OPTOGENETIC STUDY OF THE PROJECTIONS FROM THE BED NUCLEUS OF THE STRIA TERMINALIS TO THE CENTRAL AMYGDALA

Nur Zeynep Gungor, Ryo Yamamoto, Denis Pare

Center for Molecular and Behavioral Neuroscience, Rutgers University-Newark
197 University Avenue, Newark, NJ

Running head: Connections from BNST to CeA

Correspondence should be sent to:
Denis Paré
Center for Molecular and Behavioral Neuroscience
Rutgers State University
197 University Ave.
Newark, NJ 07102, USA

Tel: 973-353-3251
Fax: 973-353-1255
Email: pare@andromeda.rutgers.edu

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It was proposed that the central amygdala (CeA), particularly its medial sector (CeM) generates brief fear responses to discrete conditioned cues, whereas the bed nucleus of the stria terminalis (BNST) promotes long-lasting, anxiety-like states in response to more diffuse contingencies. Although it is believed that BNST-CeA interactions determine the transition between short- and long-duration responses, the nature of these interactions remains unknown. To shed light on this question, we used a double viral strategy to drive the expression of Channelrhodopsin (ChR2) in BNST cells that project to CeA. Next, using patch clamp recordings in vitro, we investigated the connectivity of infected cells to non-infected cells in BNST and compared the influence of BNST axons on neurons in the medial and lateral (CeL) parts of CeA. CeA-projecting BNST cells were concentrated in the anterolateral (AL) and anteroventral (AV) sectors of BNST. Dense plexuses of BNST axons were observed throughout CeA. In CeA and BNST, light-evoked EPSPs accounted for a minority of responses (0-9% of tested cells); inhibition prevailed. The incidence of inhibitory responses was higher in CeM than in CeL (66 and 43% of tested cells, respectively). Within BNST, the connections from CeA-projecting to non-CeA targeting cells varied as a function of the BNST sector: 50% vs. 9% of tested cells exhibited light-evoked responses in BNST-AL vs. BNST-AV, respectively. Overall, these results suggest that via its projection to CeA, BNST exerts an inhibitory influence over cued fear and that BNST neurons projecting to CeA form contrasting connections in different BNST subnuclei.

**Keywords:** fear, anxiety, BNST, central amygdala
The central amygdala (CeA) and bed nucleus of the stria terminalis (BNST) are thought to play different roles in the genesis of negative emotional states. For instance, lesion and inactivation studies have revealed that CeA, but not BNST (LeDoux et al. 1988; Hitchcock and Davis 1991; Walker and Davis 1997; Duvarci et al. 2009), is critically involved in the expression of conditioned fear responses to discrete sensory cues. On the other hand, BNST lesions decrease light-enhanced startle (Walker and Davis 1997) and contextual fear (Sullivan et al. 2004; Duvarci et al. 2009), leading to the hypothesis that BNST generates prolonged anxiety-like states in response to more diffuse contingencies (Walker et al. 2009).

The properties that support the differing contributions of CeA and BNST to fear and anxiety are unknown. Indeed, their connectivity is nearly identical. For instance, BNST and CeA target the same brainstem structures (Hopkins and Holstege, 1978; Holstege et al. 1985), including those known to generate the behavioral (e.g. periaque ductal gray) and cardiovascular correlates (e.g. dorsal vagal nucleus and nucleus tractus solitarius) of negative emotional states. Moreover, they both receive glutamatergic inputs from the basolateral amygdala (BLA; Krettek and Price 1978; Pare et al. 1995; Dong et al. 2001a), midline thalamic nuclei (Vertes et al. 2015) and a similar array of cortical regions (McDonald et al. 1999). However, BNST projects to the paraventricular hypothalamic nucleus whereas CeA does not (Prewitt and Herman 1998; Dong et al. 2001b; Dong and Swanson 2006).

Given that BNST and CeA receive similar inputs and mostly target the same structures, what explains their differing contributions to the genesis of negative emotional states? It was proposed that direct interactions between BNST and CeA might be involved (Walker et al., 2009). In support of this possibility, CeA sends strong GABAergic projections to BNST (Weller and Smith 1982; Sun and Cassell 1993; Shin et al. 2008) and optogenetic activation of these
projections elicits IPSPs in target BNST cells (Li et al. 2012). BNST, particularly its anterolateral (BNST-AL) and anteroventral (BNST-AV) sectors, projects back to CeA (Sun and Cassell 1993; Dong et al. 2001b; Dong and Swanson 2004) and inhibition of BNST with muscimol infusions enhances conditioned fear to cues (Meloni et al. 2006). BNST projections to CeA are strongest to its medial sector (CeM) and lighter to its lateral part (CeL) (Sun and Cassell 1993; Dong et al. 2001b).

