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Deconstructing the Role of Zona Incerta in Fear Generalization

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Deconstructing the Role of Zona Incerta in Fear Generalization

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B.Tech., Anna University, 2009
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Abstract

Deconstructing the Role of Zona Incerta in Fear Generalization
By Archana Venkataraman

The ability of an animal to assess threat and express appropriate fear is crucial for survival. Generalization of learned fear allows information from a previous experience to be used flexibly in a dynamic environment and is adaptive in nature. In contrast, persistent generalization of fear towards neutral, non-aversive cues is maladaptive and a core symptom of trauma- and anxiety-related disorders. Knowledge about the neural circuitry underlying fear generalization is primarily concentrated around the canonical tripartite neural circuit comprising of the amygdala, hippocampus and prefrontal cortex. Very few studies have attempted to understand the contribution of thalamic and sub-thalamic brain regions to fear generalization. This dissertation utilizes a rodent model of discriminative auditory fear learning to examine the role of subthalamic zona incerta (ZI) in mediating fear generalization. First, using C-FOS immunohistochemistry, we report an inverse relationship between ZI activation and fear generalization such that the animals that generalized fear had lower number of C-FOS expressing cells in the ZI. Subsequently, we demonstrate that chemogenetic activation of the ZI reduces fear generalization and chemogenetic inhibition of the ZI results in fear generalization. Given the considerable presence of GABAergic neurons in the ZI, we probed the role of these cells in mediating fear generalization. Using cell-specific chemogenetic manipulations, we demonstrate that the GABAergic neurons in the ZI bidirectionally modulate fear generalization. Further, our anterograde tracing studies reveal dense efferent GABAergic projections from the ZI to the thalamic nucleus reuniens (RE), dorsolateral periaqueducatal gray, ventral periaqueducatal gray, and posterior hypothalamus. With the RE implicated in maintaining specificity of fear memories, we chose to examine whether the ZI → RE GABAergic projections modulate fear generalization. *In vitro* electrophysiological recordings reveal that GABAergic inputs from the ZI evoke inhibitory post-synaptic currents (IPSCs) in the RE. Using cell-specific and projection-specific optogenetics, we show that activation of GABAergic projections from ZI to the RE *in vivo* prevented fear generalization. The experimental results contained in this dissertation establishes a central role for ZI in fear generalization and provides novel evidence for the influence of an inhibitory incerto-thalamic circuit in controlling fear memory specificity. This work contributes to a growing body of research on the role of thalamic and sub-thalamic influences in fear expression and inhibition and underscores the complex and dynamic nature of fear memories involving multiple, parallel neural pathways.

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CHAPTER 1: INTRODUCTION

1.1 Context for the dissertation

The sustained expression of fear toward stimuli that do not signal threat is maladaptive and a central pathological feature of trauma- and anxiety-related disorders. Inhibiting such maladaptive fear requires an understanding of the neural circuitry that maintains fear memory representations and expression of fear responses. Much of our current appreciation of such circuitry coalesces around the trisynaptic circuit comprising the amygdala, hippocampus, and prefrontal cortex. By discussing other cortical, thalamic and sub-thalamic influences on fear generalization and fear extinction, we suggest a more inclusive neurobiological framework that expands our canonical view of fear inhibition. I will begin with a brief synopsis of the prevailing understanding of the contributions of the canonical trisynaptic circuit to fear inhibition. Then, I discuss the emerging literature on the role of anterior cingulate cortex, insular cortex, nucleus reuniens, paraventricular thalamus and zona incerta in fear inhibition. Finally, I conclude with the conceptual framework for this dissertation.

1.2 The neurobiology of fear inhibition.

Fear is an emotional state that is induced when imminent danger or threat is perceived by an organism. Observed across many species, fearful behavior allows an organism to be vigilant, evaluate threat and respond appropriately. In contrast to these adaptive properties of fear, fear responses can become maladaptive when they cannot be inhibited even in the absence of threat or danger. Fear expressed toward stimuli that do not themselves signal threat (fear generalization) and fear expressed toward stimuli even after they cease to be threats (deficits in fear extinction) are two forms of deficits in the ability to inhibit fear. Such deficits of fear inhibition are highly prevalent in individuals living with trauma- and anxiety-related disorders such as post-traumatic stress disorder (PTSD) and generalized anxiety disorder (GAD) (Dunsmoor & Paz, 2015; Dymond, Dunsmoor, Vervliet, Roche, & Hermans, 2015; Jovanovic, Kazama, Bachevalier, & Davis, 2012; Jovanovic & Ressler, 2010; Kaczkurkin et al., 2017). Rescuing deficits in fear inhibition requires an appreciation for the neurobiological mechanisms that govern normative and disrupted fear inhibition.

The expression and inhibition of fear are accomplished by a network of brain regions that integrate sensory information and threat assessment with behavioral output, or the lack thereof. A wealth of research has provided strong evidence that cortico-limbic networks make important contributions to fear inhibition. More specifically, canonical fear-related neural circuitry comprising of the amygdala, hippocampus, and prefrontal cortex have received the most attention for their roles in regulating fear-related behaviors (Ehrlich et al., 2009; Gross & Canteras, 2012; Herry et al., 2010; Maren & Quirk, 2004; Orsini & Maren, 2012; Tovote, Fadok, & Luthi, 2015). New technologies like activity-based circuit mapping, optogenetics, chemogenetics and *in vivo* recordings of neural activity are making a case for more nuanced and involved roles in fear inhibition for brain regions outside of this canon. Most notably, thalamic and sub-thalamic brain

regions that have traditionally been relegated to being mere relays of information flow in the brain, are beginning to be understood for their roles in fear inhibition.

1.3 Behavioral protocols to study fear extinction and fear generalization.

Fear extinction and fear generalization are studied via the use of Pavlovian classical conditioning. To study both constructs, presentations of a neutral stimulus called the conditioned stimulus (CS) are paired with an aversive unconditioned stimulus (US) (Fig. 1.1A). For example, presentations of a specific tone or specific image are paired with a mild shock. As a consequence of the CS/US association, re-exposure to the CS after such conditioning will elicit a robust conditioned fear response. In humans, this fear response is measured in the form of an increased startle reflex or increased skin conductance and in rodents, freezing responses are used as a proxy for fear. To study the extinction of fear responses, multiple presentations of the CS are made without any negative reinforcement and the learning of this new association (CS but no aversive outcome) is assayed by measuring fear toward future presentations of the CS. Individuals living with trauma- and anxiety-related disorders like PTSD and GAD show deficits in extinction learning and a consequent inability to inhibit fear as evidenced by continued expression of fear toward the CS even after this stimulus is no longer associated with the threat of the US (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Dunsmoor & Paz, 2015; Dymond et al., 2015; Jasnow, Lynch, Gilman, & Riccio, 2017; Kaczkurkin et al., 2017; VanElzakker, Dahlgren, Davis, Dubois, & Shin, 2014; Wessa & Flor, 2007). In rodent studies, prior exposure to stress impairs extinction learning (Maren & Holmes, 2016; Maroun et al., 2013; Miracle, Brace, Huyck, Singler, & Wellman, 2006; Raio, Brignoni-Perez, Goldman, & Phelps, 2014; Raio & Phelps, 2015) and the ability to inhibit fear to the now non-threatening CS+. To study fear generalization, animals are trained to distinguish between a conditioned stimulus (CS+) paired with an aversive outcome and an unpaired neutral stimulus (Fig. 1.1B). Generalization of fear manifests as a failure to discriminate between the CS+ and the similar but non-identical CS-. Again, fear generalization is a debilitating dimension of the aforementioned neuropsychiatric conditions and can be induced in

rodents by exposure to stress. Our appreciation for neurobiological mechanisms that underlie normative and disrupted fear inhibition is centered around the contributions of the amygdala, prefrontal cortex, and hippocampus, as discussed in the next section.

1.4 Canonical view of the neurobiology of fear inhibition.

As noted above, our understanding of normative and disrupted fear inhibition comes from examination of the amygdala, prefrontal cortex, and hippocampus. In this section, I provide a broad review of the literature highlighting the contributions of the tripartite circuit to expression of appropriate fear responses. For a comprehensive analyses of the contributions of these brain regions to fear inhibition, see (Asok, Kandel, & Rayman, 2018; Dunsmoor & Paz, 2015; Dymond et al., 2015; Herry et al., 2010; Herry & Johansen, 2014; Jovanovic & Ressler, 2010; Krabbe, Grundemann, & Luthi, 2018; Likhtik & Johansen, 2019; Lissek et al., 2014; Maren & Quirk, 2004; Tovote et al., 2015).

1.4.1 Amygdala

Alterations in neuronal excitability within the amygdala have been proposed to lead to exaggerated amygdala responses to negative emotional stimuli, that is associated with symptom severity in PTSD and anxiety-related disorders (Babaev, Piletti Chatain, & Krueger-Burg, 2018; McLaughlin et al., 2014; Stevens et al., 2017). Specifically, activity-dependent synaptic plasticity at glutamatergic synapses in the basolateral nucleus of the amygdala (BLA) have been shown to be responsible for fear extinction and fear generalization (Grosso, Santoni, Manassero, Renna, & Sacchetti, 2018; G. L. Jones et al., 2015; J. Kim et al., 2007; W. B. Kim & Cho, 2017; Rajbhandari, Zhu, Adling, Fanselow, & Waschek, 2016; Walker & Davis, 2002).

(i) Fear extinction: Imaging studies in individuals suffering from PTSD have revealed increased amygdala activation during extinction learning followed by impaired retention of extinction memories (Bremner et al., 2005; Linnman, Zeffiro, Pitman, & Milad, 2011; Milad et al., 2009; Rauch, Shin, & Phelps, 2006). Work in mice and rats have unequivocally demonstrated that the BLA is critical for the acquisition of extinction memories. Intra-amygdala injections of ERK/MAPK inhibitors or NMDA antagonists impairs fear extinction (Herry, Trifilieff, Micheau,

Luthi, & Mons, 2006; Lu, Walker, & Davis, 2001; Sotres-Bayon, Bush, & LeDoux, 2007). Single-unit *in vivo* recordings from the BLA have established that distinct neuronal subpopulations are active during states of high and low fear. While 'fear neurons' show increased response to the CS with acquisition, another subpopulation of cells called 'extinction neurons' show increased response to the CS after extinction training (Botta et al., 2015; Haubensak et al., 2010; Herry et al., 2008). The behavioral shift from fear expression to extinction is accompanied by plastic changes between fear and extinction pathways in the BLA. This shift towards extinction requires inhibitory interneuron-mediated silencing of fear neurons in the BLA (Trouche, Sasaki, Tu, & Reijmers, 2013). Another BLA-specific mechanism that supports extinction is through its actions on the intercalated cell masses (ITCs). Extinction training is accompanied by potentiation of excitatory inputs from the BLA to the ITC, that in turn inhibits information flow to the CeA (central amygdala) output neurons (Amano, Unal, & Pare, 2010). However, further research into the local circuit elements within the amygdala supporting the shift between fear maintenance and extinction is needed.

(ii) Fear generalization: Neuronal responses within the BLA closely reflect behavioral fear generalization. For instance, neuronal tuning curves (neuronal firing rate as a function of CS frequency) in the primate amygdala were narrowly tuned close to the CS suggesting that the amygdala plays a crucial role in graded fear response (Resnik & Paz, 2015). Moreover, the extent of amygdala activation during conditioning was correlated with overgeneralization seen in individuals with GAD (Laufer, Israeli, & Paz, 2016). The precise functional contribution of amygdala microcircuits to fear generalization is still being uncovered. Studies in rodents have reported that distinct neuronal subsets within the BLA store cue-specific associations, facilitate the discrimination between safe and aversive experiences, and thereby serve to gate the expression or

inhibition of fear generalization (Ghosh & Chattarji, 2015; Grosso et al., 2018). Specifically, Ghosh and Chattarji (2015) showed that under normal conditions, 'cue-specific neurons' in the amygdala increase firing selectively in response to CS+ compared to CS-. However, behavioral shift to generalization leads to increased response to both CS+ and CS- and therefore, loss of cue-specificity in these neurons.

1.4.3 Prefrontal Cortex

The medial prefrontal cortex (mPFC) is considered a critical site for fear inhibition because of its inhibitory control of amygdala function. Clinical studies report reduced activation of the mPFC in PTSD patients and decreased functional connectivity between the PFC and amygdala (Bremner et al., 1999; Bremner et al., 2005; Etkin & Wager, 2007; Rauch et al., 2006; Shin et al., 2004; Shin, Rauch, & Pitman, 2006).

(i) Fear extinction: Imaging studies in healthy human volunteers indicate that the PFC becomes activated during extinction recall and engagement of the region is directly associated with successful extinction recall (Kalisch et al., 2006; Milad et al., 2005; Milad et al., 2007). Individuals diagnosed with PTSD show reduced activation of the PFC and consequently, impaired recall of extinction memories (Garfinkel et al., 2014; Milad et al., 2009). Convergent data from work in rodents implicate the prelimbic (PL) region of the mPFC in signaling fear expression (Corcoran & Quirk, 2007; Sierra-Mercado, Padilla-Coreano, & Quirk, 2011) and the infralimbic (IL) region in fear suppression during extinction (Izquierdo, Wellman, & Holmes, 2006; Laurent & Westbrook, 2009; Morawska & Fendt, 2012). Local pharmacological activation or electrical stimulation of the IL in conjunction with CS presentations enhanced fear extinction (Milad, Vidal-Gonzalez, & Quirk, 2004; B. M. Thompson et al., 2010; Vidal-Gonzalez, Vidal-Gonzalez, Rauch, & Quirk, 2006). The mere stimulation of IL alone paired with CS presentations under anesthesia, has been

shown to be sufficient to simulate extinction (Park & Choi, 2010). Inactivation of the IL, on the other hand, disrupts the ability to consolidate and retrieve extinction memories (Burgos-Robles, Vidal-Gonzalez, Santini, & Quirk, 2007; Sangha, Robinson, Greba, Davies, & Howland, 2014; Sotres-Bayon, Diaz-Mataix, Bush, & LeDoux, 2009).

(ii) Fear generalization: fMRI studies implicate the mPFC in signaling safety where activation of the region is specifically required for inhibiting responses to the inappropriate stimuli. In particular, healthy volunteers showed increased mPFC activity in response to stimuli with the least resemblance to the CS+ whereas individuals diagnosed with GAD showed an opposite activity pattern (Cha et al., 2014; Greenberg, Carlson, Cha, Hajcak, & Mujica-Parodi, 2013a, 2013b). Work in rodent models also emphasize the importance of the mPFC in fear generalization (Asok et al., 2018; Dunsmoor & Paz, 2015; Jasnow et al., 2017). Mice with targeted deletion of NMDARs in prefrontal excitatory neurons failed to discriminate between fearful and neutral stimuli (Vieira et al., 2015). Inactivation of the PL has been shown to interfere with the encoding and expression of contextual discrimination in rodents (Sharpe & Killcross, 2015). Reversible inactivation of the IL prior to testing impaired the ability of animals to distinguish between fear and safety cues in a discriminative conditioning task (Sangha et al., 2014). Therefore, activity-dependent plasticity within the mPFC is required to exhibit adaptive and flexible responses after assessment of safety or danger cues in the environment.

1.4.4 Hippocampus

The hippocampus is required for the formation of contextual representations and plays a central role in contextual modulation of fear inhibition. MRI studies have documented a strong reduction in hippocampal volume in patients suffering from PTSD and anxiety disorders (Kitayama, Vaccarino, Kutner, Weiss, & Bremner, 2005; Levy-Gigi, Szabo, Richter-Levin, & Keri, 2015;

Shin et al., 2006; Stein, Koverola, Hanna, Torchia, & McClarty, 1997). High-resolution MRI has revealed specific reduction in volume of the dentate gyrus (DG) subfields of the hippocampus in PTSD (Z. Wang et al., 2010).

(i) Fear extinction: The formation and retrieval of extinction memories relies strongly on the extinction-related contextual information from the hippocampus (Herry et al., 2010; Ji & Maren, 2007; Maren & Quirk, 2004; Wotjak & Pape, 2013). Inactivation of hippocampus prior to extinction training delayed acquisition of extinction and impaired extinction recall (Corcoran, Desmond, Frey, & Maren, 2005). While fear extinction creates a new 'inhibitory memory' that dampens previously learned fear associations, renewal of the fear occurs outside the extinction context. Inactivation of the hippocampus prior to testing impairs fear renewal. Moreover, disruption of hippocampal projections to the BLA or mPFC completely eliminated fear renewal (Orsini, Kim, Knapska, & Maren, 2011) and unambiguously establishes a crucial role for hippocampus in contextual modulation of fear extinction. A recent study by Lacagnina and colleagues (2019) explored the possibility of dedicated cell populations within the hippocampal DG subfield that control fear and extinction memories. Using activity-dependent neural tagging and targeted optogenetic manipulations, they demonstrated that extinction training involves active suppression of DG neurons encoding fear acquisition and establishment of another distinct set of DG neurons encoding the extinction memory. Further research is needed to better understand if and whether the interaction between fear and extinction representations in the DG can determine resistance to extinction.

(ii) Fear generalization: Cells in the hippocampal DG are crucial for pattern separation and completion, where representations for threat and safety are stored in a distinct, non-overlapping manner (Lacagnina et al., 2019; Yassa & Stark, 2011). NMDA depletion in DG cells causes

deficits in discrimination learning (McHugh et al., 2007). Furthermore, inhibition of neural activity in the DG during retrieval of fear memories leads to overgeneralization of fear to safe contexts (Bernier et al., 2017), arguing that pattern separation processes are crucial for fear inhibition. It is suggested that failure in pattern separation processes where a safe stimulus inaccurately activates a threat representation may result in fear generalization (Dymond et al., 2015; Kheirbek, Klemenhagen, Sahay, & Hen, 2012).

1.4.5 The tripartite synaptic circuit

Taken together, the evidence presented above suggests that fear inhibition relies on the tripartite synaptic circuit including the amygdala, hippocampus and prefrontal cortex. Each of these specialized and spatially distributed neuronal populations are functionally coupled to support fear and safety behaviors. Circuit-level communication between these regions balance the mechanisms required for signaling danger and safety (Fig. 1.2).

Cortical input to the amygdala is particularly crucial for inhibition of fear responses and safety signaling (Likhtik, Stujenske, Topiwala, Harris, & Gordon, 2014). 'Extinction neurons' in the amygdala, that encode low fear states, send long-range bi-directional projections to and from the mPFC. The extinction of fear memories requires inhibition of amygdala-dependent fear responses by the mPFC (Herry & Mons, 2004). Strong amygdala-prefrontal synchrony at the end of fear learning has been implicated in resistance to extinction of fear memories (Livneh & Paz, 2012). Activation of the IL subregion of the mPFC results in inhibition of the central nucleus of the amygdala, through direct inputs to intercalated cells (ITCs)ef of the amygdala (Asede, Bosch, Luthi, Ferraguti, & Ehrlich, 2015; Berretta, Pantazopoulos, Caldera, Pantazopoulos, & Pare, 2005; Cho, Deisseroth, & Bolshakov, 2013; Likhtik, Popa, Apergis-Schoute, Fidacaro, & Pare, 2008; Marek, Strobel, Bredy, & Sah, 2013). The ITCs are mostly GABAergic and blockade of these cells

after extinction learning results in spontaneous fear recovery (Likhtik et al., 2008), demonstrating a strong role for this circuit in fear inhibition. Moreover, the BLA sends direct projections to the PL region such that inactivation of BLA leads to reduced PL cell firing and a subsequent reduction in conditioned responses (Sotres-Bayon, Sierra-Mercado, Pardilla-Delgado, & Quirk, 2012). While the PL-projecting BLA neurons are selectively activated during fear expression, the IL-projecting BLA neurons are activated only during fear extinction. Balance of activity between these two populations of projection neurons is necessary to mediate fear extinction (Senn et al., 2014).

Gating of fear responses after extinction also rely on hippocampal inputs to PL (Bouton, 2002; Sotres-Bayon et al., 2012). Further, through reciprocal connections with the BLA, the hippocampus plays an important role in discriminating threat from safety as well as acquisition and retrieval of extinction memories. Synchronization of theta oscillations between BLA, HPC and PFC may support extinction of conditioned fear (Lesting et al., 2011).

1.5 Non-canonical circuits for fear inhibition.

Studies of the aforementioned canonical fear-related circuitry have significantly advanced our understanding of fear expression and inhibition. However, recent data obtained via the study of anterior cingulate cortex, insular cortex, thalamic and sub-thalamic brain regions emphasize the need to update our neurobiological perspective of fear inhibition. For fear to be appropriately expressed and inhibited, interoceptive and exteroceptive information must be integrated. Such integration occurs at multiple sites within the nervous system that include cortical structures such as the cingulate cortex and insula, and multi-modal thalamic and sub-thalamic neuroanatomy. Below, we discuss the contributions of these brain regions to fear extinction and fear generalization.

1.5.1 Anterior Cingulate Cortex

The ACC exerts top-down control of limbic systems and plays a crucial role in emotional and autonomic regulation. Damage to the ACC in humans is associated with blunted autonomic arousal (Critchley et al., 2003; Zahn, Grafman, & Tranel, 1999) and increased activation of the ACC has been reported in individuals suffering from PTSD (Bryant et al., 2005; Liberzon et al., 1999; van Rooij et al., 2015). In addition to neural activity in the ACC being altered in scenarios of impaired of fear inhibition like PTSD, molecular and genetic perturbations have also been documented in animal studies that model behavioral dimensions of fear- and anxiety-related disorders. For example, reduced histone H3 acetylation in neurons of the ACC is associated with high-anxiety phenotype in mice (Sah et al., 2019), and stress hormone-related genes associated with glucocorticoid regulation such as *Fkbp5*, *Crhr1* and *Crhr2* are altered in the ACC of stressed mice (Tanaka et al., 2019).

(i) Fear extinction: Increased activation of the ACC has been associated with impaired extinction recall in individuals living with PTSD (Milad et al., 2009; Shin et al., 2007; Shin et al., 2001). Although the ACC is known to be critical for formation and consolidation of recent and remote fear memories (Frankland, Bontempi, Talton, Kaczmarek, & Silva, 2004; Restivo, Vetere, Bontempi, & Ammassari-Teule, 2009; Vetere et al., 2011), very little is understood about its role in fear extinction. Hefner and colleagues reported enhanced activation of immediate early genes in the ACC following extinction recall but did not detect any differences between the normal (C57BL/6J) and extinction-resistant (129S1) mouse strains (Hefner et al., 2008).

(ii) Fear generalization: Activation of the ACC at a recent time point after learning facilitated fear generalization, whereas, inactivation at remote time points resulted in reduction of generalized fear (Bian et al., 2019; Cullen, Gilman, Winiecki, Riccio, & Jasnow, 2015). Further, AMPA/kainate receptor blockade in the ACC after reactivation of remote memories also resulted in reduction of generalized fear (Einarsson, Pors, & Nader, 2015). At the molecular level, specific knock-down of histone deacetylase 2 (HDAC2) within the ACC has been shown to reduce fear generalization (Qin et al., 2019).

Connectivity: The ACC has strong reciprocal connections with the orbitofrontal cortex, insula, and amygdala (Kobayashi, 2011; Pandya, Van Hoesen, & Mesulam, 1981; Vogt & Pandya, 1987). Moreover, ACC efferents reach the hippocampal formation (B. F. Jones & Witter, 2007; Vogt & Pandya, 1987), zona incerta (Mitrofanis & Mikuletic, 1999; Roger & Cadusseau, 1985) and the locus coeruleus (LC) through which it can modulate memory and arousal systems. Silencing the activity of ACC projections to the ventral hippocampus at a remote time point, reduces fear generalization (Bian et al., 2019). Interestingly, silencing of ACC projections to the BLA at both

recent (1 days) and remote (28 days) time points after learning, reduces generalized fear (Ortiz et al., 2019).

1.5.2 Insular Cortex

The insula receives interoceptive information and is considered to be a hub for establishing associations between sensory experiences and emotional valence. Hyperactivity of the insula has been associated with anticipatory anxiety and treatment resistance to a wide range of psychotherapeutic interventions in individuals suffering from PTSD (Aupperle, Melrose, Stein, & Paulus, 2012; Lanius et al., 2007; Lindauer et al., 2008; Rosso et al., 2014; van Rooij, Kennis, Vink, & Geuze, 2016). Lesions to the posterior IC in rats have been shown to impair the processing of safety signals (Christianson et al., 2008).

(i) Fear extinction: The identification of distinct populations of neurons in the posterior IC that exhibit modulation of firing rate during early vs late extinction, suggests a critical role for IC in extinguishing learned fear (Casanova, Aguilar-Rivera, Rodriguez, Coleman, & Torrealba, 2018).

(ii) Fear generalization: fMRI experiments have revealed that increases in neural activity in the insula tracked the generalization gradients in a conditioned fear protocol to which healthy individuals had been exposed (Greenberg et al., 2013a; Lissek et al., 2014) and that activation of the insula increased as a function of physiological arousal to a generalized stimulus (Dunsmoor, Prince, Murty, Kragel, & LaBar, 2011). Increased activation of the insula has been consistently reported in individuals suffering from PTSD (Bruce et al., 2012; Lanius et al., 2007; Lindauer et al., 2008; Simmons et al., 2008). In support of glutamatergic neurotransmission in the IC being important for safety learning, administration of an NMDAR antagonist into the posterior IC

interfered with fear inhibition in the presence of safety cues (Foilb, Flyer-Adams, Maier, & Christianson, 2016).

Connectivity: The insular cortex has extensive connectivity with the cortical and subcortical networks serving emotional and cognitive functions. The posterior IC is a major source of input to the basolateral, central and cortical nuclei of amygdala(Ottersen, 1982; C. J. Shi & Cassell, 1998). Projections of the posterior IC to the amygdala mediate anxiety-like behaviors and exert top-down inhibitory control over the limbic system to block expression of consummatory behaviors upon detection of danger (Gehrlach et al., 2019).

1.5.3 Thalamic and sub-thalamic influence on fear inhibition.

Thalamic and subthalamic regions have the potential to synchronize neural activity across multiple nodes of cortical and subcortical networks according to attentional demands in situations of safety versus danger. The normative entrenched view of the thalamus is that it merely serves as a relay center that transfers sensorimotor information from the lower brain centers to the cortex, where higher level processing occurs. This view is gradually changing with a growing literature showing that the thalamus functions as a 'switch board' where sensorimotor information is integrated and targeted to specific, segregated subsets of cortical and subcortical structures for appropriate computation and outcomes. Individuals with damage to thalamic neuroanatomy express profound impairments in inhibitory control, with the deficits spanning emotional and cognitive domains (Bogousslavsky, Regli, & Uske, 1988; Carrera & Bogousslavsky, 2006; Cheung, Lee, Yip, King, & Li, 2006; Van der Werf et al., 2003; Van Der Werf et al., 1999; Wilkos, Brown, Slawinska, & Kucharska, 2015). For instance, patients with thalamic infarcts show disinhibition syndrome characterized by failure to inhibit inappropriate behaviors and apathy (Bogousslavsky et al., 1988; Carrera & Bogousslavsky, 2006).

Tracing experiments have revealed that thalamic and subthalamic regions communicate with the canonical fear circuitry through direct or polysynaptic pathways. The midline thalamic nuclei that includes the paraventricular nucleus (PVT) and the nucleus reuniens (RE) have been referred to as the 'limbic circuitry of the thalamus' (Vertes, Linley, & Hoover, 2015). The subthalamic ZI has also been shown to contain modality-specific sectors, one of which is dedicated to processing limbic information from regions such as cingulate cortex, central amygdala and ventromedial hypothalamus (Mitrofanis, 2005; Mitrofanis & Mikuletic, 1999; Roger & Cadusseau, 1985). While very little is understood about the role of thalamic and subthalamic regions in the context of fear inhibition, this picture is beginning to change with rapidly accumulating literature that capitalizes on animal studies that model fear generalization and deficits in fear extinction (Fig. 1.3).

1.5.4 Nucleus Reuniens

RE is a ventral midline thalamic nucleus that serves as an integrative hub for interactions between the mPFC and hippocampus and controls specificity and persistence of fear memories (Ramanathan, Ressler, Jin, & Maren, 2018; Troyner, Bicca, & Bertoglio, 2018; Xu & Sudhof, 2013). It is well-positioned to exert significant control over fear extinction and fear generalization, as appreciated from the studies discussed below.

(i) Fear extinction: Activity-dependent brain mapping has revealed increased activation of RE following extinction learning as well as extinction recall (Ramanathan, Jin, Giustino, Payne, & Maren, 2018; Silva, Burns, & Graff, 2019). Muscimol-induced reversible inactivation of the RE before extinction training impaired acquisition of extinction and RE inactivation prior to retrieval impaired retrieval of extinction memories (Ramanathan & Maren, 2019). *In vivo* extracellular recordings from the RE showed that these neurons increase spike firing in response to an extinguished CS.

(ii) Fear generalization: RE neuronal activity is essential for the formation of precise memories that allow clear distinction of fear and safety in the environment. RE inactivation with muscimol after a weak fear conditioning procedure resulted in strongly consolidated and generalized fear memory (Troyner et al., 2018). Furthermore, use of two different stimulation patterns on RE neurons resulted in opposing effects on fear generalization. Phasic stimulation of the RE during fear acquisition resulted in increased fear generalization while tonic stimulation reduced generalization (Xu & Sudhof, 2013).

Connectivity: RE receives widespread projections from several cortical and subcortical regions including the BNST, PVT, ZI, VTA, raphe nuclei, and PAG (Canteras, Simerly, & Swanson, 1995; Cassel et al., 2013; Krout, Belzer, & Loewy, 2002; McKenna & Vertes, 2004). RE also serves a major source of thalamic afferents to hippocampus and supports bidirectional communication between the mPFC and hippocampus. Interestingly, activation of RE projectors to the mPFC increases arousal and enhances defensive responses to counteract threats (Salay, Ishiko, & Huberman, 2018). In contrast, silencing of prefrontal inputs to RE results in enhanced fear generalization as well as impaired fear extinction (Ramanathan, Ressler, et al., 2018; Xu & Sudhof, 2013). Taken together, these results suggest that RE could be of major clinical relevance in achieving fear inhibition in the context of PTSD and other anxiety-related disorders.

1.5.5 Paraventricular nucleus of thalamus

The PVT is a dorsal midline thalamic nucleus that is potently activated in response to stress and emotional arousal. Recent studies have demonstrated that the PVT plays a critical time-dependent role in fear learning. More specifically, the PVT appears to be required for retrieval of remote fear memories (Do-Monte, Quinones-Laracuente, & Quirk, 2015; Padilla-Coreano, Do-Monte, & Quirk, 2012).

(i) Fear extinction: Lesioning of the PVT after fear conditioning does not affect the rate of extinction (Y. Li, Dong, Li, & Kirouac, 2014). Although the PVT has direct connections with the IL, pharmacological inactivation of PVT using muscimol prior to extinction learning does not affect the acquisition or retrieval of extinction memories (Padilla-Coreano et al., 2012). However, immunohistochemical analysis on adolescent animals have revealed a potential developmental role for PVT in fear inhibition. Adolescence, in particular, is marked by impairments in ability to inhibit fear and poor extinction recall in adolescent rats has been shown to be associated with increased MAPK expression in the posterior PVT (Baker & Richardson, 2015). The observed changes in MAPK activation was specific to adolescents and not juveniles or adults (Baker & Richardson, 2015; Y. Li et al., 2014). Given the time-sensitive recruitment of PVT in fear inhibition, understanding how the PVT might differentially regulate the canonical trisynaptic circuitry through the course of development remains an unanswered question for further research.***Fear generalization:*** It has been suggested that the PVT is crucial for assessing the balance between danger and reward, but not safety evaluation. Work by Choi and McNally (2017) showed that silencing of PVT does not affect discrimination of fear memories. However, when animals were posed with an approach-avoidance conflict-based task, PVT silencing shifts the balance between threat avoidance and reward-related approach behaviors but does not affect threat avoidance in the absence of reward. Therefore, the PVT seems to be essential for resolving competing behavioral demands between danger and reward in adults, but not in inhibiting fear *per se*.

