

**THE ROLE OF THE BED NUCLEUS OF THE STRIA TERMINALIS
IN FEAR AND ANXIETY**

by

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ABSTRACT OF THE DISSERTATION

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

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The bed nucleus of the stria terminalis (BNST) is a poorly understood brain structure. Most prior behavioral studies used experimental manipulations that affected the entire BNST. However recent research has made it clear that different BNST subdivisions, as well as different cell types within these divisions, serve different functions. Also, although the roles of BNST and central amygdala (CeA) in negative emotions are well established, little is known about their influence on each other. In this thesis, using in vitro whole cell patch clamp recordings, I investigated (1) how a particular peptide, called calcitonin gene related peptide (CGRP), regulates anxiety by acting on the neurons in the anterolateral BNST (BNST –AL), (2) BNST's projections to CeA, (3) the characteristics of different cell types in anteroventral BNST (BNST-AV) and their modulation by noradrenaline (NA) .

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Preface

An abbreviated version of Chapter I is in press as a review paper (Gungor and Pare, 2016, *The Journal of Neuroscience*). Chapter III and IV are published (Gungor and Pare, 2014, *The Journal of Neuroscience*; Gungor et al., 2015, *Journal of Neurophysiology*). The work in Chapter IV resulted from collaboration between Dr. Ryo Yamamoto and myself. Chapter V is in preparation.

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List of Abbreviations

ACTH:	adrenocorticotrophic hormone
BNST:	bed nucleus of the stria terminalis
BNST-AL:	anterolateral sector of BNST
BNST-AM:	anteromedial sector of BNST
BNST-AV:	anteroventral sector of BNST
BL:	basolateral nucleus of the amygdala
BLA:	basolateral complex of the amygdala
BM:	basomedial nucleus of the amygdala
CeA:	central amygdala
CeL:	central lateral nucleus of the amygdala
CeM:	central medial nucleus of the amygdala
CGRP:	calcitonin gene-related peptide
CORT:	corticosterone
CRF:	corticotropin releasing factor
CRF-R1:	CRF receptor type 1
CS:	conditioned stimulus
D1R:	dopamine receptor type 1
DVN:	dorsal vagal nucleus
EA:	extended amygdala
EPM:	elevated plus maze
EYFP:	enhanced yellow fluorescent protein
fIR:	fast inward rectifying, Type III cell

fMRI:	functional magnetic resonance imaging
LTB:	low threshold bursting, Type II cell
LTP:	long-term potentiation
NA:	noradrenaline
NTS:	nucleus tract solitarius
HPA-axis:	hypothalamic-pituitary-adrenal axis
mPFC:	medial prefrontal cortex
PBS:	phosphate buffer saline
PTSD:	post-traumatic stress disorder
PVN:	paraventricular nucleus of hypothalamus
PVT:	paraventricular nucleus of thalamus
RS:	regular spiking, Type I cell
sEPSC:	spontaneous excitatory postsynaptic currents
sIPSC:	spontaneous inhibitory postsynaptic currents
ST:	stria terminalis
STEP:	striatal-enriched protein tyrosine phosphatase
US:	unconditioned stimulus
VGAT:	vesicular GABA transporter
VGlut2:	vesicular glutamate transporter type 2
vlPAG:	ventrolateral periaqueductal grey
VTA:	ventral tegmental area

CHAPTER I

INTRODUCTION

1.1 Significance

We frequently experience fear and anxiety in everyday life. Although unpleasant, fear and anxiety serve a protective function by preparing us to develop strategies to avoid or cope with potentially dangerous or disadvantageous events. However, in some individuals, anxiety responses become maladaptive and debilitating, leading to anxiety disorders. Anxiety disorders are the most prevalent mood disorders, affecting about 28% of the population in the USA (Kessler et al., 2005).

The first step to understand and address anxiety disorders is to characterize the brain circuits that underlie normal anxiety. Because anxiety/fear responses to real or perceived threats are essential to survival, it is commonly accepted that they depend on brain circuits that evolved early and were conserved until the appearance of humans (LeDoux, 2015). Thus, neuroscientists frequently use animal models in fear/anxiety research. Indeed, accumulating evidence show that similar brain networks underlie fear and anxiety behaviors in animals and humans (for review, Phelps and LeDoux, 2005).

The bed nucleus of the stria terminalis (BNST), along with amygdala, is a crucial node in the cerebral networks that regulate anxiety. Using animal models, the experiments described in this thesis work aimed to expand our knowledge of the physiology of BNST and its role in the regulation of anxiety.

1.2 Overview of the introduction

In Section 1.3, I will review behavioral studies that implicate BNST in negative emotional states. First, I will go over the human literature as recent functional magnetic resonance imaging (fMRI) studies demonstrate the involvement of BNST in anxiety. Furthermore, there is some preliminary evidence that BNST is hyperactive in people afflicted with an anxious temperament. Second, I will focus on rodent studies that provide evidence for BNST's involvement in the genesis of responses to unconditioned threats, learned contextual fear and responses to other long-lasting threatening stimuli. I will also present a popular model positing that BNST regulates long-duration, sustained, anxiety-like responses to diffuse environmental contingencies. However, in counterpoint to this theory, I will present data indicating that BNST also processes short-lasting aversive or rewarding cues.

In Section 1.4, I will review anatomical data pertaining to the structure and connections of BNST. This material will lead me to propose a simpler BNST parcellation than originally proposed. In summary, I will divide anterior BNST into three sectors: the anterolateral (AL), which includes the oval nucleus, as well as the anteromedial (AM) and the anteroventral (AV) sectors.

In Section 1.5, I will describe the electrophysiological cell types present in BNST, as well as in vivo spontaneous firing patterns.

BNST neurons express a variety of neuropeptides and they receive a dense peptergic innervation. In Section 1.6, I will focus on one neuropeptide, corticotrophin releasing factor (CRF), because it is thought to play a critical role in the regulation of

anxiety, in part via its effects on BNST. In the General Discussion, I will discuss the results of Chapter III in relation to CRF-expressing cells in BNST.

Section 1.7 focuses on NA inputs to BNST as NA effects in BNST-AV are thought to generate negative emotional states associated with drug addiction.

In section 1.8, I will review the evidence indicating that BNST is functionally heterogeneous.

In section 1.9, I will describe the relation between BNST and the central nucleus of the amygdala (CeA). These two structures have been hypothesized to play different roles by Walker et al., 2009. In particular, CeA generates fear responses to discrete cues whereas BNST mediates long lasting anxiety-like responses to diffuse contingencies. I will discuss this model and review evidence for and against it.

Finally, in Section 1.10, I briefly introduce the 3 data chapters.

1.3 What is the function of BNST? Behavioral evidence from anxiety research

1.3.1 Human and non-human primate studies

In humans, BNST is small and not easily identifiable. Given the low spatial resolution power of fMRI, the small size of BNST prevented many researchers from studying it. Nevertheless, accumulating evidence supports BNST's involvement in anxiety in humans. In these studies, research designs are mostly inspired from rodent research (see details below, Walker et al., 2009) and aim to elicit BNST activity by using uncertain and distant treat stimuli (Fox et al., 2015; Avery et al., in press).

For example, long anticipatory periods before seeing negative pictures (Grupe et al., 2013b), as well as unpredictable shocks (Alvarez et al., 2011) evoke activity in

BNST. In rhesus monkeys, BNST activity, measured by positron emission tomography, increases during freezing when the animal encounters unfamiliar individuals (Kalin et al., 2009). Also, BNST activity in adolescent monkeys is predictive of later trait anxiety (Fox et al., 2008; Oler et al., 2009).

In addition, there is some evidence that BNST is hyperactive in humans that suffer from greater anxiety. Relative to controls, BNST is more active in spider phobics before exposure to spider pictures (Straube et al., 2007) and tracks shock proximity in healthy individuals with greater anxiety (Somerville et al., 2010). However, these results should be approached with caution. Indeed, another study found that patients with generalized anxiety disorder and healthy controls show similar levels of BNST activity during a monetary loss game (Yassa et al., 2012).

1.3.2 Rodent studies

a. Conditioned responses to short-lasting stimuli versus unconditioned responses:

Pavlovian fear conditioning is one of the most common paradigms used in the laboratory to assess fear. In this assay, an initially neutral stimulus, such as a tone (conditioned stimulus-CS) is paired with a noxious unconditioned stimulus (US), usually a mild foot shock. As a result, the animal develops fear responses, such as behavioral freezing, to the CS presented alone. In another design, called fear-potentiated startle paradigm, fear is assessed by measuring startle responses elicited by loud noise bursts. After CS-footshock pairings, noise bursts presented during the CS elicit higher startle responses. Alternatively, the noise bursts can be presented during a naturally threatening environment, like in a brightly lit room.

Early studies investigated BNST function using the acoustic startle paradigm and classical fear conditioning. They demonstrated that electrolytic (Hitchcock and Davis, 1991; Gewirtz et al., 1998) and chemical (LeDoux et al., 1988) lesions of BNST do not affect fear responses to short lasting CSs. However, blocking glutamatergic transmission in BNST abolishes light enhanced startle (Walker and Davis, 1997). Similarly, infusions of muscimol, a GABA-A agonist, reduces fear responses to a natural treat, TMT, a component of fox feces (Fendt et al., 2003). Later on, BNST inactivation was shown to suppress fear to cat urine (Xu et al., 2012) and alarm pheromone induced defensive behaviors (Breitfeld et al., 2015). Of note, both light and predator odors are unconditioned stimuli.

The elevated plus maze (EPM) is another assay that explores unconditioned behaviors. Commonly used to assess anxiety in rodents, EPM consists of open and enclosed arms. BNST inactivation increases exploration of the open arms, which indicates a reduction in anxiety levels (Waddell et al., 2006; Kim et al., 2013, however, see Treit et al., 1998). Overall, these results show that BNST activity mediates unconditioned negative states in response to naturally threatening environments.

b. Conditioned responses to long-lasting stimuli:

In addition to mediating unconditioned fear states, there is also evidence that BNST is involved in conditioned fear responses to contexts. During Pavlovian fear conditioning, alongside the tone CS, the context where the training takes place also acquires the ability to elicit fear responses. This form of learning is called contextual fear conditioning. BNST lesions impair both acquisition (Poulos et al., 2010) and the recall of this association (Sullivan et al., 2004; Duvarci et al., 2009). BNST lesions also block

corticosterone (CORT) level elevations that are observed after contextual conditioning (Gray et al., 1993; Sullivan et al., 2004).

One aspect of context that is distinct from a short CS is that exposure to a context is, simply, longer. Studies that utilized tone CSs lasting minutes found that BNST lesions block fear responses to long CSs as well (Waddell et al., 2006; Walker et al., 2009). Interestingly, recent evidence showed that pre-training lesions of BNST do not affect contextual conditioning when animals are exposed to a context only 1 minute before footshocks (Hammack et al., 2015). Thus BNST might be involved in mediating responses to long duration aversive stimuli, not contextual conditioned stimuli per se.

Overall, the findings about unconditional treats, context and long-lasting cue conditioning led to the theory that BNST mediates long-duration, sustained, anxiety-like responses to diffuse environmental contingencies (Walker et al., 2009). This explanation is well accepted in the BNST literature and guides not only animal, but also human research. Indeed, anxiety can be distinguished from fear, despite the psychological and physiological commonalities between these two emotions. Fear-eliciting cues, like tone CSs in Pavlovian fear conditioning, signal treats with a high certainty of occurrence, imminence and distinctiveness. On the other hand, anxiety arises from the anticipation of uncertain events (Grupe and Nitschke, 2013a). Studies that showed BNST's involvement in aversive responses used CSs like contexts associated with a footshock, bright lights or predator odors, all of which are distal and unpredictable treats.

c. Sensitization:

Having made the distinction between fear and anxiety, next we need to state that anxiety is not a unitary phenomenon. A basic distinction can be made between the

associative and non-associative aspects of anxiety. After fear learning, reactions elicited by re-encounters with the learned cues (associative processes) and a state of hyperarousal unrelated to specific environmental cues (sensitization) are both part of the anxious experience (Siegmund and Wotjak, 2007a). There is some evidence that different brain regions might play a role in these two processes. For instance, dorsal hippocampus inactivation blocks fear responses to contexts that had been associated with footshocks. However, sensitization is unaffected by this procedure (Siegmund and Wotjak, 2007b).

In the acoustic startle paradigm, sensitization is operationally defined as the increase in startle observed after repetitive footshocks in contexts not associated with footshocks. In addition, animals can exhibit elevated startle responses in non-associated contexts simply because they are similar to fear-associated contexts. This phenomenon is called generalization. To disambiguate sensitization from context generalization, Davis and Walker (2014) showed that sensitization decays over time. In contrast, generalized associative startle responses persist through a learning-test delay period. Importantly, blocking AMPA receptors in BNST not only blocks contextual fear conditioning, but also sensitization. These results suggest that BNST is involved in both the associative and non associative aspects of anxiety. Given that most recall tests of contextual fear are done a day after fear conditioning (Sullivan et al., 2004; Duvarci et al., 2009), when the animals are still sensitized, BNST's role in contextual/long stimuli fear learning might be partially explained by sensitization.

d. Evidence that BNST also regulates responses to short CSs.

In contrast with earlier studies that showed BNST's lack of involvement in fear responses to short and distinct CSs, recent evidence suggests that BNST might process

short lasting stimuli as well. Unit recordings in BNST during Pavlovian fear conditioning demonstrated that about 20% of the cells modified their firing rates during short-tone CS (Haufler et al., 2013). In support to this, one study that showed muscimol injections in BNST *potentiates* fear-conditioned startle (Meloni et al., 2006b).

Direct evidence of short-cue processing by BNST is also found in the addiction literature. Indeed, BNST has been implicated in drug addiction (Wenzel et al., 2011; 2014), withdrawal and reinstatement (Aston-Jones and Harris, 2004; Koob, 2009; 2010). In drug dependence experiments, animals are trained to lever press for drug self-administration when cued with the presentation of a light and tone compound stimulus. Cue presentations are followed by time-out periods where lever presses lead to no drug delivery. Following self-administration, animals undergo an extinction period where lever responses decrease but do not completely disappear. Next, in cue-induced reinforcement, re-introduction of the light-tone CS results in an increase of lever presses even though no drug is delivered in this part of the experiment.

In this paradigm, BNST inactivation reduces short-cue induced reinstatement (Buffalari and See, 2010), implicating BNST in short-cue processing. Furthermore, Reisiger et al. (2014) showed that long term nicotine dependency established by cue induced nose pokes leads to long-term plasticity (LTP) in the infralimbic cortex (IL)-BNST pathway. LTP induction was not observed in yoked animals that were exposed to the same experimental procedures and consumed the same amount of nicotine but did not form the cue-nicotine association. Critically, LTP was diminished only in animals that went through extinction training but not in the group that abstained for the same duration. The results of this important study suggest that synaptic plasticity in BNST occurs only

with cue-reward association. Furthermore, the IL stimulation parameters that led to LTP in BNST also increased the number of nose pokes in the time-out period. Thus, animals expected drugs not only during, but also after the cessation of the cues. The phenomena where the specificity of the cue-outcome association is compromised and expectancy of outcome expands to similar cues is called stimulus generalization.

BNST's role in stimulus generalization was also shown in a study that utilized differential fear conditioning paradigm (Duvarci et al., 2009). In this protocol, two CS are presented, only one of which (CS+) signals a subsequent foot shock. Animals with high discrimination of the CS+ and CS- also exhibited low levels of anxiety in the EPM. Interestingly, BNST lesions reduced both anxiety levels in the EPM and improved discrimination of the CS+ and CS-. Consequently, similar to the generalization seen in the Reisiger et al. (2014) study, these results suggest that BNST activity promotes generalization of fear to unthreatening cues.

A recent study (Goode et al., 2015) investigating BNST's role in the renewal and reinstatement of extinguished fear to short conditioned cues demonstrated another unexpected aspect of short cue processing by BNST. Fear responses to a CS can be extinguished by repetitive CS presentations without subsequent footshocks. However, the memory of the CS as threatening stimulus is not erased by extinction training. Indeed, after extinction training, presentation of an unsignaled footshock in the conditioning context reinstates fear of the CS (reinstatement). Moreover, outside the extinction training context, the CS still elicits fear responses (renewal).

Recently, Goode et al. (2015) found that BNST inactivation does not affect renewal. Given the earlier literature showing that fear responses to short CSs persist after

BNST lesions, this result is not surprising. However, unexpectedly, Goode et al. (2015) also found that BNST lesions block reinstatement. The authors speculated that unsignaled shocks presented in the conditioning context after extinction training “refreshed” the shock-context association. Then, the enhanced shock-context association would reactivate the context-CS association. Presumably, inactivating BNST just before the reinstatement test prevents reactivation of the footshock-context-CS association.

1.3.3 SUMMARY

BNST inactivation interferes with defensive responses elicited by unconditional treats, fear conditioned contexts and long-lasting cues. A well-accepted theory states that BNST regulates long-duration, sustained, anxiety-like responses to diffuse environmental contingencies (Walker et al., 2009). This view guides not only animal but also human research. However, other results suggest that BNST is also involved in short cue processing, although in some cases, it is not *necessary* for developing cue evoked responses. Last, short cue representations in BNST play a role in stimulus generalization.

1.4 **Anatomical organization of BNST**

Based on cytoarchitectural, histochemical and hodological similarities between the amygdala and BNST, De Olmos et al. (2004) proposed the concept of extended amygdala (EA). They distinguished two sectors in the EA: lateral and medial. Later, the distinction between medial and lateral BNST was updated with anterior and posterior partitions, defined by the stria terminalis. This change was motivated by studies showing that the anterior and posterior parts of BNST have different embryonic origins (Bayer,

1987) and accumulating evidence that the posterior BNST is involved in reproductive behaviors (Simerly, 2002).

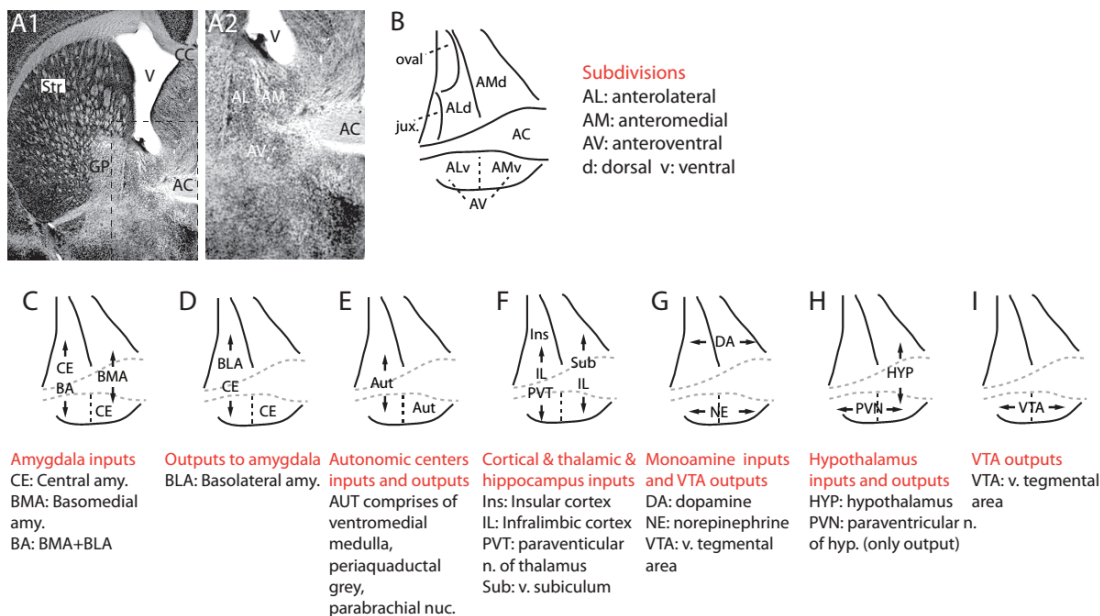


Figure 1.1 Structure and the connections of BNST. **A.** Anterior BNST at low (1) and higher (2) magnification. Coronal sections processed to reveal NeuN immunoreactivity. **B.** Nomenclature. **C-I.** Connections. Abbreviations: Str, Striatum; V, Ventricle; CC, Corpus collosum; GP, Globus pallidus; AC, Anterior commissure.