At present, it is unclear how BNST influences CeA, in part because the neurotransmitter used by CeA-projecting BNST cells has not been identified. While most BNST neurons are GABAergic, some glutamatergic cells are also present, especially in BNST-AV (Poulin et al. 2009), and little is known about their projection sites. Thus, to shed light on the impact of BNST inputs on CeA, we used a double viral strategy to selectively drive the expression of Channelrhodopsin (ChR2) in BNST cells that project to CeA. Then, using whole-cell patch clamp recordings in vitro, we investigated the influence of BNST on CeA neurons and assessed the connectivity of infected to non-infected BNST cells.
MATERIALS AND METHODS

Animals and virus injections

Procedures were approved by the Institutional Animal Care and Use Committee of Rutgers University, in compliance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services). Male Lewis rats (225-250 gr) were anesthetized with a mixture of isoflurane and oxygen and placed into a stereotaxic apparatus. Body temperature was kept at 37-38 °C. Atropine methyl nitrate (0.05mg/kg, i.m.) was administered to aid breathing. Betadine and alcohol was used to clean the scalp. Bupivacaine was injected in the region to be incised (0.125% solution, s.c.). Small burr holes were drilled above BNST (in mm, relative to bregma: AP: -0.36, ML: -1.6, DV: 6.8 and 7.4) and CeA (AP: -2.4, ML: 4.2, DV: 8.2 and 8.4). Nanoject II (Drummond Scientific Company) was used to make pressure injections (1 µL total – 0.5 µL at each DV level) at a rate of 9.6nL/5sec using glass pipettes pulled to an outer tip diameter of ~70 µm by a PE-22 puller (Narishige Instruments).

EF1a-DIO-hChR2(H134R)-EYFP was infused in BNST and EF1a-mCherry-IRES-WGA-Cre in CeA (Fig. 1A). AAV serotype 5 was used for both viruses. In the second virus, Cre recombinase is fused to the transcellular tracer protein WGA (wheat germ agglutinin), which is retrogradely transported from CeA, to neurons that project to CeA. The first virus (infused in BNST) drives the expression of ChR2 and EYFP (enhanced yellow florescence protein), but only in cells that express Cre, because they project to CeA. These viruses were obtained from University of North Carolina Vector Core, Chapel Hill, NC. After the injections, the scalp was sutured, a local antibiotic (Neosporin paste) was applied on the wound, and an analgesic was administered (Ketoprofen, 2 mg/kg, s.c. twice a day for three days). Rats were used for in vitro
experiments six weeks after the virus injections because pilot experiments had revealed that this survival time was optimal for high transgene expression.

Slice preparation

Rats were anesthetized with avertin (300 mg/kg, i.p.), followed by isoflurane. After abolition of reflexes, they were perfused with an ice-cold solution containing (in mM): 126 choline chloride, 2.5 KCl, 1 MgCl$_2$, 26 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 10 glucose. The brains were sliced with a vibrating microtome (350 µm thickness) while submerged in the same solution. The slices were then kept in an oxygenated chamber containing the same solution as above except for the substitution of 126 mM NaCl for choline chloride (pH 7.3, 300 mOsm). The temperature of the chamber was kept at 34°C for 20 min and then returned to room temperature. One hour later, a first slice was transferred to the recording chamber perfused with the latter oxygenated solution at 32°C (6 ml/min).

Electrophysiology

First, using fluorescence microscopy (Zeiss, Axioscope), we verified the location of the injection sites. A CeA injection site was considered accurate when mCherry expression covered the entire CeA, and did not spread to the neighboring BLA or medial amygdala. A BNST injection was considered accurate when EYFP expression was present in BNST and absent from adjacent structures. We defined BNST-AL as the lateral area above the anterior commissure, which corresponds to the oval, juxtacapsular and anterolateral subnuclei in the nomenclature of Ju and Swanson (1989). We defined BNST-AV as all the BNST subnuclei located below the
anterior commissure. Data from a particular animal was only considered when the injection sites met the above criteria and at least one responsive cell was recorded.

Whole-cell recordings were obtained under visual guidance using infrared differential interface contrast microscopy. We used 5–8 MΩ pipettes pulled from borosilicate glass capillaries. The intracellular solution contained (in mM): 130 K-gluconate, 10 HEPES, 10 KCl, 2 MgCl₂, 2 ATP-Mg, and 0.2 GTP-tris (hydroxymethyl) aminomethane, pH 7.2, 280 mOsm. The liquid junction potential was 10 mV with this solution. However, the membrane potential (Vm) values mentioned below were not corrected for the junction potential. We used a MultiClamp 700B Amplifier (Molecular Devices) and digitized the data at 10 kHz with a Digidata-1550 interface controlled by pClamp-10.3 (Molecular Devices).