(ii) *Connectivity:* The PVT receives widespread afferents from forebrain structures such as the mPFC, and insular cortices, and from brainstem structures including VTA, raphe nuclei, PAG and LC. Orexinergic inputs to the PVT influence arousal and anxiety (Bhatnagar, Huber, Lazar, Pych, & Vining, 2003; Y. Li et al., 2011). PVT sends projections to multiple brain regions involved in

fear regulation such as the amygdala, mPFC, hippocampus, nucleus accumbens, BNST and ZI. In particular, PVT projections to the central amygdala have been shown to be essential for fear learning and retrieval (Penzo et al., 2015). Despite this well-established connectivity between the PVT and brain regions that are involved in inhibiting fear, specific functional contribution of these PVT connections in fear inhibition remain to be uncovered.

1.5.6 Zona Incerta

The ZI, a sub-thalamic brain region, present directly beneath the thalamus, is involved in sensorimotor integration and has a limbic subsector that receives cingulate, subfornical and brainstem inputs. This makes the ZI well positioned to modulate behavioral states based on incoming sensory information. In keeping with this rationale, neurons in the ZI are activated in response to adverse experiences such as immobilization stress, social defeat stress, high foot-shock intensities, and exposure to noxious stimuli (Dopfel et al., 2019; Lkhagvasuren et al., 2014; Otake, Kin, & Nakamura, 2002; Porro et al., 2003; Ueyama et al., 2006). Recently, the ZI has been shown to be crucial for the acquisition and expression of fear memories (Chou et al., 2018; Zhou et al., 2018). Clinical studies in a subset of Parkinsonian patients with deep brain stimulation electrodes in the ZI has shown that activation of the region can ameliorate symptoms of anxiety and depression, and enhance appropriate facial fear recognition (Burrows et al., 2012).

(i) Fear extinction: In a recent study, Chou and colleagues demonstrated that inhibition of GABAergic cells in the ZI during extinction training results in reduced fear expression. Extracellular single-unit recordings from neurons in the ZI indicated consistent increase in neuronal activity specifically during fear extinction (and not during acquisition). This increase in activity within ZI might be, in part, attributed to prefrontal inputs to the region (Chou et al., 2018).

(ii) Fear generalization: In contrast to the recently demonstrated role of the zona incerta in fear extinction, nothing is known about whether zona incerta modulates fear generalization. My dissertation fills this gap in our knowledge and addresses the contributions of the ZI to expression of appropriate fear responses in the context of fear generalization.

A case for the zona incerta and fear generalization.

While generalization of memories provide flexibility in fear learning, overgeneralization of fear memories is maladaptive. This maladaptive threat processing results in excessive fear and imprecise retrieval of fear memories. Identifying circuit mechanisms underlying precision and generalization of fear memories is crucial for understanding dysregulated psychological processes. Research thus far suggests that recruitment of inhibitory networks is required for striking a balance between memory specificity and generalization (Cullen, Dulka, Ortiz, Riccio, & Jasnow, 2014; Ehrlich et al., 2009; Guo et al., 2018; Krabbe et al., 2018; Ruediger et al., 2011; Shaban et al., 2006). While local inhibitory influences within the trisynaptic circuit (amygdala, prefrontal cortex and hippocampus) are relatively well-understood in this context, knowledge of the functional contributions of thalamic and subthalamic inhibitory nodes to fear generalization, is still at its infancy.

The subthalamic ZI, in particular, is a central node of inhibition and has been implicated in sensory discrimination. Specificity and generalization of fear responses, at its core, relies on integrating relevant sensory stimuli and selecting appropriate behavioral outputs. The ZI has been postulated to fine-tune behavioral responses employing a range of mechanisms, such as:

1. *Integration:* The rich network of sensory and nociceptive inputs to the ZI allows it to function as an integrator of multi-modal signals. Signal integration is not only made possible by virtue of its location, but also by its specialized synaptic arrangement. The ZI

neurons display extensive dendritic arborization with long dendritic segments (Bartho et al., 2007), indicating that distinct information from multiple brain regions can be integrated at a cellular level.

2. *Gating:* Through its afferents, the ZI controls thalamic output such that the thalamocortical circuit dynamics match ongoing behaviors (Lavallee et al., 2005; Trageser et al., 2006; Trageser & Keller, 2004). Gating of cortico-thalamo-cortical information by the ZI allows the information to be directed towards or away from the cortex. This could allow for rapid transition of attentional, arousal or fear states.

3. *Initiator:* Cortical inputs directly reach the ZI and while the ZI sends information to the thalamus, it does not receive thalamic inputs (Bartho, Freund, & Acsady, 2002; Bartho et al., 2007; Kaelber & Smith, 1979; Roger & Cadusseau, 1985). This suggests that the ZI is set up to initiate thalamic activity but not maintain recurrent activity.

In addition to these functions, the connectivity of ZI with fear-related brain regions like the amygdala, PFC, and PAG strengthen the case for the ZI to be involved in modulating fear (Bartho et al., 2007; Chou et al., 2018; Mitrofanis, 2005; Zhou et al., 2018). Inhibitory inputs from the central amygdala to the ZI has been implicated in acquisition of fear memories and remote memory retrieval (Zhou et al., 2018). Cortical inputs from layer V pyramidal neurons in the mPFC and ACC also reach the ZI. While prefrontal projectors are required for extinction of fear memories (Chou et al., 2018), the cingulate projectors have been implicated in modulation of the aversiveness associated with painful experiences (Hu et al., 2019).

The ZI serves as a conduit between the amygdala and the midbrain PAG. GABAergic inputs from the ZI to the PAG allows direct suppression of excitatory neurons in the PAG (Chou et al., 2018) and could thereby play a crucial role in modulating fear responses. Moreover, the A13 cells

in the medial zona incerta provide a key source of dopaminergic input to the midbrain superior colliculus and brainstem locomotor regions such as the cuneiform nucleus and the pedunculopontine-tegmental-nucleus, suggesting a potential role in modulation of appropriate behavioral output (Bolton et al., 2015; Comoli et al., 2012; Sharma, Kim, Mayr, Elliott, & Whelan, 2018).

1.6 A framework for the dissertation.

Traumatic experiences pathologically manifest as stress- and anxiety-related disorders for certain individuals. Research thus far has largely focused on understanding the encoding, consolidation and retrieval of traumatic memories. One central yet largely understudied symptom of such disorders is the persistence of exaggerated fear in response to safe and benign events that merely resemble the traumatic event. This inappropriate generalization of fear responses is maladaptive and pathological. The research studies presented in this dissertation is motivated by a desire to understand the neurobiological mechanisms that could reduce the pathological generalization of fear responses.

The goal of this dissertation is to delineate the role of the subthalamic zona incerta (ZI) in fear generalization. To reveal the functional contributions of ZI to fear generalization, in the experiments contained within this dissertation, I use a mouse model of differential auditory fear conditioning. In Chapter 2, I use activity-based immunohistochemical mapping with chemogenetic manipulation of neuronal activity to identify how the ZI influences fear expression. I show that there is an inverse relationship between C-FOS based activation of ZI and generalization of fear responses. With chemogenetic manipulations, I reveal that the relationship holds true; stimulation of ZI reduces fear generalization and vice versa. In Chapter 3, I continue to probe the identity of the cells in ZI that contribute to fear generalization. I demonstrate that targeted chemogenetic manipulation of GABAergic cells in the ZI faithfully replicates the ZI-mediated effects on fear generalization. I then describe the afferent connections of these GABAergic cells in ZI obtained using anterograde tracing. In addition to revealing the presence of inhibitory connections between the ZI and thalamic reuniens (RE), in Chapter 4, I use electrophysiology to establish a functional relationship between the two regions. After characterization of these projections, I use cell type-

specific and projection-specific optogenetic stimulation of the GABAergic projections from ZI → RE to uncover the role of this pathway in fear generalization. These experiments revealed that activation of the ZI → RE inhibitory pathway prevents fear generalization. Finally, in Chapter 5, I collate the experimental findings presented in the above chapters, discuss the broader implications of the results and propose directions for future experiments. In sum, the work presented here, illuminates the function of ZI in calibrating fear responses and defines the contribution of a previously unidentified incerto-thalamic pathway in fear generalization.

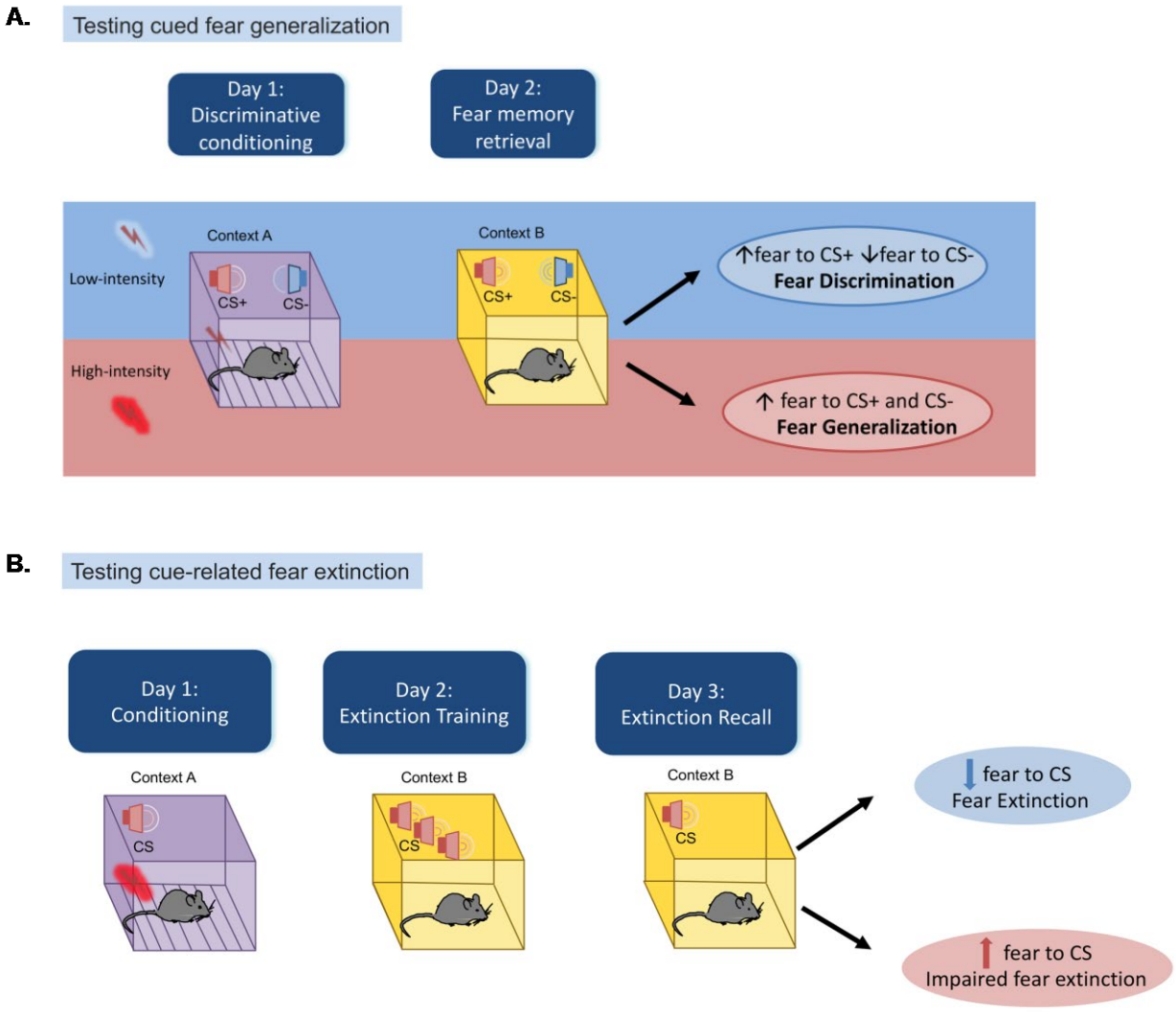

Figure 1.1: Behavioral protocols for testing fear inhibition.

(A) Experimental setup for testing cued-fear generalization. On day 1, one group of animals receive CS+ tone presentations paired with foot-shocks of low threat intensity and unpaired CS- tone presentations. Another group of mice receive CS+ tone presentations paired with foot-shocks of high threat intensity and unpaired CS- tone presentations. On day 2, fear memory to the CS+ and CS- tone presentations will be assessed in both groups of animals. Typically, animals that received low intensity foot-shocks exhibit fear discrimination while animals that received high intensity foot-shocks exhibit fear generalization. **(B)** Experimental setup for testing cued fear

extinction. On day 1, animals receive a CS tone presentation paired with aversive foot-shock. On day 2, animals receive repeated CS tone presentations in the absence of a shock. This generates a new memory as the animals learn to inhibit fear in response to the tone. On day 3, fear memory to the CS will be assessed. Reduced fear towards the CS is interpreted as successful fear extinction and persistence of fear towards the CS indicates impairments in fear extinction.

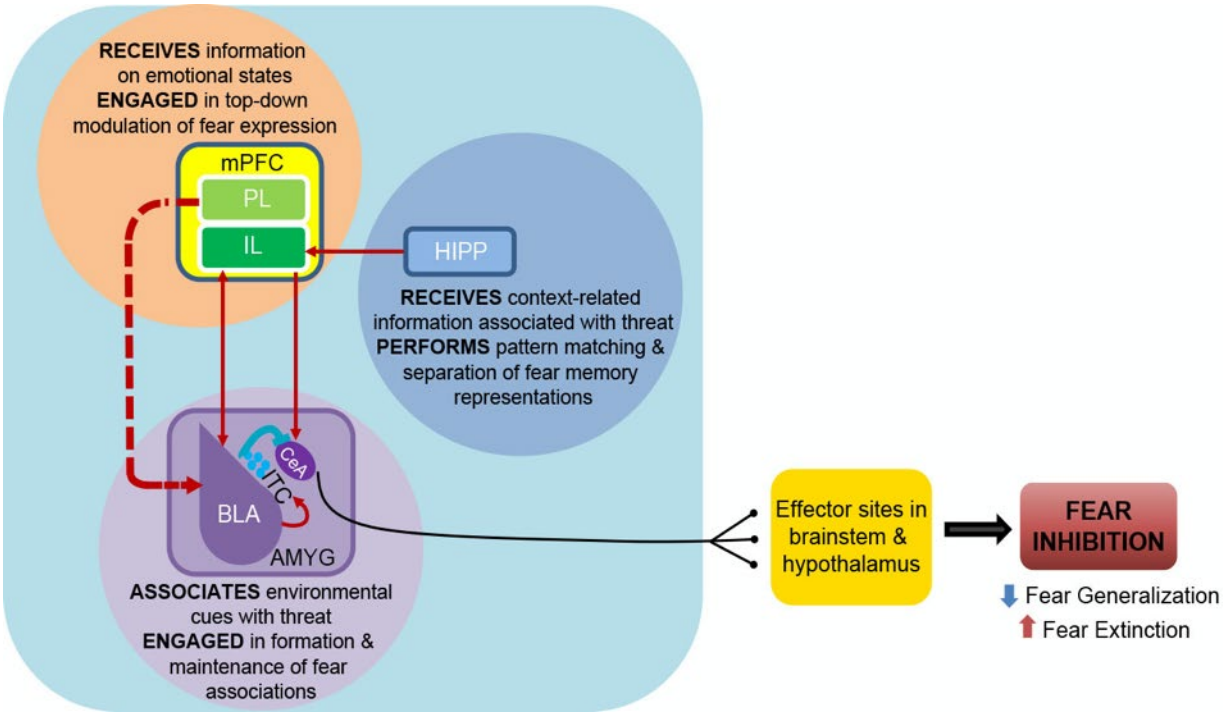

Figure 1.2: Canonical circuitry mediating fear inhibition.

The prefrontal cortex (PFC), hippocampus (HIPP) and amygdala (AMYG) are part of the canonical neural circuit mediating fear inhibition. Inhibition of fear responses requires formation and maintenance of appropriate fear memory representations in the AMYG with contextual inputs from HIPP and top-down modulation from the PFC. Solid red arrows indicate activation of an excitatory pathway and dashed arrows indicate suppression of the pathway. Solid blue lines indicate inhibitory connections.

PL: prelimbic prefrontal cortex; IL: infralimbic prefrontal cortex; BLA: basolateral amygdala; ITC: intercalated GABAergic interneurons; CeA: central amygdala

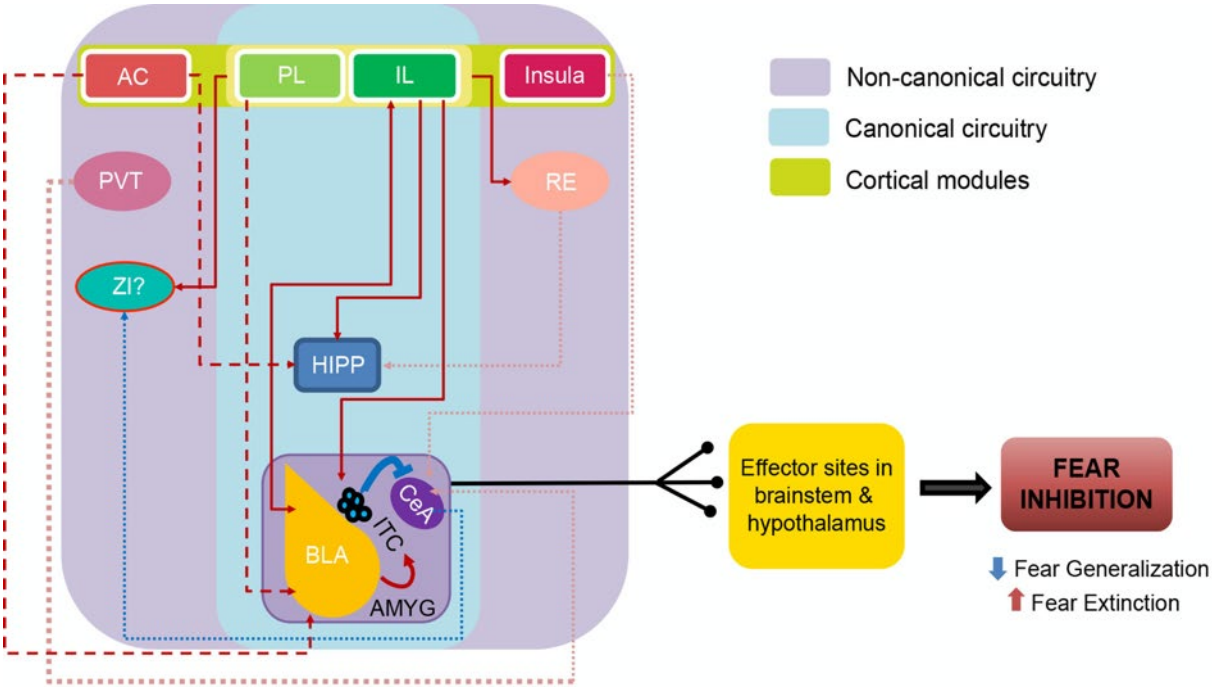

Figure 1.3: Neuronal circuits mediating fear inhibition.

The core canonical circuitry mediating fear inhibition including the PFC, HIPP, and HIPP is indicated in the blue rectangle in the center. The outer purple rectangle contains the non-canonical brain regions (AC, Insula, PVT, RE, and ZI) recently studied in the context of fear inhibition. Red lines indicate excitatory connections and blue lines indicate inhibitory connections. Dotted lines indicate dampening of the pathway to enable fear inhibition. Light red dotted connectors indicate the pathways with identified role in fear but yet to be examined in the context of fear inhibition. AC: anterior cingulate cortex; PVT: paraventricular nucleus of the thalamus; RE: thalamic nucleus reuniens; ZI: zona incerta; PL: prelimbic prefrontal cortex; IL: infralimbic prefrontal cortex; BLA: basolateral amygdala; ITC: intercalated GABAergic interneurons; CeA: central amygdala

CHAPTER 2: Bidirectional regulation of fear generalization by the zona incerta.

2.1 Context, Author's Contribution, and Acknowledgement of Reproduction

This chapter presents evidence that activity in the zona incerta (ZI) suppresses fear generalization. The study was the result of an effort to identify brain regions outside canonical fear-related circuitry that could potentially be modulated to suppress fear generalization. Presented here are data profiling activity-dependent protein expression in the sub-thalamic ZI that was associated with generalization and functional demonstration of role of the ZI in fear generalization using chemogenetics. The dissertation author designed and conducted most of the experiments with the exception of the electrophysiology data that were collected by Dr. Jidong Guo. The work was conceptualized, organized and written by the dissertation author and Dr. Brian Dias with guidance from Dr. Donald Rainnie. The chapter is reproduced with minor edits from Venkataraman, A., Brody, N., Reddi, P., Guo, J. G., Rainnie, D., Dias, B. G. (2019) Modulation of fear generalization by the zona incerta. *Proceedings of the National Academy of Sciences* 116 (8):9072-9077

2.2 ABSTRACT

Fear expressed towards threat-associated stimuli is an adaptive behavioral response. In contrast, the generalization of fear responses toward non-threatening cues is maladaptive and a debilitating dimension of trauma- and anxiety-related disorders. Expressing fear to appropriate stimuli and suppressing fear generalization requires integration of relevant sensory information and motor output. While thalamic and sub-thalamic brain regions play important roles in sensorimotor integration, very little is known about the contribution of these regions to the phenomenon of fear generalization. In this study, we sought to determine whether fear generalization could be modulated by the zona incerta (ZI), a sub-thalamic brain region that influences sensory discrimination, defensive responses, and retrieval of fear memories. To do so, we combined differential intensity-based auditory fear conditioning protocols in mice with C-FOS immunohistochemistry and DREADD-based manipulation of neuronal activity in the ZI. C-FOS immunohistochemistry revealed an inverse relationship between ZI activation and fear generalization – the ZI was less active in animals that generalized fear. In agreement with this relationship, chemogenetic activation of the ZI suppressed fear generalization in a high-intensity fear conditioning protocol that typically produces generalized fear. In contrast, chemogenetic inhibition of the ZI resulted in fear generalization in a low-intensity fear conditioning protocol that typically does not produce fear generalization. To conclude, our data suggest that stimulation of the ZI could be used to treat fear generalization that often occurs in the context of trauma- and anxiety-related disorders.

2.3 INTRODUCTION

Expressing fear toward cues that had been previously associated with trauma is adaptive (conditioned fear). Equally adaptive is the expression of fear toward stimuli that closely resemble traumatic cues (generalization). Such generalization of fear allows the organism to be "better safe than sorry". However, fear generalization can diminish quality of life and is a highly debilitating dimension of trauma- and anxiety-related disorders like Post-Traumatic Stress Disorder (PTSD) and Generalized Anxiety Disorder (GAD) (Dunsmoor & Paz, 2015; Dymond et al., 2015; Jasnow et al., 2017; Kaczkurkin et al., 2017). Reducing fear generalization while maintaining adaptive fear responses will reduce the daily burden experienced by individuals living with these disorders. Recently, introducing procedures that involve stimulus discrimination into cognitive behavioral therapy has been shown to reduce fear generalization, re-experiencing and intrusive thoughts in PTSD patients (Blechert et al., 2007; Ehlers, Clark, Hackmann, McManus, & Fennell, 2005; Lommen et al., 2017).

As discussed in Chapter 1, the canonical fear-related circuitry including the lateral amygdala (Ghosh & Chattarji, 2015; G. L. Jones et al., 2015; Rajbhandari et al., 2016), central amygdala (Ciocchi et al., 2010; Sanford et al., 2017), prefrontal cortex (Rozeske et al., 2018; Zelikowsky et al., 2013), and hippocampus (Jasnow et al., 2017; Lissek et al., 2014), have been strongly implicated in generalization of fear responses. More importantly, these regions play crucial roles in detecting threats and assigning valence to environmental stimuli (Gross & Canteras, 2012; Maren & Quirk, 2004; Orsini & Maren, 2012; Tovote et al., 2015). Therefore, while manipulating these regions could potentially reduce fear generalization, doing so might compromise threat detection, conditioned fear and survival. In this study, we set out to ask whether targeting brain

regions outside of the aforementioned canonical fear-related circuitry could reduce fear generalization.

Thalamic and sub-thalamic brain regions are ideal candidates to exert modulatory control over appropriate fear expression because they serve as hubs relaying information from sensory cortices to limbic, midbrain and brainstem nuclei (Bartho et al., 2002; Do Monte, Quirk, Li, & Penzo, 2016; Lissek et al., 2014; Tyll, Budinger, & Noesselt, 2011). While the contributions of these brain regions have largely been ignored in the context of fear-related behavior, newly emerging literature indicates that the thalamic nucleus reuniens influences fear generalization (Ferrara, Cullen, Pullins, Rotondo, & Helmstetter, 2017; Han et al., 2008; Ramanathan, Jin, et al., 2018; Xu & Sudhof, 2013) and that the paraventricular nucleus of thalamus influences fear conditioning and fear memory retrieval (Do-Monte et al., 2015; Penzo et al., 2015) (for further details, refer to Chapter 1). Most recently, the zona incerta (ZI), a sub-thalamic region, has received attention for its role in modulating defensive responses and retrieval of fear-related memories (Chou et al., 2018; Zhou et al., 2018). Notably, studies in rodents have highlighted that the ZI influences sensory discrimination (Legg, 1979; R. Thompson & Bachman, 1979) and that stimulation of the ZI in humans facilitates discrimination of fearful from non-fearful stimuli (Burrows et al., 2012). Motivated by these findings, we hypothesized a potential role for the ZI in fear generalization.

To test this hypothesis, we leveraged the fact that high threat intensities elicit excessive fear responses even towards neutral stimuli, resulting in fear generalization. We used differential auditory fear conditioning in mice at varying threat intensities to model high and low threat conditions. More specifically, during conditioning, auditory conditioned stimulus (CS+) presentations were paired with foot-shocks of low (0.3mA) or high (0.8mA) intensity, whereas a second stimulus (CS-) was not reinforced. Animals trained under low threat conditions (0.3mA)

expressed appropriately high fear responses to CS+ and relatively low fear responses to CS-. However, animals trained under high threat conditions (0.8mA) expressed high fear responses to both CS+ and CS-, exhibiting maladaptive fear generalization as is observed in individuals affected by PTSD and GAD (Blechert et al., 2007; Dunsmoor & Paz, 2015; Dymond et al., 2015; Jasnow et al., 2017; Kaczkurkin et al., 2017).

C-FOS immunohistochemistry revealed that the ZI was less active in animals that exhibited fear generalization following training under high threat conditions. To directly test whether the ZI plays a role in fear generalization, we manipulated cellular activity in the ZI using chemogenetic approaches. Stimulating cells in the ZI suppressed fear generalization in animals trained under high threat conditions, while decreasing cellular activity in the ZI resulted in fear generalization in animals trained under low threat conditions. These results provide evidence that the ZI can modulate expression of appropriate behavioral fear responses. To our knowledge, our study is the first demonstration that stimulating the ZI may be of therapeutic value in reducing fear generalization.

2.4 MATERIALS AND METHODS

2.4.1 Animals

Adult female and male mice (2-3 months of age) were group-housed under a 14:10 light/dark cycle with food and water available ad libitum. C57BL/6J (wild type) mice were originally ordered from Jackson labs and then bred in our vivarium for these experiments. All experimental procedures involving animals were approved by the Emory Institutional Animal Care and Use Committee and carried out in accordance with National Institute of Health standards.

2.4.2 Auditory fear conditioning to test fear generalization

Differential intensity-based auditory fear conditioning was used to test fear generalization as described elsewhere (Aizenberg & Geffen, 2013). Briefly, the training and testing protocol consisted of four phases on four consecutive days: (1) habituation, (2) baseline, (3) training, and (4) testing (as outlined in Fig. 2.1A). On the first day, mice were habituated to the CS+ tone in the training context (Context A) for 10 minutes. One day later, during the baseline phase in Context A, freezing levels were measured during two random presentations each of the CS+ and the CS-, followed by exposure to continuous CS+ tone for a total of 10 minutes in Context A. Pre-exposure to the tones were designed in the protocol to allow for better discrimination and has been shown to prevent generalization (Ito, Pan, Yang, Thakur, & Morozov, 2009; Rescorla, 1976). The training phase that occurred one day later, included an initial 5-minute exposure to Context A followed by 20 trials consisting of 10 CS+ presentations that co-terminated with a 0.5 sec foot-shock with randomly interleaved 10 CS- presentations that were not reinforced. Depending on the experiment, either 0.3 mA (low threat condition) or 0.8 mA (high threat condition) foot-shocks were used as the unconditioned stimulus paired with the CS+. The inter-trial intervals varied randomly between 2-6 mins. During the testing phase on day 4, mice were exposed to a new context (Context B) for 3 minutes followed by two randomized presentations each of the CS+ and CS- and freezing levels

measured during the tone presentations were used as a behavioral index of fear generalization. FreezeFrame-4 software (Actimetrics) was used for stimulus presentations and video recording of freezing behavior. Hardware associated with these experiments was purchased from Harvard Apparatus. The time spent freezing to CS+ and CS- was analyzed by an experimenter blind to the treatment conditions, using FreezeFrame software with the freezing bout length set to 0.5 secs. Context A consisted of grid flooring, illuminated with house lights and cleaned with the disinfectant, quatricide. Context B consisted of plexiglass flooring, illuminated with infra-red lights and cleaned with 70% ethanol. Sound levels were adjusted so that all tones were presented at approximately 85dB. CNO injections (where relevant) were administered intra-peritoneally at a dose of 1 mg/kg, one hour before testing for fear generalization in Context B. Discrimination index (DI) was calculated as the difference in the % of time spent freezing to the conditioned and neutral tone divided by the sum of the % of time spent freezing to both tones.

2.4.3 Stereotaxic surgeries

To manipulate cellular activity in the ZI of wild-type C57BL/6J animals, we used AAV5-hSyn-hM4DGi-mCherry (to reduce activity), AAV5-hSyn-hM3DGq-mCherry (to stimulate activity) and AAV5-hSyn-eGFP (as control) viruses. All viruses were obtained from the UNC Viral Vector Core and Addgene. Bilateral stereotaxic AAV injections into ZI were performed while the animal was under anesthesia using the following stereotaxic co-ordinates: AP: -1.52 mm, ML: 0.73 mm and DV: -4.79 mm relative to Bregma (Fig. 2.4). AAV-containing solutions were injected at the rate of 1 nl/sec using Nanoject III (Drummond Scientific) and behavioral experiments were performed after 2 weeks to allow for optimal viral expression. A final volume of 50 nl of AAV5-hSyn-eGFP, AAV5-hSyn-hM4DGi-mCherry, and AAV5-hSyn-hM3DGq-mCherry was infused. Animals were sacrificed after behavioral experiments for histological examination of viral

infusions.

2.4.4 C-FOS immunohistochemistry & Cell Counting

C-FOS protein expression was detected 90 minutes after exposure to either the CS+ or the CS- on testing day (as outlined in Fig. 2.2A) in animals trained under low or high threat conditions. Mice were trans-cardially perfused with 4% paraformaldehyde dissolved in 1X phosphate-buffered saline (PBS). Brains were collected and stored in paraformaldehyde solution for a day and transferred to 30% sucrose solution for 3-4 days before sectioning at 35µm on a freezing microtome (Leica). Brain sections were washed three times in 1X PBS for 10 minutes and incubated in 0.3% hydrogen peroxide to block endogenous peroxidase activity. Sections were blocked in 1X PBS with 5% normal goat serum for 1 hour at room temperature and then incubated in primary rabbit polyclonal anti-C-FOS antibody (1:6000 dilution, Millipore ABE 457) overnight on a shaker at room temperature. The next day, sections were washed three times in 1X PBS for 10 minutes and then incubated in secondary biotinylated goat anti-rabbit IgG antibody (1:1000 dilution, Vector Laboratories BA-1000) for 2 hours. Following this, sections were treated for 1 hour with avidin-biotin peroxidase system (Vectastain Elite ABC kit, PK-6100) and visualized using 3,3'-diaminobenzidine (Sigma-Aldrich). Sections were mounted on SuperFrost Plus slides (Fisher Scientific) and after drying, slides were coverslipped using Permount (Fisher Scientific). Images of the ZI were captured using Nikon E800 microscope at 4X magnification and C-FOS expression was quantified using MCID Core Imaging software. C-FOS immunoreactivity was quantified across three consecutive sections per animal in both left and right hemispheres.

