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Demonstrating the ability of GABAergic cells in the zona incerta to modulate motivation

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eLife Assessment

This set of studies provides **important** knowledge for the role of zona incerta neurons in motivation and food-seeking in mice. The evidence supporting the findings is **convincing**, with the use of multiple approaches and nice behavioral procedures to provide converging lines of evidence. Additional analyses and data from the current studies would further strengthen the evidence. This work will be of interest to those interested in motivation and its brain substrates.

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Abstract

Motivation to engage in goal-directed actions is crucial for survival and well-being. Dopaminergic and serotonergic systems have been the focus of efforts to understand neurobiological etiologies of normative and disrupted motivation. Understanding how the brain incorporates salient sensory cues into motivational drive outside of these neuromodulatory systems is less appreciated. We posited that given their afferent and efferent connections, GABAergic cells in the zona incerta (ZI) are ideally positioned to perform this function. Combining behavioral tasks in mice with chemogenetics we show that GABAergic ZI neurons are capable of modulating effort-based motivation and that chemogenetic activation of this cell population can rescue motivational deficits induced by chronic stress - without affecting attention, memory, locomotion, or appetite. Next, using fiber photometry we report that GABAergic cells in the ZI robustly respond to sensory cues and their response to a cue increases as reward-predictive associations are formed. Inhibiting GABAergic cells in the ZI did not abolish cue-associated motivational behavior while cue-locked optogenetic activation of these cells robustly enhanced cue-associated motivated behavior demonstrating that these cells are not necessary but sufficient to allow for sensory cues to influence action selection. These findings establish GABAergic cells in the ZI as modulators of motivation and afford us a neuroanatomical hub that could be targeted to potentially remedy treatment-resistant deficits in motivation.

Introduction

Engaging in goal-directed behavior like seeking out food or social interaction is an important dimension of general well-being. Operationally, motivation to engage in, plan and execute a task is at the core of goal-directed behavior (Salamone & Correa, 2012 ) . Regulated by neural circuits these components of motivation enable organisms to exert effort toward desired outcomes (Schultz, 2015 ) . Disruptions in motivation are central to numerous neuropsychiatric conditions (Nestler & Carlezon, 2006 ) , and the reduced pursuit of rewarding actions is exacerbated by environmental factors like exposure to stress (Russo & Nestler, 2013 ) . Most efforts to

understand neurobiological mechanisms that underlie normative motivation and remedy stress-induced disruptions of motivation have focused on mesolimbic and hypothalamic dopaminergic systems in the brain (Baik, 2020 [↗](#); Hollon et al., 2015 [↗](#); Nieh et al., 2016 [↗](#); Stanton et al., 2019 [↗](#)). Despite the progress made in these neurobiological arenas, therapeutic strategies targeting these systems frequently fail in many individuals (Al-harbi, 2012 [↗](#); Berlim & Turecki, 2007 [↗](#)). Additionally, most studies of motivation pay very little attention to sensory cues that become associated with rewards and then acquire the ability to directly evoke motivational drive and energize goal-directed actions (Berridge & Robinson, 2016 [↗](#); Cartoni et al., 2016 [↗](#); Wolff & Saunders, 2024 [↗](#)). Consequently, the circuits that integrate sensory input into motivational drive remain poorly defined. This gap limits our ability to fully map the circuits underlying adaptive motivational control and develop more effective interventions to rescue deficits in motivation. With the goal of not only expanding cellular substrates that could eventually serve as therapeutic targets for stress-induced deficits in motivation but also ensuring that sensory cues are included in such contextualization of motivation, in this study, we sought to determine the contribution of GABAergic cells in the sub-thalamic zona incerta (ZI) to motivation.

The predominantly GABAergic ZI is extensively connected to sensorimotor, limbic, and basal ganglia structures (Arena et al., 2024 [↗](#); Wang et al., 2020 [↗](#)). Through this connectivity, GABAergic cells in the ZI integrate incoming sensory information with internal states and motor outputs, and have been implicated in arousal, attention, anxiety, feeding, and reward pursuit (Ahmadlou et al., 2021 [↗](#); Chou et al., 2018 [↗](#); Zhang & van den Pol, 2017 [↗](#); Zhao et al., 2019 [↗](#)). These findings make GABAergic cells in the ZI perfect neuroanatomical substrates that could influence normative motivation, remedy stress-induced deficits in motivation and incorporate sensory cues into the drive to be engaged in tasks. Using a combination of chemogenetics, fiber photometry, operant as well as Pavlovian behavioral assays and projection-targeted neuronal manipulations in mice, we identified GABAergic cells in the ZI as important modulators of normative and stress-induced deficits in motivation while dynamically responding to reward-related sensory stimuli and incorporating these stimuli into goal-directed action. Our findings reveal a previously unrecognized role for GABAergic cells in the ZI in integrating sensory and reward information to shape goal-directed action.

Results

Chemogenetic manipulation of GABAergic neurons in the ZI bidirectionally modulates motivation

To test whether GABAergic neurons in the ZI contribute to motivational drive, we expressed inhibitory (hM4DGi) or excitatory (hM3DQG) DREADDs in ZI GABAergic neurons by bilaterally injecting Cre-dependent AAVs (AAV-hSyn-DIO-DREADD-mCherry or AAV-hSyn-DIO-eGFP) into the ZI of vGAT-CRE mice (Fig. 1A [↗](#) and 1B [↗](#)). Following surgical recovery and operant training on fixed-ratio schedules, motivation in the mice was tested on a progressive ratio (PR) schedule (Fig. 1C [↗](#)). In this well-established assay of motivational vigor, the response requirement increases exponentially with each successive reinforcer, and the breakpoint (the highest ratio completed) serves as the primary index of motivation.

In our inhibitory experiment, to account for the repeated-measures design (each animal receiving both vehicle and CNO), a linear mixed-effects model was fitted with treatment, group, and sex as fixed effects and animal as a random intercept. The model revealed a significant main effect of treatment ($F(1, 35.9) = 12.17, p = 0.001$) and, critically, a significant treatment $\times$ group interaction ($F(1, 35.9) = 9.60, p = 0.004$). No main effect of group ($F(1, 36.4) = 0.13, p = 0.72$) or sex ($F(1, 36.4) = 0.63, p = 0.43$) was observed, and no interactions involving sex reached significance (all $p > 0.28$). More specifically, chemogenetic inhibition (hM4DGi group: $n=14$: 7 males, 7 females) of GABAergic ZI neurons in the ZI after CNO administration significantly reduced breakpoints relative to VEH (vehicle) treatment ($W = 158.5, p\text{-value} = 0.005498, \text{Cohen's } d = 1.347753$) and relative to GFP controls ($n=15$: 8 males, 7 females) treated with CNO ($W = 175, p\text{-value} = 0.008851, \text{Cohen's } d = 1.08543$) (Fig. 1D [↗](#)). Importantly, this reduction in breakpoint was specific to the Gi DREADD

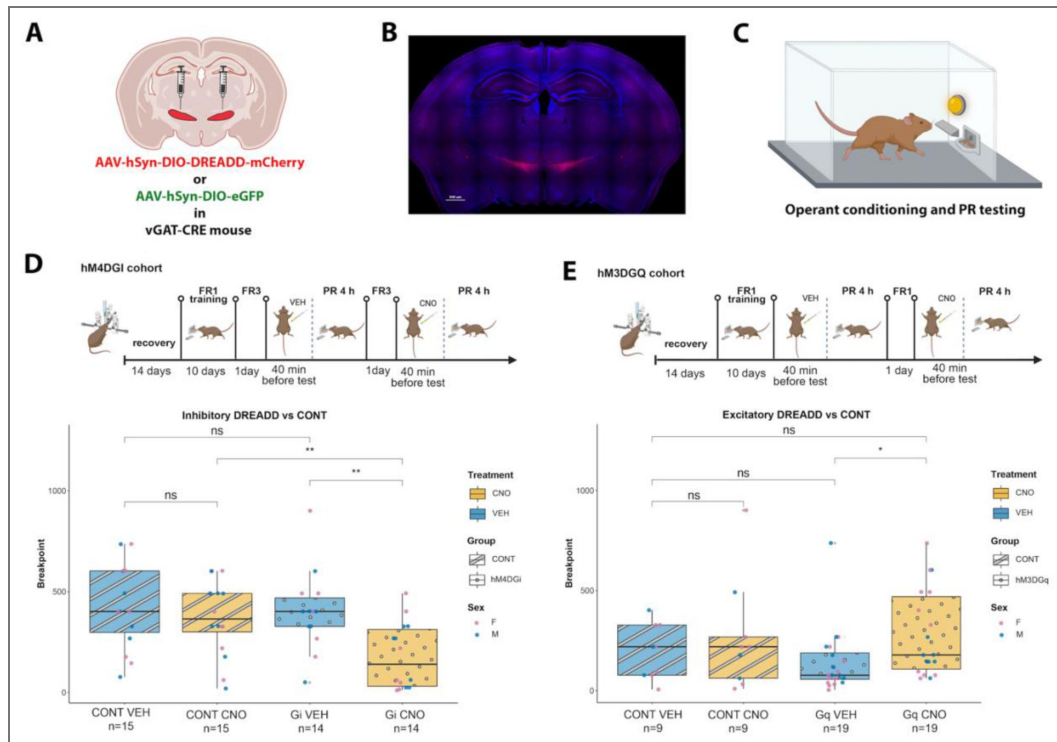


Figure 1. Chemogenetic manipulation of GABAergic neurons in the ZI bidirectionally modulates motivation.

(A) Schematic of experiment: CRE-dependent inhibitory (hM4DGi) or excitatory (hM3DGq) DREADDs or GFP control were bilaterally injected into the ZI of vGAT-CRE mice. (B) Representative histological image showing DREADD expression in the ZI. (C) Schematic of the operant conditioning box. (D) Experimental timeline and Progressive Ratio test results for the inhibitory DREADD (hM4DGi) cohort. Chemogenetic inhibition of GABAergic neurons in the ZI significantly reduced breakpoints following CNO administration relative to vehicle and relative to GFP controls. GFP controls showed no treatment-dependent change. (E) Experimental timeline and Progressive Ratio test results for the excitatory DREADD (hM3DGq) cohort. Chemogenetic activation of GABAergic neurons in the ZI significantly increased breakpoints following CNO administration compared to vehicle, with no effect in GFP controls. Whisker plots show median and interquartile range; individual data points are colored by sex. ** $p < 0.01$, * $p < 0.05$, ns = not significant.

group following CNO administration; GFP controls showed no change in breakpoint between vehicle and CNO sessions ($W = 143$, p -value = 0.3685), confirming that the effect was not attributable to CNO alone.

In the excitatory DREADD (hM3DGq) experiment, a linear mixed-effects model with treatment, group, and sex as fixed effects and animal as a random intercept revealed a significant main effect of treatment on progressive ratio breakpoint ($F(1, 52) = 4.583$, $p = 0.03699$). The treatment $\times$ group interaction did not reach significance ($F(1, 52) = 1.3613$, $p = 0.24864$), nor did any other main effects or interactions (all $p > 0.2$). Planned comparisons (Wilcoxon signed-rank test) showed that CNO significantly increased breakpoint relative to vehicle in the hM3DGq group ($n=19$, 10 males, 9 females) ($W = 82.5$, p -value = 0.0026, Cohen's $d = 1.026742$) whereas GFP controls ($n=9$, male = 3, female = 6) again showed no treatment-dependent change ($W = 40$, p -value = 1) (Fig. 1E). Together, these results demonstrate that manipulating activity of GABAergic cells in the ZI bidirectionally and causally modulates motivational effort.

Given that we saw a dramatic reduction in motivation after chemogenetically inhibiting GABAergic neurons in the ZI, we wanted to rule out confounding effects of locomotor capacity, memory, or homeostatic hunger drive. Chemogenetic inhibition of GABAergic neurons in the ZI did not alter total distance traveled in the open field ($F(3,37) = 1.082$, p -value = 0.369) (Fig. S1), had no effect on preference index in a memory task ($F(1,30) = 0.369$, p -value = 0.57025) (Fig. S2), and did not change food consumption ($W = 24.5$, p -value = 1) (Fig. S3). These data indicate that the observed changes in breakpoint reflect a specific modulation of motivational state rather than generalized alterations in motor output, cognitive function, or appetitive drive.

Chemogenetic activation of GABAergic neurons of the ZI rescues chronic stress-induced deficits in motivation

To determine whether activation of GABAergic ZI neurons is sufficient to rescue stress-induced deficits in motivation, we expressed excitatory DREADDs (AAV-hSyn-DIO-hM3DGq-mCherry) or control virus (AAV-hSyn-DIO-eGFP) in GABAergic neurons in the ZI of vGAT-CRE mice and assigned animals to one of three groups: no stress/GFP control ($n=7$, 3 males, 4 females), stress/GFP ($n=6$, 2 males, 4 females), and stress/hM3DGq ($n=8$, 4 males, 4 females) (Fig. 2A-C). Following recovery from surgery, mice in the stress/GFP and stress/hM3DGq groups were exposed to a chronic stress protocol concurrent with operant training on FR schedules (Fig. 2D). Critically, all three groups acquired the operant response at comparable rates, with no significant differences in reinforcements earned across training days, indicating that stress exposure did not impair task learning ($F(26) = 1.27$, $p > 0.05$) (Fig. S4).