To characterize the electroresponsive properties of the cells, we applied graded series of current pulses (±10 pA increments; 500 ms; 0.2 Hz). The input resistance of the cells was calculated from the voltage response to the lowest current injection. Blue light stimulation was provided by a 200-230 µm optic fiber patch cable coupled to a PlexBright Tabletop Blue LED module (Plexon, Dallas, TX). The light power density at the tip of the fibers was ~700mW/mm². The distance between the recording pipette and the fiber optic tip was ~200 µm. Post-synaptic potentials or currents were evoked from several membrane potentials. The IPSP or IPSC reversal potentials were calculated from the linear fit of fluctuations in IPSP or IPSC amplitudes as a function of membrane potential. Picrotoxin, CNQX disodium salt (6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt) and CPP (±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid) were used for abolishing GABA-A, AMPA and NMDA-dependent responses respectively. All drugs were obtained from Sigma (St. Louis, MO).
Blue light stimuli (2 or 5 ms) were generally applied at 0.05, 1, or 5 Hz. This range of stimulation frequencies was selected for the following reasons. First, we previously observed that most BNST-AL and BNST-AM neurons fire at low rates in awake freely moving rats: around 85% of the cells fired below 4 Hz and the group average was around 2-3 Hz (Haufler et al. 2013). Second, we aimed to minimize use-dependent depression of optogenetically-elicited synaptic responses, a phenomenon observed frequently at higher stimulation frequencies. However, given that the light-evoked PSPs we observed generally lasted less than 0.2 s and that BNST cells fire at low rates (Haufler et al., 2013), it is unlikely that the PSPs elicited by a single BNST axon undergo temporal summation during baseline activity. However, summation of PSPs generated by different input neurons on a common target most likely occurs.

**Imaging**

Immediately after the recordings, in vitro slices were fixed in 4% paraformaldehyde for 12 hours. The slices were then examined with Stereo Investigator v11 (MBF Biosciences) and Nikon Eclipse E800. The boundaries of BNST and CE were drawn on the brightfield images. The fluorescence images were superimposed on the brightfield images to assess virus diffusion. Confocal images were taken using Olympus Fluoview FV1000 and FV10-ASW v3. Four z-steps of 1.16 µm were collapsed to create the image stacks.

**Statistics**

We used Fisher exact tests to compare the incidence of responsive cells in different subnuclei. Unpaired t-tests were used to assess significance of differences between the electrophysiological properties of responsive and unresponsive cells.
RESULTS

Approach and database

We used a dual viral strategy to drive the expression of ChR2 and EYFP in BNST neurons that project to CeA (Fig. 1). To this end, EF1a-mCherry-IRES-WGA-Cre was infused in CeA (Fig. 1A1, red), causing the expression of Cre in neurons projecting to CeA. EF1a-DIO-hChR2(H134R)-EYFP was infused in BNST (Fig. 1A1, green), causing the expression of ChR2 and EYFP, but only in Cre-expressing BNST neurons. Six weeks after the virus infusions, coronal slices of the amygdala (Fig. 1A2) and BNST (Fig. 1A3) were prepared for whole-cell patch clamp recordings. Electrophysiological recordings from 13 animals are included in this data set. Two rats were used for anatomical observations only. Seven additional animals were excluded because of improper location of the virus injections. We obtained stable whole-cell recordings from 34 BNST-AL (4 EYFP+ and 30 EYFP− negative), 37 BNST-AV (3 EYFP+ and 34 EYFP− negative), 28 CeL, and 23 CeM neurons.

The physiological properties of BNST and CeA neurons did not appear to be have been altered by the dual viral strategy as they matched earlier descriptions from this and other laboratories (BNST: Hammack et al. 2007; Rodriguez-Sierra et al. 2013; CeA: Dumont et al. 2002; Lopez De Armentia and Sah 2004; Amano et al. 2012). Specifically, consistent with prior reports, in both BNST-AL and AV, fast inward rectifying (fIR) cells were rare (7 and 9% of recorded cells, respectively). Regular spiking (RS; AL: 57%, AV: 38%) and low-threshold bursting (LTB; AL: 37%, AV: 53%) cells prevailed in both BNST sectors, as previously reported (Hammack et al. 2007; Rodriguez-Sierra et al. 2013).

In CeA, we observed LTB, RS and late firing (LF) cells, as reported previously. In CeM,
most cells were LTB (43%) and RS (39%) neurons; LF cells accounted for a minority of the recordings (17%). These figures match the proportions seen in an earlier report in rats (Dumont et al. 2002). Also consistent with prior reports, in CeL there was a higher incidence of RS (43%) cells than LTB (10%) neurons. However, there was a higher incidence of LF cells (46%) in our sample compared to that reported in two prior studies (Dumont et al. 2002; Amano et al. 2012). However, another study (Lopez De Armentia and Sah 2004) also reported a higher incidence of this cell type in CeL.

Anatomical observations

Figure 1 provides representative examples of the distribution of EYFP+ neurons in BNST (Fig. 1B1) and of mCherry in CeA (Fig. 1B2). Higher power illustrations of labeled elements are provided in figure 1C. In all animals with successful injections (n=15), we observed that BNST to CeA connections originate from BNST-AL and BNST-AV. Invariably, very few EYFP+ cells were observed in BNST-AM. In the amygdala, EYFP+ axons were observed throughout CeA (Fig. 1C3,4). These observations are consistent with prior tracing studies (Sun and Cassell 1993; Dong et al. 2001b).