2.4.6 Histology

To validate the placement of intra-cranial virus injections, animals were anesthetized and trans-cardially perfused after behavioral experiments with 4% paraformaldehyde dissolved in 1X

phosphate-buffered saline (PBS). Brains were removed and stored in paraformaldehyde solution for a day and transferred to 30% sucrose solution for 3-4 days before sectioning on a freezing microtome (Leica). Brains were sectioned at 35µm, stained with Hoechst nuclear stain (1:1000) and mounted on slides using SlowFade Gold Antifade mountant (Life Technologies). The position of GFP or mCherry positive cells was assessed using Nikon Eclipse E800 fluorescent microscope (presented in Fig. 2.4).

2.4.7 Open field test

The open field arena (50 x 50 x 50 cm^3) was illuminated by red lights with the center defined as 16% of the total area. The mice were acclimated to the red-light conditions in the testing room for 1 hr after i.p. CNO injections (1mg/kg). The mice were then placed in the center of the arena and allowed to explore for 5 mins. Each session was videotaped using an overhead digital camera and the data were analyzed using automated video tracking system TopScan 2.0 (CleverSys Inc.).

2.4.8 Electrophysiology

Four to six weeks after intra-cranial virus injections, 300 µm mouse brain slices containing ZI were obtained as previously reported (Daniel, Guo, & Rainnie, 2017). Briefly, each mouse in this study was anesthetized with isoflurane, the brain was quickly removed from the skull, and a tissue block containing the ZI mounted on the stage of a Leica VTS-1000 vibratome (Leica Microsystems Inc., Bannockburn, IL, USA). Coronal slices were obtained and then incubated in 95%O2/5%CO2 oxygenated artificial cerebrospinal fluid (ACSF) at 32°C for 1 hr before recording.

At the start of each recording, an individual slice was transferred to a recording chamber mounted on the stage of Leica STP6000 microscope and perfused with oxygenated ACSF at 32°C at a speed of 1-2 ml/min. Individual neurons in the ZI were visualized in bright field space using an infrared sensitive Hamamatsu CCD camera connected to a Windows PC using Simple PCI

software. To identify neurons expressing the fluorescent transgene, we used epifluorescent illumination in combination with the appropriate excitation/emission filter sets. Standard whole cell patch-clamp recordings from fluorescent neurons in the ZI were performed using a MultiClamp 700B amplifier, an Axon Digidata 1550 A-D interface, and pClamp 10.4 software (Molecular Devices Corporation, Sunnyvale, CA). Recording pipettes were pulled from borosilicate glass and had resistances of 4-6 MΩ when filled with intracellular solution (in mM): 130 K-Gluconate, 2 KCl, 10 HEPES, 3 MgCl2, 5 phosphocreatine, 2 K-ATP, and 0.2 NaGTP. The patch solution was buffered to a pH of 7.3 and had an osmolarity of 280-290 mOsm. Current clamp recordings were performed to examine the effect of bath application of clozapine-N-oxide (CNO, 20 μM) on the resting membrane potential, and basic physiological properties of ZI neurons.

2.4.9 Statistical Analysis

GraphPad Prism was used to analyze the data. Unpaired t-tests were used for data sets containing only two groups and one dependent variable (C-FOS immunohistochemistry). Repeated-measures two-way ANOVA was used to analyze data sets with more than one independent variable (behavior experiments). Post-hoc tests were only performed when interaction effects between the independent variables were significant and Sidak's correction applied to account for multiple comparisons. Significance was set at $p < 0.05$.

2.5 RESULTS

2.5.1 High intensity foot-shock training leads to fear generalization.

We trained mice in a differential auditory fear conditioning protocol using low and high threat foot-shocks to study the role of the zona incerta in fear generalization (Fig. 2.1A). Wild type mice trained under low threat conditions (0.3mA foot-shocks), exhibited appropriate fear responses as indicated by increased freezing to CS+ (conditioned auditory stimulus) and reduced freezing to CS- (neutral auditory stimulus) (Fig. 2.1B). Under high threat conditions (0.8mA foot-shocks), wild type mice exhibited overgeneralization of fear as indicated by indistinguishable freezing to both the CS+ and CS- tones (Fig. 2.1B). (Low-intensity training group n = 14, High-intensity training group n = 10, Training x Tone interaction $F(1, 22) = 19.17$, $p < 0.0001$ Post-hoc tests: Low Intensity Training: CS- vs. Low Intensity Training: CS+ $p<0.0001$, Low Intensity Training: CS- vs. High Intensity Training: CS- $p<0.01$). Animals trained under high threat conditions showed poor discrimination in their fear response to the CS+ and CS- and increased generalization, as noted by their lower discrimination index compared to animals trained under low threat conditions. (Fig. 2.1C) ($p < 0.01$, $t = 3.640$, $df = 22$). Importantly, there was no statistically significant difference between the groups in their freezing response to the context alone on the day of testing (Fig. 2.1D), demonstrating a specificity of freezing responses to the tones.

2.5.2 Decreased neuronal activity in the ZI accompanies increased fear generalization.

To examine neuronal activation of the ZI in the context of fear generalization, we counted the number of cells expressing the immediate early gene, C-FOS, in the ZI after exposing animals to either CS- or CS+ tone presentations. These animals had been previously trained under low threat or high threat conditions as outlined (Fig. 2.2). Animals trained under high threat conditions expressed increased fear to CS- on the day of testing, accompanied by lower numbers of C-FOS positive cells in the ZI (Figs. 2.3 A-C). We did not find any significant differences between groups

in the numbers of C-FOS positive cells in the ZI after exposure to the CS+. (Training x Tone interaction $F(1, 19) = 4.944$, $p < 0.05$. Post-hoc tests: Low Intensity Training: CS- vs. High Intensity Training: CS- $p<0.01$. CS-: Low-intensity shock group n = 7, High-intensity shock group n = 8; CS+: Low-intensity shock group n = 4, High-intensity shock group n = 4). We found that in general, higher levels of fear expression (as measured by the freezing responses) were associated with lower numbers of C-FOS expressing cells in the ZI (Fig. 2.3D) (n = 21 animals, $p < 0.01$, $r = -0.5563$).

2.5.3 Increasing cellular activity in the ZI reduces fear generalization that manifests after conditioning with high intensity foot-shocks.

We utilized Gq-coupled DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) to increase activity of cells in the ZI (Figs. 2.4, 2.5). Bath application of 20 μM of CNO *in vitro* depolarized hM3DGq positive neurons in the ZI, increasing cellular activity (Fig. 2.5D). We queried whether stimulating cells in the ZI can reduce fear generalization observed in animals trained under high threat conditions. We injected AAV5-hsyn-hM3D(Gq)-mCherry or AAV5-hsyn-eGFP bilaterally into the ZI of wild type mice and CNO was administered intraperitoneally, one hour before testing fear generalization (Figs. 2.5 A-C). Increasing activity of the ZI reduced fear generalization in animals trained under high threat conditions (Fig. 2.5E). Specifically, High Intensity Training-hM3D(Gq)+CNO animals exhibited significantly lower freezing responses to CS- than their responses to CS+, compared to the High Intensity Training-GFP+CNO animals. (High Intensity Training-GFP+CNO group n = 7, High Intensity Training-hM3DGq+CNO n = 10, DREADD x Tone interaction: $F(1,15) = 20.16$, $p < 0.001$. DREADD treatment main effect: $F(1,15) = 19.47$, $p < 0.001$. Tone main effect: $F(1,15) = 136.6$, $p < 0.0001$. Post-hocs: High Intensity Training-GFP+CNO:CS- vs. High Intensity Training-hM3DGq+CNO:CS- $p<0.0001$, High Intensity Training-hM3DGq+CNO:CS+ vs. High Intensity Training-hM3DGq+CNO:CS-

p<0.0001, High Intensity Training-GFP+CNO:CS+ vs. High Intensity Training-hM3DGq+CNO:CS+ p<0.01). High Intensity Training-hM3DGq+CNO animals showed better discrimination in their fear response to the CS+ and CS- as noted by their higher discrimination index compared to High Intensity Training-GFP+CNO animals (Fig. 2.5F) ($p < 0.0001$, $t = 5.931$, $df = 17$). There was no statistically significant difference between the groups in their freezing responses to the context (Context B) before tone presentations on the day of testing (Fig. 2.6A), suggesting a specificity of freezing responses to the tones. These observed differences in freezing responses of animals with chemogenetic activation of ZI, were not accompanied by alterations in locomotor activity or anxiety-like behavior (Figs. 2.6 B-D).

2.5.4 Decreasing cellular activity in the ZI results in fear generalization after conditioning with low intensity foot-shocks.

We utilized Gi-coupled DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) to decrease activity of cells in the ZI (Fig. 2.7). Bath application of 20 μM of CNO *in vitro* hyperpolarized hM4DGi expressing neurons in the ZI, reducing cellular activity (Fig. 2.7D). We queried whether such reductions of cellular activity in the ZI would facilitate fear generalization in animals trained under low threat conditions that normally do not exhibit fear generalization. We injected AAV5-hsyn-hM4D(Gi)-mCherry or AAV5-hsyn-eGFP bilaterally into the ZI of wild type mice and CNO was administered intraperitoneally, one hour before testing fear generalization (Figs. 2.7 A-C). Decreasing activity of the ZI resulted in fear generalization in animals trained under low threat conditions (Fig. 2.7E). Specifically, Low Intensity Training-hM4D(Gi)+CNO animals exhibited significantly higher freezing responses to CS- than compared to freezing responses to the CS- of the Low Intensity Training-GFP+CNO animals. (Low Intensity Training-GFP+CNO group $n = 6$, Low Intensity Training-hM4DGi+CNO $n = 7$, DREADD x Tone interaction: $F(1,11) = 6.335$, $p < 0.05$. DREADD treatment main effect: $F(1,11) = 26.73$, $p < 0.001$.

Tone main effect: $F(1,11) = 91.91$, $p < 0.0001$. Post-hocs: Low Intensity Training-GFP+CNO:CS- vs. Low Intensity Training-GFP+CNO:CS+ $p < 0.0001$, Low Intensity Training-hM4DGi+CNO:CS- vs. Low Intensity Training-hM4DGi+CNO:CS+ $p < 0.01$, Low Intensity Training-GFP+CNO:CS- vs. Low Intensity Training-hM4DGi+CNO:CS- $p < 0.0001$). Low Intensity Training-hM4DGi+CNO animals showed an impaired ability to discriminate between the CS+ and CS- as noted by their lower discrimination index compared to Low Intensity Training-GFP+CNO animals (Fig. 2.7F) ($p < 0.01$, $t=3.572$ $df=14$). There was no statistically significant difference between the groups in their freezing responses to the context (Context B) before tone presentations on the day of testing (Fig. 2.8A), suggesting a specificity of freezing responses to the tones. Chemogenetic inhibition of cells in the ZI was not accompanied by alterations in locomotor activity or anxiety-like behavior (Figs. 2.8B-D).

2.6 DISCUSSION

Our results demonstrate a novel role for the zona incerta (ZI) in modulating fear generalization. First, we found reduced C-FOS activation in the ZI associated with increased fear towards a neutral auditory stimulus. Next, stimulation of cellular activity in the ZI reduced generalized fear responses observed after training animals under high threat conditions. Further, we found that reducing cellular activity in the ZI resulted in fear generalization in animals trained under low threat conditions. Taken together, our data provide evidence for a translationally relevant role for the ZI in modulating fear generalization.

Lesioning studies as well as computational models have suggested a role for thalamic and sub-thalamic brain circuits in stimulus discrimination (Antunes & Moita, 2010; Armony, Servan-Schreiber, Romanski, Cohen, & LeDoux, 1997; Heldt & Falls, 2006) – a key component of fear generalization. In particular, it is hypothesized that the broader receptive fields of thalamic and sub-thalamic neurons communicating with core fear-related circuitry could support fear generalization (Armony et al., 1997; Halassa & Acsady, 2016; Resnik, Sobel, & Paz, 2011). Increasing threat intensities has been shown to broaden generalization gradients in humans (Dunsmoor, Kroes, Braren, & Phelps, 2017). In this study, we used a threat intensity-based model of fear generalization in rodents to examine sub-thalamic contributions to fear processing. Animals when trained under high threat conditions (0.8mA foot-shocks) generalized fear to both conditioned (CS+) and neutral (CS-) tones whereas animals trained under low threat conditions (0.3mA foot-shocks) did not demonstrate such generalization. These observations agree with previous reports that conditioning using increasing shock intensities promotes cue-related fear generalization in rodents (Ghosh & Chattarji, 2015; Laxmi, Stork, & Pape, 2003). While others have reported fear generalization to occur across contexts using similar protocols (Baldi, Lorenzini, & Bucherelli, 2004; Duvarci, Bauer, & Pare, 2009; Fanselow, 1980; Poulos et al.,

2016), we do not observe the generalization of fear in testing Context B after high-intensity fear conditioning in Context A. This lack of contextual fear generalization in our high intensity training protocol could be attributed to differences in study organism and experimental design. First, most studies that have demonstrated context-related fear generalization have used rats and there may be species differences in cue- and context-related fear generalization. Second, Context A and Context B were made easily distinguishable in our experimental protocol with the use of distinct floors, odors and chamber lighting – changes that a recent study in mice suggested were sufficient to prevent the expression of contextual fear generalization (Huckleberry, Ferguson, & Drew, 2016).

To test whether the ZI is responsive to neutral stimuli and potentially involved in fear generalization, we first sought to compare C-FOS immunohistochemistry in the ZI of animals trained under low and high threat conditions. More specifically, we counted C-FOS positive cells in the ZI of animals exposed to the CS+ or CS- on testing day. Excitingly, we found fewer C-FOS positive cells in the ZI of animals that generalized fear to the CS-. Additionally, we found reduced C-FOS expression in the ZI after exposure to the CS+ in animals trained under low as well as high threat conditions. Could the ZI modulate fear generalization associated with high threat conditions? The ZI is ideally positioned to convey information regarding the salience of specific sensory stimuli and orchestrate appropriate fear-related behavioral responses. First, the ZI receives projections from sensory cortices (including the auditory cortex) and can coordinate activity across cortical networks according to attentional demands (Bartho et al., 2007; Mitrofanis & Mikuletic, 1999). Additionally, the ZI innervates midbrain regions like the periaqueductal gray that plays an important role in orchestrating fearful behaviors (Gross & Canteras, 2012; Mitrofanis, 2005; Mota-Ortiz, Sukikara, Felicio, & Canteras, 2009). Second, the ZI has been implicated in sensory discrimination and can modulate incoming sensory information (Trageser et al., 2006). Finally,

stimulating the ZI in humans facilitates the discrimination of fearful faces from non-fearful ones (Blomstedt et al., 2012; Burrows et al., 2012). It is possible that stressful states like those created by high-intensity threat conditioning directly perturb cellular function in the ZI, rendering fear generalization as a behavioral outcome. Alternatively, generalized fear responses could arise indirectly due to amygdala→ZI connectivity (Mitrofanis, 2005; Zhou et al., 2018). Loss of cue-specificity and widening of the memory trace in the amygdala occurring during fear generalization (Ghosh & Chattarji, 2015) could alter ZI's influence on modulating fear responses. Future experiments will need to examine how cellular and molecular niches in the ZI are impacted by stress as well as amygdala function, resulting in generalization of fear responses.

Building on our observations from the C-FOS study, we used DREADD-based strategies to test whether manipulating cellular activity in the ZI affected fear generalization. Reducing cellular activity in the ZI resulted in animals trained under low threat conditions showing fear generalization. Conversely, increasing the activity of cells in the ZI of animals trained under high threat conditions distinctly reduced fear generalization. It is important to note that the caudal ZI has been associated with motor function due to its connections with the basal ganglia network and has been investigated as a potential target for deep brain stimulation treatment for patients with Parkinson's disease (PD) (Blomstedt et al., 2012; Burrows et al., 2012; Mitrofanis, 2005). Therefore, alterations in locomotor behavior could have potentially contributed to the observed effects on fear generalization following the bidirectional chemogenetic manipulations of cellular activity in the ZI. However, we did not observe any significant differences in total distance traveled and velocity during open field tests performed after chemogenetic manipulation of ZI (Figs. 2.6, 2.8). Freezing to the testing context (Context B) remained unaltered after stimulating activity in

the ZI (Figs. 2.6A, 2.8A), further emphasizing that the observed effects on fear generalization were specific to the CS+ and CS- tones presented.

Overall, the experimental results described here bolster the recently demonstrated link between ZI activity and fearful behavior and its role in calibrating fearful behavior toward environmental stimuli (Chou et al., 2018; Zhou et al., 2018). Our study makes a novel contribution to this body of work by demonstrating a role for the ZI in fear generalization. To conclude, our work suggests that stimulating the ZI in the clinic during exposure therapy could potentially reduce fear generalization, while leaving adaptive fear responses intact.

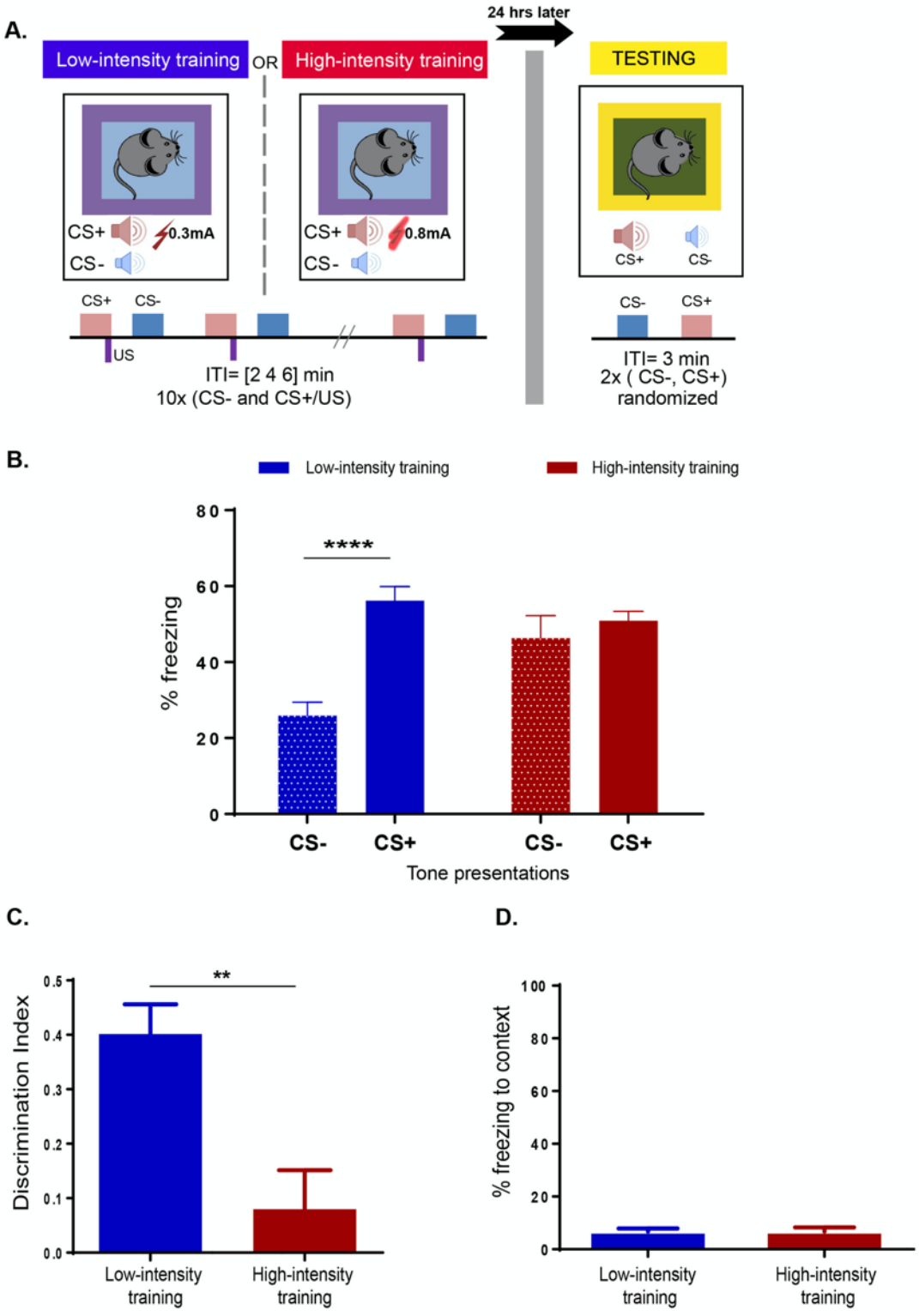

Figure 2.1: Increasing shock intensities promotes fear generalization.

(A) Outline of the differential auditory fear conditioning protocol used in the study. On day 1, one group of mice received CS+ tone presentations paired with 0.3mA foot-shocks (low threat intensity) and unpaired CS- tone presentations. Another group of mice received CS+ tone presentations paired with 0.8mA foot-shocks (high threat intensity) and unpaired CS- tone presentations. On day 2, freezing responses in both groups of animals were recorded for the CS+ and CS- tone presentations. **(B)** Animals trained under low threat conditions show low freezing response to CS- and high freezing response to CS+ (no fear generalization). In contrast, animals trained under high threat conditions show increased freezing response to both CS- and CS+ (fear generalization). **(C)** Discrimination indices calculated for the two groups reveal significant fear generalization in the animals trained under high threat conditions. **(D)** Freezing responses in both groups are specific to the tone presentations on testing day. No significant differences were observed in freezing to Context B on testing day between animals trained under low and high threat intensities. $^{**}p<0.01$, $^{****}p<0.0001$. Data represented as Mean ± S.E.M.

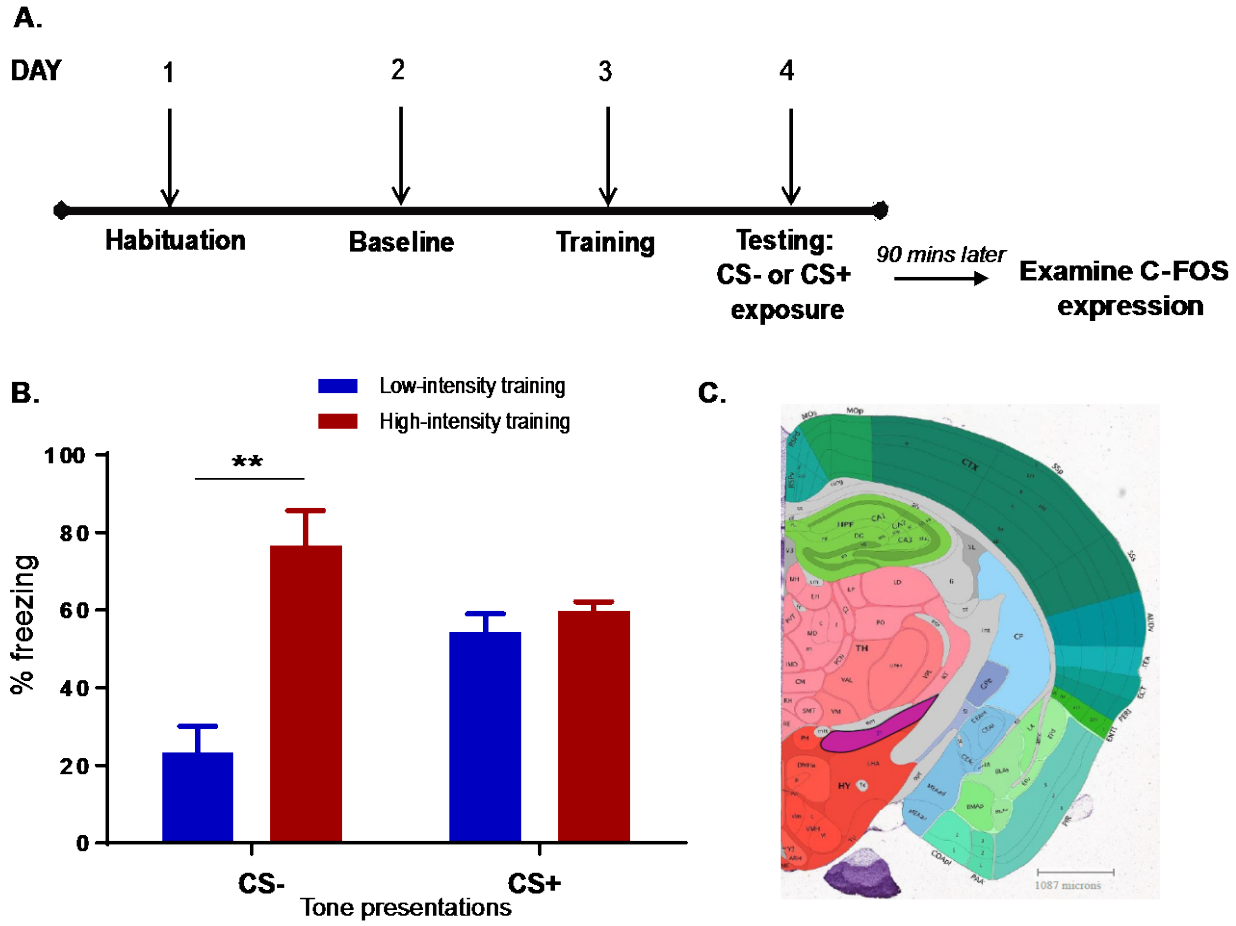

Figure 2.2: Animals trained under high threat conditions express increased fear to the neutral stimulus alone.

(A) Experimental design for C-FOS study: After habituation and baseline recording of stimulus responses, animals were split into four different groups. One group of mice received CS+ tone presentations paired with 0.3mA foot-shocks (Low-intensity training) and unpaired CS- tone presentations. Another group of mice received CS+ tone presentations paired with 0.8mA foot-shocks (High-intensity training) and unpaired CS- tone presentations. On day 2, each group was further divided in to two and freezing responses were recorded for CS+ or CS- tone presentations alone (Low-intensity training/CS-; Low-intensity training/CS+; High-intensity training/CS-; High-intensity training/CS+). **(B)** Animals trained under high threat conditions show

significantly increased freezing response to CS+ compared to animals trained under low threat conditions. No significant differences were observed in animals' response to CS+, when trained under the different threat intensities. **(C)** Reference image from Allen Brain Atlas showing position of ZI shaded in purple. $^{**}p<0.01$. Data represented as Mean ± S.E.M.

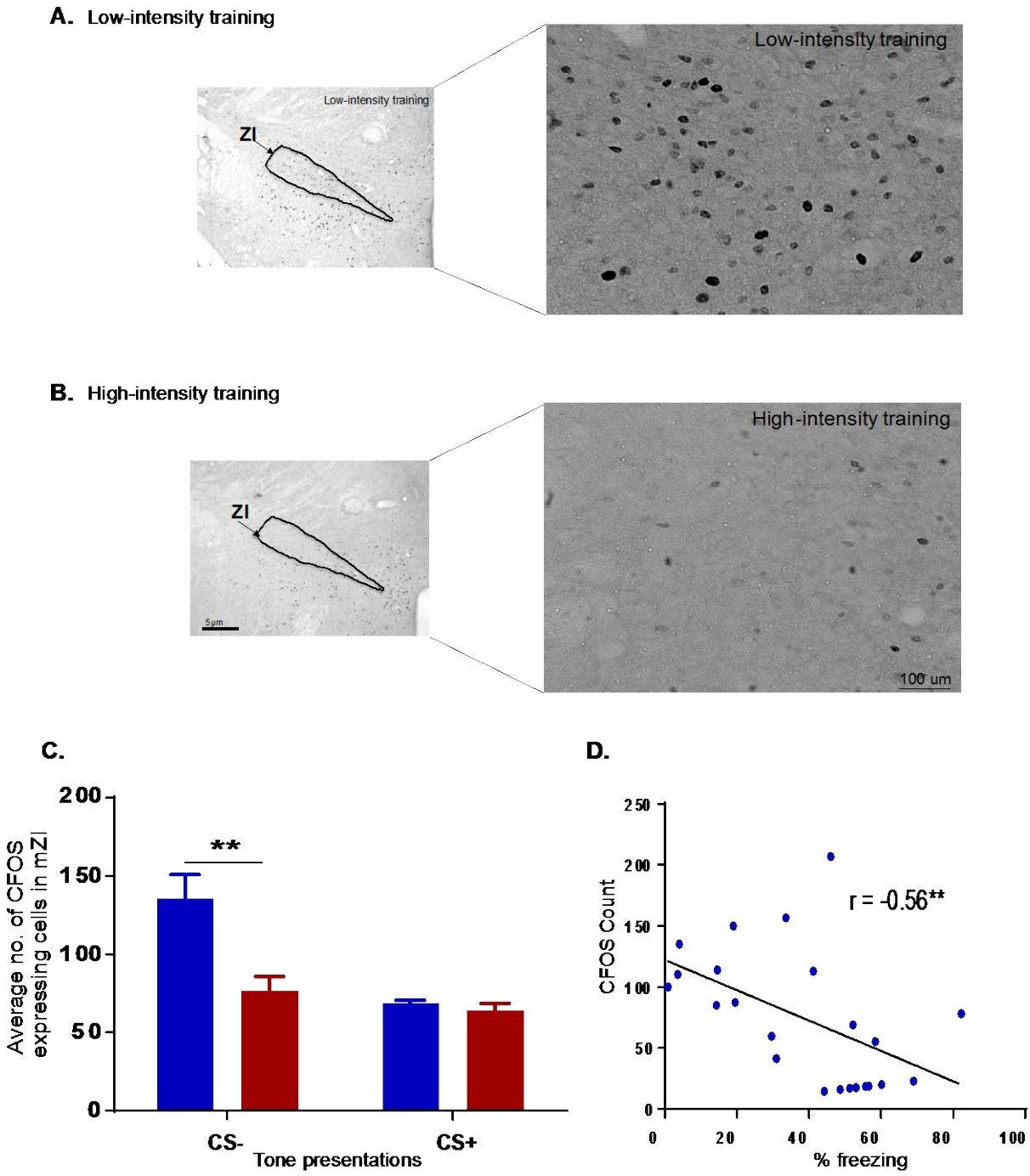

Figure 2.3: Fear generalization is associated with decreased neuronal activation in the ZI.

(A) Left: Representative images of C-FOS expression in the ZI in response to tone presentations during testing day after training under low threat conditions. Right: Representative image of darkly stained neuronal nuclei expressing relatively higher levels of C-FOS, at 20X magnification. **(B)** Left: Representative images of C-FOS expression in the ZI in response to tone presentations during testing day after training under high threat conditions. Right: Representative image of darkly stained neuronal nuclei expressing relatively lower levels of C-FOS, at 20X magnification. **(C)** Decreased C-FOS expression was observed in the ZI of animals that showed increased fear to CS- presentation on testing day. **(D)** Significant correlation was found between C-FOS expression in the ZI and behavioral fear responses. $^{**}p<0.01$. Data represented as Mean ± S.E.M.