A linear mixed-effects model was fitted with treatment (vehicle vs. CNO), group (control, stress/GFP, stress/hM3DGq), and sex as fixed effects and animal as a random intercept. The model revealed a significant main effect of treatment ($F(1, 15) = 10.89$, $p = 0.005$) and, critically, a highly significant treatment $\times$ group interaction ($F(2, 15) = 11.93$, $p < 0.001$), indicating that the effect of CNO on breakpoint differed across groups. A trending main effect of group was also observed ($F(2, 15) = 2.82$, $p = 0.091$), while sex and all sex-related interactions were non-significant (all $p > 0.69$). During the PR test under vehicle conditions, mice exposed to stress exhibited significantly lower breakpoints compared to non-stressed controls (stress/GFP vs no stress/GFP: $W=38$, p -value = 0.01399, Cohen's $d = 1.497416$; stress/ hM3DGq vs no stress/GFP: $W=53.5$, p -value = 0.003628, Cohen's $d = 1.740187$), confirming that chronic stress reduced motivational effort (Fig. 2E, left). However, upon CNO administration, hM3DGq mice exposed to stress showed a marked increase in breakpoint that was no longer significantly different from non-stressed controls ($W=23.5$, p -value = 0.6419), whereas stressed GFP mice still exhibited significant reduction in motivation ($W=36$, p -value = 0.03726, Cohen's $d = 1.264385$) (Fig. 2E, right). These findings demonstrate that chemogenetic activation of GABAergic neurons of the ZI is sufficient to rescue motivation following chronic stress.

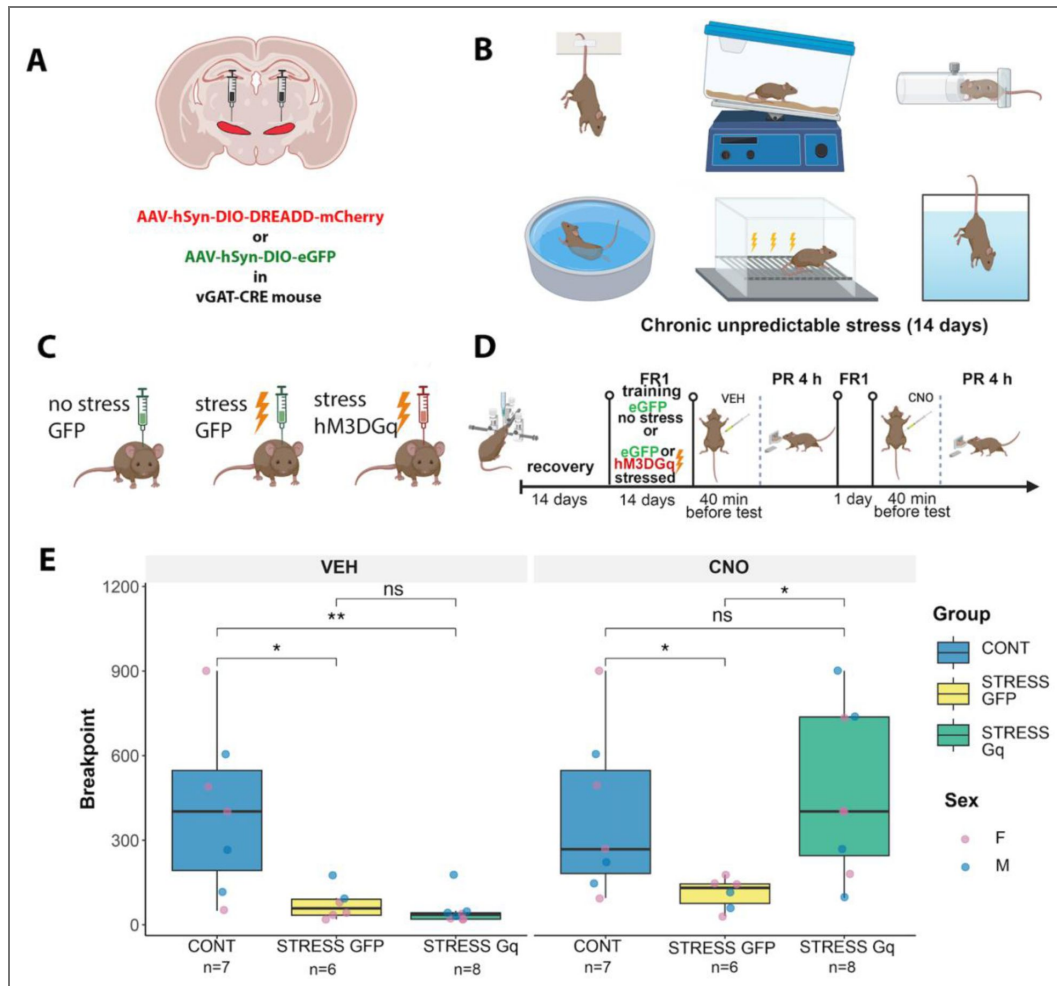


Figure 2. Chemogenetic activation of GABAergic ZI neurons rescues stress-induced deficits in motivation.

(A) Schematic of viral strategy: excitatory DREADDs (hM3DGq) or GFP control were bilaterally injected into the ZI of vGAT-Cre mice. (B) Schematic of the chronic unpredictable stress protocol. (C) Experimental groups: no stress/GFP control, stress/GFP, and stress/hM3DGq. (D) Experimental timeline showing concurrent stress exposure and operant training on FR schedules, followed by PR testing under vehicle and CNO conditions. (E) Breakpoint data under vehicle (left) and CNO (right) conditions. Under vehicle, both groups exposed to stress showed significantly reduced breakpoints compared to non-stressed controls. Following CNO administration, hM3DGq mice exposed to stress showed breakpoints no longer significantly different from controls. Whisker plots show median and interquartile range; individual data points are colored by sex. **p < 0.01, *p < 0.05, ns = not significant.

GABAergic neurons of the ZI fire in response to sensory stimuli across multiple modalities

Motivation is not a unitary process but rather a temporally extended sequence of responses: detection of reward-predictive cues, initiation of goal-directed action, execution of instrumental responses, and evaluation of reward outcomes. While our findings established GABAergic neurons in the ZI as regulators of motivated behavior, the specific components of motivation that these neurons govern were left unresolved. The ZI receives convergent sensory input from diverse brain regions. However, whether GABAergic neurons in the ZI respond to moments of intention, action, or sensory events during motivated behavior has not been directly examined. To dissect the contribution of the ZI to distinct phases of motivational processing, we expressed GCaMP in GABAergic neurons in the ZI of vGAT-CRE mice and performed fiber photometry recordings during operant behavior. Real-time calcium dynamics were recorded while animals performed an operant task on a modified FR1 schedule designed to isolate sensory, motor, and reward-related components of motivated behavior by disabling lever retraction after press and delivering reward after a delay (Fig. 3A). This design temporally separates neural responses associated with the instrumental action from those evoked by reward delivery.

During FR1 training, we observed robust calcium transients in GABAergic ZI neurons time-locked to both lever press and reward delivery, with a characteristic two-component response: an initial signal increase upon lever press followed by a sustained activation upon reward retrieval (Fig. 3B).

To assess whether the response of GABAergic ZI neurons was specific to reward delivery, we tested how these neurons respond to neutral sensory stimuli of different modalities. Mice naïve to the operant chamber were exposed to brief auditory and visual stimuli while calcium dynamics were recorded (Fig. 3C). GABAergic ZI neurons exhibited time-locked increases in z-scored $\Delta F/F$ in response to these sensory modalities, with responses emerging rapidly after stimulus onset and returning to baseline within approximately one second (Fig. 3D). Individual animal traces ($n=3$) confirmed the consistency of these responses across subjects (Fig. S5). These data reveal that GABAergic ZI neurons are broadly tuned to sensory input across modalities.

GABAergic neurons of the Zona Incerta encode the learned motivational valence of sensory cues

Given that GABAergic ZI neurons respond to sensory stimuli and are involved in regulation of motivated behavior, we next asked whether responses to sensory cues are modulated by the learned motivational significance of the cue. To test this, we employed a Pavlovian discrimination paradigm in which one visual cue (CS+; e.g., light on the right) was paired with reward delivery and a second visual cue (CS-; e.g., light on the left) was presented without reward, while recording calcium transients in GABAergic ZI neurons via fiber photometry (Fig. 4A, 4B).

GABAergic ZI responses were quantified as the area under the curve (AUC) of z-scored $\Delta F/F$ during the 1-second window following CS presentation. A three-way ANOVA examining the effects of CS, time (pre- vs post-training), and sex on GABAergic ZI activity revealed a significant CS $\times$ training interaction ($F(1, 28) = 5.66$, p -value = 0.025), with no significant interactions involving sex ($F(1, 28) = 0.482$, p -value=0.4932). Before conditioning, GABAergic ZI neurons responded comparably to both cues, consistent with the observed general sensory responsiveness ($W=33$, p -value = 0.5457) (Fig. 4C, top). However, after Pavlovian training, a striking divergence emerged: the CS+ elicited a larger calcium transient than the CS- ($W=65$, p -value = 0.03147, Cohen's $d=1.178327$) (Fig. 4C, bottom). Quantification of the responses confirmed that, before training, there was no significant difference in GABAergic ZI responses between the rewarded and non-rewarded visual cues, whereas after training, responses of GABAergic neurons of the ZI to the CS+ were significantly greater than those to the CS- (Fig. 4D). Behavioral evidence of successful discrimination was further supported by a progressive decrease in inter-response time (IRT) between light onset and detector entry selectively for the CS+ across training days (LME, p -value <

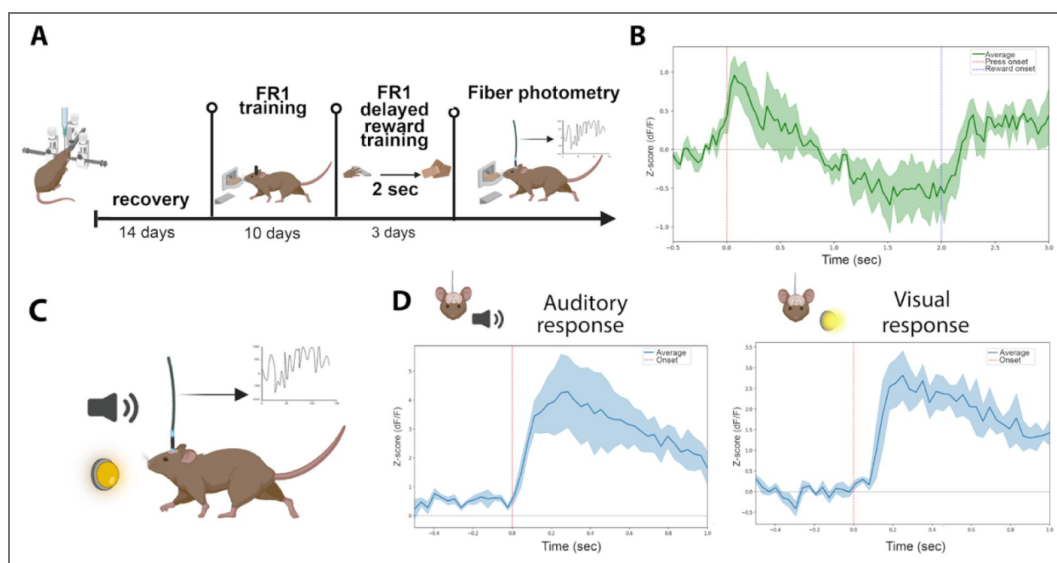


Figure 3. GABAergic neurons in the ZI respond to sensory stimuli across multiple modalities.

(A) Experimental timeline: vGAT-CRE mice expressing GCaMP in GABAergic neurons in the ZI underwent FR1 operant training followed by a modified FR1 task with delayed reward delivery, during which fiber photometry recordings were performed. **(B)** Mean z-scored calcium signal (dF/F) from GABAergic neurons in the ZI time-locked to lever press and reward delivery during the delayed-reward FR1 task. Shaded regions represent SEM. **(C)** Schematic of multimodal sensory stimulation paradigm in naive mice. **(D)** Mean z-scored calcium transients in ZI-located GABAergic neurons in response to auditory (left) and visual (right) stimuli, showing significant time-locked responses to both sensory modalities.

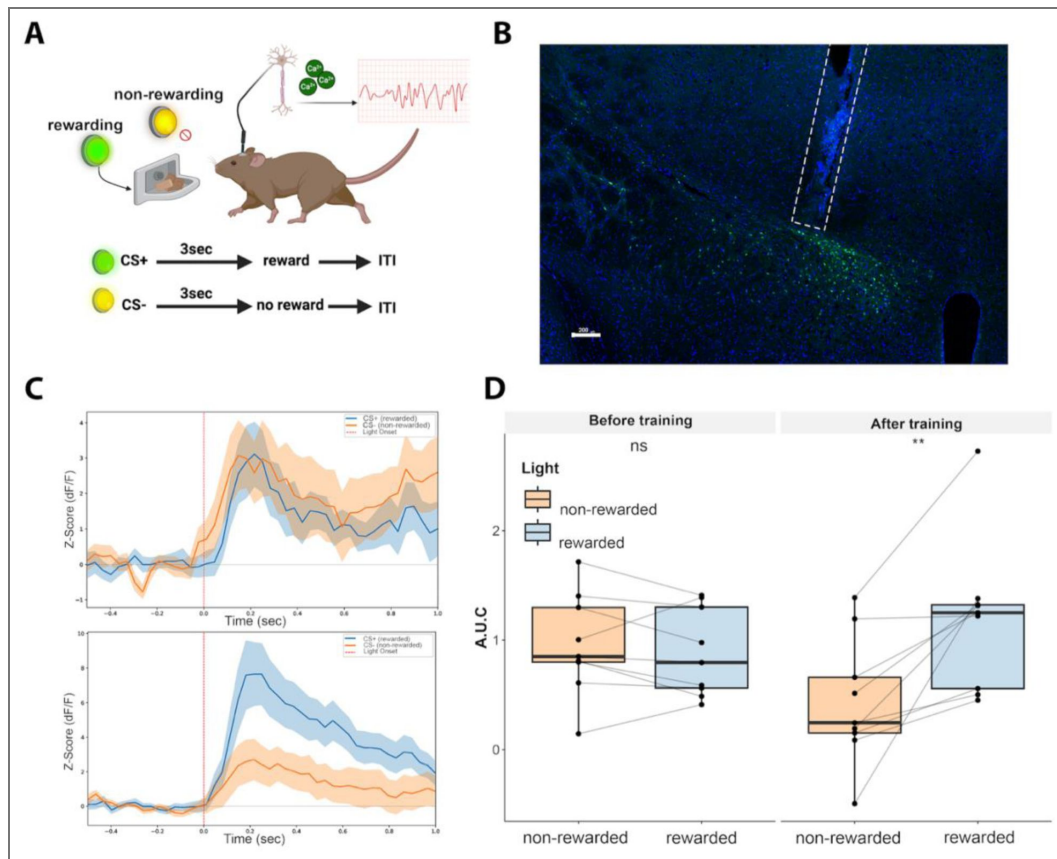


Figure 4. GABAergic ZI neurons encode the learned motivational valence of sensory cues.

