Neural Circuits of Contextual Fear and Extinction: Contributions of the Basal Amygdala and Bed Nucleus of the Stria Terminalis

A thesis

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Abstract

Feelings of fear and anxiety are evolutionarily conserved traits which have been selected to be protective against dangerous situations, but their effects can be debilitating when uncontrolled. Examples of uncontrolled fear responses include post-traumatic stress disorder (PTSD) and a variety of fear and anxiety disorders that afflict approximately 18% of the adult US population. Many of these disorders are characterized by having an impairment in the learning or extinction of fear, thus inhibiting the gradual decrease of the fear response that normally occurs over time. Understanding the functional synaptic connections between brain regions that participate in the fear and extinction circuits will offer insight into the neural mechanisms of fear and provide a basis for therapies of a dysfunctional circuit.

The basal amygdala (BA) has been established as the primary control center in the brain that underlies contextual fear learning and memory. The bed nucleus of the stria terminalis (BNST), a limbic structure in the forebrain, has been implicated to preferentially support contextual fear, although its precise functional connectivity with the amygdala in contextual fear remains poorly understood. Here, we used a c-fos based reporter mouse (TetTag mouse) to investigate the activity of the BNST in contextual fear learning and memory. We found that the oval BNST subdivision (ovBNST) was activated during contextual fear conditioning while the anterodorsal BNST subdivision (adBNST) was not, underscoring the divergent functionality of these two dBNST subdivisions. To further characterize amygdalar input to the ovBNST, we used anterograde and retrograde tracers to identify a direct projection from the BA to the ovBNST. We then combined retrograde tracer injections with the TetTag mouse to investigate the activity of a BA-BNST pathway during
the acquisition and retrieval of contextual fear. In support of a direct functional interaction between the BA and the BNST during the learning of fear, we identified BNST-projecting neurons in the posterior subdivision of the BA that were activated during contextual fear conditioning without reactivation during retrieval. We also identified a separate, non-BNST-projecting population of neurons in the anterior subdivision of the BA that were preferentially reactivated during fear retrieval. Our results suggest that a direct BA-ovBNST pathway might support the acquisition and/or consolidation of contextual fear, while BA neurons that do not project to the BNST support the storage of the contextual fear memory and are incorporated into the memory engram.

We additionally used the TetTag system to investigate the extinction-induced synaptic changes that occur around neurons in the BA after contextual fear extinction. We found that extinction training leads to a decrease in fear response concomitant with a decreased reactivation of fear neurons in the mouse amygdala. This decrease in reactivated neurons was not seen in the hippocampus or medial prefrontal cortex, suggesting that the silenced BA neurons were a selective site of extinction-induced suppression. We found that extinction led to an increase in inhibitory perisomatic PV selectively around these silenced fear neurons in the BA, as well as an increase in CB1R localized to CCK perisomatic synapses around active fear neurons. These modulatory synaptic changes matched the silent or active state of the postsynaptic cell, inferring a target-specific mechanism through which the extinction circuit can directly interact with and suppress the fear circuit in the BA. Together, our findings add to the understanding of the complex fear circuit and the synaptic mechanisms underlying fear extinction.
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List of Abbreviations

AAV, adeno-associated virus

BA, basal amygdala
  aBA, basal amygdala, anterior division
  pBA, basal amygdala, posterior division

BLA, basolateral complex of the amygdala

BDNF, brain-derived neurotrophic factor

BNST, bed nucleus of the stria terminalis
  adBNST, bed nucleus of the stria terminalis, anterodorsal division
  dBNST, bed nucleus of the stria terminalis, dorsal division; inclusive of adBNST, mdBNST, and ovBNST
  mdBNST, bed nucleus of the stria terminalis, mediodorsal division
  ovBNST, bed nucleus of the stria terminalis, oval division

CB1R, cannabinoid 1 receptor

CCK, cholecystokinin

CeA, central amygdala

CamK2a, calcium/calmodulin-dependent protein kinase II alpha

CTB, cholera toxin subunit B

DOX, standard doxycycline chow

DRD1, dopamine receptor D1

EXT, extinction

FC, fear conditioned

GABA, gamma-aminobutyric acid

GAD67, 67kDa glutamic acid decarboxylase
GFP, green fluorescent protein
His-GFP, histone2B-GFP
HC, home cage
IEG, immediate-early gene
LA, lateral amygdala
PAG, periaqueductal gray
PFC, prefrontal cortex
  IL, infralimbic prefrontal cortex
  PL, prelimbic prefrontal cortex
PSD95, postsynaptic density protein 95
PV, parvalbumin
SOM, somatostatin
SR2C, serotonin receptor 2C
ST, stria terminalis
TrkB, tropomyosin-related kinase B
tTA, tetracycline-transactivator
SynI, synapsin I
Zif, immediate-early gene zif268/Egr1
Chapter 1:

Introduction
1.1 Contextual fear

Contextual fear conditioning and extinction overview

Fear is a negative emotion that is often implemented as an adaptive response to stimuli that may threaten survival or otherwise cause harm to the organism. The amygdala, a bilateral almond-shaped structure in the temporal lobe of the brain, has been well established as the primary component of the neural circuitry underlying fear learning and memory (LeDoux 2003; Duvarci & Pare 2014; LeDoux 2000). Conditioned fear, in which an aversive stimulus is paired with a neutral stimulus or environment, relies on the amygdala and can generally be classified as either contextual fear or auditory fear. Contextual fear differs from auditory or tone fear in that the aversive stimulus is not associated with a discrete, predictive cue, but rather with a polymodal integration of sensory components present in the surroundings. These sensory components can include vision, smell, and touch in addition to sound, and together create a representation of the context that can then be associated with the fear-inducing stimulus. The encoding of the contextual representation within an initial exploratory period is necessary for the subsequent acquisition of the conditioned fear memory (Landeira-Fernandez et al. 2006). Furthermore, contextual fear differs from auditory fear at the fundamental level of processing, with known distinctions in circuitry that establish and maintain the memory (Phillips & LeDoux 1992; Sullivan et al. 2004; Onishi & Xavier 2010).