Subsequently, two prominent anatomical studies from the laboratories of L. Swanson and C. Saper divided the anterior BNST into numerous subnuclei according to cytoarchitectural criteria (Ju and Swanson, 1989; Moga et al., 1989). These studies pointed out that BNST is not a homogenous structure and hinted that some subnuclei, like the oval nucleus in anterolateral BNST, might carry out distinct functions. However, the two parcellations differed in many ways. Moreover, the proposed BNST subnuclei cannot be reliably distinguished in live tissue, nor can they be targeted individually in the live

animal due to their small size. More importantly, many of the adjacent subnuclei form similar connections. Thus, for practicality, we subdivide BNST into three larger subdivisions, namely BNST-AL, BNST-AM and BNST-AV, based on differences in connectivity. Table 1.1 shows the correspondence between our nomenclature, Swanson's and Saper's.

Table 1.1 Correspondence of proposed BNST subnuclei with the previous parcellations

Proposed nomenclature	Ju and Swanson (1989)	Moga, Saper, Gray (1989)
BNST-AL (dorsal)	Oval Juxtacapsular Anterolateral Anterior portions of rhomboid	Dorsolateral Anterolateral Juxtacapsular Anterior part of the posterior lateral
BNST-AM (dorsal)	Anterodorsal	Anteromedial
BNST-AV	Anteroventral Fusiform Parastrial Dorsomedial Subcommissural zone Dorsolateral Magnocellular	Ventromedial Ventrolateral Preoptic Parasagittal

As summarized in figure 1.1 and detailed below, the division of BNST into anterolateral and anteromedial sectors is supported by their contrasting connections. Indeed, in contrast with BNST-AL, BNST-AM receives no CeA inputs, it does not project to brainstem autonomic centers, and it is innervated by largely distinct cortical areas and receives different thalamic inputs. Last, relative to BNST-AL, BNST-AM contributes massive hypothalamic projections. *It is important to note that BNST-AL*

includes the lateral part of BNST-AV. Similarly, BNST-AM includes the medial part of BNST-AV. However, other factors suggest that BNST-AV should be considered as a separate subnucleus. Below, I first explain why BNST-AL and BNST-AM should be considered as two distinct sub-regions of BNST. Then, I discuss BNST-AV.

1.4.1 Connections of BNST-AL and BNST-AM

a. Connections with the amygdala (see section 1.9.1 for amygdala anatomy):

BNST-AM contributes negligible projections to CeA (Bienkowski and Rinaman, 2013), BNST-AL and BNST-AV project strongly to the medial sector of central amygdala (CeM), and lightly to the lateral sector (CeL) (Sun and Cassell, 1993; Dong et al., 2001b). In the opposite direction, CeA projections to BNST mostly originate in CeL and mainly target BNST-AL, sparing the juxtacapsular region (Dong et al., 2001a). CeM appears to contribute minimally to BNST's innervation (Bienkowski and Rinaman, 2013; Sun and Cassell, 1993) and it is clear that BNST-AM receives much weaker CeA inputs than BNST-AL (Krettek and Price, 1978; Weller and Smith, 1982; Sun et al., 1991).

Whereas BNST-projecting CeA neurons are GABAergic (Sun and Cassell, 1993), only glutamatergic cells of the basolateral nucleus of the amygdala (BL) innervate BNST (Kim et al., 2013). However, the three BLA nuclei contribute differentially to this pathway, with the lateral amygdala having no projections, and the basal nuclei contributing prominently (Krettek and Price, 1978; Weller and Smith, 1982; Dong et al., 2001a). Although both basal nuclei project to BNST's three sectors, their projections are complementary. The basomedial nucleus (BM) preferentially targets BNST-AM and the medial part of BNST-AV whereas the basolateral (BL) preferentially projects to BNST-

AL (Dong et al., 2001a). With respect to the latter projection, there is disagreement in the literature with some reporting no BLA projection to the oval region of BNST-AL (Dong et al., 2001a) and others describing a strong projection to the same site (Krettek and Price, 1978). BNST projections to BLA are sparse and originate from BNST-AL (Swanson and Cowan, 1979; Dong et al., 2000; 2001b; 2003; 2004).

b. Connections with brainstem autonomic centers: In contrast with BNST-AM, BNST-AL and BNST-AV form reciprocal connections with CeA and autonomic centers of the brainstem (Dong et al., 2003; Shin et al., 2010; Bienkowski and Rinaman, 2013). BNST-AL and BNST-AV are heavily and reciprocally connected with the ventromedial medulla (nucleus tract solitarius, NTS and dorsal vagal nucleus, DVN) (Ricardo and Koh, 1978; Schwaber et al., 1982; Sofroniew, 1983; Gray and Magnuson, 1987), the parabrachial nucleus (Norgren, 1976; Saper and Loewy, 1980; Panguluri et al., 2009) and the ventrolateral periaqueductal grey (vlPAG) (Gray and Magnuson, 1992; Shin et al., 2010; Bienkowski and Rinaman, 2013).

c. Cortical and thalamic connections: Dygranular and agranular insula projections target dorsal BNST-AL (McDonald et al., 1999; Reynolds et al., 2005; Shin et al., 2010; Bienkowski and Rinaman, 2013). The paraventricular nucleus of the thalamus (PVT) projects to BNST-AL (Li and Kirouac, 2008). In contrast, BNST-AM is not innervated by these cortical areas or by PVT. Another source of cortical inputs to BNST is IL. IL inputs are distributed among BNST-AL, AM and AV (Dong et al., 2001a; Vertes, 2004).

d. Inputs from the hippocampus: A major input to BNST-AM arises from the ventral subiculum (Cullinan et al., 1993), but BNST-AM does not reciprocate this

connection (Dong et al., 2006a; 2006b; 2006c). In contrast, BNST-AL is devoid of hippocampal inputs.

e. Connections with the hypothalamus: Compared to BNST-AL, BNST-AM is heavily connected with the hypothalamus. BNST-AM projects to almost all subregions of hypothalamus, except the core of the ventromedial nucleus (Kita and Oomura, 1982b; Dong et al., 2006a; 2006b; 2006c). So far, few studies have examined hypothalamic projections to BNST. They showed that the VMH, the preoptic area and the anterior medial hypothalamus innervate BNST-AM (Conrad and Pfaff, 1976a; 1976b; Saper et al., 1976; Swanson, 1976; Swanson and Cowan, 1976). Hypothalamus innervation from BNST-AL targets the paraventricular nucleus of the hypothalamus (PVN), lateral hypothalamus and dorsomedial hypothalamus (Kita and Oomura, 1982a; 1982b; Dong et al., 2000; 2001b; 2003; 2004). Projections from the hypothalamus back to BNST-AL are weak with one exception, the PVN (Conrad and Pfaff, 1976a).

1.4.2 Differences between dorsal and ventral BNST

a. Monoamine inputs: All anterior BNST sectors receive dopaminergic inputs from the ventral tegmental area (VTA) and vlPAG (Shin et al., 2010) and as well as NA axons from the locus coeruleus and other brainstem NA nuclei (most prominently A1 and A2) (Woulfe et al., 1988). However, dopaminergic inputs to dorsal BNST are stronger (Freedman and Cassell, 1994; Meloni et al., 2006a). Furthermore, the NA innervation of BNST-AV is one of the densest in the entire brain (Fallon and Moore, 1978; Forray et al., 2000).

b. Projections to VTA: Most BNST projections to the VTA arise from BNST-AV. (Georges and Aston-Jones, 2002; Dong et al., 2001b)

c. *Projections to PVN:* Most BNST projections to the PVN arise from BNST-AV (Sawchenko and Swanson, 1983; Moga and Saper, 1994).

1.4.3 Special case of the oval nucleus in BNST-AL

The oval nucleus (terminology of Swanson, 1989) is located dorsally in BNST-AL. Although its connections are similar to the rest of the BNST-AL, there are significant differences. For instance, BL and BM do not project to the oval nucleus (Dong et al., 2001a). On the other hand, it receives denser projections from the insula than the rest of BNST-AL (McDonald et al., 1999). Moreover, most of BNST's peptidergic neurons, in particular CRF-positive cells, are found in the oval nucleus (Gray and Magnuson, 1987; Ju et al., 1989; Moga et al., 1989). For these various reasons, the oval nucleus stands out as a special component of BNST-AL.

Most of the anatomical studies reviewed in this section were conducted in rats. Although we assume that the same connectivity patterns are present in mice, they might not. For instance, the rat's oval nucleus covers about one third of BNST-AL, whereas in mice, the oval nucleus and BNST-AL are almost synonymous.

1.4.4 Cell types and intrinsic connections

Most BNST neurons are GABAergic cells with small to medium somata that resemble medium spiny cells of the striatum (Day et al., 1999; McDonald, 1983; Poulin et al., 2009; Sun and Cassell, 1993). Some glutamatergic cells (positive for vesicular glutamate transporter-VGluT2) are also present in BNST-AV and AM (Poulin et al., 2009). Most intrinsic connections are inhibitory, although some excitatory transmission also exists (Turesson et al., 2013).

Anterograde tracing studies showed that there are connections from dorsal BNST to BNST-AV (Dong et al., 2001b). There is also evidence for connections from BNST-AL to BNST-AM (Kim et al., 2013). Using glutamate uncaging (GU), Turesson et al. (2013) also showed that BNST-AL to BNST-AM connections are more frequent than connections from BNST-AM to BNST-AL. Furthermore, BNST-AV cells receive inputs from both BNST-AM and BNST-AL, however they do not project back to dorsal sectors.

1.4.5 SUMMARY OF BNST ANATOMY

BNST-AL is connected with BL, CeA, autonomic centers, insular cortex and PVT. In contrast, BNST-AM forms connections with the BM, hippocampus and hypothalamus. We consider BNST-AV, which is comprised of ventral BNST-AL and ventral BNST-AM, as a distinct subnucleus because of its heavy NA innervation and connections with PVN and VTA. Intrinsic information flow in BNST is predominantly from dorsal BNST to ventral BNST.

1.5 **Physiological properties of BNST neurons**

A number of studies have characterized BNST neurons according to their electro-responsive properties in rats (Rainnie, 1999; Egli and Rainnie, 2003; Hammack et al., 2007; Rodriguez-Sierra et al., 2013). In summary, there are three main cell types. Type I (or regular spiking, RS) cells exhibit a regular firing pattern in response to depolarizing current injection and often display a depolarizing sag during membrane hyperpolarization, indicative of HCN currents. When hyperpolarized, Type II (or low threshold bursting, LTB) cells also display a depolarizing sag. However, in contrast to Type I cells, Type II cells exhibit burst firing in response to positive current injections

applied from hyperpolarized levels, due to the activation of the low-threshold calcium current I_T . Type III (or fast inward rectifying, fIR) cells fire regularly in response to positive current injection. When hyperpolarized, they exhibit a fast inward rectification indicating that they express an inwardly rectifying potassium current $I_{K(IR)}$.

Importantly, in BNST-AL, these three cell types are accurately clustered according to the expression of mRNA for different ion channel subunits, as revealed by single-cell RT-PCR (Hazra et al., 2011). Furthermore, Type III cells express mRNA for CRF (Dabrowska et al., 2013a). In line with the immunohistochemical studies, CRF is only expressed in the oval nucleus, Type III cells are selectively found in the oval nucleus (Rodriguez-Sierra et al., 2013). In contrast to this, patch recordings in a transgenic mouse line, CRF-tomato, showed that CRF neurons do not belong to a consistent electrophysiological cell type (Silberman et al., 2013). However, it is important to note that CRF expression in this mouse line does not closely match the distribution of CRF positive neurons, as assessed by immunohistochemistry.

Although the distribution of cell types differs between BNST subregions, overall Type II cells are the most numerous and Type III cells the least common. One rare cell type identified by Rodriguez-Sierra et al. (2013) fires spontaneously and these cells are only found in BNST-AV.

In vivo, the firing rates of BNST cells are very low. Casada and Dafny (1993) showed that median firing rate of rat BNST is 0.125 Hz (0-7.5 Hz range) under urethane anesthesia. One third of the recorded cells exhibited no spontaneous firing at all and their presence was detected by glutamate infusions. In behaving rats, both Henke (1984) and Haufler et al. (2013) reported average baseline firing rates below 4 Hz (Haufler et al.,

2013-range 0-32 Hz). Jennings et al. (2014) reported a range of 0.5 to 25 Hz in mice BNST-AV.

In accord with the low spontaneous firing rates of BNST neurons, no tonic glutamatergic release was observed in BNST (Forray et al., 1999; 2004). Although the reason for the low spontaneous firing of BNST cells has not been thoroughly investigated, the inhibitory influence of NA is a good candidate. Indeed, Forray et al. (1997) showed that NA is tonically released in BNST. Moreover, NA inhibits glutamatergic transmission through $\alpha 2$ adrenergic receptors in most BNST neurons (Casada and Dafny, 1993; Forray et al., 1999; Egli et al., 2005; for more details, see section 1.7)

1.6 Corticotrophin releasing factor in BNST

BNST is very rich in neuropeptides. For instance, BNST-AL contains one of the densest CRF immunoreactive cell populations outside the PVN. (Sakana et al., 1987; Phelix and Paull, 1990). Also present are neurotensin, enkephalin, somatostatin and vasoactive intestinal peptide (Gray and Magnuson, 1987; Ju et al., 1989; Moga et al., 1989). Furthermore, BNST receives axons immunopositive for various peptides from extrinsic sources (Woodhams et al., 1983).

CRF, also known as corticotrophin releasing hormone, is one of the major stress hormones/transmitters in the brain and controls the hypothalamic-pituitary-adrenal (HPA)-axis. In response to stress, CRH is released from neuroendocrine PVN cells into the anterior pituitary gland where it stimulates the release of adrenocorticotrophic hormone (ACTH). ACTH is released into bloodstream, stimulating the adrenal cortex of the

adrenal gland to produce glucocorticoid hormones (corticosterone in rodents). The role of CRF outside of the HPA axis is less well defined, but has received a lot of attention.

1.6.1 Characteristics of local CRF neurons

The oval nucleus contains a dense CRF cell population. Also, some CRF cells are located in BNST-AV (Sakana et al., 1987; Phelix and Paull, 1990). These cells contribute to BNST's projections to various brainstem and neuroendocrine centers such as the PBN (Moga et al., 1989; Panguluri, 2009), dorsal vagal nucleus (Gray and Magnuson, 1987), central gray (Gray and Magnuson, 1992), PVN (Moga et al., 1994) and VTA (Rodaros et al., 2007; Vranjkovic et al., 2014). These cells are not only projection cells: they make synaptic contacts within the BNST. In the dorsal part of BNST-AL, some neuromodulators like dopamine and NA act through the activation of local CRF cells to regulate synaptic transmission (Kash et al., 2008; Silberman et al., 2013).

As I mentioned in Section 1.5, single cell RT-PCR studies showed that all type III and some type II cells express CRF mRNA. The GABAergic cell marker GAD67 is co-expressed in all dorsal CRF cells. (Dabrowska et al., 2013a). On the other hand, information on the ventral CRF cell population is limited. For instance, we do not yet know whether these cells show distinct electrophysiological properties. Furthermore, no data is available on the co-expression of CRF and GABAergic cell markers in BNST-AV. However, Radley et al. (2009) showed that selective ablation of GABAergic cells in BNST-AV left CRF mRNA levels intact; suggesting that the CRF cells of BNST-AV might be glutamatergic.

1.6.2 Activation of oval nucleus/CRF neurons

Stressors like footshocks increase CRF mRNA expression in both dorsal and ventral BNST, indicating that CRF cells are activated during stress (reviewed in Daniel and Rainnie, 2016). Also, repeated restraint stress facilitates LTP of glutamatergic transmission selectively in Type III cells in a STEP (striatal-enriched protein tyrosine phosphatase) dependent manner (Dabrowska et al., 2013b). STEP regulates synaptic plasticity by inactivating several kinases and dephosphorylating NMDA and AMPA subunits. In BNST, it is selectively expressed in CRF cells (Dabrowska et al., 2013b). Restraint stress also increases anxiety and reduces STEP mRNA levels in dorsal BNST. Reduced levels of STEP leads to increased LTP selectively in Type III/CRF cells, indicating that stress removes the STEP-brake on plasticity in Type III/CRF cells (Dabrowska et al., 2013b).

What is the behavioral outcome of activating CRF cells? So far, two studies have addressed this question. First, an optogenetic study showed that inhibition of the oval nucleus resulted in decreased anxiety in the EPM (Kim et al., 2013). To selectively target the oval nucleus, the authors used *Drd1a::cre* mice, which express Cre only in cells that express dopamine receptor type 1 (D1R). In their review paper, Daniel and Rainnie (2015) mention that they have found preliminary evidence for the selective expression of D1R by type III/CRF cells. Together, these results suggest that CRF cells in the oval nucleus might regulate anxiogenesis. However, the distribution of D1R expression in BNST is controversial. A recent report showed that oval BNST is completely devoid of D1R (Krawczyk et al., 2010). However, other studies reported the presence of both D1R and D2R in BNST-AL (Scibilia et al., 1992; Hurd et al., 2001).

In support of the view that dorsal CRF population causes anxiogenic effects, a recent study found that chemogenetic inhibition of CRF neurons decreased anxiety in mice that went through binge like alcohol drinking (Pheil et al., 2015). At present, it is not clear if CRF cells exert this effect through their local connections in BNST or by directly affecting the autonomic centers they project to.

1.6.3 Sources of CRF and effects of increased CRF in BNST

As I noted above, stress activates local CRF cells. Activation of these cells might increase CRF levels within BNST. However, local CRF cells are not the only source of CRF. Lesions of CeA drastically reduce CRF immunoreactive axons in BNST (Sakana et al., 1986), suggesting that extrinsic CRF inputs to BNST play a prominent role in this region.

What are the behavioral effects of increased CRF in BNST? Lee and Davis (1997) showed that ventricular infusions of CRF increase startle and that this effect is blocked by BNST lesions or CRF receptor type1 (CRF-R1) antagonist infusions. Their results are supported by the finding that intra-ventricular CRF injections also increase c-fos expression in dorsal BNST-AL (Arnold et al., 1992). Moreover, intra-BNST CRF infusions increase anxiety in the EPM and induce conditioned place aversion (Sahuque et al., 2006), suggesting that CRF exerts anxiogenic effects.

1.6.4 CRF receptors and the physiological effects of CRF

Despite the high number of CRF cells in BNST and the dense CRF innervation it receives from CeA, only moderate levels of CRF-R1 mRNA expression have been detected in BNST (Potter et al., 1994; van Pett et al., 2000). This puzzling discrepancy between high levels of CRF and low levels of CRF-R1 (dominant receptor in anterior

BNST) mRNA might be explained by a study that used a BAC transgenic mice line which tag CRF-R1. They demonstrated that despite moderate expression of CRF-R1 in cell bodies, BNST-AL is heavily innervated by axons expressing CRF-R1 (Justice et al., 2008). In support of this evidence, electron microscopic studies showed a high prevalence of CRF-R1 on axons as well as on cell bodies (Jaferi et al., 2008; 2009). Taken together, these results suggest that the physiological effects of CRF should be both post and presynaptic.

Currently, it is not known whether CRF-R1 are expressed selectively by a specific pathway, or ubiquitously located on all afferent and local axons. Given that glutamatergic cells are rare in BNST, evidence of CRF mediated presynaptic modulation of glutamatergic transmission would suggest that CRF-R1 are expressed by afferent axons. In support of this possibility, in vitro slice recordings showed that CRF presynaptically potentiates glutamatergic transmission in dorsal BNST-AL. Specifically, in the presence of picrotoxin, CRF increases the frequency of spontaneous excitatory postsynaptic currents (sEPSC) via a CRF-R1 dependent mechanism (Kash et al., 2008; Nobis et al., 2011; Silberman et al., 2013).

Currently, it is unknown whether CRF-R1 is differentially expressed in different cell types. Although a single cell RT-PCR analysis study attempted to address this question, they showed very little expression of CRF-R1 throughout Type I-III cells (Dabrowska et al., 2013a), which is in contradiction with immunohistochemical and electron microscopic studies that consistently reported moderate levels.

Nevertheless, there is some evidence that CRF exerts postsynaptic effects. Recently, Ide et al. (2013) showed that CRF depolarizes Type II cells through CRF-R1 in

dorsal BNST-AL neurons, an effect that might explain why CRF increases spike-dependent inhibitory inputs to Type III neurons in the oval nucleus (Nagano et al., 2015).

