Threat detection in the bed nucleus of stria terminalis: dissection of specific neuronal populations mediating innate and learned fear

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Background and aims

The extended amygdala (amygdalar nuclei and the bed nucleus of stria terminalis-BNST) has been shown to process sensorial-contextual information in order to interpret the valence of environmental stimuli (e.g. rewarding or threatening). Accordingly, it orchestrates defensive responses (both innate and learned fear), however, to date few studies investigated how BNST complements amygdalar functions, which has been described in details. To better understand how BNST circuits modulate certain aspects of fear responses, we aimed to identify how specific BNST pathways and cell types modulates fear-driven/defensive behaviors. Since growing evidence suggests that BNST modulates defensive responses for specific threats (e.g. anticipatory), we manipulated threat intensity and quality in systematic manner in our studies, with additional systematic testing of its impact on specific phases of fear learning, i.e. acute fear reaction, fear acquisition, consolidation, and recalls.

Since the feasibility of manipulation of specific inputs of BNST from amygdalar nuclei faced significant technical issues in Year 1 (retrograde virus infection, efficacy of virus expression, and lack of behavioral outcomes), we mapped amygdala-BNST innervation and focused on target cells from Year 2 (Decision point) in our further chemogenetic studies. It also gave us an opportunity to be more specific on target cell types. Accordingly, we tested major (GABAergic) and specific subpopulations, i.e. somatostatin (SST) and cortiocotropin releasing hormone (CRH) expressing neurons in a detailed manner as mentioned above (specific phases).

Results

First we mapped the activity of amygdala and BNST nuclei during innate fear expression (i.e. by predator odor) and learned fear recall (Pavlovian fear conditioning) with additional mapping of afferent inputs from the amygdala using choleratoxin B tracing in combination with c-Fos immunochemistry (labeling c-Fos as acute neuronal activation marker). As mentioned, we found limited impact of amygdala-BNST projection manipulation on both c-Fos activation and behavioral outcomes using projection-specific chemogenetic technique (retrograde canine adeno-

associated virus 2 combined with cre-dependent designer receptors exclusively activated by designer drugs (DREADD) virus constructs). It was likely due to underpowered amygdala-BNST pathway activation during innate and learned fear states as we showed that only 3-5% of CTB+neurons expressed c-Fos. At this point, we turned to local (BNST) modulation, where we could induce robust activation and inhibition of BNST circuits using stimulatory or inhibitory DREADDs, respectively. We showed this by both patch clamp recordings (effect of CNO on firing and membrane potential characteristics of DREADD+ neurons) and c-Fos immunohistochemistry (Fig.1A).

After confirming and validating our DREADD methodology, we aimed to dissect when BNST neurons are recruited and modulate fear responses (i.e. which phases). Accordingly, we assessed BNST activity during fear acquisition/conditioning and fear recall using c-Fos immunohistochemistry. We found that BNST exhibits strong activation during acquisition-consolidation phase, but not during fear expression/recall (Fig.1B). In line with this activity pattern, we found that chemogenetic stimulation of major proportion of BNST (GABAergic neurons) during fear acquisition or consolidation enhanced subsequent fear responses during recall tests, but it had no acute/direct impact when stimulation occurred during fear expression/recall (Fig.1C). We also showed that this effect could be recapitulated by cell type specific chemogenetic stimulation of SST, but not CRH, neurons of the BNST during fear memory consolidation (Fig.1D). Importantly, we also observed elevated fear generalization when SST+ neurons were stimulated as mice spent more time with freezing in the altered ‘Safe’ context (Fig.1D, BL block without cues). Importantly, these effects were again specific to fear consolidation since the same manipulation during fear recall resulted in no alteration of the fear response (similarly to GABAergic manipulations). These findings imply that BNST SST+ neurons are important modulators of fear learning processes and may contribute to fear generalization (core symptom of anxiety disorders).