Due to overlapping circuits between fear and anxiety (Adhikari 2014; Janak & Tye 2015; Davis et al. 2010), the distinction between the two emotional behaviors should be defined. Conditioned fear occurs when a previously neutral stimulus (conditioned
stimulus, CS) is associated with an aversive stimulus (unconditioned stimulus, US), leading to a discrete defensive response at the presentation of the CS alone. This fear response will subside in the absence of the CS. Anxiety, on the other hand, is defined as a long-duration behavioral response that arises from general and non-specific stimuli and is characterized by hypervigilance and a diffuse perception of an unspecified threat (Sylvers et al. 2011). Within conditioned fear, contextual fear is viewed as a more sustained and anxiety-like behavioral response when compared to cued or auditory fear, which evokes an acute and phasic response (Walker & Davis 2008; Davis et al. 2010). Since different behavioral tests and analyses are used in assessing the fear or anxiety state of rodent models in the laboratory, it is especially important to distinguish between the two when ultimately dissecting the function and circuitry of contextual fear.

Once consolidated, fear memories are stable and can endure over the lifetime of an animal (Gale et al. 2004). Fear extinction, the reduction of a fear response to a conditioned stimulus or context in the absence of the aversive stimulus, is a form of new learning that gates the expression of fear (Herry et al. 2010). Generally, multiple sessions of re-exposure to the context in the absence of the noxious unconditioned stimulus is required to effectively produce the extinction memory, reducing the physiologic fear response. The amygdala, along with other brain structures that are involved in the processing of fear, plays an active and integral role in the extinction process (Pape & Pare 2010; Laurent et al. 2008; Portero-Tresserra et al. 2012). The basis of fear and extinction memories is formed by a hierarchy of neural systems, including activation of multiple circuits between brain regions, adaptive synaptic plasticity, and signaling by complex chemical components.
As in the broader field of learning and memory, expression of contextual fear memory requires a collaboration of multiple stages and processes that support the learning, storage, and recall of the memory. These stages are dependent upon precise circuit activation, memory engrams stored in distributed neuronal ensembles, synaptic plasticity, and molecular and chemical signaling. Many of these events occur in concert to form a fear memory, with the amygdala at the core of the action.

**Fear disorders in humans**

While the natural fear response is evolutionarily beneficial and generally promotes survival when implemented in an appropriate context, fear can be extremely debilitating when deregulated in its pathological state. Fear and anxiety disorders are prevalent in the United States and together affect approximately 18% of the adult population to varying degrees of severity (Kessler et al. 2005). Disorders that fall within this classification include post-traumatic stress disorder (PTSD), panic disorder, specific phobias, and generalized anxiety disorder. While the generation of a fear disorder is likely the result of multiple contributing factors, including genetics and environment, strong evidence suggests that maladaptive changes in the neural circuitry underlying fear and extinction is an integral mechanism of pathology. Imaging studies in human patients suggest that hyper- and/or hypo-activation of brain regions and between neural circuits are a causal link to the dysregulation of fear (Gonzalez-Pardo et al. 2011; Admon et al. 2013; Rougemont-Bücking et al. 2011). PTSD is a salient example of a fear disorder that can be debilitating and severely affect quality of life. The disorder manifests when a person who has been exposed to a traumatic event develops an inability to properly regulate the fear response in safe contexts. Whether a trauma-exposed individual will
develop PTSD depends on a number of risk factors, including genetics, environment, childhood background, severity of trauma, and vulnerability or resilience to the trauma (Pitman et al. 2012). There is a lifetime prevalence of 7.8% of PTSD in the general population, with higher rates seen in women (Kessler 1995; Haskell et al. 2010).

Neuroimaging studies that compare PTSD subjects with healthy trauma-exposed subjects show altered brain activity in multiple regions, including the amygdala and medial prefrontal cortex, when both groups are exposed to fear and extinction paradigms (Liberzon & Sripada 2008; Rougemont-Bücking et al. 2011; Lanius et al. 2006). These findings emphasize the importance of understanding the regulation of these brain regions and their specific contributions to the generation of fear disorders.

Current treatment and therapy

Treatment is not effective in all fear disorder patients and is rarely permanent, underscoring the need to better understand the neural substrates of fear and its therapy. Currently, one of the most widely used non-pharmacological treatments for fear disorders is exposure therapy, which is based on the process of fear extinction (Quirk et al. 2010). Evidence that fear memories are labile after recall provides a temporal opportunity to disrupt the fear memory during the protein synthesis-dependent phase of reconsolidation (Nader et al. 2000; Schiller et al. 2010). Indeed, behavioral disruption during the reconsolidation phase has been shown to lead to fear suppression and attenuated activity in the human amygdala (Agren et al. 2012). Many antidepressants and mood stabilizing drugs are currently used as a pharmaceutical approach to treating some fear disorders, such as PTSD. However, the high comorbidity of fear disorders with other neurological disorders makes pharmacological treatment challenging due to off-target effects and
interactions. In addition to behavioral and pharmacological treatment, recent developments in neurosurgical techniques like deep brain stimulation (DBS) have proved to be promising (Rodriguez-Romaguera et al. 2012), although progress has been cautious since the mechanism of action of the technique is not well understood. Overall, despite the multiple classes of therapy currently available, few are widely effective or permanent. The treatment of fear disorders would greatly benefit from a fuller understanding of underlying physiological and pathological mechanisms.