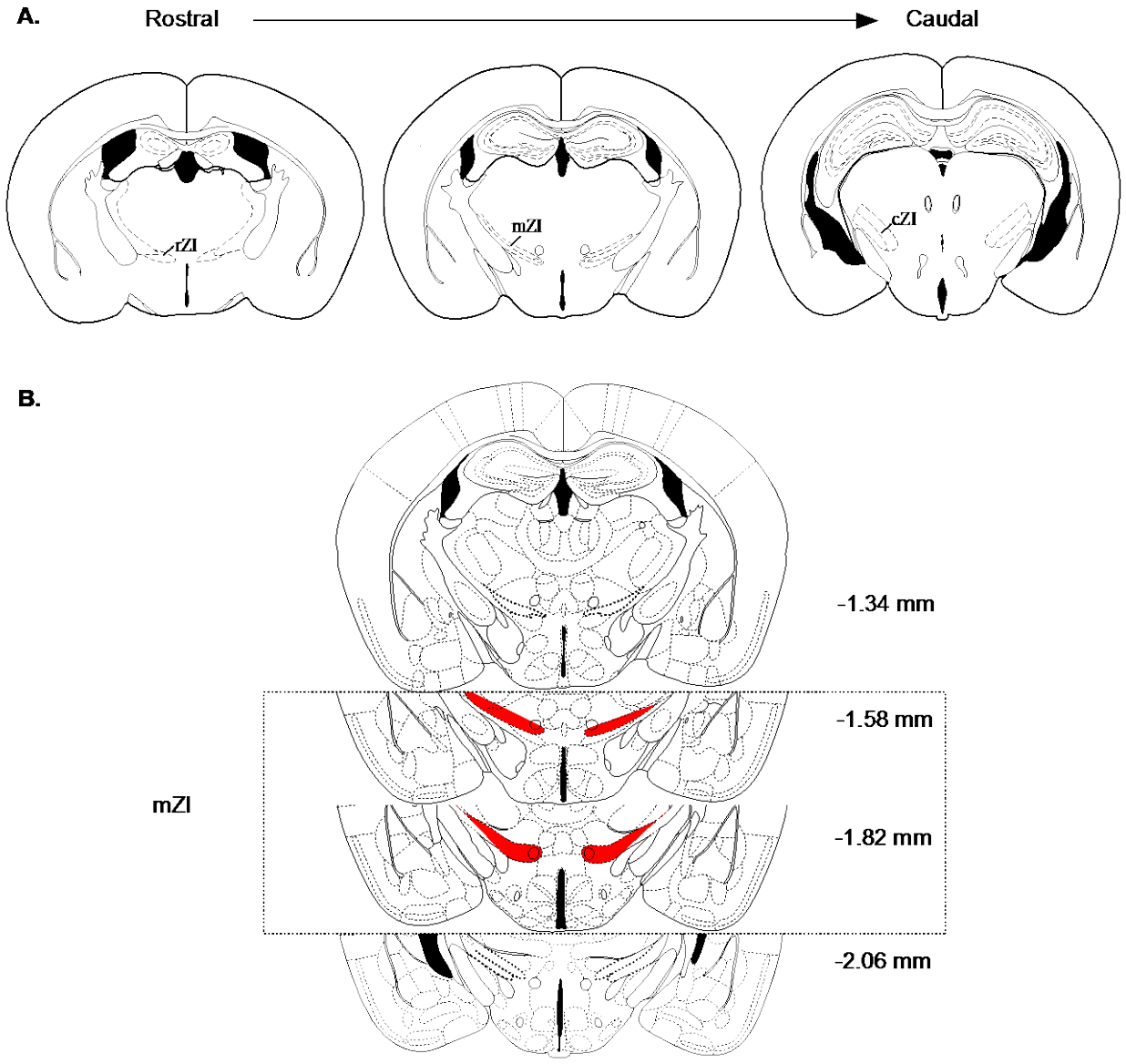

Figure 2.4: Schematic of ZI target placements in this study

(A) Rostrocaudal extent of the zona incerta showing rostral (rZI), medial (mZI) and caudal (cZI) subdivisions. (B) Injection sites were restricted to portions of the medial zona incerta (mZI) within the coordinates noted in the Methods section.

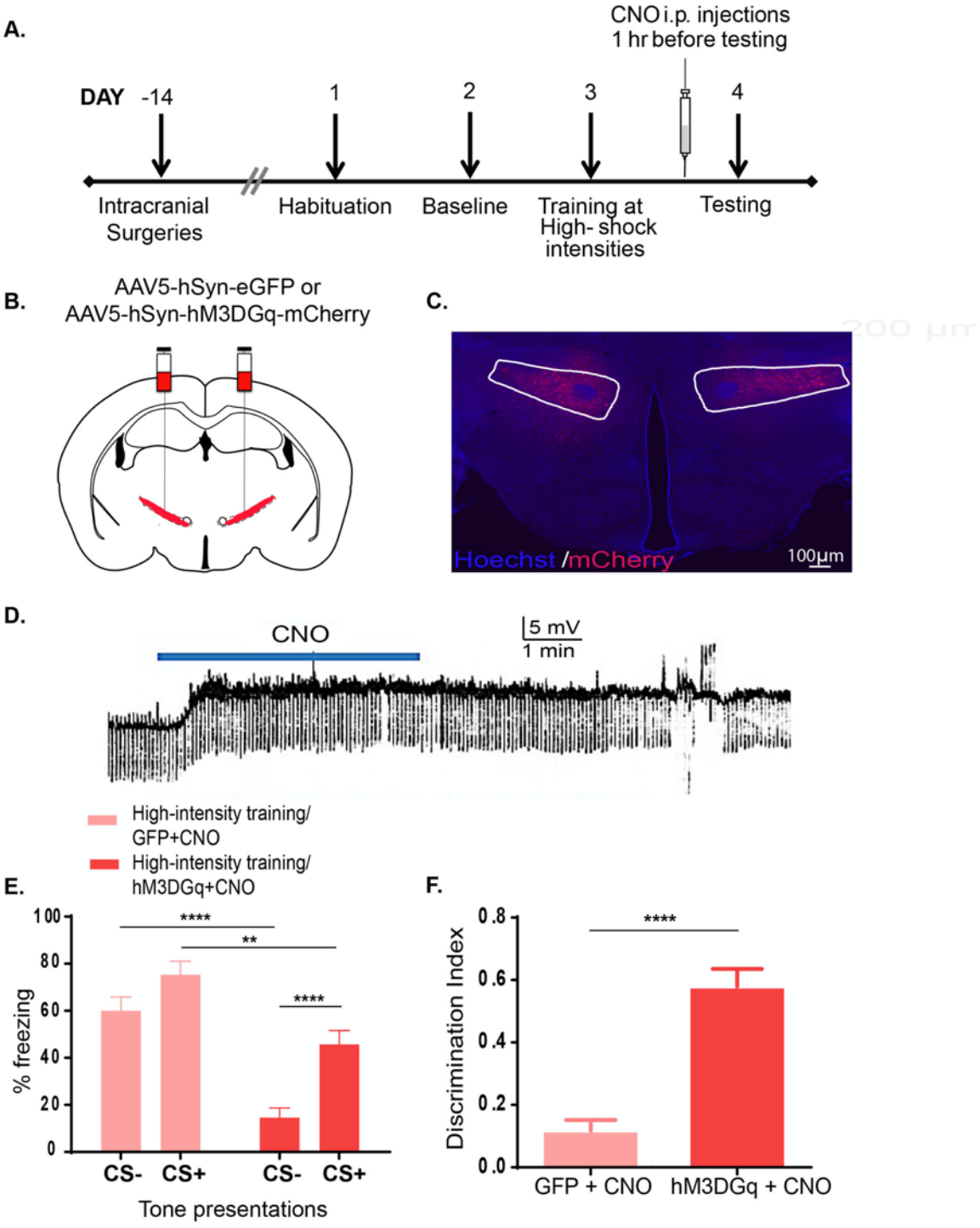

Figure 2.5: Increasing cellular activity in the ZI prevents fear generalization.

(A) Experimental protocol for chemogenetic activation. Two weeks after intracranial injection of the control or DREADD virus, animals were conditioned to high threat intensities. The next day, CNO was administered intraperitoneally 1 hour before testing fear generalization. **(B)** Wild-type animals were injected with either the control virus (AAV5-hSyn-eGFP) or excitatory DREADDs (AAV5-hSyn-hM3DGq-mCherry) at -1.5mm posterior to bregma. **(C)** Representative image of the ZI targeted with intra-cranial infusions of DREADD-expressing mCherry viruses. **(D)** Patch-clamp recording of hSyn-hM3DGq-mCherry expressing cells in the ZI showing membrane depolarization during CNO exposure. **(E)** Training using high intensity foot-shock causes fear generalization as seen by high freezing to both CS+ and CS-. Chemogenetic activation of the ZI (hM3DGq+CNO) resulted in a significant decrease in fear response to CS+ as well as CS- compared to controls (GFP+CNO). **(F)** Chemogenetic activation of the ZI (hM3DGq+CNO) resulted in a better ability to discriminate between the CS+ and the CS-. $^{**}p<0.01$, $^{****}p<0.0001$. Data represented as Mean ± S.E.M.

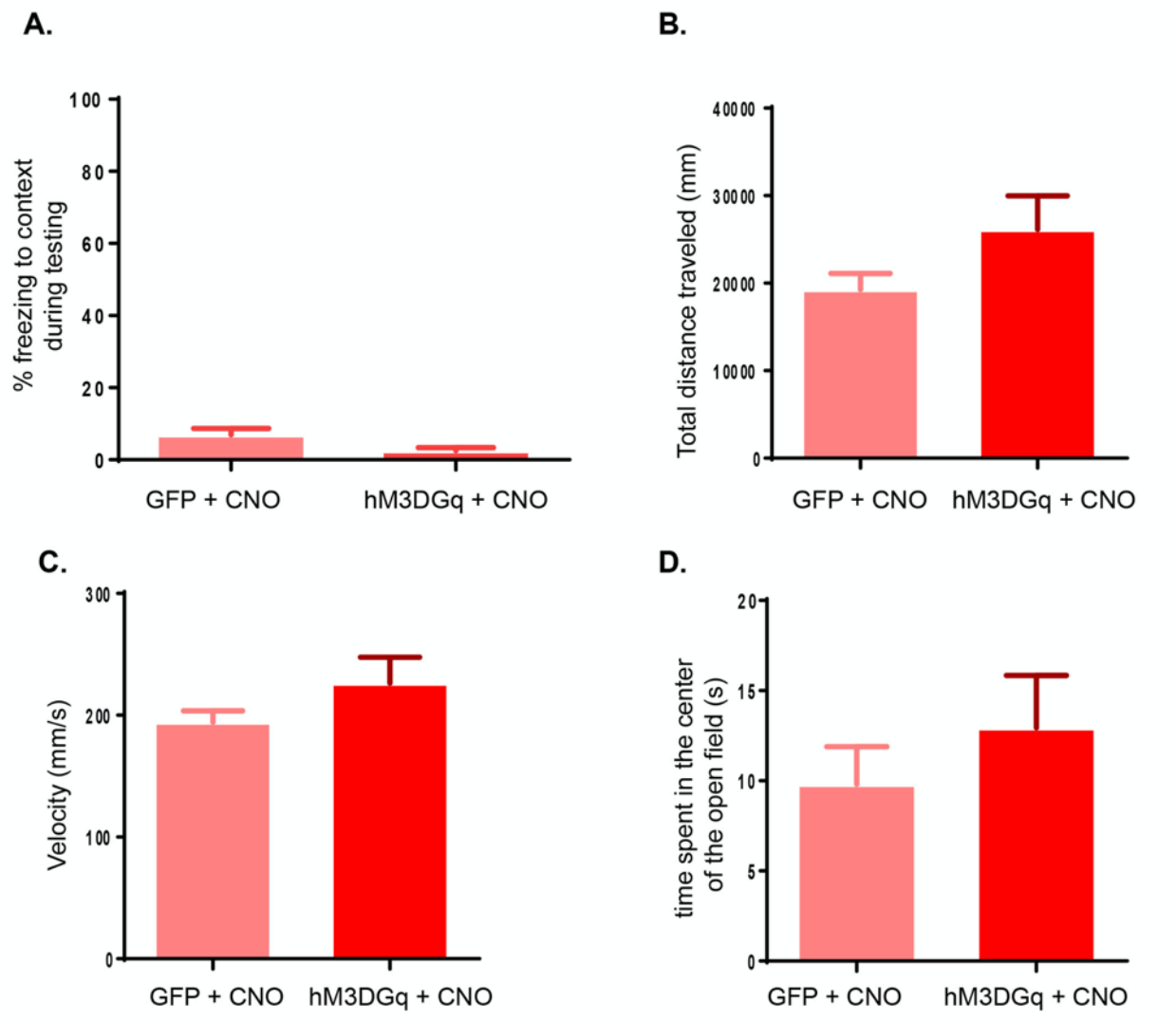

Figure 2.6: Chemogenetic activation of the ZI does not produce non-specific increase in freezing responses and does not affect general locomotor function or anxiety-like behavior. **(A)** No significant differences were observed in freezing to Context B with chemogenetic activation of ZI on testing day**,** demonstrating that the observed changes in freezing responses were specific to the auditory stimuli. **(B-D)** In the Open Field Test performed one hour after CNO injections, chemogenetic activation (hM3DGq+CNO) of the ZI in wild type animals did not produce detectable changes in **(B)** total distance traveled (in mm), **(C)** velocity (mm/sec), and **(D)** time spent in center of open field, compared to controls (GFP+CNO). Data represented as Mean ± S.E.M.

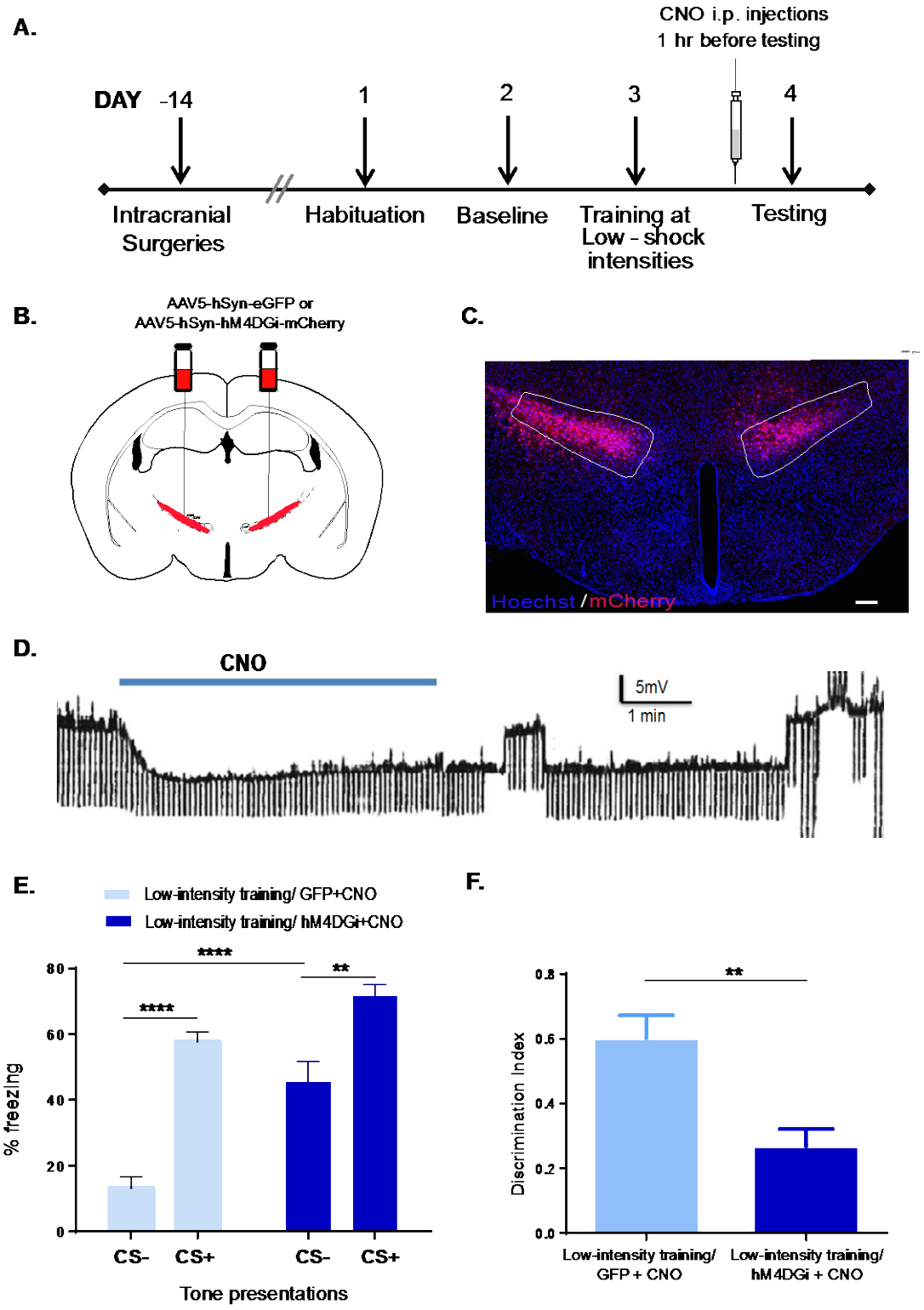

Figure 2.7: Decreasing cellular activity in the ZI results in fear generalization.

(A) Experimental protocol for chemogenetic inhibition. Two weeks after intracranial injection of the control or DREADD virus, animals were conditioned to low threat intensities. The next day, CNO was administered intraperitoneally 1 hour before testing fear generalization. **(B)** Wild-type animals were injected with either the control virus (AAV5-hSyn-eGFP) or inhibitory DREADDs (AAV5-hSyn-hM4DGi-mCherry) at -1.5mm posterior to bregma. **(C)** Representative image of the ZI targeted with intra-cranial infusions of DREADD-expressing mCherry viruses. **(D)** Patch-clamp recording of hSyn-hM4DGi-mCherry expressing cells in the ZI showing membrane hyperpolarization during CNO exposure. **(E)** Chemogenetic inhibition of the ZI (hM4DGi+CNO) resulted in a significant increase in fear response to CS- compared to controls (GFP+CNO). **(F)** Chemogenetic inhibition of the ZI (hM4DGi+CNO) resulted in an impaired ability to discriminate between the CS+ and the CS-. $^{**}p<0.01$, $^{****}p<0.0001$. Data represented as Mean ± S.E.M.

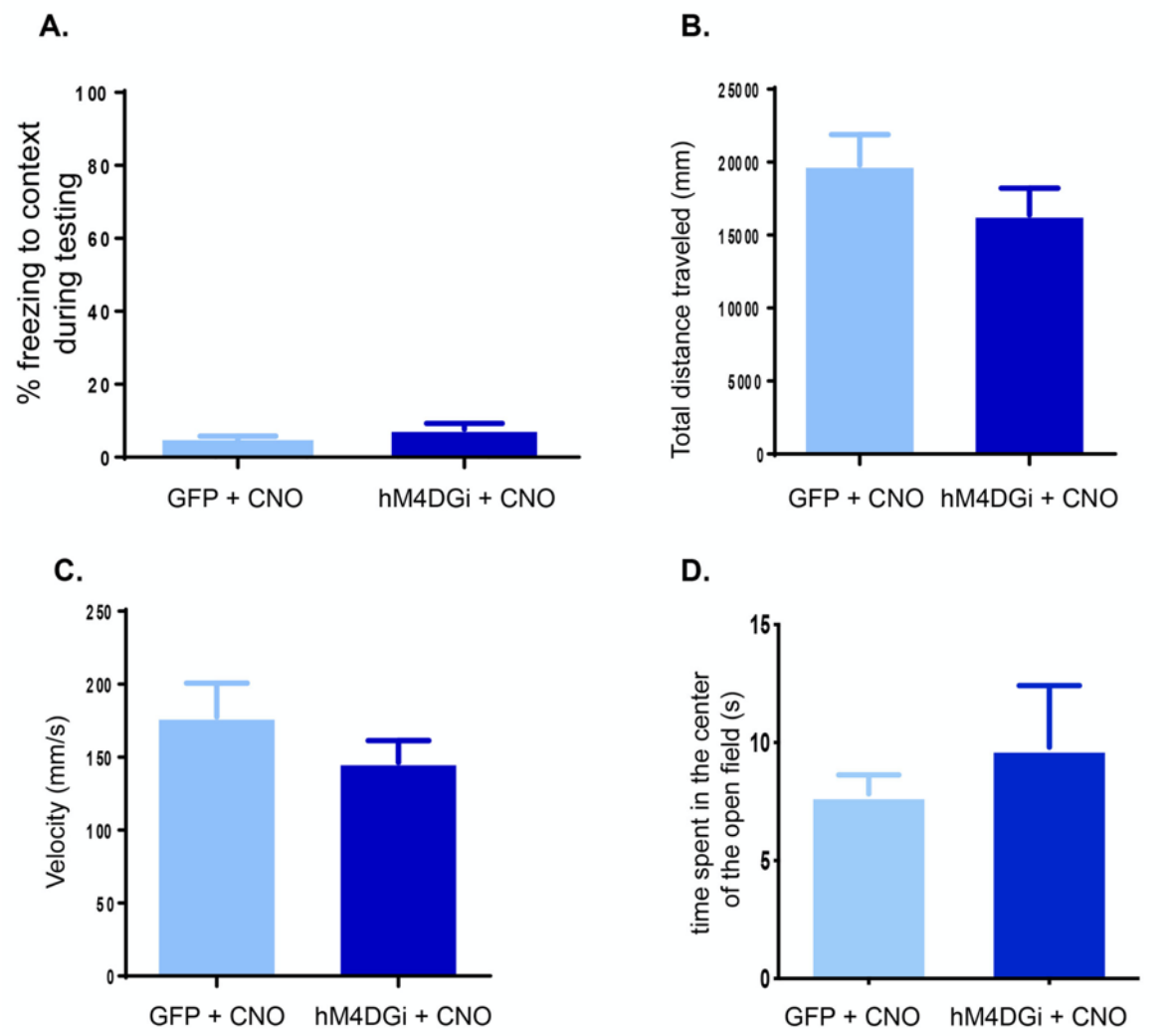

Figure 2.8: Chemogenetic inhibition of the ZI does not produce non-specific alterations in freezing responses and does not affect general locomotor function or anxiety-like behavior. **(A)** No significant differences were observed in freezing to Context B with chemogenetic inhibition of the ZI on testing day**,** demonstrating that the observed changes in freezing responses were specific to the auditory stimuli. **(B-D)** In the Open Field Test performed one hour after CNO injections, chemogenetic inhibition (hM4DGi+CNO) of the ZI in wild type animals did not produce detectable changes in **(B)** total distance traveled (in mm), **(C)** velocity (mm/sec), and **(D)** time spent in center of open field, compared to controls (GFP+CNO). Data represented as Mean ± S.E.M.

CHAPTER 3: GABAergic cells in the zona incerta mediate fear generalization

3.1 Context, Author's Contribution, and Acknowledgement of Reproduction

The following chapter presents evidence for modulation of fear generalization by GABAergic cells in the zona incerta. The investigation was performed to examine whether ZI GABAergic cells alter fear generalization. The functional contribution of ZI GABAergic cells to fear generalization was assessed using chemogenetics followed by neuroanatomical tracing of projections from these cells. The dissertation author designed and conducted most of the experiments with the exception of the electrophysiology data that were collected by Dr. Jidong Guo. The work was conceptualized, organized and written by the dissertation author with editorial feedback from Dr. Brian Dias. A portion of the chapter presented here is reproduced with edits from Venkataraman, A., Brody, N., Reddi, P., Guo, J. G., Rainnie, D., Dias, B. G. (2019) Modulation of fear generalization by the zona incerta. *Proceedings of the National Academy of Sciences* 116 (8):9072-9077

3.2 ABSTRACT

Generalization of fear responses is a pathological characteristic prevalent in trauma- and anxiety-related disorders. Very little is understood about the thalamic and subthalamic neural substrates that govern fear generalization. We previously reported that activity of the medial zona incerta (ZI) can suppress fear generalization. Given the predominant presence of GABAergic neurons in the medial ZI, here we examined whether the GABAergic cells in ZI modulate fear generalization. Mice were trained in an auditory fear discrimination task using low intensity or high intensity foot-shocks. Levels of freezing measured to the tone presentations 24 hours later served as an index of fear generalization. Chemogenetic activation of GABAergic cells in the ZI prior to retrieval strongly impairs fear generalization. Conversely, chemogenetic inhibition of these neurons prior to retrieval promotes fear generalization. To further understand how GABAergic cells in the ZI might modulate fear generalization, we examined the efferent projections from these cells using unilateral anterograde tracer injections. Results indicate that the GABAergic cells in the ZI send strong projections to the nucleus reuniens, dorsolateral periaqueducatal gray, ventral periaqueducatal gray, and posterior hypothalamus. With all these regions having been implicated in orchestrating different aspects of a fear response, GABAergic cells in the ZI are well-positioned to regulate appropriate fear expression. Together, our data establishes a critical role for GABAergic ZI in fear generalization.

3.3 INTRODUCTION

Learning to associate stimuli with danger during adverse situations is crucial. The ability to generate defensive responses to a range of stimuli similar to a previously encountered threat is adaptive and termed 'fear generalization'. This ability to generalize must be balanced with the ability to discriminate stimuli dissimilar from previously encountered threats. This balance between discrimination and generalization is essential to maximize survival. However, in humans, the inability to inhibit exaggerated, inappropriate fear responses leads to fear overgeneralization and is recognized as a central feature of trauma- and anxiety-related disorders. Decades of research have elucidated the brain regions governing fear behaviors (Gross & Canteras, 2012; Maren & Quirk, 2004; Orsini & Maren, 2012; Tovote et al., 2015), but very little is understood about the circuits mediating fear generalization.

Understanding the brain circuits that mediate normal and pathological fear states is required to develop effective treatments. Studies thus far have largely focused on examining fear generalization in the context of the canonical fear-related circuitry like the amygdala, prefrontal cortex and hippocampus that continuously assess threats in the environment (Ciocchi et al., 2010; Ghosh & Chattarji, 2015; Jasnow et al., 2017; G. L. Jones et al., 2015; Lissek et al., 2014; Sanford et al., 2017). However, very little is understood about the role of thalamic and subthalamic regions in modulating fear generalization. Thalamic and sub-thalamic brain regions have traditionally been considered hubs that relay information from sensory cortices to limbic, midbrain and brainstem nuclei. Emerging literature suggests that some of these nuclei contribute to fear-related behaviors (Do-Monte et al., 2015; Penzo et al., 2015; Ramanathan, Jin, et al., 2018; Ramanathan, Ressler, et al., 2018; Xu & Sudhof, 2013).

The zona incerta, in particular, is a subthalamic brain region that integrates multimodal sensory information and has extensive connections along the entire nervous system (C. Kolmac &

Mitrofanis, 1999; C. I. Kolmac, Power, & Mitrofanis, 1998; Mitrofanis, 2005; Mitrofanis & Mikuletic, 1999; Roger & Cadusseau, 1985; Shaw & Mitrofanis, 2002). ZI does not innervate primary sensory thalamic nuclei that carry information from the sensory periphery, but higher order thalamic nuclei involved in thalamocortical communication. This has been verified by recent electrophysiological studies that demonstrate extensive inhibitory control of ZI over thalamic and cortical networks (Bartho et al., 2007; J. Liu et al., 2015; Weitz, Lee, Choy, & Lee, 2019) and therefore, can influence sensorimotor control, attention, arousal and emotional regulation. The rostral, medial (dorsal and ventral), and caudal sectors of the ZI contain a constellation of neurochemicals (C. Kolmac & Mitrofanis, 1999; Mitrofanis, 2005). In keeping with the diverse chemoarchitecture of this region, it has been implicated in controlling a range of behaviors including hunting, feeding, sleep, and recollection of fear memories (Chou et al., 2018; K. Liu et al., 2017; X. Zhang & van den Pol, 2017; Zhao et al., 2019; Zhou et al., 2018). Combining discriminative auditory fear conditioning in mice with C-FOS mapping and chemogenetic manipulation of neuronal activity, our findings reported in the previous chapter, demonstrate a vital role for the ZI in suppressing fear generalization. Given the extensive inhibitory control of ZI over its downstream targets, we examined the functional contribution of GABAergic cells in the ZI mediating fear generalization.

In this study, we use cell-type specific targeting technique with vesicular GABA transporter (vGAT)-CRE transgenic mice to selectively manipulate GABAergic neurons in the ZI *in vivo*. In extension of our previous work, we examined whether suppression or expression of fear generalization requires activity within GABAergic cells in the ZI. To address this, we employed differential auditory fear conditioning in mice where high intensity foot-shocks elicit fear generalization and low intensity foot-shocks elicit fear discrimination. We found that activation of

GABAergic cells in the ZI reduced fear generalization, while inhibition of these cells impaired fear discrimination, resulting in overgeneralization of fear responses. To better understand the downstream targets of the GABAergic cells, we traced their projection patterns using viral-mediated anterograde tract tracing. Examination of anterograde terminals revealed dense projections to the thalamus (posterior thalamic nucleus, nucleus reuniens, lateral geniculate nucleus), posterior hypothalamus and midbrain (dorsolateral subdivision of periquedutal gray, ventrolateral subdivision of periaqueductal gray). Taken together, these results indicate that amidst the rich chemoarchitecture of the ZI, GABAergic cells modulate fear generalization and could do so by exerting inhibitory control over distinct thalamic and mid-brain regions that have been implicated in fear expression and inhibition.

3.4 MATERIALS AND METHODS

3.4.1 Animals

2-3-month-old adult female and male vGAT-CRE mice were used in this study. The transgenic mice were obtained from Jackson labs, and bred in our vivarium for the experiments described here. The animals were group-housed under a 14:10 light/dark cycle with food and water available ad libitum. The Emory Institutional Animal Care and Use Committee approved this study in compliance with the National Institute of Health standards.

3.4.2 Discriminative auditory fear conditioning to test fear generalization

Discriminative auditory fear conditioning protocol to test fear generalization was followed as previously described in the Methods section of Chapter 2. In brief, after habituation and baseline recording of freezing to CS+ and CS- tones, animals were trained to discriminate between the two tones. During training, after an initial 5-minute exposure to Context A, the mice received 10 CS+ presentations that co-terminated with a 0.5 sec foot-shock with randomly interleaved 10 CS- presentations that were not reinforced (CS+: 15kHz at 85dB, CS-: 6kHz at 85dB). In the low threat conditioning experiments, the CS+ was paired with 0.3 mA foot-shocks and in high threat conditioning experiments, the CS+ was paired with 0.8 mA foot-shocks. The ITI (inter-trial interval) between the tones was set to vary between 2-6 minutes. 24 hours later, freezing levels of the mice to two randomized presentations each of the CS+ and CS- were tested in a new context (Context B). The percentage of time spent freezing to the tone presentations were used as a behavioral index of fear generalization. FreezeFrame-4 software (Actimetrics) was used for tone presentations and video recording of the animals' fear responses. The hardware used in these experiments was acquired from Harvard Apparatus. Freezing was quantified using the FreezeFrame software, with the length of freezing bout set to 0.5 seconds. For context A with the grid floors, the house lights were turned on and cleaned with the quatricide. For context B with the

plexiglass floors, the infra-red lights were turned on and cleaned with 70% ethanol. For the DREADD experiments, 1 mg/kg clozapine-N-oxide (CNO; Sigma-Aldrich) injections were administered one hour before testing for fear generalization in Context B. Discrimination index (DI) was calculated as below.

$$DI = \frac{\text{freezing to } CS^{+} - \text{freezing to } CS^{-}}{\text{freezing to } CS^{+} + \text{freezing to } CS^{-}}$$

3.4.3 Stereotaxic viral injections

AAV5-hSyn-DIO-mCherry, AAV5-hSyn-DIO-hM3DGq-mCherry and AAV5-hSyn-DIO-hM4DGi-mCherry viruses were obtained from the University of North Carolina Viral Vector Core and Addgene. Mice were anesthetized using ketamine and dexdomitor. 80 nl of AAV viruses (AAV5-hSyn-DIO-mCherry or AAV5-hSyn-DIO-hM3DGq-mCherry) were delivered bilaterally through a pulled glass pipette at the rate of 1 nl/sec using Nanoject III (Drummond Scientific). The following stereotaxic co-ordinates were used for targeting the ZI: AP: -1.52 mm, ML: 0.73 mm and DV: -4.79 mm relative to Bregma. Mice were allowed to recover for 2 weeks from the surgery before performing behavioral experiments. For anterograde tracing of projections from the vGAT-expressing GABAergic cells in the ZI, 300 nl of AAV-DIO-eGFP was infused unilaterally into the ZI of vGAT-Cre mice and animals were sacrificed 4-5 weeks later for histological examination.