Finally, in BNST-AV, Kash and Winder (2006) found that CRF potentiates evoked GABA-A mediated inhibitory postsynaptic current (IPSC) amplitudes via postsynaptic CRF-R1. They reported that evoked EPSCs are not affected by CRF application.

In conclusion, in dorsal BNST-AL, CRF presynaptically enhances spontaneous glutamate release from currently unknown afferents. At the same time, in BNST-AV, it enhances inhibitory transmission with no effect on evoked EPSCs. Surprisingly, Nagano et al. (2015) showed that CRF increased sIPSCs on Type III/CRF cells. As reviewed above, both CRF injections in BNST and activation of CRF cells lead to anxiety-like behavior. In this context, this auto-inhibition of CRF cells hints to a negative feedback mechanism.

1.6.5 SUMMARY

CRF immunoreactive cells are located in the oval nucleus and BNST-AV. They are projections cells, but also make local synaptic contacts. Dorsal CRF cells are GABAergic and largely overlap with the Type III cell population. Activation of these neurons has anxiogenic effects. Local CRF cells, along with CRF inputs from CeA are the main sources of CRF in BNST. Increased CRF levels in BNST have anxiogenic effects. Most CRF effects are mediated by CRF-R1. These receptors are found not only on cell bodies but also on axon terminals. In dorsal BNST-AL, CRF increases glutamatergic transmission. In BNST-AV, CRF potentiates inhibitory transmission.

Despite the commonly believed anxiogenic role of CRF in BNST, some studies imply that anxiety might be correlated with an increased inhibition of CRF cells.

1.7 NA in BNST

NA is a member of the catecholamine family along with dopamine and adrenaline. NA is the major neurotransmitter/hormone used by the sympathetic nervous system to mobilize the body in a ‘fight or flight’ mode. In the central nervous system, NA neurons are concentrated in small brainstem nuclei that project to large areas of the brain. Mostly, NA activation is associated with attention, alertness and arousal.

1.7.1 Sources of NA inputs to BNST

BNST receives one of the densest NA innervation in the brain, mainly from the A1 cell group in ventrolateral medulla and A2 cell group in the NTS and DVN (Moore, 1978; Woulfe et al., 1988; Forray et al., 2000; Shin et al., 2008; Bienkowski and Rinaman, 2013). NA fibers are much denser in BNST-AV than dorsal BNST (Moore, 1978; Phelix et al., 1994; Egli et al., 2005), regardless NA modulation of synaptic transmission was also observed in BNST-AL (Egli et al., 2005; Kash et al., 2008; Nobis et al., 2011).

1.7.2 Effects of increased NA levels in BNST

A number of studies found that NA is released in BNST-AV during stress, predator threat and pain. In addition, it might facilitate fear learning. For instance, NA is elevated during immobilization stress (Pacak et al., 1995; Cecchi et al., 2002), exposure to the fox-odor component TMT (Fendt et al., 2005), formalin-induced pain (Deyama et al., 2008) and aversive tastes (Park et al., 2012). The role of NA in contextual fear

conditioning was also tested but conflicting results have been reported. Delfs et al. (2000) found that lesions of the ventral NA bundle, which relays NA axons of the A1 and A2 cell groups to BNST, did not affect contextual fear. However, Hott et al. (2012) demonstrated that pretest infusions of NA antagonists in BNST block expression of contextual fear. Also, immediate post training infusions of NA in BNST enhanced memory in the inhibitory avoidance paradigm (Liu et al., 2009).

1.7.3 NA and BNST in addiction

Both positive and negative reinforcement processes motivate drug use; not only the rewarding qualities of the drug, but also the negative consequences of drug use and withdrawal (Wenzel et al., 2011, 2014). These negative consequences are both somatic (like teeth chattering and eye twitching) and affective. As reviewed in section 1.4, having connections with the cortex, amygdala as well as autonomic and neuroendocrine centers, BNST has the capacity to integrate stress-related responses. Furthermore, due to its reciprocal connections with VTA, it is viewed as a hub that relays stress-related information to reward centers of the brain, causing stress-related relapses to drug-seeking. Furthermore, it was hypothesized that the negative affective state induced by state drug use or withdrawal is regulated by BNST and leads to the negative reinforcement of drug use. The role of NA in BNST during drug abuse has received much attention. BNST's involvement in multiple stages of addiction has been documented. Below, I summarize these findings (for more extended reviews, see Aston-Jones and Harris, 2004; Koob, 2009; 2010; Mantsch et al., 2014; Stamatis et al., 2014).

First, BNST is implicated at the early stages of addiction: drug use potentiates glutamatergic transmission in BNST-AV, an effect required for certain kinds of drug

seeking behavior. For instance, chronic intermittent ethanol exposure leads to LTP of glutamatergic transmission by increasing NMDA receptor efficacy (Kash et al., 2009). Likewise, cocaine self-administration increases the AMPA/NMDA ratio in BNST-AV neurons, suggesting an increase in the efficacy of glutamatergic transmission (Dumont et al., 2005). Furthermore, BNST lesions block drug-seeking behavior in contexts that had been paired with cocaine (Sartor and Aston-Jones, 2012, tested in BNST-AV) and ethanol use (Pina et al., 2015).

Second, BNST is involved in drug withdrawal. After opiate withdrawal, injecting NA antagonists in BNST-AV eliminates the affective components of withdrawal but not its somatic manifestations. Also, A1 and A2 cells retrogradely labeled from BNST show high *cfos* signaling indicating NA release is enhanced in BNST during withdrawal (Delfs et al., 2000). Likewise, using fast scan cyclic voltammetry, Park et al. (2013) showed that NA levels are elevated in BNST-AV during extinction of cue-dependent intracranial self-stimulation. Interestingly, even though NA levels are not altered by cue presentations, they increase when the animal expects a reward, but does not receive it during the extinction period.

Third, BNST is necessary for the reinstatement of drug-seeking behavior. As reviewed in section 1.3.2, BNST is critical for cue induced reinstatement of drug seeking (Buffalari and See, 2011). In lieu of cues, reinstatement of extinguished drug seeking behavior can also be achieved by exposing animals to stress, such as a footshock and ample evidence indicates that NA is involved in this process. Inactivating BNST-AV (McFarland et al., 2004), as well as antagonizing CRF-R1 (Erb and Steward, 1999) or

NA receptors in BNST (Leri et al., 2002, whole BNST; Vranjkovic et al., 2012) interfere with stress induced reinstatement of drug-seeking behavior.

1.7.4 NA receptors and their physiological effects

A number of studies showed that $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ receptors are important for addiction and other stress-related behaviors. In vitro studies showed that the influence of these receptors is complex. In the table 1.2, I summarize these results (adapted from Flavin and Winder, 2013; Daniel and Rainnie, 2016).

Table 1.2 Behavioral and physiological effects of NA

	<i>Behavioral effect</i>	<i>Physiological effect</i>
all (NA)	Whole BNST: -NA is elevated during immobilization stress (Pacak et al., 1995)	Dorsal BNST-AL: -Increase (62% of cells tested) and decrease (34%) in glutamatergic transmission through $\alpha 2AR$ (Egli et al., 2005)
	BNST-AV: NA is elevated during: - Immobilization stress (Cecchi et al., 2002) -Exposure to fox-odor component TMT (Fendt et al., 2005) -Formalin-induced pain (Deyama et al., 2008) -Aversive tastes (Park et al., 2012)	BNST-AV: -70% decrease, 2% increase in firing rate in vivo (Casada and Dafny, 1993) -Decrease in glutamatergic transmission through $\alpha 2AR$ (Egli et al., 2005) -Depolarization in non-VTA projecting neurons & Hyperpolarization in VTA-projecting neurons along with increase in frequency of sIPSC thru $\alpha 1AR$ (after morphine treatment, this effect also becomes βAR dependent) (Dumont and Williams, 2004)

βAR	Whole BNST: -Stress induced reinstatement of cocaine seeking (Leri et al., 2002) -Expression of contextual fear (Hott et al., 2012) -Increases inhibitory avoidance learning (Liu et al., 2009)	Dorsal BNST-AL: -Depolarizes CRF neurons (Silberman et al., 2013) -Increases sEPSC in a CRF dependent manner, through β 1AR (Nobis et al., 2011) -Increases in glutamatergic transmission, through β 2AR (Egli et al., 2005)
	BNST-AV: -Anxiety like behavior on EPM after acute immobilization stress (Cecchi et al., 2002) -Affective but not somatic components of formalin induced pain (Deyama et al., 2008) -Stress induced reinstatement of cocaine seeking – through β 2AR not β 1AR (Vranjkovic et al., 2014)	BNST-AV: -No effect on glutamatergic transmission (Egli et al., 2005)
α1AR	Whole BNST: -Expression of contextual fear (Hott et al., 2012) BNST-AV: -Anxiety like behavior on EPM after acute immobilization stress and ACTH levels (Cecchi et al., 2002)	Whole BNST: -LTD of glutamatergic transmission (McElligott and Winder, 2008) -Increases NA levels -No change in glutamate levels -No change in GABA levels (Forray et al., 1999)
α2AR	BNST-AV: -Blocks expression of TMT induced fear (Fendt et al., 2005) Note: See Conrad et al. (2012) for NA independent processing of yohimbine in BNST, a traditional α 2AR antagonist.	Whole BNST: -Inhibits glutamate release -Does not change GABA release (Forray et al., 1999) -Inhibits NA release (Forray et al., 1997; 1999) -Decreases glutamatergic transmission (Egli et al., 2005)

		Dorsal BNST-AL: -Decreases glutamatergic and inhibitory transmission (Shields et al., 2009)
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1.7.5 SUMMARY

BNST-AV receives a dense NA innervation and NA is necessary for various stress and addiction-related behaviors. Various microdialysis, in vitro and in vivo studies showed that the main effect of NA in BNST-AV is inhibitory and depends on the activation of $\alpha 2AR$. In contrast, in dorsal BNST-AL, βAR activation enhances of glutamatergic transmission in a subpopulation of cells but this effect might be mediated by CRF-R1 (Nobis et al., 2011; Silberman et al., 2013).

1.8 **Distinct roles of BNST-AL, BNST-AM and BNST-AV**

As reviewed in section 1.3.2 and 1.7.3, global BNST lesions or inactivations reduce negative affective states, implying that BNST activity promotes anxiety. However, BNST is a heterogeneous structure and its subnuclei might have opposing influences on behavior. In section 1.4, I proposed a BNST parcellation in three sectors based on connectivity. In this section, I will review the data indicating that these three regions are functionally heterogeneous.

1.8.1 BNST –AL

In contrast to BNST-AM, which receives hippocampal inputs and forms reciprocal connections with the hypothalamus, BNST-AL is innervated by the BL, insular cortex and PVT. BNST-AL forms reciprocal connections with brainstem autonomic centers and CeA. Moreover, whereas BNST-AM contains glutamatergic and GABAergic neurons, BNST-AL only contains GABAergic cells. Thus, activation of BNST-AL is

expected to *inhibit* the autonomic brainstem nuclei that are thought to mediate negative emotional states.

Due to the small size of BNST sub-nuclei, few behavioral studies have selectively manipulated BNST-AL activity. A few studies hinted that BNST-AL might exert an anxiolytic influence. For instance, Dunn (1987) found that stimulating BNST-AL reduces CORT levels. In agreement with this, BNST-AL lesions increase gastric erosions after stress exposure (Henke, 1984). Recently, it was demonstrated that about 25% of BNST-AL cells increase their firing rate during low fear states whereas only 10% fire more during high fear states (Haufler et al., 2013).

Dabrowska et al. (2013b) showed chronic restraint stress causes LTP of glutamatergic transmission selectively in Type III/CRF cells. With the exception of this study, various stressors were found to suppress glutamatergic transmission in BNST-AL. For example, chronic restraint stress causes $\alpha 1$ AR dependent LTD (McElligott et al., 2010). Chronic cortisol administration and social isolation blunts LTP (Conrad et al., 2011) and withdrawal from various drugs of abuse reduces the intrinsic excitability of BNST-AL neurons (Francesconi et al., 2009). Together, these findings suggest that BNST-AL exerts an anxiolytic influence.

Opposite to this conclusion, an optogenetic study targeting the oval nucleus in BNST-AL showed that inhibition of this region results in *decreased* anxiety in elevated plus maze (Kim et al., 2013). As reviewed in section 1.6.2, it is probable that Kim et al. (2013) selectively manipulated the CRF cell population in this nucleus. This data, along with others that showed stress-dependent activation of CRF cells (section 1.6.2), led to

the widely accepted view that the CRF cell population in the oval nucleus exerts an anxiogenic influence.

Yet, we still do not know if this influence of CRF cells depends on its projections within BNST or to brainstem effectors. To expand on the first possibility, local CRF-R1 actions are implicated in anxiety (Sink et al., 2013). Also, stimulation of the oval nucleus triggers GABA-A mediated inhibition in neighboring regions (Kim et al., 2013; Turesson et al., 2013). At the same time, CRF application mostly exerts an excitatory influence in the nucleus (Kash et al., 2008; Nobis et al., 2011, Silberman et al., 2013; Ide et al., 2013). Further studies are needed to disentangle how CRF and non-CRF cells of BNST-AL interact and determine what mechanisms gains the upper hand during stress and anxiety.

Accordingly, many studies have examined how various peptides and modulators alter cellular excitability and synaptic transmission in BNST-AL. For example, as I pointed in section 1.7.4, NA application was found to either increase or decrease excitatory transmission, depending on the NA receptor activated. Likewise, the effects of dopamine and serotonin are not uniform (reviewed in Daniel and Rainnie, 2016), leading to the idea that the regulation of negative affective states by BNST-AL depends on the integration of multiple modulatory influences.

1.8.2 BNST-AM

Few studies have focused on BNST-AM. For instance the effects of CRF, NA and many other peptides and modulators have not been tested in BNST-AM. Being the only BNST region that receives projections from the ventral subiculum, understanding BNST-AM is important.

Haufler et al. (2013) demonstrated that about 30% of BNST-AM cells increase their firing rate in high fear states, versus around 15% that are inhibited. Consistent with this, in a rat model of post-traumatic stress disorder (PTSD) model, animals that show PTSD-like symptoms after a predator stress display increased excitability in BNST-AM, compared to resilient animals (Rodriguez-Sierra et al., in press). Together, these results suggest that BNST-AM exert anxiogenic effects.

Opposite to this, it was reported that optogenetically activating BL inputs to BNST reduces anxiety-like behaviors (Kim et al., 2013). Because the oval nucleus is devoid of inputs from BL, this effect was ascribed to BNST-AM and ventral BNST-AL activation. However, these results are surprising given the well-established role of the BL in processing threat information (reviewed in Pape and Pare, 2010). Because BNST lacks inputs from the sensory thalamus and cortices it depends on the BLA to receive threat information. Moreover, BL projections to BNST are massive and constitute its major excitatory drive. While it is possible that BNST receives threat information from other structures, like the hypothalamus and insular cortex, it is hard to conceive that a naturalistic activation of BL projections to BNST would cause anxiolysis. Thus, further studies are needed to investigate the nature of BL-BNST connections with other techniques.

Having said this, glutamatergic inputs from different afferents might have contrasting effects in BNST-AM. Thus, the activating BLA to BNST-AM pathway does not necessarily define the general function of BNST-AM.

1.8.3 BNST-AV

BNST projections to PVN mainly originate from BNST-AV (Sawchenko and Swanson, 1983; Moga and Saper, 1994) and these projections are hypothesized to play an important role in regulating the HPA-axis (Herman et al., 2005). Although most BNST-AV cells are GABA-ergic and are expected to inhibit PVN cells, PVN also receives glutamatergic (Csaki et al., 2000) and CRF (Moga and Saper, 1994) inputs from BNST.

A few studies have investigated the effects of BNST lesions on PVN activity. BNST-AV lesions reduce the increase in *cfos* expression induced in PVN by systemic interleukin injection (an immune system activator), as well as restraint and airpuff stress (Choi et al., 2007; Crane et al., 2003; Spencer et al., 2005). Also, anterior BNST lesions reduce baseline CRF mRNA levels in PVN (Herman et al., 1994). In contrast, selective ablation of GABAergic cells in BNST-AV increase adrenocorticotrophic hormone (ACTH) and CORT levels, indicators of an activated PVN, after restraint stress (Radley et al., 2009). Overall, these findings suggest that BNST-AV, as a whole, exerts an excitatory influence on PVN whereas the GABAergic cells of BNST-AV inhibit it. Together, these observations suggest that glutamatergic BNST-AV cells are the source of the facilitatory effect and that, although fewer in number, their influence dominates.

BNST-AV contains PVN-projecting cells that receive inputs from the medial prefrontal cortex (mPFC) and hippocampus (Radley et al., 2009; Radley and Sawchenko, 2011). Of note, these cells rarely receive inputs from CeA (Prewitt and Herman, 1998). mPFC (Radley et al., 2009) and hippocampal lesions (Radley and Sawchenko, 2011) decrease *cfos* activity in GABAergic cells of BNST-AV, while increasing the *cfos* expression in PVN, further supporting the idea that the GABAergic cells of BNST-AV exerts an inhibitory influence over PVN.

There is a parallel between BNST's control of the HPA-axis and of the VTA. Indeed, a recent study showed that VTA-projecting VGlut2 cells of BNST-AV increase their firing rates during both aversive unconditioned and conditioned stimuli. In contrast, GABAergic cells are inhibited by both. Optogenetically activating VGlut2 cells produces place aversion and anxiogenic effects, whereas activation of the GABAergic cells produces place preference and anxiolytic effects (Jennings et al., 2013).

1.9 BNST-amygdala interactions

1.9.1 Brief overview of amygdala

The amygdala consists of a heterogeneous group of cortex and striatum-like nuclei (LeDoux, 2000). The areas most relevant for Pavlovian fear conditioning are BLA, which includes the lateral (LA), BL and BM nuclei; as well as the CeA, which is commonly divided in lateral (CeL) and medial (CeM) sectors.

Most sensory inputs about CSs arise from thalamus or cortex and terminate in LA. On the other hand, contextual information are thought to be relayed by ventral hippocampus projections to BM and BL (Canteras and Swanson, 1992; Cullinan et al., 1993; McDonald, 1998). Thus, BLA receives sensory information and associates threat value to it. CeM is considered to be the amygdala's main output structure to fear effector neurons due to its projections to various brainstem structures (Veening et al., 1984; Holstege et al., 1985; Gray and Magnuson, 1987), that mediate autonomic and behavioral correlates of fear. CeL, on the other hand, receives inputs from BLA and projects to CeM, thus acting as one of the intermediate steps between input and output structures of

the amygdala (see Pape and Pare, 2010 and Duvarci and Pare, 2014 for amygdala interconnectivity that control the expression of learned fear).

1.9.2 Different roles for CeA and BNST: the Walker et al. (2009) model

Early studies demonstrated that lesions of CeA but not BNST (LeDoux et al., 1988; Hitchcock and Davis, 1991; Walker and Davis, 1997) blocks fear responses to short lasting CSs during Pavlovian fear conditioning. On the other hand, as I reviewed in section 1.3.2, BNST lesions disrupt anxiety responses to various long and diffuse environmental cues.

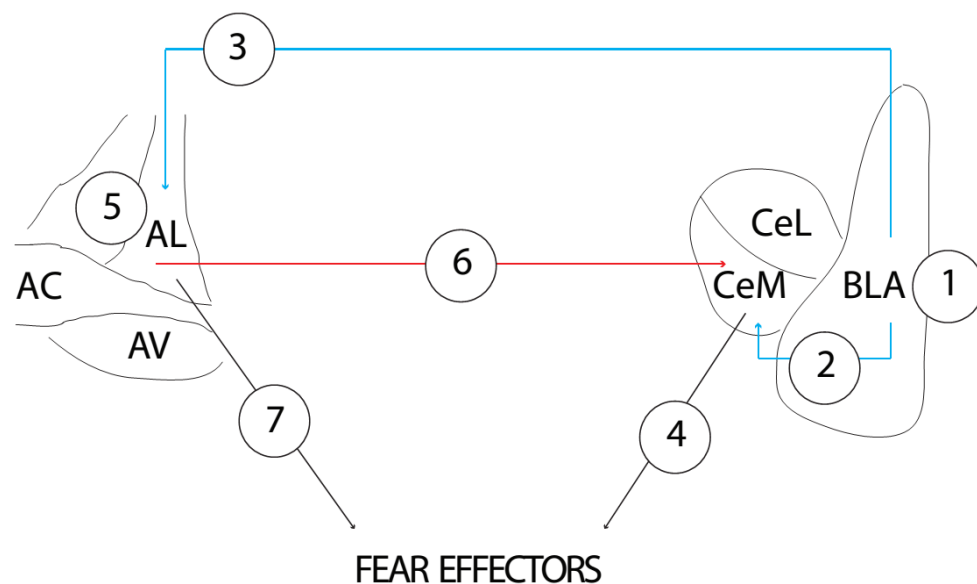


Figure 1.2. Walker et al. (2009) model of BLA-BNST-CeA interactions. Blue and red indicate excitatory and inhibitory connections, respectively.