**Animal models**

The limbic system, including the amygdala, is highly conserved throughout evolution. This not only emphasizes the importance of the amygdala and the fear circuit to survival, but it also provides practical model systems in which we can study and dissect the fear circuit. A context previously associated with an aversive experience can induce a sustained and anxiety-like fear that is associated with similar brain activation patterns across species (Alvarez et al. 2008). Contextual fear conditioning in animals can therefore be used as a model for studying mechanisms that might underlie anxiety and fear disorders in humans (Davis et al. 2010). In modeling contextual fear for scientific study, rodents can be conditioned to fear a specific context in which they receive the aversive stimulus. Rodents display a defensive behavior of immobilization, or freezing, in response to a fear-inducing stimulus, and the percentage of time the rodent spends freezing can be used to quantify fear expression. This behavioral paradigm can be combined with an array of techniques available in animal models, including slice electrophysiology, optogenetics, and immunohistochemistry, to dissect the neural correlates underlying fear at a level far beyond what is accessible in human patients.
alone. To date, studies using lesion techniques, cellular activity markers, and optogenetic stimulation or inhibition have contributed greatly to our understanding of the fear circuit. Furthermore, pharmacological treatment and therapy can also be investigated to a much greater extent, and findings can often be translated to the clinic (Milad et al. 2006; Goode & Maren 2014). In addition to gaining insight into the pathology of fear and anxiety disorders, research on fear memory in animal models provides an attractive and accessible model for understanding the fundamental processes underlying learning and memory.

1.2 Neural circuits of contextual fear

The amygdala

The amygdala has been well established as an essential component of the fear circuit, with its subdivisions contributing to specific functions in the acquisition, storage, retrieval, and extinction of fear. Lesions or pharmacological inactivation of the amygdala have acute and long-lasting effects on the generation and retention of fear memory (Maren et al. 1996; Goosens & Maren 2001; Anglada-Figueroa & Quirk 2005; Poulos et al. 2009). The core amygdala can be divided into multiple subdivisions with distinct functions, including the basolateral complex (BLA), which consists of the lateral amygdala (LA) and basal amygdala (BA), and the central amygdala (CeA).

The BLA is analogous to the main control center of the fear circuit, and is believed to be the primary integration site between sensory information and emotional valence (Fanselow & LeDoux 1999). This complex is similar in cellular composition to the cortex, however it lacks structured layers and instead consists of a mixed distribution
of the cell types. The BLA is primarily composed of spiny glutamatergic neurons that make up 80-85% of the structure’s cellular composition. These excitatory pyramidal neurons almost exclusively account for the population of cells projecting from the BLA to the CeA and other brain regions (McDonald 1992). The remaining 15-20% of cells are GABAergic neurons, the majority of which are interneurons that form inhibitory synapses within the BLA. These interneurons can further be classified by their chemical composition, with two of the main types being parvalbumin-positive (PV+) and somatostatin-positive (SOM+) (McDonald & Betette 2001; McDonald & Mascagni 2002; McDonald & Mascagni 2001a). The cell-type markers and differential protein expression of the distinct classes of interneurons make them readily identifiable and are highly correlative with their physiologic profile (Freund 2003; McDonald & Mascagni 2001a). The CeA, in contrast to the BLA, contains a majority of GABAergic cells that send a significant portion of long-range projections downstream of the amygdala (Sun & Cassell 1993). The CeA is further divided into the lateral subdivision (CeL) and the medial subdivision (CeM), with the CeM serving as the primary output source of the amygdala (LeDoux et al. 1988). The amygdala also contains intercalated cell masses (ICMs), which are clusters of GABAergic cells that are located in the fiber tracts that border the lateral side of the BLA (the external capsule) and the medial side between the BLA and CeA (internal capsule) (Paré & Smith 1993a; Paré & Smith 1993b; Nitecka & Ben-Ari 1987).

*Internal circuits of the amygdala*

Within the amygdala, the basic flow of information is generally represented by projections running from the LA to the BA, the LA and BA to the CeA, and from the
CeA to other downstream non-amygdalar structures (Duvarci & Pare 2014). The circuitry underlying auditory fear acquisition and consolidation has been extensively studied and has been well described in literature (Pape & Pare 2010; Duvarci & Pare 2014; Janak & Tye 2015). Briefly, in cued fear, sensory information from the thalamus and cortex are integrated in the LA, and the behavioral output is primarily regulated by way of the CeA. Since there are few projections between the LA and CeA, it is likely that information passes through the BA en route, although a direct pathway between the LA and CeL has also been shown to contribute to the fear circuit (Li et al. 2013). The GABAergic cells of the ICM also provide an important level of feed-forward inhibitory regulation within the amygdala circuit (Royer et al. 1999).

Although contextual fear does share fundamental similarities with the auditory conditioned fear circuit, it has been shown to recruit different brain regions and pathways that are essential for the acquisition and expression of context-based fear. Contextual fear requires input from the hippocampus to form the memory engram, and contextual fear memory is abolished when the hippocampus is lesioned (Phillips & LeDoux 1992; Maren et al. 1995). Furthermore, ibotenate lesions of the BA, which spares fibers of passage, selectively impairs contextual fear and not auditory fear (Onishi & Xavier 2010), emphasizing a difference between the intra-amygdalar circuits of the two types of conditioned fear.
Mechanisms underlying fear memory in the amygdala

The fear memory trace within the BLA can further be localized to individual cells and synapses that are essential in supporting the memory. Neurons in the BA that are activated during contextual fear conditioning are preferentially reactivated during the recall of the fear memory (Reijmers et al. 2007), and selective ablation of LA cells activated by auditory fear memory blocks subsequent fear expression to the conditioned cue (Han et al. 2009). These findings support the hypothesis that the association between the conditioned and aversive stimuli is made and stored via the activation of specific cells. These cells can be preferentially activated or inhibited through mechanisms of synaptic plasticity, such as Long-Term Potentiation (LTP) and Long-Term Depression (LTD), respectively. Synaptic plasticity within the BLA can be induced by fear conditioning (Rogan et al. 1997; McKernan & Shinnick-Gallagher 1997; Ostroff et al. 2010) and extinction training (Amano et al. 2010), and is likely a critical mechanism contributing to the learning and modulation of the memory (Sigurdsson et al. 2007). Furthermore, protein synthesis in the amygdala is necessary for the reconsolidation of a fear memory after its retrieval (Nader et al. 2000).

