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Programvezető: Dr. Fekete Csaba, DSc

Témavezető: Dr. Tóth Máté, PhD

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THE ROLE OF THE BED NUCLEUS OF STRIA TERMINALIS IN LEARNED AND INNATE FEAR RESPONSES

PhD thesis

Bíborka Bruzsik

Semmelweis University

Janos Szentagothai Doctoral School of Neurosciences

Supervisor: Máté Tóth, Ph.D.

Official reviewers: István Gyertyán, Ph.D.
István Hernádi, Ph.D.

Head of the Complex Examination Committee:
Dániel Bereczki, D.Sc.

Members of the Complex Examination Committee:

László Acsády, D.Sc.

Gergely Zachar, Ph.D.

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Abbreviations

2MT	2-methyl-thiazoline
5-HT2C	5-hydroxytryptamine receptor 2C subtype
AAV	adeno-associated virus
BLA	basolateral amygdala
BNST	bed nucleus of the stria terminalis
CCK	cholecystokinin
CeL	the lateral subdivision of the central amygdala
CNO	clozapine-N-oxid
CRH	corticotropin releasing hormone
CRHR1	corticotropin releasing hormone receptor type 1
CS	conditioned stimulus
DA	dopamin
DIO	double-floxed inverted open reading frame
DMT	dorsal medial thalamus
DR	dorsal raphe
DREADDs	Designer Receptors Exclusively Activated by Designer Drugs
DSM	Diagnostic and Statistical Manual of Mental Disorders
DYN	dynorphin
ENK	enkephalin
fMRI	functional magnetic resonance imaging
FS	footshock
GABA	gamma-aminobutyric acid
GPCR	G-protein-coupled receptor
HC	hippocampus
HPA	hypothalamic–pituitary–adrenal axis
ITI	inter-trial interval
LC	locus coeruleus
LH	lateral hypothalamus
NAc	nucleus accumbens core region
NPY	neuropeptid Y
NT	neurotensin
PAG	periaqueductal gray

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PFC	prefrontal cortex
PKC-δ	protein kinase C delta
PTSD	posttraumatic stress disorder
PVN	paraventriculer nucleus of hypothalamus
PVT	paraventriculer nucleus of thalamus
SI	substantia innominata
SST	somatostatin
TMT	2,3,5-trimethyl-3-thiazoline
US	unconditioned stimulus
VTA	ventral tegmental area

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Introduction

1.1. Distinguishing fear from anxiety

Fear is one of the six basic emotions (along with anger, disgust, surprise, sadness, and pleasure) common in the animal kingdom (Ekman, 1970). There is no scientific consensus regarding the definition of emotions, but generally, they are considered as a summation of cognitive and physiological processes in response to environmental challenges (Anderson and Adolphs, 2014). On the level of the brain, emotions are associated with changes in arousal, attention, memory, and decision making, while somatically they affect endocrine, automatic and motor processes (LeDoux, 1996; Quirk, 2017). Fear responses can be determined as 'a set of behavioral defense sequences protecting individuals from environmental dangers, social aggressions or abiotic aversive stimuli' (Misslin, 2003). Fear-inducing stimuli are innately aversive or the association between a neutral stimulus and an aversive outcome is formed by prior negative experiences (LeDoux, 1996). Threats that co-exist with the species for a long evolutional time scale (e.g. insects, snakes, height) can elicit innate fear responses, likely involving genetically determined mechanisms (Mineka and Öhman, 2002; LoBue and Rakison, 2013; Anderson and Adolphs, 2014). Among innately aversive stimuli, the heritability of predator odor avoidance and the underlying genetic mechanisms have been explored in details (Dias and Ressler, 2014; Wang et al, 2018). Nevertheless, the adaptation to the constantly changing environment demands the individual to react to novel dangers on a shorter timescale (e.g. novel predators, predicting cues to physical harm etc.). Hence, animals developed the ability to avoid threatening cues by learning from their previous experiences or from other conspecifics (Griffin, 2004; Olsson and Phelps, 2007).

Both innate and learned aversive cues represent actual threat only in certain contexts and proximity. Therefore, emotional responses are correlated with threat imminence, where anxiety, fear and panic form a continuum of defensive behavior (Fanselow and Lester, 1988; Perusini and Fanselow, 2015). Anxiety is future-oriented emotional state, which can help the organism to prepare for potential threats, whereas fear is an alarm response to present or imminent danger, which can turn into panic under uncontrollable circumstances (Zoellner et al, 2020). As such, anxiety is an adaptive response when individuals are confronted with unfamiliar stimuli or indicators of future threats. The

increased alertness in both autonomic and behavioral level (enhanced startle reflexes, sweating, increased heart rate, stress hormone release, pupil dilution, avoidant or cautious behavior) help the individual to effectively prepare for defense in need (Fanselow and Lester, 1988; Zoellner et al, 2020). When threat become imminent (e.g. a predator is detected by a prey animal), it elicits instant defensive responses (flight, freezing, tonic immobilization, submissive postures or hypoalgesia). These immediate defensive responses are predominantly evolutionary hard-wired and less sensitive to the internal state of the animals (Misslin, 2003). Fearless behavior as well as sustained anxiety could be detrimental for the survival. Thus, risk avoidance and foraging drives force the animals to a constant behavioral trade-off conflict, where appropriate evaluation and early detection of threats are necessary to promote adaptive decision-making.

1.2. The clinical relevance of basic fear research

Excessive fear responses could underlie anxiety disorders, phobias, panic disorder, and posttraumatic stress disorder (PTSD), with a high (~ 7.3%) global prevalence of these disorders (Baxter et al, 2013). Distinguishing abnormal fear from anxiety is challenging since both phenomena have overlapping characteristics (Grillon, 2008). The Diagnostic and Statistical Manual of Mental Disorders defines anxiety as sustained anticipation of future threats, associated with muscle tension, vigilance, and avoidant behavior. Anxiety disorders are characterized by persistent anxiety (e.g., typically lasting 6 months or more) induced by stress and inappropriate threat evaluation (DSM-V, 2013). Trauma and stress-related disorders form a separate group of disorders with a history of traumatic or stressful events as a diagnostic criterion. These disorders can be characterized by anhedonia, aggressive, and dissociative symptoms among anxiety- and fear-based symptoms (Watson et al, 2005; Knight and Depue, 2019). Commonly, patients with PTSD have recurrent, involuntary, and intrusive recollections of the traumatic event (Liberzon and Abelson, 2016). Patients can experience intense psychological distress when exposed to settings that resemble an aspect of the traumatic event, suggesting dysfunction in contextual discrimination and fear extinction (DSM-V, 2013). Hypervigilance and threat reactivity in PTSD is considered as an anxiety-related symptom, while enhanced fear conditioning refers to impairments in fear regulation as well (Grillon, 2008; Liberzon and Abelson, 2016; Knight and Depue, 2019). Phobias and panic attacks also show mixed features of fear and anxiety. Panic attacks or confrontation with the object of the phobia

elicits excessive fear reactions, while the anticipation of future reoccurrences can maintain sustained anxiety (Grillon, 2008; Knight and Depue, 2019). In sum, all anxiety/fear-related disorders involve abnormal interpretation of actual and potential threats: (1) lack of differentiation between safe and danger signals; (2) low thresholds for threat perception, i.e. even ambiguous signals are interpreted as threatening, (3) biased attention for threat searching (4) persistent, extinction-resistant fear responses, i.e. rigid behavioral coping (Öhman et al, 2001; Liberzon and Abelson, 2016). Accordingly, better understanding of the neurobiological basis of these pathological fear characteristics are essential: it is still not well understood how pathological fear memories, fear generalization, or inadequate threat evaluation are formed and maintained.

1.3. Innate fear responses in laboratory animals

The majority of preclinical research use rodents to study the neuronal underpinnings of fear responses and to model human anxiety disorders. The ecologically most relevant threats to rodents are the risk of predation by other animals (Kats and Dill, 1998). To maximize the effectiveness of early detection of predators, prey animals can perceive several sensory stimuli as indicators of predator proximity: visual cues (e.g. looming stimulus), auditory signals from predator and conspecifics, and olfactory cues (Shelley and Blumstein, 2005; Takahashi et al, 2005; Yilmaz and Meister, 2013). Nocturnal animals (like laboratory rodents) dominantly rely on odors as predator signals (Kats and Dill, 1998). In experiments with rodents, predator-related odorants are commonly used to study innate fear responses. Such odorants are derived from predator urines (bobcat, cat, ferret urine, etc.), faces (fox), or fur (i.e. primarily from saliva on the fur) (Apfelbach et al, 2015). In natural settings, alarm pheromones from these products decay over time and thus might indicate the spatial and temporal proximity of predators (Hegab et al, 2014; Apfelbach et al, 2015). The laboratory use of these products could be problematic because of the latter phenomenon. Single, fear-inducing alarm compounds were isolated to allow standard, reproducible application of predator odors. The first and widely used single-molecule compound was 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox faeces (Vernet-Maury et al, 1984; Rosen et al, 2015). Recently, a synthetic analogue of TMT, 2-methyl-thiazoline (2MT) become commercially available with an even more robust fear-inducing potential in rodents (Tomoko Isosaka et al, 2015; Wang et al, 2018; Cruz et al, 2020). TMT and 2MT elicit freezing behavior; interestingly 2MT has a fear-potentiating

property even in microsmatic humans (Taylor et al, 2020). Single compound predator odorants enable us to increase threat intensity and study dose-dependent defensive responses. High doses of predator odor indicate the presence of imminent danger and elicit acute fear responses (Fanselow and Lester, 1988). When threat is more ambiguous (i.e. weak scent of predator odor is detectable), it might not reach the threshold to elicit freeze or flight responses, but animals display increased vigilance, exploratory, and risk assessment behavior (Lever et al, 2006; Andraka et al, 2021). Individuals exhibit various extent of defensive responses to ambiguous threats based on their pre-determined anxious traits, thus low intensity threats are suitable to study anxiety-like characteristics. The understanding of the neuronal mechanism of threat evaluation and action selection between active or passive defensive response can have a translational value in disorders characterized by exaggerated threat avoidance (anxiety disorders, phobias) and threat-reactivity (panic disorders, depression, etc.; DSM-V, 2013).

1.4. Associative fear learning- the Pavlovian fear conditioning

Immediate dangers elicit fast learning processes for successful avoidance of similar future threats. In rodents, the neural background of fear learning is widely studied by using Pavlovian fear conditioning (Maren, 2008; Quirk, 2017). Classical conditioning developed by Ivan Pavlov refers to a procedure, where a biologically relevant stimulus (the so-called unconditioned stimulus, US; e.g. food) is repeatedly paired with a neutral stimulus and subsequently animals showed a conditioned response to the neutral stimulus that is similar to the response for the US (e.g. salvation). During fear conditioning, an aversive stimulus, for example footshock or air-puff is paired with a neutral sensory stimulus (conditioned stimulus, CS; e.g. tone, light, or odor). The conditioned fear response is freezing, potentiation of startle, analgesia and hypertension (Perusini and Fanselow, 2015). Fear learning is a complex neurobiological process that can be subdivided to several phases. The initial phase of memory formation is called ‘acquisition’ when the first associations between two cues are integrated within a memory engram. After this encoding, the elongated phase of memory consolidation results in the strengthening of the engram. Consolidation involves several molecular processes underlying synaptic strengthening and synaptogenesis to strengthen connection between neuronal hubs integrated in fear engrams (Rodrigues et al, 2004; Maren, 2005; Pape and Pare, 2010; Johansen et al, 2011; Quirk, 2017). Memory consolidation spans from

minutes to several hours, and it is continued during sleep. During slow wave sleep the reactivation of recently encoded neuronal memory is crucial for its integration into long-term memory (Diekelmann and Born, 2010; Rasch and Born, 2013). Animals encode the details of the environment where the CS-US took place, called as the conditioned context. When the individual is presented again with the same context or other US-associated salient cue (i.e. the CS), the consolidated memory trace is retrieved and elicits the learned/conditioned fear response (e.g. freezing) in order to avoid the aversive outcome.

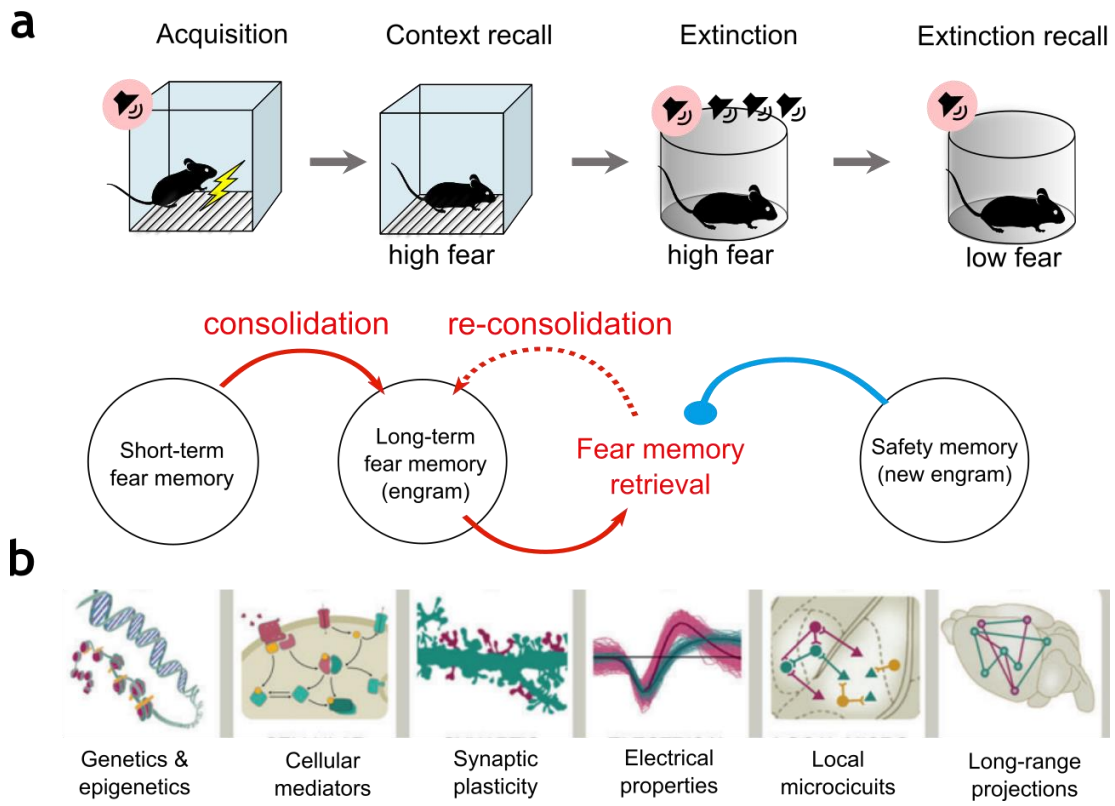

Figure 1. Schematics of Pavlovian fear conditioning procedure and fear memory formation. A*, The initial phase of fear learning is the acquisition, where the association between the aversive signal (unconditioned stimulus [US], e.g. footshock) is associated with the context and a paired neutral stimulus (e.g. an auditory tone, later becoming the conditioned stimulus [CS]). The early association is encoded in short-term memory and during the consolidation phase, it is strengthened to a fear memory engram. Fear recalls (re-exposure to the context or CS) re-activates the memory engrams, which elicit high fear (freezing). Based on the outcome of the re-exposure, the association could be reinforced (re-consolidation of the engram) or in the absence of the US fear memory can be extinguished over time (new extinction memory formed).* ***B****, The strengthening of memory engrams involves several biological processes on the genetic, cellular, synaptic levels, as well as altering micro-and mesoscale connectivity in the fear circuitry. (Images were adopted and modified from Flores et al, 2018; Maddox et al, 2019)*

To dissect the neurobiological underpinnings of contextual and cue-dependent fear recalls, CS recalls may be presented in a novel (i.e. safe) context, which represents more realistic, clinically relevant situation (i.e. patient exhibiting fear response in non-dangerous context). Importantly, recalling a memory triggers a new wave of consolidation (called re-consolidation), which makes memory traces and its neural bases (synaptic alterations, potentiation etc.) labile and sensitive for change (Sara, 2000; Dudai, 2004). Accordingly, CS-US associations may be strengthened or weakened based on reinforcement or lack of aversive stimuli, respectively. The extinction of fear memory involves a formation of a new 'safety' engram instead of erasing previous fear memory (**Figure 1**.(Maren et al, 2013)). In rodents, conditioned cues induced fear can be measured with muscle tension on electromyography, sound induced startle response, panic-like behavior (e.g. frequencies of escape jumps). The most convenient and applicable for different context, is measuring the time spent with freezing behavior (the absence of all non-respiratory movements) (Fanselow, 1980).

1.5. The role of bed nucleus of stria terminalis in fear and anxiety

The neuroanatomy of fear has been extensively studied in the past few decades. Early works established the indispensable role of amygdala nuclei in acute fear responses and fear learning (Klüver and Bucy, 1937; Bucy and Klüver, 1955). Subsequently, the function of specific amygdala nuclei in fear learning, expression, and extinction has been revealed (Janak and Tye, 2015; Tovote et al, 2015; Beyeler and Dabrowska, 2020). Sensory information from threatening cues are rapidly conveyed to the amygdala through brainstem and midbrain structures, as well as through thalamic inputs (**Figure 2.**). Higher-order cortical areas also process thalamic information and exert top-down regulation of subcortical defensive centers. The information within the amygdala flows from cortical to striatal subnuclei and the main output from the medial part of the central amygdala (CeM) innervates autonomic and motor centers of the hypothalamus, midbrain and brainstem to orchestrate defensive behavior (Davis and Whalen, 2001). This scheme of fear network is fine-tuned and controlled by several other nodes and modulatory system, which is not detailed here for reasons of space limitations. However, these modulatory networks of anxiety are more complex and harder to identify underlying neuronal substrates, since anxiety is a sustained state (i.e. constant monitoring, evaluation,

prediction involving several processes) and not initiated by direct threat confrontation. However, brain areas implicated in fear and anxiety show a great overlap and utilizing similar executory pathways (Tovote et al, 2015). While there is a consensus for the hyperactivation and impaired cortical regulation of amygdala in anxiety disorders, these characteristics can be also observed in other conditions such as depression and intermittent explosive disorder. Therefore, amygdala deficits are possibly not responsible solely for the development of anxiety disorders (Knight and Depue, 2019). Recent studies have drawn the attention to a previously overlooked forebrain structure in fear and anxiety research. The bed nucleus of stria terminalis (BNST) is anatomically and functionally coupled to the amygdala, forming a unit called the extended amygdala. More precisely, the extended amygdala refers to network forming by the BNST, central amygdala (CeA), the shell of the nucleus accumbens and the posterior limb of the anterior commissure (Alheid & Heimer, 1988). Within this network, the BNST is well positioned to process higher-order cognitive and motivational information and regulate downstream stress, neuroendocrine and motor executive centers (Jalabert et al, 2009; Torrisi et al, 2015; Daniel and Rainnie, 2016; Gorka et al, 2017). In line with this, recent evidence suggest that during threat anticipation the BNST shows sustained activity and involved in threat monitoring as well as mediating anxiety-like and stress responses (Shackman and Fox, 2016; Knight and Depue, 2019).