These results led Walker et al. (2009) to hypothesize a complex relationship between BLA, CeA and BNST (Fig. 1.2). According to their model, BLA (1) sends threat

signals to both CeM (2) and BNST (3). In turn, CeM responds immediately, activating downstream brainstem effectors (4). By contrast, BNST would also require CRF inputs from CeL, in addition to BLA inputs (5). Due to this difference, BNST activation would be delayed with respect to CeM, explaining why CeM generates rapid responses to discrete cues whereas BNST mediates long lasting anxiety-like responses to diffuse contingencies. This model also proposes that BNST, once active, inhibits CeM (6). Thus, CeM would not contribute to generation of anxiety-like states.

This model is well accepted in the fear literature, because it provides a parsimonious explanation for prior behavioral findings. However, as discussed below, several of its key assumptions are incompatible with the available evidence.

1.9.3 Is BNST activation delayed in a threatening environment?

At present, there is no evidence that BNST activation in a threatening environment is delayed relative to CeM activation. To the contrary, as I reviewed in section 1.3.2, there is accumulating evidence, from both addiction and aversive conditioning fields that BNST is involved in short-cue processing. In vivo recordings showed that BNST cells are responsive immediately after the onset of short (Haufler et al., 2013) or long (Jennings et al., 2014) cues. Importantly, Hammack et al. (2015) showed that the difference in freezing between sham and BNST-lesion animals remains constant throughout the duration of a long fear conditioned context. In total, these results demonstrate that BNST activation in threatening environments is (1) immediate at the onset of both short and long CS, (2) not necessarily more important the longer the animal remains in a threatening environment. In this context, given that both anxiogenic and

anxiolytic networks exist in BNST, it is very important to understand what kind of information is transmitted from BNST to CeA.

1.9.4 Are brainstem effectors modulated by BNST and CeA with a delay?

No study has yet shown that brainstem effectors are modulated first by CeM, and then by BNST. To the contrary, Nagy and Pare (2008) showed that the anatomical organization of connections between BLA, CeA, BNST and brainstem might allow for synchronization of CeA and BNST impulses to the brainstem when activated by BLA. First, CeA efferents to the brainstem follow two routes: (a) short one through the ventral amygdalofugal pathway and (b) a longer one by the stria terminalis. Paralleling this, the antidromic response latency of CeA neurons to brainstem stimulation is bimodal. In contrast, BNST responses fit a unimodal distribution. Second, the orthodromic spiking latency of BNST and CeA neurons to BLA stimulation differed with longer latencies in BNST neurons, introducing a delay in the impulse flow in the BLA-BNST-brainstem pathway. Interestingly, this delay is matched by the latency difference between the brainstem evoked antidromic responses of BNST neurons and CeA neurons with long conduction times.

The significance of this synchronized BNST and CeA output to the brainstem is not yet understood. Anatomical studies have shown that BNST (BNST-AL and AV) and CeA (CeM) send projections to the same brainstem effectors (Veening et al., 1984; Holstege et al., 1985; Gray and Magnuson, 1987). However, it is not clear whether they target the same cells within these structures. The influence of BNST and CeA neurons in the brainstem could be complementary or opposite. Understanding these pathways will be

critical to decipher the role of these structures in the regulation of negative emotional states.

1.9.5 CeA involvement in long-cues signaling threats

Walker et al. (2009) also hypothesized that once BNST is active, it will suppress CeA neurons. Thus, according to the model, CeA is not involved in modulating anxiety-like responses to diffuse and uncertain threats. While this prediction found experimental support for unconditioned threats like bright lights (Walker and Davis, 1997) or predator odors (Fendt et al., 2003; Li et al., 2004; Rosen, 2004), it did not for the fear of open spaces. Indeed, CeA lesions reduce anxiety-like behavior in the EPM (Moller et al., 1997; Moreira et al., 2007). Similarly, contradictory results were reported for the impact of CeA lesions on conditioned negative associations to long cues or contexts. Although CeA lesions do not block fear-potentiated startle to long cues (Walker et al., 2009a), many found that they reduce freezing to an aversive context (Sullivan et al., 2004; Goosens and Maren, 2001, 2003). However, some failed to find an effect of CeA lesions (Fanselow and Kim, 1994) or concluded that CeA is not involved in the expression but in the consolidation of contextual fear memories (Pitts et al., 2009).

1.9.6 Connections between BNST and CeA

Whereas BNST-AM contributes negligible projections to CeA (Bienkowski and Rinaman, 2013), BNST-AL and BNST-AV project strongly to CeM, and lightly to CeL (Sun and Cassell, 1993; Dong et al., 2001b). BNST to CeA projections prevalently arise from GABAergic neurons, although a few glutamatergic and Type III (presumed CRF) neurons also contribute (Gungor et al., 2015). In the opposite direction, CeA projections to BNST mostly originate in CeL and mainly target BNST-AL, sparing the juxtacapsular

region (Dong et al., 2001a). CeM contributes less to BNST's innervation (Bienkowski and Rinaman, 2013; Sun and Cassell, 1993) and BNST-AM receives far weaker inputs from CeA than BNST-AL (Krettek and Price, 1978a; Weller and Smith, 1982; Sun et al., 1991).

1.9.7 SUMMARY

It is believed that CeM generates brief fear reactions in response to discrete and short lasting conditioned cues, whereas BNST generates long lasting anxiety-like states in response to more diffuse contingencies. Yet, CeA and BNST receive threat information from BLA and are reciprocally connected. Moreover, there is accumulating evidence that BNST is involved in the processing of short and discrete cues and that these cues activate BNST immediately. Thus, although BNST is not *necessary* for responding to such threats, it is in a position to *modulate* them either through its projections to CeA or the brainstem. On the other hand, CeA's role in anxiety-like responses is not well characterized, and how BNST and CeA interact in anxiety promoting environments is not known.

1.10 **Introducing the data chapters**

Research presented in Chapter III aims to broaden our knowledge on BNST-AL. Here, I investigated the effects of a particular peptide, calcitonin gene-related peptide (CGRP), on synaptic transmission of this subnucleus. CGRP is expected to act on BNST-AL due to selective targeting of BNST-AL by CGRP inputs from PBN. As explained in the previous sections, most BNST-AL cells are GABAergic and they are expected to inhibit their projection targets. In contrast with this, infusion of CGRP into BNST was reported to *potentiate* anxiety while *activating* BNST targets. To understand how

GABAergic cells excite their targets, I examined the effect of CGRP on BNST-AL neurons.

In Chapter IV, I investigated the influence of BNST projections to CeA to improve our understanding of how these two structures contribute to fear and anxiety. To this end, I combined optogenetic methods with whole cell recordings in brain slices kept in vitro.

As reviewed above, NA levels in BNST-AV are elevated during stress. Moreover, the previous literature suggests that glutamatergic and GABAergic cells in BNST-AV might exert anxiogenic and anxiolytic influences, respectively. In Chapter V, I investigated the electrophysiological characteristics of these cell types and how they respond to NA.

CHAPTER II

GENERAL METHODS

2.1 Animals

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 (DHHS). Male Lewis rats were used for experiments described in Chapter III and IV. For Chapter V, we crossed Vglut2-ires- Cre (Stock no: 016963) and Vgat-ires- Cre knock-in mice (Stock no: 01696) with Ai6 reporter mice (Stock no: 007906), expressing ZsGreen1. All animals have a homozygous mutation and are purchased from Jackson Labs. Both female and male animals are used for these experiments.

2.2 Virus injections

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 μ L total – 0.5 μ L at each DV level) at a rate of 9.6nL/5sec using glass pipettes pulled to an outer tip diameter of $\sim$ 70 μ m 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. 4.1A). 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 fluorescence 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.

2.2.1 Confocal microscopy

Immediately after the in vitro recordings, the 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 CeA 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.

2.3 In vitro whole cell patch clamp electrophysiology

2.3.1 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 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 10 glucose. The brains were sliced with a vibrating microtome (300 μ m) while submerged in the same solution. The slices were then kept in an oxygenated chamber containing artificial cerebrospinal fluid (aCSF; as above except for the substitution of 126 mM NaCl for choline chloride; pH 7.2, 300 mOsm). The temperature of the chamber was kept at 34°C for 20 min and then returned to room temperature. One hour later, slices were then transferred to a recording chamber perfused with oxygenated aCSF at 32°C (6 ml/min).

Mice were anesthetized with isoflurane. After abolition of reflexes, they were perfused with ice-cold solution containing (in mM) 103 NMDG, 2.5 KCl, 10 MgSO₄, 30 NaHCO₃, 1.2 NaH₂PO₄, 0.5 CaCl₂, 25 glucose, 20 HEPES, 2 Thiourea, 3 Na-pyruvate, 12 N-acetyl-L-cysteine; pH 7.3, 300 mOsm. The brains were sliced with a vibrating microtome (400 μ m) while submerged in the same solution. The slices were then kept in an oxygenated chamber containing 126 mM NaCl, 2.5 KCl, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 10 glucose; pH 7.3, 300 mOsm.

2.3.2 Electrophysiological recordings

Whole-cell recordings were obtained under visual guidance using 5-8 M Ω pipettes pulled from borosilicate glass capillaries. The intracellular solution contained (in mM) 130 K-gluconate, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (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, membrane potential (V_m) values mentioned below were not corrected for the junction potential. In CGRP experiments, we used an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA) and digitized the data at 10 kHz with a Digidata-1200 interface controlled by pClamp-8.1 (Molecular Devices, Sunnyvale, CA). In other experiments, 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 series of current pulses (± 10 pA increments; 500 ms; 0.2 Hz) from -55 and -70 mV; this revealed that all of the neurons recorded in these studies correspond to the previously described Type-I, II, III neurons (Hammack et al., 2007; Rodriguez-Sierra et al., 2013).

2.3.3 Electrical stimulation

For the CGRP and NA experiments, a pair of tungsten stimulating electrodes (inter-tip spacing, 200 μ m) was placed in the stria terminalis (ST, Fig. 3.1A) and used to deliver brief current pulses (0.1 ms; 0.03 Hz). Cells were kept at -55 mV unless stated otherwise. When testing the effects of CGRP and NA on ST-evoked EPSP amplitudes, stimulation intensity (0.1-0.8 mA) was adjusted to obtain the highest subthreshold response amplitudes. When testing CGRP effects on IPSPs, the stimulation intensity was adjusted to elicit IPSPs of about 5 mV amplitude so that the IPSP peak would not approach the GABA-A reversal potential. Input resistance (R_{in}) was calculated as the average voltage response to -10 pA current injections during the stimulation protocol. A 10-min baseline recording was obtained before drug application. Drugs were then applied

for 10 minutes. Before vs. after peptide comparisons were made using responses obtained 5-10 minutes before vs. 15-20 minutes after onset of drug application (separate averages of ten ST-evoked responses). Antagonists were added to the perfusate solution 15 min before drug application, and were present throughout the recordings.

2.3.4 Drugs

Picrotoxin, CNQX disodium salt (6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt) and CPP ($\pm$)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid) were used for abolishing GABA-A, AMPA and NMDA-dependent responses respectively. CGRP (rat), SB-268262 (N-Methyl-N-(2-methylphenyl)-3-nitro-4-(2-thiazolylsulfinyl)-benzamide), picrotoxin, CNQX disodium salt, CPP and NA (catalog number N5785) were obtained from Sigma (St. Louis, MO). CGRP₈₋₃₇ from Tocris (Minneapolis, MN). NA was aliquoted and stored at -80° C with 100 μ M ascorbic acid to prevent oxidization.

2.3.5 Fluorescence microscopy

For optogenetic experiments, we used Lewis rats that were injected with AAV virus, as described in the section 2.2. 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.

For experiments using transgenic mice, the glutamatergic and GABAergic cells expressed ZsGreen and easily visualized with fluorescence microscopy.

2.3.6 Light stimulation for optogenetic experiments

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 coupled to a PlexBright Tabletop Blue LED module (Plexon, Dallas, TX). The light power density at the tip of the fiber was $\sim 700\text{mW/mm}^2$. The distance between the recording pipette and the fiber optic tip was $\sim 200\text{ }\mu\text{m}$. Postsynaptic 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.

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.

2.4 Data analysis

Data was analyzed offline using Clampfit-9.2 or Clampfit 10. All data are reported as averages $\pm$ SEM. SEM calculations were modified for repeated designs as described in Cousineau (2005). For statistical analyses, we conducted one way ANOVAs with Tukey's HSD (Honestly significant difference) paired post-hoc tests as well as paired t-tests.

In optogenetics experiments, 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.

In Chapter V, we used Fisher's exact test to compare the incidence of fIR and spontaneously active cells among glutamatergic and GABAergic neurons. Unpaired t-tests were used to assess significance of differences between the electrophysiological properties of glutamatergic and GABAergic cells and p values were adjusted by Holm-Bonferroni corrections for six tests (conducted for input resistance, time constant, rheobase, spike threshold, latency and firing rate). Paired t-tests were used to assess the effects of NA on the electrophysiological properties (alpha level corrections for 5 tests per each cell phenotype). 2x3 mixed ANOVA design was used to assess the effects of cell type (between subjects) and NA (within subjects) on EPSP amplitudes and input resistance. Paired t-tests were used as post-hoc analysis to determine the time dependence

of the changes observed (2 tests per cell phenotype: control vs. 5 min NA, control vs. 10 min NA, corrections apply). Last, to assess whether time-dependent percentage change differed between glutamatergic and GABAergic cells, we used unpaired t-tests (6 different time points; corrections apply).

CHAPTER III

CGRP INHIBITS NEURONS OF THE BED NUCLEUS OF THE STRIA TERMINALIS: IMPLICATIONS FOR THE REGULATION OF FEAR AND ANXIETY

3.1 Rationale

Currently, it is unclear whether activation or inhibition of BNST is required to produce anxiety-like behaviors. On the one hand, the fact that lesion or inactivation of BNST produces anxiolytic effects suggests that increased BNST activity generates negative emotional states. On the other, BNST is mainly comprised of GABAergic neurons (Esclapez et al., 1993; Hur and Zaborszky, 2005; Poulin et al., 2009) and therefore presumably exerts inhibitory effects on its targets. In contrast with this however, Sink et al. (2011) reported that intra-BNST injections of calcitonin gene-related peptide (CGRP) *augments* acoustic startle while *increasing* activity in targets of BNST. The present study was undertaken to address this apparent contradiction.

CGRP is a 37 amino acid peptide involved in autonomic functions and pain processing (reviewed in van Rossum et al., 1997). The sole CGRP input to BNST originates in the pontine parabrachial nucleus (Shimada et al., 1985), which projects heavily to the anterolateral portion of BNST (BNST-AL; Gustafson and Greengard, 1990; Alden et al., 1994; Dobolyi et al., 2005). Thus, to shed light on how BNST regulates anxiety, we studied the effect of CGRP on BNST-AL neurons recorded with the patch method in brain slices *in vitro*.

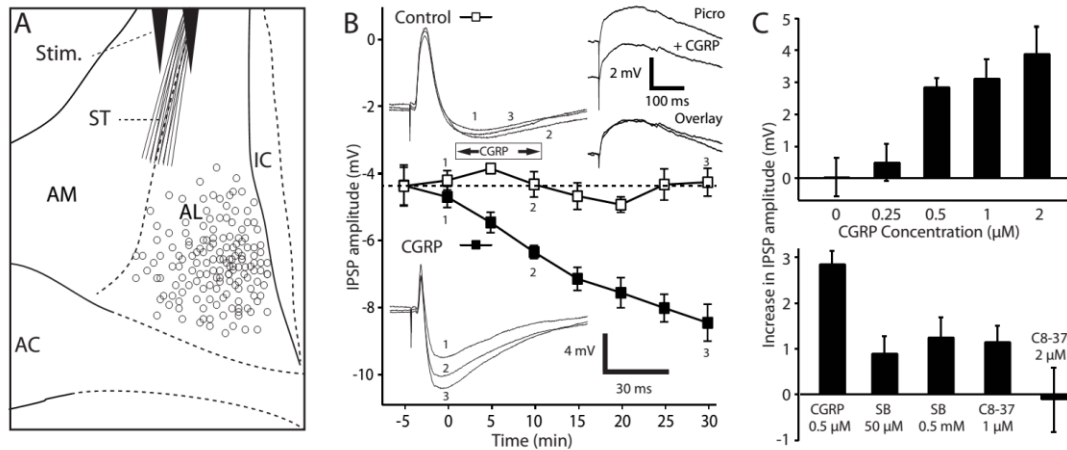


Figure 3.1. CGRP potentiates ST-evoked IPSPs. **(A)** Scheme showing stimulating (stim.) and recording (circles) sites. **(B)** Time course of CGRP effect on IPSPs (solid squares, CGRP 0.5 μM; empty squares, control cells). CGRP steadily increases ST-evoked IPSP amplitudes and this effect outlasts the period of CGRP application. **Insets on left** show examples of ST-evoked responses in a control cell (top) and one exposed to CGRP (bottom). **(C, top)** Dose dependency of CGRP effect on IPSPs. After CGRP application (15 min), IPSP amplitudes increased to $260 \pm 49\%$ for 2 μM ($n=9$, $p=0.002$), $174 \pm 22\%$ for 1 μM ($n=4$, $p=0.01$), $170 \pm 15\%$ for 0.5 μM ($n=5$, $p=0.0005$) of pre-application values. 250 nM CGRP had no significant effect ($n=8$, $p=0.41$). Eight cells were tested with no CGRP application. **Inset on top right:** examples of EPSPs isolated with picrotoxin (100 μM) before vs. after a 10 min application of CGRP (500 nM). **(C, bottom)** Two CGRP receptor type-1 antagonists reduced the effect of CGRP, compared to when CGRP (500 nM, $n=5$) was applied alone (SB268262, 50 μM, $n=12$, $p<0.001$; 500 μM, $n=7$, $p<0.01$; CGRP₈₋₃₇, 1 μM, $n=4$, $p<0.001$; 2 μM, $n=5$, $p=0.01$). Abbreviations: AC: anterior commissure; AM: Anteromedial BNST; AL: Anterolateral BNST; IC, internal capsule; ST, stria terminalis.

3.2 Overview of methods

Whole cell patch clamp recordings were obtained from BNST-AL neurons of Lewis rats (4-7 weeks old). Electroresponsive properties of recorded cells fit with the Type I and II characterization, with no difference of CGRP responsiveness. Stimulation electrodes were positioned over stria to evoke postsynaptic potentials.

3.3 Results

3.3.1 Effect of CGRP on the electroresponsive properties and synaptic responses of BNST-AL cells

We obtained patch recordings of 184 BNST-AL cells (Fig. 3.1A) that had stable resting potentials and generated overshooting action potentials upon depolarization. We first tested whether CGRP (0.5-2 μ M) alters the passive properties, firing pattern, or spike characteristics of BNST-AL cells but found no effect (n=26; Table 3.1). We restricted our analysis of passive properties and spike characteristics to cells tested in the same conditions (before and after CGRP), with no other drugs present. Other cell groups, not considered in Table 3.1, were recorded in the presence of picrotoxin (n = 40), CGRP receptor antagonists (n = 28), glutamate receptor antagonists (n = 20), KCC2 blocker (n = 9), or GABA-B agonists or antagonists (n = 31). Finally, there were control recordings performed to look for time-dependent effects (independent of CGRP; n=22) and 8 additional cells tested with 250 nM CGRP, which were not included because this dose had no effect. Finally, for the spike characteristics, in 14 of the 26 cells meeting our selection criteria, no responses to positive current pulses were recorded.

Because it was reported that CGRP increases EPSP amplitudes in central amygdala neurons at their resting potential (Han et al., 2005, 2010), we next examined the impact of CGRP on ST-evoked EPSPs in the presence of picrotoxin (100 μ M). Picrotoxin completely abolished ST-evoked IPSPs in 90% of tested neurons (36 of 40), allowing us to examine the influence of CGRP (1 μ M) on isolated EPSPs. However, EPSP amplitudes were unaffected by CGRP (difference of -0.7 ± 0.6 mV, $n=12$, $p=0.25$; Fig. 3.1B, top right inset).