External circuits of the amygdala

In addition to the complex microcircuitry within the amygdala that supports fear learning and memory, the importance of circuits between the amygdala and other brain regions is becoming increasingly apparent in the regulation of fear. The amygdala has many efferent and afferent connections that play a potential role in the fear circuit, among many other behavioral outputs. The projection targets of the BA are tightly coupled with
the activity and function of the BA neurons in a variety of emotional and behavioral states. Particularly within the field of fear and anxiety research, the long-range projections of BA neurons have been shown to target distinct structures that confer a functional specificity and are necessary for the generation of the behavior (Kim et al. 2013; Senn et al. 2014). As such, knowing the projection target of fear neurons in the BA is critical in determining their function in contextual fear learning, memory and extinction. Here I will briefly review the structures that form circuits with the BA that have been implicated to preferentially influence the regulation or expression of contextual fear: the hippocampus (HPC), the medial prefrontal cortex (mPFC), the central amygdala (CeA), and the bed nucleus of the stria terminalis (BNST).

**BLA-hippocampus**

The hippocampus is known to play an important role in contextual processing (Sutherland & McDonald 1990; Rudy & Sutherland 1989), and has strong reciprocal projections with the BA (Canteras et al. 1992; Pitkanen et al. 2006). This connection between the hippocampus and the BA has proved to be integral in associating the contextual elements with the aversive stimulus in contextual fear (Phillips & LeDoux 1992; Anagnostaras et al. 2001). High frequency stimulation of hippocampal efferents induced long-term potentiation (LTP) in BA synapses, and excitotoxic lesions of the BA-projecting areas of the ventral hippocampus led to an abolishment of contextual fear acquisition (Maren et al. 1995). Notably, auditory conditioned fear does not seem to be
hippocampal dependent, and recall of auditory fear is not impaired by hippocampal damage or inactivation (Phillips & LeDoux 1992).

**BLA-medial prefrontal cortex**

A number of recent studies have implicated the medial prefrontal cortex (mPFC) in contextual fear and processing (Rozeske et al. 2014; Senn et al. 2014; Lemos et al. 2010; Sah et al. 2013). The mPFC, which includes the infralimbic cortex (IL) prelimbic cortex (PL), medial precentral cortex (PC), and anterior cingulate cortex (AC), receives strong input from the BLA and also sends significant input back to the BLA and CeA (McDonald et al. 1996). The AC is believed to play a role in the acquisition of contextual fear, likely during the consolidation phase (Tang et al. 2005; Vetere et al. 2011). PL-projecting BA neurons showed a selective increase in cfos, an immediate-early gene, in response to fear conditioning, whereas IL-projecting BA neurons were cfos responsive to fear extinction (Senn et al. 2014). Inhibition of parvalbumin-positive (PV) inhibitory interneurons in the mPFC disinhibits excitatory projections from the mPFC to the BLA, which is then believed to contribute to fear expression (Courtin, Chaudun, et al. 2013). The reciprocal connectivity between the amygdala and mPFC sets the stage for a tight and adaptive regulatory circuit.

**BLA-CeA**

The BLA projects heavily to the adjacent CeA (Paré et al. 1995). The CeA sends a significant number of projections to hypothalamic, midbrain, and brainstem nuclei that are associated with the behavioral expression of conditioned fear (Petrovich & Swanson 1997; LeDoux et al. 1988; Penzo et al. 2014). This includes the ventrolateral
periaqueductal gray (vIPAG), which is critical to the freezing behavior as a fear response in rodents (LeDoux et al. 1988). Additionally, the CeA contains reciprocal projections with the BNST, particularly the oval BNST subdivision (Dong, Petrovich & Swanson 2001; Dong, Petrovich, Watts, et al. 2001). Lesion or pharmacological inactivation of the CeA impairs learning and memory in both contextual and auditory conditioned fear (Sullivan et al. 2004; Goosens & Maren 2001; Zimmerman et al. 2007), indicating an important role for the CeA in the fear circuit. However, the majority of studies implicating the CeA in contextual fear use lesion techniques that may also destroy fibers of passage. This is of particular importance since the BA sends projections to the BNST through the stria terminalis, which courses directly adjacent to the CeA, and would likely be disrupted by electrolytic lesions. Indeed, a study comparing the inactivation of the CeA through the use of electrolytic lesions versus fiber-sparing neurotoxic lesions found significant impairment of conditioned fear in only the first group (Koo et al. 2004). Both electrolytic and neurotoxic lesion methods in the BA were sufficient to successfully abolish conditioned fear. This suggests that some of the functions originally attributed to the CeA in the fear circuit may be erroneous due to the disruption of fibers of passage adjacent to the CeA. Functional similarities between the CeA and BNST have often been inferred due to the overlap in their afferent and efferent projections, making the BNST an attractive candidate for contributing to the contextual fear circuitry.
1.3 The bed nucleus of the stria terminalis