(A) Schematic of Pavlovian discrimination paradigm: one visual cue (CS+) was paired with reward delivery and a second visual cue (CS-) was presented without reward, while calcium transients were recorded via fiber photometry. (B) Representative histological image showing DIO-GCaMP expression and fiber placement in the ZI. (C) Mean z-scored calcium traces in response to CS+ and CS- before training (top) and after training (bottom). Data represented as mean $\pm$ SEM (shaded area). Dashed red line indicates light onset. (D) Quantification of calcium responses (area under the curve of z-scored dF/F) to rewarded and non-rewarded cues before and after training ($n=9$, 5 males, 4 females). After training, responses to the CS+ were significantly greater than to the CS-. Data represented as boxplots (median, interquartile range, whiskers) with individual data points connected across conditions by lines. ** $p < 0.01$, ns = not significant.

0.01) (Fig. S6 [↗](#)). These results demonstrate that GABAergic ZI neurons do not merely relay sensory information but dynamically update their responses to reflect the acquired motivational valence of environmental cues.

Inhibition of GABAergic ZI neurons does not suppress reward-associated conditioned responding

Our photometry data revealed that ZI GABAergic neurons encode the learned valence of reward-predictive cues. We therefore asked whether silencing these neurons would disrupt the ability of a conditioned stimulus to evoke goal-directed responding. vGAT-CRE mice expressing inhibitory DIO-DREADDs (Gi) or DIO-GFP control virus in the GABAergic neurons of the ZI underwent Pavlovian conditioning in which an auditory cue (CS+) was paired with reward and a visual cue (CS-) was unreinforced (Fig. 5A-B [↗](#)). Following conditioning during which all groups acquired operant training (Fig. S7 [↗](#)), mice were trained on an FR1 operant schedule and subsequently underwent an operant probe test in which the CS+ and CS- were presented in alternating blocks without reinforcement upon CNO condition (Fig. 5C [↗](#)).

A linear mixed-effects model examining pressing rate during the probe test under CNO condition, with block, group (Gi vs GFP), and sex as fixed factors and animal as a random effect revealed significant block $\times$ group interaction ($F(4, 104) = 9.66$, $p < 0.001$), indicating that the two groups responded differently across block types. There were no significant interactions involving sex (all $p > 0.41$). During the probe test, GFP controls exhibited a progressive decline in pressing rate over successive blocks, with no differential response to CS+ versus CS- presentations ($W = 73.5$, $p\text{-value} = 0.4094$) (Fig. 5C [↗](#)). In contrast, the Gi DREADD group, despite an expectedly low initial pressing rate during acclimation, showed selective increase of motivated behavior during CS+ blocks, with pressing rates significantly elevated relative to both acclimation and CS- blocks (when averaged across presentations, CS+ vs acclimation: $W = 261.5$, $p\text{-value} = 0.0009$; CS+ vs CS-: $W = 246$, $p\text{-value} = 0.007$) (Fig. 5C [↗](#)). These data indicate that, although chemogenetic inhibition of GABAergic ZI neurons reduces the general motivational drive to work for reward (Fig. 1D [↗](#)), it does not abolish previously established cue-reward associations or the capacity of conditioned stimuli to selectively enhance instrumental responding.

Temporally precise optogenetic activation of GABAergic neurons of the ZI during reward-predictive cues is sufficient to enhance motivation

To establish a causal link between sensory-evoked ZI activity and motivational drive with temporal precision, we employed optogenetic stimulation of GABAergic ZI neurons time-locked to reward-predictive sensory cues during progressive ratio testing. Following Chr2 (AAV5-Ef1a-DIO hChr2(E123T/T159C)-EYFP) injection and unilateral surgical implantation of an optical fiber above the ZI (Fig. 6A [↗](#)), mice were first trained on FR1 - one lever press gives one reward, then on a contingent FR1 schedule in which a visual cue (light) signaled reward availability (Fig. 6B [↗](#)). During this contingent FR1 schedule, the lever was available to be pressed throughout the entire session, but presses were reinforced with reward only during light-on periods (each 10 seconds long). After acquiring the contingent association, evidenced by progressively higher pressing rates during light-on versus light-off periods (Fig. 6C [↗](#)), mice were tested on a PR schedule for an hour under three counterbalanced conditions: optogenetic stimulation coincident with the light cue, temporally random stimulation, and no stimulation.

In female mice, contingent optogenetic stimulation time-locked to the reward-predictive light cue significantly increased total lever presses on the PR schedule compared to both the no-stimulation and random stimulation conditions (stim at light vs random stim: $V = 0$, $p\text{-value} = 0.01563$; stim at light vs no stim: $V = 2$, $p\text{-value} = 0.04688$) (Fig. 6D, left [↗](#)). Notably, random stimulation did not significantly increase lever pressing relative to the no stimulation condition ($V = 9$, $p\text{-value} = 0.4688$), demonstrating that the enhancement of motivational output required temporal coincidence between GABAergic ZI activation and the sensory cue rather than generalized

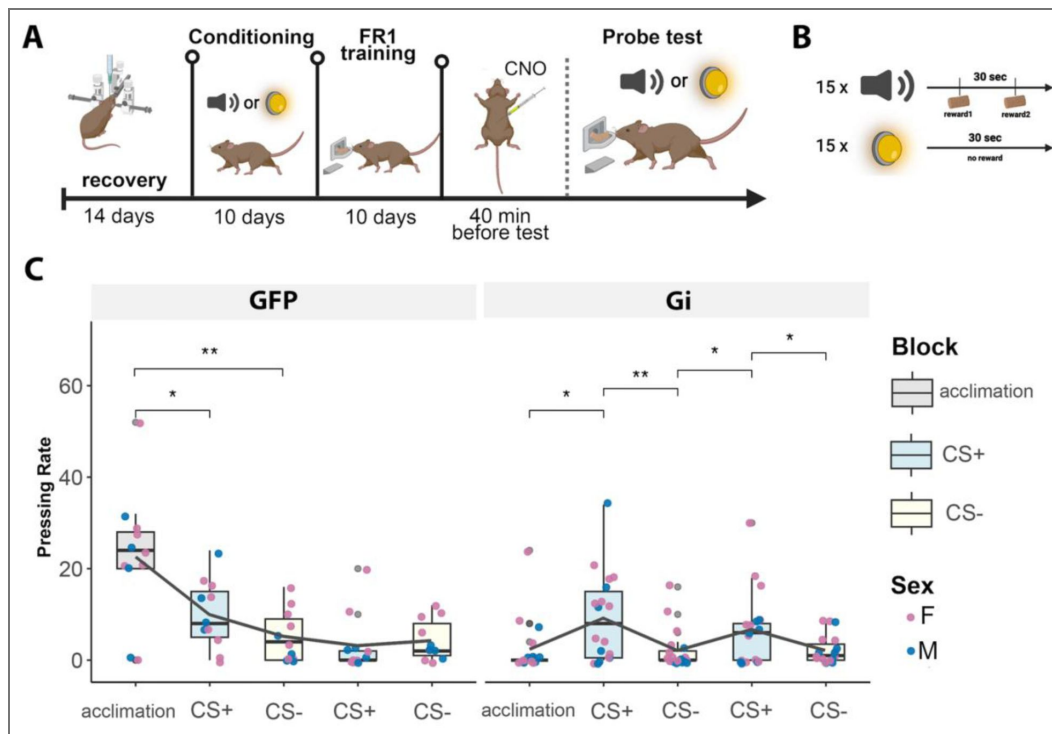


Figure 5. Inhibition of GABAergic ZI neurons does not suppress reward-associated conditioned responding.

(A) Experimental timeline: vGAT-CRE mice expressing inhibitory DIO-DREADDs (Gi) or DIO-GFP control underwent Pavlovian conditioning, FR1 operant training, and an operant probe test with CS presentations under CNO. (B) Pavlovian conditioning design: an auditory cue (CS+) was paired with reward and a visual cue (CS-) was unreinforced. (C) Pressing rate during the probe test across alternating CS+, CS-, and acclimation time blocks for GFP controls (left) and Gi DREADD mice (right). GFP controls showed a progressive decline in pressing with no differential response to CS+ versus CS-. Gi DREADD mice showed selective increases in pressing rate during CS+ blocks relative to both acclimation and CS-blocks. (Gi: n=18, 7 males, 11 females; GFP: n=11, 4 males, 7 females). Comparisons tested: acclimation: CS+ (first presentation); acclimation: CS- (first presentation); CS+ (first presentation): CS- (first presentation); CS+ (first presentation): CS- (second presentation); CS+ (second presentation): CS- (second presentation). **p < 0.01, *p < 0.05. Only significant comparisons are shown. Data represented as boxplots (median, interquartile range, whiskers) with individual data points colored by sex and group mean connected by lines.

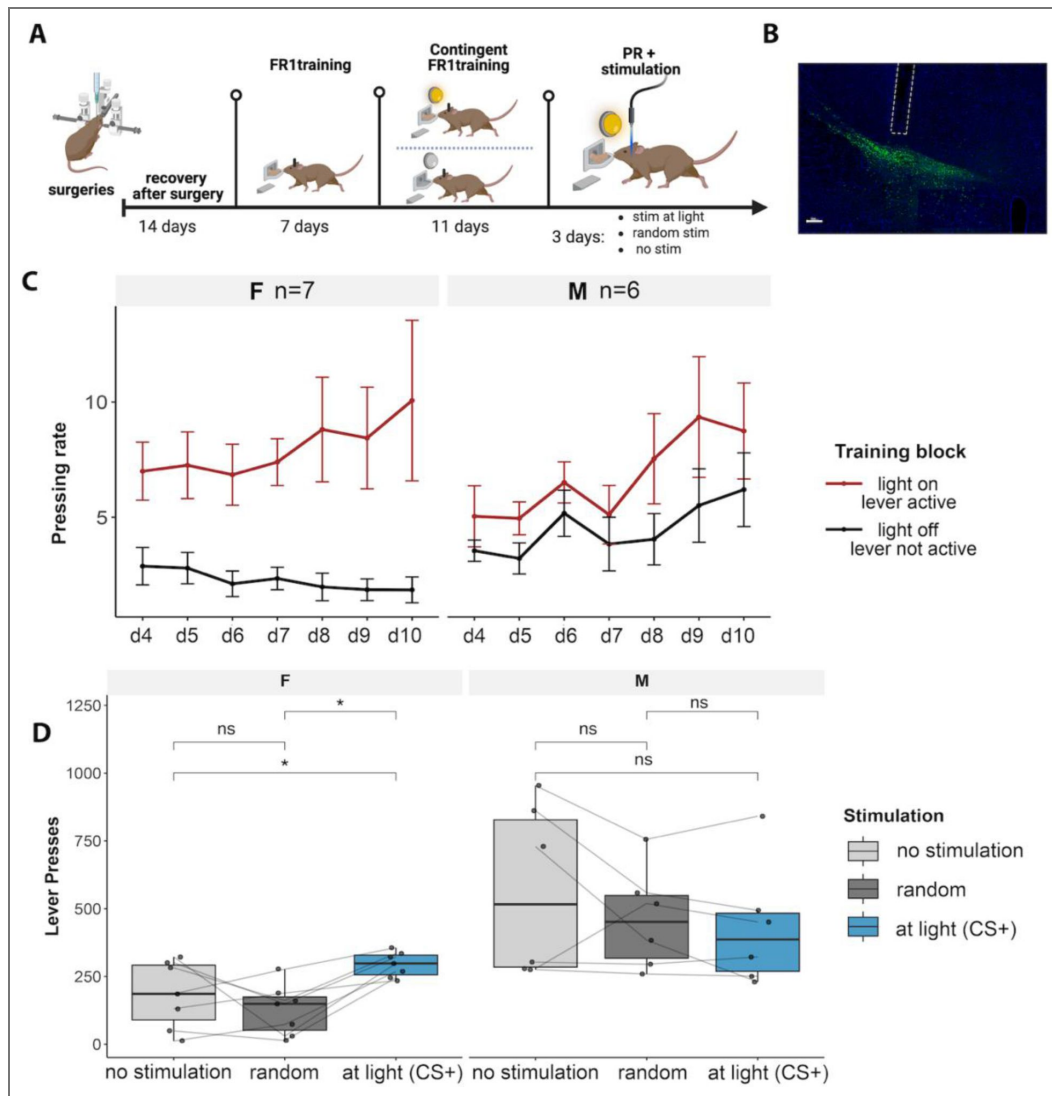


Figure 6. Temporally precise optogenetic activation of GABAergic ZI neurons during reward-predictive cues enhances motivation.