Local BNST connections

With the methods we used, BNST cells that project to CeA express EYFP and ChR2 (Fig. 1B,C1-2). EYFP- cells are assumed not to contribute projections to CeA. We first verified whether blue light stimuli could elicit firing in EYFP+ cells. As expected, blue light stimuli (5 ms) reliably elicited spiking in all tested EYFP+ cells (Fig. 2A, n=7). Trains of blue light stimuli (40 Hz train of 5 ms light stimuli for 1 sec) elicited spiking that persisted for the duration of the train (Fig. 2A1). In response to isolated light stimuli (5 ms at 2 Hz), all EYFP+ cells generated
action potentials, either single spikes, spike doublets, or high-frequency spike bursts (4-5 spikes at 150-300 Hz, Fig. 2A2; respectively 2, 2, and 3 of 7 tested cells).

Although none of the tested EYFP− BNST cells (n=64) showed light-evoked spiking, many showed sub-threshold synaptic responses (Fig. 2B). In BNST-AL, 15 of 30 tested EYFP− cells responded to blue light stimulation (Fig. 3A), implying they receive inputs from the BNST cells that project to CeA. In thirteen of these cells, blue light stimuli elicited IPSPs (Fig. 2B1); only two cells with excitatory responses were observed (Figs. 2B2, 3A). In BNST-AV, only three of 34 cells were responsive and all of these had inhibitory responses (Fig. 3B). The proportion of responsive EYFP− cells was significantly lower in BNST-AV than BNST-AL (Fig. 3E; Fisher exact test; p= .0003). The leftmost two columns of Table 1 summarize the properties of the responses evoked in BNST-AL and AV neurons. Although the incidence of responses was markedly lower in BNST-AV than AL, in both cases IPSP prevailed and exhibited similar properties, including a reversal potential around -77 mV.

Table 2 compares the electrophysiological properties of responsive and unresponsive cells in BNST-AL. At rheobase, responsive cells had a significant longer firing latency than unresponsive cells [unpaired t-test; t(28)= -2.87; p=.008]), despite having similar membrane time constant, input resistance, and spike threshold. This difference suggests that the distance between the soma and spike initiation zone is longer in responsive cells. In terms of the dynamics of current-evoked spiking, we observed no significant difference in the incidence of flR, LTB, and RS cells between responsive and unresponsive cells (Table 5; BNST-AL: χ² (2, N=30) = 4.29, p = 0.11).
BNST inputs to CeA

Blue light stimulation of BNST axons evoked synaptic responses in 53% of tested CeA cells (CeL, 12 of 28; CeM, 15 of 23). Figure 2C-D depicts examples of light-evoked synaptic responses observed in CeL and CeM neurons, respectively. As in EYFP+ BNST cells, most light-evoked responses were inhibitory in CeA cells (Figs. 2C,D2 and 3C,D). Excitatory responses were observed in only four of 51 tested CeA cells and in two of these, they were superimposed on IPSPs or IPSCs (Fig. 2D1).

Consistent with prior tracing studies indicating that BNST projections are stronger to CeM than CeL (Sun and Cassell 1993; Dong et al. 2001b), the incidence of CeA cells with inhibitory responses was significantly higher in CeM than CeL (Fig. 3E, Fisher exact test; \( p = .05 \)). However, compared to BNST neurons, light-evoked IPSPs had a significantly less negative reversal potential in CeA cells (CeA, -70.7 ± 1.8 mV; BNST, -78.2 ± 2.7 mV; unpaired t-test, \( t(33)=5.55, \ p=0.02 \)), suggesting that chloride homeostatic mechanisms differ in the two cell types or that the light-activated inputs end more distally in the dendritic tree of BNST than CeA cells.

The two rightmost columns of Table 1 compare the properties of light-evoked responses in CeL and CeM neurons. In both regions, IPSPs were more frequent than EPSPs. IPSPs had a similar latency, and reversal potential. Consistent with the higher incidence of inhibitory responses in CeM than CeL neurons, the amplitude of light-evoked IPSPs tended to be higher in CeM than CeL cells. However, the amplitude difference did not reach significance [unpaired t-test; \( t(22)=2.52; \ p =.13 \)].

To test whether BNST axons target a specific subset of CeA cells, we compared the physiological properties of responsive and unresponsive CeA cells (CeL, Table 3; CeM, Table
4). In both sectors of CeA, no differences were observed between responsive and unresponsive neurons. This statement was true of their passive properties, the amplitude and duration their action potentials, or the dynamics of current-evoked spiking. With respect to the latter point, we observed no significant differences in the incidence of RS, LTB, and LF cells (Table 5) between responsive and unresponsive CeL ($\chi^2 (2, N=28) = 0.8, p = 0.67$) or CeM neurons ($\chi^2 (2, N=23) = 2.84, p = 0.24$).

Last, we tested the pharmacological sensitivity of light-evoked synaptic responses in nine cells (Fig. 2B,C). Irrespective of the recording site, all inhibitory responses were abolished or nearly obliterated by picrotoxin (100 µM; n = 7) whereas excitatory responses were eliminated or largely reduced by CNQX and CPP (both 10 µM, n = 2).
DISCUSSION

This study examined the physiology of BNST projections to CeA. The significance of this question stems from behavioral studies indicating that BNST and CeA play different roles in negative emotional states and the hypothesis that direct interactions between them explain their differing functions. Overall, we found that BNST exerts a prevalently inhibitory influence over CeA and that BNST neurons projecting to CeA form contrasting intrinsic connections in different BNST subnuclei. Below, we consider the significance of these findings in light of previous studies about the regulation of fear and anxiety.