1.5.1. Threat-processing within the extended amygdala

Early models suggested that phasic and sustained fear were evolved on distinct neuronal levels and initiated by different modalities of threatening stimuli. Davis and Walker proposed a working hypothesis on distinct processing of acute and sustained fear based on their pioneer work with extended amygdala lesions. Lesion of the central amygdala impairs auditory fear conditioning and fear-potentiated startle, while BNST lesion only disrupts contextual conditioned fear and light enhanced startle. These observations led researchers to the conclusion that the BNST process diffuse or long-duration threats and may convert acute stress into long-term anxiety-like behavior (Davis et al, 2010; Dabrowska et al, 2013a; Daniel and Rainnie, 2016). According to the Davis-Walker model, information about potential learned threats flows from the basolateral amygdala (BLA) to the lateral division of the central amygdala (CeL), which in turn initiates an acute fear response by disinhibiting autonomous and motor outputs from the

CeM. After this acute response, the CeL activates the BNST, which sustain fear reaction over a longer period and sends feedback inhibition to the CeA, terminating the acute fear response. When rodents are confronted with a predator, the activation of the CeA mediates the circa-strike defense response (considered as a fear reaction), on the other hand, indicators of a potential predator threat elicit sustained cautious behavior (risk assessment, freezing), which is rather similar to anxiety response (Fanselow, 1986; Blanchard et al, 1993). The experimental affirmation of this temporal dynamic between the CeA and BNST, and the consequent transition between fear and anxiety is still elusive. Although the hypothesis of Davis and Walker remained highly influential, recent research challenged this strict dichotomy between CeA and BNST-dependent functions (Shackman and Fox, 2016).

Emerging evidence shows that the CeA regulates sustained, anxiety-like responses as well as contextual fear (Kolber et al, 2008; Pitts et al, 2009; Asok et al, 2017; Pomrenze et al, 2019). Moreover, different circuits in the CeA are recruited by remote or imminent threats (Andraka et al, 2021). On the other hand, BNST manipulations often result in mixed effects on anxiety test, several BNST inhibition experiments failed to affect anxiety-like behavior (Kim et al, 2013; Mazzone et al, 2018). The latter findings suggest that BNST may regulate prior stress or fear potentiated anxiety-like behavior rather than anxiety-trait in naïve animals. Some preclinical studies also provided evidence for BNST activation to discrete conditioned cues, but BNST manipulations resulted in mixed effects on auditory fear conditioning (Jennings et al, 2013; Daldrup et al, 2016; Marcinkiewcz et al, 2016; Moaddab and Dabrowska, 2017; Glover et al, 2020). Gungor and Pare suggested that BNST may not be indispensable for phasic fear responses, but BNST can play a modulatory role in acute fear (Gungor and Pare, 2016). Recent research nuanced the picture, suggesting that stimulus unpredictability and ambiguity could be a determining factor, which recruits the BNST in conditioned fear recalls (Goode and Maren, 2017; Goode et al, 2019, 2020). Confusing results might origin from different timing of BNST manipulation in studies (i.e. acquisition vs recalls) or the lack of cell-type specific investigations, thus the temporal regulation of BNST-dependent fear processes and their exact microcircuits are still not known.

Although the functional imaging of the human BNST is challenging because of its small size (approximately 190 mm^3 – the size of sunflower seed in humans), fMRI studies in the past few years provided useful insights to better understand BNST-related functions (Avery et al, 2016; Shackman and Fox, 2016). These studies can follow the task- and

emotional state-related temporal changes of BNST activity and connectivity with better controllability of threat ambiguity and anticipation. Overall, the majority of studies reported sustained BNST activity during monitoring of prospective threats, evaluating threat proximity both spatially (e.g. distance of an approaching tarantula; (Mobbs et al, 2010)) and temporally (e.g. during anticipation of a future aversive stimulus (Somerville et al, 2010; Klumpers et al, 2017). While the amygdala shows transient activation during confrontation with explicit threats or at the onset of threat anticipation, the BNST maintain elevated activity during threat anticipation, especially if threats are ambiguous (Klumpers et al, 2017; Naaz et al, 2019). Since the main driving forces of human anxiety are future-oriented hypothetical threats, revealing the involvement of BNST in threat anticipation could provide useful insights to understand and treat anxiety disorders. Clinical observations on patients with phobias and anxiety disorders also confirm that hypervigilant threat processing or dealing with uncertain threats is associated with elevated BNST activity in these conditions (Mobbs et al, 2010; Somerville et al, 2010; Yassa et al, 2012; Buff et al, 2017). Importantly, threat evaluation and recruitment of the BNST is probably regulated by prefrontal regions, as ambiguous threats can increase the functional connectivity between the PFC and BNST, while voluntary suppressing feelings of fear or anxiety both significantly downregulate the activity of the BLA and the BNST (Knight and Depue, 2019).

Several studies on the CeA and the BNST showed that these regions are also implicated in multiple functions such as the stress response, reward-seeking, addiction, compulsivity, and sexual behavior (Daniel and Rainnie, 2016; Shackman and Fox, 2016; Ch ’ng et al, 2018; Fadok et al, 2018; Li, 2019). This functional diversity supposes a potential role in organizing and selecting adequate behavior according to environmental challenges. Indeed, the CeA has been reported to manage the hierarchy between conflicting drives of innate and learned fear responses, as well as the choice between active and passive fear responses i.e. freeze or flight (Isosaka et al, 2015; Fadok et al, 2017). The BNST might be involved in threat evaluation and modulate behavioral (dis)engagement accordingly along with the amygdala (Paré et al, 2017). Disruption of these circuits can cause a mismatch between the environmental challenges and the behavioral response, which is a trademark of anxiety disorders. The similarities and slight differences between the CeA and BNST are still require a unified theory of their function in orchestrating defensive behavior. The great neuronal heterogeneity within each

structure demands cell-type specific manipulations of specific extended amygdala circuits during different environmental challenges.

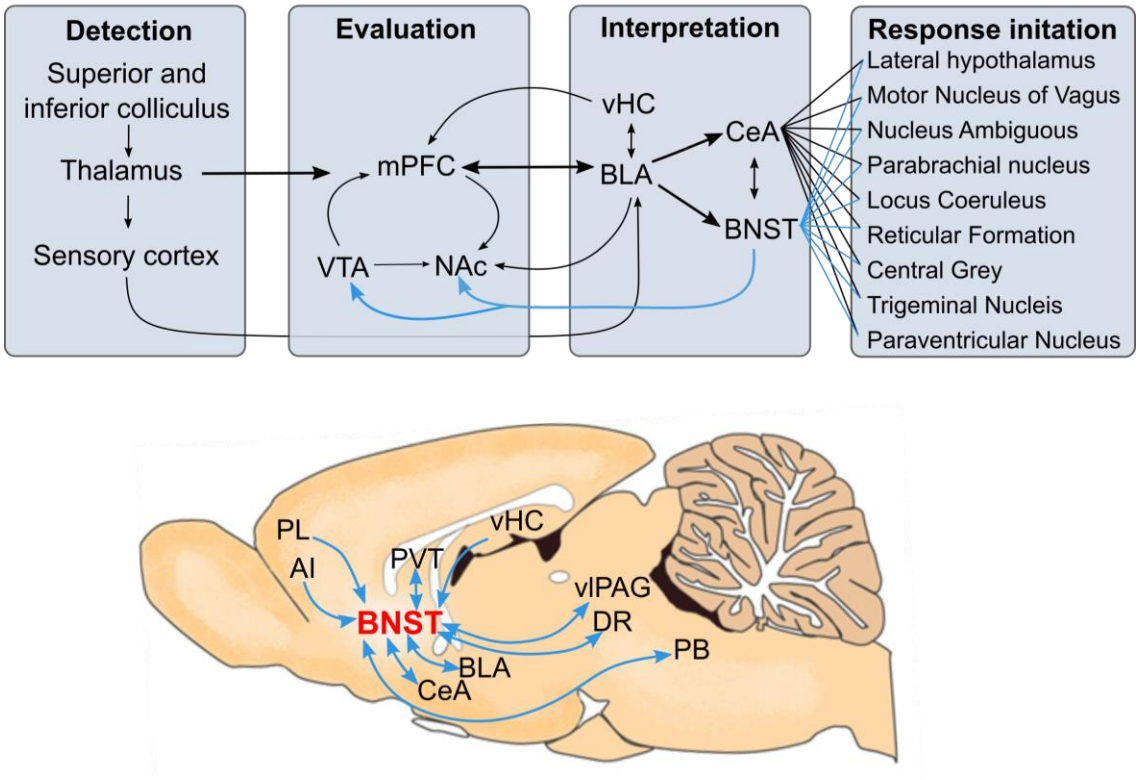

Figure 2. Circuit organization of threat processing and fear responses. *Upper panel: four-step model of threat detection, evaluation, interpretation and response initiation on neuronal level (adopted from Calhoon and Tye, 2015). Lower panel: connections of the BNST with brain regions involved in fear responses.*

1.5.2. Cytoarchitecture and neurochemical heterogeneity of the BNST

The BNST is located ventral to the septum and lateral ventricle, lying between the ventral striatum and thalamus, surrounding the anterior commissure. Rodent BNST is a heterogeneous structure, certain classifications distinguish 16 BNST subnuclei (Larriva-Sahd, 2006). These subnuclei can be grouped into anterior/posterior or medial/lateral divisions based on different classifications. Here, I used the anterior/posterior terminology adapted from the work of Swanson and Dong (Dong et al, 2001a). The anterior BNST surrounds the decussation of the anterior commissure and has been classified into the anteromedial (amBNST), antero/dorsolateral (alBNST) and anteroventral (avBNST) nuclei. The alBNST is further divided into the juxtacapsular, oval and rhomboid nuclei. The posterior division is separated from the anterior by the fiberbunds of the stria terminalis and consists of 3 subnuclei, the principal, transverse and interfascicular nuclei (Ju and Swanson, 1989; Moga et al, 1989). In humans, the BNST is

divided into medial, central, lateral and lateroventral regions based on distinct immunolabelling profiles.

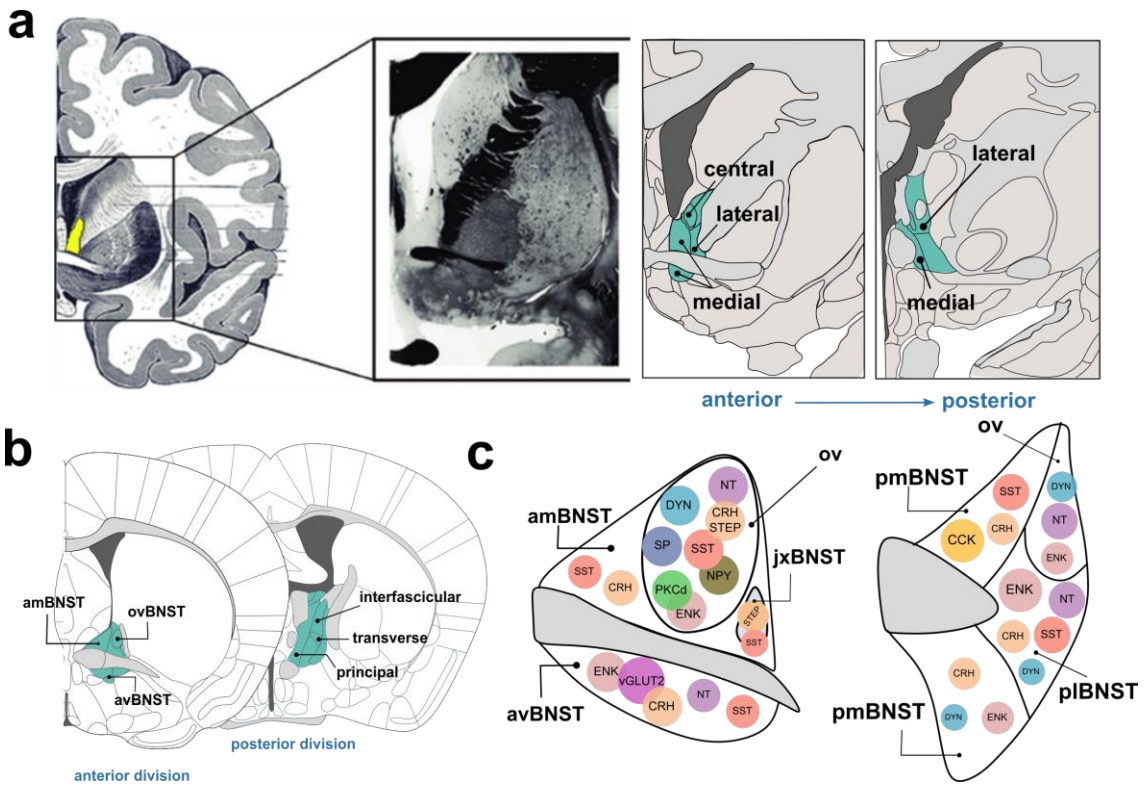

Figure 3. Anatomy of the BNST in humans and rodents. *a, Localization and divisions of the human BNST. b, Major subnuclei of the mouse BNST in the anterior and posterior division. c, Neurochemical heterogeinity of the rat BNST. Overlapping circles indicate neuropeptides that can be co-expressed. Images were adopted and modified from Avery et al 2016, Beyeler & Dabrowska, 2020, Ortiz-Juza et al, 2021 and the Allen Brain mouse reference atlas (https://mouse.brain-map.org/static/atlas). Abbrevations: amBNST-anteromedial BNST, avBNST-anteroventral BNST, jxBNST: juxtacapsular BNST, ov- oval nucleus,CCK-cholecystokinin, CRH- corticotropin-releasing hormon, DYN-dynorphin, ENK-enkephalin, NPY-neuropeptid Y, NT- neurotensin, PKCδ- protein kinase delta, pmBNST-posteromedial BNST, plBNST-posterolateral BNST, SP-substance P, SST-somatostatin, STEP-striatal enriched protein, vGLUT2- vesicular glutamate transporter 2.*

The neurochemical composition of the BNST shows remarkable similarities to the CeA, since they both share a common striatal origin. The majority of BNST neurons are GABA-ergic, with a small population of glutamatergic neurons in the avBNST (Nguyen et al, 2016). Besides fast major amino acid neurotransmitters, BNST neurons co-express various neuropeptides (**Figure 3.**): cholecystokinin (CCK), corticotropin-releasing factor (CRH), dynorphin (DYN), enkephalin (ENK), neuropeptide Y (NPY), neurotensin (NT),

protein kinase C delta (PKCδ), somatostatin (SST), substance P (SP), etc. (Hammack et al, 2015; Beyeler and Dabrowska, 2020; Ortiz-Juza et al, 2021). In rats, the majority of these peptidergic cells show the highest density in the ovBNST, while in mice neuropeptide expression disregards the boundaries of BNST subnuclei (Ju and Swanson, 1989; Beyeler and Dabrowska, 2020). Interestingly, in humans, the central nucleus of the BNST is characterized by SST expression, while the lateral division exhibits the most heterogeneity similar to rodents (SST, NT, NPY, ENK, CCK, etc.(Lesur et al, 1989)). In rodents, most of these cell types can project locally or outside the BNST, making an incredibly complex BNST microcircuit. The functional and anatomical examination of intra-BNST connections is difficult because of the small size and proximity of the subnuclei. Previous data demonstrated that the ovBNST sends strong projections to the amBNST and avBNST, suggesting a gating function of BNST outputs (Gungor and Pare, 2016). However, the extra-BNST connectivity of genetically distinct neuronal populations is still needs to be clarified.

1.6. CRH and SST neurons in the extended amygdala

In this paragraph, I introduce the CRH and SST neurons of the extended amygdala in more detail, since our experiments focused on these two major cell population. BNST is one of the major source of extra-hypothalamic CRH, which target hypothalamic, midbrain and brainstem nuclei (Dabrowska et al, 2013a; Pomrenze et al, 2015; Dabrowska et al, 2016). In rodents, projection mapping of CRH neurons of BNST ($BNST^{CRH}$) revealed that they share highly overlapping projection areas with CeA^{CRH} neurons. $BNST^{CRH}$ neurons also have intra-BNST connections, preferentially targeting non-CRH neurons (Ch ’ng et al, 2018). However, CRH is also released from the CeL to the ovBNST, which projection critically contributes to stress and anxiety-like responses (Erb et al, 2001; Asok et al, 2017; Pomrenze et al, 2019). CRH neurons in the rat BNST are clustered to the oval nucleus, while CRH expression in mouse shows a more scattered distribution throughout the antero-posterior and dorso-ventral axis of the BNST (Dabrowska et al, 2013a; Nguyen et al, 2016). In contrast to hypothalamic CRH neurons, the majority of $BNST^{CRH}$ cells are GABAergic, while some CRH neurons in the avBNST are co-expressed with vesicular glutamate transport-2 (vGLUT2) (Dabrowska et al, 2013a; Pomrenze et al, 2015). Functional investigation of $BNST^{CRH}$ neurons is still elusive, most of the studies investigated CRH receptor signalling within the BNST.

CRHR1 signaling has been implicated in stress response, light enhanced startle as well as anxiety-like behavior (Dabrowska et al, 2013a). In contrast, CRHR2-expressing neurons in the posterior BNST exert an anxiolytic effect (Henckens et al, 2017). Similar to the CeA, $BNST^{CRH}$ neurons are recruited by aversive stimuli and also implicated in stress-mediated substance abuse (Marcinkiewcz et al, 2016; Vranjkovic et al, 2017; Giardino et al, 2018). Based on their overlapping projections, a functional similarity between BNST and CeL^{CRH} neurons is presumed. Extensive research on CeL^{CRH} neurons provided evidence on the role of these neurons in fear extinction and flight responses, indicating a complementary function with CeL SST neurons, i.e. promoting active-passive defensive responses, respectively (Fadok et al, 2018; Li, 2019). Recent findings indicate that these cells may also regulate learning of weak threats (Sanford et al, 2017). Similarly, $5\text{-}HT_{2C}$ receptor-expressing $BNST^{CRH}$ neurons seem to mediate serotonin-induced enhancement of cued fear, but the general role of $BNST^{CRH}$ neurons in learned and innate fear responses has not been investigated in detail yet (Ravinder et al, 2013; Marcinkiewcz et al, 2016; Pelrine et al, 2016).