(A) Representative histological image showing DIO-ChR2 expression and fiber placement in the ZI. **(B)** Experimental timeline: vGAT-Cre mice expressing ChR2 in the ZI underwent FR1 training, contingent FR1 training with a visual cue signaling reward availability, and PR testing under three counterbalanced stimulation conditions. **(C)** Pressing rates during light on (lever active) and light off (lever not active) periods across contingent FR1 training days for females (left) and males (right), showing acquisition of cue-contingent responding. **(D)** Total lever presses on the PR schedule under no stimulation, random stimulation, and stimulation time-locked to the light cue (CS+) for females (left) and males (right). In females, contingent optogenetic stimulation significantly increased lever pressing compared to both random and no stimulation conditions. This effect was not observed in males. Data represented as boxplots (median, interquartile range, whiskers) with individual data points, * $p < 0.05$, ns = not significant.

excitation. To rule out the possibility that these effects were driven by order or practice effects rather than stimulation condition, we reanalyzed the data by testing day rather than stimulation pattern (Fig. S8 [↗](#)).

This effect was not observed in male mice, for whom no stimulation condition significantly altered PR performance ($V = 10$, $p\text{-value} = 1$) (Fig. 6D, right [↗](#)). To explore the basis of this sex difference, we examined behavioral strategies during training. Female mice progressively suppressed responding during light-off periods across training, developing a clear discrimination between cue-on and cue-off states ($W = 45$, $p\text{-value} = 0.004$) (Fig. 6C, left [↗](#)). Male mice, by contrast, maintained substantial responding during both light on and light off periods throughout training, with no significant trend of divergence between conditions ($W = 20$, $p = 0.06$) (Fig. 6C, right [↗](#)), despite exhibiting comparable learning trajectories to females as measured by decreasing inter-trial response rates between cue presentation and lever press (sex:day $F(6,183) = 0.6540$, $p\text{-value} = 0.6868$, and increasing success rate (proportion of retrieved reward) over sessions (sex:day $F(6,36) = 2.0355$, $p\text{-value} = 0.08606$, with a trend of lower success rate in females) (Figs. S9 and S10 [↗](#)).

Discussion

The brain must continuously evaluate which environmental stimuli require attention and action. This computation requires more than reward prediction alone. While dopaminergic signaling is critical for encoding reward value and prediction errors (Coddington & Dudman, 2018 [↗](#); Schultz, 2015 [↗](#)), it is insufficient to explain how the nervous system responds to salient cues in stochastic sensory environments and translates those cues into appropriate motivational responses. Here, we first identified GABAergic cells in the ZI as a subcortical cell population that are both necessary and sufficient to modulate effort-based motivation and their stimulation being sufficient to rescue stress-induced deficits in motivation. Next, we determined that these cells function as sensory-motivational integrators. They respond to multi-modal environmental cues, accumulate the significance of these cues over time and are sufficient but not necessary to drive cue-associated motivational output.

Our chemogenetic manipulation of the activity of GABAergic neurons while working for food rewards during progressive ratio testing established that these neurons are both necessary and sufficient for effort-based motivational effort. Inhibition of these neurons reduced progressive ratio breakpoints with large effect sizes, while stimulation enhanced the willingness to press levers for reward. Critically, these effects were specific to motivational state: inhibiting their activity did not alter locomotion, memory, or food consumption, ruling out confounds that the lower breakpoints were a result of movement and memory deficits or satiation. This specificity distinguishes the ZI from nodes such as the ventral tegmental area or nucleus accumbens, where manipulations frequently produce intertwined changes in motor output and hedonic reactivity (Hughes et al., 2020 [↗](#); Kim et al., 2012 [↗](#)). The bidirectional control observed suggests that tonic GABAergic ZI activity sets a gain on motivational drive, with the level of ZI GABAergic output scaling the willingness to exert effort for reward (Fig. 1D, 1E [↗](#)). These findings align with converging evidence that ZI GABAergic neurons promote appetitive and investigatory behaviors across multiple contexts. For example, optogenetic activation of ZI GABAergic neurons strongly promoted hunting of both live and artificial prey, while photoinhibition or deletion of GABA function severely impaired hunting (Ahmadlou et al., 2021 [↗](#); Zhao et al., 2019 [↗](#)), indicating that the motivational role of ZI GABAergic signaling extends beyond operant effort to encompass a broad repertoire of goal-directed appetitive behaviors.

With stress-induced deficits in motivation being an endophenotype of depression (Russo & Nestler, 2013 [↗](#); Treadway & Zald, 2011 [↗](#)), the rescue of motivational deficits following chronic unpredictable stress via chemogenetic activation of GABAergic ZI neurons carries both mechanistic and translational significance. This result is noteworthy given that a substantial proportion of patients with depression do not respond to conventional monoaminergic treatments (Al-harbi, 2012 [↗](#); Berlim & Turecki, 2007 [↗](#)). The rescue of stress-induced deficits in motivation by modulating activity of a GABAergic node suggests the existence of parallel motivational pathways

that may be therapeutically accessible when dopaminergic or serotonergic interventions fail. Notably, a GABAergic mechanism for stress-induced anhedonia has precedent. GABAergic, rather than dopaminergic regulation of the VTA is able to mediate depression-like symptoms (Lowes et al., 2021), establishing that non-monoaminergic inhibitory circuits can be causal drivers of motivational impairment under stress. Our finding that ZI activation rescues stress-induced deficits suggests a parallel, non-monoaminergic route for restoring motivation. The ZI's position as a convergence point for diverse sensory inputs and its long-range inhibitory projections to cortical and subcortical targets (Béreau et al., 2023; Hormigo et al., 2020; Wang et al., 2020; Wilt et al., 2025) make it a particularly attractive candidate for circuit-based interventions in treatment-resistant anhedonia and apathy.

Fiber photometry recordings revealed that GABAergic ZI neurons are not passive sensory relays. These neurons responded to stimuli across auditory and visual modalities, consistent with the known multimodal convergent input architecture of the ZI (Arena et al., 2024; Wang et al., 2020). However, the critical observation was that initially neutral cues acquired the ability to drive differential ZI responses only after Pavlovian conditioning: CS+ cues elicited significantly larger calcium transients than CS- cues after, but not before, training (Fig. 4D). This learning-dependent divergence demonstrates that ZI neurons encode the acquired motivational significance of stimulus salience rather than its physical properties. This finding positions the ZI as a node where sensory representations are transformed into motivational signals, a computation that is essential for adaptive behavior. While the amygdala and orbitofrontal cortex are known to encode cue-outcome associations (Herry & Johansen, 2014; Paton et al., 2006; Schoenbaum et al., 2009), the ZI's role is distinct in that it operates through long-range GABAergic projections capable of directly suppressing activity in downstream targets. Importantly, this role of the ZI in decoding stimulus valence through sensory integration has been corroborated across modalities and behavioral contexts. Visual or whisker sensory deprivation reduced ZI calcium responses to prey and impaired hunting, yet ZI photoactivation largely corrected these sensory deprivation-induced deficits, demonstrating that ZI activity can substitute for degraded sensory input to maintain motivated behavior. In novelty-seeking contexts, optogenetic activation of medial ZI GAD2 neurons dramatically increased interaction duration with novel objects and conspecifics relative to familiar stimuli, elevating arousal levels and deepening investigatory action sequences (Ahmadlou et al., 2021). Strikingly, this discriminative function of the ZI appears to emerge early in development and extends into adulthood. Recent work in pre-weaning pups identified a defined ZI – SST population that integrates multimodal dam-associated cues and selectively responds during mother-infant social interactions (Li et al., 2024). In adulthood, the ZI contributes to sensory discrimination and modulation of sensory information in fear contexts (Venkataraman et al., 2019, 2021). These findings collectively show that the ZI is capable of encoding the learned value of specific stimulus classes and converting them into motivational and state changes.

The optogenetic experiments revealed a surprising and instructive pattern: cue-locked stimulation of GABAergic cells in the ZI enhanced motivation specifically in animals whose behavior was under strong cue control. Female mice, which developed robust discrimination between cue-on and cue-off periods during contingent training, showed significant enhancement of progressive ratio performance when optogenetic stimulation was time-locked to the reward-predictive cue but not when stimulation was delivered randomly. Male mice, who maintained high response rates regardless of cue state, showed no significant effect of any stimulation pattern. This strategic difference is critical for interpreting the optogenetic results. Females developed strong behavioral contingency on the cue, effectively gating their instrumental responding to periods of cue availability. For males, activating GABAergic ZI neurons during cue presentation may not have significantly altered behavior because their pressing was already less contingent on cue information, and they engaged in high levels of operant responding throughout the session regardless of cue presence. Together, these findings demonstrate that activation of GABAergic ZI neurons at the precise moment of a reward-predictive sensory cue is sufficient to boost motivational effort, and that this effect depends not only on the temporal alignment of neural activation with cue processing but also on the strength of the behavioral contingency between cue and action, as revealed by the sex difference in cue-gated responding. This interaction between ZI

activation and behavioral strategy suggests that the ZI does not simply add a fixed motivational boost. Rather, it amplifies the signals that are currently controlling action selection. This finding resonates with theoretical frameworks proposing that motivation arises from the interaction of internal states with environmental information (Puglisi-Allegra & Ventura, 2012), and provides a circuit-level mechanism for how this interaction might be implemented. The dependence of ZI effects on behavioral strategy is also consistent with work by Berridge and Warlow (Warlow & Berridge, 2021), who demonstrated that the same circuit node - in their case, the central amygdala - can generate divergent motivational outputs depending on context and the strategy an animal adopts. Our data extend this principle to the ZI, suggesting that the motivational impact of subcortical circuit activation is not fixed but is shaped by the behavioral policy through which an animal engages with environmental cues. The sex difference we observed likely reflects divergent behavioral strategies rather than a categorical sex difference in ZI function per se, as both sexes showed comparable learning trajectories. Nevertheless, this finding highlights the importance of considering individual variation in behavioral strategy when interpreting circuit manipulation experiments and may have implications for understanding sex differences in motivation-related psychopathology.

A seemingly paradoxical finding was that chemogenetic inhibition of GABAergic ZI neurons did not suppress reward-associated conditioned responding. Instead, Gi DREADD mice showed selective increases in pressing during CS+ blocks relative to both acclimation and CS-periods, while GFP controls exhibited a progressive, non-discriminative decline. This dissociation suggests that the ZI operates within a redundant motivational network. When ZI-mediated drive is attenuated, alternative circuits capable of supporting cue-reward associations, such as amygdala-to-nucleus accumbens, thalamo-striatal, or cortico-striatal pathways (Jennings et al., 2013; Zorrilla & Koob, 2013), may be released from ZI-mediated inhibition or may compensate to maintain cue-directed behavior (Fig. 5D). This interpretation also aligns with evidence that VTA GABA neurons can themselves disrupt responding to reward-predictive cues (Wakabayashi et al., 2019), underscoring that cue-driven behavior is supported by multiple inhibitory nodes whose contributions can dissociate under perturbation. This architectural redundancy is consistent with the evolutionary importance of maintaining motivated behavior: a system critical for survival would not depend on a single node. It also suggests that the ZI's normal role may involve integrating and prioritizing among multiple motivational signals, with its tonic inhibitory output serving to suppress competing or contextually inappropriate responses. When this inhibitory regulation is removed, previously gated cue-reward associations may gain unimpeded access to motor output.

While our work makes a compelling case for the ZI to be included in existing neuroanatomical discussions of effort-based motivation and cue-associated motivational salience, some limitations of our work bear mentioning. Our chemogenetic manipulations targeted all GABAergic ZI neurons, and emerging evidence suggests molecular heterogeneity within this population that may map onto distinct functional roles (Arena et al., 2024; Wilt et al., 2025). Future work to first profile the molecular heterogeneity of GABAergic cells in the ZI and then using intersectional strategies to target defined ZI sub-populations will be essential to providing a more nuanced view of ZI GABAergic influences on motivation. Our fiber photometry recordings captured aggregate population activity, and single-cell resolution approaches will be needed to determine whether individual ZI neurons encode appetitive salience, aversive salience, or both. The sex difference in the optogenetic experiment warrants further investigation with larger cohorts and additional behavioral protocols to determine whether it reflects a genuine sex difference in ZI circuit function or a difference in cue-utilization strategies that may vary across task contexts. Finally, identifying the specific downstream targets through which the ZI exerts its motivational effects will be critical for building a complete circuit model.

This work establishes the GABAergic Zona Incerta as a sensory-motivational integration hub that transforms multimodal sensory information into motivational signals. The ZI does not merely relay sensory information but computes motivational salience - the learned significance of stimuli for guiding adaptive behavior. Through bidirectional control of motivational drive, encoding of

learned cue valence, state-dependent amplification of cue-driven behavior, and operation within a redundant motivational network, the ZI solves a fundamental problem in adaptive behavior: how the brain translates environmental stimuli into appropriate motivational responses. That activation of this GABAergic node is sufficient to rescue stress-induced motivational deficits opens a new avenue for understanding and potentially treating disorders of motivation through non-dopaminergic circuit mechanisms. Through this work, the “zone of uncertainty” is revealed as a hub where sensory signals acquire meaning and environmental cues are translated into the drive to act.

Materials and methods

Animals

vGAT-IRES-CRE mice (Slc32a1^{tm2(cre)}Lowl/J, Strain #:016962, Jackson) were maintained on a C57BL/6J-Aw-J/J B6 agouti background. Mice were 8–10 weeks old at the time of stereotaxic surgery and were group-housed on a 12:12 h light/dark cycle with *ad libitum* access to water. For operant behavioral experiments, mice were food-restricted to maintain 90–93% of their free-feeding body weight. Both male and female mice were included in all experiments, and sex was examined as a biological variable in all analyses. The number of mice used in experiments are listed in [Supplementary Table 1](#). All experiments were approved by the Institutional Animal Care and Use Committee of Children’s Hospital Los Angeles in accordance with the National Institutes of Health Guide and followed NIH standards.