Table 3.1. Effect of CGRP (1 μ M) on the electroresponsive properties of BNST-AL neurons (values are means $\pm$ SEM)

	Control	CGRP	p	n
Resting potential (mV)	-61.5 ± 1.5	-60.9 ± 1.4	.27	26
Input resistance ($m\Omega$)	715.7 ± 74.1	731.2 ± 74.6	.38	26
Time constant (ms)	46.9 ± 4.6	50.6 ± 4	.25	26
Rheobase (pA)	40.8 ± 6.1	42.5 ± 5.7	.44	12
Spike threshold (mV)	-48.1 ± 2.2	-49.4 ± 2.3	.31	12
Spike latency (ms)	98.2 ± 13.3	104.3 ± 15.9	.7	12
Spike amplitude (mV)	87.8 ± 3	83.6 ± 3.63	.16	12
Spike duration at half amplitude (ms)	$.48 \pm .03$	$.49 \pm .01$	.8	12
Firing rate at rheobase (Hz)	$.75 \pm .21$	$.75 \pm .14$	1	12

In contrast, in the absence of picrotoxin, CGRP significantly increased the amplitude of ST-evoked IPSPs (Fig.3.1B, solid squares and bottom inset) in a dose-dependent fashion ($n=26$; ANOVA, $F(4,29)=5.48$, $p=0.02$; Fig.3.1C,top). The potentiation of ST-evoked IPSPs outlasted the CGRP application period by >20 min (Fig.3.1B). Because the increase in IPSP could have resulted from the gradual equilibration of the intracellular Cl^- concentration with that of the pipette solution, we examined whether the IPSP amplitudes increased spontaneously over time ($n=8$), with no peptide application. However, no significant time-dependent changes were observed (Fig.3.1B, empty squares,inset; $n=8$, $p=0.35$). Furthermore, two different CGRP type-1 receptor antagonists,

SB268262 (n=19) and CGRP₈₋₃₇ (n=9), reduced or completely abolished the IPSP potentiation produced by CGRP (Fig.3.1C, bottom).

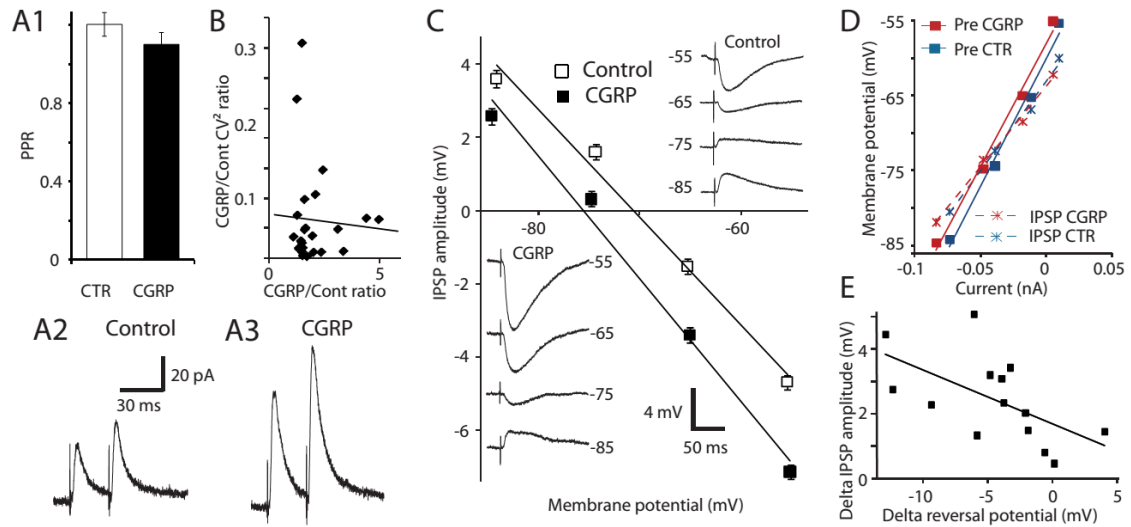


Figure 3.2. CGRP potentiates ST-evoked IPSPs through a postsynaptic mechanism. **(A1)** Paired pulse ratio (PPR) before and 15 min after CGRP application (n=6). **(A2-3)** Average traces from same cell before (2) and after (3) CGRP application. Despite strong ST-evoked IPSC potentiation, there is no difference in the PPR. **(B)** CV² analysis. Ratio of CV² during CGRP to control (y-axis) vs. ratio of CGRP to control IPSP amplitudes (x-axis, n=18). **(C)** CGRP causes a negative shift of the GABA-A reversal potential. CNQX (10 μ M) and CPP (10 μ M) present throughout. Linear fits of IPSP amplitude (y-axis) as a function of V_m (x-axis) before (empty squares) and after (solid squares) CGRP (average $\pm$ SEM, 14 cells). **Insets** show average IPSP amplitude of a representative cell before (top right) and after (bottom left) CGRP. **(D)** Linear fits of V_m (y-axis) vs. current (x-axis) before the ST stimulus (solid squares) and at the IPSP peak (stars), before (blue)

and after (red) CGRP. (E) CGRP-induced changes in IPSP amplitudes (y-axis) and GABA-A reversal potentials (x-axis) are correlated.

3.3.2 Mechanisms underlying the potentiation of ST-evoked IPSPs by CGRP

To investigate whether the increase in ST-evoked IPSPs produced by CGRP is dependent on pre- or postsynaptic mechanisms, we conducted three analyses. First, we compared the paired-pulse ratio (PPR) of ST-evoked IPSCs before vs. after CGRP application (Fig.3.2A). In such analyses, two stimuli of equal intensity are applied in brief succession (50 ms), leading to an enhancement or reduction of the response elicited by the second stimulus. Changes in PPR are commonly thought to reflect alterations in transmitter release probability (Creager et al., 1980; Manabe et al., 1993). PPR tests were performed in the presence of the glutamate receptor antagonists CNQX (10 μ M) and CPP (10 μ M). In control conditions, a small average paired-pulse facilitation was observed but it did not reach significance ($p=0.32$). Addition of CGRP (1 μ M) did not alter the PPR of ST-evoked IPSPs (Fig.3.2A; PPR difference of 0.1 ± 0.1 ; $n=6$; $p=0.43$), suggesting that CGRP acts postsynaptically to potentiate the IPSPs.

Second, we studied IPSP variability using the data obtained in the dose-response experiments of figure 3.1C. This variability is known to reflect the probabilistic process underlying transmitter release and can be estimated by computing the coefficient of variation (CV, standard deviation/mean). By plotting the ratio of experimental to control CV^2 against the ratio of experimental to control response amplitudes, the dependence of presynaptic vs. postsynaptic function can be determined (Bekkers and Stevens, 1990; Manabe et al., 1993). In these prior studies, a positive correlation between the two was

shown to reflect a presynaptic mechanism, while a horizontal regression is indicative of a purely postsynaptic action. We found no significant relationship between CV^2 and IPSP amplitudes (Fig.3.2B; $r=-0.06$, $p=0.77$, $n=14$), again pointing to a postsynaptic locus of CGRP action.

Third, we tested whether CGRP alters the reversal potential (E_{GABA-A}) of ST-evoked IPSPs in the presence of the glutamate receptor antagonists CNQX (10 μ M) and CPP (10 μ M). CGRP (1 μ M) caused a significant negative shift of E_{GABA-A} (Fig.3.2C; Difference 4.31 ± 1.25 mV; Control, -72.66 ± 1.9 mV; CGRP, -76.97 ± 1.99 mV; $p=0.004$, $n=14$). Importantly, this effect was not associated with a change in the R_{in} drop caused by the IPSPs (Fig.3.2D; -11 ± 11 M Ω ; $p=0.33$), suggesting that the CGRP-induced augmentation in IPSP amplitude is largely dependent on an increased Cl^- driving force. Indeed, there was a significant correlation between the CGRP-induced changes in IPSP amplitudes and E_{GABA-A} (Fig.3.2E; $n=14$, $r=-0.59$, $p=0.03$). In contrast, in a different sample of control cells without CGRP application, no time-dependent shift in GABA-A reversal potential was observed (-0.1 ± 0.9 mV, $n=14$, $p=0.89$).

If CGRP acts postsynaptically to enhance IPSPs by increasing the Cl^- driving force, one would expect manipulations that interfere with the cells' Cl^- homeostasis to disrupt CGRP's effects. A major regulator of the intracellular Cl^- concentration in neurons (reviewed in Kaila, 1994) is the potassium-chloride cotransporter (KCC), which mediates Cl^- extrusion (Misgeld et al., 1986; Thompson et al., 1988). Consistent with this, VU-0240551 (40 μ M), a selective KCC2 blocker, depolarized E_{GABA-A} by 4.1 ± 1.1 mV ($n=9$, $p=0.007$) and prevented the effects of CGRP on IPSP amplitudes (0.5 ± 0.5 mV; $n=7$, $p=0.31$) and E_{GABA-A} (-0.3 ± 0.7 mV; $p=0.72$; Fig.3.3).

An alternative interpretation for CGRP effects, namely that it is due to an enhancement of GABA-B IPSPs, appears unlikely for the following reasons. First, as mentioned just above, inhibiting KCC2 blocked the CGRP effect. Second, our IPSP measurements were performed at the peak of the GABA-A IPSPs, about 30 ms from response onset, well before the development of GABA-B responses in other cell types. Third, most BNST-AL cells lacked overt GABA-B responses, with picrotoxin abolishing ST-evoked IPSPs in 36 of 40 cells. Last, in the rare BNST-AL cells with picrotoxin-resistant IPSP components, addition of CGRP failed to alter the residual IPSP amplitudes (reduction of 0.4 ± 0.04 mV, $n=2$).

3.4 Summary of results

CGRP did not alter the passive properties of BNST-AL cells but increased the amplitude of IPSPs evoked by stimulation of the stria terminalis. However, IPSP paired-pulse ratios were unchanged by CGRP and there was no correlation between IPSP potentiation and variance, suggesting that CGRP acts postsynaptically. Consistent with this, CGRP hyperpolarized the GABA-A reversal of BNST-AL cells. These results indicate that CGRP increases ST-evoked GABA-A IPSPs and hyperpolarizes their reversal potential through a postsynaptic change in Cl^- homeostasis. Overall, our findings suggest that CGRP potentiates anxiety-like behaviors and increases neural activity in BNST targets, by inhibiting BNST-AL cells, supporting the conclusion that BNST-AL exerts anxiolytic effects.

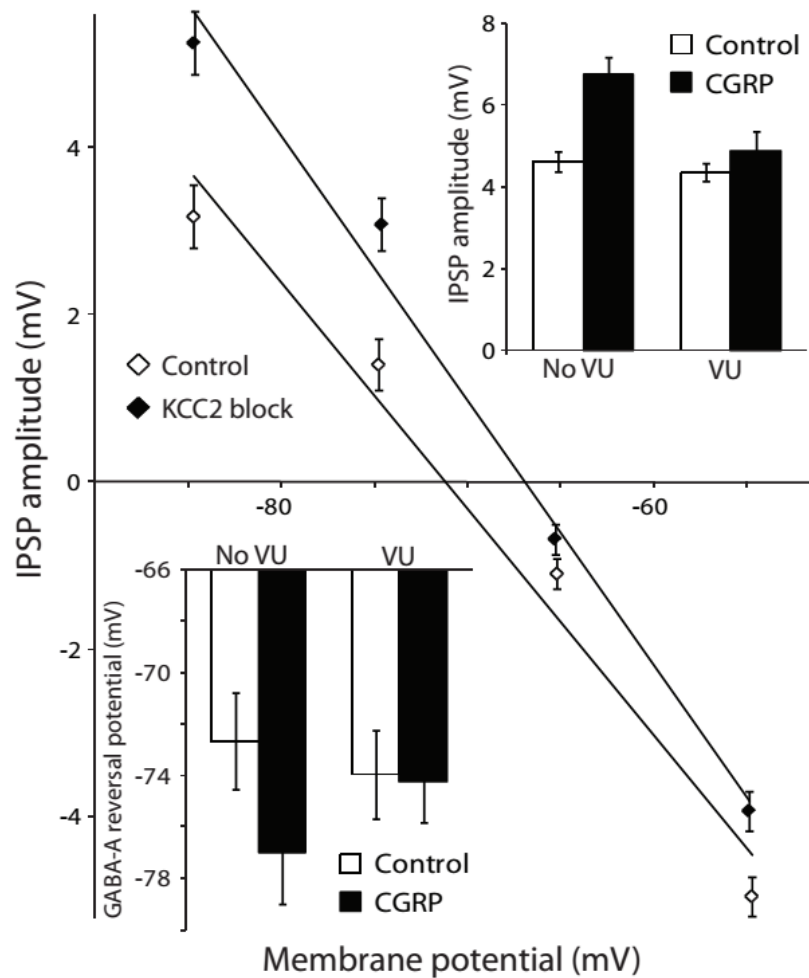


Figure 3.3. KCC2 antagonist VU-0240551 (VU) prevents the effects of CGRP on IPSP amplitudes and reversal potentials. IPSP amplitude (y-axis) as a function of membrane potential (x-axis) in cells recorded in the absence (empty diamonds) vs. presence (filled diamonds) of the KCC2 antagonist VU-0240551 (40 μ M). **Top right inset:** Amplitude of IPSPs before (empty bars) vs. after CGRP (filled bars) in the absence (left) vs. presence (right) of VU-0240551. **Bottom left inset:** GABA-A reversal potential before (empty

bars) vs. after CGRP (filled bars) in the absence (left) vs. presence (right) of VU-0240551.

CHAPTER IV

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

4.1 Rationale

As I reviewed in the Section 1.9, CeA and BNST are thought to play different roles in the genesis of negative emotional states. CeA is thought to generate brief fear reactions in response to discrete and short lasting conditioned cues; whereas BNST generates long lasting anxiety-like states in response to more diffuse contingencies.

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. periaqueductal 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.

4.2 Overview of methods

Male Lewis rats (225-250 gr) underwent virus injection surgeries. Approximately 6 weeks later, they were sacrificed for *in vitro* slice recordings. BNST and CeA slices were optogenetically stimulated to evoke postsynaptic potentials.

4.3 Results

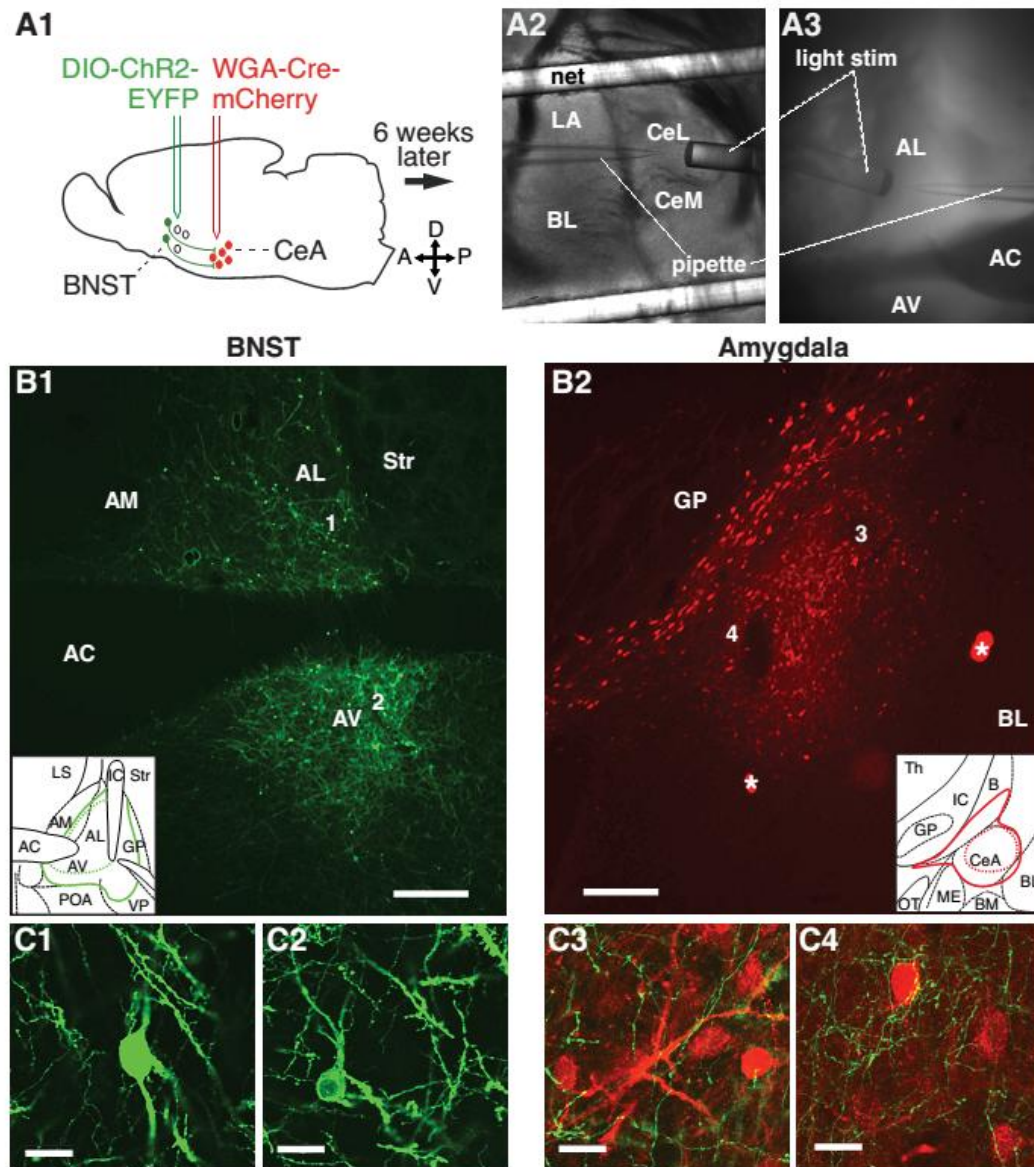


Figure 4.1. (A) Experimental design. (A1) Dual viral strategy for selectively driving ChR2 expression in BNST neurons that project to the central amygdala. Six weeks after the virus infusions, coronal slices of the amygdala (A2) and BNST (A3) were prepared for whole-cell patch clamp recordings. Blue 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 μ m, 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.

4.3.1 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. 4.1). To this end, EF1a-mCherry-IRES-WGA-Cre was infused in CeA (Fig. 4.1A1, red), causing the expression of Cre in neurons projecting to CeA. EF1a-DIO-hChR2(H134R)-EYFP was infused in BNST (Fig. 4.1A1, 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. 4.1A2) and

BNST (Fig. 4.1A3) 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 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).