3.4.4 Histology

Mice were trans-cardially perfused with 1X phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were harvested and stored in 4% PFA solution overnight and transferred to 30% sucrose solution until the tissue completely sank. Brains were sectioned at 35µm on a freezing microtome (Leica), stained with Hoechst nuclear stain (1:1000) and mounted on slides using SlowFade Gold Antifade mountant (Life Technologies). Viral expression in the ZI

was assessed using Nikon Eclipse E800 fluorescent microscope.

3.4.5 Open field test

The open field arena was a 50 x 50 x 50 cm^3 white acrylic chamber with the center defined as 16% of the total area. Mice were allowed to habituate to red-light conditions in the testing room for 1 hr after intraperitoneal CNO injections (1mg/kg). At the beginning of each testing session, a mouse was individually placed in the center of the arena and exploratory activity was recorded for 5 minutes using an overhead digital infrared camera. The mouse position and velocity were analyzed using automated video tracking system TopScan 2.0 (CleverSys Inc.).

3.4.6 Slice preparation and recording

To confirm DREADD function, vGAT-CRE mice injected with the DREADD viruses were sacrificed 4-6 weeks later and electrophysiological recordings were performed as previously reported (Daniel et al., 2017). Briefly, mice were anesthetized with isoflurane and the brains were quickly removed from the skull. Coronal brain slices (300 μm thick) containing the ZI were prepared using a Leica VTS-1000 vibratome (Leica Microsystems Inc., Bannockburn, IL, USA). Brain slices were then transferred to 95%O2/5%CO2 oxygenated artificial cerebrospinal fluid (ACSF) at 32°C for 1 hour before recording.

Following this, each slice was transferred to a recording chamber mounted on the stage of Leica STP6000 microscope and continuously perfused with oxygenated ACSF at 32°C at a speed of 1-2 ml/min. The ZI was located using differential interference contrast (DIC) optics and an infrared sensitive Hamamatsu CCD camera. Neurons in the ZI expressing the fluorescent transgene (mCherry) were visually identified using epifluorescent illumination in combination with the appropriate emission filter sets. A subset of the recorded neurons was filled with patch solution containing 0.3% biocytin for visualization of neurons in the ZI. Patch recording electrodes were

pulled from borosilicate glass and filled with solution consisting of the following (in mM): 130 K-Gluconate, 2 KCl, 10 HEPES, 3 MgCl2, 5 phosphocreatine, 2 K-ATP, and 0.2 NaGTP, buffered to a pH of 7.3 and an osmolarity of 280-290 mOsm. The patch electrode resistance was 4-6 MΩ. Whole cell patch-clamp recordings were performed on the mCherry-expressing neurons in the ZI using a MultiClamp 700B amplifier, in conjunction with an Axon Digidata 1550 A-D interface and pClamp 10.4 software (Molecular Devices, Sunnyvale, CA). Current clamp recordings were obtained 0-30 s before CNO was added to the perfusion medium. The effect of bath application of 20 μM CNO on the resting membrane potential, and basic physiological properties of ZI neurons, was examined.

3.4.7 Statistical Analysis

All results are presented as mean values ± S.E.M. and statistical analysis was performed using GraphPad Prism. Comparisons of means between two groups were conducted using unpaired t-tests when appropriate. Repeated-measures two-way ANOVA was used to compare data from multiple experimental groups with post-hoc Holm-Sidak multiple comparisons in the case of significant interactions or main effects. Differences were considered statistically significant at $p < 0.05$.

3.5 RESULTS

3.5.1 Selective targeting of GABAergic neurons in the ZI

GABAergic neurons in the ZI were targeted using stereotaxic injections of AAVs encoding CRE-dependent DREADDs in to the ZI of vGAT-CRE mice (Fig. 3.1). Injection of the AAVs encoding the mCherry reporter resulted in robust expression in the dorsal and ventral sections of the medial ZI (Fig. 3.1). To further confirm DREADD function, we performed whole-cell patch clamp recordings(Figs. 3.2 A-C). Bath application of 20 μM CNO produced depolarization in DIO-hM3DGq-mCherry expressing vGAT neurons in the ZI (Fig. 3.2C). Post hoc visualization of biocytin enabled reliable identification of the mCherry expressing GABAergic cells in the ZI and revealed the soma of these recorded cells with dense local axons and spiny dendrites.

3.5.2 Increasing activity of GABAergic cells in the ZI reduces fear generalization.

To determine whether stimulation GABAergic cells in the ZI affects fear generalization, we injected AAV5-hSyn -DIO-hM3D(Gq)-mCherry (stimulatory DREADDs) or AAV5-hSyn -DIO-mCherry bilaterally into the ZI of vGAT-CRE mice (as described in Fig. 3.3A). The mice were then trained in a differential auditory fear conditioning protocol using high intensity foot-shocks that has been shown to produce overgeneralization of fear responses for the neutral CS- and aversive CS+ tones (as previously described in Chapter 2.6.1). Approximately, 24 hours after training, CNO was administered intraperitoneally before testing fear generalization. Increased fear generalization was observed in vGAT-CRE animals that received control viruses. However, increasing activity of GABAergic cells in the ZI alone, drastically reduced fear generalization (Fig. 3.3B). Specifically, vGAT-CRE:DIO-hM3D(Gq)-mCherry+CNO animals exhibited significantly lower freezing responses to CS- than their responses to CS+, compared to vGAT-CRE:DIO-mCherry+CNO animals. (vGAT-CRE:DIO-mCherry+CNO group n = 9, vGAT-CRE:DIO-hM3D(Gq)-mCherry+CNO n = 11, DREADD x Tone interaction: $F(1,18) = 21.48$, $p < 0.0001$.

DREADD treatment main effect: $F(1,18) = 26.03$, $p < 0.0001$. Tone main effect: $F(1,18) = 50.84$, $p < 0.0001$.Post-hocs: High Intensity Training-DIO-hM3DGq+CNO:CS+ vs. High Intensity Training-DIO-hM3DGq+CNO:CS- $p<0.0001$, High Intensity Training-DIO-GFP+CNO:CS- vs. High Intensity Training-DIO-hM3DGq+CNO:CS- $p<0.0001$, High Intensity Training-DIO-GFP+CNO:CS+ vs. High Intensity Training-DIO-hM3DGq+CNO:CS+ $p<0.05$). High Intensity Training-DIO-hM3DGq+CNO animals showed better discrimination in their fear response to the CS+ and CS- as noted by their higher discrimination index compared to High Intensity Training-DIO-GFP+CNO animals (Fig. 3.3C) ($p < 0.0001$, $t = 9.151$, $df = 18$). There was no statistically significant difference between the vGAT-CRE groups in their freezing to the context (Context B) before tone presentations on the day of testing (Fig. 3.4A), suggesting a specificity of freezing responses to the tones. These observed differences in freezing responses of animals with chemogenetic stimulation of GABAergic cells, were not accompanied by alterations in locomotor activity or anxiety-like behavior (Figs. 3.4 B-D).

3.5.3 Decreasing activity of GABAergic cells in the ZI induces fear generalization.

Next, we asked whether reducing activity of GABAergic cells in the ZI can produce fear generalization. To this end, we injected AAV5-hSyn-DIO-hM4D(Gi)-mCherry or AAV5-hSyn-DIO-mCherry bilaterally into the ZI of vGAT-CRE mice (as described in Fig. 3.5A). The mice were then trained in a differential auditory fear conditioning protocol using low intensity foot-shocks that has been shown to result in clear discrimination of the neutral CS- tone from the aversive CS+ tone (as previously described in Chapter 2.6.1). Approximately, 24 hours after training, CNO was administered intraperitoneally before testing fear generalization. vGAT-CRE animals that received control viruses expressed the ability to discriminate between the CS+ and CS-. However, decreasing activity of GABAergic cells in the ZI robustly increased fear

generalization (Fig. 3.5B). Specifically, vGAT-CRE:DIO-hM4D(Gi)-mCherry+CNO animals exhibited significantly higher freezing responses to CS- compared to vGAT-CRE:DIO-mCherry+CNO animals. (vGAT-CRE:DIO-mCherry+CNO group n = 8, vGAT-CRE:DIO-hM4D(Gi)-mCherry+CNO n = 10, DREADD x Tone interaction: $F(1,16) = 12.15$, $p < 0.01$. DREADD treatment main effect: $F(1,16) = 4.78$, $p < 0.05$. Tone main effect: $F(1,16) = 25.83$, $p = 0.001$. Post-hocs: Low Intensity Training- DIO-mCherry +CNO:CS+ vs. Low Intensity Training- DIO-mCherry +CNO:CS- $p<0.0001$, Low Intensity Training-DIO-mCherry+CNO:CS- vs. Low Intensity Training-DIO-hM4DGi+CNO:CS- $p<0.001$. Low Intensity Training-DIO-hM4DGi+CNO animals showed overgeneralization of fear responses to the CS+ and CS- tones as noted by their poor discrimination index compared to Low Intensity Training-DIO-mCherry+CNO animals (Fig. 3.5C) ($p < 0.001$, $t = 4.662$, $df = 16$). There was no statistically significant difference between the vGAT-CRE groups in their freezing to the context (Context B) before tone presentations on the day of testing (Fig. 3.6A), suggesting a specificity of freezing responses to the tones. These observed differences in freezing responses of animals with chemogenetic inhibition of GABAergic cells, were not accompanied by alterations in locomotor activity or anxiety-like behavior (Figs. 3.6 B-D).

3.5.4 GABAergic projections from the ZI target the thalamus, hypothalamus and midbrain.

To better understand how GABAergic cells in the ZI could potentially modulate fear generalization, we sought to map their projections. Figures 3.7 A and B depict the injection site where CRE recombinase-dependent GFP was unilaterally injected in the ZI of vGAT-CRE mice. The GFP-labelled GABAergic neuronal cell bodies were mainly observed in the medial portion of the zona incerta covering the dorsal and ventral subdivisions. The GABAergic cells originating in the medial mZI sent projections to the caudal ZI. At the level of the thalamus, the

entirety of the midline thalamic nucleus reuniens (RE) including the anterior and posterior subdivisions contained GFP-labelled terminals (Figs. 3.8 A-B). GABAergic fibers were also observed in the laterodorsal thalamic nucleus, lateral posterior medio-rostral thalamic nucleus (LPMR) and parvicellular part of the ventral lateral geniculate nucleus (vlGPC) (Figs. 3.8 C-F). The dense fibers travel through the periventricular fiber system (pv) of the hypothalamus to reach the posterior hypothalamus (PH). Labelling was also present in the supramamillary region (data not shown). In the midbrain, GABAergic fibers were concentrated in the dorsolateral and ventrolateral subdivision of periaqueductal gray (Figs. 3.9 A-B). Moderate labelling was also observed in the pretectal region of the midbrain (data not shown).

3.6 DISCUSSION

The generalization of fear responses toward neutral stimuli is a highly prevalent and debilitating dimension of trauma- and anxiety-related disorders. There is significant translational relevance in trying to understand the neural circuits that underlie the ability to suppress fear generalization. Inhibitory networks have been hypothesized to play a dominant role in carefully controlling the memory specificity and fear suppression (Ehrlich et al., 2009; Herry et al., 2010; Marin, 2012). However, studies on inhibitory circuits in fear behaviors have largely focused on microcircuits within the amygdala. Our results presented here demonstrate a crucial role for the GABAergic cells of the zona incerta (ZI) in modulating fear generalization. We used a chemogenetic strategy to probe the function of genetically defined cell population in fear generalization. We found that the GABAergic cells within the ZI bidirectionally modulate fear generalization – stimulation of these GABAergic cells reduced the generalization of fear responses that manifests after conditioning with high intensity foot-shocks and inhibition of the cells increased fear generalization even after exposure to low threat training conditions. Anterograde tracing studies revealed that the GABAergic cells in the ZI strongly innervates thalamus, hypothalamus and midbrain regions. Taken together, our data establishes a novel function for GABAergic cells of the ZI in regulating fear generalization.

Building on our observations from the C-FOS study and DREADD-based experiments in the ZI described in Chapter 2, and given the complex neurochemical profile of the region (C. Kolmac & Mitrofanis, 1999), we wanted to determine the cell populations responsible for suppressing fear generalization. The GABAergic neurons of the ventral ZI were of particular interest, since they have been shown to gate ascending sensory information by fast feed-forward inhibition of higher order thalamic nuclei (Bartho et al., 2002; Lavallee et al., 2005; Trageser et al., 2006; Trageser & Keller, 2004). More recently, GABAergic cells in the ZI have been shown to be important for

defensive responses as well as acquisition and retrieval of fear memories (Chou et al., 2018; Zhou et al., 2018). Therefore, in our study, we tested whether manipulating cellular activity of GABAergic cells in the ZI affected fear generalization. We found that stimulating GABAergic cells in the ZI reduced fear generalization while inhibiting GABAergic cells in the ZI increased fear generalization. It is important to note that the caudal ZI (cZI) has been posited to be part of a basal ganglia motor network and is involved in posture, locomotion and other motor behaviors (Mitrofanis, 2005; Ossowska, 2019; Trageser et al., 2006; Zhao et al., 2019). Clinical studies have suggested cZI as a potential therapeutic target for deep brain stimulation treatment in patients with Parkinson's disease (PD) (Blomstedt et al., 2012; Burrows et al., 2012; Ossowska, 2019). There is a possibility that alterations in locomotor behavior contributed to the effects on fear generalization observed with the chemogenetic manipulations of the ZI GABAergic cells. However, we did not observe any significant differences in total distance traveled and velocity during open field tests performed after chemogenetic activation or inhibition of ZI (Figs. 3.4 and 3.6). Freezing to the novel testing context (Context B) did not change after stimulating or inhibiting activity in the ZI (Figs. 3.4 and 3.6), further emphasizing that the observed effects on fear generalization were specific to the CS+ and CS- tones presented.

Stimulation of GABAergic cells and the paravalbumin (PV)-expressing cells in the ZI has been demonstrated to reduce fearful behavior (Chou et al., 2018; Zhou et al., 2018). In line with these findings, we find that stimulating GABAergic cells within the ZI results in reduced fear responses toward the conditioned stimuli (DREADD treatment main effects reported in Figs. 3.3 and 3.5). However, it should be pointed out that the reduction in fear responses to the CS- that we observe after stimulating GABAergic cells in the ZI are of a qualitatively greater magnitude than the decrease in fear responses to the CS+ after such stimulation. Moreover, animals continue to show

a high fear response to the CS+ even after activation of the ZI suggesting that our results are not a consequence of all fear responses being lowered from the ceiling levels of fear observed after training with high intensity foot-shocks. Further support for this perspective comes from the significant differences in the discrimination index between the control and DREADD treated groups. Therefore, stimulating the ZI still leaves room for adaptive fear responses to the CS+ to be expressed while reducing fear to the neutral CS-.

Anterograde tracing of projections from GABAergic cells in the ZI reveal the extensive network of connections to the thalamus, hypothalamus and midbrain regions from this distinct cell population. In line with immunohistochemical studies in rodents and primates that have revealed the presence of GABAergic cells in both the dorsal and ventral subdivisions of the ZI (C. Kolmac & Mitrofanis, 1999; Nicolelis, Chapin, & Lin, 1992; Watson, Lind, & Thomas, 2014), we observed GFP-labelled cell bodies throughout the dorsal and ventral subdivision of the medial zona incerta. . We identified intrinsic connectivity within the ZI as reported previously in rats (Power & Mitrofanis, 1999). This interconnectivity in ZI has been suggested to be essential for integrating diverse information sent from the afferents to the distinct subdivisions (rostral, medial and caudal) of ZI. The fibers originating from the GABAergic cells in the ZI then innervate several "higher order" nuclei of the thalamus. In particular, GABAergic cells in the ZI sent dense axonal projections to the midline thalamic nucleus reuniens (RE) that serves as a conduit between mPFC and hippocampus. The RE has been implicated in suppressing fear responses in the context of fear generalization as well as fear extinction (Ramanathan, Jin, et al., 2018; Troyner et al., 2018; Xu & Sudhof, 2013). Moreover, the GABAergic cells in the ZI communicate with the posterior hypothalamus (PH) that controls the sympathetic division of the autonomic nervous system, the hypothalamic-pituitary-adrenal axis (Biagioni, Silva, & Coimbra, 2012; Canteras & Graeff, 2014;

Shekhar, Hingtgen, & DiMicco, 1990), and is potentially involved in unconditioned fear-induced behavioral responses. Further, strong efferent GABAergic projections from the ZI innervate both the dorsolateral and ventral subdivisions of the periaqueductal gray (PAG) that has been implicated in conditioned and unconditioned fear responses. The dorsolateral subdivision of the PAG modulates fear responses to predator-related cues (Cezario, Ribeiro-Barbosa, Baldo, & Canteras, 2008; Pavesi, Canteras, & Carobrez, 2011; Sukikara, Mota-Ortiz, Baldo, Felicio, & Canteras, 2010) and the ventrolateral subdivision of the PAG has the ability to suppress ongoing appetitive behaviors and enhancing fear responses (Bittencourt, Carobrez, Zamprogno, Tufik, & Schenberg, 2004; Johansen, Tarpley, LeDoux, & Blair, 2010; Tovote et al., 2016). The ZI has been shown to exert direct inhibitory control over PAG through monosynaptic GABAergic connections and activation of the ZI-PAG pathway has been specifically implicated in suppressing sound-triggered flight responses and promoting hunting behavior (Chou et al., 2018; Zhao et al., 2019). In summary, these tracing data reveal strong connectivity arising from the zona incerta to fear-responsive regions in the brain. Further examination of these pathways using chemogenetic and optogenetic tools can help us dissociate the independent roles of these incertal pathways in calibrating fear towards aversive and neutral stimuli.

As alluded to previously, the ZI is chemo-architecturally diverse and future experiments will be needed to determine the functional contribution of specific neuromodulators (e.g. parvalbumin or somatostatin or both) present in the GABAergic cells in the ZI and the role of their downstream targets in tuning specificity of fear responses. It would also be informative to elucidate the neurotransmitter/neuromodulator profiles of the ZI cells that are activated in animals that suppress fear versus the animals that express fear generalization. While blocking synaptic transmission in the ZI has also been shown to alter anxiety-related measures (Zhou et al., 2018), we did not find

similar effects with chemogenetic manipulations of the ZI (Figs. 3.4D, 3.6D), a discrepancy possibly explained by cell-type specific influences on behavior. Taken together, future studies that target more specific sub-populations of activated GABAergic cells in the ZI will be required to achieve a finer-grained resolution of how the ZI influences varied dimensions of fear and anxiety.

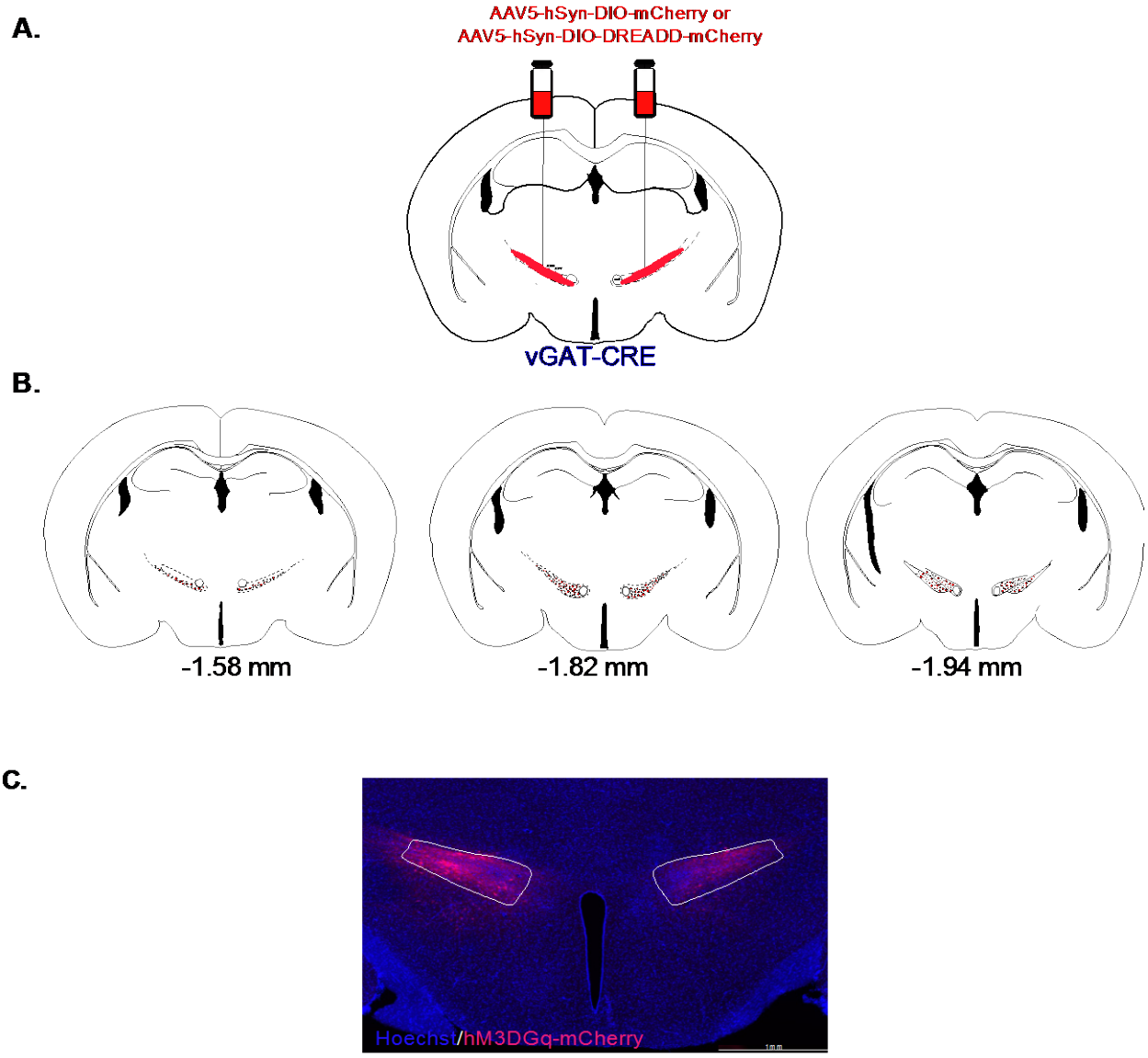

Figure 3.1: Stereotaxic delivery of CRE-dependent AAVs in the ZI of vGAT-CRE animals. **(A)** vGAT-CRE animals were injected with either the control virus (AAV5-hSyn-DIO-mCherry) or DREADD containing viruses (AAV5-hSyn-DIO-hM3DGq-mCherry or AAV5-hSyn-DIO-hM4DGi-mCherry) into the ZI. **(B)** mCherry expressing cells were visualized in the dorsal and ventral ZI. **(C)** Representative image of the GABAergic cells within the ZI infected with mCherry-expressing (red) excitatory DREADDs. Nuclei stained in blue (Hoechst).

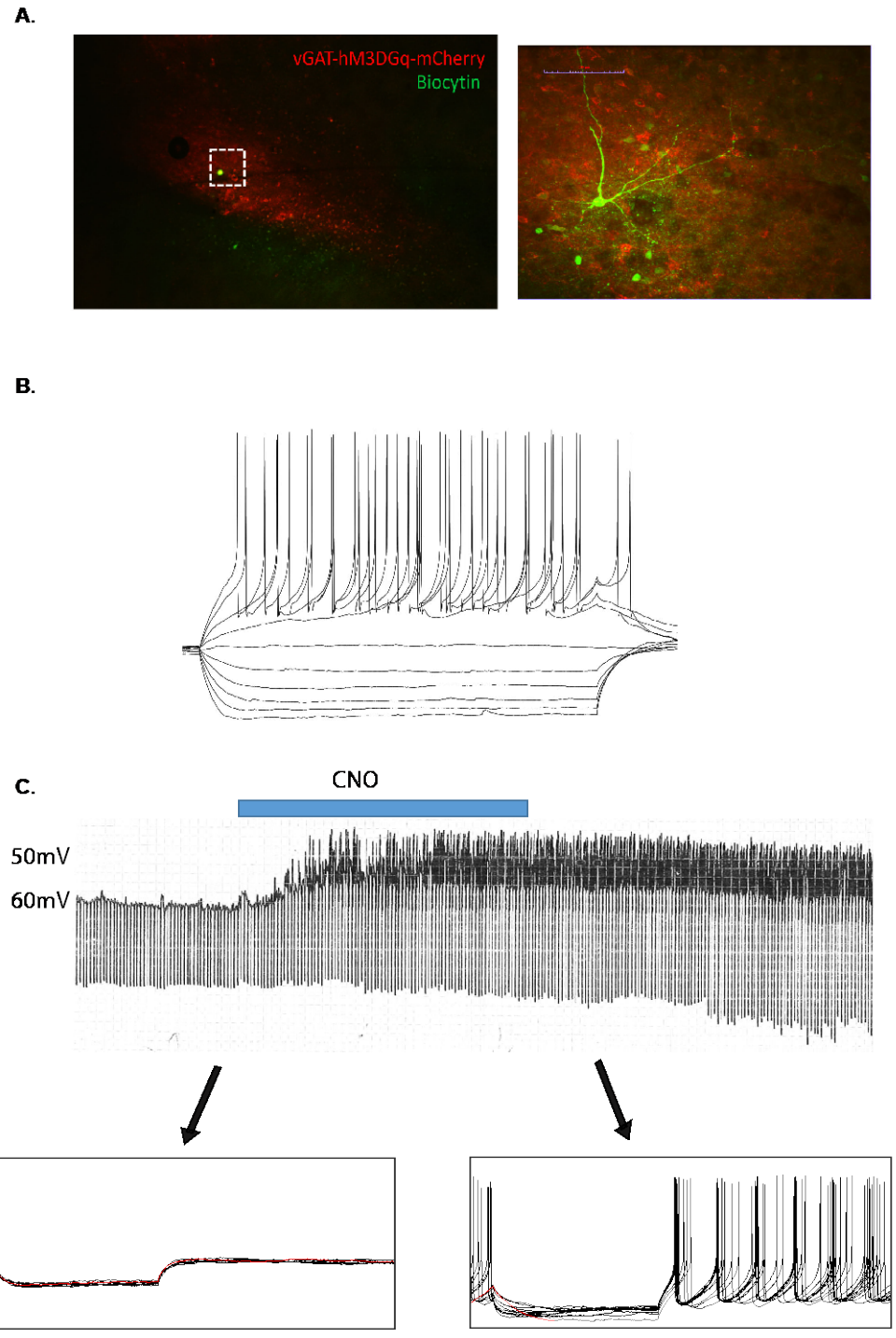

Figure 3.2: Targeted modulation of ZI vesicular GABA transporter (vGAT) expressing neurons using designer receptors exclusively activated by designer drugs (DREADDs).

(A) Left: Representative section showing expression of Cre-dependent hM3DGq virus in ZI of vGAT-CRE mouse. Right: Example of hM3DGq receptor-expressing mCherry+ neuron (in red)

recovered from whole-cell patch clamp recordings and filled with biocytin (in green) **(B)** Representative voltage responses to a series of hyperpolarizing and depolarizing current steps. **(C)** Representative trace showing depolarization of hM3DGq-expressing ZI GABAergic neuron in response to bath application of 20 μM of CNO.

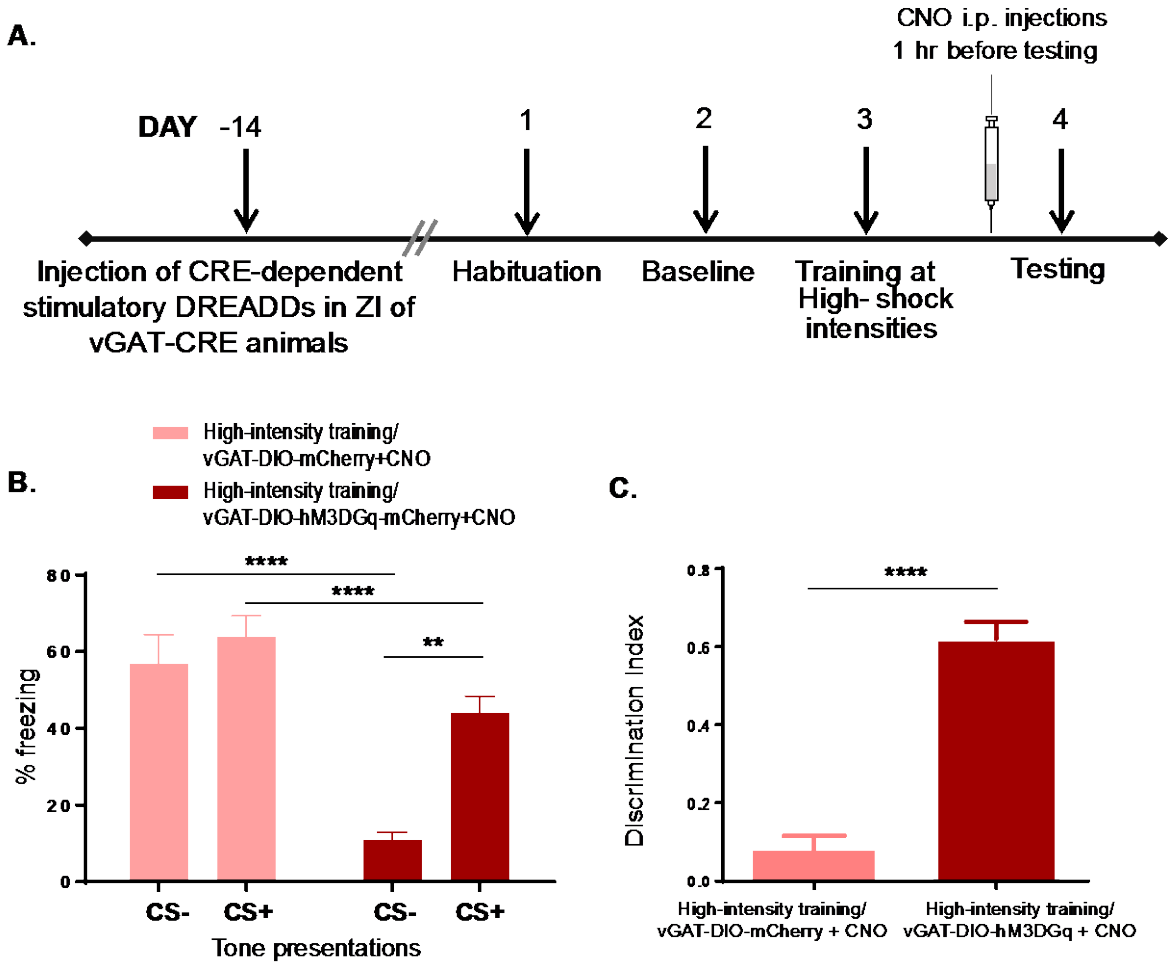

Figure 3.3: Targeted chemogenetic activation of GABAergic cells in the ZI can reduce fear generalization.

(A) Experimental design: vGAT-CRE animals received intracranial injections of CRE-dependent control or DREADD virus and after two weeks, were conditioned to high threat intensities. One day post-training, CNO was administered intraperitoneally 1 hour before testing for fear generalization. **(B)** Animals with expression of DIO-hM3DGq virus in vGAT-CRE expressing GABAergic cells in the ZI and injected with CNO (DIO-hM3DGq+CNO) one hour before testing for fear generalization showed a significant decrease in fear response to CS- compared to animals that were infused with the DIO-mCherry virus in vGAT-CRE expressing GABAergic cells in the ZI and injected with CNO (DIO-mCherry+CNO). **(C)** Chemogenetic activation of GABAergic

cells in the ZI (DIO-hM3DGq+CNO) resulted in a better ability to discriminate between the CS+ and the CS-. *$p<0.05$ **** $p<0.0001$. Data represented as Mean ± S.E.M.