Viral Vectors

The following adeno-associated viral (AAV) vectors were used: AAV5-hSyn-DIO-DREADD-mCherry (inhibitory hM4DGi: Addgene # 44362-AAV5, excitatory hM3DGq: Addgene # 44361-AAV5), AAV5-hSyn-DIO-EGFP (Cre-dependent GFP control: Addgene # 50457-AAV5), AAV5-Syn-FLEX-GCaMP8f (for fiber photometry: Addgene # 162379-AAV5), and AAV5-Ef1a-DIO-hChR2(E123T/T159C)-EYFP (for optogenetics: Addgene # 35509-AAV5). All viruses had titers of $\geq 1 \times 10^{12}$ vg/mL.

Stereotaxic Surgery

Mice received analgesia (meloxicam (Metacam), 1.5 mg/ml, 1 unit, PO) preoperatively. Next, they were anesthetized with isoflurane (1–2% in O₂, induction at 3%), and placed in a motorized stereotaxic frame (Stoelting 51730M). Bilateral injections targeting the zona incerta (ZI) were made at the following coordinates relative to bregma: AP -1.52, ML ± 0.74 , DV -4.68. A volume of 80 nL of virus was delivered per hemisphere at a rate of 40 nL/min using a Nanoject III. The injection needle was left in place for 10 minutes following injection to minimize backflow and then slowly withdrawn. For fiber photometry and optogenetic experiments, a 200 μ m optical fiber (0.37 NA; Neurophotometrics) was implanted unilaterally above the ZI at DV -4.6mm and cemented in place with GC FujiCEM 2. Mice were administered oral meloxicam perioperatively and allowed to recover for 14 days before behavioral testing.

Chemogenetic Manipulation

Clozapine-N-oxide (CNO; Sigma-Aldrich # C0832-5mg) was dissolved in 2% DMSO sterile saline and administered intraperitoneally at a dose of 1.5 mg/kg 40 minutes prior to testing. Vehicle injections consisted of an equivalent volume of 2% DMSO sterile saline. All mice received both vehicle and CNO injections in a within-subjects with a minimum of 1 day between test sessions.

Operant Conditioning

Operant conditioning was performed in standard mouse operant chambers (Harvard Apparatus) equipped with two retractable levers, a food pellet dispenser and magazine, a house light, and lights above the levers. Reinforcers were 20 mg grain-based dustless precision food pellets (Bio-Serv). Behavioral events were recorded and controlled by Panlab PACKWIN software.

Fixed-Ratio (FR) Operant Training

Following food restriction, mice were trained on a fixed-ratio 1 (FR1) schedule in which a single press on the active lever resulted in delivery of one food pellet. Sessions lasted 40 minutes or until a maximum number of reinforcements (40) was earned. Training continued for 14 days. The inactive lever was retracted. Mice were considered to have acquired the operant response when they earned ≥ 30 reinforcers in a session for at least 3 consecutive days.

Progressive Ratio (PR) Testing

Following acquisition of operant responding, mice were tested on a progressive ratio schedule in which the response requirement for each successive reinforcer increased according to a geometric progression (defined as $n_j = 5e^{(j/5)} - 5$) (Killeen et al., 2009 [↗](#)). Sessions lasted 4 hours or until the mouse stopped pressing for 5 minutes. The breakpoint was defined as the highest ratio completed (i.e., the last ratio at which the animal earned a reinforcement).

Chronic Unpredictable Stress Protocol

For the stress rescue experiment, a subset of mice was exposed to a 14-day chronic unpredictable stress (CUS) protocol concurrent with FR operant training (Willner, 2017 [↗](#)). The CUS protocol consisted of repeated exposure to varying stressors including water submersion (7 seconds, 3 exposures), forced swimming (3 minutes, 2 exposures), restraint (1h, 3 exposures), cage tilt (3h, 3 exposures), tail suspension (5 min, 2 exposures) and mild footshock (0.05mA, 1 exposure). Stressors were presented in a pseudorandom order such that animals could not predict the next stressor. Non-stressed control animals were handled daily but otherwise remained undisturbed in their home cages. FR1 training sessions began 30 minutes after the end of each stressor. Following CUS and operant training, all groups underwent PR testing under vehicle and CNO conditions as described above.

Open Field Test

To assess locomotor activity, mice were placed in an open field white plexiglass arena (70 × 70 × 50 cm) and allowed to explore freely for 10 minutes. Total distance traveled (cm) was recorded by an overhead camera and quantified using LimeLight software (ActiMetrics). Mice received either vehicle or CNO 40 minutes prior to testing.

Memory Test

To evaluate whether chemogenetic inhibition of ZI GABAergic neurons affected memory for the operant contingency, mice were trained on an FR1 schedule in which two levers were available but only one was active (a single press resulted in reinforcement delivery). The correct lever index, defined as the proportion of presses on the previously rewarded lever relative to total lever presses, was calculated following vehicle or CNO administration.

Food Consumption Test

To assess whether changes in breakpoint reflected alterations in appetitive drive, a food consumption test was conducted. Following CNO administration, food-restricted mice previously trained on the FR1 schedule were placed in the operant chamber. Each visit to the food dispenser resulted in pellet delivery. The total number of pellets consumed was recorded.

Fiber Photometry

Fiber photometry recordings were performed using Neurophotometrics FP300 system. GCaMP fluorescence was excited at 470 nm (calcium-dependent signal) and 415 nm (isosbestic control). Excitation light was delivered through a 200 μm optical fiber patch cord (0.37 NA) connected to the implanted fiber via a ceramic sleeve. Emitted fluorescence was collected through the same fiber. Signals were digitized at 30 Hz and recorded using Bonsai software. The isosbestic signal was used

to correct for motion artifacts and photobleaching. Calcium transients were expressed as z-scored $\Delta F/F$ and quantified as the area under the curve (AUC) during the 1-second window following CS presentation.

Delayed-reward FR1 task

To temporally dissociate lever-press and reward-delivery responses, mice were tested on a modified FR1 schedule in which the lever did not retract following a press and reward delivery occurred after a 2-second delay. Fiber photometry recordings were performed throughout the session, and calcium signals were time-locked to lever press and reward delivery events. Timestamps for synchronization were obtained via BNC connectors between the operant chambers and the fiber photometry system. Mice were tested individually.

Multimodal sensory stimulation

Naïve mice (not previously exposed to operant contingencies) were placed in the operant chamber and exposed to brief auditory (sound of lever retraction) and visual (2-s illumination of the above lever light) stimuli while calcium dynamics were recorded. Stimuli were delivered in a pseudorandom order with a variable inter-trial interval of 20–40 s.

Pavlovian Discrimination Conditioning

Mice expressing GCaMP in ZI GABAergic neurons underwent a Pavlovian discrimination protocol in which one visual cue (CS+; light on the right side) was paired with food reward delivery and a second visual cue (CS−; light on the left side) was presented without reward. Each conditioning session consisted of 15 CS+ trials and 15 CS− trials presented in a pseudorandom order. Each CS was presented for 3 seconds, followed by reward delivery (CS+ only) and a variable inter-trial interval. Calcium transients were recorded via fiber photometry before training (first session) and after 7 days of conditioning. Responses were quantified as the area under the curve (AUC) of z-scored $\Delta F/F$ during the 1-second window following CS presentation.

Pavlovian Conditioning with Chemogenetic Inhibition

vGAT-CRE mice expressing inhibitory DIO-DREADDs (hM4DGi) or DIO-GFP control virus in ZI GABAergic neurons underwent Pavlovian conditioning in which an auditory cue (CS+, 6kHz, 70 dB) was paired with reward and a visual cue (CS−) was unreinforced. Following 10 days of conditioning, mice were trained on an FR1 operant schedule for 10 days. Mice then received CNO (0.3 mg/kg, i.p.) 40 minutes prior to a probe test in which CS+ and CS− were presented in alternating blocks without reinforcement. Pressing rate was recorded during each block type (CS+, CS−, and acclimation periods).

Optogenetic Stimulation Experiment

Viral expression and fiber implantation

vGAT-CRE mice received bilateral injections of AAV5-Ef1a-hChR2(H134R)-EYFP into the ZI, and unilateral optical fibers were implanted above the injection sites as described above.

Contingent FR1 training

After initial FR1 training (7 days), mice were transitioned to a contingent FR1 schedule in which a visual cue (light) signaled reward availability. The lever was available throughout the session, but lever presses were reinforced only during 10-second light-on periods. Light-off periods served as a signal that reward was unavailable.

PR testing with optogenetic stimulation

Following contingent FR1 training, mice were tested on a PR schedule for 1 hour across three counterbalanced sessions: (1) optogenetic stimulation time-locked to the reward-predictive light cue (CS+), (2) temporally random stimulation, and (3) no stimulation. Optical stimulation

parameters were: power 5–7 mW; frequency 20 Hz; pulse width 10 ms; duration 10 s. The order of conditions was counterbalanced across mice. Total lever presses served as the primary dependent measure.

Histology

Following the completion of behavioral experiments, mice were deeply anesthetized with an intraperitoneal injection of a ketamine/dexmedetomidine (Dexdomitor) cocktail (ketamine 100 mg/kg; dexmedetomidine 0.5 mg/kg) and, upon confirmation of loss of pedal and corneal reflexes, transcardially perfused with PBS followed by ice-cold 4% paraformaldehyde (PFA) in PBS. Brains were extracted, post-fixed in 4% PFA for 24 hours at 4°C, and cryoprotected in 30% sucrose in PB for 48 h. Coronal sections of 40 µm were cut on a cryostat and mounted on glass slides. Viral expression (mCherry, eGFP, GCaMP, or Chr2-EYFP reporter fluorescence) and fiber placement were verified by fluorescence microscopy. Animals with off-target viral expression or fiber placements outside the ZI were excluded from analyses.

Quantification and Statistical Analyses

All statistical analyses were performed using R (version 2022.07.1 Build 554). Data are presented as median and interquartile range unless otherwise noted. A two-way ANOVA with treatment:group and sex as factors and Wilcoxon rank sum test were used to assess effects, with Cohen's d reported as a measure of effect size.

For the Pavlovian probe test with chemogenetic inhibition, a linear mixed-effects model was fitted with block (CS+, CS-, acclimation), group (Gi vs. GFP), and sex as fixed factors and animal as a random effect, examining pressing rate as the dependent variable. Post-hoc comparisons between block types within groups were performed using Wilcoxon signed-rank tests.

For the optogenetic experiment, within-subjects comparisons of total lever presses across the three stimulation conditions (cue-locked, random, no stimulation) were performed separately by sex using Wilcoxon signed-rank tests. Inter-response times and success rates across training days were analyzed using repeated-measures ANOVAs with sex and day as factors.

Photometry data were processed using custom Python scripts (Python 3.x; NumPy, SciPy, Pandas). The first 1 second and final second of each recording were removed to eliminate edge artifacts, and frames were trimmed to ensure equal samples per channel. The 470 nm and 415 nm signals were deinterleaved. To correct for photobleaching and motion artifacts, a biexponential decay function was fit to the 415 nm isosbestic signal using nonlinear least-squares optimization (`scipy.optimize.curve_fit`); in cases where the biexponential fit did not converge, a 4th-degree polynomial was used as a fallback. The fitted isosbestic signal was then aligned to the 470 nm signal using iterative robust linear regression. The corrected signal ($\Delta F/F$) was calculated as $100 \times (F_{470} - F_{\text{fitted}}) / F_{\text{fitted}}$, where F_{fitted} is the robustly fitted isosbestic prediction. $\Delta F/F$ values were then z-scored across the entire session ($z = (\Delta F/F - \text{mean}) / \text{SD}$).

Behavioral events (lever press, reward delivery, or stimulus onset) were identified from TTL digital input transitions (0 → 1). For each event, a peri-event segment was extracted from the z-scored trace spanning a 0.5–1 s pre-event baseline window and a 1–2 s post-event window (exact windows varied by experiment: 1 s baseline / 2 s post-event for the delayed-reward FR1 task; 0.5 s baseline / 1 s post-event for sensory stimulation and Pavlovian discrimination experiments). Each segment was baseline-normalized by subtracting the mean z-scored $\Delta F/F$ of the pre-event window. Individual trial traces were aligned to event onset (time = 0) and averaged within each animal to generate mean peri-event traces. For the Pavlovian discrimination experiment, responses were quantified as the area under the curve (AUC) of z-scored $\Delta F/F$ during the 1-second window following CS presentation.

For all analyses, significance was set at $\alpha = 0.05$. Sample sizes for each experiment are reported in the results and figure legends. Both sexes were included in all experiments; where no significant main effect or interaction involving sex was detected, data were pooled across sexes for visualization.

Data availability

Data will be deposited on Dryad once a decision is made to send the manuscript out for review.

Supplementary tables and figures

Experiment	Female	Male	Total	Groups
GI DREADD - Progressive Ratio	15	15	30	Cont GFP (n=16), GI (n=14)
GI DREADD - Open Field	10	14	24	Cont GFP (n=7), GI (n=17)
GI DREADD - Memory	8	9	17	Cont GFP (n=7), GI (n=10)
Food consumption	6	9	15	Cont GFP (n=8), GI (n=7)
GQ DREADD - Progressive Ratio	14	14	28	Cont GFP (n=9), GQ (n=19)
Stress GQ DREADD - Progressive Ratio	12	9	21	Cont GFP (n=7), Stress GFP (n=6), Stress GQ (n=8)
Reward-associated sound conditioning	18	11	29	Cont GFP (n=11), GI (n=18)
Fiber Photometry - Reward Discrimination	4	5	9	NA
Fiber Photometry - Sensory Response	2	1	3	NA
Optogenetic - Progressive Ratio	7	6	13	NA

Supplementary Table 1..