Impact of BNST inputs on CeA neurons

Prior tracing studies indicated that BNST projections to CeA mainly originate in BNST-AL and BNST-AV (Sun and Cassell 1993; Dong et al. 2001b). Replicating these findings, our dual viral strategy led to strong EYFP expression in numerous BNST-AL and AV neurons, but in very few BNST-AM cells. Earlier studies also noted that the majority of BNST neurons are GABAergic (Cullinan et al. 1993; Polston et al. 2004; Poulin et al. 2009) and that BNST projections are denser to CeM than CeL (Dong et al. 2001b). Consistent with this, we found that activation of BNST axons typically elicited inhibitory responses in CeA neurons and that their incidence was higher in CeM than CeL.

However, CeM also receives GABAergic projections from CeL (Pitkanen et al. 1997) raising the possibility that via CeL, BNST disinhibits CeM, opposing the inhibitory influence exerted by direct BNST inputs. A possible solution to this conundrum comes from recent reports indicating that different subsets of CeL neurons reciprocally inhibit each other and form contrasting connections with CeM (Viviani et al. 2011; Ciocchi et al. 2010; Haubensak et al.
2010; Li et al. 2013). For instance, CeL cells expressing somatostatin (SOM⁻) send inhibitory projections to CeM whereas SOM⁺ neurons do not (Li et al. 2013). While it is currently unclear whether BNST axons form differential connections with SOM⁻ and SOM⁺ neurons, a preferential innervation of SOM⁺ cells by BNST axons would, via the disinhibition of SOM⁻ cells, potentiate the impact of direct BNST projections to CeM (Fig. 4A).

Although GABAergic cells prevail in BNST, some glutamatergic cells are also present, mostly in BNST-AV (Poulin et al. 2009). However, there is little data on their projection site(s). Some target the ventral tegmental area (Georges and Aston-Jones 2001, 2002; Kudo et al. 2012; Jennings et al. 2013) but it remains unclear whether they also project to CeA, although earlier observations hinted to this possibility (Sun and Cassell 1993). Supporting this, we observed light-evoked glutamatergic responses in CeA cells, but their incidence was very low. Nevertheless, it is possible that GABAergic and glutamatergic BNST neurons are targeted by different inputs allowing for their independent activation. In this context, it should be noted that optogenetic activation of glutamatergic or GABAergic BNST-AV neurons elicits anxiogenic or anxiolytic effects, respectively (Jennings et al. 2013). In light of the low incidence of EPSPs in BNST-CeA connections, it seems unlikely that the negative emotional states evoked by activation of glutamatergic BNST-AV cells depend on BNST-CeA connections.

While optogenetic methods are well suited to characterize neuronal connections and their role in behavior, it has so far proven difficult to study neuropeptide release driven by opsin activation. Although the light-evoked responses we observed were abolished by ionotropic receptor antagonists, neurons in BNST-AL and CeL express many neuropeptides (Gray and Magnuson 1987, 1992; Woodhams et al. 1983) that likely modulate fast inhibitory and excitatory neurotransmission (McElligott and Winder 2009; Kash et al. 2015). For example, Francesconi et
al. (2009) demonstrated that CRF impaired the long-term potentiation of intrinsic excitability in juxtacapsular BNST-AL neurons, mimicking the consequences of drug withdrawal. This effect may lead to a reduced inhibitory control of CeA, contributing to the negative emotional state experienced during drug abstinence.

Implications for the regulation of fear and anxiety by the extended amygdala

It is widely accepted that CeM is the main output station of the amygdala for conditioned fear. Nearly all brainstem projections of the amygdala stem from CeM (Hopkins and Holstege 1978; Veening et al. 1984; Petrovich and Swanson 1997). In particular, CeM is the sole source of amygdala projections to the periaqueductal gray, which generates freezing (LeDoux et al. 1988), the most common index of conditioned fear. Moreover, CeM neurons fire at high rates during fear-inducing conditioned stimuli (Ciocchi et al. 2010; Duvarci et al. 2011) and optogenetic activation or inactivation of CeM triggers or impairs freezing, respectively (Ciocchi et al. 2010).

According Walker et al. (2009), upon receiving threat signals from the BLA, CeM would immediately activate downstream brainstem effectors, generating brief fear reactions in response to discrete and short lasting conditioned cues. By contrast, BNST activation, in addition to requiring BLA afferents, would depend on CRF inputs from CeL (Sakana et al. 1986, 1987; Lee and Davis 1997). Consequently, BNST would be activated more slowly and persistently, explaining its involvement in the generation of long-lasting anxiety-like states. This model also proposed that once activated, BNST inhibits CeM. In support of this, it was reported that intra-BNST infusion of muscimol enhanced cued conditioned fear (Meloni et al., 2006).