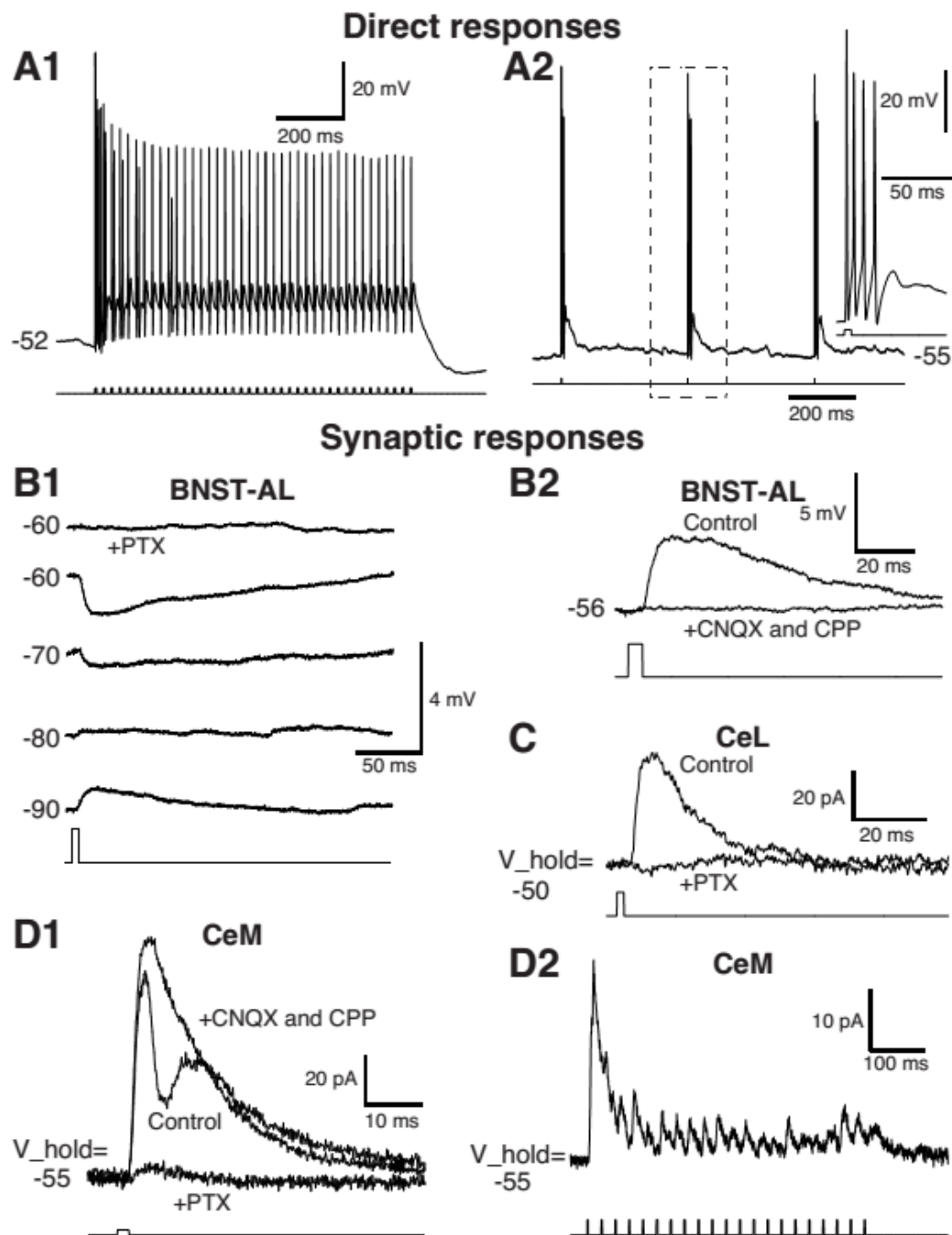


Figure 4.2. Blue light evoked responses in BNST and CeA neurons. (A) Direct responses in ChR2-expressing BNST neurons that project to CeA. (A1) Train of light stimuli (bottom) reliably eliciting spikes (top). (A2) At a lower frequency, each light stimulus (bottom) elicits a spike burst (top). **Inset** 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 μ M) 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 μ M) and CPP (10 μ M). **(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.

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.

4.3.2 Anatomical observations

Figure 4.1 provides representative examples of the distribution of EYFP⁺ neurons in BNST (Fig. 4.1B1) and of mCherry in CeA (Fig. 4.1B2). Higher power illustrations of labeled elements are provided in figure 4.1C. 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. 4.1C3,4). These observations are consistent with prior tracing studies (Sun and Cassell, 1993; Dong et al., 2001b).

4.3.3 Local BNST connections

With the methods we used, BNST cells that project to CeA express EYFP and Chr2 (Fig. 4.1B,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. 4.2A, 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. 4.2A1). 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. 4.2A2; 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. 4.2B). In BNST-AL, 15 of 30 tested EYFP⁻ cells responded to blue light stimulation (Fig. 4.3A), implying they

receive inputs from the BNST cells that project to CeA. In thirteen of these cells, blue light stimuli elicited IPSPs (Fig. 4.2B1); only two cells with excitatory responses were observed (Figs. 4.2B2, 4.3A). In BNST-AV, only three of 34 cells were responsive and all of these had inhibitory responses (Fig. 4.3B). The proportion of responsive EYFP⁻ cells was significantly lower in BNST-AV than BNST-AL (Fig. 4E; Fisher exact test; $p = .0003$). The leftmost two columns of Table 4.1 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 4.1. Properties of light-evoked responses in BNST and CeA neurons. Values are means $\pm$ SEM.

	BNST-AL	BNST-AV	CeL	CeM
IPSP incidence	13/30	3/34	10/28	15/23
IPSP latency (ms)	4.31 $\pm$.3	3.07 $\pm$.9	5.51 $\pm$ 1.02	4.1 $\pm$.58
IPSP amplitude (mV)	-2.94 $\pm$.52	-5.12 $\pm$ 3.07	-1.77 $\pm$.38	-3.27 $\pm$.75
IPSP reversal (mV)	-78.6 $\pm$ 3.3	-76.79 $\pm$ 2.9	-68.8 $\pm$ 1.4	-71.59 $\pm$ 2.54
EPSP incidence	2/30	0/34	2/28	2/23
EPSP latency (ms)	7.27 $\pm$.03	N/A	2.17 $\pm$.03	4.08 $\pm$.98
EPSP amplitude (mV)	1.72 $\pm$.74	N/A	2.8 $\pm$.87	3.41 $\pm$ 1.35

Table 4.2 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 fIR, LTB, and RS cells between responsive and unresponsive cells (Table 4.5; BNST-AL: χ^2 (2, N=30) = 4.29, p = 0.11).

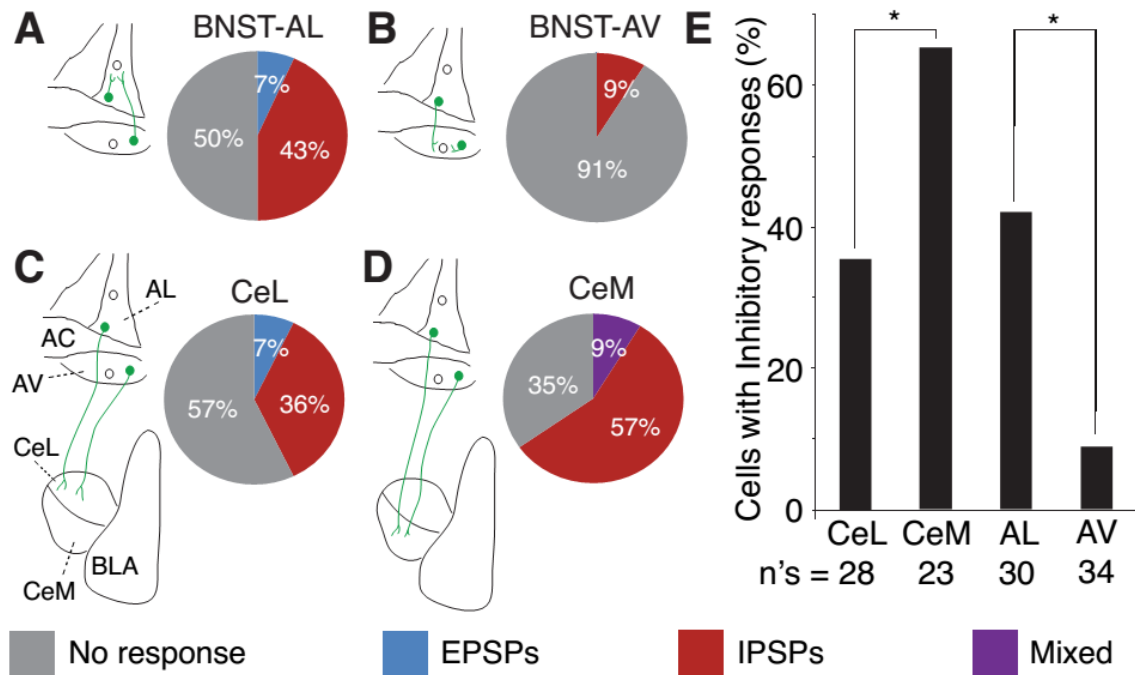


Figure 4.3. Incidence and types of responses elicited by blue light stimuli in BNST-AL (A), BNST-AV (B), CeL (C), and CeM (D) neurons. The schemes on the left of each pie chart illustrate the pathway stimulated and recording sites examined. In the pie charts, grey indicates the percentage of unresponsive 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.

4.3.4 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 4.2C-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. 4.2C,D2 and 4.3C,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. 4.2D1).

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. 4.3E, 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 4.1 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 4.3;

CeM, Table 4.4). 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 4.5) between responsive and unresponsive CeL (χ^2 (2, N=28)= 0.8, p = 0.67) or CeM neurons (χ^2 (2, N=23)= 2.84, p = 0.24).

Last, we tested the pharmacological sensitivity of light-evoked synaptic responses in nine cells (Fig. 4.2B,C). Irrespective of the recording site, all inhibitory responses were abolished or nearly obliterated by picrotoxin (100 μ M; n = 7) whereas excitatory responses were eliminated or largely reduced by CNQX and CPP (both 10 μ M, n =2).

Table 4.2. Physiological properties of responsive and non responsive BNST-AL neurons. Values are means $\pm$ SEM.

	Responsive cells (n=15)	Unresponsive cells (n=15)	p
Resting potential (mV)	-62.9 $\pm$ 2.4	-62.5 $\pm$ 1.9	.91
Input resistance (M Ω)	706.9 $\pm$ 51.3	658.3 $\pm$ 50.6	.5
Time constant (ms)	46.9 $\pm$ 5.3	51.6 $\pm$ 6.9	.6
Rheobase (pA)	15.3 $\pm$ 2.4	18.7 $\pm$ 2.2	.31
Spike threshold (mV)	-43.1 $\pm$ 1.1	-45.5 $\pm$ 1.7	.24
Spike latency (ms)	94.7 $\pm$ 12.6	50 $\pm$ 9.2	.008*
Spike amplitude (mV)	81.4 $\pm$ 4.3	78.2 $\pm$ 3.9	.59
Spike duration at half amplitude (ms)	0.62 $\pm$.06	0.69 $\pm$.06	.41
Firing rate at rheobase (Hz)	5.1 $\pm$ 0.7	4.7 $\pm$ 0.7	.6

Table 4.3. Physiological properties of responsive and non responsive CeL neurons. Values are means $\pm$ SEM.

	Responsive cells (n=12)	Unresponsive cells (n=16)	p
Resting potential (mV)	-62.3 $\pm$ 2.3	-61.4 $\pm$ 1.1	.72
Input resistance (M Ω)	506.2 $\pm$ 84.5	413.4 $\pm$ 26.8	.25
Time constant (ms)	60 $\pm$ 6.9	54.8 $\pm$ 5.5	.56
Rheobase (pA)	35 $\pm$ 8.2	33.1 $\pm$ 3.4	.82
Spike threshold (mV)	-43.2 $\pm$.9	-42.8 $\pm$ 1	.81
Spike latency (ms)	67.6 $\pm$ 14.6	118.8 $\pm$ 28	.15
Spike amplitude (mV)	94.1 $\pm$ 2.2	89.5 $\pm$ 2	.13
Spike duration at half amplitude (ms)	0.6 $\pm$.05	0.56 $\pm$.04	.7
Firing rate at rheobase (Hz)	6.8 $\pm$.7	7.6 $\pm$ 1.3	.64

Table 4.4. Physiological properties of responsive and non responsive CeM neurons. Values are means $\pm$ SEM.

	Responsive cells (n=15)	Unresponsive cells (n=8)	p
Resting potential (mV)	-62.8 $\pm$ 2.4	-64.38 $\pm$ 2.8	.68
Input resistance (M Ω)	486.3 $\pm$ 82.5	487.7 $\pm$ 67.6	.99
Time constant (ms)	53.5 $\pm$ 8.5	46.8 $\pm$ 14.9	.67
Rheobase (pA)	31.7 $\pm$ 5.8	37.1 $\pm$ 4.7	.52
Spike threshold (mV)	-42.3 $\pm$ 1.2	-42.6 $\pm$ 1.3	.87
Spike latency (ms)	108.3 $\pm$ 29.9	83.6 $\pm$ 35.5	.61
Spike amplitude (mV)	92.7 $\pm$ 2.2	96.4 $\pm$ 2.6	.3
Spike duration at half amplitude (ms)	.5 $\pm$.03	.42 $\pm$.04	.16
Firing rate at rheobase (Hz)	4.8 $\pm$.7	6 $\pm$ 1.9	.5

Table 4.5. Incidence of different physiological cell types among responsive (r) and unresponsive (nr) BNST (top) and CeA (bottom) neurons.

	RS			LTB			fIR		
	Total	r	nr	Total	r	nr	Total	r	nr
BNSTAL	17	11	6	11	4	7	2	0	2
BNSTAV	12	1	11	17	1	16	3	1	2

	RS			LTB			LF		
	Total	r	nr	Total	r	nr	Total	r	nr
CeL	12	5	7	3	2	1	12	5	8
CeM	9	4	5	10	8	2	4	3	1

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

4.4 Summary of results

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.

CHAPTER V

CHARACTERIZING THE PHYSIOLOGICAL PROPERTIES AND NA RESPONSIVENESS IN GLUTAMATERGIC AND GABAERGIC BNST-AV CELLS

5.1 Rationale

Recently, it was shown that glutamatergic and GABAergic BNST-AV neurons play opposite roles in negative emotional states. VTA-projecting VGlut2 cells increase their firing rate during aversive unconditioned and conditioned stimuli. In contrast, GABAergic cells are inhibited by both. Optogenetically activating VGlut2 cells produces place aversion and anxiogenic effects, whereas activation of the GABAergic cells produces place preference and anxiolytic effects (Jennings et al., 2013).

In line with above, much data suggests that these two cell groups control the HPA-axis in opposite ways. For instance, global BNST-AV lesions reduce stress-induced fos expression in PVN (Choi et al., 2007; Crane et al., 2003; Spencer et al., 2005). In contrast, selective ablation of GABAergic cells increases adrenocorticotrophic hormone and corticosterone levels after restraint stress (Radley et al., 2009). Likewise, mPFC (Radley et al., 2009) and hippocampal lesions (Radley and Sawchenko, 2011) decrease the number of fos-positive GABAergic cells in BNST-AV, while increasing fos expression in PVN. These findings indicate that while GABAergic BNST-AV neurons inhibit PVN, the overall influence of BNST-AV over PVN is excitatory. Together, these observations suggest that glutamatergic BNST-AV cells are the source of the facilitatory

effect and that their influence dominates. This conclusion is surprising, given that few BNST-AV cells are glutamatergic (Poulin et al., 2009).

Another factor regulating BNST-AV's influence over negative emotional states is NA. Negative events like immobilization stress (Pacak et al., 1995; Cecchi et al., 2002), exposure to the fox-odor component TMT (Fendt et al., 2005), formalin-induced pain (Deyama et al., 2008) and aversive tastes (Park et al., 2012) increase NA levels in BNST-AV. The NA innervation of BNST-AV, arising from A1 and A2 cell groups in the brainstem (Moore, 1978; Woulfe et al., 1988; Shin et al., 2008; Bienkowski and Rinaman, 2013), is among the strongest in the brain (Forray et al., 2000) and strongly inhibits BNST-AV neurons (in vivo, Casada and Dafny, 1993; in vitro, Egli et al., 2005).

To shed light on how glutamatergic BNST-AV cells generate negative emotional states, we first set out to identify their electrophysiological and characteristics. We also compared the influence of NA on the excitability of glutamatergic and GABAergic neurons.

5.2 Overview of the methods

We prepared transgenic mice that express fluorescent protein in either glutamatergic or GABAergic cells for whole cell patch clamp recordings in brain slices kept in vitro. We investigated their electroresponsive characteristics. We also investigated the effects of NA on EPSPs triggered by electrically stimulating the stria terminalis fibers.

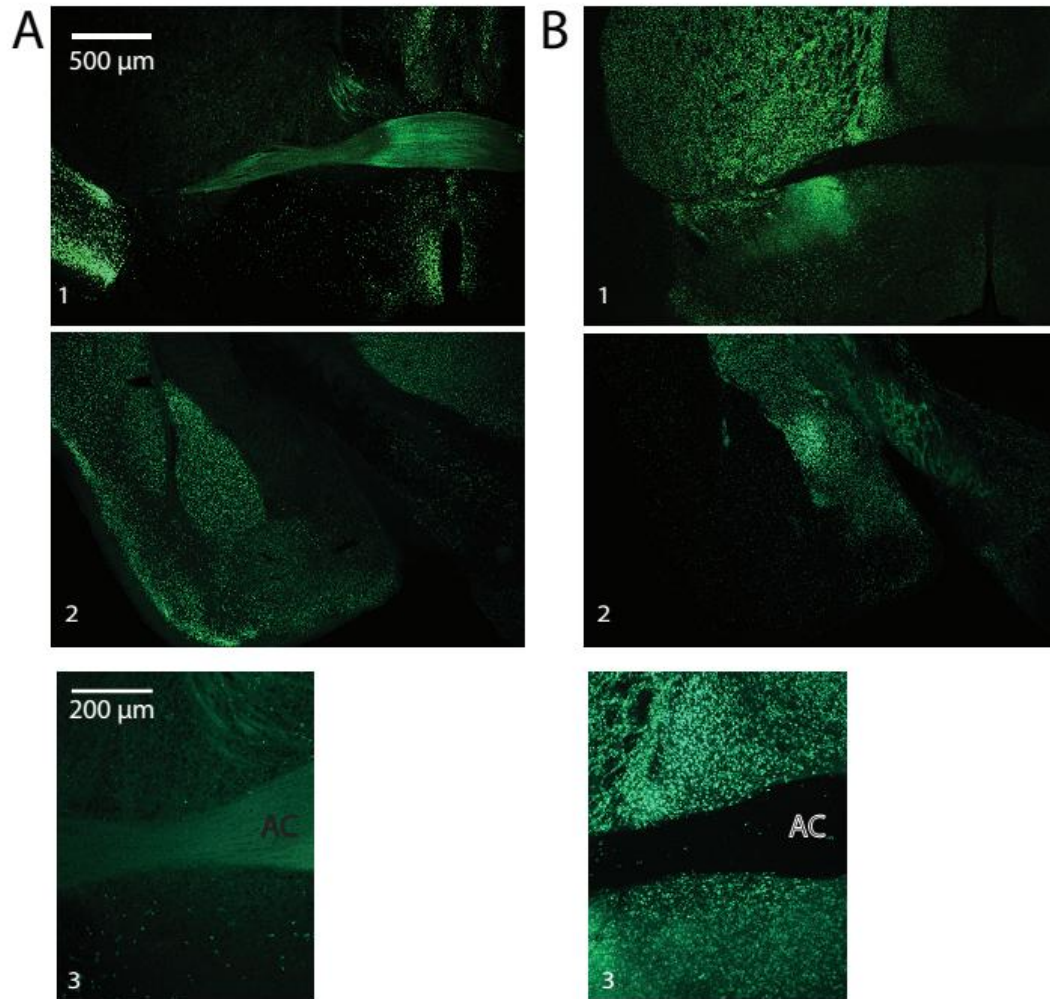


Figure 5.1. Contrasting the distribution of glutamatergic and GABAergic neurons in BNST and the amygdala. **A.** Vglut2-Cre-IRES-knockin mice crossed with reporter ROSA. **C.** Vgat-Cre-IRES-knockin mice crossed with reporter ROSA. **(1)** BNST and surrounding areas. **(2)** CeA and surrounding areas. **(3)** BNST at higher magnification.