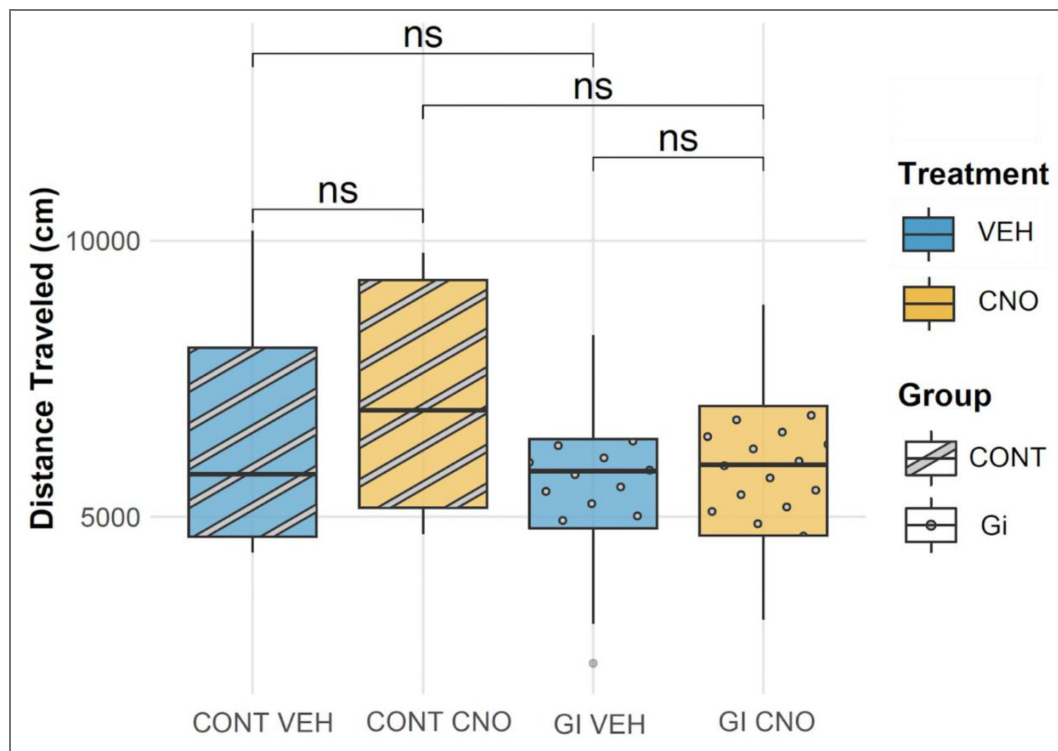


Figure S1. Chemogenetic inhibition of GABAergic ZI neurons does not affect locomotion.

Total distance traveled (cm) in the open field for GFP controls and inhibitory DREADD (Gi) mice under vehicle and CNO conditions. No significant differences were observed between any group-treatment combinations, indicating that the reduced pressing after inhibition of GABAergic neurons in the ZI is not attributable to changes in locomotor activity. (Gi: n=18, 10 males, 8 females; GFP: n=17, 9 males, 8 females). ns = not significant.

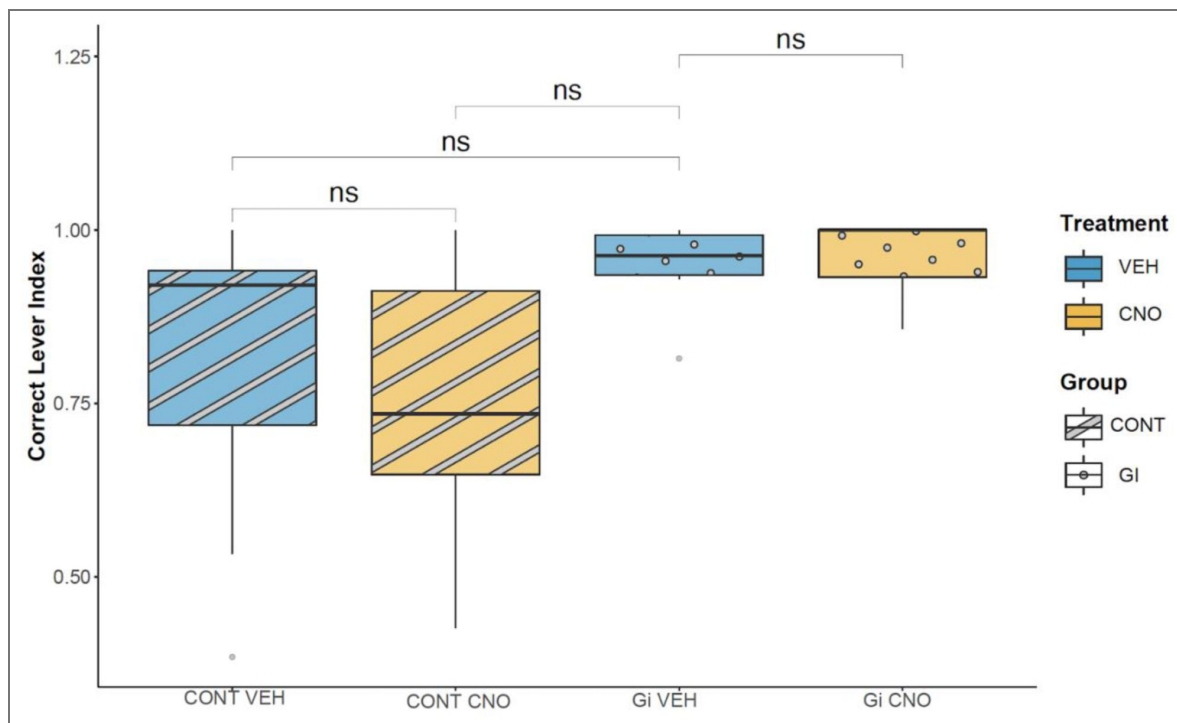


Figure S2. Chemogenetic inhibition of GABAergic ZI neurons does not affect memory.

Correct lever index (proportion of presses on the correct (rewarding) lever for GFP controls and inhibitory DREADD (Gi) mice under vehicle and CNO conditions. During training, animals were presented with two levers, only one of which was paired with a food reward; the second non-rewarding lever was present but inactive. No significant differences were observed between any group-treatment combinations, indicating that inhibition of GABAergic ZI neurons does not impair memory. ns = not significant. (GFP: n=7, 4 males, 3 females; Gi: n=10, 5 males, 5 females)

Figure S3. Chemogenetic inhibition of GABAergic ZI neurons does not affect food consumption.

Number of pellets consumed by GFP controls and inhibitory DREADD (Gi) mice following CNO administration. No significant difference was observed between groups, indicating that the reduction in breakpoint in PR test is not because the mice become less hungry. (Gi: n=7, 4 males, 3 females, GFP: n=8, 5 males, 3 females).

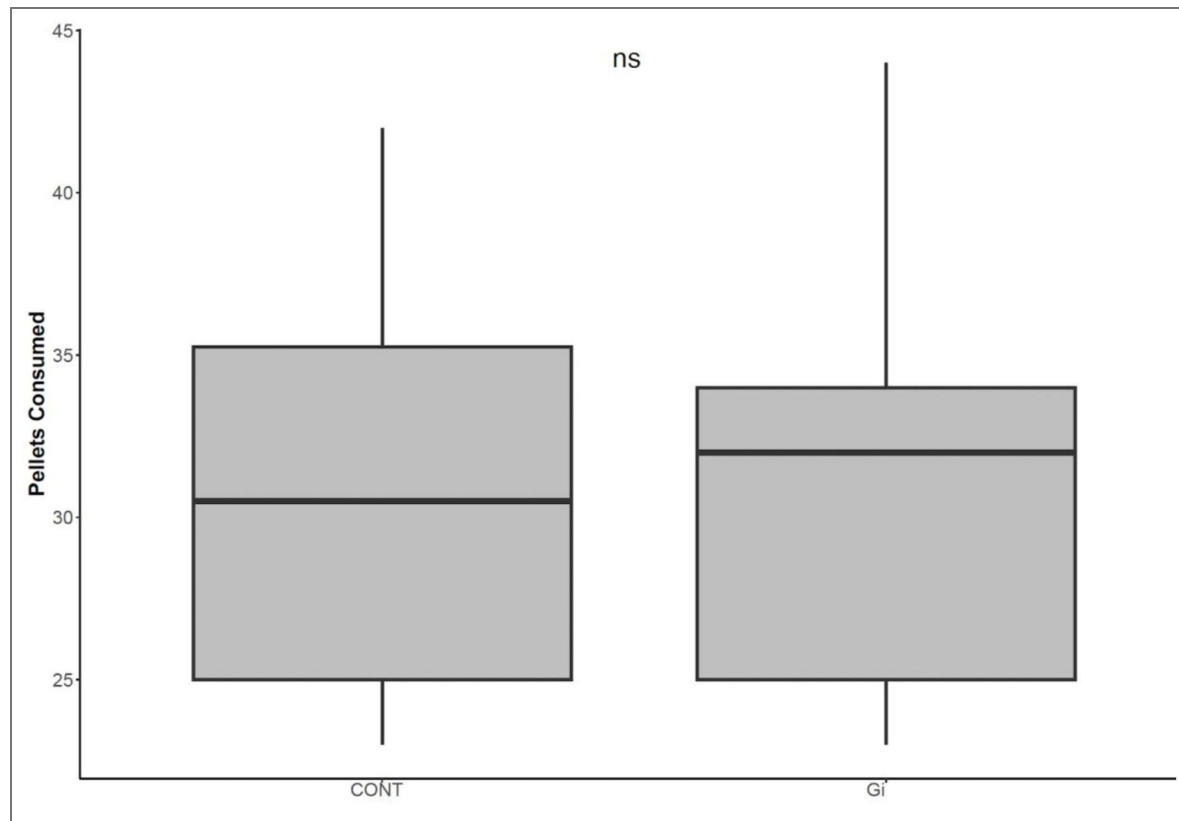


Figure S4. Chronic stress does not impair acquisition during operant conditioning.

Number of reinforcements earned across 14 training days for no stress/GFP control, stress/GFP, and stress/hM3DGq groups, representing learning. Separated by sex (females, left; males, right). All three groups acquired the operant response at comparable rates, with no significant differences in reinforcements earned across training days, indicating that stress exposure did not impair task learning.

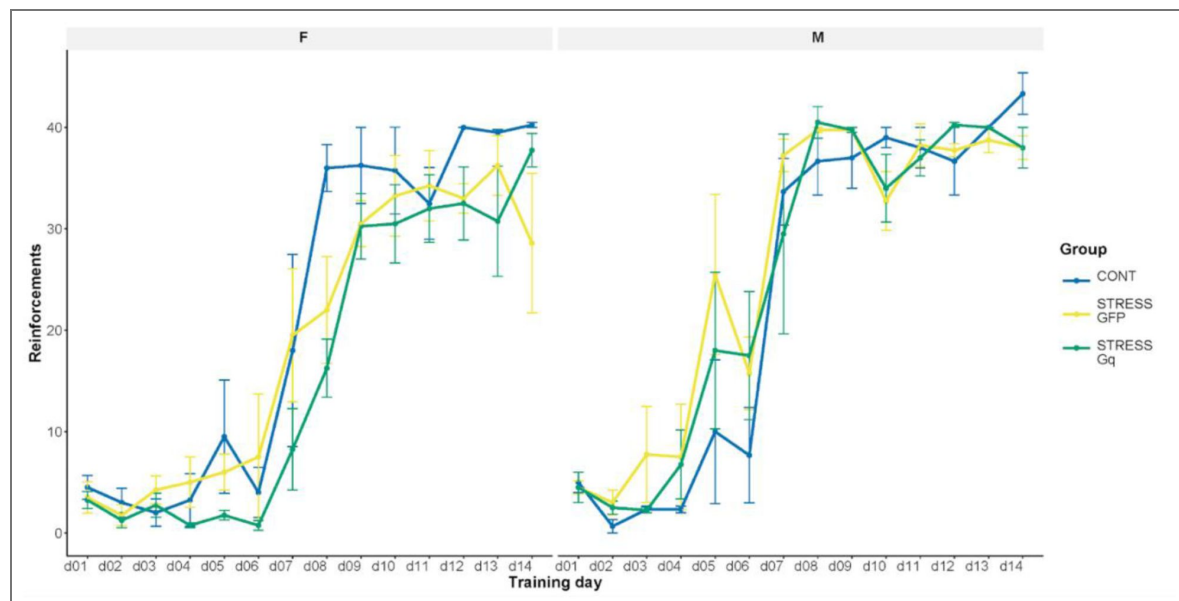


Figure S5. Calcium responses of GABAergic neurons in the ZI of individual animals to auditory and visual stimuli.

Z-scored calcium traces (dF/F) from three individual animals in response to auditory (top row) and visual stimuli (bottom row). Individual trial traces (colored lines) and mean response (black line) are shown for each animal, confirming consistent sensory-evoked calcium transients in GABAergic ZI neurons across subjects.