The bed nucleus of the stria terminalis (BNST) shares many similar output pathways as the CeA, including connections with the periaqueductal gray (PAG), hypothalamus, and ventral tegmental area (VTA), and is often referred to as part of the “extended amygdala” (De Olmos & Heimer 1999). Many of these downstream structures participate in the behavioral expression of stress, anxiety, and fear, putting the BNST in a suitable position to modulate salient fear memory and response. Similar to the CeA, the BNST is largely composed of inhibitory GABAergic projection neurons, with the remaining minority of the population made up of glutamatergic neurons (Sun & Cassell 1993). In addition to fast neurotransmitters, the neurons of the BNST express a wide array of neuropeptides, which contribute to the functional heterogeneity of the cells and subdivisions (Moga et al. 1989; Gungor & Pare 2014; Kash & Winder 2006). This provides an accessible point of neuromodulation within the BNST and its connected circuits. Neuropeptides expressed in the BNST include Corticotropin-Releasing Factor (CRF), Dynorphin, Neuropeptide Y (NPY), and Oxytocin (Kash et al. 2015). Studies aimed at characterizing the dorsal BNST neuronal population based on physiological properties have distinguished at least three major cell types, however these properties do not seem to be readily correlated with morphology (Rodriguez-Sierra et al. 2013; Hammack et al. 2007), making it challenging to identify distinct cell populations. This heterogeneity leads to a complex intra-BNST micro-circuitry, as well as multiple parallel interactions with connecting structures, that ultimately contributes to the many reported functions of the structure and its circuits (Turesson et al. 2013; Kash & Winder 2006; Kim et al. 2013).
Subdivisions of the BNST

The BNST is composed of many subnuclei with a high level of cellular and functional heterogeneity. While nomenclature has varied over time, the anterior portion of the dorsal BNST can be classified as three distinct subregions: the oval BNST (ovBNST), anterodorsal BNST (adBNST), and mediodorsal BNST (mdBNST) (Fig. 1.4). The ovBNST and adBNST are also sometimes referred to together as the anterolateral BNST, particularly in electrophysiological studies where the two subdivisions are not readily distinguishable from each other (Haufler et al. 2013), and the mdBNST as the anteromedial BNST. Each subregion is attributed with unique intrinsic connectivity within the BNST and extrinsic connectivity with upstream and downstream brain regions, though characterization of defining cell types and functions is still underway. Early studies have largely described the global BNST as an anxiogenic structure, however more recent research shows that individual subnuclei within the BNST can have unique and opposing functions. Specifically, the ovBNST was found to exert an anxiogenic function when stimulated, while the adBNST generated anxiolytic behavioral properties (Kim et al. 2013). With this in mind, it is particularly important to distinguish between subdivisions when describing the functions and circuits of the BNST.
Figure 1.1

Subdivisions of the anterior portion of the dorsal BNST. (A) Diagram of a coronal section of a mouse brain. The location of the anterior BNST is outlined in red. (Modified from Paxinos & Franklin 2001) (B) Identification of dorsal BNST subdivisions, including the oval BNST (ovBNST), anterodorsal BNST (adBNST), and mediodorsal BNST (mdBNST).

The BNST in contextual fear

The BNST has been well established in the generation and expression of anxiety (Adhikari 2014), however the precise role of the BNST in contextual fear has not yet been fully elucidated. Early studies of the structure implied a selective participation of the BNST in sustained fear and not shorter-duration phasic fear (Walker & Davis 2008), leading researchers to investigate the function of the BNST in contextual fear paradigms. Whereas post-training lesions of the CeA were found to abolish both tone and contextual
fear, lesions of the BNST only impaired the contextual fear memory (Sullivan et al. 2004). This finding led to the hypothesis that the BNST played a selective supporting role in contextual fear, however the relationship between the BA, CeA, and BNST in the different types of conditioned fear remained unclear. Later pharmacological experiments focused on determining the cellular mechanisms underlying the BNST’s function in contextual fear. Infusion of cannabidiol, an anxiolytic compound, into the BNST directly attenuated contextual fear expression and likely involved the activation of serotonin-1A receptors (Gomes et al. 2010). Infusion of cobalt chloride, a reversible non-selective synapse blocker, in the BNST had a similar effect of impairment on contextual fear (Resstel et al. 2008). More recently, a study revealed that calcitonin gene-related peptide (CGRP) antagonist infused into the BNST selectively impairs acquisition and expression of contextual fear, while leaving cued fear intact (Sink et al. 2013). These studies further confirm the participation of the BNST in contextual fear, however its precise place within the fear circuit is unknown. It has also been shown that lesion or inactivation of the BNST in rats can prevent the BLA-independent learning of contextual fear after overtraining, suggesting that the BNST could play a compensatory role in fear learning after BLA inactivation, or perhaps participate in a redundant fear pathway (Poulos et al. 2010). This finding is intriguing, since it suggests the BNST may participate in a fear circuit that is not directly downstream of the BA. However, given the many subdivisions and connections of the BNST, it is entirely possible that the BNST could participate in both the normal contextual fear circuit through a BA-BNST pathway, as well as a compensatory BLA-independent circuit. When discussing the BNST and its subdivisions, it should be noted that the lesions and pharmacological infusions in the studies discussed
here largely targeted both the medial and lateral subdivisions of the BNST, making it impossible to differentiate between possible varying functions of the subdivisions.

In contrast to the findings that describe a selective participation of the BNST in contextual fear, a recent study has cast doubt on this model. An electrophysiological study comparing the anterolateral and anteromedial BNST in response to both context and cued fear found differential activity in the two subdivisions, however, no difference was seen in their functional response to context versus cued fear (Haufler et al. 2013). The authors of this study believe the discrepancy could be due to a few different possibilities; either a different BNST subdivision not included in the study is responsible for the structure’s preferential contribution to contextual fear, or prior studies generated effects on contextual fear by way of inadvertently manipulating an adjacent non-BNST structure. It is important to keep this finding in mind when interpreting the role of the BNST in conditioned fear. Nevertheless, it still seems clear that the BNST is active during contextual fear, although it is unknown precisely which subdivisions of the BNST might participate and to what extent and function.

*Amygdalar projections to the BNST*

The major projections from the amygdala to the BNST have been described through the use of anatomical tracers. The CeA sends strong projections to the lateral BNST, including the oval, rhomboid, and fusiform nuclei. The BA, which also sends dense projections to the BNST, seems to target different subdivisions than the CeA, though with some overlap. While the BA most significantly sends projections to the anterodorsal BNST and rhomboid nucleus, some processes were also seen in oval and
fusiform nuclei (Dong, Petrovich & Swanson 2001). The BA projections to the BNST are glutamatergic and preferentially originate from the posterior subdivision (Dong, Petrovich & Swanson 2001; McDonald 1991), while the CeA projections are primarily GABAergic and CRF-containing (Sakanaka et al. 1986; Dong, Petrovich & Swanson 2001).