Another major type of GABAergic neurons in the extended amygdala express somatostatin (SST), which form bidirectional inhibitory connections with CRH neurons and they presumably mediate distinct behavioral responses (Fadok et al, 2017; Hartley et al, 2019). CeL^{SST} neurons are critical regulators of fear learning and repress active avoidance (Li et al, 2013; Penzo et al, 2014; Yu et al, 2016). Optogenetic stimulation of SST neurons is sufficient to induce freezing without threatening stimulus (Li et al, 2013). Furthermore, enhancing the activity of CeL^{SST} neurons increase anxiety through projections to the extended amygdala, including the BNST (Ahrens et al, 2018; Sun et al, 2020). The function of $BNST^{SST}$ neurons has not been well-characterized yet and selective modulation of these neurons is still lacking besides a recent paper showing an anxiolytic effect of $BNST^{SST}$-NAc projections (Xiao et al, 2020). The involvement of $BNST^{SST}$ neurons in fear responses and the entire input-output connectivity of these cells have not been described yet.

1.7. Chemogenetic tools to study the neurobiological mechanism of fear

In the last decades, a variety of techniques were developed to genetically target and reversibly modulate neurochemically-identified cells. Mapping the connections and

functions of these circuits have significantly improved the understanding of the neuronal mechanisms of fear responses. Chemogenetics is an approach when macromolecule proteins (e.g. ionotropic or metabotropic receptors) are engineered to interact with an otherwise biologically inactive exogenous chemical ligand (Michaelides and Hurd, 2016). The second generation of chemogenetic strategies, the so-called DREADDs (designer receptors exclusively activated by designer drugs) was developed by Roth and his colleagues (Armbruster et al, 2007). One of the most widely used DREADD is hM3Dq, which was produced by directed molecular evolution of the human M3 muscarinic receptor (hM3) in yeast (Armbruster et al, 2007). hM3Dq receptor activation triggers G_q signalling cascade (**Figure 4.**), which results in increased intracellular Ca^{2+} levels and depolarization (Urban and Roth, 2015; Aldrin-Kirk and Björklund, 2019). In 2007, Armbruster and colleagues also created a G_i-targeting DREADD from the human M4 muscarinic receptor, known as hM4Di receptors. Enhanced G_i signalling facilitates K^+ influx through protein inwardly rectifying potassium channels, consequently hyperpolarize neurons, and inhibits presynaptic neurotransmitter release. The efficacy of hM3Dq and hM4Di modulated neuronal activity was validated both in vitro and in vivo (Urban and Roth, 2015).

hM3Dq and hM4Di receptors can be activated by the pharmacologically inert clozapine metabolite CNO (clozapine-n-oxide) by means of intraperitoneal, intracranial or oral administration (Aldrin-Kirk and Björklund, 2019). CNO has a nanomolar potency for hM3Dq and hM4Di receptors, in contrast to endogenous M3 and M4 receptors. Vice versa, hM3Dq, and hM4Di are insensitive to acetylcholine and show low constitutive muscarinic receptor activity. Although it has been demonstrated that in the brain, CNO can be metabolized to clozapine, this turnover could be avoided when low doses of CNO is applied (i.e. < 50 mg/kg) (Gomez et al, 2017; Aldrin-Kirk and Björklund, 2019). Additionally, control animals (expressing inactive DREADD receptors) should be also treated with CNO to unmask possible clozapine-related effects. Notably, chemogenetics are suited for elongated modulation of neuronal activity (from minutes to hours) since the temporal dynamics of GPCR receptors are much slower than fast ion-channel mediated responses, with additional low clearance of CNO enabling slow-dynamic modulation of neuronal activity for approximately 10 hours (Urban and Roth, 2015). Latter was optimal for us to manipulate fear responses on an elongated timescale with minimal invasive disruptions during behavioral testing.

DREADDs can be expressed in specific cell populations via two strategies: (1) by the use of transgenic mice expressing DREADDs under certain promoters, or (2) via local infusions of viral vectors carrying DREADD transgenes (**Figure 3.**). For the latter method, most of the studies using the flip-excision (FLEX)-switch approach [also known as double-inverted open reading frame (DIO) viral vectors]. In this, researchers inject viral vectors [usually adeno-associated viruses (AAV)] carrying the inverted transgene of DREADDs inserted between two pairs of heterotypic, antiparallel loxP-type recombination sites. In mice with cell type–specific Cre recombinase activity, this transgene undergoes an inversion of the coding sequence followed by the excision of two loxP sites and results in a cell type–specific expression of DREADDs (Michaelides and Hurd, 2016).

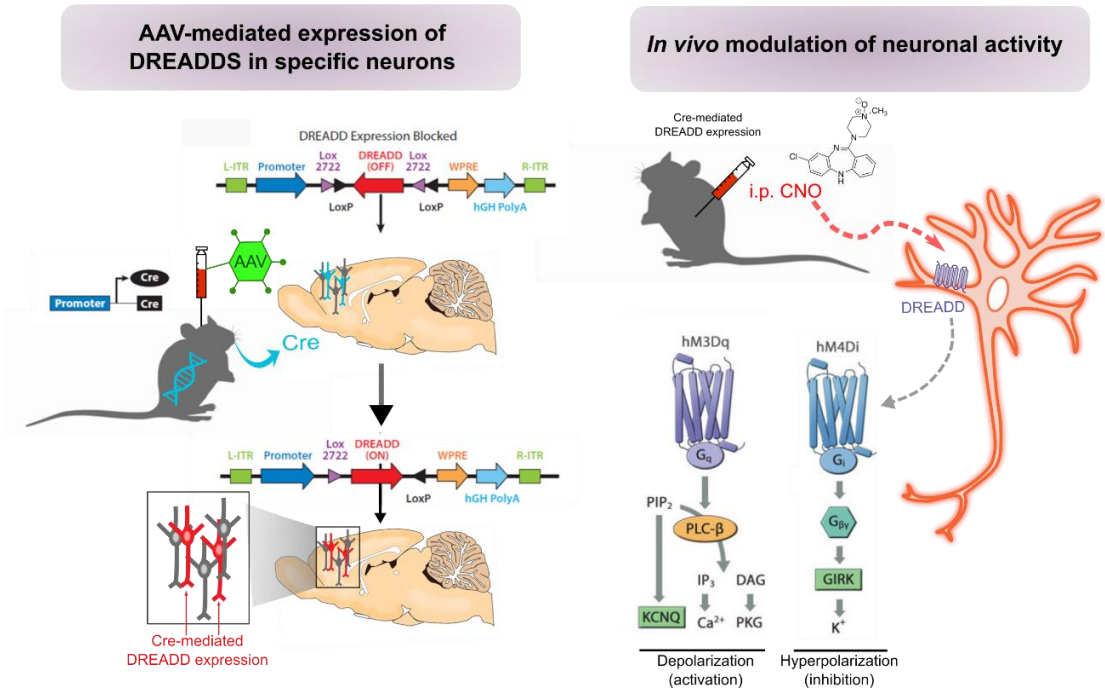

Figure 4. Overview of chemogenetic strategy to manipulate genetically identified neurons. *Figures of DREADD receptors were adopted from* Michaelides and Hurd, 2016.

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2. Objectives

In our experiments, we aimed to clarify how learned and innate fear responses are modulated by the bed nucleus of stria terminalis (BNST) with a special focus on somatostatin and corticotropin-releasing hormone expressing neurons based on their specific and opposing roles in central amygdala functions.
Therefore, we applied chemogenetic modulation of genetically defined BNST cell populations in two sets of experiments to answer the following questions:

I. <u>The involvement of BNST in Pavlovian fear learning:</u>

1. Which phases of the conditioned fear are modulated by the BNST, (i.e. acquisition, consolidation, recall)?
2. Which aspects of fear learning or recall are modulated by BNST circuits (i.e. contextual, CS-related, generalization to safe context, extinction)?
3. Which specific cell-types ($BNST^{SST}$ and $BNST^{CRH}$ neurons) mediate these functions?
4. Finally, which downstream brain regions are modulated by BNST circuits?

II. <u>The role of BNST in innate fear responses:</u>

1. How threat certainty determines recruitment of BNST circuits in defensive responses? Does predator odor intensity as indicator of danger proximity determines the impact of BNST?
2. How do $BNST^{SST}$ neurons modulate low vs. high predator odor-induced fear responses?
3. How do $BNST^{CRH}$ neurons modulate low vs. high predator odor-induced fear responses?

3. Results

3.1. The involvement of BNST in Pavlovian fear learning

3.1.1. BNST is recruited during fear acquisition, but not during cued fear recall

First, we investigated the engagement of BNST in different phases of conditioned fear response by mapping c-Fos expression in BNST subregions during fear learning (cued fear acquisition) and cue-dependent fear recall in adult male C57Bl/6J mice. In the first experiment, fear-conditioned mice (n=10) underwent Pavlovian auditory fear conditioning, when seven 30 s pure tones (7 kHz) were presented and co-terminated with footshocks (1s duration, 0.7 mA). Control animals (n=10) were exposed to the same auditory cues in the test chamber without footshocks. Mice were sacrificed for c-Fos staining 90 min after fear conditioning to measure c-Fos expression in the BNST. As expected, footshock induced significant (gradually increasing) freezing response compared to controls (**Figure 5A**, $F_{1,18}$=153.944, p<0.001). Fear conditioning activated all investigated BNST subregions, indicated by significantly higher c-Fos expression in shocked mice (**Figure 5C and F**; all regions: $F_{1,18}$>5.55, p=0.029). In a second experiment, mice underwent fear conditioning with a similar experimental design and a consequent cue-dependent fear recall 2 days later in an altered context ('safe context B'). Again, shocked-mice exhibited increasing freezing levels during acquisition and during exposure to context B and auditory CS (**Figure 5B**, $F_{1,19}$=9.777 and p<0.0001). Despite that cue recall elicited high levels of freezing in shocked mice, the experimental groups (control: n=10, shocked: n=11) showed no significant difference in c-Fos expression in the BNST (**Figure 5G**, all regions: $F_{1,19}$>1.008, p>0.327). These data suggest that BNST is recruited during fear acquisition, but not when conditioned fear is recalled. Next, we aimed to test if enhanced BNST activity contributes to enhanced fear memory formation based on observation of BNST hyperactivity in fear-related and anxiety disorders.

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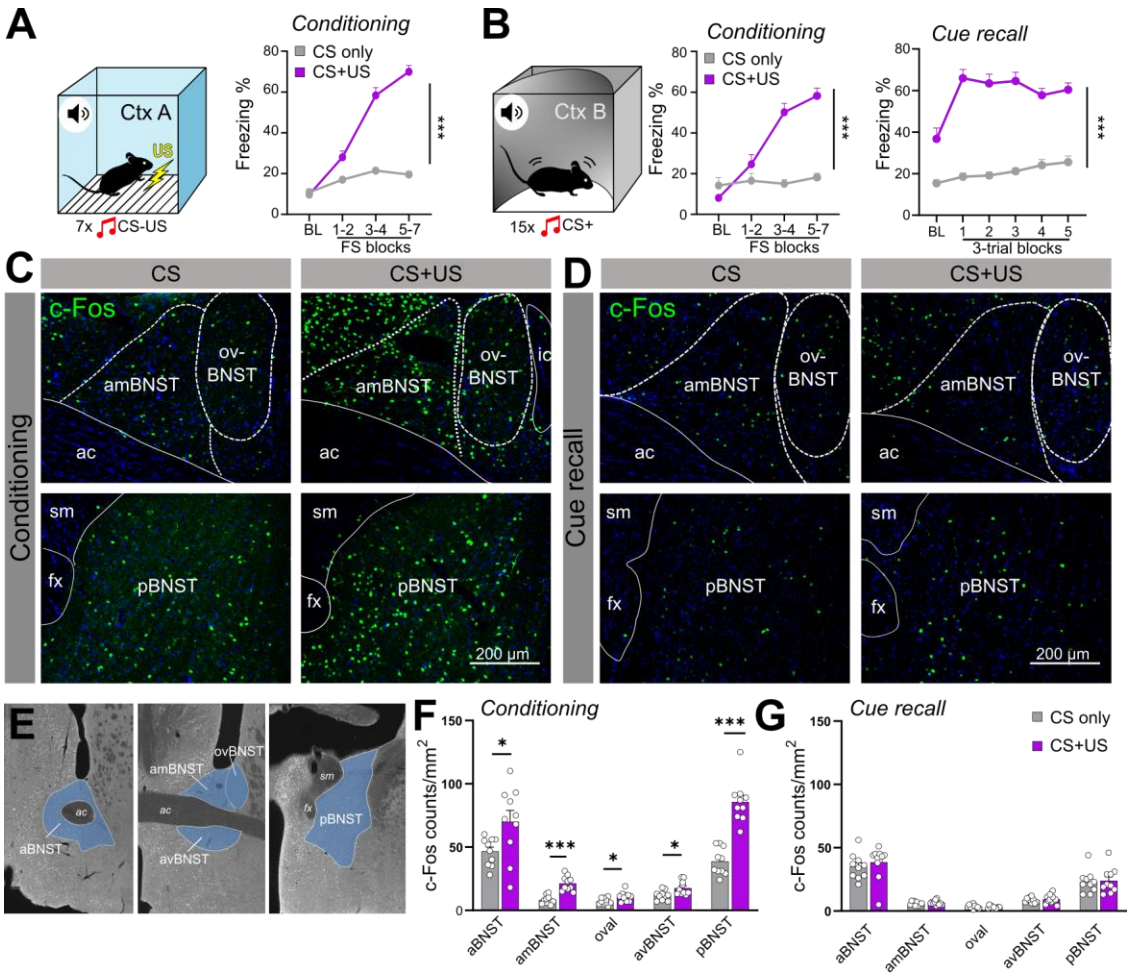

Figure 5. c-Fos expression in the BNST following fear conditioning and auditory fear recall. *A, Freezing levels during auditory fear acquisition in non-shocked control (CS-exposed) and shocked (CS+US) mice. B, Freezing levels during acquisition and CS-dependent fear recall test. C, Representative photomicrographs of fear conditioning-induced c-Fos expression in the BNST. D, Representative photomicrographs of c-Fos expression in the BNST during auditory fear recall. E, Illustration of BNST subregions analysed for c-Fos expression. F and G, Average c-Fos counts in the BNST following conditioning and CS recall. On freezing time curves, each major tick depicts two to three footshock (FS) blocks in case of conditioning, and a 180-s block, starting with a 150-s pretone baseline period (BL) in case of recall test. *p<0.05, **p<0.01, ***p<0.001.*

3.1.2. Chemogenetically enhanced BNST activity strengthens cue-dependent fear memory formation without directly affecting fear memory recalls

To enhance BNST activity during fear acquisition, we expressed stimulatory hM3Dq DREADDs in the BNST of adult male vgat-ires-cre mice to target the major neuronal population, i.e. GABAergic BNST neurons ($BNST^{vGAT}$, **Figure 6A**). Control animals were injected with viral vector carrying only mCherry fluorophore protein without active hM3Dq receptor. To confirm the depolarizing effect of hM3Dq on neuronal activity, we performed whole-cell patch clamp recordings from acute brain slices (n=4/groups). hM3Dq expressing $BNST^{vGAT}$ neurons exhibited significant resting

membrane potential depolarization after CNO administration compared to baseline recordings (4.18 ± 0.60 mV vs. -0.54 ± 1.11 mV in controls; $F_{1,7}$=17.319, p=0.004; t=9.286, p<0.001) and increased firing rate (30 pA pulse: from 0.9 ± 0.23 action potentials (APs) to 4.4 ± 0.37 APs, $F_{1,9}$=169.615, p<0.001; 60 pA pulse: from 3.2 ± 0.29 APs to 16.5 ± 0.80 APs, $F_{1,9}$=214.846, p<0.001) (**Figure 6B-C**). *In vivo* intraperitoneal administration of CNO (1 mg/kg) also significantly increased c-Fos expression in hM3Dq expressing $BNST^{Vgat}$ neurons under undisturbed conditions (**Figure 6D**: $F_{1,4}$=145.377, p<0.001).

Next, we chemogenetically activated $BNST^{vGAT}$ neurons during fear acquisition to study the outcome of BNST hyperactivity on fear learning (**Figure 6E**). Experimental groups (control: n=8, hM3Dq: n=9) showed similar levels of freezing during acquisition (**Figure 6F**, $F_{1,15}$=0.041, p=0.841), suggesting a lack of effect of BNST activity on acute freezing response and pain perception. Whereas BNST stimulation had no impact on contextual fear recall ($F_{1,15}$=0.025, p=0.875, **Figure 6G**), it increased freezing levels during CS-dependent fear recall in an altered context (context B, ($F_{1,15}$=6.774, p=0.019), which effect diminished by the next extinction session 1 day later (i.e. Extinction recall: $F_{1,15}$=1.275, p=0.276; **Figure 6H**). Experimental groups showed no significant difference in freezing during pre-CS period in context B, indicating no effect on $BNST^{vGAT}$ stimulation on contextual fear generalization (; $F_{1,15}$=0.961, p=0.342, respectively; **Figure 6G and H**).

Since chemogenetic stimulation can exert effect on neuronal activity for several hours, these effects may be explained by facilitating memory consolidation and not acquisition itself. To dissect the temporal dynamics of our effect, in the next experiment CNO was injected immediately after fear conditioning to activate $BNST^{vGAT}$ neurons specifically during fear memory consolidation (**Figure 6I**). Consolidation-specific stimulation replicated the enhancement of CS-induced fear recall (**Figure 6K**: $F_{1,15}$=5.320, p=0.035), with no change in contextual recall (**Figure 6J**: $F_{1,17}$=1.560, p=0.228). Importantly, fear acquisition was similar between groups as indicated by freezing (**Figure 6I**: $F_{1,17}$=0.311, p=0.584, n=11-9/groups).