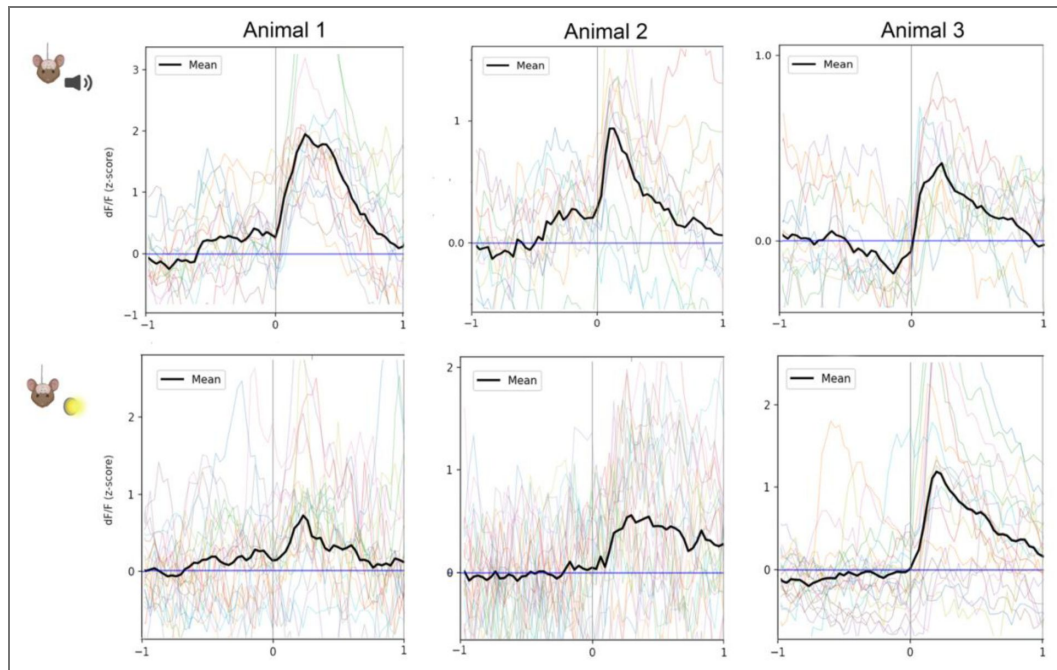
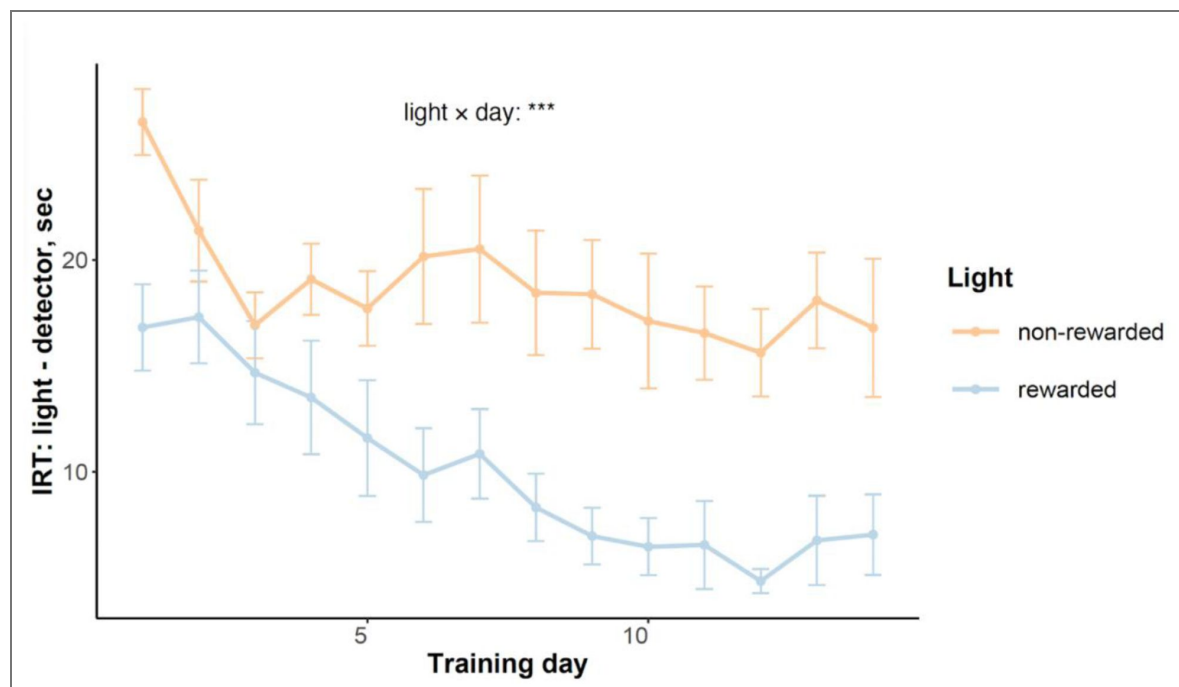
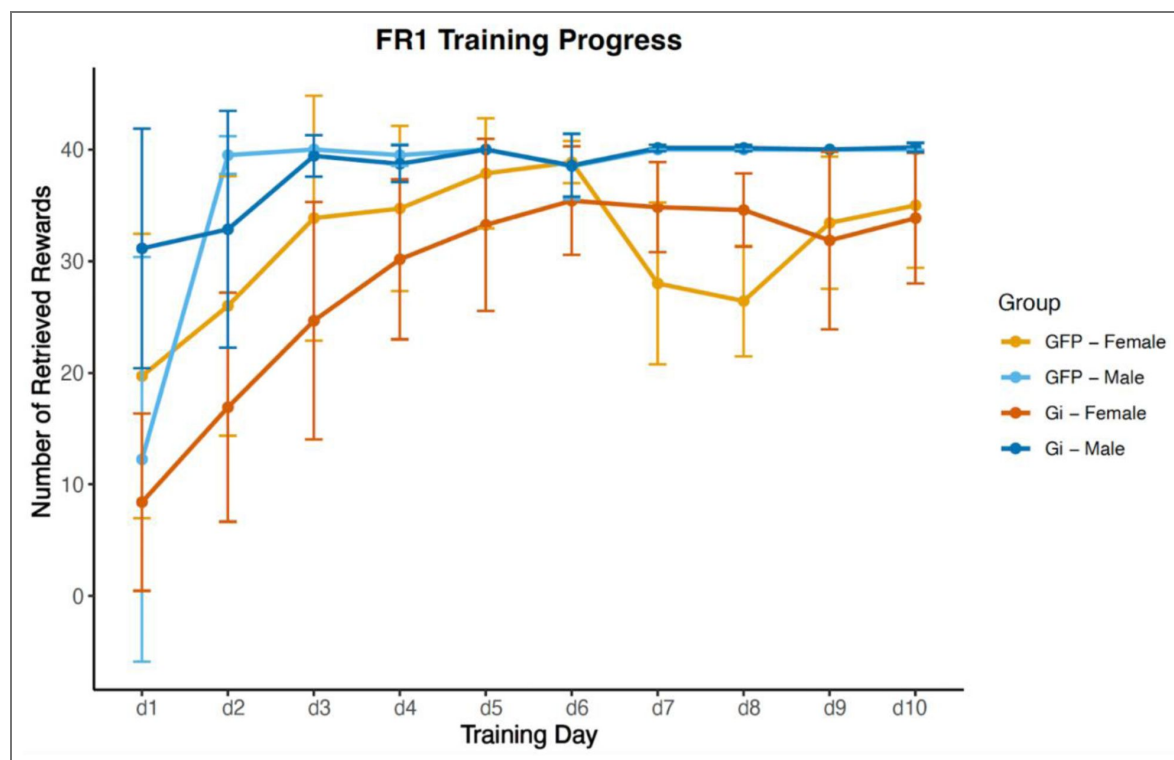


Figure S6. Behavioral evidence of Pavlovian discrimination during training.

Inter-response time (IRT) between light onset and detector entry across training days for rewarded (CS+) and non-rewarded (CS-) cues. IRT decreased selectively for the CS+ across training days (significant light $\times$ day interaction, $p < 0.001$), confirming successful acquisition of cue-reward discrimination. ($n=9$, 5 males, 4 females). Data represented as mean $\pm$ SEM.



FR1 training progress showing number of retrieved rewards across 10 training days for GFP and inhibitory DREADD (Gi) groups, separated by sex. (Gi: 7 males, 11 females; GFP: 4 males, 7 females). All groups acquired the operant response at comparable rates. Data represented as mean $\pm$ SEM.



Total lever presses across three testing days (d1, d2, d3) for females (left) and males (right). No significant differences were observed across days for either sex, ruling out order or practice effects on the optogenetic stimulation results. ns = not significant.

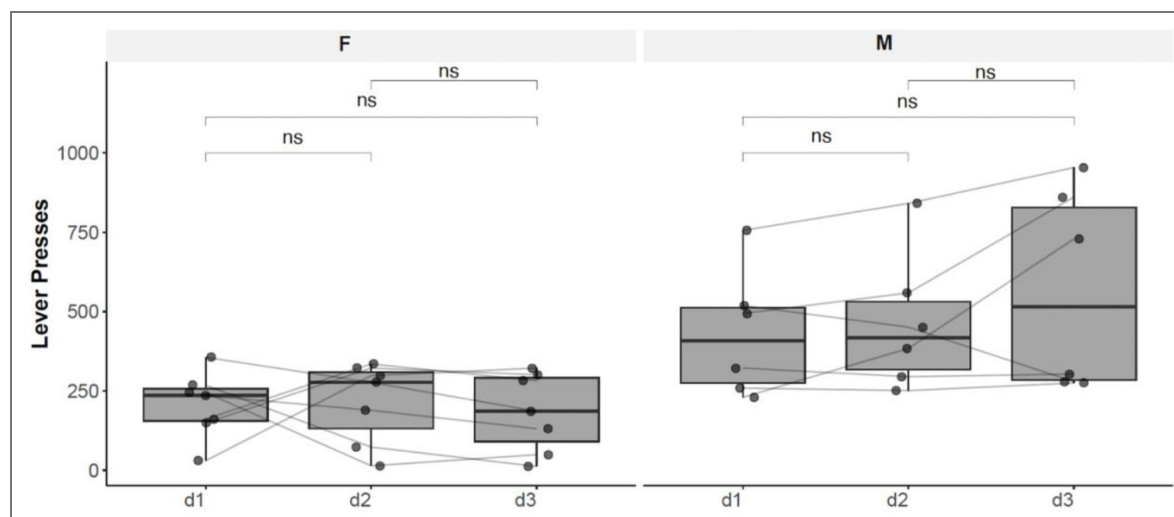


Figure S9. Inter-response time during contingent FR1 training.

Inter-response time (IRT, seconds) between light onset and lever press across contingent FR1 training days (d5-d11) for females and males. Both sexes showed comparable decreases in IRT over training, indicating similar learning trajectories. (6 males, 7 females). Data represented as mean $\pm$ SEM.

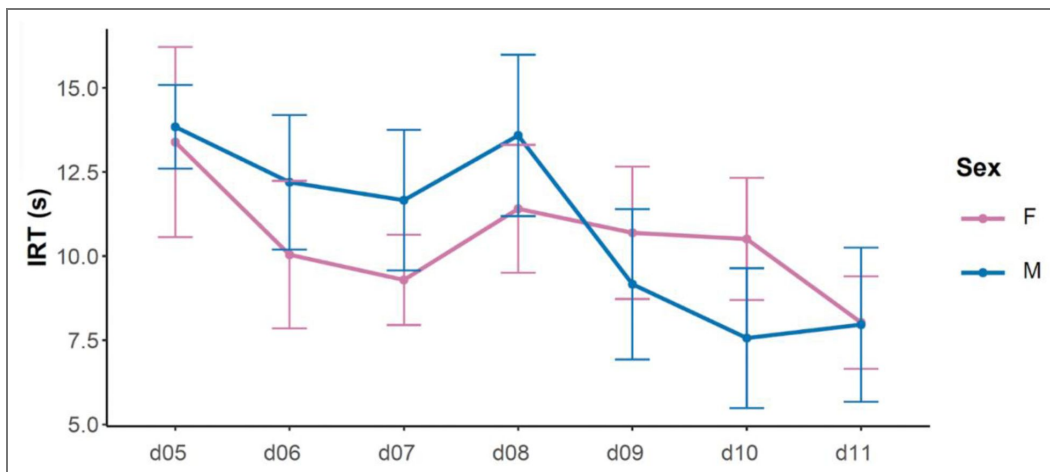
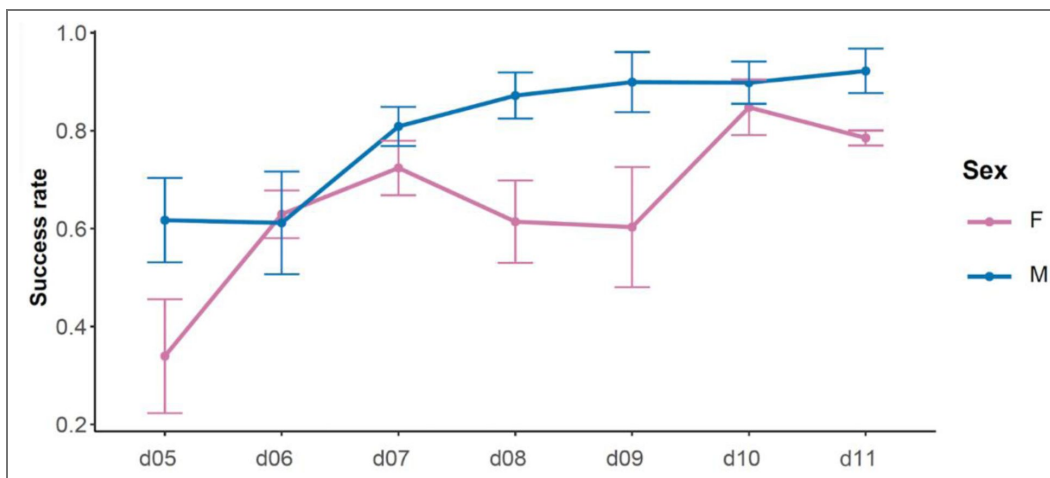


Figure S10. Success rate during contingent FR1 training.