5.3 Results

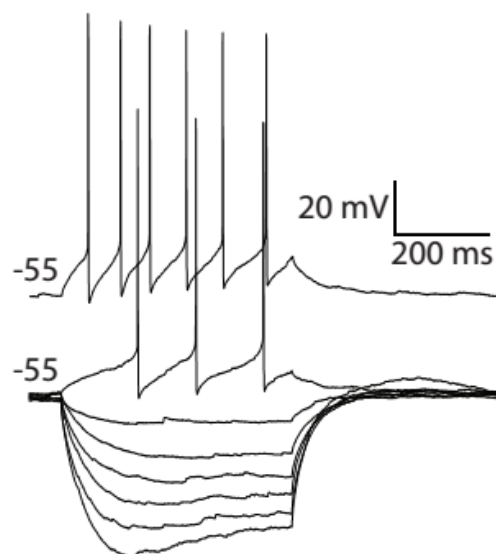
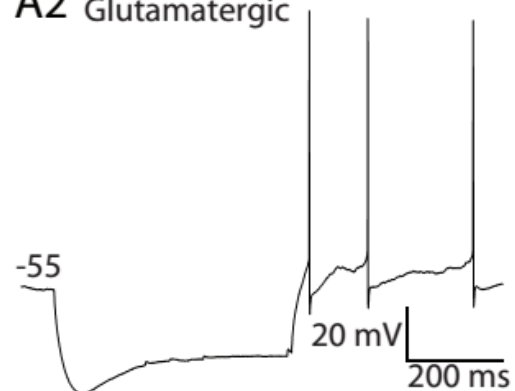
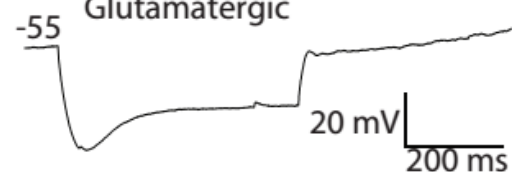
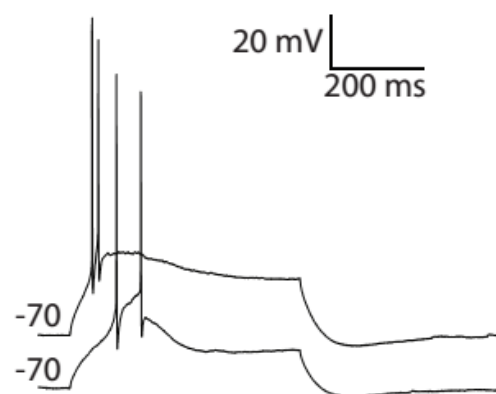
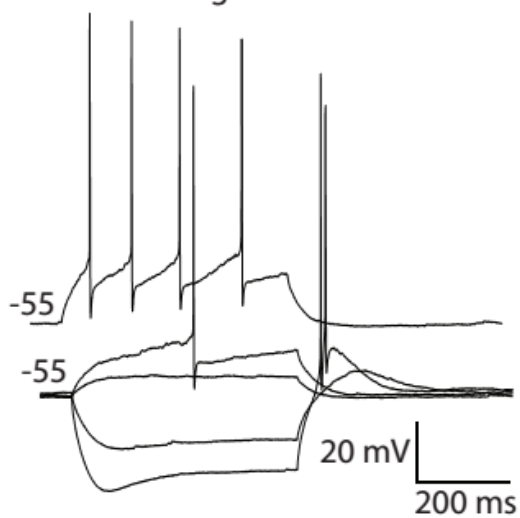
In order to visualize glutamatergic or GABAergic cells in BNST-AV, we used transgenic mice that express the fluorescent reporter ZsGreen in either cell group (Fig 5.1). In total, 33 glutamatergic and 72 GABAergic cells were recorded from 23 and 40

animals, respectively. Between the two types of cells, we found differences in the prevalence of spontaneously firing cells, intrinsic excitability, and incidence of fIR cells, as detailed below.

5.3.1 Physiological differences between glutamatergic and GABAergic cells

Previously, three main BNST cell types have been distinguished in rats based on their physiological properties (Hammack et al., 2007; Rodriguez-Sierra et al., 2013): Regular spiking (RS- Type I), low threshold bursting (LTB- Type II) and fast inward rectifying (fIR- Type III), the latter being the least common. The physiological properties of BNST-AV neurons in our database matched these earlier descriptions (Fig 5.2A,B,C); however we observed a difference in the incidence of the cell types between glutamatergic and GABAergic neurons (Fig 5D). Although the prevalence of RS (glutamatergic: 12/33, GABAergic: 35/72) and LTB (glutamatergic: 16/33, GABAergic: 35/72) cells was similar in both populations, fIR cells (Fig 5.2C) were significantly less frequent among GABAergic than in glutamatergic neurons (glutamatergic: 5/33, GABAergic: 2/72; Fisher's exact test, fIR vs. non-fIR; $p = .009$).

A second difference between glutamatergic and GABAergic cells was the magnitude of the depolarizing sag observed in response to negative current injections (-60pA). Commonly observed in RS and LTB cells (Fig 5.2A, B), this sag is thought to result from the activation of the hyperpolarization-activated mixed cationic current I_h (Hammack et al., 2007). Although the incidence of the depolarizing sag (>2 mV) was similar between the two cell phenotypes (glutamatergic: 96%, GABAergic: 91%), its magnitude was significantly higher in glutamatergic cells (glutamatergic: 9.9 ± 4.9 , GABAergic: 7.5 ± 3.9 , $t(68) = 2.2$, $p = .03$).

A1 Glutamatergic**A2** Glutamatergic**A3** Glutamatergic**B1** GABAergic**B2** GABAergic

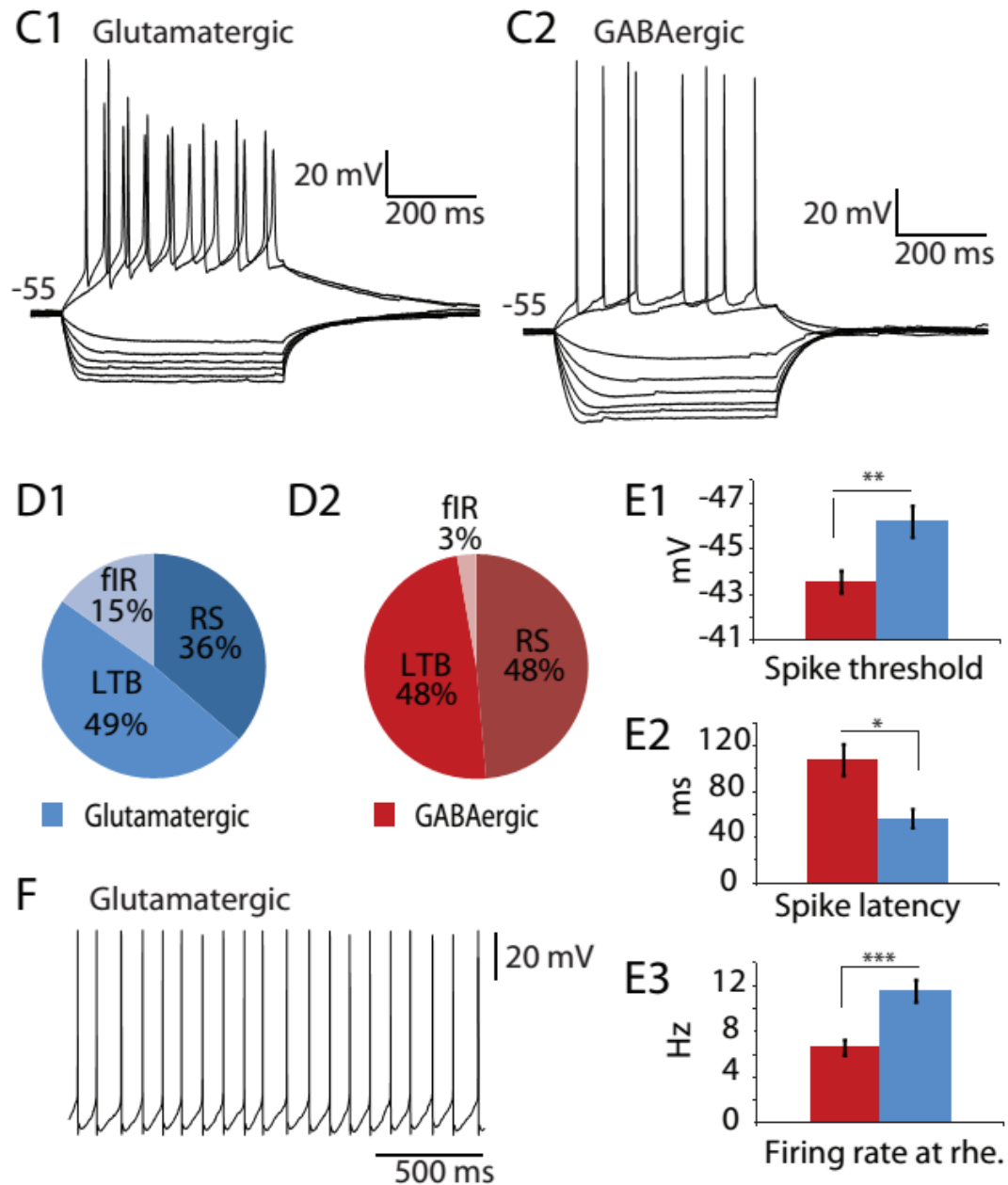


Figure 5.2. Cell types in BNST-AV. **A.** Regular spiking (Type I) cells. **A1.** A glutamatergic cell with small depolarizing sag. Voltage responses to current injections from -60pA to 20pA in 10pA increments are depicted. **A2.** A rare example of rebound firing by a RS cell at the end of a -60pA current injection. At the end of the negative current pulse, note the lack of a slow rebound depolarization that characterizes LTB cells. **A3.** Example of a glutamatergic cell that lacks rebound firing. Cells in A2 and A3 both

show strong depolarizing sags. **B1.** A GABAergic LTB (Type II) cell that shows spike doublets in response to depolarization from -70mV. **B2.** The same cell displays regular firing at -55mV. At the end of hyperpolarizing current pulses, the same neuron generates a slow depolarizing current that may cause spike doublets or burst firing. Note that the slow depolarization outlasts firing. In some cases, rebound firing was also observed when the hyperpolarizing current pulses were applied at -70mV. **C1.** A glutamatergic fIR (Type III cell) cell. These cells displayed fast inward rectification in response to hyperpolarizing current pulses and regular firing in response to depolarization. In a subset of fIR cells, a low amplitude depolarization sag (<2mV at -60pA current injection) was observed. **C2.** A GABAergic fIR cell. In A1, B1 and B2, traces showing 20pA injection have been offset for clarity. **D.** Incidence of physiological cell types among glutamatergic (**D1**) and GABAergic cells (**D2**). **E.** Differences between glutamatergic and GABAergic cells in spike threshold (**E1**), latency to fire at rheobase (**E2**) and firing rate at rheobase (**E3**). **F.** A glutamatergic cell spontaneously firing at 8.5 Hz at rest. * $p < .05$, ** $p \leq .01$, ***, $p \leq 0.001$, corrected.

Third, glutamatergic cells were more excitable than GABAergic cells (Fig 5.2E). They had lower spike thresholds ($t(80)=3.2$; see Table 5.1 for p values), shorter latencies to spiking at rheobase ($t(80)=2.86$), and higher firing rates at rheobase ($t(80)=4.3$). Thus, excitatory inputs are more likely to trigger action potentials in glutamatergic than GABAergic cells.

Table 5.1. Physiological properties of glutamatergic and GABAergic neurons (mean $\pm$ SD).

	Glut. cells (n=33)	GABAergic cells (n=49)	<i>p</i>	<i>p</i> corr'd
Input resistance (M Ω)	1177 $\pm$ 601	1145 $\pm$ 547	.8	.9
Time constant (ms)	72.3 $\pm$ 75.3	54.2 $\pm$ 24.4	.13	.39
Rheobase (pA)	12.1 $\pm$ 4.8	13.1 $\pm$ 5.8	.45	.9
Spike threshold (mV)	-46.2 $\pm$ 3.9	-43.6 $\pm$ 3.4	.002	.01
Spike latency (ms)	56.8 $\pm$ 46.5	108 $\pm$ 94.3	.005	.02
Firing rate at rheobase (Hz)	11.6 $\pm$ 5.6	6.6 $\pm$ 4.8	<.001	.0003

The last difference between the two phenotypes was the incidence of spontaneously firing cells (firing rates >3 Hz at rest). A markedly higher proportion of spontaneously firing cells was observed among the glutamatergic cells (68%; Fig 5.2F) compared to GABAergic cells (24%; Fishers exact test; $p=.0001$). For comparison, we recorded 16 GABAergic cells in dorsal BNST and did not encounter any spontaneously active cell.

5.3.2 NA effects on electroresponsive properties

The effects of NA on the electroresponsive properties of BNST-AV cells were tested in a subset of neurons (glutamatergic, $n=5$; GABAergic, $n=8$). In these cells, NA (100 μ M) application did not change the time constant, rheobase or firing characteristics (Table 5.2), except for a small but highly significant negative shift in the spike threshold of the glutamatergic cells ($t(4)=11$, $p<.001$; Fig. 5.3A). As mentioned above, glutamatergic cells are intrinsically more excitable than GABAergic cells in control conditions. NA enhances this difference.

Table 5.2. NA (100 μ M) effects on electroresponsive properties of BNST-AV cells (mean $\pm$ SD).

	Glut. cells (n=5)		<i>p</i>	GABAergic cells (n=8)		<i>p</i>
	Control	NA		Control	NA	
Time cons.(ms)	130.8 $\pm$ 149.3	93.4 $\pm$ 45.7	.66	66.5 $\pm$ 23.8	78.5 $\pm$ 55.7	.44
Rhe. (pA)	14 $\pm$ 8	12 $\pm$ 4	.37	12.5 $\pm$ 4.3	13.8 $\pm$ 4.8	.35
Sp. thres. (mV)	-45 $\pm$ 3.1	-47.2 $\pm$ 2.9	<.001* ¹	-41.5 $\pm$ 2.8	-42.6 $\pm$ 2.6	.15
Sp. lat. (ms)	61.4 $\pm$ 49.5	109.8 $\pm$ 100.5	.39	144 $\pm$ 4 $\pm$ 83.7	111.3 $\pm$ 78.1	.39
FR at rhe. (Hz)	8 $\pm$ 5.1	10.8 $\pm$ 9	.45	6.8 $\pm$ 6.2	9.3 $\pm$ 6.8	.14

¹Corrected *p* value is .002.

5.3.3 NA effects on glutamatergic transmission

Next, in the presence of picrotoxin (100 μ M), we assessed whether NA modulates ST-evoked EPSPs in BNST-AV. Consistent with previous findings (Sawada and Yamamoto, 1981; Matsui and Yamamoto, 1984; Casada and Dafny, 1993; Egli et al., 2005; Krawczyk et al., 2011), we observed a reduction in EPSP amplitudes upon NA application ($F_{\text{within}}(2,42)=77.05$, $p<.0001$) and this effect was seen in both glutamatergic (control 6.26 $\pm$ 1.13, NA 3.37 $\pm$.49, $t(10)=3.16$, $p=.01$, $p \text{ corr'd}=.02$) and GABAergic (control 6.34 $\pm$.69, NA 2.10 $\pm$.33, $t(11)=6.93$, $p<.0001$, $p \text{ corr'd}=.0002$, Fig. 5.3C4) cells. However, the EPSP amplitude reduction caused by NA was significantly less pronounced in glutamatergic cells (41% decrease) than GABAergic (66%) cells at its peak. Furthermore, the effect was shorter lasting in the glutamatergic cells. Difference was significant at various time points (5 min: $t(21)=3.24$, $p=.004$, $p \text{ corr'd}=.02$; 10 min: $t(21)=2.62$, $p=.02$, $p \text{ corr'd}=.06$; 25 min: $t(10)=2.41$, $p=.03$, $p \text{ corr'd}=.11$; 30 min: $t(8)=3.77$, $p=.006$, $p \text{ corr'd}=.03$, Fig. 5.3C1).

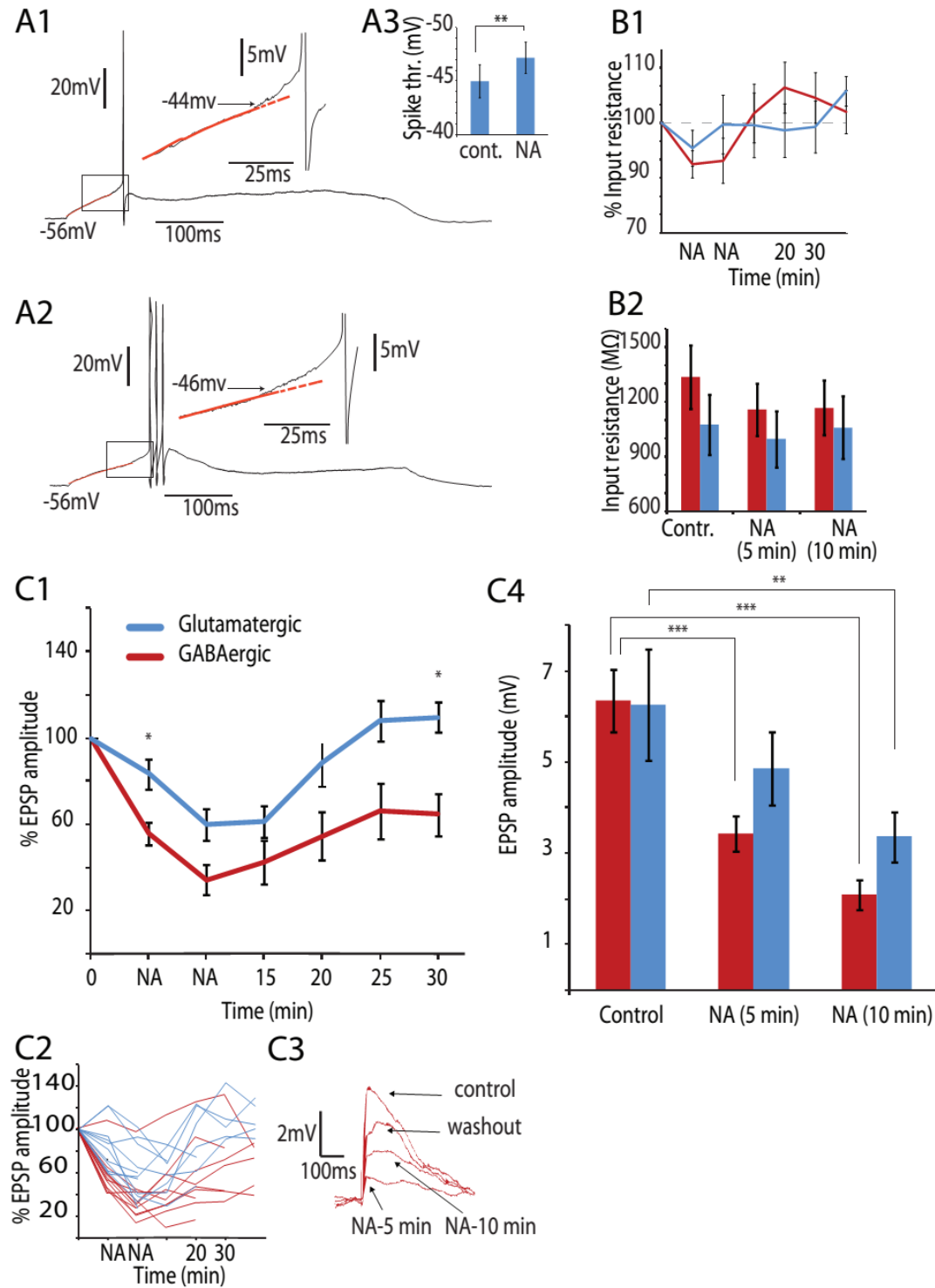


Figure 5.3. NA effects on the intrinsic properties and ST-evoked EPSP

amplitudes of BNST-AV cells. **A.** Spike threshold. **A1.** A glutamatergic cell firing at

rheobase before NA. An exponential curve is fit to reveal the spike threshold (-44mV). Magnified trace is shown in the inset. **A2.** After NA, the spike threshold is decreased to -46mV. **A3.** Summary graph for all glutamatergic cells ($n=5$). Note that y-axis is reversed.

B. Input resistance. B1. Percentage change in input resistance after NA. There was no difference in the change in input resistance between the glutamatergic and GABAergic cells at any time point. **B2.** Input resistance before and during NA application. There was no difference between the glutamatergic or GABAergic cells before NA application (glutamatergic 1075 ± 165 , GABAergic 1338 ± 174 , $t(21)=1.07$, $p=.3$). A statistically significant time dependent reduction in input resistance upon NA application was observed ($F_{\text{within}}(2,42)=4.58$, $p=.01$). However, the decrease in input resistance in GABAergic cells in the first 5 minutes of NA application was not significant in the multi-test corrected post-hoc analysis (control 1338 ± 174 , NA 1159 ± 142 , $t(11)=2.77$, $p=.02$, corr'd $p=.07$).

C. ST-evoked EPSP amplitudes. C1. Percentage change in EPSP amplitudes after NA. Significant differences in the change in EPSP amplitude between glutamatergic and GABAergic cells are marked by asterisks. **C2.** Percent change in EPSP amplitudes for each cell recorded. Note that the outlier among the GABAergic cells was kept in all analyses. **C3.** Example traces from a GABAergic cell. **C4.** EPSP amplitudes before and during NA application. There was no difference in EPSP amplitudes before NA application (glutamatergic 6.26 ± 1.13 , GABAergic $6.34 \pm .69$, $t(21)=.06$, $p=.95$). * $p < .05$, ** $p \leq .01$, *** $p \leq 0.001$, corrected.