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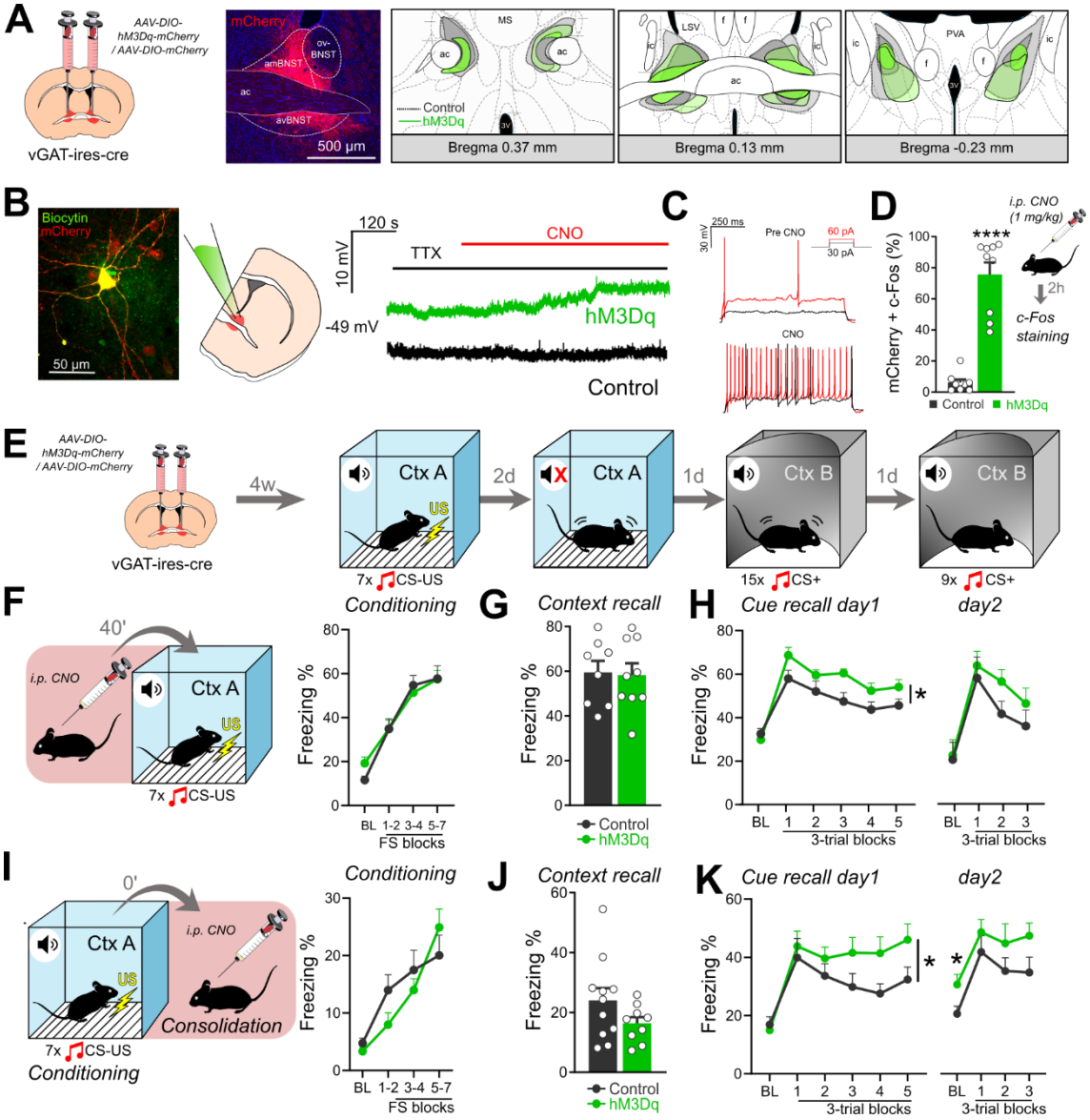

Figure 6. Stimulation of BNST^{vGAT} neurons facilitates CS-dependent fear learning. *A, Schematics of cre-inducible AAV-hM3Dq-mCherry injections to the BNST of vgat-cre mice and representative photomicrograph of mCherry expression. Right panels show minimum (filled) and maximum (outlined) extensions of mCherry expression. B-C, Photomicrograph of a biocytin-filled mCherry+ neuron and representative traces of whole-cell patch clamp recordings from BNST slices. D, Mean percentage of c-Fos expressing mCherry+ BNST^{vGAT} neurons after in vivo CNO administration. E, Experimental schematics of Pavlovian auditory fear conditioning tests. F, Schematics of CNO administration before fear conditioning and freezing levels during acquisition. G, Freezing levels during contextual fear recall. H, Baseline (pre-tone) freezing in the context B and CS-induced freezing behavior during cue recall tests. I, Schematics of CNO administration after fear conditioning (i.e. during memory consolidation) and freezing rates during acquisition. J, Time spent with freezing in the contextual recall test. K, Baseline (pre-tone) freezing in the context B and CS-induced freezing behavior during cue recall tests. *p<0.05*

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Finally, we tested if BNST^{vGAT} stimulation could acutely modulate CS recall or CS generalization (**Figure 7A**). We used differential auditory fear conditioning, where two alternating auditory cues were presented, one was co-terminated with footshock (CS+) and the other was not coupled with the US and thus representing a safety cue (CS-). For auditory cues 30 s duration 7 kHz tones or white noise were used, randomly assigned as CS- or CS+ between mice (control: n=8, hM3Dq: n=12). Fear acquisition was similar between groups ($F_{1,19}$=0.001, p=0.987), and both groups could similarly well differentiate between CS+ and CS- indicated by freezing levels during a brief recall test (CS-/CS+: $F_{1,18}$=0.451, p=0.510) (**Figure 7B-C**). BNST^{vGAT} stimulation during the subsequent CS+/CS- recall test did not affected freezing level (**Figure 7D**: CS+-induced recalls: $F_{1,19}$=0.303, p=0.588; CS-/CS+: $F_{1,19}$=0.043, p=0.837). These data and our results from c-Fos activity during fear recall (Fig.) suggest that BNST is not recruited during fear recalls.

Taken together, our findings suggested that BNST is recruited during fear acquisition and BNST hyperactivity can strengthen CS-dependent fear memory consolidation specifically.

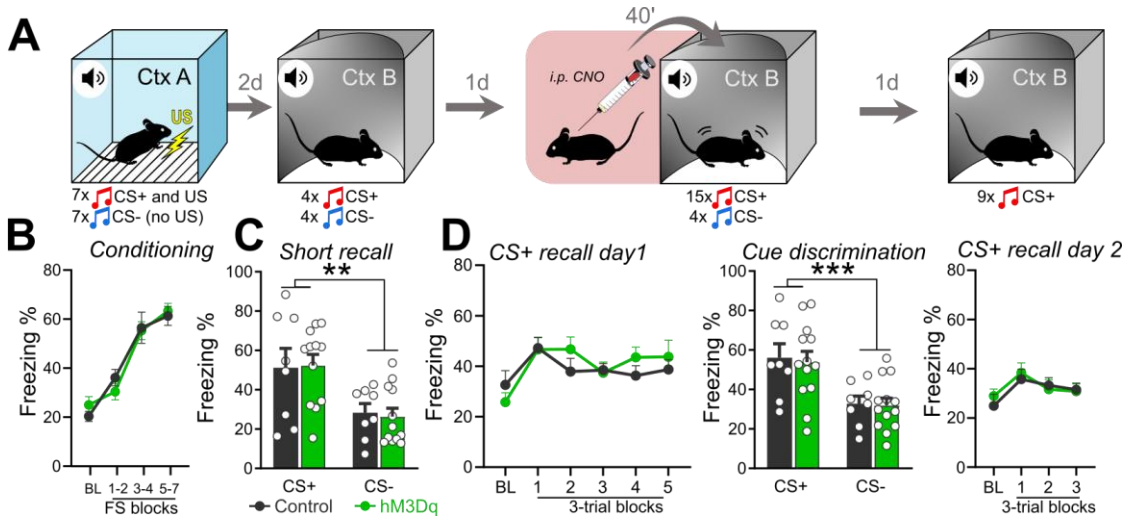

Figure 7. Chemogenetic stimulation of BNST^{vGAT} neurons did not affect cued fear expression and cue discrimination.

*A, Experimental design for differential auditory fear conditioning with chemogenetic activation of the BNST during cued fear recall test. B, Freezing levels during fear conditioning. C, Freezing levels to conditioned (CS+) and safety (CS-) cues during a brief cue-recall test. D, Freezing time curve to CS+ following chemogenetic activation of BNST^{vGAT} neurons. E, Freezing to the first 4 CS+ and CS- following CNO administration. F, Time curve showing freezing levels in the pre-tone period and after CS+ presentations during the next cue recall day (after CNO washout). **p<0.01, ***p<0.001.*

3.1.3. c-Fos mapping of BNST downstream regions during chemogenetically enhanced fear consolidation

Next, we mapped c-Fos expression in BNST target areas 6 hours after fear conditioning in order to identify key regions, which could potentially mediate our behavioral effects, and to describe how BNST-coupled fear network may be altered by BNST stimulation (**Figure 8A**). In the BNST, hM3Dq-expressing neurons (from n=10 mice) showed markedly enhanced c-Fos expression even 6 hours after fear acquisition (with additional chemogenetic activation) compered to control mice (86.36% vs. 6.09%, n=6, **Figure 8C-D**; $F_{1,8}$=1117.353, p<0.001). We selected major BNST projection areas based on previous reports and our observation of axonal mCherry immunoreactivity (**Figure 8B**, (Dong et al, 2001b; Dong and Swanson, 2004; Kudo et al, 2012; Dabrowska et al, 2013a). Accordingly, c-Fos-ir neurons were counted in the nucleus accumbens (NAc) shell, dorsal midline thalamus (DMT), central amygdala (CeA, medial nucleus-CeM), lateral hypothalamus (LH), paraventricular nucleus of the hypothalamus (PVN), substantia nigra pars compacta (SNc), ventral tegmental area (VTA), dorsal raphe (DR), ventrolateral periaqueductal gray (vlPAG).

Among these regions, DMT, VTA (interfascicular part-ifVTA), and vlPAG exhibited increased c-Fos expression (**Figure 8C and F**, $F_{1,13}$=7.516, p=0.016; $F_{1,13}$=8.191, p=0.013; $F_{1,14}$=18.919, p<0.001, respectively), whereas PVN exhibited reduced c-Fos expression in hM3Dq mice (**Figure 8C and E**, $F_{1,14}$=5.208, p=0.038). Additionally, we observed a trend for increased activity in the DR ($F_{1,14}$=4.013, p=0.064).

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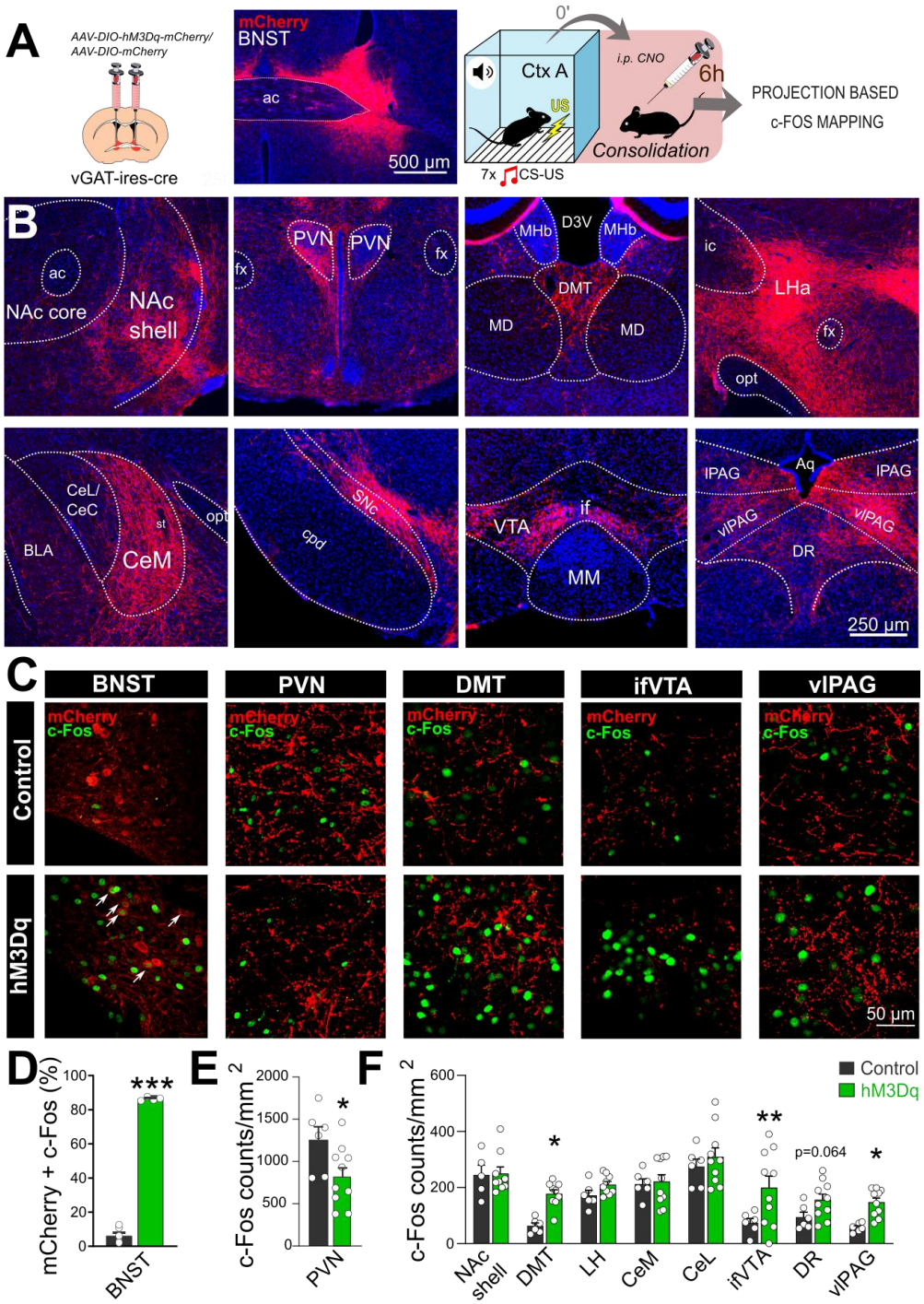

Figure 8. Consolidation-specific BNST stimulation modulates the activity of several brain regions innervated by the BNST. *A, Representative photomicrograph of hM3Dq-mCherry expression in BNST^{vGAT} neurons (left) and illustration of experimental design (right). B, Representative wide-field fluorescence photomicrographs depicting major projection areas of BNST^{vGAT} neurons. C, Representative single-plane confocal photomicrographs showing c-Fos expression during consolidation in the BNST and downstream regions. White arrows indicate c-Fos+ activated hM3Dq-expressing BNST^{vGAT} neurons. D, Percentage of c-Fos+ mCherry neurons in the BNST. E-F) Average c-Fos counts during memory consolidation in the downstream regions of BNST^{vGAT} neurons.*

3.1.4. Distribution and efferent connections of BNST^{SST} and BNST^{CRH} neurons

Next, we aimed to elaborate our findings further by identifying the subtypes of GABAergic neurons involved in cue-dependent fear memory formation. The BNST contains a great variety of neuropeptide expressing GABAergic neurons with a remarkable resemblance to the peptidergic neurons in the CeL. In the CeL, several data showed that SST and CRH neurons can regulate fear learning, but the function of these cell types in the BNST is still poorly understood. First, we quantified the intra-BNST distribution of CRH and SST positive neurons using cre-dependent reporter mouse lines (Gt(ROSA)26Sor-CAG/LSL-ZsGreen1 x sst-cre or crh-cre mice, n=3/strain). We observed similar density of SST and CRH neurons in the anterior and medial divisions (~15-20%), and a significant dominance of SST neurons in the oval nucleus and posterior regions compared to CRH (**Figure 9A-B**). While, efferent connections of BNST^{CRH} neurons have been previously documented (Dabrowska et al, 2016; Giardino et al, 2018), the projections of BNST^{SST} neurons have not been investigated in detail. Using viral expression of mCherry in BNST^{SST} and BNST^{CRH} neurons, we observed that the projection areas of these cell types largely overlap with the projections of BNST^{vGAT} neurons (**Figure 9C-D**). We observed dense projections of SST and CRH neurons in the substantia innominate (SI), lateral hypothalamic area (LHa), medial amygdala (MeA), medial part of the central amygdala (CeM), ventral premammillary nucleus (PMv), parasubthalamic nucleus (PSTh), paraventricular thalamic nucleus (PVT). We found sparse projections in the paraventricular hypothalamic nucleus (PVN), with more prominent innervation in its posterior parts. Interestingly, midbrain projections of CRH and SST neurons are particularly abundant in monoaminergic regions, i.e. the ventral tegmental area (VTA), the substantia nigra pars compacta (SNc), the retrorubral field (RRF), the dorsal raphe/ventrolateral periaqueductal gray (DR/vlPAG), and the locus coeruleus (LC).