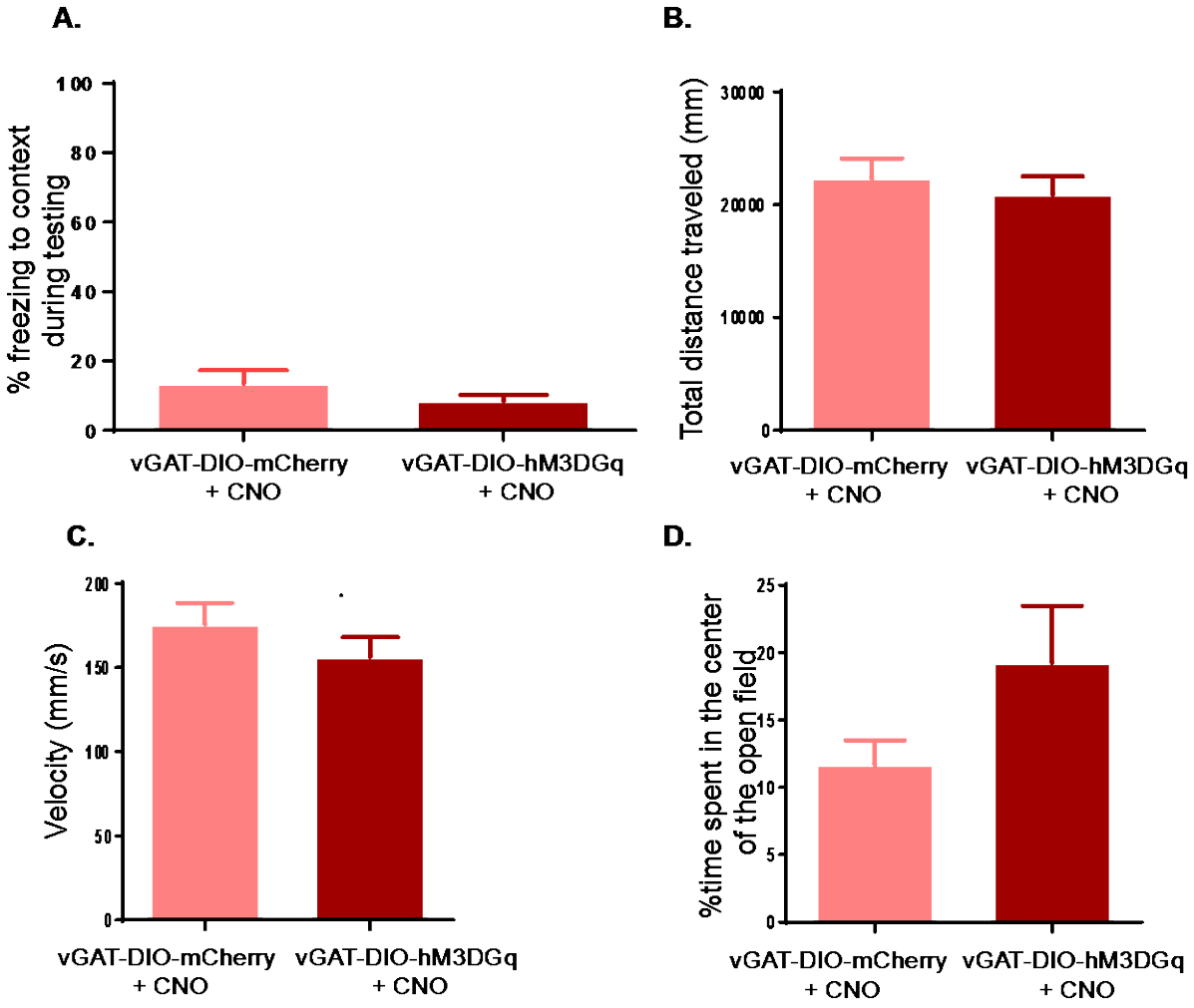

Figure 3.4: Chemogenetic activation of GABAergic cells in the ZI does not produce non-specific increase in freezing responses and does not affect general locomotor function or anxiety-like behavior.

(A) No significant differences were observed in freezing to Context B with chemogenetic activation of GABAergic cells in the ZI on testing day. **(B-D)** In the Open Field Test performed one hour after CNO injections, chemogenetic activation (DIO-hM3DGq+CNO) of GABAergic cells in the ZI in vGAT-CRE animals did not produce detectable changes in **(B)** total distance traveled (in mm), **(C)** velocity (mm/sec), and **(D)** time spent in center of open field, compared to controls (DIO-mCherry+CNO). Data represented as Mean ± S.E.M.

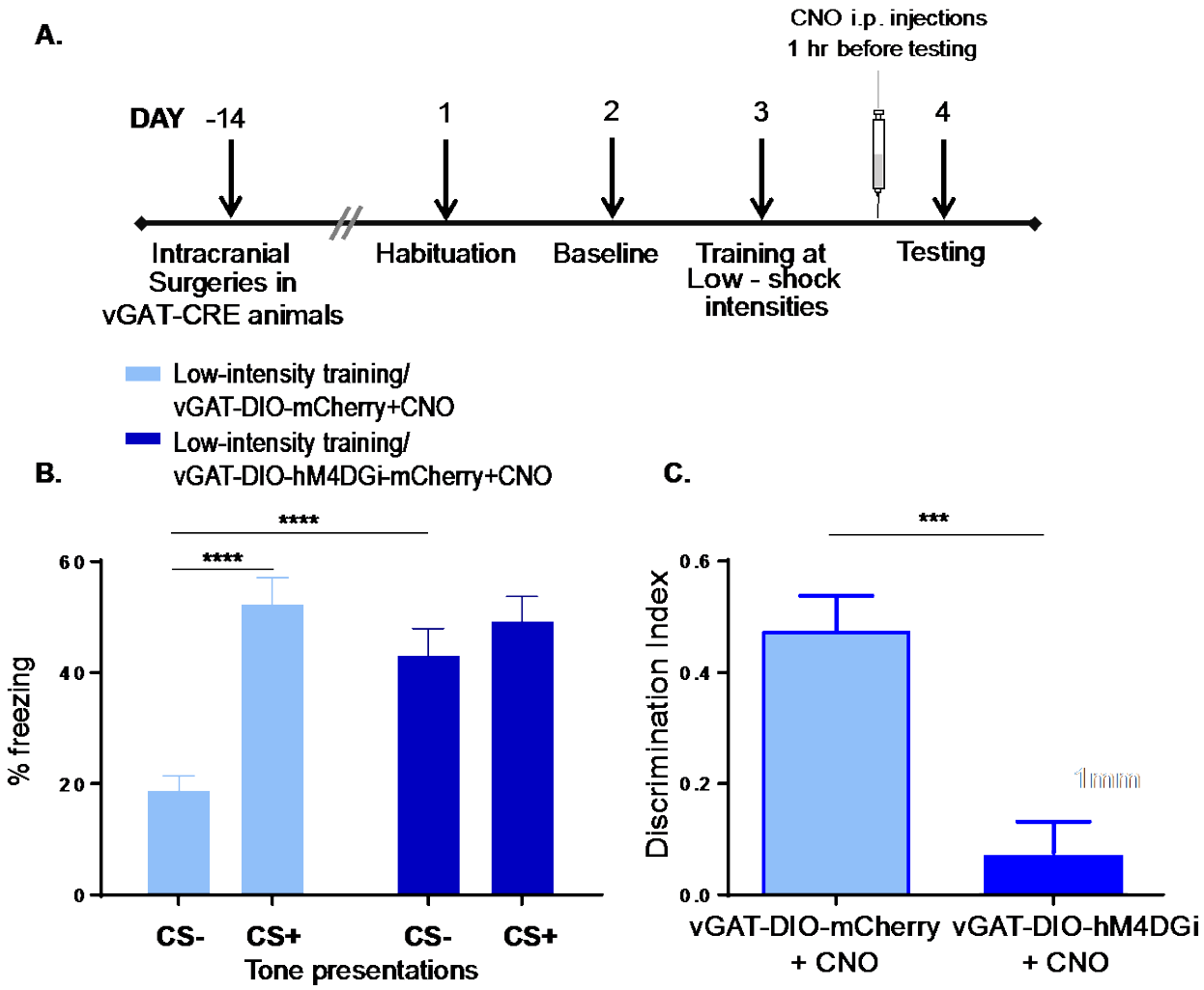

Figure 3.5: Targeted chemogenetic inhibition of GABAergic cells in the ZI can reduce fear generalization.

(A) Experimental design: vGAT-CRE animals received intracranial injections of CRE-dependent control or DREADD virus and after two weeks, were conditioned to low threat intensities. One day post-training, CNO was administered intraperitoneally 1 hour before testing for fear generalization. **(B)** Animals with expression of DIO-hM4DGi virus in vGAT-CRE expressing GABAergic cells in the ZI and injected with CNO (DIO-hM4DGi+CNO) one hour before testing for fear generalization showed a significant increase in fear response to CS- compared to animals that were infused with the DIO-mCherry virus in vGAT-CRE expressing GABAergic cells in the ZI and injected with CNO (DIO-mCherry+CNO). **(C)** Chemogenetic inhibition of GABAergic

cells in the ZI (DIO-hM4DGi+CNO) resulted in reduced ability to discriminate between the CS+ and the CS-. *$p<0.05$ **** $p<0.0001$. Data represented as Mean ± S.E.M.

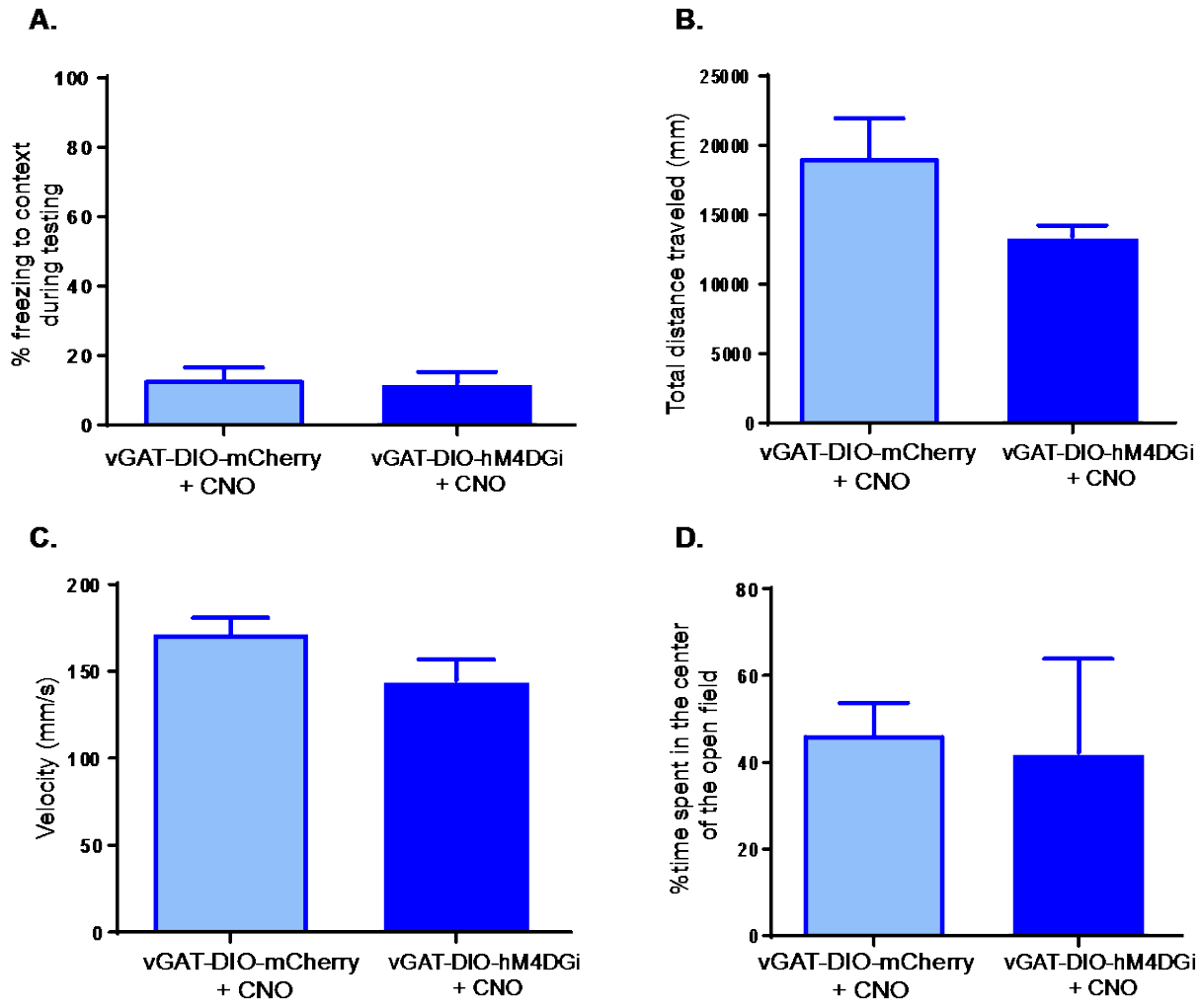

Figure 3.6: Chemogenetic inhibition of GABAergic cells in the ZI does not produce non-specific increase in freezing responses and does not affect general locomotor function or anxiety-like behavior.

(A) No significant differences were observed in freezing to Context B with chemogenetic inhibition of GABAergic cells in the ZI on testing day. **(B-D)** In the Open Field Test performed one hour after CNO injections, chemogenetic inhibition (DIO-hM4DGi+CNO) of GABAergic cells in the ZI in vGAT-CRE animals did not produce detectable changes in **(B)** total distance traveled (in mm), **(C)** velocity (mm/sec), and **(D)** time spent in center of open field, compared to controls (DIO-mCherry+CNO). Data represented as Mean ± S.E.M.

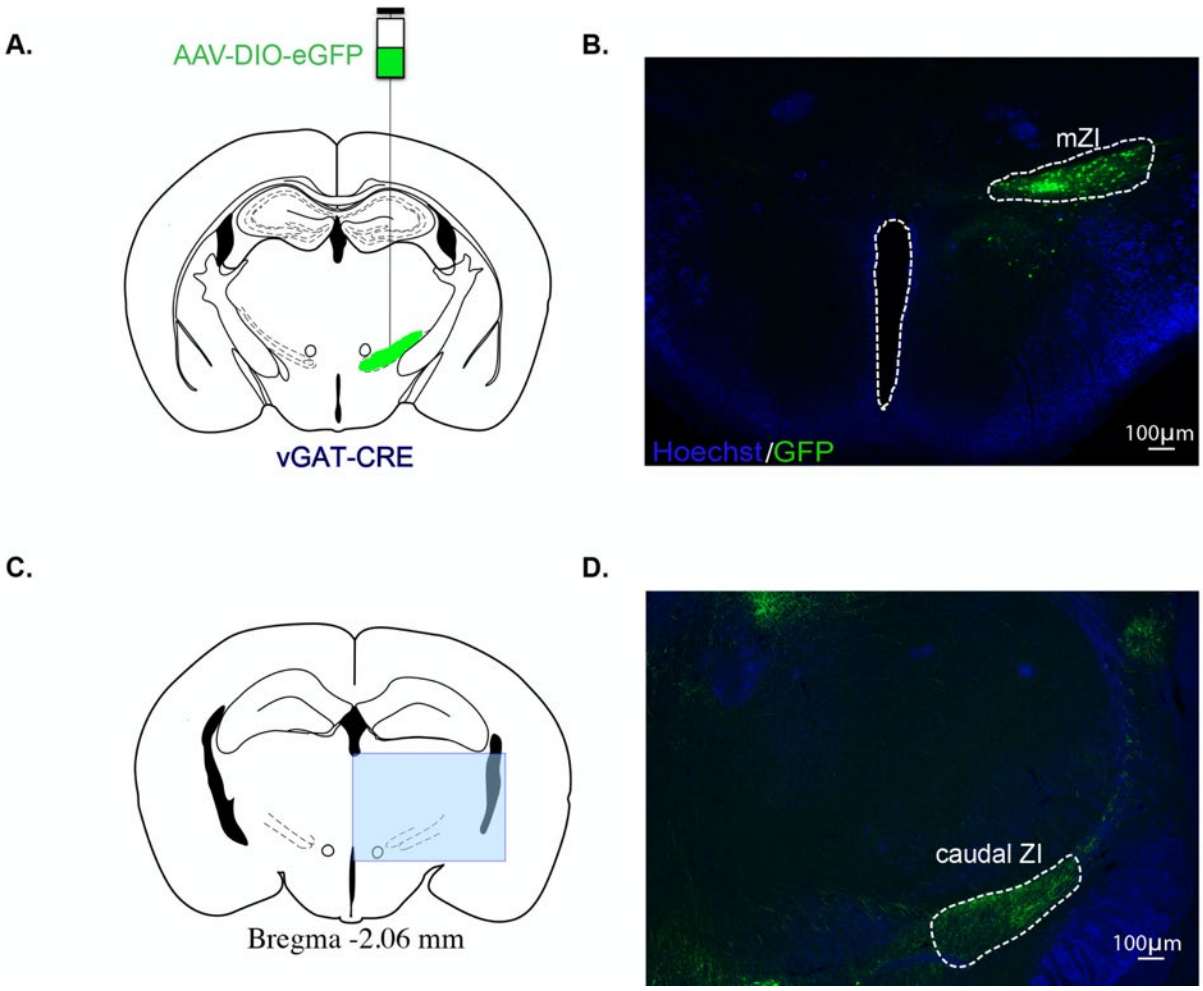

Figure 3.7: Tracing GABAergic projections from the zona incerta (ZI).

(A) Schematic of unilateral injection of CRE-dependent eGFP virus into the ZI of vGAT-CRE mice. **(B)** Injection site showing tracer-labeled (eGFP) GABAergic neuronal cell bodies in the medial zona incerta (mZI) encompassing the dorsal and ventral subdivisions. **(C)** Identification of GABAergic projections in the caudal subdivision of the zona incerta (cZI) 2.06 mm posterior to Bregma. **(D)** Image showing GFP expression (indicating GABAergic projections) in cZI.

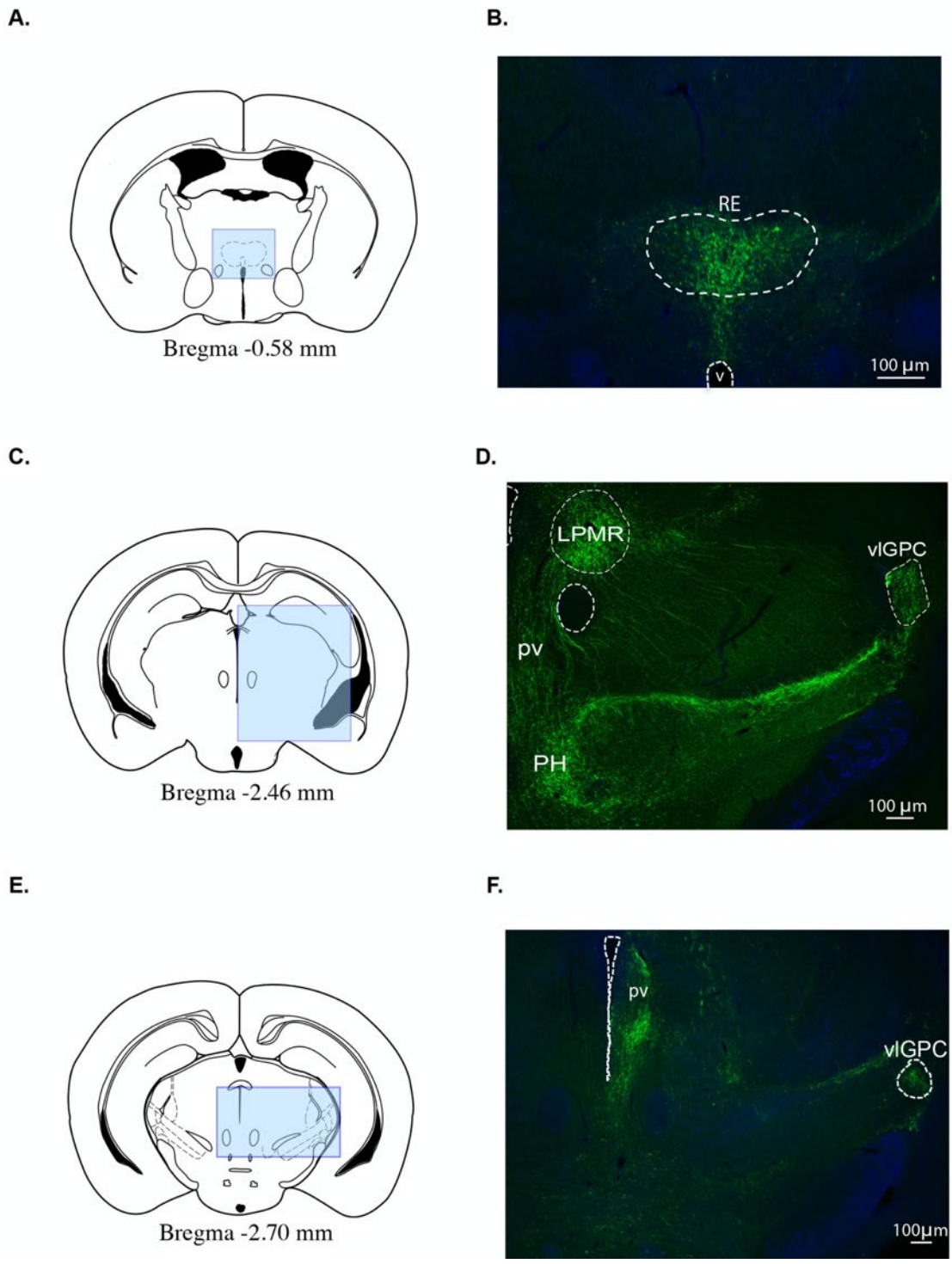

Figure 3.8: GABAergic projections from the ZI to thalamus and hypothalamus.
(A) Identification of GABAergic projections in the midline thalamic nucleus reuniens (RE) 0.58 mm posterior to Bregma. **(B)** Image showing extensive GABAergic projections (green) in RE. **(C**

& E) Identification of GABAergic projections at 2.46 mm and 2.70 mm posterior to Bregma. **(D & F)** Images showing dense labelling in the lateral posterior medio-rostral thalamic nucleus (LPMR), parvicellular part of the ventral lateral geniculate nucleus (vlGPC), periventricular fiber system, and posterior hypothalamus (PH).

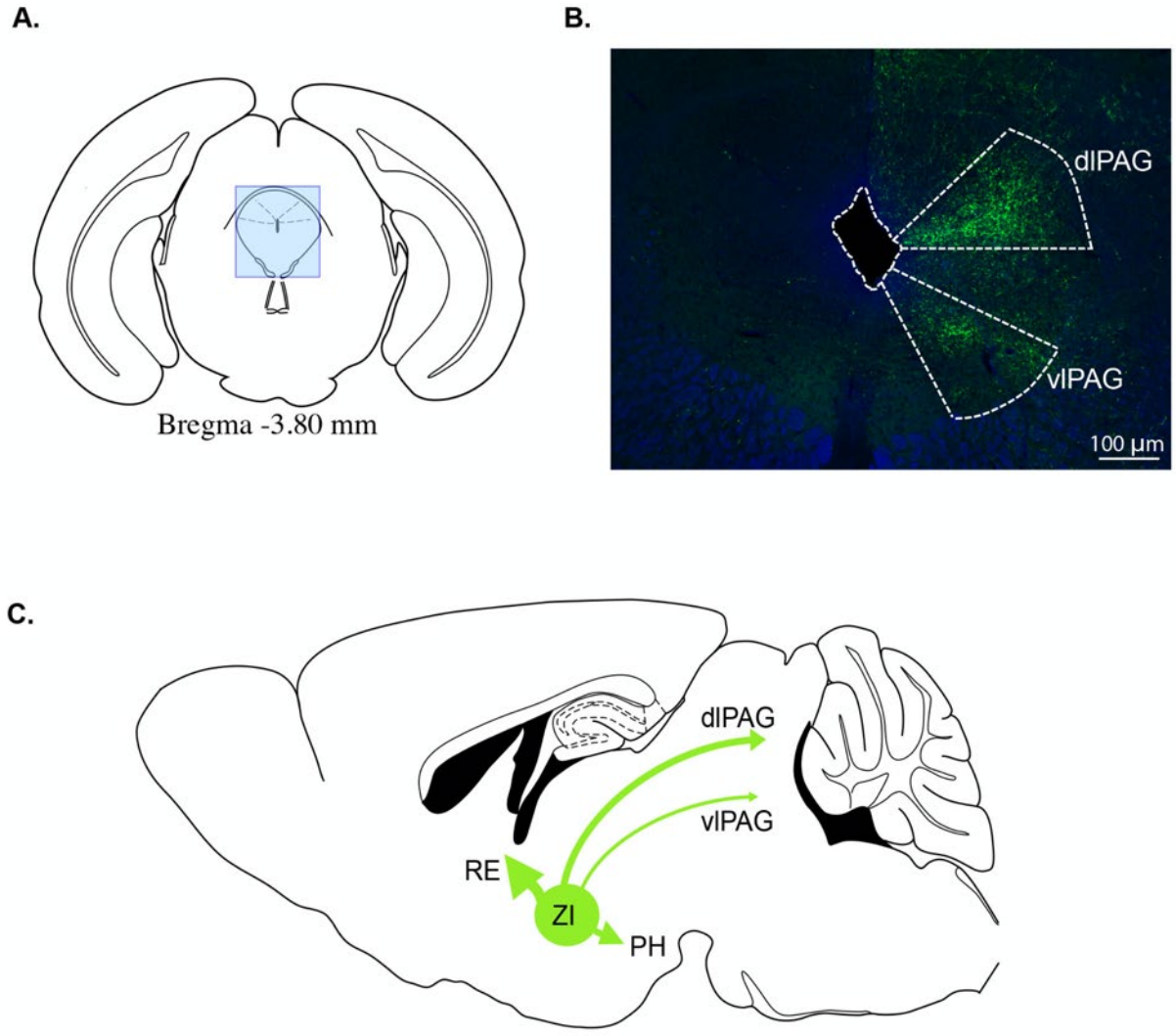

Figure 3.9: GABAergic projections from the ZI to fear-responsive regions.
(A) Identification of heavy labelling of GABAergic projections in the midbrain periaqueductal gray 3.8 mm posterior to Bregma. **(B)** Image showing distribution pattern of GABAergic fibers in the dorsolateral and ventrolateral subdivisions of the periaqueductal gray (dlPAG, vlPAG). **(C)** Schematic summary depicting key fear-responsive regions (RE, dlPAG, vlPAG and PH) receiving GABAergic neurons from the ZI.

CHAPTER 4: GABAergic projections from zona incerta to thalamic reuniens regulate fear generalization

4.1 Context, Author's Contribution, and Acknowledgement of Reproduction

This chapter presents evidence for the role of ZI → RE GABAergic projections in fear generalization. The investigation was performed to characterize the function of GABAergic pathway from ZI to thalamic RE in fear generalization. The functional connectivity between the two regions was confirmed using whole-cell patch clamp recordings. Following this, we used projection-based optogenetic strategy to uncover the role of the ZI-RE pathway in generalization of fear responses. The majority of the experiments in the following chapter were designed and conducted by the dissertation author. All of the electrophysiology data presented here were collected by Dr. Jidong Guo. The work was conceptualized, organized and written by the dissertation author, with guidance from Dr. Brian Dias.

4.2 ABSTRACT

Fear of neutral stimuli that are distinct from stimuli that had been previously associated with aversive outcomes is maladaptive. This process, fear generalization, is a central feature of stress- and anxiety-related disorders such as post-traumatic stress disorder (PTSD) and generalized anxiety disorder (GAD). Although cortical and subcortical contributions to fear generalization have received considerable attention, thalamic and subthalamic contributions to this phenomenon are yet to be understood. In the previous chapter, I demonstrated that GABAergic cells in the ZI can bidirectionally modulate fear generalization and that these cells send extensive projections to fear-responsive regions. In particular, the ZI sends dense efferents to the thalamic nucleus reuniens (RE), a brain region that is known to play a critical role in balancing the specificity and generalization of fear memories. Here, I delineate a functional role for GABAergic projections from the zona incerta to the reuniens in fear generalization. Optogenetic activation of GABAergic incertal inputs induced inhibitory post-synaptic currents (IPSCs) in the RE in *in vitro studies*. Targeted *in vivo* optogenetic activation of GABAergic incertal projections to the RE during memory retrieval, attenuated fear generalization. Together, these findings demonstrate that the ZI-RE circuits inhibit the expression of inappropriate fear responses, a function that is of cardinal importance for adaptive emotional regulation.

4.3 INTRODUCTION

Failure to inhibit fear is one of the core symptoms of stress- and anxiety-related disorders such as PTSD and GAD. One form of such an inability to inhibit fear is the overgeneralization of fear responses beyond established fear associations. More specifically, fear being expressed toward neutral stimuli even though these are unrelated to stimuli that had been directly associated with threat. Understanding the neural circuitry that regulates generalization of fear responses can help us design effective therapeutic strategies to alleviate fear-related symptoms in these disorders.

Thus far, my dissertation has built a case for GABAergic cells in the zona incerta being able to modulate fear generalization. As discussed in chapters 2 and 3, we have shown that the stimulation of the ZI, specifically, the GABAergic cells results in reduced fear generalization and enhanced fear discrimination between neutral and aversive cues. In agreement with these findings, Chou et al. (2018) have shown that suppressing ZI activity dampens fear expression during retrieval as well as extinction of fear memories. Nevertheless, the mechanisms supporting incertal modulation of fear generalization remains to be clarified. To address this gap, in this chapter, I turn my attention to the connectivity between the zona incerta and the nucleus reuniens, a thalamic brain region that controls formation, maintenance and retrieval of fear memories (Ramanathan, Ressler, et al., 2018; Troyner et al., 2018; Xu & Sudhof, 2013). My focus on the ZI→RE pathway is motivated by the connectivity that I observed between GABAergic cells in the ZI and the RE, and literature that has demonstrated a role for the RE specifically in fear generalization.

The ZI sends strong GABAergic projections to the 'higher order' thalamic nucleus called the nucleus reuniens (RE) (see Chapter 3). The RE serves as a critical hub that connects the medial prefrontal cortex (mPFC) to the hippocampus and plays a pivotal role in emotional regulation (Anderson, Bunce, & Barbas, 2016; Cassel et al., 2013; Ramanathan, Ressler, et al., 2018; Troyner

et al., 2018; Vertes et al., 2015; Xu & Sudhof, 2013). Importantly, studies have demonstrated that the RE is necessary for the maintenance as well as specificity of fear memories, thereby playing a role in fear generalization (Troyner et al., 2018; Xu & Sudhof, 2013). Based on the anatomical observations from our studies and others, and the established functions of RE, we hypothesized a modulatory role for the ZI→RE GABAergic pathway in fear generalization.

In this study, whole-cell patch clamp recordings from RE neurons indicated that the GABA+ neurons in the ZI send functional inhibitory projections to the RE. Next, to investigate the role of the incertal-thalamic pathway in fear generalization, we used high-intensity shock auditory fear conditioning in combination with cell-type specific and projection-specific optogenetics. The high intensity auditory fear conditioning was used to model fear generalization where tone-shock associations were formed at high threat intensities and resulted in increased fear response towards neutral as well as aversive stimuli. Targeted optogenetic activation of incertal GABAergic projections to the RE attenuated fear generalization typically observed in animals trained under high threat conditions. Notably, activation of the GABAergic afferents to the RE enhanced discrimination between the CS- and CS+ cues, while retaining appropriately high fear toward the CS+ cue. Together these findings reveal an incerto-thalamic circuit that regulates fear generalization.

4.4 MATERIALS AND METHODS

4.4.1 Animals

vGAT-CRE mice were acquired from Jackson labs and then bred in our vivarium with controlled temperature, humidity and pressure. Adult female or male vGAT-CRE mice (2-3 months of age) were group-housed and kept on a 14:10 light/dark cycle with *ad libitum* access to standard chow and water. All experimental procedures were performed during the light cycle and were approved by the Emory Institutional Animal Care and Use Committee, in accordance with National Institute of Health guidelines.

4.4.2 Virus injection and fiber optic implantation

For optogenetic stimulation of the ZI-RE pathway, vGAT-CRE mice were injected with AAV5-hSyn-DIO-mCherry or AAV5-EF1α-DIO-ChR2(H134R)-mCherry obtained from the University of North Carolina Viral Vector Core.