Since the number of processes between the BA and ovBNST is significantly less than between the BA and adBNST, this pathway is generally discounted in studies that consider BA-BNST circuitry. This may lead to an oversimplification of the BA-BNST circuit, thus neglecting to account for the finer effects on behavior from alternative BA-BNST pathways. It is also important to note that the anterograde tracing method used in these original anatomical studies, phaseolus vulgaris leukoagglutinin (PHAL), does not identify sites of synaptic contact originating from the labeled processes. As such, it is not possible to distinguish axons that make synaptic contact with a particular region versus fibers of passage.

**Efferent projections of the BNST**

The BNST sends a significant number of projections to structures involved in mediating autonomic and endocrine functions. In particular, the BNST projects to many of the brain regions that together produce the various aspects of the behavioral fear response (Dong, Petrovich, Watts, et al. 2001; Dong & Swanson 2004). Many of these overlap with projections from the CeA, putting the BNST in an ideal location to participate in the fear circuit (Walker et al. 2003). The ovBNST supplies many intrinsic circuits within the BNST, including projections to the adBNST, rhomboid, and fusiform
nuclei. Outside of the BNST, the ovBNST and adBNST both send significant projections to the CeA, parabrachial nucleus, nucleus accumbens, and ventrolateral PAG (Dong, Petrovich, Watts, et al. 2001; Dong & Swanson 2004). The ovBNST has been recently shown to send monosynaptic projections to serotonin neurons in the dorsal raphe nucleus (DRN) (Weissbourd et al. 2014; Pollak Dorocic et al. 2014). The adBNST additionally sends projections to the ventral tegmental area (VTA) and dorsal lateral hypothalamic area (Dong & Swanson 2004).
1.4 Mechanisms of fear extinction

Contextual extinction is the decrease of a conditioned fear response over time with the repeated exposure of the animal to the context in the absence of the aversive stimulus. As extinction training progresses, levels of fear behavior, such as freezing in mice, will decrease. The possibility of the sudden unprompted occurrence of the fear response after extinction, known as spontaneous recovery, reveals that the process of extinction at least partly relies on new learning rather than the total erasure of the original fear memory (Courtin, Karalis, et al. 2013; Bouton & Bolles 1979; Bouton 2002). Indeed, researchers have shown that extinguished animals will reacquire conditioned fear responses faster than naïve animals, providing strong evidence that the original memory remains intact after extinction training (Bouton & Bolles 1979). Like fear learning and memory, extinction is the result of cellular, molecular, and circuit-level activities that interface with the fear circuit to produce the realized behavioral effect (Pape & Pare 2010). Cell and circuit-specific manipulations of the brain can lead to different effects on fear conditioning and extinction (Anglada-Figueroa & Quirk 2005), underscoring that the two behaviors are regulated by distinct circuits and processes. An imbalance in the activity of the fear and extinction circuits, caused by either excessive fear or impaired extinction, could underlie pathological fear expression. Understanding how and where the fear circuit and extinction circuit interact will provide valuable insight into the process of extinction and points of dysfunction.
Neural circuits of extinction

While new research is continuously shedding light on the brain regions involved in fear extinction, it is clear that three key structures crucial to contextual extinction are the basal amygdala, hippocampus, and medial prefrontal cortex. The neural circuits between these structures interact with the fear circuit and provide regulation, with the BA as the primary site of extinction-induced regulation and plasticity. It has been shown that a distinct subset of neurons in the BA is active during extinction, in contrast to a separate population of fear-responsive neurons, and that the relative activity of the two subsets correlates with the level of fear expression after extinction (Herry et al. 2008). The amygdala is reciprocally connected with both the PL and IL subdivisions of the mPFC, and the extinction neurons in the BA have been shown to preferentially project to the mPFC (Herry et al. 2008). Within the mPFC, neurons projecting from the BA to the IL form a circuit that is believed to be critical in the consolidation of extinction (Senn et al. 2014; Quirk & Mueller 2008). The hippocampus is believed to be important for the contextual specificity of extinction memory, and studies show impaired extinction when the hippocampus is pharmacologically inactivated (Corcoran et al. 2005). Determining the projection target of BA neurons that are modulated by extinction provides greater insight into their role in manipulating behavioral and emotional states. Furthermore, understanding how these neurons are themselves regulated will be critical in dissecting the actions of extinction within the amygdalar fear circuit.
Inhibitory synaptic plasticity within the BA is believed to be a key component in the acquisition and consolidation of a fear extinction memory (Ehrlich et al. 2009; Herry et al. 2010; Maren 2014). Perisomatic synapses exist where inhibitory neurons synapse directly onto the soma of another cell, rather than dendritically, and are in a unique position to modulate the functional activity of excitatory neurons due to their targeted inhibitory regulation (Miles et al. 1996; Freund & Katona 2007). In the hippocampus, inhibitory perisomatic synapses preferentially gate the efferent signaling of the target neuron, whereas inhibitory dendritic synapses are better positioned to suppress afferent signals (Miles et al. 1996). In addition to suppressing the activity of a cell, perisomatic synapses from an interneuron can selectively target cells with a higher degree of specificity than through dendritic inhibition, as seen in the entorhinal cortex (Varga et al. 2010). Within the BA, two classes of GABAergic interneurons, parvalbumin-positive (PV) and cholecystokinin-positive (CCK), both make perisomatic synapses onto local excitatory pyramidal neurons. Since inhibitory interneurons in the BA have been widely implicated in the process of fear extinction (Heldt & Ressler 2007; Ehrlich et al. 2009; Heldt et al. 2012), perisomatic inhibition of pyramidal neurons that support the fear memory is an attractive mechanism of extinction-induced regulation.