CHAPTER VI

GENERAL DISCUSSION

The work presented in this thesis was undertaken to investigate BNST's role in negative emotional states. First, I showed that CGRP, a peptide that produces anxiogenic effects *in vivo*, inhibits neurons in BNST-AL. The results of this study supports the view that BNST-AL exerts anxiolytic influences. Second, I showed that most of the projections from BNST to CeA are inhibitory and are more prevalent in CeM. The anxiogenic cells of BNST-AV contribute little to this projection. Furthermore, in BNST-AL, the CeA-projecting cells do not send collaterals to their non-projecting neighbors. Third, compared to GABAergic cells, glutamatergic cells of BNST-AV are highly excitable. While NA reduced ST-evoked EPSPs in GABAergic cells, this reduction was negligible in glutamatergic cells.

6.1 CGRP effects in BNST-AL

The study presented in Chapter III aimed to characterize the influence of CGRP on BNST neurons. Pontine parabrachial neurons constitute the sole source of CGRP to BNST and they project to its anterolateral sector (Alden et al., 1994) where there are no glutamatergic, only GABAergic/peptidergic cells (Poulin et al., 2009). In light of this data, the finding that intra-BNST infusions of CGRP enhance startle and neuronal activation in BNST-AL targets (Sink et al., 2011), suggested that CGRP inhibits BNST-AL neurons. However, this inference is in apparent contradiction with the generally accepted view that BNST activity exerts an anxiogenic influence (Davis et al., 2010). Here, we observed that CGRP inhibits BNST-AL neurons. Below, I consider the

mechanisms and significance of CGRP's inhibitory influence on BNST-AL for the regulation of fear and anxiety.

6.1.1 CGRP potentiates GABA-A inhibition through a postsynaptic regulation of Cl^- homeostasis

To the best of our knowledge, there are no prior reports of CGRP's influence on BNST neurons. However, in other parts of the nervous system, a variety of cell-type specific effects were reported. For example, CGRP inhibits high-threshold voltage-gated Ca^{2+} currents in neurons of nucleus tractus solitarius (Hosokawa et al., 2010) but enhances them in dorsal root ganglia cells (Ryu et al., 1988). It causes a membrane hyperpolarization in some cell types (Kajekar and Myers, 2008) and the opposite (Gokin et al., 1996) or no change in others (Meng et al., 2009). In CA1 pyramidal cells, CGRP inhibits the slow Ca^{2+} -dependent K^+ current (Haug and Storm, 2000). Consistent with this, in central amygdala neurons, CGRP reduces spike frequency adaptation. In the same cell type, CGRP also causes a postsynaptically-mediated increase in glutamatergic EPSCs at rest (Han et al., 2005, 2010).

Given the functional kinship and anatomical similarities between BNST and the central amygdala, one might expect CGRP to exert similar effects at the two sites. Yet, this is not what we observed. In BNST-AL cells, firing rate/pattern, passive properties, spike characteristics, and EPSP amplitudes were unaffected by CGRP. Instead, CGRP produced a robust potentiation of ST-evoked IPSPs and this effect was reduced or blocked by CGRP Type-1 receptor antagonists.

Several observations indicate that the IPSP potentiation produced by CGRP is dependent on a postsynaptic mechanism. First, CGRP did not alter the PPR and there was

no correlation between the ratios of experimental to control IPSP variance vs. amplitude. Second, the R_{in} drop associated with the IPSP was not altered by CGRP. Third, CGRP shifted E_{GABA-A} negatively and the amplitude of this shift correlated with the magnitude of IPSP potentiation. Last, CGRP's effect on IPSP amplitudes and reversal potentials was blocked by prior application of a KCC inhibitor. Although the mechanisms of CGRP action on chloride homeostasis are currently unclear, given the results obtained in other cells types (reviewed in Khale et al., 2010), an upregulation of potassium chloride transporters by phosphoregulation is likely involved.

6.1.2 Alternative routes for CGRP's anxiogenic effects

A different interpretation than the above was recently offered for CGRP's anxiogenic effects. Sink et al. (2013) reported that systemic or intra-BNST infusions of CRF-R1 antagonists as well as virally mediated knockdown of CRF expression interfered with the startle potentiation produced by intra-BNST CGRP infusions. These results imply that CGRP acts by *increasing the activity of CRF-positive BNST neurons*. On the surface, these results seem inconsistent with our findings, as we demonstrated that CGRP *inhibits BNST-AL cells* by the changing intracellular chloride concentration.

As I reviewed in Section 1.6, activation of CRF neurons in BNST-AL induce anxiogenic states. Sink et al. (2013) demonstrated that CRF cells are a downstream target of CGRP actions. How can their results be reconciled with ours? The answer might lie in the types of neurons we sampled. Stimulating electrodes were positioned on the dorsal portion of BNST-AL, where the stria terminalis is thick. However, this area partially overlaps with the oval nucleus. Consequently, most of the recorded cells were positioned in the ventral part of BNST-AL. This recording configuration prevented us from

recording cells in the oval nucleus, where CRF cells are located. Accordingly, we did not have any Type III neurons in our sample, which express CRF mRNA (Dabrowska et al., 2013).

Our findings indicate that CGRP increases the inhibitory tone on type I-II cells. Speculatively, it might also selectively activate CRF cells. Alternatively, assuming that Type I-II cells make synaptic contacts on CRF cells, when CGRP inhibits the type I-II cells, CRF cells might be *disinhibited*. In this plausible scenario, which BNST neuron population causes activation of BNST's output targets remains to be determined. Both CRF cells and non-CRF cells have been shown to be projection neurons (Moga et al., 1989; Gray and Magnuson, 1987; Gray and Magnuson, 1992; Moga and Saper, 1994; Rodaros et al., 2007; Panguluri et al., 2009 Vranjkovic et al., 2014). Thus, *cfos* activation might arise from either CRF release onto the target structures, or from a reduction of GABA release by Type I-II neurons.

6.2 Connections from BNST to CeA

In the study presented in Chapter IV, we 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, I consider the significance of these findings in light of previous studies about the regulation of fear and anxiety.

6.2.1 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; Ciochi 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. 6.1A).

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.

6.2.2 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. 6.1B).

6.2.3 BNST 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.

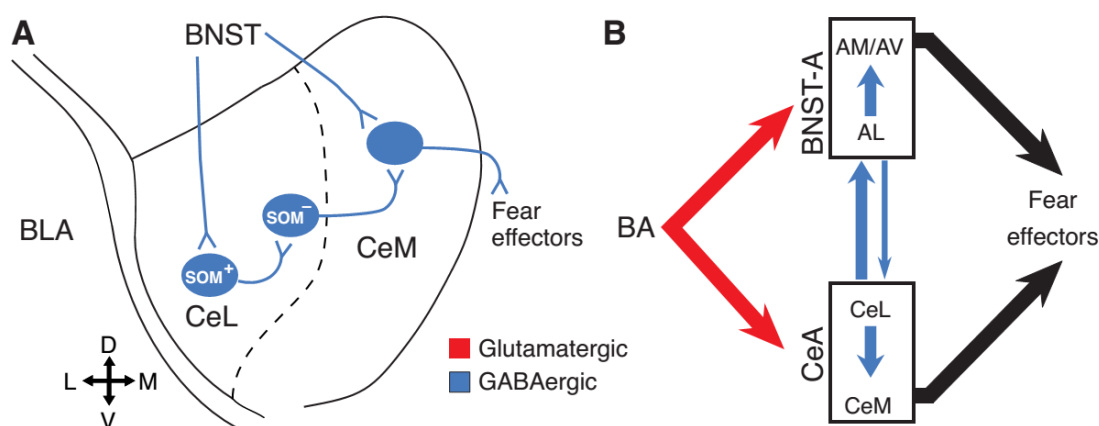


Figure 6.1. Hypothetical schemes of BNST-CeA interactions. **(A)** Differential innervation of SOM⁺ and SOM⁻ CeL neurons by BNST axons. The direct inhibitory effects of BNST projections to CeM neurons are increased by the inhibition of SOM⁺ CeL cells, leading to the disinhibition of SOM⁻ CeL neurons. **(B)** Overall organization of the reciprocal BNST-CeA connections.

6.3 NA effects on glutamatergic and GABAergic cells of BNST-AV

6.3.1 General characteristics of BNST-AV neurons in mice

Previously, BNST-AV cells were characterized in rats by Rodriguez-Sierra et al. (2013). They reported that LTB cells are the most common cell type in BNST-AV, followed by RS and FIR neurons. Although we observed the same distribution in glutamatergic cells, we found that RS cells are as frequent as LTB cells in GABAergic neurons. Second, we observed that the incidence of cells displaying a depolarizing sag is much higher (glutamatergic: 96%, GABAergic: 91%) in mice than in rats (50-60%). Third, the incidence of spontaneously firing cells is much higher in mice (54%) than in

rats (observed only in BNST-AV; 8%). Furthermore, in rats, these cells do not display depolarizing sags or LTBs, whereas in mice they commonly exhibit these features. Although these differences probably reflect species differences, we cannot rule out the possibility that the expression of a fluorescent reporter in our experiments altered the cells' physiological properties.

6.3.2 Glutamatergic cells are more excitable than GABAergic cells

Converging evidence summarized in the introductions suggests that glutamatergic cells exert anxiogenic effects. Puzzlingly, although few in number, their effect seems to dominate over the anxiolytic influence of the predominant GABAergic cells. The present study provides a possible explanation for this paradox. Indeed, we found that glutamatergic cells are intrinsically more excitable. For instance, they exhibit higher firing rates at rheobase, shorter firing latency, and more negative spike thresholds. Although it is currently unknown whether glutamatergic and GABAergic cells receive common afferents, given these differences in intrinsic excitability, upon receiving an excitatory input of similar strength, glutamatergic cells should be activated more readily and quickly. In turn, they will activate their downstream targets, such as PVN, and trigger an anxiogenic response.

6.3.3 Possible overlap between glutamatergic and CRF cell populations

Previously, it was reported that all fIR and some LTB cells in BNST-AL express CRF mRNA (Dabrowska et al., 2013a). Whether the same overlap exists in BNST-AV awaits confirmation. Despite this uncertainty, it is interesting that in our database fIR cells were more commonly observed in glutamatergic cells, suggesting that CRF cells might be glutamatergic. This possibility has been suggested by Radley et al. (2009), who

found that the selective ablation of GABAergic cells in BNST-AV left CRF mRNA levels intact. In contrast to this, the CRF cells in BNST-AL are GABAergic (Dabrowska et al., 2013a), and exert anxiogenic effects (Daniel and Rainnie, 2015). If indeed CRF cells in BNST-AV are glutamatergic, interesting questions arise regarding the interaction between the two CRF cell populations in BNST.

Dumont and Williams (2004) reported that VTA projecting BNST-AV cells display an inwardly rectifying potassium current, characteristic of fIR cells. Although we have demonstrated that most of these cells are glutamatergic, the phenotype of VTA-projecting cells has been debated. While early reports suggested that VTA projecting BNST-AV cells are glutamatergic (Georges and Aston-Jones, 2001, 2002), later studies showed that GABAergic cells also project to the VTA (Kudo et al., 2012; Jennings et al., 2013).

6.3.4 Noradrenergic effects on the intrinsic properties of BNST-AV neurons and ST-evoked EPSPs

We found that NA application causes a negative shift in the spike threshold of glutamatergic neurons. This small but highly significant change might be important in terms of regulating the stress response. Indeed, it was shown that NA is released in BNST-AV during stress and that it affects defensive behaviors (see introduction). However, the physiological mechanisms mediating these effects remained unclear. Here, we demonstrate that NA increases the excitability of glutamatergic BNST-AV neurons, which are already more excitable than GABAergic cells in control conditions. Thus, in time of stress, NA might exert anxiogenic effects by activating glutamatergic BNST-AV neurons.

Furthermore in the current study, we found that NA reduces ST-evoked EPSP amplitudes in both glutamatergic and GABAergic cells. However, this reduction is stronger and longer lasting in GABAergic cells. Thus, while NA release in BNST-AV at times of stress decreases the effectiveness of glutamatergic inputs to GABAergic cells, glutamatergic cells are partially spared from this modulation, again positioning them to dominate BNST-AV outputs to downstream effectors in stressful conditions.

6.3.5 NA reduces glutamatergic transmission through an $\alpha 2$ AR dependent mechanism

There is converging evidence that the major effect of NA in BNST is inhibitory. In vivo, NA reduces firing rates (Casada and Dafny, 1993). In vitro, NA reduces evoked excitatory field potentials (Sawada and Yamamoto, 1981; Matsui and Yamamoto, 1984; Egli et al., 2005) and EPSCs (Egli et al., 2005; Krawczyk et al., 2011) These effects were reduced or abolished in the presence of general α AR antagonists (Sawada and Yamamoto, 1981; Matsui and Yamamoto, 1984) or selective $\alpha 2$ AR blockers (Matsui and Yamamoto, 1984; Krawczyk et al., 2011, for complex effects of yohimbine, see below). Consistent with these observations, $\alpha 2$ AR agonists reduce glutamate release (Forray et al., 1999), excitatory field potentials and EPSCs (Egli et al., 2005; Shields et al., 2009; Krawczyk et al., 2011).

$\alpha 2$ ARs are $G_{i/o}$ coupled receptors whose effects include a reduction in cAMP production, augmentation of K^+ currents and decrease of Ca^{2+} currents (Stanford, 2001). Known as autoreceptors, they inhibit the release of NA by NA neurons. Though most abundant in the locus coeruleus, $\alpha 2$ AR mRNA is expressed widely in the brain suggesting that these receptors serve postsynaptic functions in non-NA cells as well (Scheinin et al., 1994).

In BNST, despite dense $\alpha 2AR$ labeling by autoradiography (Unnerstall et al., 1984; Boyajian et al., 1987; Hudson et al., 1992), $\alpha 2AR$ mRNA expression is low (Scheinin et al., 1994), suggesting that most of $\alpha 2AR$ expression is axonal. Interestingly, $\alpha 2AR$ and VGlut1 expression appear to colocalize (Shields et al., 2009). In line with this, $\alpha 2AR$ agonists reduce glutamate levels (Forray et al., 1999) and change paired pulse ratio of EPSCs in BNST-AL (Egli et al., 2005, a trend in BNST-AV is also observed; Shields et al., 2009; Krawczyk et al., 2011).

6.3.6 $\alpha 2AR$ s also act as autoreceptors

Overall, the results reviewed above suggest that NA inhibits glutamate release in BNST through activation of $\alpha 2AR$ s. Given that high BNST-AV levels of NA are observed in stressful conditions (Pacak et al., 1995; Cecchi et al., 2002; Fendt et al., 2005; Deyama et al., 2008), infusions of $\alpha 2AR$ agonists in vivo are expected to induce aversive behavioral states. Instead, behavioral studies found that *$\alpha 2AR$ agonists reduce* negative emotional states and *$\alpha 2AR$ antagonists* are effective stressors. For instance, systemic $\alpha 2AR$ agonist injections block stress induced reinstatement of cocaine seeking (Erb et al., 2000) and place preference (Mantsch et al., 2000). Intra BNST-AV $\alpha 2AR$ agonist infusions produce similar results with stress induced reinstatement of morphine conditioned place preference (Wang et al., 2001). This treatment also reduce TMT-evoked freezing (Fendt et al., 2005) as well as light enhanced and fear potentiated startle (Schweimer et al., 2005).

On the flip side, $\alpha 2AR$ antagonists are effective stressors, as their systemic or i.c.v administration activates the HPA-axis (Banihashemi and Rinaman, 2006), increase avoidance behavior (Morilak et al., 2003), induces reinstatement of cocaine-induced

place preference (Mantsch et al., 2010) and drug-seeking (Brown et al., 2009; Buffalari and See, 2011).

These behavioral effects are usually not attributed to $\alpha 2$ AR effects on glutamatergic transmission in BNST. Rather they are thought to arise from changes in NA levels due to the autoreceptor activity of $\alpha 2$ ARs (Palij and Stamford, 1993; Forray et al., 1997). Blocking $\alpha 2$ ARs increases NA efflux and this effect is also observable in slice recordings (Palij and Stamford, 1993). Likewise, $\alpha 2$ AR antagonists increase NA release in vivo (Forray et al., 1999). Thus, $\alpha 2$ AR agonists, which decrease NA levels, reduce behavioral responses to stress, whereas $\alpha 2$ AR antagonists induce stress responses.

In summary, $\alpha 2$ AR agonists reduce NA levels by acting as autoreceptors. At the same time, $\alpha 2$ AR activation underlies the most conspicuous physiological effect of NA: a reduction of glutamatergic transmission. Then, how come increased NA levels in BNST-AV occur during stress, when agonists of $\alpha 2$ ARs, the main mediator of NA effects, reduce behavioral responses to stress? In behavioral tests involving $\alpha 2$ AR agonist injections, observing either of the two opposing behavioral effects should be possible: First, a reduction of anxiety because of a decrease in NA efflux, and second, a replica of NA effects. This scenario was found in only one study. Mantsch et al. (2009) found that systemic injections of clonidine, an $\alpha 2$ AR agonist, blocked stress-induced reinstatement of cocaine conditioned place preference, but only at low doses. In contrast, given at high doses, clonidine induced reinstatement by itself.

Alternatively, it is possible to imagine that NA's major behavioral effects are not exerted through an $\alpha 2$ AR dependent reduction of glutamatergic transmission. Instead,

NA activation might trigger long-term intracellular effects that drive the observed behavioral influences.

6.3.7 Notes on yohimbine, an α 2AR antagonist

Another noteworthy problem is that yohimbine, a well known α 2AR antagonist, exerts multiple effects in BNST. In fact, α 2ARs have a high affinity for yohimbine (Stanford, 2001). In humans, its administration generates anxiety panic states, hypertension and activation of the HPA axis (cf. Banihashemi and Rinaman, 2006). Similarly, it acts as a stressor in rodents (see above).

Physiologically, as expected from α 2AR antagonists, yohimbine blocks the NA induced reduction of glutamatergic transmission (Matsui and Yamamoto, 1984; Krawczyk et al., 2011). However, yohimbine's effects in BNST might be more complex. It has been shown that by itself, yohimbine depresses glutamatergic transmission (Egli et al., 2005); a paradoxical finding given that α 2AR agonists do the same and these effects are orexin-dependent (Conrad et al., 2012). Although yohimbine's effects on glutamatergic transmission was not replicated in other studies (Krawczyk et al., 2011), interestingly, the behavioral effects of yohimbine are not obtained with other α 2AR antagonists (Davis et al., 2008) and are not blocked by clonidine, an α 2AR agonist (Brown et al., 2009; Mantsch et al., 2010).

6.3.8 Possible effects of β ARs

Intra-BNST infusions of β AR antagonists attenuate opiate withdrawal-induced conditioned place aversion (Aston-Jones et al., 1999; Delfs et al., 2000), stress-induced reinstatement of cocaine seeking (Leri et al., 2002; Vranjkovic et al., 2014), expression of contextual fear (Hott et al., 2012), pain-induced place aversion (Deyama et al., 2008) and

anxiety-like behaviors (Cecchi et al., 2002). The β AR antagonist injection sites in some of these studies were in or just above BNST-AV (Cecchi et al., 2002; Deyama et al., 2008; Hott et al., 2012; Vranjkovic et al., 2014).

Contrasting with these pharmacological-behavioral results, earlier physiological studies reported null effects. For instance, the NA induced inhibition of excitatory transmission in BNST is insensitive to β AR antagonists (Sawada and Yamamoto, 1981). Later, Egli et al. (2005) also reported that β AR agonists have no effect on glutamatergic transmission in BNST-AV.

On the other hand, in BNST-AL, where NA innervation is sparser (Moore, 1978; Phelix et al., 1994; Egli et al., 2005), β AR agonists have excitatory effects such as increases in EPSC slopes (Egli et al., 2005) and sEPSC frequencies (Nobis et al., 2011), as well as the depolarization of CRF neurons (Silberman et al., 2013). Furthermore, Egli et al. (2005) showed that in dorsal BNST, NA generally increases EPSC amplitudes (as opposed none in BNST-AV) through a β 2AR-dependent mechanism (note that in the Nobis et al., 2011 study, the β AR effects were β 1AR mediated).

Thus, future research is needed to determine whether β AR effects are indeed absent in BNST-AV and whether the behavioral effects of intra BNST-AV β AR antagonist infusions might depend on β AR activation in BNST-AL, secondary to diffusion from the injection site.

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