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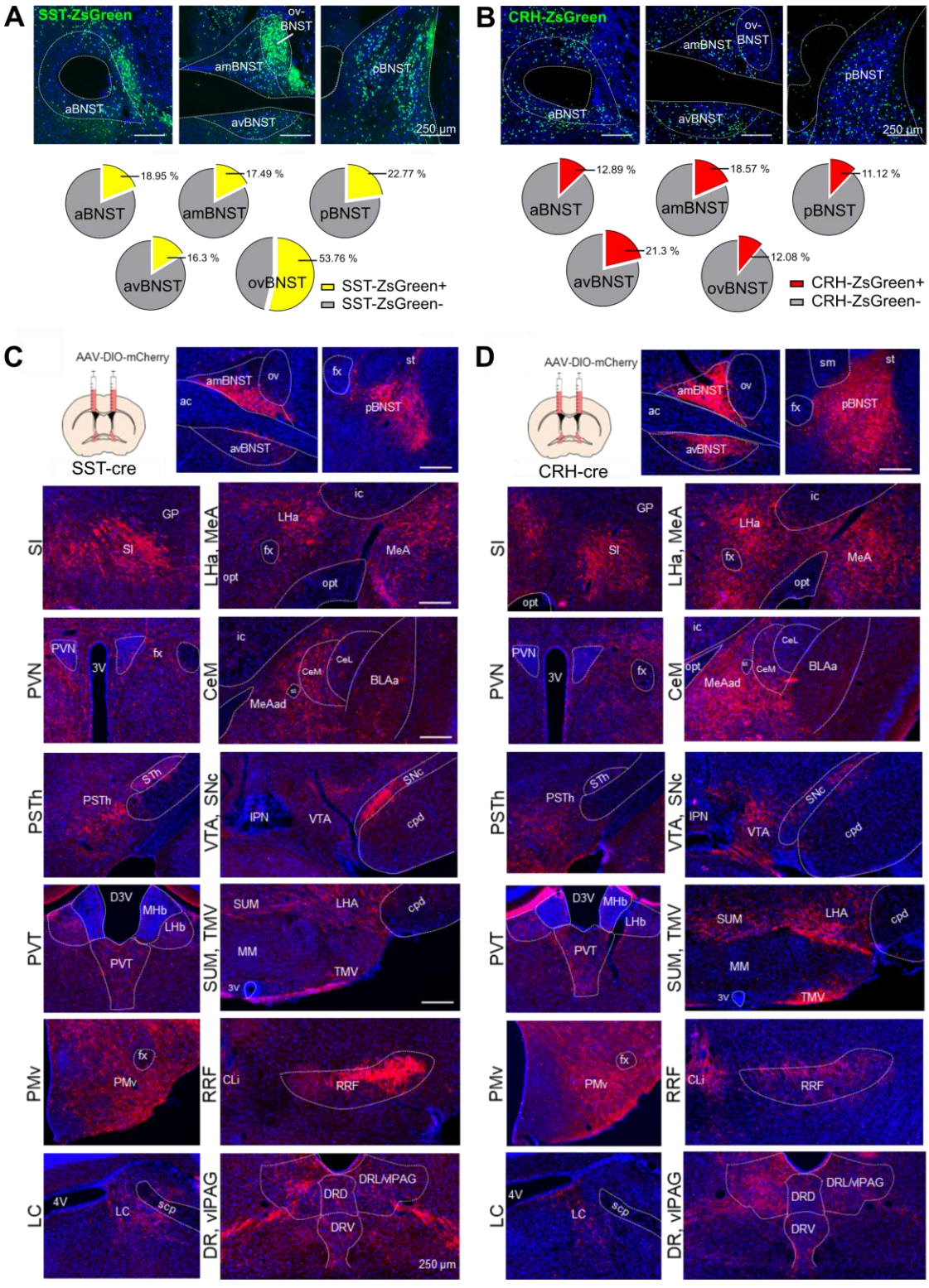

Figure 9. Quantification and output mapping of BNST^{SST} and BNST^{CRH} neurons. *A-B, Distribution of SST and CRH neurons across major BNST subnuclei in SST-Zsgreen and CRH-Zsgreen reporter mice. C, Projection areas of BNST^{SST} neurons. D, Projections areas of BNST^{CRH} neurons.*

3.1.5. BNST^{SST} neurons promote the consolidation and the contextual generalization of CS-induced fear

To study the role of BNST^{SST} neurons in fear learning, we bidirectionally modulated SST neuronal activity during fear memory consolidation by cre-dependent hM4Di and hM3Dq activation in sst-cre mice (**Figure 10A-B**). CNO administration reliably modulated neuronal activity *in vivo* (**Figure 10C**): hM3Dq expressing SST neurons (n=5) showed significantly higher c-Fos expression compared to mCherry expressing controls (n=3; $F_{1,2}$=93.361, p<0.001, Tukey post hoc p<0.001), whereas c-Fos expression was significantly reduced in hM4Di expressing neurons (n=5, Tukey post hoc p=0.008).

Selective chemogenetic stimulation of BNST^{SST} neurons during consolidation produced a more pronounced and persistent facilitation of CS-induced freezing during later recalls (**Figure 10F-G**, Cue recall day1: $F_{2,30}$=8.067, p=0.001, Tukey post hoc p=0.001 and p=0.391 control vs. hM3Dq and hM4Di, respectively; day2/Extinction recall: $F_{2,29}$=5.547, p=0.009, Tukey post hoc p=0.021 and p=0.773 control vs. hM3Dq and hM4Di, respectively; CtxB BL's for day1: $F_{2,31}$=0.769, p=0.471 and day2: $F_{2,29}$=4.225, p=0.024). Again, freezing during conditioning and contextual recall was similar between groups (**Figure 10D-E**, $F_{2,29}$=1,512, p=0.237 and $F_{2,31}$=0.160, p=0.852, respectively; control: n=16, hM3Dq: n=8, hM4Di: n=9). Interestingly, chemogenetic inhibition did not affect any forms of fear recall, despite its potential to lower neuronal activity indicated by c-Fos ($F_{2,10}$=93,361, p<0.001, Tukey post hoc p=0.008; **Figure 10E-F**). Similarly to BNST^{vGAT} stimulation, hM3Dq-BNST^{SST} mice showed significantly higher freezing levels during the baseline period in the safe context B compared to controls (BL freezing for day 1: $F_{2,31}$ = 0.769, p=0.471 and day 2: $F_{2,29}$ = 4.225, p=0.024; **Figure 10F**), indicating a contextual generalization effect on the second recall day. hM3Dq mice also showed a deficit in CS/ITI discrimination, indicated by similar levels of freezing during the CS presentations and ITIs (discrimination index - controls: 1.15 ± 0.04, t=4.037, p=0.001; hM4Di: 1.24 ± 0.05, t = 4.400, p = 0.002; hM3Dq: 1.06± 0.05; t = 1.080, p=0.316, **Figure 10G**).

Based on the above tendencies to fear generalization in context B, we aimed to test whether our effect represents contextual generalization independent of CS. We tested a separate cohort of mice (n=9-11/groups) with chemogenetic stimulation of BNST^{SST} neurons during consolidation with subsequent longer exposure to the safe context without CS-presentation (**Figure 10H**). Experimental groups showed no difference in freezing

levels during conditioning ($F_{1,17=}$ 0.088, p=0.769, **Figure 10I**) and context exposures (context A: $F_{1,18=}$0.131, p=0.720, context B: $F_{1,18=}$0.275, p=0.606; **Figure 10J-K**), suggesting that presence of CS was necessary for fear generalization effects (potentially ambiguous signal as a CS in a safe context).

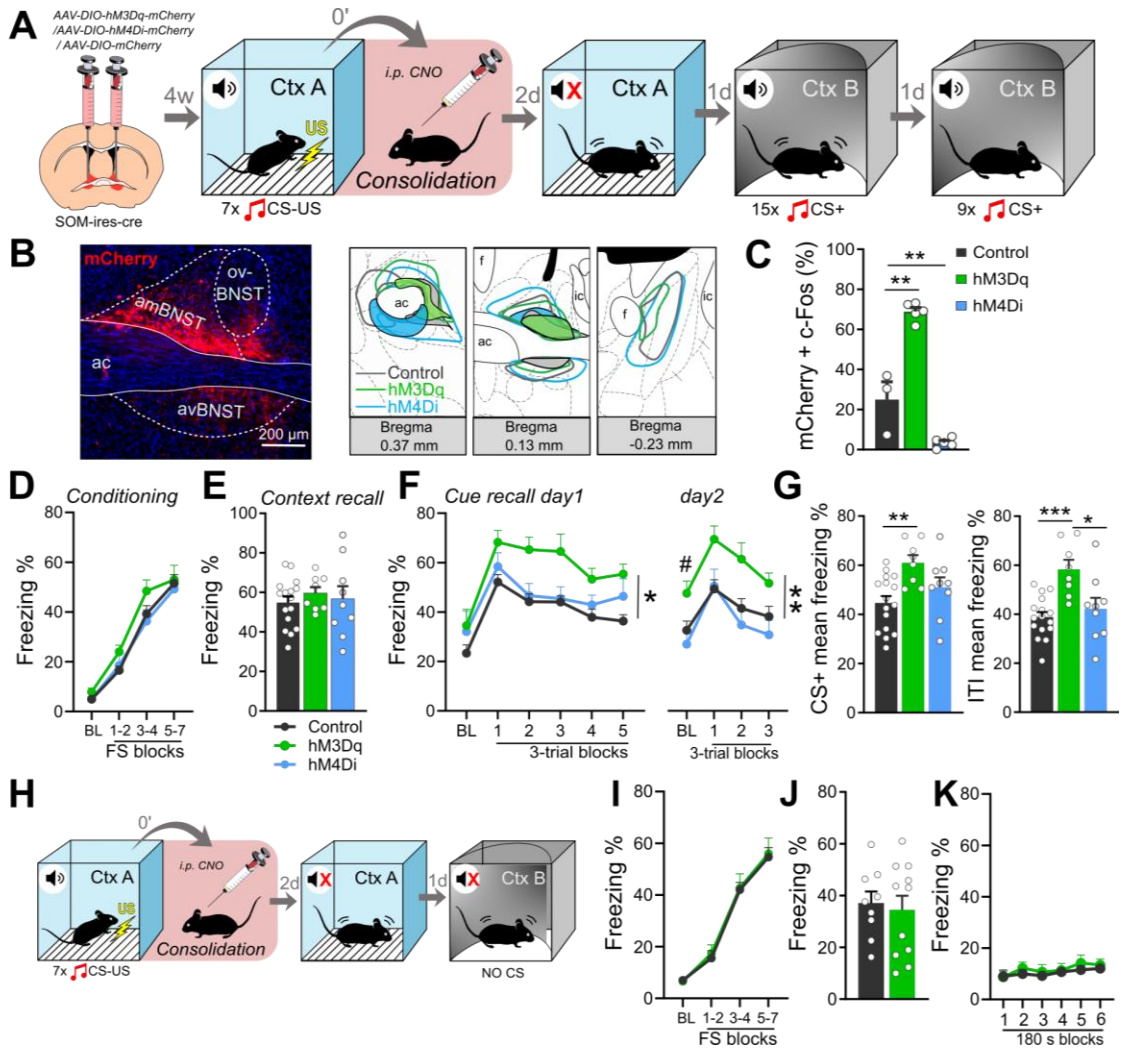

Figure 10. Chemogenetic stimulation of BNST^{SST} neurons facilitates cue-dependent fear learning. *A, Experimental design for chemogenetic modulation of BNST^{SST} neurons during fear memory consolidation. B) Representative photomicrographs of mCherry expression in SST neurons and illustrations of the virus extensions. C, Percentage of c-Fos expressing mCherry+ neurons following CNO administration in homecage condition. D, Freezing timecurves of experimental groups during acquisition. E, Freezing behavior during contextual recall. F, Freezing time curves of cue-dependent fear recall tests. G, Mean time percentage spent with freezing during CS+ representation and it is. H, Experimental design to study the cue-dependency of contextual generalization elicited BNST^{SST} stimulation. I-J, Freezing behavior during acquisition and contextual recall, respectively. K, Freezing behavior during exposure to the context without CS representations.*

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3.1.6. *Chemogenetic modulation of BNST^{CRH} did not affect fear memory consolidation*

Next, we investigated the role of CRH cells in fear learning by chemogenetic modulation of BNST^{CRH} neurons (**Figure 11A**). Intraperitoneal CNO induced significant c-Fos expression in hM3Dq-mCherry BNST neurons ($F_{2,17}$ = 199.509, Tukey's post hoc test: p<0.001, n=7 mice), but we could not detect significant difference in c-Fos and mCherry co-expression between hM4Di and control groups (n=6 and n=7, respectively) in homecage condition (Tukey's post hoc test: p=0.786) likely due to minimal baseline activity in control mice (**Figure 11B**).

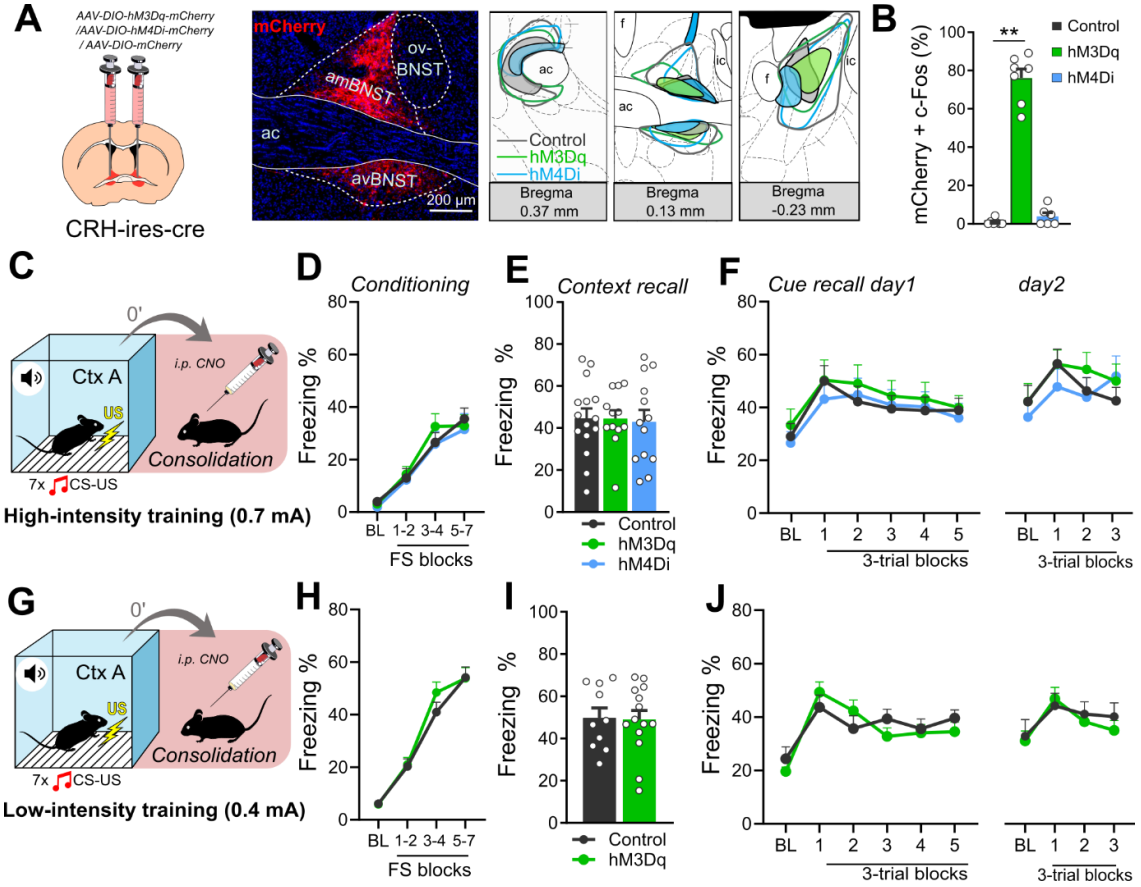

Figure 11. Chemogenetic modulation of BNST^{CRH} neurons did not affect fear memory formation. A, *Representative photomicrographs of mCherry expression in CRH neurons and illustrations of the virus extensions. B, Percentage of c-Fos expressing mCherry+ neurons following CNO administration in homecage condition. C, Experimental design for chemogenetic modulation of BNSTCRH during memory consolidation (after fear conditioning with high-intensity US). D-F, Freezing levels during acquisition, contextual and cue-dependent recall, respectively. G, Experimental design for chemogenetic modulation of BNSTCRH during memory consolidation after low-intensity fear training. H-J, Freezing levels during acquisition, contextual and cue-dependent recall, respectively.*

Chemogenetic modulation of $BNST^{CRH}$ neurons (n=12-15/groups) had no effect on contextual (**Figure 11C-D**, $F_{2,37}$=0.041, p=0.959) and CS-induced fear recalls (**Figure 11E-F**, Cue recalls: F<0.270, p>0.765), or fear generalization (CtxB BL's: F<0.416, p>0.662). Fear acquisition was similar between groups as indicated by freezing ($F_{2,37}$=0.205, p=0.815; **Figure 11D**). Since previous reports showed that CRH neurons in the CeL specifically regulate learning to weak threat (i.e. CS coupled with low-intensity footshocks, (Sanford et al, 2017)), we re-tested the effect of chemogenetic modulation of $BNST^{CRH}$ neurons after fear conditioning with low-intensity footshocks (0.4 mA, **Figure 11G-H**). Similar to high-intensity (0.7 mA) conditioning, we did not observe alteration in fear memory consolidation indicated by similar contextual or CS-dependent fear recalls between groups ($F_{1,22}$ = 0.045, p = 0.833 and $F_{1,22}$ = 0.117, p = 0.734, respectively; n=10-14/groups **Figure 11I-J**). Latter findings suggest that $BNST^{CRH}$ neurons are not or minimally involved in the memory-enhancing effect of the BNST.

3.2. The role of BNST in innate fear responses

3.2.1. Establishing a scalable innate fear paradigm

In a second set of experiments, we investigated the role of $BNST^{SST}$ and $BNST^{CRH}$ neurons in innate fear responses. First, we established a scalable innate fear paradigm in our laboratory in order to manipulate threat intensity/certainty. First, we exposed adult male C57BL/6J mice to either H_2O (n=11) or undiluted 2MT (5 µl, n=12) to confirm the robust fear-inducing potential of 2MT (Wang et al, 2018; Matsuo et al, 2021). Since predator odors represent a more complex threat signal evoking different forms of defensive behavior, we quantified multiple behavioral variables to describe the whole defensive repertoire and shifts in active-passive fear responses and shifts in the approach-avoidance dimension. We observed that mice exposed to 2MT (5 µl) showed markedly decreased locomotor activity (i.e. distance moved: t=-12.911, p<0.001), decreased exploratory rearing (t=-7.432, p<0.001), increased avoidance of the odor source (indicated by reduced entries to the odor zone and higher mean distance from the odor source (t=-7.805, p<0.001; t=5.027, p<0.001) with increased freezing response (t=12.563, p<0.001) (**Figure 12A-B**).

Since increasing threat intensity could shift the defensive behavioral response from active to passive strategies, we assessed the dose-response curve of 2MT-induced fear to

provide a range for bidirectional manipulations and to study the role of BNST in passive and active defensive responses. We tested four decreasing doses on a nearly logarithmic scale, i.e. from 250µl (equivalent with the undiluted dose used above) to 1/125 dose (250, 50, 10, 2 µl of a 50x dilution of 2MT, n=8 mice/groups). A gradual decrease in the 2MT dose had significantly increased locomotion ($F(1,35)=43.41$, $p<0.001$) and exploratory rearing ($F(1,35)=29.52$, $p<0.001$), reduced the avoidance of 2MT source (i.e. increased entries to the odor zone ($F(1,35)=40.19$, $p<0.001$) and reduced mean distance from the odor source ($F(1,35)=6.21$, $p<0.001$)), and finally reduced the time spent with freezing ($F(1,35)=67.90$, $p<0.001$, **Figure 12C**). We found a floor effect and a plateau with 2 and 50 µl dose, respectively (Tukey's posthoc for all variables: $p>0.54$; except decreased approach: $p=0.025$; $p>0.52$; except somewhat lower freezing levels, $p=0.072$, respectively). Based on these results, we selected 10 µl and 250 µl doses for further experiments as low and high stimulus intensities (referred as 'low- and high-dose'). These two doses were effective inducer of the fear response indicated by all variables (Tukey's posthoc for all variables of both doses: $p<0.001$, except mean distance from the odor source for 10 µl dose: $p=0.57$).