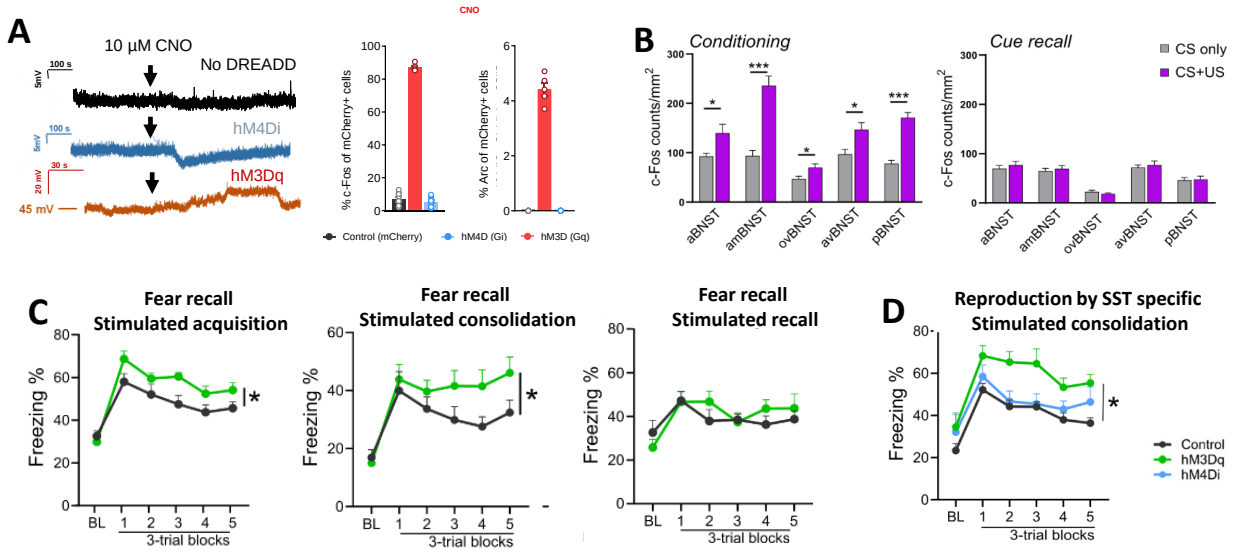

Figure 1. (A) Chemogenetic activation and inhibition of BNST neurons using DREADD constructs. Left panel shows electrophysiological recordings (depolarization and hyperpolarization), whereas right panel shows c-Fos expression in DREADD expressing neurons induced by stimulatory and inhibitory DREADDs (hM3Dq and hM4Di, respectively). (B) C-Fos expression in BNST nuclei during fear conditioning and fear recall. (C) Enhanced fear responses following chemogenetic activation/stimulation of GABAergic neurons of the BNST during three phases, i.e. stimulated during acquisition, stimulated during consolidation, and stimulated during actual recall. (D) Enhanced fear response following chemogenetic activation/stimulation of somatostatin (SST+) neurons of the BNST during consolidation. Inhibition (hM4Di) had no effect. *p<0.05, **p<0.01, ***p<0.001. aBNST, anterior; amBNST, anteromedial; avBNST, anteroventral BNST. CS: conditioned stimulus (auditory tone cue paired with foothshock), US: unconditioned stimulus (footshock), CNO: clozapine-N-oxide as activator of DREADD receptors, hM3Dq: stimulatory DREADD, hM4Di: inhibitory DREADD, Control: no active DREADD, only mCherry reporter protein.

Importantly, we also described the projection patterns of these BNST cell types (GABAergic, SST+ and CRH+) along the whole brain, showing highly similar projection profiles between cell types, i.e. minimal regional difference, and some difference in abundance across regions (Fig.2A). We also mapped how neuronal activity changes in these innervated target regions when BNST is chemogenetically activated during fear memory consolidation phase. We found significant changes (mostly enhanced activity) in relevant regions of the stress-fear circuitry (Fig.2B).