While our findings are consistent with the idea that BNST inhibits CeM, how BNST also generates anxiety-like states is unclear. Indeed, at odds with the above model, activation of
GABAergic BNST-AV cells induces place preference and anxiolytic effects (Jennings et al. 2013). The anxiolytic influence of BNST-AV extends to the negative regulation of the hypothalamic-pituitary-adrenal axis (Radley and Sawchenko, 2011, 2015). Similarly, BNST-AL, which only contains GABAergic neurons, also suppresses fear and anxiety. For instance, BNST-AL stimulation reduces corticosterone levels (Dunn 1987) and BNST-AL lesions increase stress-induced gastric erosions (Henke 1984). Furthermore, most BNST-AL cells fire at higher rates in low compared to high fear states (Haufler et al. 2013). Last, CGRP infusions in BNST, which elicit anxiety-like responses, increase inhibitory tone in BNST-AL (Gungor and Pare 2014).

Overall, these results suggest that BNST-AL and the GABAergic cells of BNST-AV act as a fear/anxiety suppressing system. Opposite to this, stimulation of BNST-AM increases circulating corticosterone levels (Dunn 1987) and most BNST-AM cells fire at higher rates in high compared to low fear states (Haufler et al. 2013). However it is unclear how BNST-AM would promote fear and anxiety as it contributes sparse projections to the amygdala (Bienkowski and Rinaman 2013). A hypothalamic locus of action is possible (Gross and Canteras 2012) but remains to be tested.

One neglected point in the Walker et al. (1997) model is the importance of GABAergic CeA projections to BNST, which mainly arise from CeL and are especially dense in BNST-AL (Krettek and Price 1978; Weller and Smith 1982; Sun et al. 1991; Sun and Cassell 1993; Bienkowski and Rinaman 2013). A prior study reported that CeA axons elicit IPSPs in 81% of BNST-AL cells (Li et al. 2012) while we observed that 57% of CeM cells receive inhibitory inputs from BNST. Furthermore, we found that the GABA-A reversal potential was 8 mV more negative in BNST than CeA neurons. Given the higher incidence of inhibitory connections from CeA to BNST than in the opposite direction and the more negative reversal potential of IPSPs in
BNST cells, it is likely that CeA gains the upper hand in reciprocal BNST-CeA interactions, determining the intensity of negative emotional responses (Fig. 4B).

*BNS T cells projecting to CeA form contrasting connections in different BNST subnuclei*

Besides BNST projections to CeA, our dual viral strategy presented us with the opportunity to examine the intrinsic BNST network. Indeed, EYFP-expressing (that is, CeA-projecting) neurons were intermingled with EYFP\(^-\) (that is, non-CeA projecting) cells, allowing us to study the connections from the former to the latter. Previously, a glutamate uncaging study had concluded that the intrinsic BNST-AL and AV networks were similar (Turesson et al. 2013). However, the projection sites of recorded cells were unidentified. Thus, the null hypothesis in our experiments was that the connections formed by CeA-projecting neurons with EYFP\(^-\) cells would be similar in the two regions. In contrast, we observed a marked difference between the incidence of responsive EYFP\(^-\) neurons in BNST-AL and AV. In particular, activation of CeA-projecting cells elicited synaptic responses in 50% EYFP\(^-\) BNST-AL cells compared to 9% in BNST-AV. This is surprising given that the glutamate-uncaging study had found that projections from BNST-AL to AV were stronger than in the opposite direction (Turesson et al. 2013). These results suggest that in BNST-AV at least, neurons with different projection sites form different intrinsic connections. A challenge for future studies will be to extend these analyses to other projection sites of BNST while considering the transmitter phenotype of the cells.
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The authors declare that they have no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS:

NZG and DP designed the study. NZG and RY conducted the experiments. NZG did the data analysis. NZG and DP wrote the manuscript.


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FIGURE LEGENDS

Figure 1. (A) Experimental design. (A1) Dual viral strategy for selectively driving ChR2 expression in BNST neurons that project to the central amygdala. Six weeks after the virus infusions, coronal slices of the amygdala (A2) and BNST (A3) were prepared for whole-cell patch clamp recordings. Blue light stimuli were applied through optic fibers positioned at proximity of the recorded cells. We studied the impact of inputs from CeA-projecting BNST neurons onto CeA cells and other BNST cells that do not project to CeA. (B1) EYFP and ChR2-expressing BNST neurons that project to CeA. (B2) Amygdala neurons expressing mCherry. Insets in B1 and B2 indicate the largest (solid colored lines) and smallest (dashed colored lines) region containing cells expressing EYFP and ChR2 (green, B1) or mCherry (red, B2), respectively. The white numbers in B mark the approximate location of the higher power pictures provided in C. (C1,2) EYFP+ BNST cells. (D1-2) EYFP+ BNST axons (green) in close proximity to mCherry+ CeA neurons (red). Scale bars in B and C correspond to 300 and 20 µm, respectively. Asterisks in B2 mark artifacts. Abbreviations: AC, anterior commissure; AL, anterolateral sector of BNST; AM, anteromedial sector of BNST; AV, anteroventral sector of BNST; B, nucleus basalis; BL, basolateral nucleus of the amygdala; BM, basomedial nucleus of the amygdala; CeA, central nucleus of the amygdala; CeL, lateral sector of CeA; CeM, medial sector of CeA; GP, globus pallidus; IC, internal capsule; LA, lateral septum; OT, optic tract; POA, preoptic area; Th, thalamus; Str, striatum; VP, ventral pallidum.