Success rate (proportion of retrieved rewards) across contingent FR1 training days (d5-d11) for females and males. Both sexes showed increasing success rates over training, with a trend toward lower success rates in females. (6 males, 7 females). Data represented as mean $\pm$ SEM.



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Peer reviews

Reviewer #1 (Public review):

Summary:

This manuscript by Laura Korobkova and Brian Dias describes an interesting study of the role of GABAergic neurons in the zona incerta (ZI) in incentive motivation for reward.

The authors report that DREADD inhibition of ZI neurons reduced the effort breakpoint in a progressive ratio task, which measures the intensity of incentive motivation to obtain food rewards. In other tests, chemogenetic inhibition did not alter food consumption or memory.

Conversely, DREADD excitation of ZI neurons increased incentive motivation in the progressive ratio task, expressed as a higher breakpoint for food rewards.

Korobkova and Dias report that prior stress exposure to a series of stressors (e.g., forced swim & water submersion, restraint, mild footshock) by itself reduced the breakpoint for food reward under vehicle, though it did not impair the ability to learn an instrumental response. However, DREADD excitation of ZI neurons in previously stressed mice increased the breakpoint to normal levels equivalent to the never-stressed group. This important finding indicates the ability of ZI stimulation to rescue the incentive motivational deficit induced by prior stress.

In fiber photometry studies using vGAT-CRE mice to specifically identify GABA neurons, Korobkova and Dias report that ZI GABA neurons are excited by sensory signals, including neutral cues. However, after reward conditioning, ZI GABA neurons increase their activation to the CS+ cue that predicts reward, but not to the CS- cue that doesn't. ZI neurons also respond in an instrumental reward task during both lever press and reward delivery. The authors conclude that ZI neurons respond to sensory stimuli, but specifically code the motivational significance of reward-related stimuli.

In optogenetic studies, the authors find that ZI GABA neuron stimulation during a reward CS+ enhances motivated responding to obtain reward, particularly in females, but not stimulation outside the CS+. This suggests the ZI stimulation in females may specifically enhance the incentive salience of the CS+, namely the cue's ability to trigger an increase in 'wanting' for the reward. However, that effect was not found here in males.

Altogether, this is a fine contribution to the literature, and the authors deserve congratulations on their study and manuscript.

Strengths:

This is a powerful and creative set of studies that clarifies the roles of ZI neurons in sensory processing and especially in incentive motivation for rewards. The use of multiple methods and test situations to triangulate on reward motivation functions gives a well-rounded perspective on ZI function. The discovery of incentive motivation roles for ZI neurons is intriguing and improves understanding of ZI, which traditionally has been a relatively understudied brain structure. The finding that ZI stimulation may rescue stress-induced deficits in motivation is especially notable and may have therapeutic implications.

Weaknesses:

Minor: This version of the manuscript focuses the introduction and discussion specifically on ZI GABA neurons. The ZI may be primarily GABAergic, but also contains other neurons, and DREADD studies may have used the hSyn promoter, which would impact all types of ZI neurons. Other studies here did more specifically target GABA neurons using vGAT Cre mice and specific targeting. The manuscript might be slightly improved by distinguishing in the discussion a bit more clearly which effects implicate GABA neurons specifically, and which effects might include other neurons too, to more clearly parse out the relative roles of GABA vs broader neuronal populations in ZI.

<https://doi.org/10.7554/eLife.112218.1.sa2>

Reviewer #2 (Public review):

Summary:

This paper describes a study that uses a combination of observational and experimental techniques to investigate the hypothesis that the zona incerta is a neural loci where sensory information is integrated to interpret the motivational value of reward-associated cues. They show that manipulation of GABAergic neurons in this region bidirectionally modulates responding during a progressive ratio test, that activating these neurons recovers motivational deficits incurred by chronic stress, and that they fire in response to reward-associated visual or auditory cues. They also showed that activity in these neurons is not necessary for incentive salience of reward-associated cues, because inactivating them did not prevent Pavlovian-instrumental transfer. However, activating them did enhance responding during the presentation of reward-associated cues in females but not in males.

Strengths:

The study has a very systematic and elegant approach to assess how this region responds first to intrinsic motivation and then to motivation-enhancing effects of reward-associated cues.

Weaknesses:

Males and females are used throughout, but sample sizes are generally too small to make a meaningful interpretation of sex differences (which is not the focus of the study, but is worth bearing in mind). In the last experiment, the lack of discrimination between CS+ and CS- conditions across training for males confounds any interpretation of sex-differences in the outcomes.

The ZI is known to be a region where there is notable convergence of neural inputs from a diverse and heterogeneous range of sensory and other cortical inputs. To my knowledge, this is the first study that has directly tested whether it may serve to encode motivational/incentive properties of reward-associated cues. The outcomes are not definitive - it appears that they are sufficient but not necessary. However, this study represents an important first step - the ZI also has notable heterogeneity in the genetic identity of neurons, and properly dissecting the function of ZI microcircuits will likely require characterising function based on more than one molecular marker. This is addressed by the authors in the discussion.

In summary, this study will have a significant impact on our understanding of how motivation is calculated based on complex environmental signals.

<https://doi.org/10.7554/eLife.112218.1.sa1>

Reviewer #3 (Public review):

Summary:

The authors investigated the role of the zona incerta in motivation and cue-reward associations. Using chemogenetic and optogenetic manipulations of the ZI, they altered motivation in cued and uncued variants of the progressive ratio task and rescued deficits in motivation induced by chronic stress. They further use fiber photometry to demonstrate that the ZI tracks the formation of cue-reward associations.

Strengths:

- (1) The authors fill an important gap in the literature linking sensory input to motivation via the zona incerta.
- (2) The authors demonstrate that ZI tracks cue value rather than just tracking sensory input.
- (3) The authors demonstrate that the ZI excitation rescues stress-induced suppression of motivation.
- (4) The authors perform several important control tasks, demonstrating that their findings are not a result of alterations in locomotor activity, food consumption, or memory.

Weaknesses:

In Figure 1D and E (inhibitory vs excitatory DREADDS), the control groups in the Gi group appear to have more elevated breakpoints than the control groups in the Gq group, although a statistical comparison between the two is not reported. It is not clear if this is because the two groups were given a different reinforcement schedule, this should be made clearer.

In Figure 1E, it is important to note that although the authors found a significant planned comparison between Gq VEH and Gq CNO, the interaction was not significant, nor were comparisons to mice injected with control virus. Thus, activation of ZI GABA neurons appears to be a relatively weak effect.

In Figure 5, the authors see what is likely a significant difference in lever presses during acclimation between the Gi and GFP groups, which they state is an expected difference. However, it is difficult to see why this would be expected. While Gi:CNO manipulation yielded lower breakpoints in Figure 1D, it did not yield lower FR1 responding for food in Fig S3 (although this was FR1 for food dispenser visits rather than lever press). One reason I ask is that the authors highlight the differences in CS+/CS- between groups, but the biggest difference between groups appears to be in acclimation, which may be driving the group x block interaction.

In Figure 6, the authors demonstrate that optogenetic stimulation during cue light increases the breakpoint in females, but not in males. They suggest that this may be because the males did not sufficiently discriminate the cue light before optogenetic manipulation began. If this were the case, then the authors would need to use "cue discrimination" as a factor to determine if it is a better predictor than sex.

The authors' work demonstrates that chemogenetic inhibition of GABAergic ZI cells reduces uncued motivation for reward but enhances cued responses under extinction. The authors state that this is a paradoxical finding that suggests that the ZI operates within a redundant motivation network. However, a critical difference between the two tasks is that one measures motivation for food while the other measures persistent responding under food extinction, which are not the same process. Thus, a simpler explanation is that ZI inhibition reduces motivation and impairs extinction.

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Author response:

Public Reviews:

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This is a powerful and creative set of studies that clarifies the roles of ZI neurons in sensory processing and especially in incentive motivation for rewards. The use of multiple methods and test situations to triangulate on reward motivation functions gives a well-rounded perspective on ZI function. The discovery of incentive motivation roles for ZI neurons is intriguing and improves understanding of ZI, which traditionally has been a relatively understudied brain structure. The finding that ZI stimulation may rescue stress-induced deficits in motivation is especially notable and may have therapeutic implications.

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The current version of our manuscript notes “Our chemogenetic manipulations targeted all GABAergic ZI neurons, and emerging evidence suggests molecular heterogeneity within this population that may map onto distinct functional roles (Arena et al., 2024; Wilt et al., 2025). Future work to first profile the molecular heterogeneity of GABAergic cells in the ZI and then using intersectional strategies to target defined ZI sub-populations will be essential to providing a more nuanced view of ZI GABAergic influences on motivation.” Our revision will make sure to discuss newer literature demonstrating cellular heterogeneity of ZI and emphasize that this heterogeneity will provide more nuanced contributions of ZI cells beyond the studied GABAergic population on motivation.

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This paper describes a study that uses a combination of observational and experimental techniques to investigate the hypothesis that the zona incerta is a neural loci where sensory information is integrated to interpret the motivational value of reward-associated cues. They show that manipulation of GABAergic neurons in this region bidirectionally modulates responding during a progressive ratio test, that activating these neurons recovers motivational deficits incurred by chronic stress, and that they fire in response to reward-associated visual or auditory cues. They also showed that activity in these neurons is not necessary for incentive salience of reward-associated cues, because inactivating them did not prevent Pavlovian-instrumental transfer. However, activating them did enhance responding during the presentation of reward-associated cues in females but not in males.

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Weaknesses:

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The goal of our study was not to determine sex differences in motivation mediated by the ZI but rather demonstrate a general role of GABAergic cells in the ZI on motivation. As such, while all our experiments used male and female mice and our statistical analyses did not uncover any sex differences in most experiments, our sample sizes of each sex are too small to definitively make any statements about sex differences. As noted already in our Discussion, the sex-specific cue-utilization behavioral strategy in our optogenetic experiment warrants further investigation as a contributing factor to motivation that may or may not be influenced by GABAergic cells in the ZI. It bears mentioning that, to our knowledge, none of the recently published literature on the role of the zona incerta in learning, memory and appetitive behavior that is cited in this manuscript (including our own prior work) has uncovered sex differences in the contributions of the zona incerta to these behaviors.

The ZI is known to be a region where there is notable convergence of neural inputs from a diverse and heterogenous range of sensory and other cortical inputs. To my knowledge, this is the first study that has directly tested whether it may serve to encode motivational/incentive properties of reward-associated cues. The outcomes are not definitive - it appears that they are sufficient but not necessary. However, this study

represents an important first step - the ZI also has notable heterogeneity in the genetic identity of neurons, and properly dissecting the function of ZI microcircuits will likely require characterising function based on more than one molecular marker. This is addressed by the authors in the discussion.

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Weaknesses:

In Figure 1D and E (inhibitory vs excitatory DREADDS), the control groups in the Gi group appear to have more elevated breakpoints than the control groups in the Gq group, although a statistical comparison between the two is not reported. It is not clear if this is because the two groups were given a different reinforcement schedule, this should be made clearer.

In our revision, we will be sure to insert language re-emphasizing that the Gi and Gq experiments were performed using different reinforcement schedules, FR3 and FR1, respectively.

In Figure 1E, it is important to note that although the authors found a significant planned comparison between Gq VEH and Gq CNO, the interaction was not significant, nor were comparisons to mice injected with control virus. Thus, activation of ZI GABA neurons appears to be a relatively weak effect.

In our revision, we will insert language to acknowledge that reducing motivation after inhibiting GABAergic ZI cell activity is stronger than increasing motivation seen after stimulating the activity of these cells.

In Figure 5, the authors see what is likely a significant difference in lever presses during acclimation between the Gi and GFP groups, which they state is an expected difference. However, it is difficult to see why this would be expected. While Gi:CNO manipulation yielded lower breakpoints in Figure 1D, it did not yield lower FR1 responding for food in Fig S3 (although this was FR1 for food dispenser visits rather than lever press). One reason I ask is that the authors highlight the differences in CS+/CS- between groups, but

the biggest difference between groups appears to be in acclimation, which may be driving the group x block interaction.

In a revision, we will revise the language to state that the acclimation difference that is more pronounced in the GFP control group vs the Gi group is to be expected because we had already shown that inhibition of GABAergic cells in the ZI would reduce lever pressing. We will also report a planned comparison of CS+ versus CS- responding that omits the acclimation block, which shows discrimination between sound (CS+) and light (CS-) in the Gi group but not in the GFP group, confirming that the cue effect is not driven by the acclimation difference. We will also note that acclimation responding is non-reinforced and effortful, whereas FR1 dispenser visits (Fig. S3) are reinforced and low-effort, which is why the two measures dissociate.

In Figure 6, the authors demonstrate that optogenetic stimulation during cue light increases the breakpoint in females, but not in males. They suggest that this may be because the males did not sufficiently discriminate the cue light before optogenetic manipulation began. If this were the case, then the authors would need to use "cue discrimination" as a factor to determine if it is a better predictor than sex.

In a revision, we will add an analysis that includes cue discrimination as a factor, to test whether it is a better predictor of the optogenetic effect on breakpoint than sex.

The authors' work demonstrates that chemogenetic inhibition of GABAergic ZI cells reduces uncued motivation for reward but enhances cued responses under extinction. The authors state that this is a paradoxical finding that suggests that the ZI operates within a redundant motivation network. However, a critical difference between the two tasks is that one measures motivation for food while the other measures persistent responding under food extinction, which are not the same process. Thus, a simpler explanation is that ZI inhibition reduces motivation and impairs extinction.

Our revision will include discussion of this important point and tie it into our previous work (Venkataraman et al. 2019 and 2021 – cited in this version) that included extinction-like protocols, albeit in classical (not operant) conditioning protocols.

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