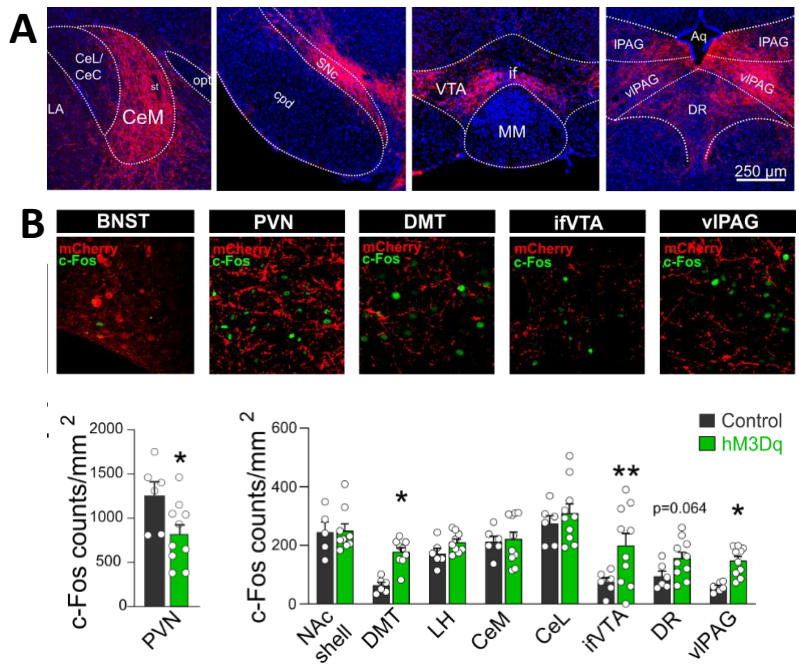

Figure 2. (A) Efferent projections of GABAergic BNST neurons. (B) C-Fos activation in the BNST and in its downstream regions following chemogenetic activation (hM3Dq) during the fear consolidation phase, when behavioral effects manifested. BLA, basolateral amygdala; CeL/CeC, central amygdala, lateral/capsular part; CeM, central amygdala, medial part; cpd, cerebral peduncle; DMT, dorsal midline thalamus; DR, dorsal raphe; fx, fornix; ic, internal capsule; LH, lateral hypothalamic area; MM, medial mammillary nucleus; NAc, nucleus accumbens; PVN, paraventricular hypothalamic nucleus; SNc, substantia nigra, pars compacta; vlPAG/lPAG, periaqueductal gray, ventrolateral/lateral part; ifVTA, ventral tegmental area, interfascicular nucleus. *p<0.05, **p<0.01.

In the second part of our project, we investigated innate fear modulation by BNST circuits. To establish a proper model with adjustable threat levels, we validated a scalable threat version of predator avoidance test by using and dosing the synthetic derivate of a fox urine molecule compound, 2-methyl-thiazoline (2MT). We defined proper doses to elicit low and high threat conditions and investigated fear responses under these circumstances, i.e. when responses dominated by inhibition and freezing vs. more anticipatory-like responses like avoidance alternating with approaches. We also quantified multiple behavioral variables to measure detailed defensive behavioral profile: active exploration-approach, rearing, avoidance of predatory stimulus, active and passive defensive reactions such as freezing and escape runs/jumps (Fig.3A). We also validated if cat odor (as an ecologically valid stimulus) can reliably induce innate fear, and assessed its behavioral profile. Indeed, cat odor induced significant anxiety-like behavior in mice, but with a different behavioral outcome compared to 2MT. It mostly lowered exploratory activity and increased freezing, although the effect size was rather small, i.e. weak stimulus resulting in a ‘cautious’-like phenotype.

We found differential effects of BNST inhibition between the two threat exposures, i.e. low and high doses of 2MT. Chemogenetic inhibition had an impact on defensive/fear responses only under low threat conditions, but no behavioral alterations was observed during high threat exposure (high 2MT dose). Another differential observation was that SST+ and CRH+ neurons exerted opposing effects on fear responses. Inhibition of SST neurons decreased fear by increasing odor approaches (Fig.3B). In contrast, inhibition of CRH neurons increased fear levels by decreasing approaches, but it occurred only in cat odor exposure test and not 2MT (Fig.3C). Latter may suggest that qualitative nature of predator odor as threatening stimuli can be also an important point besides its weaker stimulus nature as cat odor elicits less avoidance and defensive responses.