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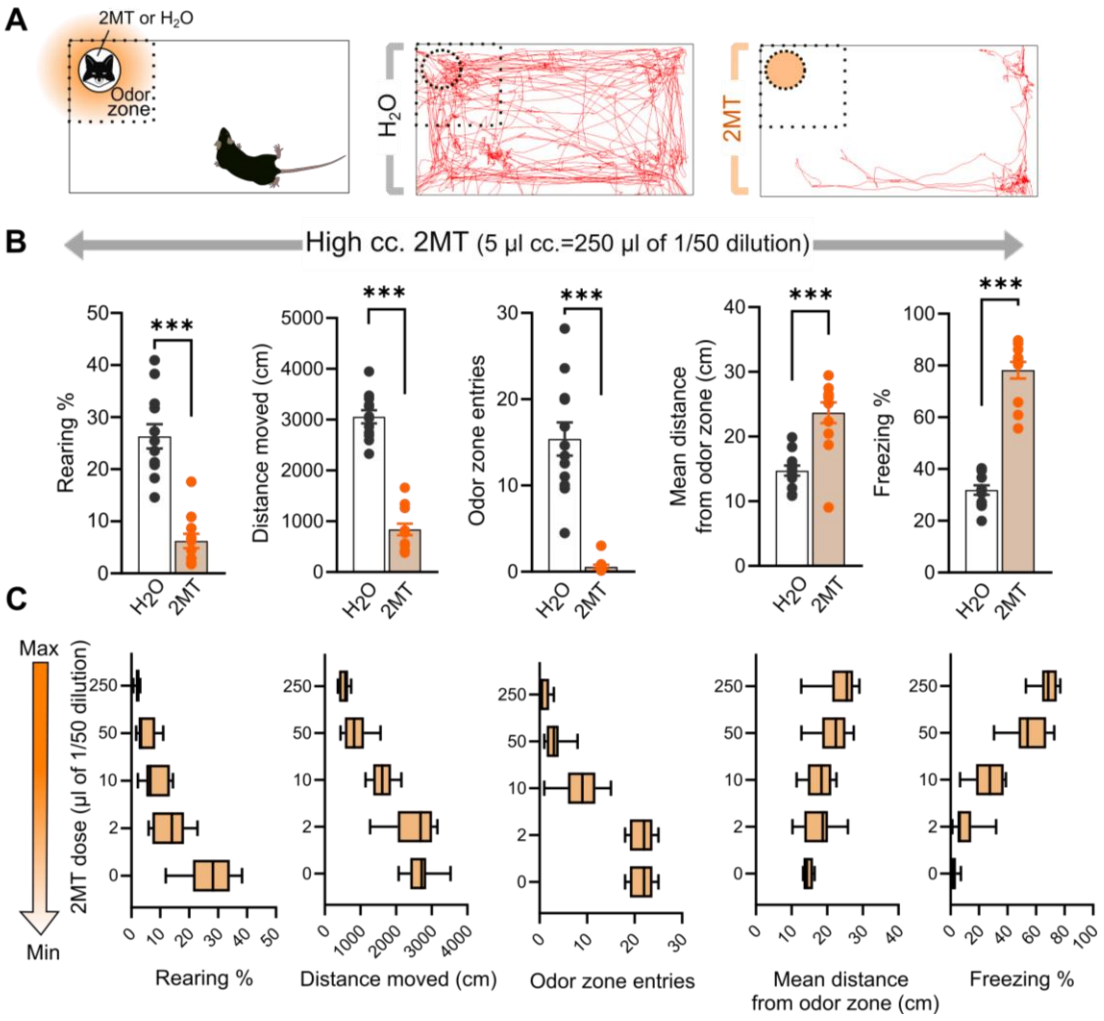

Figure 12. Predator odor 2MT elicits robust and dose-dependent innate fear responses in mice*. (A) Schematics of the apparatus used for the predator odor avoidance test (left) and representative trajectory plots of mice exposed to H_2O or 2MT. (B) Indices of active behavioral responses (exploratory rearing, approaches) and passive defensive responses (avoidance and freezing) to high dose of 2MT. (C) Dose-response curve of 2MT-induced behavioral changes. ***p<0.001.*

3.2.2. BNST^{SST} neurons facilitate innate fear responses to low intensity innate threat

Next, we tested whether chemogenetic inhibition of BNST^{SST} neurons modulate predator odor avoidance of low or high-dose of 2MT. We observed an amelioration of innate fear in hM4Di mice (n=10), when exposed to low-dose 2MT (control: n=9, **Figure 13A and B**). Inhibition of BNST^{SST} neurons significantly increased locomotion and rearing (**Figure 13B**, distance moved: t=-2.203, p=0.041, rearing time%: t=-2.392, p=0.029), reduced avoidance of the odor source (entries to the odor zone: t=-2.348, p=0.031, mean distance from the odor zone: t= 3.203, p=0.005). Noteworthy, freezing levels were not significantly affected by inhibition of BNST^{SST} neurons in this experiment

(t= 1.436, p=0.168). Less rigorous statistical analysis indicates that the effect in freezing may be obscured by an extreme value that lies 2.12 SD away from the mean, since hM4Di group showed significant reduction in freezing compared to control with the exclusion of this individual data point (p = 0.034).

In line with the behavioral effects, we confirmed a significantly lower c-Fos expression in the mCherry+ neurons of the BNST in hM4Di mice compared to controls (n=6/group, t=5.165, p<0.001) (**Figure 13C and D**).

Exposure to high-dose 2MT resulted in higher ratio of passive defensive behaviors, but interestingly experimental groups showed no significant difference in the analyzed behavioral parameters (distance moved, t=0.039, p=0.969; entries into the odor zone: t=0.244, p=0.808; mean distances from the odor zone: t=-0.078, p=0.938; rearing: t=1.639, p=0.114; freezing: t=-0.671, p=0.508; n=9-11/group) **(Figure 13E)**.

Next, we were interested if the observed effect under the weak threat condition was indeed reflected to blunted innate fear responses to a specific predator cue or it rather resulted from a general anxiolytic or motor effect. To answer this question, mice were tested in an open field arena with accompanied chemogenetic inhibition of $BNST^{SST}$ neurons (**Figure 13F**). Our findings showed clearly that BNST inactivation was ineffective to modulate defensive behavior in the absence of predator odor (i.e. no significant differences in the time percentage of rearing and freezing: t=0.140, p=0.890 and t=0.201, p=0.843, respectively; distance moved: t=-0.506, p=0.619 and time spent in the center of the arena: t=-0.741, p=0.468; n=9-10/group).

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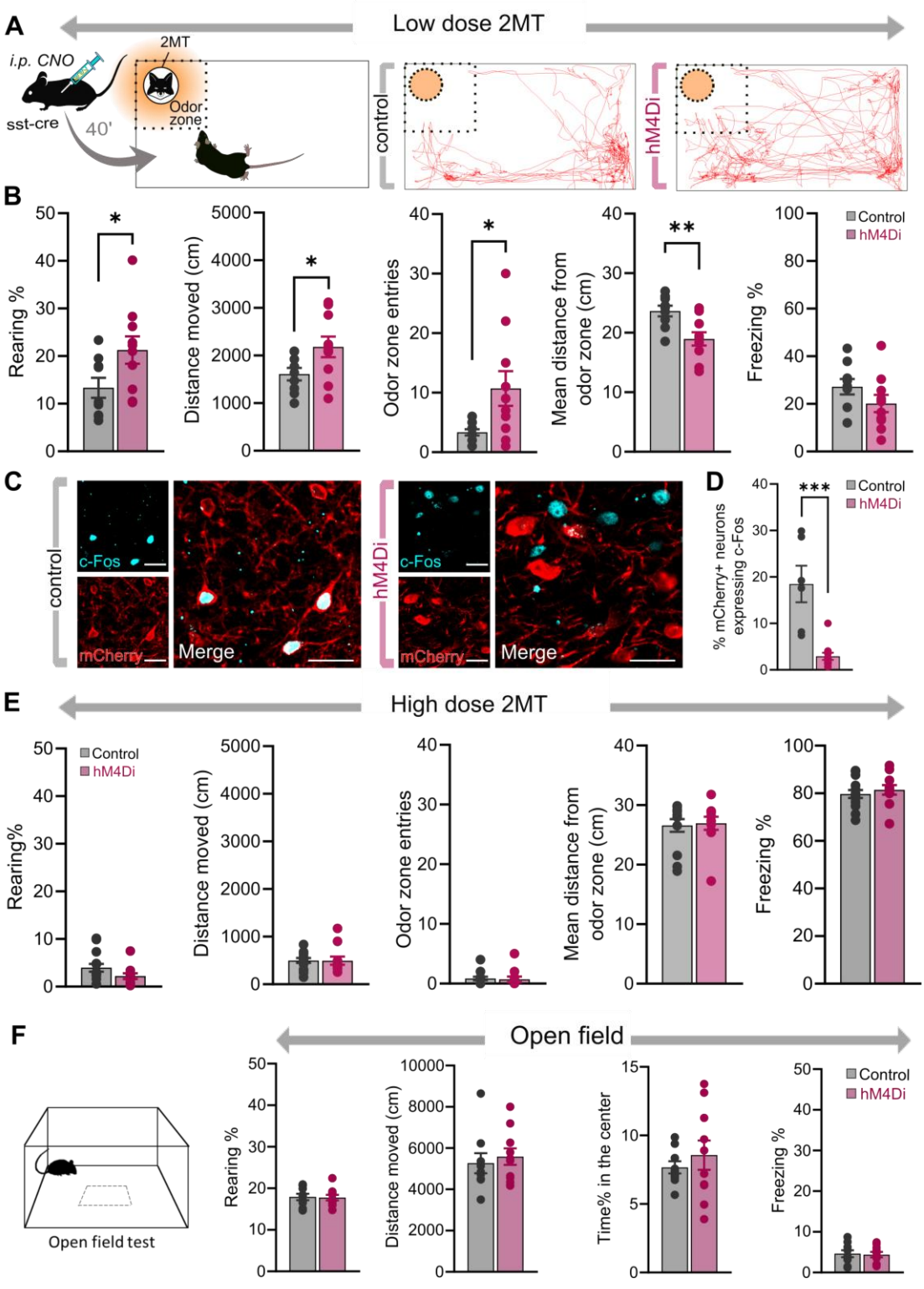

Figure 13. Chemogenetic inhibition of BNST^{SST} neurons reduces fear response under weak predator odor threat. *(A) Representative trajectory plots of control and hM4Di mice exposed to low dose of 2MT after chemogenetic inhibition of BNST^{SST} neurons. (B) Behavioral responses to low-dose 2MT during BNST^{SST} inhibition. (C) Representative confocal microscopic images of c-Fos expression in mCherry+ BNST^{SST} neurons from 2MT-exposed mice. (D) Percentage of c-Fos expressing mCherry+ neurons*

in control and hM4Di mice. (E) Behavioral responses to high-dose 2MT during BNST^{SST} inhibition. (F) Behavior in the open field test with inhibition of BNST^{SST} neurons.

3.2.3. Chemogenetic inhibition of BNST^{CRH} neurons has no impact on innate fear response evoked by the predator odor 2MT.

Next, we explored the impact of BNST^{CRH} inhibition on 2MT- induced innate fear responses. Similar to BNST^{SST} inhibition, inhibition of CRH neurons did not affect baseline anxiety in the open field test (**Figure 14A-B**; center time%: t=0.217, p=0.829; distance moved: t=-1.400, p=0.175; rearing: t=-0.297, p=0.768; freezing: t=1.636, p=0.116; n=8-16/group).

Chemogenetic inhibition of BNST^{CRH} neurons (control: n=9, hM4Di: n=14) had no effect on the fear response evoked by low-dose 2MT as indicated by unaltered distance moved (t=0.710, p=0.484), entries into the odor zone (t=-0.884, p=0.386), time spent with rearing (t= 0.643, p=0.526), freezing (t=-0.399, p=0.693), and mean distance from the odor zone (t= -0.418, p=0.679) (**Figure 14C**). Similarly, high-dose 2MT evoked fear response did not show significant difference between groups (control: n=11, hM4Di: n=14): total distance travelled (t=-1.805, p=0.084), entries into the odor zone (t=0.894, p=0.380), rearing (t=-1.086, p=0.288), freezing (t=1.600, p=0.123), and mean distance from the odor zone (t=0.264, p=0.793) (**Figure 14D**).

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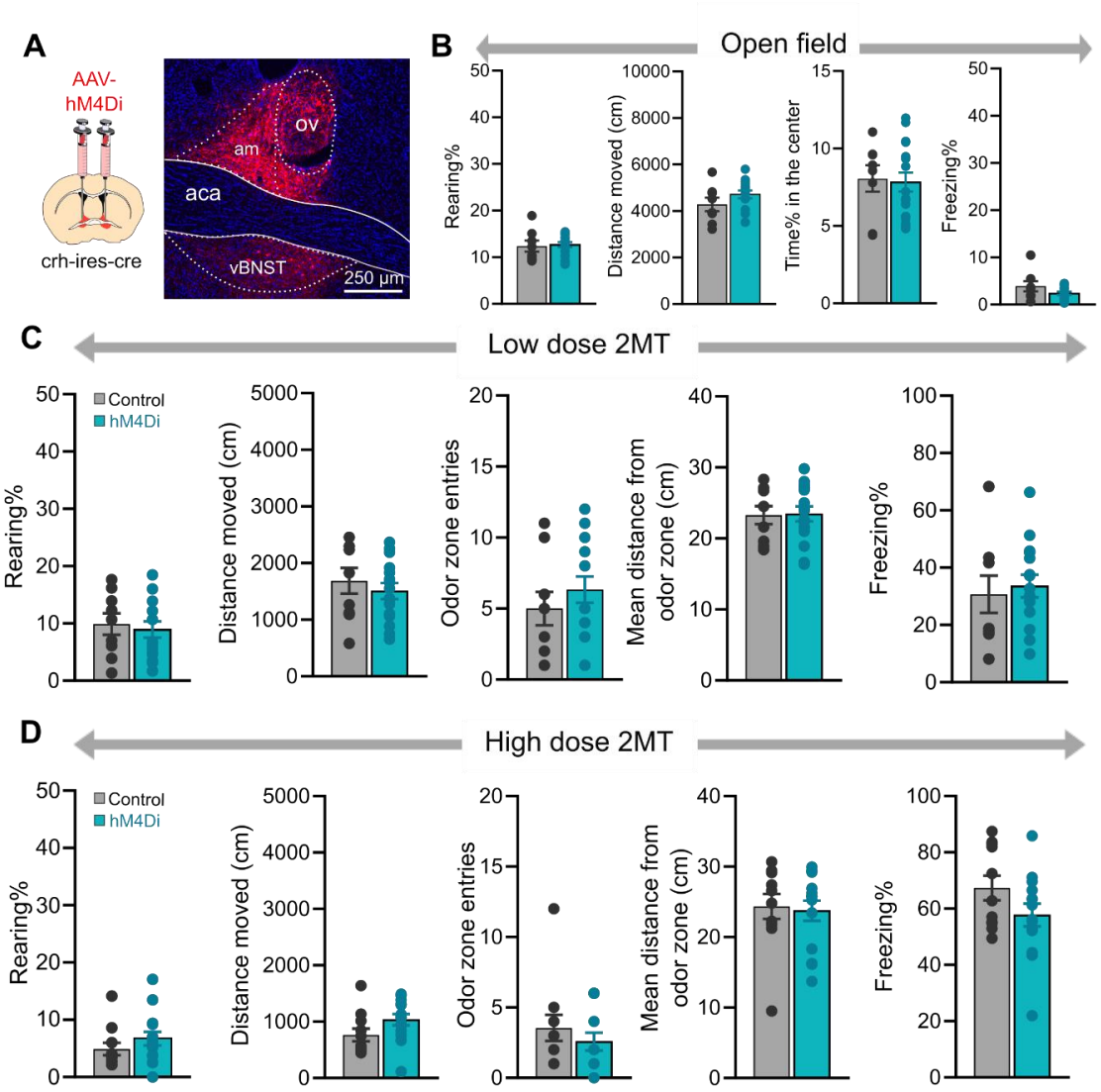

Figure 14. Chemogenetic inhibition of BNST^{CRH} neurons has no impact on innate fear response evoked by 2MT. *(A) Illustration of viral delivery of hM4Di to crh-cre mice and representative photomicrograph of mCherry expression in BNST^{CRH} neurons. (B) Behavior in the open field test with BNST^{CRH} inhibition. (C-D) Behavioral responses to low and high-dose 2MT during BNST^{CRH} inhibition, respectively.*

3.2.4. Inhibition of BNST^{CRH} neurons enhances the innate fear response evoked by cat odor.

Since previous studies showed that BNST^{CRH} neurons are activated by predator odors (Butler et al, 2016; Giardino et al, 2018), we aimed to confirm in another paradigm if our effects are indeed negative. Noteworthy, in our paradigm even low-dose of 2MT is a potent fear-eliciting agent resulting in ~30% of test time spent with freezing, and marked avoidance of the odor zone (3-4 entries/10 min) in control mice (**Figure 12C).** Since

stimulus intensity has been reported as a crucial factor in case of CRH neurons of the amygdala (Sanford et al, 2017), we tested an even lower threat conditions, i.e. cat odor evoked innate fear.

In naïve adult male C57BL/6J mice we found that cat urine (presented as soiled cat litter) is a mild stressor (**Figure 15A-B**), increasing the time spent with immobility (distance travelled: t=3.183, p=0.003; time% of freezing: t=-2.790, p=0.008), without a significant effect on the active exploration of the test arena and the odor source (odor zone entries: t=1.126, p=0.216, rearing time%: t=0.296, p=0.768, mean distance from the odor zone: t=-0.237, p=0.813) compared to clean litter exposed control mice (n=8-10/groups).