Animals were anesthetized with ketamine/dexdomitor i.p. injection and placed in a stereotaxic device for virus placement and optic fiber insertion. AAV constructs were bilaterally injected at using Nanoject III (Drummond Scientific) into the ZI using the following stereotaxic co-ordinates: AP: -1.52 mm, ML: 0.73 mm and DV: -4.79 mm relative to Bregma. The total volume of AAV-containing solutions injected into the ZI was 150 nl per side, at the rate of 1 nl/sec. After injection, the needle was left in place for an additional 10 mins and slowly withdrawn over 1 min. Mice were allowed to recover for at least 6 weeks to allow for optimal expression of the opsin. To stimulate the ZI GABAergic cell terminals in RE, animals were anesthetized again and implanted with the fiber optic cannula (200 μm diameter, NA 0.39, Thorlabs) at midline position above the RE at AP: -0.38 mm, ML: 0 mm and DV: -4.5 mm relative to Bregma. Mice were allowed to recover for 1 week before the start of the behavioral experiments. After the recovery period, animals were handled for 5 mins each day for 5 days before the start of behavioral experiments.

4.4.3 Behavioral procedures

Behavioral sessions were conducted in the conditioning chambers (Coulbourn Instruments) connected to a tone generator. The high-intensity auditory fear conditioning procedure was used to induce fear generalization as previously described in chapter 2.

Cue-dependent fear conditioning: Briefly, mice were pre-exposed to context A 2 days before training. On training day, following a 5-min exposure to context A, mice received 10 paired CS+ tone presentations (30s, 75-80 dB) that co-terminated with high intensity foot-shocks (0.5s, 0.8mA) alternating pseudo-randomly with 10 unpaired CS- presentations (30s, 80-85 dB). The inter-trial intervals (ITIs) were set to vary between 2-6 mins. Conditioning sessions were conducted in Context A illuminated with house lights and consisted of grid floor cleaned with the disinfectant, quatricide.

Cued fear testing: The next day, during the testing session, mice were exposed to context B for 3 minutes. Mice then received two randomized presentations of the CS+ and CS- tones (30s, 80-85 dB, 2.5 min ITI) in the laser OFF condition and two randomized presentations of the CS+ and CS- tones (30s, 80-85 dB, 2.5 min ITI) in the laser ON condition. Two trials with laser stimulation alone (in the absence of tone) were also randomly presented for assessing non-specific behavioral effects of laser stimulation.

Behavioral analyses: All behavioral sessions were video recorded and freezing behavior was analyzed using FreezeFrame-4 software (Actimetrics). The total amount of time spent freezing (in seconds) to the tones, context or laser stimulation alone was analyzed in 30-s bins using FreezeFrame software by an experimenter blind to the treatment conditions. Discrimination index (DI) was calculated using the following formula:

$$DI = \frac{\text{freezing to } CS^{+} - \text{freezing to } CS^{-}}{\text{freezing to } CS^{+} + \text{freezing to } CS^{-}}$$

4.4.4 Optogenetic stimulation

The implants placed above the RE consisted of Ø200µm optic fiber (NA=0.39, Thor Labs) held in a ceramic ferrule (1.25 mm, Thor Labs). The optic fibers were cut and polished to a length of 5 mm from the bottom of the ferrules, so as to reach the RE. The optic fibers were connected to patch cables (Ø200µm, NA=0.22) that were in turn connected to a laser light source (473-nm lasers, DPSS Systems, Shanghai Laser & Optics Century). For optogenetic stimulation during cued fear testing, 20-ms pulses of blue laser light was delivered at a frequency of 20Hz. The blue laser pulses were delivered for the entire 30-s period during the CS+ and CS- tone presentations.

4.4.5 Slice preparation and electrophysiological recording

To confirm ChR2 function and determine the optimal stimulation paradigm, vGAT-CRE mice injected with the ChR2 viruses were sacrificed 8-10 weeks later and electrophysiological recordings were performed as previously reported in Daniel et al. (2017). Briefly, 300 µm-thick coronal brain slices containing the ZI and/or RE were prepared using a VTS-1000 vibrating blade microtome (Leica Microsystems Inc., Bannockburn, IL, USA) from adult vGAT-CRE mice anesthetized with isoflurane before decapitation. Brain slices were removed and placed in 95%oxygen-5%carbon dioxide oxygenated artificial cerebrospinal fluid (ACSF) at 32°C for 1 hour before transfer to the recording chamber mounted on the stage of Leica STP6000 microscope. The slices were completely submerged and continuously perfused with oxygenated ACSF at 32°C at a speed of ~2 ml/min.

The brain regions were located using differential interference contrast (DIC) optics and an infrared sensitive CCD camera (Orca ER, Hamamatsu, Tokyo, Japan). GABAergic ZI neurons and their projections were visually identified by the expression of mCherry fluorescent transgene using epifluorescence microscope. Patch pipettes were pulled from thin-walled borosilicate glass capillary tubes and filled with solution made up of the following components (in mM): 130 K-

Gluconate, 2 KCl, 10 HEPES, 3 MgCl2, 5 phosphocreatine, 2 K-ATP, and 0.2 NaGTP, buffered to a pH of 7.3 and an osmolarity of 280-290 mOsm. The resistance of the pipettes varied between 4-6 MΩ. The current and voltage signals were recorded using a MultiClamp 700B amplifier, in conjunction with an Axon Digidata 1550 A-D interface and pClamp 10.4 software (Molecular Devices, Sunnyvale, CA).

Light stimulation of ZI neurons: Laser illumination (wavelength 473 nm, 2–5 $mWmm^{-2}$) was delivered through an optic fiber positioned above the brain tissue connected to a solid-state laser (Shanghai Laser & Optics Century) and oriented directly towards the recorded neurons. Single light pulses were delivered at increasing intensities to obtain intensity-response curve and threshold for action potentials. Stimulus trains of 1-s light pulses (1 ms pulse width, frequencies of 10, 20, 30 and 50 Hz) at 1.0-1.2fold of the spike threshold were delivered to induce spike trains. In coronal slices containing both ZI and RE, a laser fiber was placed above RE neurons receiving the GABAergic projections from ZI to characterize the ZI-RE pathway. The effect of bath application of AMPA/kainite receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) and GABA-A receptor antagonist gabazine on light-evoked IPSCs, was examined. All drugs were purchased from Tocris and applied by gravity perfusion in the circulating ACSF medium.

4.4.6 Histology and immunohistochemistry

vGAT-CRE mice were trans-cardially perfused with 4% paraformaldehyde (PFA) dissolved in 1X phosphate-buffered saline (PBS). The removed brains were fixed in paraformaldehyde solution for a day and equilibrated in 30% sucrose solution for 3-4 days. Brains were sectioned at 35µm on a freezing microtome (Leica). For verification of virus expression and optic fiber placement, the 35µm sections were stained with Hoechst nuclear stain (1:1000) and mounted on slides using SlowFade Gold Antifade mountant (Life Technologies). The position of the mCherry

positive cells was assessed using Nikon Eclipse E800 fluorescent microscope.

4.4.7 Statistical Analysis

Statistical data analyses were performed using GraphPad Prism. Single-variable differences in data sets containing only two groups were discerned using unpaired t-tests. Group differences were discerned using two-way repeated measures ANOVA where appropriate. Significant interactions in the ANOVAs were analyzed for multiple comparisons using Holm-Sidak test. For all analyses, significance level was set at $p < 0.05$ and significance for post-hoc comparisons were set at *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

4.5 RESULTS

4.5.1 Selective optogenetic targeting of GABAergic projections from the ZI to RE.

To identify GABAergic projections from the ZI-RE, we injected a CRE-dependent adeno-associated viral vector expressing ChR2-mCherry into the ZI of vGAT-CRE mice (as illustrated in Fig. 4.1A). This allows expression of the stimulatory opsin ChR2 in the GABAergic cells of the ZI and more importantly, in the regions directly innervated by these GABAergic cells. Four to six weeks later, we found dense ChR2-mCherry expression in the GABAergic cell bodies in the ZI (Figs. 4.1 B,C) and terminals that innervate the RE (Figs. 4.2 A,B). To further confirm function of the stimulatory opsin ChR2, we performed whole-cell patch clamp recordings on ChR2-mCherry expressing GABAergic neurons in the ZI (Figs. 4.3 A-E). Optical stimulation (1-s light pulses) resulted in depolarization of opsin expressing vGAT neurons in the ZI. The GABAergic neurons in the ZI fire action potentials reliably in reponse to stimulation frequencies of 10 and 20 Hz (Figs. 4.3 A,B), while at frequencies greater than 20 Hz (Figs. 4.3 C,D), the firing success rate decreased with increase of frequency. Moreover, the amplitude of light induced membrane depolarization in the ChR2-mCherry expressing GABAergic neurons increased with increases in light intensity (0.4-5.0 mW/mm^2), and evoked action potentials upon reaching threshold (Fig. 4.3 E).

4.5.2 Functional validation of GABAergic projections from the ZI to RE.

Next, to validate the functional connectivity between GABAergic cells in the ZI and thalamic RE, we performed whole-cell patch clamp recordings from RE neurons. Optical stimulation (473 nm-blue light, 1.6 mW/mm^2) of ChR2-mCherry expressing GABAergic projection fibers originating from the ZI, induced inhibitory postsynaptic currents (IPSCs) in RE (Figs. 4.4 A, B). The evoked IPSCs had a reversal potential of -65 mV, which is close to chloride equilibrium potential. Blocking glutamatergic transmission with AMPA receptor antagonist DNQX (20 μM) had no effect on the evoked IPSCs. Next, when we recorded in the presence of both DNQX (20

μM) and GABA antagonist gabazine (5 μM) the IPSCs were completely abolished (Fig. 4.4C). These results confirmed the presence of a distinct inhibitory pathway from subthalamic ZI to thalamic RE.

4.5.3 Optical activation of ZI → RE GABAergic projections reduces fear generalization.

To determine whether stimulation of GABAergic projections from the ZI to RE affects fear generalization, we injected AAV5-EF1α-DIO-ChR2-mCherry (stimulatory opsins) or AAV5-hSyn-DIO-mCherry bilaterally into the ZI of vGAT-CRE mice followed by optic fiber implantation in the RE (as described in Figs. 4.5 A,B). The mice were then trained in a high-intensity auditory fear conditioning protocol (Fig. 4.5C) that has been shown to produce generalization of fear associations to both the aversive CS+ as well as the neutral CS- tones (as previously described in Chapters 2 & 3). One day after training, mice were tested for fear generalization in laser ON and laser OFF conditions. Increased fear generalization was observed after optical stimulation of RE in vGAT-CRE animals that received control DIO-mCherry viruses. However, increasing activity of GABAergic projections in the RE originating from the ZI with laser stimulation, drastically reduced fear generalization (Fig. 4.5D). Specifically, vGAT-CRE: DIO-ChR2-mCherry+stim animals exhibited significantly lower freezing responses to CS- than their responses to CS+, compared to vGAT-CRE:DIO-mCherry+stim animals (vGAT-CRE:DIO-mCherry group n = 12, vGAT-CRE:DIO- ChR2-mCherry n = 11, Laser stim x Tone interaction: $F(1,21) = 24.20$, $p < 0.0001$. Laser stimulation main effect: $F(1,21) = 11.29$, $p < 0.01$. Tone main effect: $F(1,21) = 74.52$, $p < 0.0001$). Post-hocs: vGAT-CRE: DIO-ChR2-mCherry+stim:CS+ vs. vGAT-CRE: DIO-ChR2-mCherry+stim:CS- $p<0.0001$, vGAT-CRE:DIO-mCherry:CS- vs. vGAT-CRE: DIO-ChR2-mCherry+stim:CS- $p<0.0001$. vGAT-CRE:DIO- ChR2-mCherry+stim animals showed better discrimination in their fear response to the CS+ and CS- tones as noted by

their higher discrimination index compared to vGAT-CRE:DIO-mCherry+stim animals (Fig. 4.5E) ($p < 0.0001$, $t = 7.601$, $df = 21$).

4.5.4 Optical activation of ZI → RE GABAergic projections does not produce non-specific changes in fear responses.

In the absence of optical stimulation, both vGAT-CRE:DIO-mCherry animals as well as vGAT-CRE: DIO-ChR2-mCherry animals expressed increased fear generalization (Fig. 4.6A). Subsequently, the discrimination index for CS+ compared to CS- remained indistinguishable between the two groups of animals (Fig. 4.6B). Further, we found no statistically significant difference in contextual freezing to the new context B between the vGAT-CRE groups on the day of testing in the LASER OFF condition (Fig. 4.7A), suggesting a specificity of fear generalization responses to the tones. Further, to test whether optical activation of the GABAergic projections fibers in the RE alone can produce non-specific effects on locomotor activity, we administered light pulses in the absence of tone presentations. This did not produce any significant difference in freezing levels between the two groups (Fig. 4.7B).

4.6 DISCUSSION

Using whole-cell patch clamp recordings and cell-type specific optogenetics in this study, our results demonstrate for the first time that an inhibitory incerto-thalamic circuit modulates fear generalization. The ZI sends dense inhibitory inputs to the thalamic midline nucleus RE. Light-induced *in vitro* stimulation of GABAergic cells in the ZI produces IPSCs in RE neurons. Selective *in vivo* optogenetic activation of GABAergic projection fibers in the RE originating from the ZI during memory retrieval, abolished fear generalization observed after training animals under high threat conditions. Together, these data provide functional evidence for the pivotal role played by an incerto-thalamic circuit in fear generalization and suggests that the ZI is a key inhibitory node that controls appropriate fear expression.

Given that the ZI receives multimodal sensory inputs (auditory, visual and somatosensory), and has potent inhibitory control over the thalamus (Bartho et al., 2002; Mitrofanis, 2005; Mitrofanis & Mikuletic, 1999; Nicolelis et al., 1992; Roger & Cadusseau, 1985; R. Thompson & Bachman, 1979; Trageser & Keller, 2004), it is well-positioned to act as a synaptic interface that connects diverse sensory channels to appropriate behavioral responses. Selection and expression of relevant cue-specific and context-specific fear responses is crucial for emotional regulation. In the context of fear behaviors, the ZI has been implicated in expression of active (such as avoidance or defensive flight behaviors) as well as passive (such as freezing) coping strategies when faced with threats (Chou et al., 2018; Kaelber & Smith, 1979; Loskutova, Vinnitskii, & Il'yuchenok, 1981; Zhao et al., 2019; Zhou et al., 2018). Moreover, animals in a high stress/anxiety state induced by exposure to predator scent, show lower ZI connectivity in an fMRI study (Dopfel et al., 2019). As we have seen previously from the anterograde tracing studies in chapter 3, the ZI sends strong GABAergic efferents to fear-responsive regions. Of the several thalamic and hypothalamic regions

that receive afferents from the ZI, the thalamic RE is of particular interest due to its demonstrated role in fear generalization. This midline thalamic nucleus reuniens (RE) lies at the crossroads connecting the prefrontal cortex to the hippocampus and mediates emotional and cognitive processes associated with fear learning and expression. In this study, we anatomically and functionally verified the presence of an inhibitory pathway from the ZI to RE. Suppression of firing activity in the RE target neurons occurs through hyperpolarization mediated by activation of GABAergic cells in the ZI (Fig. 4.3B). Further, we show that increase in firing of GABAergic cells in the ZI through optic stimulation activates $GABA_A$ receptor-mediated IPSPs in the postsynaptic RE neurons (Fig. 4.3C), thereby preventing them from firing action potentials. This is in agreement with previous research that has demonstrated that incertal projections to the thalamus can impede sensory transmission and drastically reduce spontaneous firing in thalamic neurons (Lavallee et al., 2005; Trageser & Keller, 2004). These findings have opened up avenues to begin understanding how thalamic and subthalamic pathways could gate information flow in cortico-thalamo-cortical information loops that support emotional regulation.

Aberrant functional connectivity of the thalamus has been reported in individuals suffering from PTSD and GAD (Bremner et al., 1999; Kennis, Rademaker, van Rooij, Kahn, & Geuze, 2013; Lanius et al., 2001; Lanius et al., 2003; Qiao et al., 2017; Yin et al., 2011; Y. Zhang, Chen, H., Long, Z., Cui, Q., Chen, H., 2016). In particular, generalization of fear responses has been associated with increased thalamic activity (Dunsmoor et al., 2011; Morey et al., 2015). Inhibitory control of the thalamic nuclei is crucial in shaping appropriate behavioral responses and dysregulation of thalamic inhibition could lead to pathological states that support overgeneralization of fear responses. One of the major sources of inhibitory control of the thalamus arises from the subthalamic ZI. With the identification of the distinct inhibitory pathway arising

from the ZI to thalamic RE, we asked whether this circuit contributes to appropriate fear expression, i.e., inhibition of fear responses in safety conditions and expression of fear responses in threatening conditions. As established earlier in Chapters 2 & 3, we first trained animals at high threat conditions (0.8mA foot-shocks) that reliably induced fear generalization. This is evident from the generalized fear responses expressed by animals in the control vGAT:DIO-mCherry group (Fig. 4.4D), in line with previous reports that conditioning at high shock intensities promotes cued fear generalization (Ghosh & Chattarji, 2015; Jo, Heymann, & Zweifel, 2018; Laxmi et al., 2003). Next, with the use of cell-type specific and projection-specific optogenetic strategy, we were able to achieve temporal control over activation of GABAergic projections in the ZI-RE circuit. The testing sessions were designed to present the CS+ and CS- tones in laser ON as well as laser OFF conditions. In the absence of laser stimulation, animals in the vGAT:DIO-ChR2-mCherry group expressed fear generalization to both CS+ and CS- tones. Stimulation of GABAergic projection fibers from ZI-RE abolished fear generalization in the vGAT:DIO-ChR2-mCherry animals trained under high threat conditions. This is in accordance with previous findings discussed in chapter 3 where we found that stimulating GABAergic cells in the ZI reduced fear generalization in animals trained under high threat conditions. Notably, the observed blockade of fear generalization with stimulation of the GABAergic ZI → RE pathway is due to an observed decrease in fear responses expressed *specifically* towards the CS- but not the CS+. Even with laser stimulation, these animals continue to express high levels of fear to the CS+ tone presentations. These data suggest that the ZI → RE pathway plays a critical role in modulating adaptive fear responses such that the fear towards the neutral stimulus is dampened while the fear towards the aversive stimulus remains intact. This interpretation is further bolstered by the significant differences observed in the discrimination index between the vGAT: DIO-mCherry and vGAT:

DIO-ChR2-mCherry groups. Lastly, to ensure that alterations in locomotor behavior did not contribute to the observed effects on fear generalization following the optogenetic manipulations of the ZI→ RE projections, we performed laser stimulation alone in the absence of tone presentations. Freezing responses remained unaltered with the stimulation (Fig. 4.6B), emphasizing that the observed effects on fear generalization were specific to the CS+ and CS- tones presented.

Previous studies have demonstrated that the RE is critically important for maintaining the specificity and generalization of fear memory representations (Ramanathan, Ressler, et al., 2018; Troyner et al., 2018; Xu & Sudhof, 2013). Ramanathan, Ressler, et al. (2018) showed that pharmacological inactivation of the RE in rats with muscimol injections before retrieval, resulted in generalization of contextual fear to a novel context. Independent of the potential species differences in cue- and context-related fear generalization between the two studies, this apparently contradictory finding could be attributed to key methodological differences: a) use of prolonged inactivation of the RE with muscimol compared to brief ZI-mediated inhibition of RE using optogenetics, and b) inactivation of entire RE compared to inhibition of only the RE neurons that are post-synaptic partners of GABAergic projections from the ZI. Moreover, it is unclear how distinct calbindin- or calretinin- expressing neuronal subpopulations within the RE contribute to fear generalization based on incoming afferents. Future studies using c-fos based labeling of neuronal ensembles during retrieval of context-dependent and cue-dependent memories can help delineate the specific functional contributions of the ZI→RE circuit to fear generalization.

In summary, the present study demonstrates that the ZI-RE inputs mainly drive an inhibitory response in the RE and dampens fear generalization, while leaving conditioned fear intact. The finding that this pathway can suppress fear expression during neutral conditions suggests that

activity in this circuit might be crucial for safety signaling. Suppression of pathological trauma-related fear memories is at the core of exposure-based cognitive behavioral therapy for affective disorders; and future investigations into altered thalamic and subthalamic connectivity underlying the psychopathology are needed.

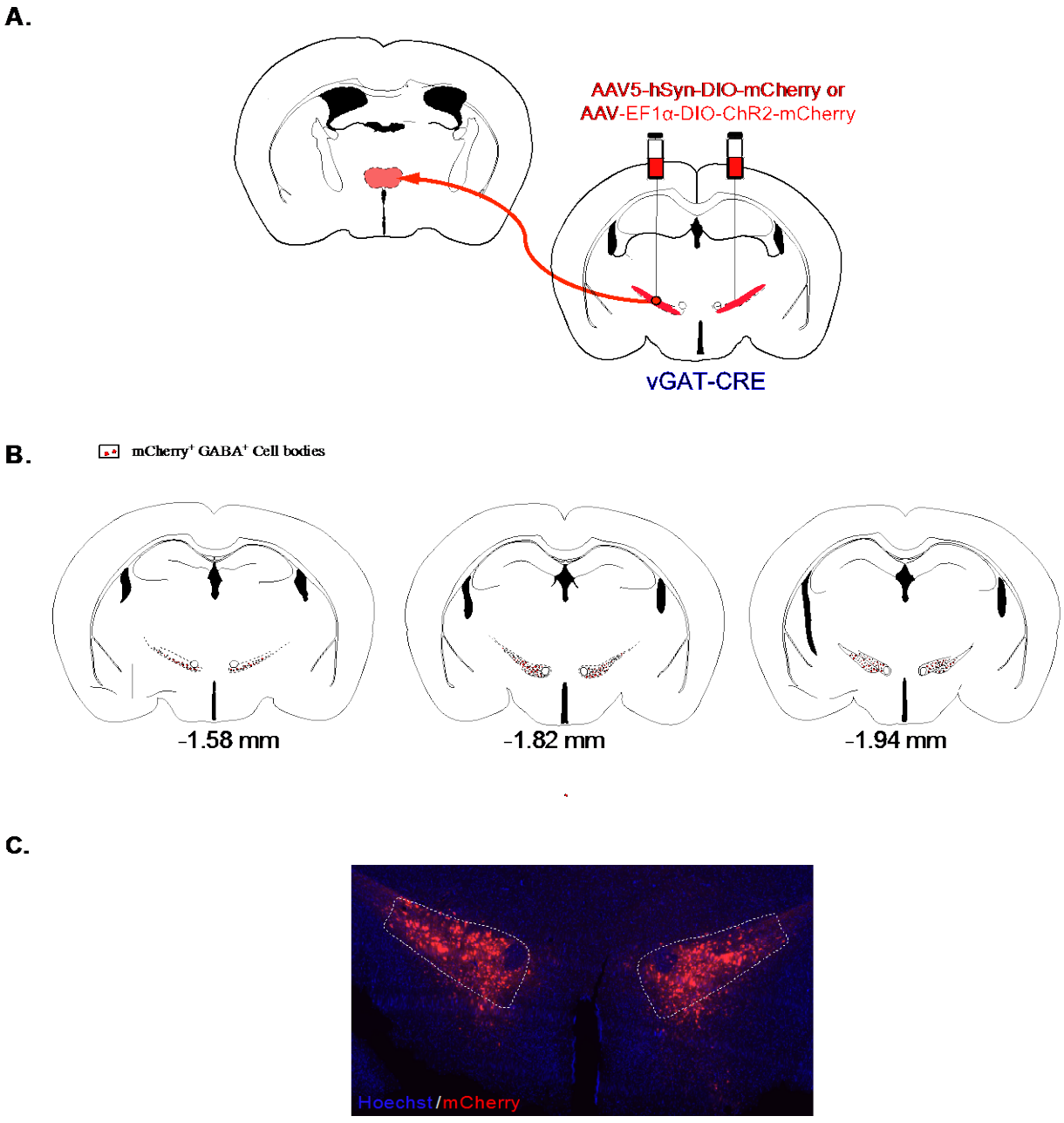

Figure 4.1: Optogenetic targeting of GABAergic projections from the ZI to RE in vGAT-CRE mice.

(A) vGAT-CRE mice were injected with either the control virus (AAV5-hSyn-DIO-mCherry) or Cre-dependent ChannelRhodopsin2 (AAV-EF1α-DIO-ChR2-mCherry) at -1.5mm posterior to bregma and the optic fiber was placed above the RE at -0.38 mm posterior to bregma. **(B)**

Injection sites were restricted to the medial portion of the ZI within the coordinates noted in the Methods section. **(C)** Representative image of the ZI targeted with intra-cranial infusions of ChR2-expressing mCherry viruses.

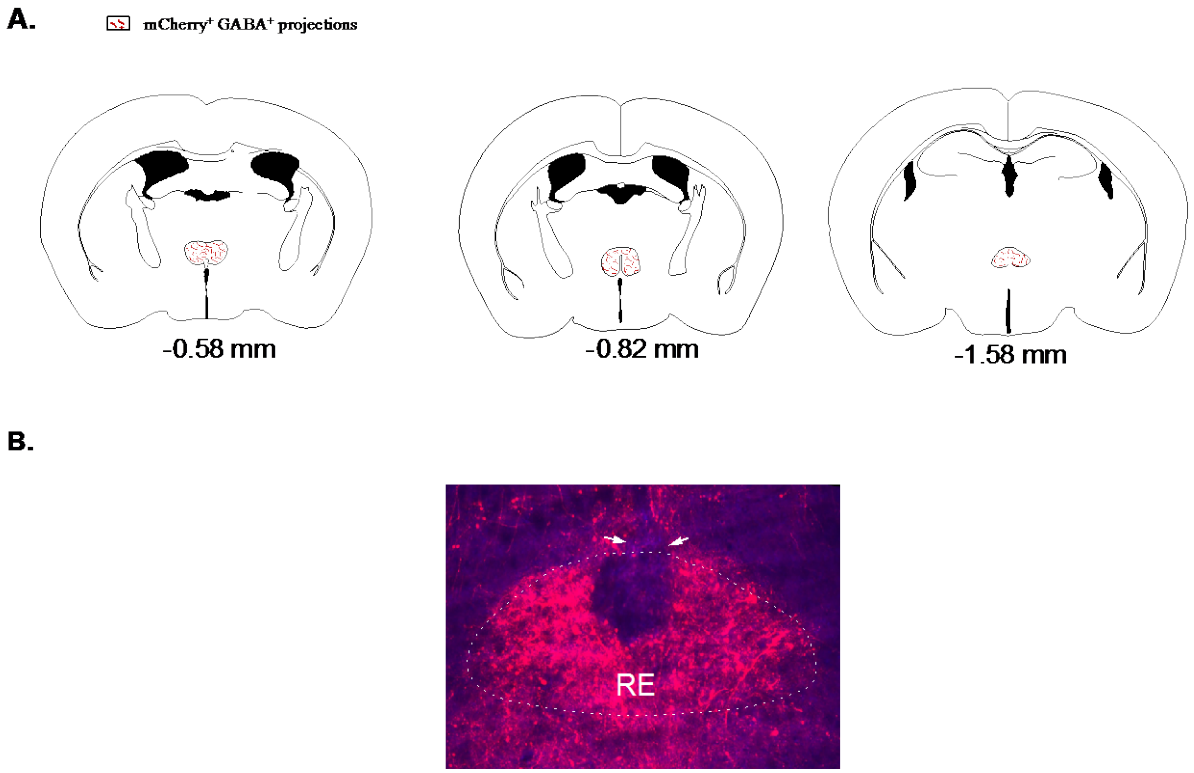

Figure 4.2: Schematic of GABAergic projections in RE of vGAT-CRE mice.

(A) mCherry expressing GABAergic projection fibers were visualized in the RE as indicated. **(B)** Representative image of the mCherry expressing GABAergic projection fibers in the RE with cannula placed above the region (indicated by arrows).

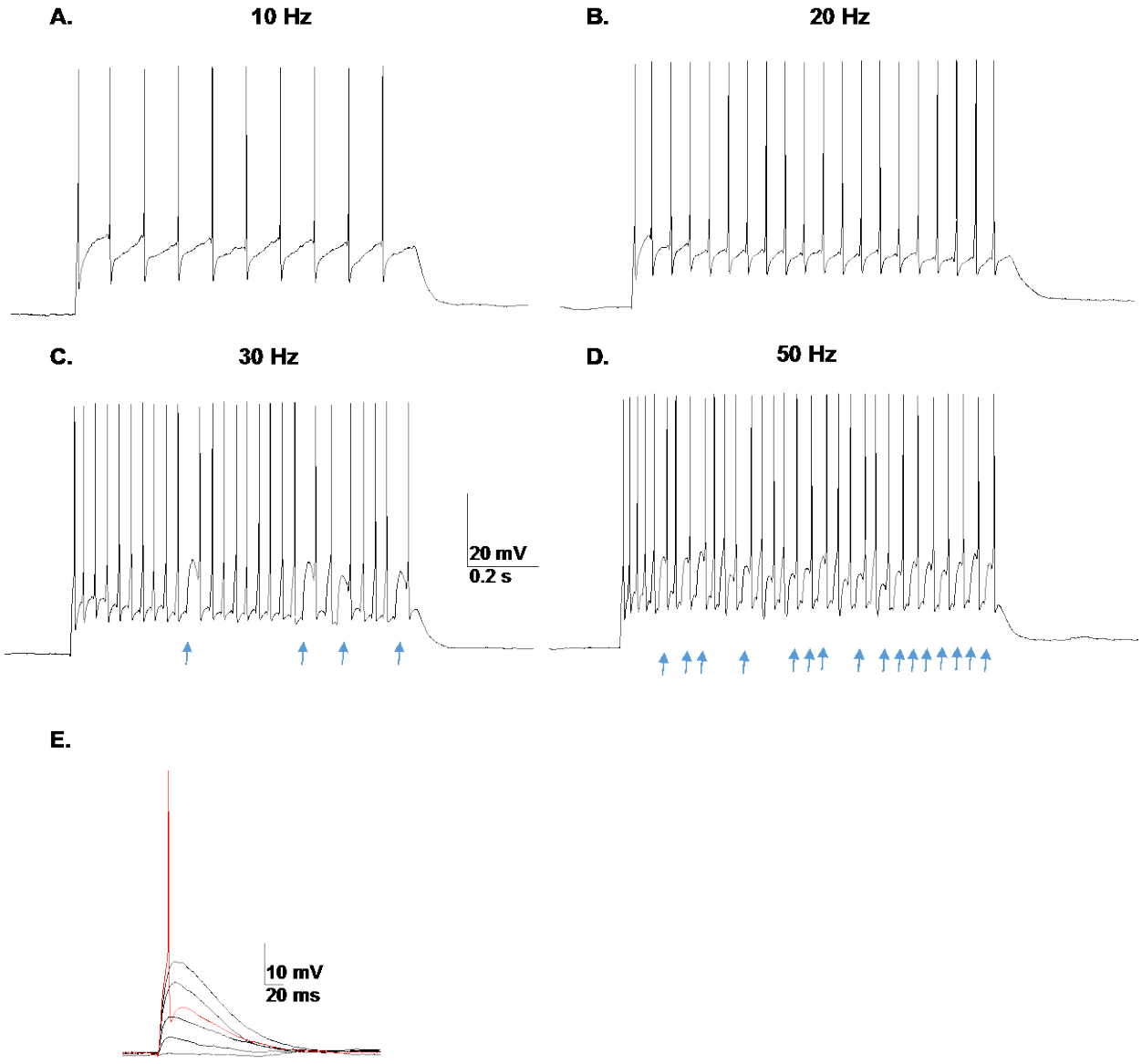

Figure 4.3: Optogenetic control of action potential firing in Zona Incerta neurons transfected with Channel Rhodopsin2 (ChR2)

(A-D) Responses of the ZI neurons to light pulse trains of different stimulation frequencies. In response to **(A)** 10 Hz **(B)** and 20 Hz light pulse trains, the ZI neuron fires an action potential reliably following stimulation frequency, while at higher frequencies **(C&D)**, the firing success rate decreased. **(E)** Representative sweeps showing light induced membrane depolarization that

increased in amplitude with increases in light intensity (0.4-5.0 mW/mm^2). An action potential was evoked when threshold was reached.