Figure 2. Blue light evoked responses in BNST and CeA neurons. (A) Direct responses in ChR2-expressing BNST neurons that project to CeA. (A1) Train of light stimuli (bottom) reliably eliciting spikes (top). (A2) At a lower frequency, each light stimulus (bottom) elicits a
spike burst (top). **Inset** on right illustrates a light-evoked spike bursts with an expanded time base. **(B)** Examples of light-evoked responses in two different EYFP^* BNST-AL neurons. **(B1)** Light-evoked activation of CeA-projecting BNST axons elicits IPSPs in a BNST-AL cell. Responses were elicited from different membrane potentials (numbers on left in mV). Picrotoxin (PTX, 100 µM) application abolished the response (top) consistent with a mediation by GABA-A receptors. **(B2)** A rare case of light-evoked EPSP (current clamp mode). Light-evoked EPSP (Control) is abolished by addition of CNQX (10 µM) and CPP (10 µM). **(C)** Example of light-evoked responses in a CeL neuron. Voltage-clamp mode (holding potential of -50 mV). Light-evoked IPSC (Control) is abolished by picrotoxin (+PTX). **(D)** Examples of light-evoked responses in two different CeM neurons (voltage-clamp mode; holding potential of -55 mV). **(D1)** Mixed excitatory-inhibitory response. Addition of CNQX and CPP to the perfusate abolishes the EPSC. Subsequent application of picrotoxin almost completely abolishes the residual response. **(D2)** Apparently pure inhibitory response to 40 Hz train of blue light stimuli. The response amplitude decreases during the train of light stimuli.

**Figure 3.** Incidence and types of responses elicited by blue light stimuli in BNST-AL (**A**), BNST-AV (**B**), CeL (**C**), and CeM (**D**) neurons. The schemes on the left of each pie chart illustrate the pathway stimulated and recording sites examined. In the pie charts, grey indicates the percentage of unresponsive cells whereas red, blue and purple indicate the percentages of neurons with IPSPs, EPSPs, or mixed responses, respectively. **E.** Proportion of cells with inhibitory responses in the different regions examined.
Figure 4. Hypothetical schemes of BNST-CeA interactions. (A) Differential innervation of SOM$^+$ and SOM$^-$ CeL neurons by BNST axons. The direct inhibitory effects of BNST projections to CeM neurons are increased by the inhibition of SOM$^+$ CeL cells, leading to the disinhibition of SOM$^-$ CeL neurons. (B) Overall organization of the reciprocal BNST-CeA connections.
### Table 1. Properties of light-evoked responses in BNST and CE neurons. Values are means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>BNST-AL</th>
<th>BNST-AV</th>
<th>CeL</th>
<th>CeM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPSP incidence</td>
<td>13/30</td>
<td>3/34</td>
<td>10/28</td>
<td>15/23</td>
</tr>
<tr>
<td>IPSP latency (ms)</td>
<td>4.31 ± .3</td>
<td>3.07 ± .9</td>
<td>5.51 ± 1.02</td>
<td>4.1 ± .58</td>
</tr>
<tr>
<td>IPSP amplitude (mV)</td>
<td>-2.94 ± .52</td>
<td>-5.12 ± 3.07</td>
<td>-1.77 ± .38</td>
<td>-3.27 ± .75</td>
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<tr>
<td>IPSP reversal (mV)</td>
<td>-78.6 ± 3.3</td>
<td>-76.79 ± 2.9</td>
<td>-68.8 ± 1.4</td>
<td>-71.59 ± 2.54</td>
</tr>
<tr>
<td>EPSP incidence</td>
<td>2/30</td>
<td>0/34</td>
<td>2/28</td>
<td>2/23</td>
</tr>
<tr>
<td>EPSP latency (ms)</td>
<td>7.27 ± .03</td>
<td>N/A</td>
<td>2.17 ± .03</td>
<td>4.08 ± .98</td>
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<tr>
<td>EPSP amplitude (mV)</td>
<td>1.72 ± .74</td>
<td>N/A</td>
<td>2.8 ± .87</td>
<td>3.41 ± 1.35</td>
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### Table 2. Physiological properties of responsive and non responsive BNST-AL neurons. Values are means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Responsive cells (n=15)</th>
<th>Unresponsive cells (n=15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>-62.9 ± 2.4</td>
<td>-62.5 ± 1.9</td>
<td>.91</td>
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<tr>
<td>Input resistance (MΩ)</td>
<td>706.9 ± 51.3</td>
<td>658.3 ± 50.6</td>
<td>.5</td>
</tr>
<tr>
<td>Time constant (ms)</td>
<td>46.9 ± 5.3</td>
<td>51.6 ± 6.9</td>
<td>.6</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>15.3 ± 2.4</td>
<td>18.7 ± 2.2</td>
<td>.31</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-43.1 ± 1.1</td>
<td>-45.5 ± 1.7</td>
<td>.24</td>
</tr>
<tr>
<td>Spike latency (ms)</td>
<td>94.7 ± 12.6</td>
<td>50 ± 9.2</td>
<td>.008*</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>81.4 ± 4.3</td>
<td>78.2 ± 3.9</td>
<td>.59</td>
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<tr>
<td>Spike duration at half amplitude (ms)</td>
<td>0.62 ± .06</td>
<td>0.69 ± .06</td>
<td>.41</td>
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<tr>
<td>Firing rate at rheobase (Hz)</td>
<td>5.1 ± 0.7</td>
<td>4.7 ± 0.7</td>
<td>.69</td>
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### Table 3. Physiological properties of responsive and non responsive CeL neurons. Values are means ± SEM.