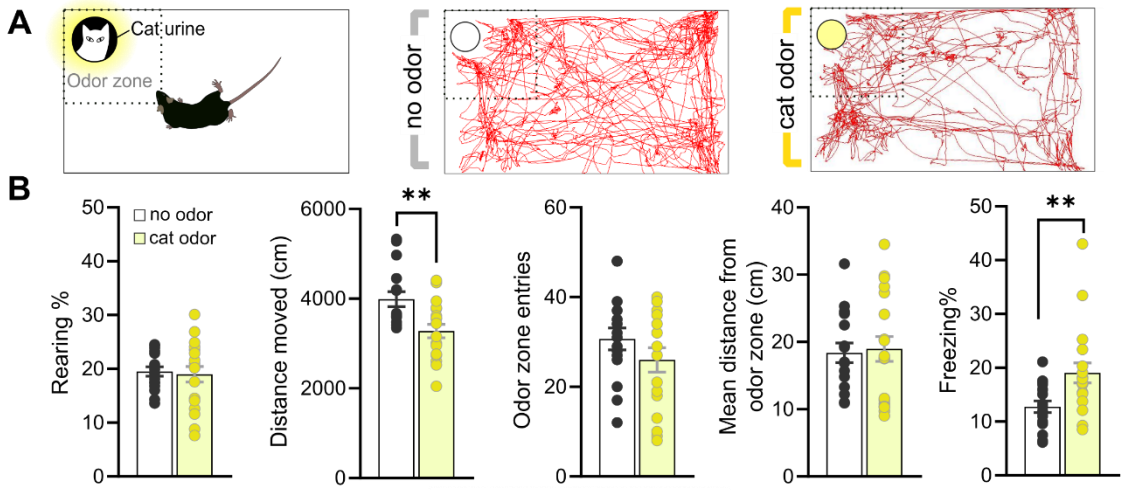

Figure 15. Behavioral responses of wild-type mice exposed to cat urine.
(A) Illustration of experimental settings and representative trajectory plots of individual mice exposed to clean or soiled cat litter. (B) Defensive behavior profile of mice exposed to cat odor.

Under this low threat condition, chemogenetic inhibition of BNST^{CRH} increased all defensive behaviors: reduced distance moved (t=4.167, p<0.001) and time spent with rearing (4.965, p<0.001), less entries into the odor zone (t=1.820, p=0.08), as well as higher mean distance from the cat odor (t=-2.696, p=0.015) and increased freezing levels (t=-2.648, p=0.017) (**Figure 16A-B**). The effect of chemogenetic inhibition on BNST activity was also detectable on the neuronal level indicated by reduced c-Fos expression in BNST^{CRH} cells (n=8-10/groups) (**Figure 16C-D**).

In contrast to BNST^{CRH} neurons, inhibition of BNST^{SST} neurons had no impact on cat urine induced fear: distance moved (t=0.239, p<0.813), rearing (t=-0.636,

p<0.534), entries into the odor zone (t=-1.246, p=0.231), mean distance from cat odor (t=0.180, p=0.859) and freezing levels (t=0.010, p=0.992) (n=8-9/groups) (**Figure 16E**).

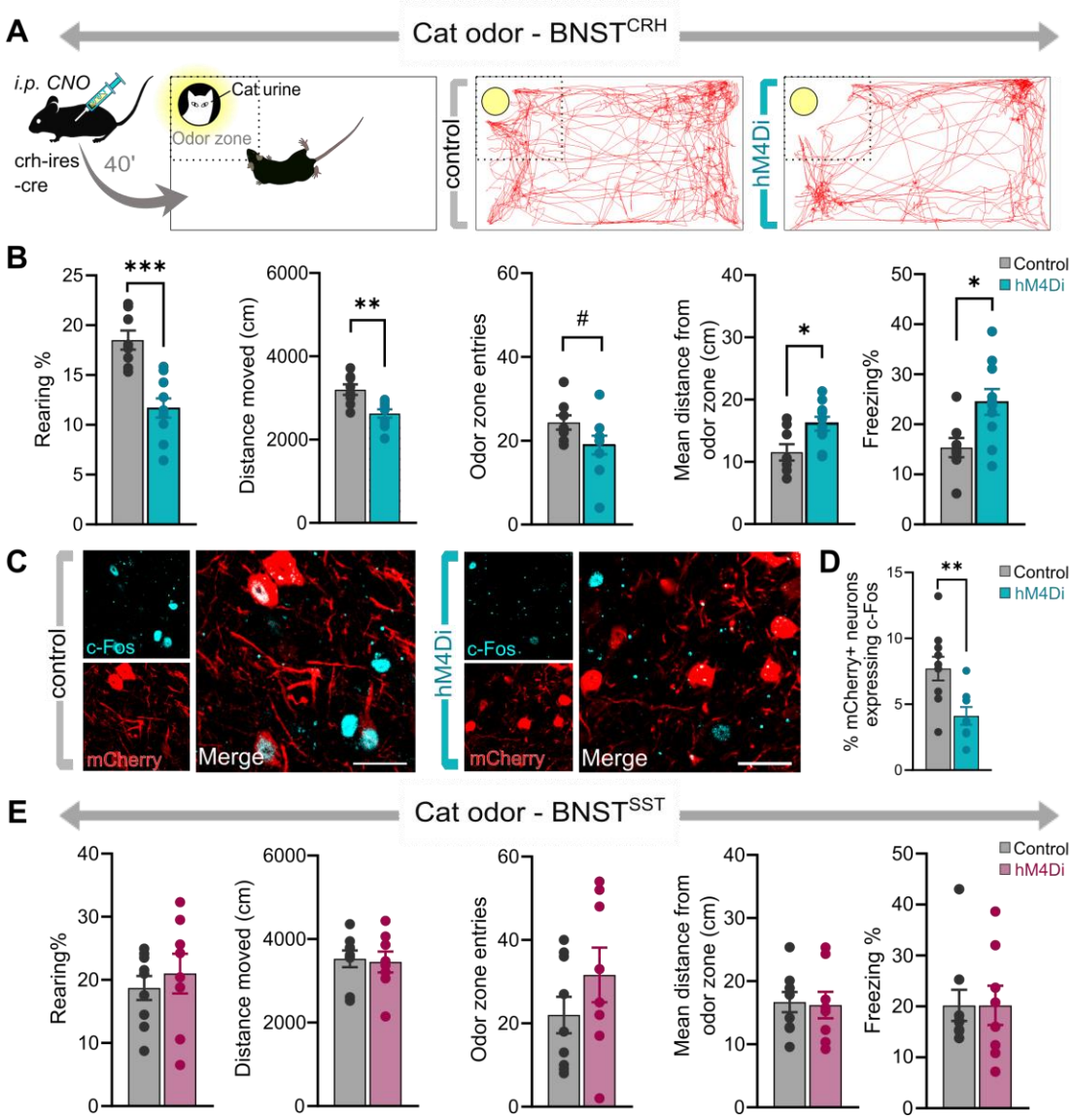

Figure 16. Chemogenetic inhibition of BNST^{CRH} neurons enhances innate fear response evoked by cat odor. *(A) Representative trajectory plots of control and hM4Di mice exposed to cat odor during chemogenetic inhibition of BNST^{CRH} neurons. (B) Behavioral responses to cat odor during BNST^{CRH} inhibition. (C) Representative confocal microscopic images of c-Fos expression in mCherry+ BNST^{CRH} neurons from cat odor-exposed mice. (D) Percentage of c-Fos expressing mCherry+ neurons in control and hM4Di mice. (E) Behavioral responses to cat odor during BNST^{SST} inhibition.*

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4. Discussion

Our experiments pointed out that increased activity of $BNST^{vGAT}$ neurons during fear learning and subsequent memory formation contributes to enhanced fear response during later re-exposure of footshock-associated auditory cues. Interestingly, we did not observe acute changes in freezing behavior during acquisition or cue-recall coupled with BNST stimulation, suggesting that the BNST did not affect fear expression or memory retrieval per se. With selective manipulations of genetically identified subtypes of $BNST^{vGAT}$ neurons, we showed that stimulation of $BNST^{SST}$ neurons could result in a similar enhancement in memory formation with an additional tendency to promote fear generalization in cue associated safe contexts. We showed that $BNST^{SST}$ neurons target several brain areas involved in defensive behavior, stress response and fear regulation, hence they are all well positioned to modulate fear learning. In line with this, chemogenetic stimulation of BNST indeed had a significant effect on the activity of its downstream targets several hours after fear acquisition. In contrast to $BNST^{SST}$ neurons, manipulation of $BNST^{CRH}$ cells- another major population of $BNST^{vGAT}$ neurons- failed to affect fear memory consolidation in our experiments.

In a second set of experiments, we revealed that $BNST^{SST}$ neurons also promote innate fear responses elicited by the predator odor 2MT in a concentration-dependent manner, i.e. increasing fear response to low-dose 2MT exposure. We showed an opposite role of $BNST^{CRH}$ neurons in cat urine-induced fear, i.e. these neurons promote approaches and decrease the fear response. Importantly, BNST manipulations were ineffective, when mice were exposed to high threat intensity (high-dose of 2MT), suggesting that BNST regulates innate defensive responses to more ambiguous threats and has minimal effect in manifested danger situations (e.g. high proximity of predator).

4.1. BNST exerts a modulatory effect on fear learning

One of our main findings is that the BNST not only mediates sustained, anxiety-like responses as previously suggested, but also capable to modulate long-term behavioral outcomes by regulating aversive learning. Previous studies investigating the BNST in conditioned fear paradigms primarily focused on the fear recall phase or applied pre-training lesions that affect both learning and recall phases. Accordingly, the exact role of BNST in specific phases of fear learning remained unclear (LeDoux et al, 1988;

Hammack et al, 2015; Kiyokawa et al, 2015). Nevertheless, a recent study found that enhanced serotonergic signaling in the BNST supports auditory fear acquisition (Marcinkiewcz et al, 2016). On the other hand, BNST deactivation experiments often failed to modulate auditory cue-dependent fear expression (LeDoux et al, 1988; Sullivan et al, 2004; Zimmerman and Maren, 2011; Goode et al, 2019). Along with these observations, we found that stimulation of BNST during recall test did not affect fear responses to previously learnt aversive or safety cues. Neuronal activation in the BNST of naïve mice (indicated by the immediate early gene c-Fos expression) further confirmed that BNST is preferably recruited during fear acquisition and remain silent during cue-dependent fear recall. Together these data suggest that the memory engrams about CS-US association are stored outside of the BNST and their retrieval is not BNST-dependent.

The lack of effect on contextual fear learning in our experiments is rather surprising based on previous observations of impaired contextual fear recalls with BNST inactivation (Sullivan et al, 2004; Resstel et al, 2008; Nagaya et al, 2015; Goode et al, 2020). However, only a few studies used reversible inactivation of the BNST during fear acquisition (Ressler et al, 2020), thus there is no solid evidence on whether the BNST is inevitable for contextual fear memory formation. A recent study showed that the posterior BLA- dorsal BNST pathway is active during fear conditioning, but not incorporated into the contextual fear engram (Russell et al, 2020). Since our first experiments with BNST manipulation revealed a selective effect on cue-dependent fear memory formation, we did not investigate further the involvement of BNST in contextual recalls, although the dense BNST innervation arising from the ventral hippocampus suggest that contextual information processed here as well (Radley and Sawchenko, 2011; Glangetas et al, 2017).

4.2. BNST stimulation during consolidation alters fear network activity

Since both of our c-Fos activity data and chemogenetic manipulations showed that cued fear expression per se is not mediated by the BNST, we concluded that BNST facilitates fear memory consolidation via efferent pathways. Comparative projections of BNST subnuclei previously showed that several brain areas of the fear network are targeted by the BNST, e.g. CeA, lateral hypothalamus, substantia innominata, paraventricular nucleus of the thalamus (PVT) and the hypothalamus (PVN), substantia nigra pars compacta, ventral tegmental area (VTA) and the dorsal raphe (DR), ventrolateral periaqueductal gray (vlPAG), locus coeruleus etc. (Dong et al, 2001b; Dong and

Swanson, 2004, 2006). We found that among these regions, the dorsal midline thalamus (including the PVT), the interfascicular nucleus of the VTA and the vlPAG showed significantly higher c-Fos expression during fear consolidation even six hours after chemogenetic stimulation of BNST. Although the functional characteristics of these BNST projections in fear responses have not been investigated yet, some of these downstream targets have been implicated in conditioned fear. Among these the role of PVT in fear learning is established (Penzo et al, 2015). Interestingly, the PVT both sends and receive projections from the BNST and CeA, suggesting a bidirectional regulation of fear learning within this network (Mátyás et al, 2014; Hua et al, 2018). A similar bidirectional connection exists between the vlPAG and the BNST (Petit et al, 1995; Meloni et al, 2006). In the past few years, the PAG has received increased research interest, since its role seem to be more complex than a motor executor region. Feedback projections from the vlPAG to the CeA control aversive memory strength by calibrating learning signals according to sensory feedbacks (Ozawa and Johansen, 2018). The PAG may integrate inputs from the BNST also for fear learning. In support of this idea, the avBNST-vlPAG pathway was recently shown to modulate fear memory consolidation in the active avoidance paradigm (Lingg et al, 2020). Midbrain and brainstem monoaminergic pathways also provide bottom-up regulation of fear learning, which is presumably under the control of BNST (Johansen et al, 2011; Marcinkiewcz et al, 2016; Groessl et al, 2018). Important to note, that BNST projections closely follow the distribution of dopaminergic neurons (i.e. VTA, substantia nigra, retrorubral and dorsal tegmentum dopaminergic neurons). Despite this coincidence, the regulation of dopaminergic signaling by the BNST is hitherto gain little attention to date. Among these regions, the midbrain dopaminergic neurons, VTA and the substantia nigra have been shown to regulate fear learning and memory consolidation (Baldi et al, 2007; Pignatelli et al, 2017; Groessl et al, 2018). BNST may also modulate stress reactivity during footshock-conditioning by efferents to the PVN. Surprisingly, we observed a significant decrease in c-Fos expression in the PVN, which confirms the previous observations that the BNST is a negative regulator of the HPA-axis, however contradicts the well-known memory-enhancing effects of glucocorticoids (de Quervain et al, 2009; Radley and Sawchenko, 2011; Lingg et al, 2020). On the other hand, we cannot exclude the possibility that general changes in PVN c-Fos expression does not necessarily reflect to the activity of CRH neurons or may resulted from a compensatory effect. Although we aimed to assess c-Fos activity in CRH neurons, technical limitation made it unfeasible to

reliably conduct this assessment: (1) we could not reliably immunolabel CRH+ cell bodies for co-labeling and counting (without colchicine treatment); and (2) CRH+ signal in the PVN in ZsGreen transgenic mice was quite dense and not feasible for exact counting. It is also important to note, that we assessed c-Fos labelling six hours after fear training to eliminate foot-shock induced neuronal activity, which not tightly related to the BNST stimulation. Within this time window, a significant negative feedback on PVN CRH neurons could be occurred. These changes in postsynaptic c-Fos activity observed in our experiments could point out several potential BNST targets mediating the fear-enhancing effect, but future studies with projection-specific manipulations should confirm the functional relevance of these circuits in aversive learning.

4.3. BNST^{SST} neurons facilitate fear memory consolidation

Our findings showed that the above-described effects of BNST stimulation on fear memory encoding and later recalls could be replicated by the selective stimulation of BNST^{SST} neurons, suggesting a common function of extended amygdala SST neurons in aversive learning. The identification of downstream targets where BNST^{SST} neurons modulate fear learning was out of the scope in our study, however, our anterograde viral tracing provided detailed mapping of BNST^{SST} projections. We found mCherry+ SST fibers in the lateral hypothalamus, medial amygdala, substantia innominata, substantia nigra, posterior subthalamic nucleus, paraventricular thalamus, lateral habenula, retrorubral field, central amygdala, ventrolateral PAG, dorsal raphe and locus coeruleus. Interestingly, CeL^{SST} neurons show more restricted extra-amygdalar projections by targeting the vlPAG, subthalamic nucleus, LH and LHb, parabrachial nucleus, zona incerta, medial geniculate nucleus and lateral mediodorsal thalamus (Hartley et al, 2019). Our anterograde tracing indicates that BNST^{SST} neurons avoid the CeL, suggesting that they not form connections with CeL^{SST} neurons. In contrast, a recent paper demonstrated that BNST^{SST} neurons are under tonic inhibition by CeL^{SST} neurons, but acute footshock stress elicits disinhibition of BNST^{SST} neurons, which may modulate stress induced anxiety or fear learning (Ahrens et al, 2018). To understand the function of extended amygdala SST cells in fear learning, the afferent connections of these neurons should be also take into account. Glutamatergic inputs arising from the BLA, PVT and vHC to CeA are essential to induce plasticity-related changes during fear learning (Li et al, 2013; Penzo et al, 2015; Xu et al, 2016). Interestingly, the same areas also send parallel

projections to the BNST, which may also control fear acquisition via BNST circuits (Mátyás et al, 2014; Glangetas et al, 2017; Russell et al, 2020). Poulos and his colleagues showed that contextual fear could be acquired even with BLA or BNST lesion with overtraining, but combined BLA and BNST lesions entirely impairs fear learning. Moreover, the BNST showed elevated conditioning-induced activity in rats with BLA lesion and inhibition of protein synthesis in the BNST impaired fear memory formation in these animals (Poulos et al, 2010). This result suggests that the BNST may represent a parallel/complementary circuit, which could promote fear learning independently of the BLA.

Recent studies showed that CeL^{SST} and CRH neurons play mutually exclusive function in fear responses, the first mediate fear learning and passive fear responses, while the latter regulate extinction learning and flight behavior (Fadok et al, 2017; Hartley et al, 2019). However, the results on the function of CRH neurons are not conclusive, since CeL^{CRH} neurons also mediate fear learning to weak threats (Sanford et al, 2017). These finding suggest that CRH neurons may exhibit greater functional diversity. In confirmation, Hartley and colleagues showed that CeL^{CRH} neurons show molecular heterogeneity, some co-express SST or PKCδ (Hartley et al, 2019). Here, we found that $BNST^{CRH}$ neurons did not regulate fear learning (in contrast to CeL^{CRH} neurons), not even during weak footshock intensity. However, we cannot rule out their potential involvement in extinction learning or fear expression, which was not tested here. Interestingly, the general pattern of efferent projections of $BNST^{CRH}$ neurons largely mirrors the outputs of $BNST^{SST}$ neurons (Dabrowska et al, 2016; Dedic et al, 2018). In light of this, the lack of effect of $BNST^{CRH}$ modulation in our fear conditioning paradigm is rather surprising, although we cannot exclude the possibility that SST and CRH neurons target distinct neuronal populations within the same brain regions or alternatively, they might be recruited by different stimuli as we showed in our predator odor paradigm. It is important to note that despite the functional heterogeneity of CeL^{SST} and CeL^{CRH} neurons, they also show remarkable overlaps in their efferent projections (Hartley et al, 2019).