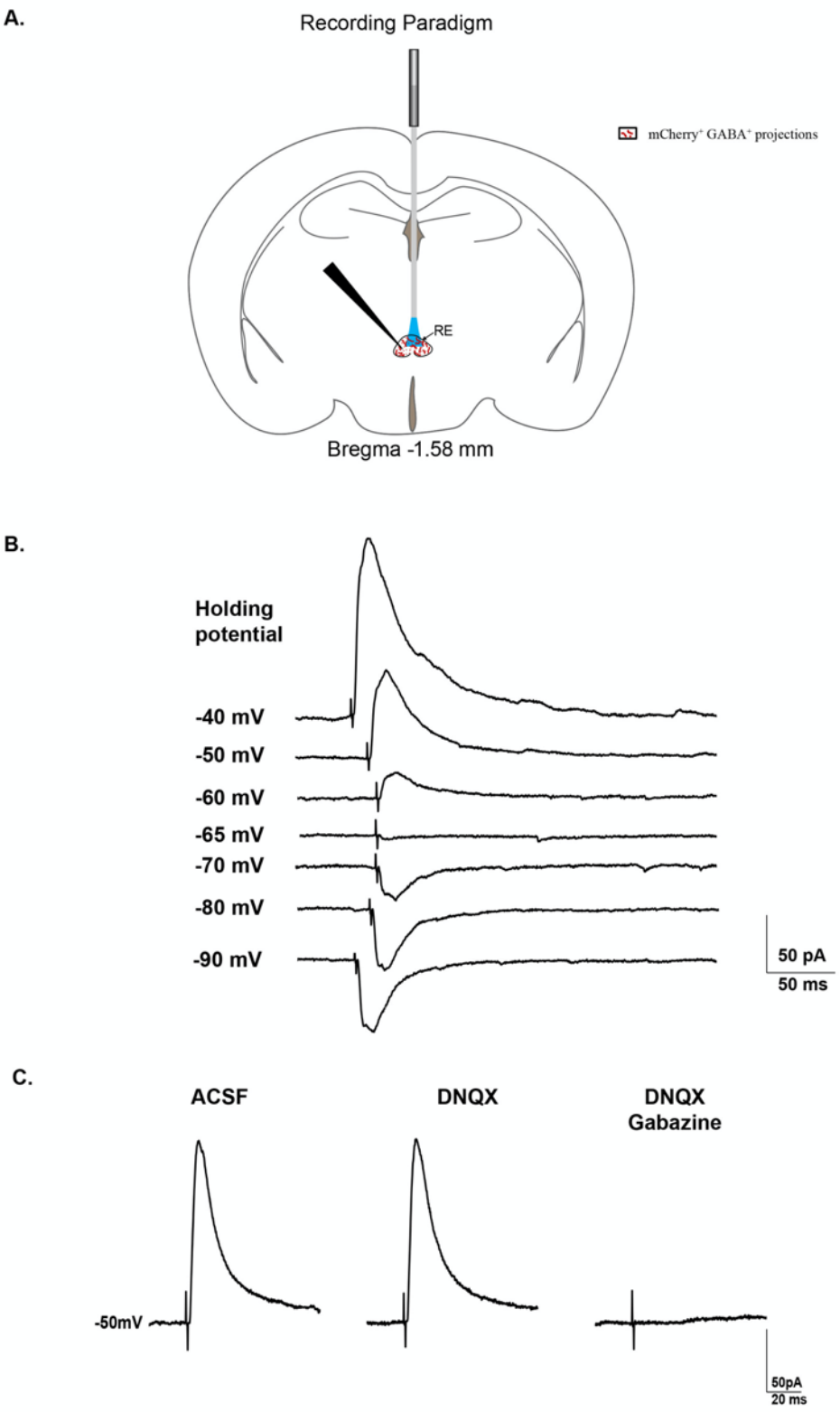

Figure 4.4: Optogenetic stimulation of GABAergic cells in ZI induced IPSCs in RE neurons.

(A) Illustration of experimental configuration for optic stimulation and in vitro patch clamp recordings from RE neurons. Optic stimulation (473 nm blue light, 1.6 mW/mm^2) was directed at ChR2-expressing GABAergic projections in RE. **(B)** Sample traces of superimposed light evoked currents recorded at the indicated holding potentials shown to the left of each trace. The evoked inhibitory post-synaptic currents (IPSCs) had a reversal potential of -65 mV, close to chloride equilibrium potential. **(C)** Bath application of 20 μM AMPA receptor antagonist DNQX had no effect on light evoked IPSCs while 5 μM of GABA receptor antagonist gabazine completely blocked light evoked IPSCs.

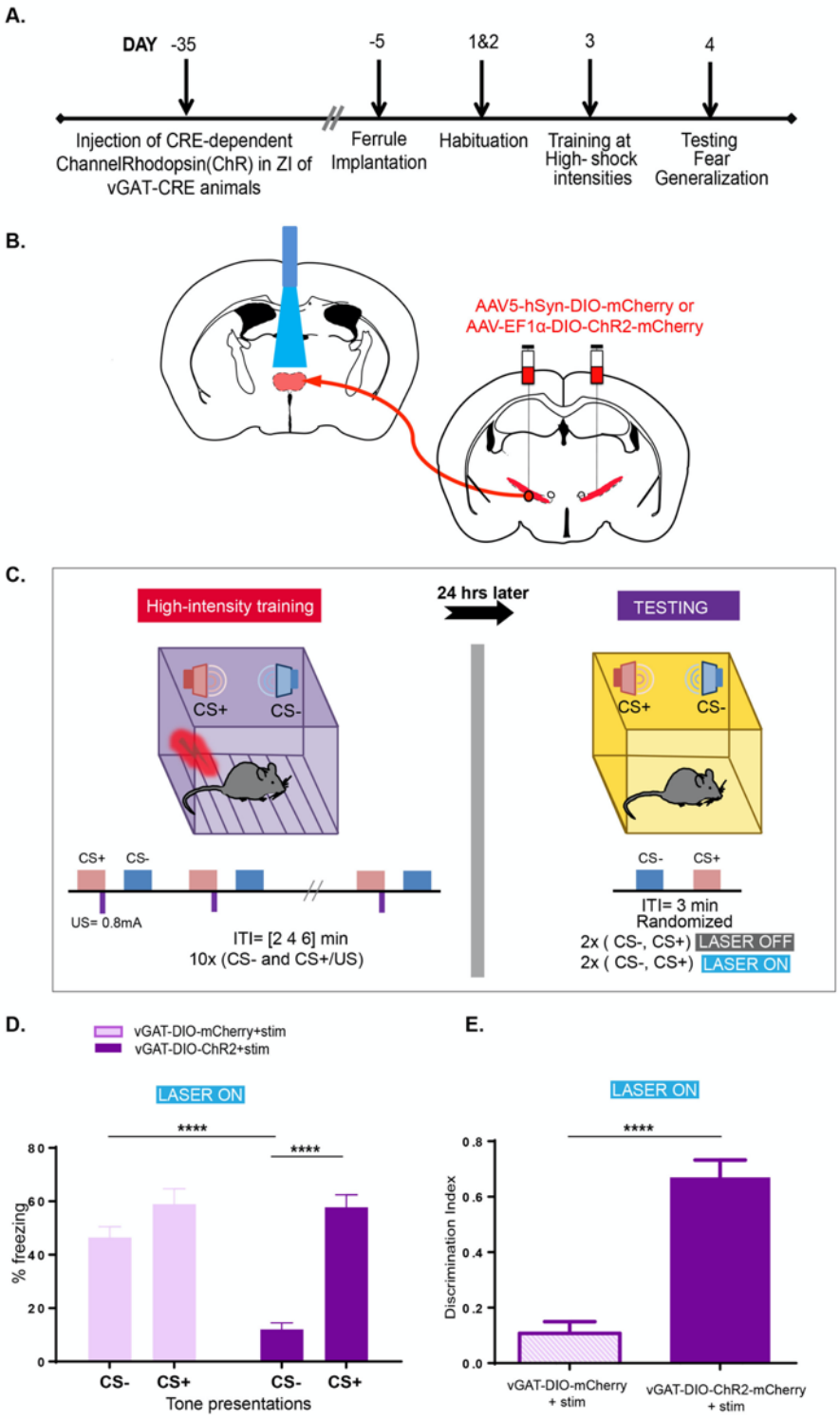

Figure 4.5: Targeted optogenetic stimulation of ZI-RE GABAergic projections reduces fear generalization.

(A) Experimental design: vGAT-CRE animals received intracranial injections of CRE-dependent control or ChR2 (ChannelRhodopsin2) virus in the ZI and after 4 weeks, were implanted with fiber

optic cannula in the RE. Post-recovery from the implantation surgeries, animals were first habituated and then fear conditioned to tones using high shock intensities. 24 hours later, animals were tested for fear generalization. **(B)** vGAT-CRE animals were injected with either the control virus (AAV5-hSyn-DIO-mCherry) or stimulatory opsins (AAV5-EF1α-DIO-ChR2-mCherry) at -1.5mm posterior to bregma. The fiber optic cannula was implanted above the RE at -0.38 mm posterior to bregma. **(C)** Outline of the high-intensity auditory fear conditioning protocol used in the study. On training day, both control and treatment groups of mice received CS+ tone presentations paired with 0.8mA foot-shocks (high threat intensity) and unpaired CS- tone presentations. On testing day, freezing responses in both groups of animals were recorded for the CS+ and CS- tone presentations in LASER ON and LASER OFF conditions. **(D)** Animals injected with DIO-ChR2 virus in vGAT-CRE expressing GABAergic cells in the ZI and optic cannula in the RE receiving GABAergic projections (vGAT-DIO-ChR2+laser stim) showed a significant decrease in fear response to CS- compared to animals that were infused with the DIO-mCherry virus in vGAT-CRE expressing GABAergic cells in the ZI and optic cannula in the RE receiving GABAergic projections (vGAT-DIO-mCherry+laser stim). **(E)** Optogenetic activation of ZI-RE GABAergic projections (vGAT-DIO-ChR2+laser stim) resulted in a better ability to discriminate between the CS+ and the CS-. **** $p<0.0001$. Data represented as Mean ± S.E.M.

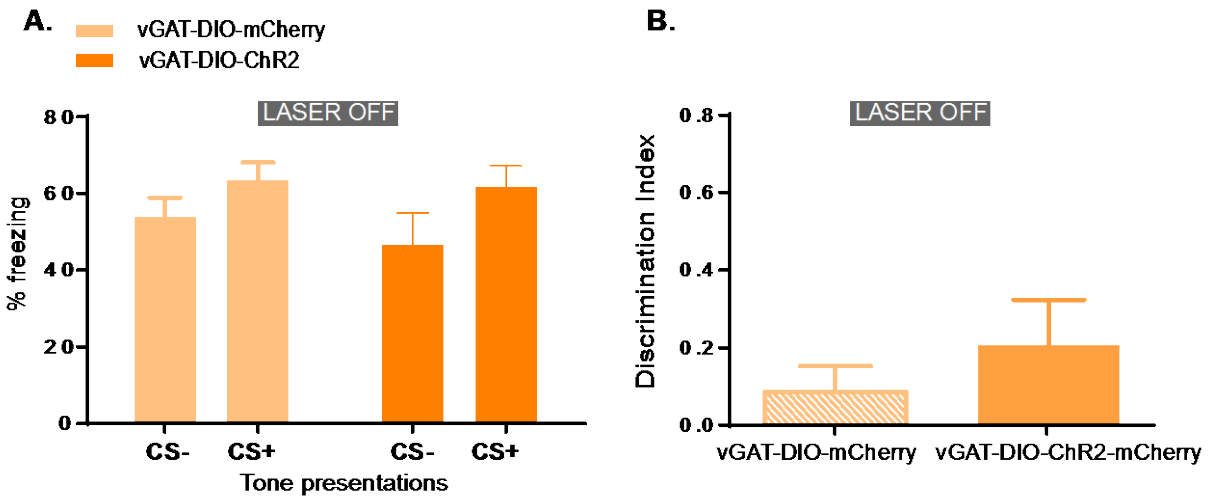

Figure 4.6: Animals trained under high threat conditions express fear generalization in the absence of optogenetic stimulation of the ZI-RE GABAergic circuit.

Without optogenetic stimulation in the LASER OFF condition, vGAT-CRE animals with DIO-ChR2 expressing cells in the ZI did not express any detectable changes compared to controls (DIO-mCherry) in **(A)** freezing responses to the CS+ and CS- tone presentations, and **(B)** discrimination index (DI) for the CS+ compared to the CS- during the testing session. Data represented as Mean ± S.E.M.

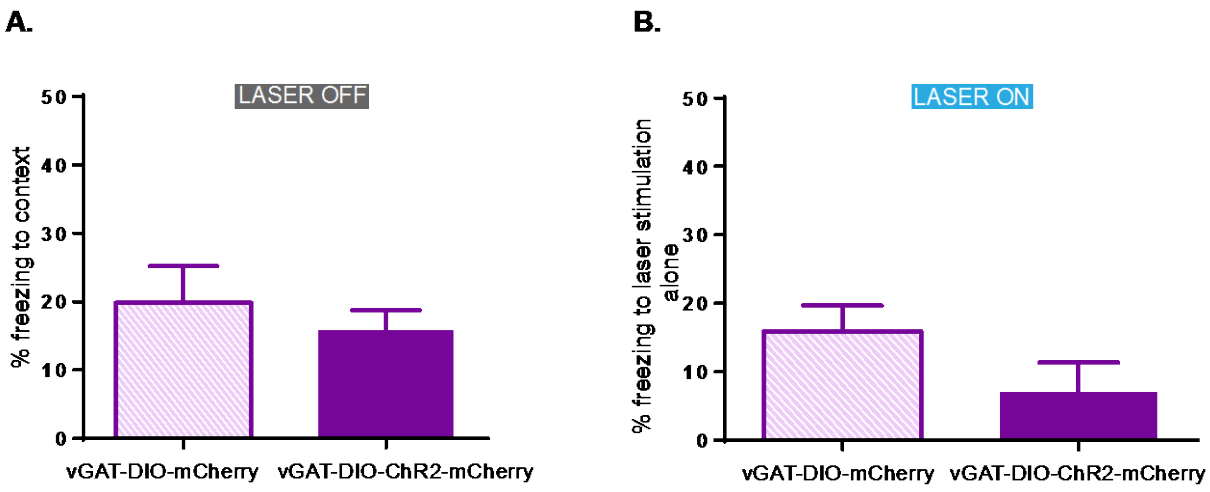

Figure 4.7: Optogenetic activation of ZI-RE GABAergic projections does not produce non-specific increase in freezing responses.

(A) No significant differences were observed in freezing to Context B with optogenetic activation of GABAergic projections from ZI to RE on testing day. These data show that the observed changes in freezing responses following optogenetic stimulation of GABAergic projections from ZI to RE (Fig. 4.4) were specific to the auditory stimuli. **(B)** Laser stimulation of GABAergic projections from ZI to RE on testing day (in the absence of tones) did not produce any significant differences in freezing between the two groups. Data represented as Mean ± S.E.M.

CHAPTER 5: DISCUSSION

5.1 Summary of results

Overgeneralization of fear responses is a hallmark of stress- and anxiety-related disorders. The persistence of fear responses in the presence of a neutral stimulus resembling the aversive stimulus, is maladaptive. Understanding the neural mechanisms underlying the emergence of fear generalization is essential for developing effective therapeutics. Although cortical and subcortical circuitry have received substantial attention in relation to their role in fear generalization, the contributions of thalamic and sub-thalamic brain regions to fear generalization are yet to be fully understood. Based on recent literature on the role of zona incerta (ZI) in multisensory integration, defensive responses, and memory retrieval, this dissertation sought to determine whether ZI modulates fear generalization and further dissect the cell type-specific and projection-specific functional contributions of ZI to fear generalization.

In the first study (Chapter 2), we discovered a novel role for the ZI in calibrating fear responses. Using a mouse model of differential auditory discrimination, we performed a brain-wide screen of C-FOS based neuronal activity in discriminators and generalizers. C-FOS immunohistochemical examination revealed reduced activity in the ZI of animals that generalized fear. Extending these correlational findings, we performed targeted and reversible manipulation of neuronal activity in the ZI using excitatory and inhibitory DREADDs. While chemogenetic activation of the ZI suppressed generalized fear, chemogenetic silencing of the ZI resulted in fear generalization. These observed effects of ZI manipulations on fear generalization were cue-related and did not affect contextual fear responses. Notably, these data revealed that the ZI plays an active role in promoting inhibition of maladaptive fear towards neutral, non-predictive cues. Work from other labs have found that activation of ZI blocks sympathetic responses, causing drastic reductions in heart rate and arterial pressure (Spencer, Sawyer, & Loewy, 1988; Ueyama, 2013).

Further, blocking synaptic transmission in the ZI has been shown to result in increased anxiety-like behavior and altered motor learning (Zhou et al., 2018). Hence it is clear that the ZI is a crucial site for integration of fear-related sensory, autonomic and locomotor behaviors. Given this expanded role for the ZI, we probed whether our chemogenetic manipulations of ZI altered measures of anxiety and locomotion. Open field testing after chemogenetic stimulation or silencing of ZI indicated no changes in velocity, the amount of distance traveled or anxiety-like behaviors. Altogether, these findings suggest the possibility that cue-specific information is processed in the ZI. Cue-related sensory information reaches distinct neuronal subpopulations in the ZI (Mitrofanis, 2002, 2005; Mitrofanis & Mikuletic, 1999; Nicolelis et al., 1992; Power, Leamey, & Mitrofanis, 2001) and the diverse chemoarchitecture of the ZI could allow for selection of appropriate fear responses.

The ZI is a sensorimotor integrator composed of multiple neurochemicals and therefore, delineating the specific functional contributions of these distinct subpopulations of cells to fear generalization was crucial. Pathological fear generalization has been attributed to GABAergic dysfunction in a number of cases (Bergado-Acosta et al., 2008; Bremner et al., 2000; Cullen et al., 2014; Geuze et al., 2008; Sangha et al., 2009; Schur et al., 2016; Shaban et al., 2006; W. H. Zhang et al., 2017). GABA release mediates fast inhibitory neurotransmission and such neurotransmission plays an important role in local inhibitory circuits of the amygdala and cortex; brain regions that are known to be involved in fear generalization. With the ZI extending across a wide rostro-caudal portion of the brain, GABAergic cells in the ZI are undoubtedly a potent source of inhibitory drive that could modulate fear generalization. Therefore, we examined the role of these neuronal populations in fear generalization as reported in Chapter 3. Using cell-specific chemogenetic stimulation, we demonstrated that stimulating the GABAergic cells in the ZI alone

was sufficient to restore fear inhibition, whereas, silencing these GABAergic cells promoted fear generalization. Upon ascertaining GABAergic control of fear generalization in the ZI, we were interested in identifying the postsynaptic partners contacted by these cells. Anterograde tracing experiments revealed that the GABAergic cells in the ZI innervate higher-order thalamic nuclei such as the nucleus reuniens (RE), laterodorsal thalamic nucleus and posterior medio-rostral thalamic nucleus. Further, brain regions involved in defensive responses such as the dorsolateral periaqueducatal gray, ventral periaqueducatal gray and posterior hypothalamus also received strong inputs from the ZI. Interestingly, these target regions have been implicated in different aspects of innate and conditioned fear behaviors. These findings highlight the distinct fear-associated functional connectivity of ZI and further emphasizes its role in modulating fear responses.

Among the fear-related brain regions contacted by the ZI, the thalamic RE is of particular interest for its recently established role in dictating the specificity and generalization of fear memory representations. In chapter 4, we provide anatomical and electrophysiological evidence for inhibitory control of ZI over RE neurons. Using whole-cell patch clamp recordings, we found that stimulation of GABAergic afferents from ZI evoked inhibitory post-synaptic currents (IPSCs) in the RE neurons, that were completely blocked with the application of GABA receptor antagonist gabazine. Further, to pinpoint the functional role of this inhibitory circuit in fear generalization, we employed a cell-specific and projection-specific optogenetic strategy. Optogenetic activation of the ZI→ RE inhibitory projections during memory retrieval after fear conditioning using high-intensity threats, abolished generalization of learned fear responses. Remarkably, the fear response to the neutral stimulus was dampened with no change in fear response to the aversive stimulus.

These experiments provide definitive evidence for the ZI-RE inhibitory circuit in expression of graded fear responses to stimuli.

Together, the data I present in this dissertation demonstrate that (1) reduced C-FOS activation in the ZI accompanies fear generalization, (2) activation of ZI, GABAergic cells in particular, prevents fear generalization, (3) inactivation of the GABAergic cells in ZI causes fear generalization, and (4) the ZI-RE inhibitory circuit is engaged in the inhibition of inappropriate fear responses.

5.2 Integration of key findings

Increased activity in the ZI is associated with appropriate fear discrimination, as evident from the CFOS expression studies in Chapter 2. Reducing the activity of ZI after low-intensity threat conditioning resulted in enhanced fear to the neutral stimulus, but the animals were still able to differentiate between neutral and aversive stimuli. Further, targeted silencing of GABAergic cells in the ZI (chapter 3) produced increased fear generalization, with a complete loss of discrimination between the neutral and aversive tones. It is important to note that the silencing of GABAergic cells in the ZI did not induce a generalized state of anxiety as evident from the lack of increased contextual freezing or any change in open-field measures.

Fear generalization induced by high-intensity threat conditioning is associated with decreased activity in the ZI, as reported in Chapter 2. Stimulation of the ZI alone produces overall dampening of fear towards both the neutral and aversive stimuli and prevents fear generalization as well. Targeted stimulation of GABAergic cells in the ZI (chapter 3) replicated the effects observed with non-specific stimulation of ZI. Taken together, these results from chapters 2 and 3 highlight the crucial role of ZI in modulating fear generalization.

High-intensity and low-intensity training protocols used in this dissertation represent high and low fear states in the animals respectively. These states of high and low fear could activate fundamentally distinct neuronal pathways related to pain, autonomic responses and locomotor behaviors that converge on the ZI. High fear states are characterized by decreased activity in the ZI and vice versa. Suppression of ZI activity in the high fear state could originate from the central amygdala (CeA) inputs. Somatostatin (SOM)-expressing neurons of the CeA send inhibitory projections to the parvalbumin (PV)-expressing cells in the ZI and have been implicated in expression of fear memories (Zhou et al., 2018). Genetic and functional analysis of SOM-

expressing cells in CeA have revealed that these “fear-on” cells control formation and expression of fear memories (Haubensak et al., 2010; H. Li et al., 2013). It is possible that in a state of high fear, an overactive CeA suppresses activity in the ZI resulting in fear generalization that can be reversed by direct chemogenetic activation of the ZI. Future cell-specific and projection-specific studies should decipher the role of this putative pathway in fear generalization.

Prior work (Haubensak et al., 2010; H. Li et al., 2013; Sanford et al., 2017) has shown that cells with different neurochemical profiles within the same brain region could modulate distinct aspects of fear behavior during the high and low fear states. The ZI consists of primarily GABAergic neurons that exert inhibitory control over behavioral expression of fear. In particular, these neurons have been implicated in defensive responses like freezing and avoidance as well as in retrieval of aversive memories (Chou et al., 2018; Zhou et al., 2018). Experiments described in chapters 3 and 4 highlight the strong inhibitory control exerted by the GABAergic cells of ZI over higher order thalamic nuclei to potentially modulate fear generalization. These GABAergic cells might largely overlap with somatostatin (SOM) and/or parvalbumin (PV) expressing cells in the ZI. Work by Zhou et al. (2018) showed that silencing of PV-containing cells in ZI impaired acquisition of contextual as well as cued fear memories, and increased anxiety-like behaviors. Although the function of SOM-containing cells in fear behaviors is unclear, they are known to play a critical role in dendritic arborization of cortical neurons early in development (Chen & Kriegstein, 2015). Akin to the interneurons in the cortex (Jang et al., 2019; Tremblay, Lee, & Rudy, 2016), the PV- and SOM- containing neurons in the ZI could contribute differently to feedforward and feedback inhibition mechanisms that support thalamocortical synchronization, thereby allowing for precise encoding and discrimination of sensory inputs. Therefore, the ZI is well-positioned to exert inhibitory control over thalamocortical loops and gate fear responses.

GABAergic input to the thalamus has been traditionally presumed to originate from the reticular nucleus, however, few instances of "extra-reticular" sources of GABAergic innervation have been described in the literature so far (Bokor et al., 2005; Churchill, Zahm, & Kalivas, 1996). Among the extra-reticular source of thalamic inhibition is the ZI that does not innervate first-order relay nuclei of thalamus but instead innervates higher-order thalamic nuclei; an indication for ZI's potential role in sensorimotor integration and other higher order functions such as attention, arousal and emotional regulation. The experiments in chapter 4 of this dissertation characterizing the inhibitory inputs from the ZI to thalamic reuniens (RE), have contributed to further our understanding of incertal-thalamic interactions. Activation of inhibitory inputs from ZI → RE in animals trained under high-threat conditions, reverses fear generalization and allows appropriate expression of fear responses to the neutral and aversive stimuli. Notably, activation of this pathway does not dampen fear expression towards the aversive stimulus, but only to the neutral stimulus. This suggests that this ZI-RE pathway contributes to cue-specific modulation of fear. The RE is a critical mediator of communication between the cortex and hippocampus; it receives monosynaptic inputs from pyramidal neurons in layer V and VI of the medial prefrontal cortex (mPFC) and selectively targets the CA1 and subiculum regions of the hippocampus (McKenna & Vertes, 2004; Varela, Kumar, Yang, & Wilson, 2014). Cortical inputs shape RE activity differently across behavioral states and by virtue of its connectivity, the RE influences fear learning, threat processing and emotional regulation (Kafetzopoulos et al., 2018; Ramanathan, Jin, et al., 2018; Ramanathan, Ressler, et al., 2018; Salay et al., 2018; Xu & Sudhof, 2013). Overall, our work along with others suggests that ZI-mediated inhibition of RE could be crucial for efficient refinement of stimulus-relevant neural representations to facilitate expression of appropriate fear responses.

5.3 Implications

The evidence for cue-specific modulation of fear by the ZI is intriguing. Results from the C-FOS study and chemogenetic manipulations (reported in Chapters 2 & 3) suggest that increased activity within GABAergic cells in the ZI could promote selective inhibition of fear towards neutral, non-predictive cues. How could fear discrimination be restored with activation of GABAergic cells in the ZI? One possibility is that the ZI could selectively gate information flow to target regions, thereby modulating appropriate stimulus-specific fear responses. The inhibitory control that ZI exerts over its postsynaptic target neurons might allow for selective flow of information through specific neuronal networks. Consistent with this view, prior research has shown that the ZI inhibits spontaneous activity in target thalamic nuclei in a state-dependent manner and exerts strong feed forward inhibition, temporally limiting the glutamatergic influence on thalamus (Lavallee et al., 2005; Trageser & Keller, 2004). Similar to the cholinergic control of ZI based on arousal states, activity in ZI could be differentially modulated by cortical and subcortical inputs from the mPFC and CeA (Bartho et al., 2007; Chou et al., 2018; Zhou et al., 2018) based on high or low fear states.

Alternatively, safety and threat processing might be mediated by parallel, partially non-overlapping circuits that converge on the fundamental canonical fear circuitry (amygdala, hippocampus and PFC). In this case, neuronal pathways involving the thalamic and subthalamic circuits such as the ZI could be recruited only under safety conditions to allow for suppression of fear-related memory representations and not for expression of learned, fear under dangerous conditions. Consistent with this proposition, accumulating evidence indicates that multiple parallel pathways might be responsible for mediating behavioral responses to threat (innate fear vs conditioned fear, high vs low fear states) (Gross & Canteras, 2012; C. Shi & Davis, 1999; Silva, Gross, & Graff, 2016; Silverstein & Ingvar, 2015; You & Li, 2016). For instance, C. Shi and Davis

(1999) found that during fear conditioning, shock information is conveyed to the amygdala through parallel, independent cortical and thalamic routes such that lesioning of both pathways hampers fear acquisition but does not have the same effect when either pathways were lesioned separately. Recently, parallel and distinct modules for fear expression and fear inhibition has become a subject of intense investigation. High fear (fear expression or renewal) and low fear (fear extinction) states require activity in discrete populations of neurons within the canonical circuitry including the amygdala, hippocampus and prefrontal cortex (Busti et al., 2011; Herry et al., 2008; Lacagnina et al., 2019). Identifying the mechanisms underlying communication between these distinct neuronal ensembles within the canonical circuits and the thalamic/subthalamic brain regions is crucial for understanding how the brain deciphers fear and safety.

Activation of the ZI → RE inhibitory pathway eliminated fear generalization in animals trained at high-intensity threat conditions. GABAergic inputs from ZI potentially gates response of RE neurons associated with specific fear memory representations. In addition to the ZI, the RE receives inhibitory inputs from the thalamic reticular nucleus (TRN). Interestingly, work by Wanaverbecq et al. (2008) has demonstrated that GABAergic inputs to the thalamus from two distinct sources exhibit different synaptic arrangements that is mirrored in the synaptic plasticity of IPSCs between the pathways. Hence, morphological and electrophysiological characterization of the synaptic arrangement of the incerto-thalamic and reticulo-thalamic pathways might further our understanding of the functional salience of the inhibitory drive exerted by ZI on RE. The RE neurons are primarily glutamatergic and contain calretinin and/or calretinin. Presence of these calcium-binding proteins differentially influence neuronal firing patterns and therefore understanding the neurochemical identity of the RE neurons contacted by ZI is important.

5.4 Future directions

Aberrant functional connectivity between cortical and subcortical regions is thought to underlie severe and recurrent fear associated with psychiatric conditions such as phobia, post-traumatic stress disorder (PTSD) and generalized anxiety disorder (GAD). The persistence and generalization of emotional memories long after the traumatic event is a characteristic feature of these disorders and research indicates that this form of memories formed in the past termed as "remote memories" is associated with pathological activity in the mPFC. Prefrontal inputs to key downstream regions are believed to mediate maintenance of remote memories. Based on the findings in this dissertation, I propose that the ZI plays a pivotal role in the retrieval of cue-specific remote memories during both early and remote time points. Investigation of the cortico-incertal mechanisms in generalization of remote fear memories, entail the following objectives:

1. Examine mPFC-ZI synapses using brain slice electrophysiology complemented by mPFC layer-specific analyses of immediate early gene responses related to generalization of immediate and remote fear memories.
2. Employ projection-specific optogenetics in freely-behaving animals to test the role of mPFC-ZI circuit in immediate and remote fear generalization.
3. Analyze stimulus related change in activity in the ZI in response to optogenetic activation and inactivation of inputs from mPFC using calcium-based imaging.

Results from these future experiments will help define mechanisms by which the zona incerta contributes to the retrieval and long-lasting maintenance of emotional memories. Understanding the function of cortico-incertal circuits that can potentially modulate fear memories might open novel treatment avenues in individuals suffering from affective disorders.

5.5 Conclusions

With the implication of ZI in a wide range of behaviors, it has been proclaimed to be a global modulator of exteroceptive and interoceptive states with profound influence on behavioral output (Mitrofanis, 2005; X. Wang, Chou, Zhang, & Tao, 2019). The extensive cortical and limbic connectivity of the ZI combined with the heterogenous chemoarchitecture, makes it a critical hub for emotional regulation. The findings presented in this dissertation adds further to our understanding of the mechanisms by which the ZI modulates behavioral fear responses.

The focus of uncovering the neurobiological mechanisms underlying fear inhibition thus far, has been limited predominantly to the canonical circuit comprising the hippocampus, amygdala and prefrontal cortex. However, our work along with others have identified parallel thalamic and subthalamic pathways (outside the canonical circuitry) that regulate fear. These discoveries have opened new targets for therapeutic interventions in treating symptoms of stress- and anxiety-related disorders such as PTSD and GAD.

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