mCherry (BrainVTA Technology) will be injected into the ZI. To anterogradely trace the axonal projection patterns of ZI innervated-BMA neurons: AAV1-Syn-Cre (Taitool Bioscience) will be injected into the ZI and Cre-dependent AAVef1a-DIO-EGFP (Brain Case Technology) will be injected into the BMA.

In addition, we plan to implement targeted manipulation of a specific neural subpopulation within this pathway, based on the outcomes of previous experimental steps.

Addressing potential issues: **(**1) Assessing ZI→BMA Projections: To investigate whether ZI→BMA projections modulate behavioral and HPA-axis responses to PSS, we will employ a CRE recombinase system to express DREADDs in neurons projecting from the ZI to the BMA. Currently, we are utilizing the retro-AAVCRE/double-floxed DREADD system in a small sample size without encountering technical difficulties. However, if no significant behavioral changes are detected, we will transition to using CRE-expressing rats or mice.

(2) If manipulation of the ZI→BMA pathway does not result in behavioral changes or altered c-FOS expression across groups, we will redirect the experiment to investigate another potential ZI pathway. Anatomical and physiological evidence indicates that the ZI receives inputs from the dorsomedial prefrontal cortex (dmPFC) (82). However, the role of the dmPFC→ZI circuit in regulating fear, anxiety, or PTSD-related behaviors remains unclear. We will assess whether the dmPFC→ZI pathway influences behavior and HPA-axis responses to PSS.

**Treatment of the data:** Due to the multidisciplinary nature of the methodologies, which need to be integrated to evaluate their interactions, we will use a mathematical tool to complement and verify the validity of the conceptual constructs underlying the variables assessed here (in addition to the regular data analysis by analysis of variance). Unsupervised fuzzy clustering will thus be applied to mathematically defined conceptual structures in the multi-dimensional feature space comprising all synchronized measured variables. We expect the clustering to yield 2–3 groups with a fair degree of overlap. The same clustering will be performed post-exposure with/without treatment using the cut-off behavioral criteria classification. We expect the PSS to increase the number of clusters, up to 4–5 more distributed clusters (increased degree of membership in the major hosting cluster) (70, 83).

**C.iv. CONDITIONS AND RESOURCES:**

A state-of-the-art animal facility located within the Mental Health Center operates according to the NIH Guide for the Care and Use of Laboratory Animals. It is managed by a veterinarian and is supervised by both the veterinarian of Ben-Gurion University of the Negev and the Ministry of Health's Veterinary Department. The PI’s laboratory has extensive expertise with animal models and is fully equipped with all necessary behavioral apparatus, a large vivarium, and molecular and morphological equipment.

**C.v. EXPECTED RESULTS AND PITFALLS:**

Refer to the text under each specific aim. In addition, although we are currently using the DREADD system in male rats, we have not yet begun work with female rats. Given that stress and PTSD-related behaviors differ between sexes (22), it is possible that the effects of manipulation could be less pronounced in females. If temporary silencing of the ZI in female rats does not lead to behavioral changes, we will employ bilateral injections of tetanus toxin light chain (Tet-tox) to completely block evoked synaptic transmission in the ZI, using adeno-associated viruses (AAVs) as described by Zhou (62). Furthermore, if ZI manipulation in female rats does not result in behavioral changes or altered c-FOS expression across groups, we will adjust the experiment to target a different ZI subdivision. Alternatively, if no changes are observed, we will modify the timing of the manipulation relative to stress exposure. As our preliminary data suggest that the PTSD phenotype is associated with reduced ZI activation following trauma reminders, we plan to manipulate the ZI earlier, either before or during the trauma reminder, using DCZ injection.

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1. \* [↑](#footnote-ref-2)