Another confounding factor in our experiments is the lack of bidirectional effect on fear learning by chemogenetic inhibition of $BNST^{SST}$ neurons. The latter finding suggests that the BNST may not be inevitable for CS-dependent fear learning, but rather represent a modulatory or alternative pathway as suggested by the data of Fanselow lab (Poulos et al, 2010). Noteworthy, several experiment failed to modulate fear learning or anxiety in naïve mice with BNST inhibition, which only ameliorated anxiety in stressed animals

indicating that BNST might mediate stress-induced anxiety (Regev et al, 2012; Pomrenze et al, 2019). Based on this, our stimulatory experiments might mimic the consequences of sustained BNST activity observed in several conditions, e.g. after chronic stress, trauma exposure, or anxiety disorders (Knight and Depue, 2019). Future studies need to determine how elevated BNST activity contributes to extinction-resistant fear memory formation under these conditions.

3.4. $BNST^{SST}$ and $BNST^{CRH}$ neurons modulate approach-avoidance behavior to ambiguous predator threat in a complementary manner

In our second set of experiments, we studied the involvement of SST and CRH neurons in defensive behavior to innately aversive stimulus (i.e. predator odor). We took advantage of single compound predator odor, 2MT as a dosable threat stimulus (Wang et al, 2018). In our experiments, several behavioral variables of naïve mice showed dose-dependent response to 2MT. High volumes of 2MT gradually elicit avoidant behavior with a general shift towards passive defensive behavior (e.g. freezing). In contrast, when 2MT represented a weaker threat (i.e. in lower doses), we observed higher incidence of approaches and exploratory rearing behavior. We concluded that higher concentrations of predator kairomones might indicate higher probability of temporally/spatially imminent presence of the predator. In agreement with our results, previous papers also showed that mice exhibit frequent rearing during potential predator risk and exploratory rearing is negatively modulated by threat imminence (Hegab et al, 2014; Andraka et al, 2021). Nonetheless, rearing is generally not classified as risk assessment activity, but rather considered as a non-defensive behavior (Lever et al, 2006). Compared to classic low-to-ground risk assessment behavior in rodents, rearing does not minimize visual detection; therefore, it occurs at lower perceived levels of direct threat. Accordingly, Gray and McNaughton proposed that rearing could be described with an inverse U shape depending on threat intensity. Notably, rearing is more frequent during low levels of fear (and relatively high levels of anxiety), while it is suppressed during high levels of fear (Gray and McNaughton, 1982). Additionally, rearing activity does not simply reflect locomotor drive: (1) rearing declines over time in novel environments in contrast to horizontal activity; (2) rearing and ambulation are pharmacologically dissociable behavioral variables (Lever et al, 2006). In summary, one can interpret our predator risk-dependent changes of behavioral variables along the approach-avoidance continuum

(which separate high fear vs. anxiety-like behavior), or alternation between active and passive coping (which can be considered as distinct behavioral strategies to avoid stressors).

Our results revealed that $BNST^{SST}$ neurons exert an anxiogenic effect by decreasing non-defensive behavior (exploratory rearing) and enhancing avoidance to low-intensity 2MT. These observations along with our data from conditioned fear paradigm suggest that $BNST^{SST}$ neurons enhance defensive responses to fearful stimuli. Similar functions have been associated with the CeL^{SST} neurons, although it is not known whether these cells are also recruited by innate threats (Fadok et al, 2018; Li, 2019). Moreover, stimulation of SST neurons is sufficient to induce freezing without any threats (Li, 2019), suggesting that these neurons can directly regulate the executive centers of defensive behaviors, e.g. PAG outputs (Penzo et al, 2014). In contrast, our chemogenetic modulation of $BNST^{SST}$ neurons had an effect on freezing only when mice were confronted with low-dose of 2MT, suggesting that the effect of SST modulation on freezing is associated with a decreased fearful state rather than simply reflected to altered locomotion. Furthermore, $BNST^{SST}$ inhibition did not affect baseline anxiety, exploration and locomotion in the open field test, indicating that SST neurons indeed modulate defensive responses to specific fear-inducing stimuli. This conclusion is also supported by previous studies reporting minimal or no effect of chemogenetic manipulation of BNST on anxiety-like behavior and exploratory activity in the elevated plus-maze and open field tests without additional stressors (Marcinkiewcz et al, 2016; Mazzone et al, 2018).

We also found that $BNST^{CRH}$ neurons exerted an opposing effect compared to $BNST^{SST}$ cells, i.e. $BNST^{CRH}$ inhibition reduced approaches, exploration and increased freezing to mild predator threat, suggesting that these cells may promote exploration during potential conflicts. Again, these behavioral changes were absent when $BNST^{CRH}$ neurons were inhibited during the open field test ruling out locomotor confounds. Despite the well-documented anxiogenic role of CRH as a canonical stress neuropeptide, several recent findings suggest that extrahypothalamic CRH neurotransmission can also promote reward seeking and active coping, which are also considered as important stress coping strategies. Accordingly, CeA^{CRH} neurons regulate conditioned flight responses and active defensive behavior to weak threats (Fadok et al, 2017; Andraka et al, 2021). Moreover, tracking of neuronal activity with Ca2+ imaging revealed that $BNST^{CRH}$ neurons are

active during active struggling during restraint stress, further supporting the role of these cells in active coping (Luchsinger et al, 2021). BNST^{CRH} neurons may exert an anxiolytic effect by innervating the VTA and enhancing mesolimbic DA signaling in the NAc and in the PFC (Refojo et al, 2011; Lemos et al, 2012; Dedic et al, 2018). Interestingly, the BNST→ VTA pathway are activated by stress, but optogenetic stimulation of this circuit elicits real-time place preference. These contradictions suggest that this pathway exert a negative feedback on stress reactivity or promote active coping (Briand et al, 2010; Jennings et al, 2013). Acute stress also enhance CRH signaling in the NAc, which directly facilitates DA release and consequently drives exploratory and appetitive behavior (Lemos and Alvarez, 2020). In contrast, prolonged/severe stress diminish this effect of CRH signaling and shift behavior from reward seeking to aversion and anxiety (Lemos et al, 2012).

It is important to note that our results obtained from inhibition of BNST^{CRH} (and BNST^{SST}) neurons do not necessarily reflect the role of CRH (or SST)-mediated signaling per se. It is not uncommon that main neurotransmitter and neuropeptides can modulate behavioral response in distinct ways (Hartley et al, 2019). Generally, peptidergic neurotransmission requires high frequency stimulation and GABAergic signaling may prevails under baseline conditions. Unpredictable or chronic stress could induce plasticity related changes in CRH neurons condition and increased CRH release could influence the behavioral shift from anxiolytic to anxiogenic state (Dabrowska et al, 2013b; Partridge et al, 2016; Hammack et al, 2021). Further studies are now needed to clarify how peptidergic and local interneurons circuits interact within the BNST to gate the appropriate behavioral responses to specific aversive stimuli. Taken together, these results suggest that CRH neurons in the extended amygdala are recruited by aversive stimuli (e.g. restrain stress, predator odor exposure) and thus might be involved in negative valence monitoring (Lebow and Chen, 2016; Shackman and Fox, 2016). However, the functional consequences of CRH neuronal activation, especially in the BNST are less clear. In acute challenges, the activation of BNST^{CRH} neurons might promote active coping, risk assessment and stress-induced reward seeking (Lemos and Alvarez, 2020; Luchsinger et al, 2021). On the other hand, sustained CRH activity elicited by prolonged or severe stress might induce aversion, anhedonia and a depressive-like phenotype (Vranjkovic et al, 2017; Hu et al, 2020; Baumgartner et al, 2021). Our results underline the role of BNST^{CRH} neurons in behavioral response to ambiguous, mild innate threats. We cannot exclude the possibility that these cells might recruited by weak conditioned threats as well. It is

possible, that our inescapable shock paradigm prevented us to monitor the effect of $BNST^{CRH}$ manipulation on active defensive responses even with mild shock, which could manifest to a certain extent in the predator odor avoidance test.

Another contradictory finding in our experiments is the selective effects of CRH and SST modulation on cat urine vs. 2MT induced fear, respectively. It is not unusual that chemically distinct predator kairomones are processed by parallel olfactory pathways and brain regions (Pérez-Gómez et al, 2015), but unfortunately no whole brain data is available for cat urine and 2MT induced activity. Some studies suggest that odors associated with positive and negative valence, as well as odorants from cat fur vs. TMT are processed by different subnuclei at the level of BNST (Kobayakawa et al, 2007; Staples et al, 2008). Both TMT and cat urine-induced freezing can be blocked by muscimol injection to the BNST, however the BNST cell types involved in TMT and cat odor induced freezing are not known (Fendt et al, 2003; Xu et al, 2012). Some previous results showed that $BNST^{CRH}$ neurons are activated by the 2MT analogue TMT (Butler et al, 2016; Giardino et al, 2018), while others showed that TMT exposure only increase CRH levels in the CeA and PVN, but not in the BNST (Asok et al, 2013). Regarding cat urine induced fear, optogenetic stimulation of medial amygdalar inputs in the mouse pBNST increased the investigation time and frequency of cat urine. In line with our results, it is possible that these afferents mediate predator odor investigation by activating CRH neurons (Miller et al, 2019). Noteworthy, a major aversive compound of cat urine is felinine, which is a non-volatile molecule, thus might require closer and frequent investigation for the olfactory perception than 2MT (Apfelbach et al, 2015). In contrast to 2MT, cat urine did not elicit avoidance and reduced exploratory rearing in our experiments supporting the differential effects of different predator odorants. Previous reports also showed that 2MT promotes robust freezing and physiological changes (e.g. hibernation-like state) by acting on the trigeminal Trpa1+ neurons, which probably helps prey animals to stay unnoticed in the presence of predators (Wang et al, 2018; Matsuo et al, 2021). Taken together different defensive profile to neurochemically distinct predator odors are modulated by complex circuits also at the level of BNST.

4.5. BNST as a threat monitoring system- implications for threat ambiguity

Human imaging studies from the past few years highlighted the role of BNST in threat monitoring (Somerville et al, 2010; Avery et al, 2016; Lebow and Chen, 2016; Knight and Depue, 2019). Unfortunately, the precise role of BNST in threat evaluation has been investigated with limitations in rodents so far. The BNST receives inputs from several brain areas involved in attention, motivational behavior and arousal (e.g. PVT, LC, PFC, etc.), thus it is well-positioned to regulate threat induced vigilance (Winsky-Sommerer et al, 2005; Kodani et al, 2017; Hua et al, 2018). Complementary BNST circuits may mediate rapid behavioral switching between approach and avoidance adjusted to threat imminence. Indeed, it has been showed that BNST is engaged during the avoidance of unpleasant stimuli (e.g. heat and pain) (Minami, 2019; Kanai et al, 2022). Similarly, the CeA also mediates/suppresses nociception, freeze or flight responses and appetitive behaviors by local mutually inhibitory circuits (Fadok et al, 2017, 2018; Kim et al, 2017). Such rapid shifting between behavioral strategies could be essential during ambiguous challenges. In line with the fact that uncertain threats are particularly evocative for human BNST activity (Lebow and Chen, 2016), we found that BNST modulate innate fear responses to ambiguous, low intensity predator odor. Despite that the concentration of predator odors (kairomones) could carry ecologically relevant information about threat proximity for the rodents (Apfelbach et al, 2015), so far only a few study investigated the relationship between odor intensities and defensive responses (Wallace and Rosen, 2000; Takahashi et al, 2005; Pérez-Gómez et al, 2015). Much more studies investigated the BNST in the conditioned fear paradigm comparing predictable and unpredictable US-CS associations. A series of experiments from the laboratory of Stephen Maren have showed that threat imminence and predictability are key factors, which recruit BNST to conditioned fear (Goode and Maren, 2017; Goode et al, 2019, 2020). Their findings support our observation that BNST manipulation do not affect fear expression to CS which was associated with 100% shock probability (Goode et al, 2019). However, our results indicated that elevated BNST activity influence the fear memory formation of reliable US predictor cues as well. Although, in our fear conditioning paradigm we used forward conditioning (the CS co-terminated with the US), we cannot exclude the possibility that the random time periods between the CSs (ITI: ranged between 60–90 s) affected the predictability of the shock. Another interesting finding is that BNST

stimulation did not affect contextual fear memory formation here, but increased the freezing in the pre-tone period of the second cue recall day. To clarify if this effect represent a higher tendency for contextual fear generalization, we ran a separate experiment with $BNST^{SST}$ stimulation during acquisition, where mice were exposed to the context B for longer without CS presentation. The null effect in this experiment indicated that enhanced pre-tone fear in previous cohorts may origins from newly formed context B- CS associations. In confirmation with this, previous data showed that an initially neutral stimulus (such as a pure tone) could be a reinforcer for later fear learning due to prior aversive learning (Gewirtz and Davis, 2000; Rescorla, 2014). Although in our case, CS-shock reinforcement have never occurred in the safe context B, mice without extinguished fear memories could develop an aversion to the context B, which is associated with CS reminders. Again, ambiguity could be an important factor here, since the CS become an uncertain threat predictor in a new environment. On the other hand, these results may confirm that mice with BNST stimulation during acquisition exhibit higher aversion to later CS encounters that in turn promote second-order conditioning in this group.

In summary, our results show that BNST plays an important role in organizing defensive behavior during ambiguous settings. Based on this function, it is possible that higher BNST reactivity to uncertain challenges is a vulnerability factor for increased threat avoidance and fear generalization. Future research on BNST activity in animals with prior life adversities could contribute significantly to the understanding of the etiology of anxiety or trauma-induced disorders.

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5. Conclusion

Major conclusions of our studies are as follows:

I. BNST hyperactivity facilitates cue-dependent fear learning and memory consolidation:
   1. BNST modulates auditory fear consolidation, but not fear expression
   2. Contextual fear learning and cue discrimination is not affected by enhanced BNST activity
   3. Neuronal populations expressing somatostatin ($BNST^{SST}$), but not corticotropin-releasing hormone ($BNST^{CRH}$) can enhance auditory fear memory formation and increase freezing response to later reminders
   4. BNST stimulation during consolidation modulates the activity of the paraventricular nucleus, dorsal midline thalamic nuclei, ventral tegmental area and central grey.

II. BNST regulates innate fear responses to mild predator odor threat:
   1. BNST neurons primarily regulate innate fear responses to low intensity, ambiguous predator odor
   2. $BNST^{SST}$ neurons promote passive defensive responses and avoidance of low-intensity threat
   3. $BNST^{CRH}$ neurons facilitates non-defensive, exploratory behavior and exert an anxiolytic effect in response to weak threats

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6. Summary

Our results in male mice showed that the bed nucleus of stria terminalis (BNST) as a part of the extended amygdala is recruited during Pavlovian auditory fear conditioning. In contrast, the BNST is not activated during auditory fear recalls. Accordingly, we proved that increased BNST activity during fear acquisition contributes to enhanced encoding of auditory fear memories. We demonstrated that chemogenetic stimulation of BNST during fear memory consolidation per se is sufficient to increase cue-dependent fear without any acute impact on fear expression. Next, we selectively modulated molecularly identified neuronal populations of the BNST. We found that stimulation of somatostatin expressing ($BNST^{SST}$) neurons recapitulated the above fear memory-enhancing effect with additional tendency for CS-dependent fear generalization in a safe context.

The second part of our investigations aimed to investigate the role of these BNST related to innate fear. We differentiated the impact of BNST on innate fear by applying strong and weak threat conditions in a predator odor avoidance paradigm. We found that BNST predominantly regulates innate fear responses to weak or ambiguous threats only, with no impact under high-threat conditions. Regarding cell types, inhibition of $BNST^{SST}$ neurons increased the frequency of non-defensive behavioral elements, i.e. exploration of predator odor with reduced avoidance of potential danger, but only when predator odor was applied in low concentration. In contrast, inhibition of BNST neurons expressing corticotropin-releasing hormone ($BNST^{CRH}$) shifted the behavioral repertoire towards defensive responses (increased fear and avoidance) and decreased exploration under low predator risk. Our results also suggest that $BNST^{SST}$ and $BNST^{CRH}$ neurons may be differently recruited by chemically distinct kairomones (e.g. fox vs cat odor, respectively). Altogether, our data indicate that BNST regulates fear memory formation as well as threat evaluation under ambiguous settings.

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8. Bibliography of the candidate's publications

8.1. List of publications used for the thesis

Bruzsik B, Biro L, Zelena D, Sipos E, Szebik H, Sarosdi KR, Horvath O, Farkas I, Csillag V, Finszter CK, Mikics E, Toth M.

Somatostatin Neurons of the Bed Nucleus of Stria Terminalis Enhance Associative Fear Memory Consolidation in Mice. J Neurosci. 2021 Mar 3;41(9):1982-1995.

Bruzsik B, Biro L, Sarosdi KR, Zelena D, Sipos E, Szebik H, Török B, Mikics E, Toth M.

Neurochemically distinct populations of the bed nucleus of stria terminalis modulate innate fear response to weak threat evoked by predator odor stimuli. Neurobiol Stress. 2021 Oct 29;15:100415.

8.2. List of publications *not* used for the thesis

Biro L, Sipos E, **Bruzsik B**, Farkas I, Zelena D, Balazsfi D, Toth M, Haller J.

Task Division within the Prefrontal Cortex: Distinct Neuron Populations Selectively Control Different Aspects of Aggressive Behavior via the Hypothalamus. J Neurosci. 2018 Apr 25;38(17):4065-4075.

Mikics E, Toth M, Biro L, **Bruzsik** B, Nagy B, Haller J.

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Biro L, Toth M, Sipos E, **Bruzsik B**, Tulogdi A, Bendahan S, Sandi C, Haller J.

Structural and functional alterations in the prefrontal cortex after post-weaning social isolation: relationship with species-typical and deviant aggression. Brain Struct Funct. 2017 May;222(4):1861-1875.

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