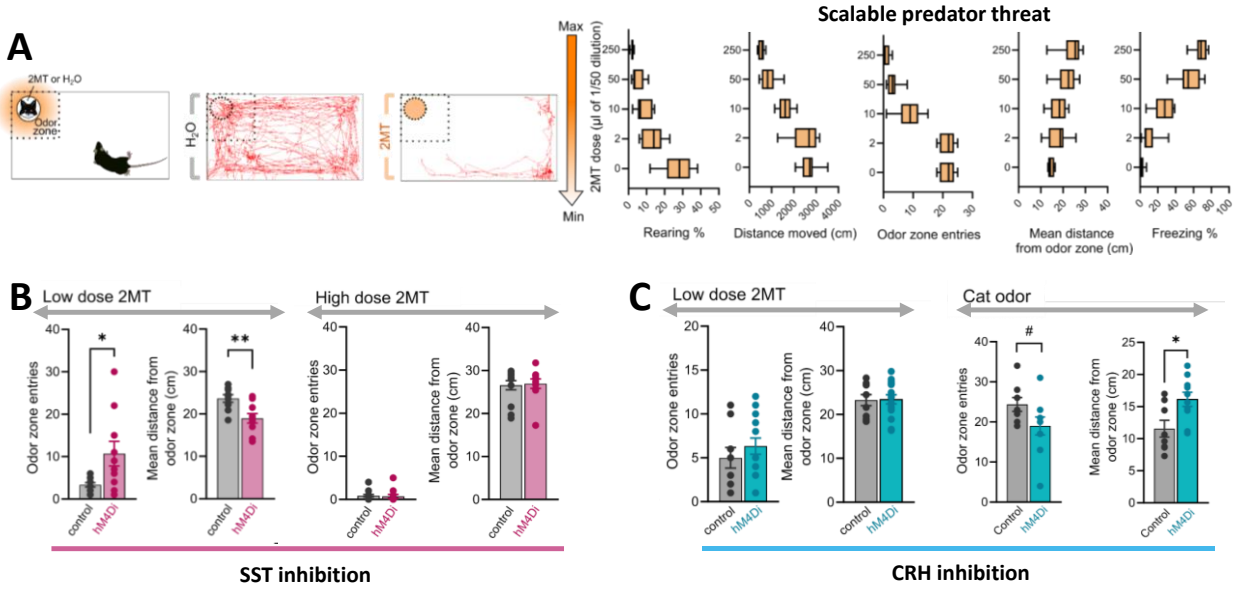

Figure 3. (A) Illustration of the predator odor test, and behavioral responses (five variables quantified) under different doses of 2MT (from 2 to 250µl). Low and high threat condition for further experiments was based on these exploratory analysis (10 µl and 250 µl were selected). (B) Chemogenetic inhibition of SST neurons reduced fear responses and increased approaches under low dose of 2MT, but it had no effect under high dose of 2MT. (C) Chemogenetic inhibition of CRH neurons had no effect on fear responses under 2MT exposure, but it significantly increased fear responses under exposure to cat odor (weaker stimulus).

These findings are in line with an articulated hypothesis in the literature that BNST is rather active under ambiguous threat conditions, when aversive stimuli are less imminent and less predictable. Our selective effects under low threat (low dose 2MT) support this as low predator

odor concentration represent lower predator/danger proximity and ambiguous situation between exploration vs avoidance drives. Similarly, fear responses under both conditioning and exposure to conditioned context (direct fear eliciting exposures) were not affected by BNST manipulations, whereas the consolidation and subsequent interpretation of fearful/threatening stimuli and contexts were modulated by BNST activity.

Summary

In the present project, we aimed to clarify how the bed nucleus of stria terminalis (BNST) modulates different types of fear responses, namely odor-related innate fear (predator) and learned forms. Using local chemogenetic manipulations, we found that BNST regulates innate fear in a stimulus-dependent and opposing manner. It can increase and decrease defensive fear responses via somatostatin (SST) and corticotropin releasing hormone (CRH) positive neurons, respectively. Importantly, this effect is manifested under low threat conditions only (low odor dose), not under imminent threat (high concentration of predator odor representing closeness of danger stimulus), which is line with previous findings reporting that BNST modulates anxiety-like responses when threat (anxiogenic stimuli) are rather ambiguous and danger is anticipatory.

In respect to modulation of learned fear, we found that again BNST has no direct effect on fear responses when evoking stimuli are closely present/imminent, i.e. during fear conditioning and exposure to conditioning context. However, BNST significantly modulated how fear experience (conditioning) is processed or interpreted. Namely, stimulation of BNST during or after fear conditioning (i.e. during consolidation of fear memory) increased subsequent fear recall and its generalization to safe contexts that is a major/core problem in fear/anxiety disorders. We also showed that this effect was mediated by SST neurons of the BNST.

Dissemination

We published our findings as two original research articles in high-impact journals. Our results on conditioned/learned fear manipulations have been published in the Journal of Neuroscience (Bruzsik et al, 2021, 10.1523/JNEUROSCI.1944-20.2020 ; grant acknowledged). Our results on innate fear responses in the predator odor paradigm have been published in the journal of Neurobiology of Stress (Bruzsik et al, 2021, 10.1016/j.ynstr.2021.100415 ; grant acknowledged).

We also presented our results as poster presentations on international conferences such as the 16th Annual Conference of the Hungarian Neuroscience Society in Debrecen, Hungary; the 2nd Munich Winter Conference on Stress in Garmisch-Patenkirchen, Germany; the Annual Meeting of the Society for Neuroscience (Chicago, October 2019); and the 49th Meeting of the European Brain and Behaviour Society (Lausanne, Hybrid-virtual meeting, September 2021), (grant acknowledged on all presentations).

Published articles based on this research:

1. Bruzsik B, Biro L, Sarosdi KR, Zelena D, Sipos E, Szebik H, Török B, Mikics E, Toth M: (2021) 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. doi: 10.1016/j.ynstr.2021.100415. IF: 5.441
2. Bruzsik B, Biro L, Zelena D, Sipos E, Szebik H, Sarosdi KR, Horvath O, Farkas I, Csillag V, Finszter CK, Mikics E, Toth M. (2021) Somatostatin Neurons of the Bed Nucleus of Stria Terminalis Enhance Associative Fear Memory Consolidation in Mice. J Neurosci; 41(9): 1982-1995. IF: 6.167