The predominant type of GABAergic interneuron in the BA is classified as expressing calcium-binding protein parvalbumin (PV) (Kosaka et al. 1987; Mascagni et al. 2009), and accounts for nearly half of the region’s inhibitory cell population (McDonald & Mascagni 2001a). PV+ cells are fast-spiking, synchronous, and precise; they are often described as operating as clockworks that drive network oscillations
(Freund & Katona 2007). Within the BA, the majority of PV enervation of pyramidal neurons occurs via perisomatic synapses (Pitkänen & Amaral 1993). This places them in an ideal position for inhibiting the activity of target BA neurons, and provides a potential mechanism of extinction-induced inhibition of fear neurons. PV+ interneurons additionally synapse onto other interneuron subtypes, such as SOM+ cells, and can contribute to fear learning through indirectly disinhibiting pyramidal neurons in the BA during the association of the cue and aversive stimulus (Wolff et al. 2014).

Another major group of inhibitory interneurons in the BA is characterized as containing the neuropeptide cholecystokinin (CCK). CCK+ interneurons are regular-spiking, asynchronous, and associated with the fine-tuning of activity via inhibition, with implications in anxiety and mood disorders (Freund & Katona 2007). The CCK+ presynaptic terminals are enriched for cannabinoid receptor 1 (CB1R), a protein crucial to endocannabinoid signaling (Katona et al. 2001; McDonald & Mascagni 2001b; Yoshida et al. 2011). Many studies have indicated that CB1R may play an important role in the process of fear extinction (Marsicano et al. 2002; Chhatwal et al. 2005). Extinction training leads to an increase in endocannabinoid levels in the BA in mice, and deletion of CB1R impairs extinction (Marsicano et al. 2002). Furthermore, manipulation of the endocannabinoid system does not affect fear acquisition or consolidation, suggesting a role for CCK+ perisomatic synapses specifically in extinction via CB1R. Since the process of extinction is generally associated with an increase in inhibition of pyramidal neurons in the BA, it was initially surprising that extinction would be positively correlated with CB1R, as endocannabinoid action through CB1R suppresses GABAergic inhibition (Katona et al. 2001). It has thus been hypothesized that these CCK+ synapses
enriched in CB1R preferentially make contact with extinction neurons or extinction-resistant neurons in the BA. The increase in endocannabinoid signaling during extinction would therefore coincide with a decrease in inhibition of the pyramidal neurons that may positively contribute to the extinction network, placing CB1R in a significant position to regulate extinction learning.
1.5 Contributions of this thesis

Despite extensive research dissecting the neural circuitry of contextual fear, a complete and comprehensive understanding of the contributing pathways and points of regulation remains to be elucidated. While the BNST has been implicated to support contextual fear learning, its precise function and relationship with the basal amygdala is not well understood. Specifically, further dissection of the BNST subdivisions, their anatomical connectivity, and unique network functions in contextual fear is needed. To gain insight into the population of neurons that are active during contextual fear, we used a cfos-based transgenic mouse called the TetTag mouse (Reijmers et al. 2007) to tag neurons in the BA and the BNST that were activated during contextual fear conditioning. This system allowed us to define a subset of neurons by their activity during fear conditioning and again during the retrieval of the fear memory, through the added use of immediate-early gene markers. In being able to observe the activity of the same neurons during two temporal windows, we could identify whether particular cells in a brain region may participate in the storage of the fear memory by being preferentially activated by both events. If fear neurons, or neurons active during fear conditioning, are significantly reactivated during the retrieval of the fear memory, this would suggest that these neurons are likely incorporated into the memory engram that supports the lasting endurance of the fear memory. Additionally, we combined the TetTag approach with retrograde circuit-tracing to identify functional activity of direct projections from the BA to the BNST during the acquisition of contextual fear. Research has shown that the long-range projection of BA neurons is tightly coupled with functionality in a number of BA-regulated emotional and behavioral states. Our findings point towards a lesser-known direct projection from the posterior BA to the dorsal BNST that might play a
role in the acquisition and/or consolidation of contextual fear. These data emphasize the complex circuitry of fear and highlight a novel pathway that may be modulated to gate the learning of contextual fear in health and disease.

We further utilized the distinct advantages of the TetTag mouse to look at molecular and synaptic changes that coincide with the silencing of fear neurons after extinction. The mechanisms underlying the suppression of fear expression after extinction training are of particular interest due to potential therapeutic applications in human fear disorders. As described above, the TetTag mouse allows us to visualize cells reactivated by fear expression during the retrieval test, thus providing information about neurons that participate in the memory trace. Furthermore, we can use the TetTag mouse to focus on fear neurons that are silenced after fear extinction. To this end, we submitted TetTag mice to extinction training and focused on silenced fear neurons in brain regions specifically implicated in the extinction circuit. We analyzed the inhibitory synaptic input onto the silenced fear neurons in the BA to further probe the potential point of extinction-induced modulation that may have suppressed the activity of the fear neuron. Finally, in an effort to further explore the molecular underpinnings of the targeted synaptic plasticity observed after extinction, we investigated the effect of BDNF depletion on the perisomatic inhibitory synapses in the BA using virally targeted cre-mediated knockout of BDNF in the BA of Floxed-BDNF mutant mice. These findings significantly contribute to the understanding of the interaction between the fear circuit and extinction circuit via synaptic inhibition in the BA.

In addition to the specific advances our findings contribute to the field of contextual fear and extinction, our research further provides an example of how the
TetTag mouse system can be used to determine functional activation of long-range projections, as well as synaptic modulation of target-specific cell types. The non-invasive nature of the neuronal tagging allows the visualization of physiologically relevant cellular activation in response to observed behavior. This provides an integral link between underlying brain mechanisms and behavioral manifestation, and can be applied to the interrogation of a variety of biological questions.
2.1 Animals

All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Tufts University Animal Care and Use Committee. Mice had access to food and water ad libitum and were socially housed until a specified period of time prior to the start of the behavioral experiment. Mice were kept on a standard 14 hr/10hr light-dark cycle, and all experimental manipulations were performed during the light phase. For transferring mice to or from their cage during experiments, mice were handled by the tail with mouse holding forceps fitted with rubber tips.