<table>
<thead>
<tr>
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<th>Responsive cells (n=12)</th>
<th>Unresponsive cells (n=16)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>-62.3 ± 2.3</td>
<td>-61.4 ± 1.1</td>
<td>.72</td>
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<tr>
<td>Input resistance (MΩ)</td>
<td>506.2 ± 84.5</td>
<td>413.4 ± 26.8</td>
<td>.25</td>
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<td>Time constant (ms)</td>
<td>60 ± 6.9</td>
<td>54.8 ± 5.5</td>
<td>.56</td>
</tr>
<tr>
<td>Rheobase (pA)</td>
<td>35 ± 8.2</td>
<td>33.1 ± 3.4</td>
<td>.82</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-43.2 ± .9</td>
<td>-42.8 ± 1</td>
<td>.81</td>
</tr>
<tr>
<td>Spike latency (ms)</td>
<td>67.6 ± 14.6</td>
<td>118.8 ± 28</td>
<td>.15</td>
</tr>
<tr>
<td>Spike amplitude (mV)</td>
<td>94.1 ± 2.2</td>
<td>89.5 ± 2</td>
<td>.13</td>
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<tr>
<td>Spike duration at half amplitude (ms)</td>
<td>0.6 ± .05</td>
<td>0.56 ± .04</td>
<td>.7</td>
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<tr>
<td>Firing rate at rheobase (Hz)</td>
<td>6.8 ± .7</td>
<td>7.6 ± 1.3</td>
<td>.64</td>
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</table>
TABLE 4. Physiological properties of responsive and non responsive CeM neurons. Values are means ± SEM.

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<th>Responsive cells (n=15)</th>
<th>Unresponsive cells (n=8)</th>
<th>p</th>
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<tbody>
<tr>
<td>Resting potential (mV)</td>
<td>-62.8 ± 2.4</td>
<td>-64.38 ± 2.8</td>
<td>.68</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>486.3 ± 82.5</td>
<td>487.7 ± 67.6</td>
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<tr>
<td>Time constant (ms)</td>
<td>53.5 ± 8.5</td>
<td>46.8 ± 14.9</td>
<td>.67</td>
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<tr>
<td>Rheobase (pA)</td>
<td>31.7 ± 5.8</td>
<td>37.1 ± 4.7</td>
<td>.52</td>
</tr>
<tr>
<td>Spike threshold (mV)</td>
<td>-42.3 ± 1.2</td>
<td>-42.6 ± 1.3</td>
<td>.87</td>
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<tr>
<td>Spike latency (ms)</td>
<td>108.3 ± 29.9</td>
<td>83.6 ± 35.5</td>
<td>.61</td>
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<tr>
<td>Spike amplitude (mV)</td>
<td>92.7 ± 2.2</td>
<td>96.4 ± 2.6</td>
<td>.3</td>
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<tr>
<td>Spike duration at half amplitude (ms)</td>
<td>.5 ± .03</td>
<td>.42 ± .04</td>
<td>.16</td>
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<tr>
<td>Firing rate at rheobase (Hz)</td>
<td>4.8 ± .7</td>
<td>6 ± 1.9</td>
<td>.5</td>
</tr>
</tbody>
</table>

TABLE 5. Incidence of different physiological cell types among responsive (r) and unresponsive (nr) BNST (top) and CeA (bottom) neurons.

<table>
<thead>
<tr>
<th></th>
<th>RS</th>
<th>LTB</th>
<th>fIR</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>r</td>
<td>nr</td>
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<tr>
<td>BNST-AL</td>
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<td>6</td>
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<tr>
<td>BNST-AV</td>
<td>12</td>
<td>1</td>
<td>11</td>
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<table>
<thead>
<tr>
<th></th>
<th>RS</th>
<th>LTB</th>
<th>LF</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>r</td>
<td>nr</td>
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<tr>
<td>CeL</td>
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<td>7</td>
</tr>
<tr>
<td>CeM</td>
<td>9</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviations: RS, regular spiking; LTB, low-threshold bursting; fIR, fast inward rectifying; LF, late firing.
Direct responses

Synaptic responses

A1

B1

BNST-AL

-60

+PTX

-60

-70

-80

-90

V_hold=

B2

BNST-AL

Control

-56

+CNQX and CPP

V_hold=

C

CeL

Control

+PTX

D1

CeM

V_hold=

D2

CeM

V_hold=
Cells with Inhibitory responses (%)

n's = 28

**No response**  
**EPSPs**  
**IPSPs**  
**Mixed**