TetTag Histone-GFP Mice

The TetTag mouse line used was heterozygous for two transgenes: a tetracycline transactivator (tTA) protein under a c-fos promoter, and a histone2B-GFP (His-GFP) fusion protein under a tet-operator (tetO) promoter. TetTag mice were backcrossed to a C57Bl6/J background. Male and female TetTag mice were at least 8 weeks of age at the start of the experiment. Mice were raised on regular doxycycline chow (40mg/kg dox) and remained on dox chow until 4 days prior to fear conditioning, when dox chow was removed and replaced with standard chow. High dox chow (1g/kg) was provided to the mice immediately following the last fear conditioning session until the following morning, when it was replaced with regular dox chow. Expression of the TetTag transgenic reporter can be widely detected throughout the mouse brain (Reijmers et al. 2007; Tayler et al. 2013; Trouche et al. 2013; Matsuo et al. 2008).
Wildtype C57Bl/6 mice

Male and female C57Bl/6 mice were supplied by The Jackson Laboratory at 9 weeks of age. Mice were socially housed until surgery, or 4 days prior to fear conditioning if they did not undergo surgical manipulation.

Floxed-BDNF mice (BDNF<sub>2L/2L</sub>)

BDNF<sub>2L/2L</sub> mice (kind gift of Dr. M. Rios) were generated with loxP sites inserted around the single coding exon of Bdnf (Rios et al. 2001). Adult male and female BDNF<sub>2L/2L</sub> mice were at least 8 weeks of age at the start of the experiment. Mice were socially housed until undergoing surgery for viral delivery of AAV-GFP or AAV-GFP-Cre in the basal amygdala, at which point they were individually housed until the conclusion of the experiment.

2.2 Behavioral paradigms

Fear conditioning, extinction, and retrieval sessions were done in groups of 4, as there were 4 separate fear conditioning boxes/isolation chambers available. The mice were transferred in their cages from the housing room to a separate room with the fear conditioning chambers. At the beginning of the day and in between sessions, the grid floors, trays, and fear conditioning boxes were wiped down with 70% ethanol. All urine and feces were removed to prevent providing a cue to the next group of mice. At the end of each day, the grid floors and trays were washed with mild soap and warm water.
**Contextual fear conditioning**

On day 1 of the behavioral experiment, mice were subjected to contextual fear conditioning, which consisted of 3 training sessions each separated by 3 hours (FC group). At the start of each fear conditioning session, the mouse was transferred to a plexi-glass box with a metal grid floor (Coulbourn Instruments, H10-11RTC, 120W x 100D x 120H) contained within an isolation chamber. Mild foot shocks (2 s each, 0.70 mA) were delivered at 198 s, 278 s, 358 s, and 438 s, with a total session time of 500 s. Mice were returned to their home cage and brought back to their normal housing room in between each session. On day 4, mice were subjected to a 500 s retrieval test to assess their fear memory of the context. Mice were placed in the context used for fear conditioning but did not receive foot shocks during the testing session.

**Contextual fear extinction**

On days 2 and 3 of the behavioral experiment, mice were subjected to extinction training, which consisted of 4 training sessions per day each separated by 2 hours, with a total of 8 extinction training sessions. Mice were placed in the context used for fear conditioning but did not receive foot shocks during the extinction session, with a total time of 30 min per session. Mice were returned to their home cage and brought back to their normal housing room in between each session.

**Quantification of freezing**

Freezing behavior was measured using a digital camera fixed at the top of each fear conditioning box and connected to a computer with Actimetrics FreezeFrame
software. The bout length of freezing was set to 1 s. The threshold for freezing behavior was set for each mouse based on baseline freezing and was determined by an experimenter blind to animal group. Final freezing percentages during a session for each mouse were obtained by averaging freezing during the second and third minutes of the trial.

2.3 Stereotaxic surgeries

All survival surgeries were performed aseptically in a designated clean room within the animal facility. Mice were initially anesthetized with 4% isoflurane, followed by 1.5-2% isoflurane during surgery and monitored for breathing rates and absence of pain reflexes. All coordinates are relative to bregma in mm. A handheld dental drill was used to create a small hole in the skull at the site of injection. After the infusion of tracer, an equal volume of sterile deionized H₂O (dH₂O) was taken up and expelled through the needle to ensure proper flow of liquid. Following the second bilateral injection, 1-2 drops of sterile PBS was applied to the skull and the incision was closed with 4-5 individual nylon sutures. A bactrin antibiotic was topically applied to the incision site. Mice were given a 1g/kg dose of buprenorphine analgesic subcutaneously near the incision site immediately after surgery. Mice were weighed and monitored for recovery daily for three days following the surgery, with additional administration of analgesic as needed. All mice were individually housed post-surgery until the end of the experiment when they were sacrificed. At the beginning of the surgery day and again at the end of the day following the last surgery, dilute bleach was taken up into the needle followed by extensive rinsing with sterile dH₂O.
**AAV-GFP anterograde tracer**

10 week old C57BL6 mice were injected with 300nL of AAV2-CMV-GFP (Virovek, 0.625E+13 vg/mL) under stereotaxic guidance. A new frozen aliquot of the virus was defrosted on ice at the beginning of each day of surgeries. Remaining virus at the end of the day was sterilized with bleach and discarded. The AAV aliquot was kept on ice and protected from light in between use. Bilateral injections were performed to target the posterior basal amygdala (AP -1.35, ML +/-3.45, DV -5.2) using a 5 uL syringe (Hamilton, 75RN) fitted with a 33 gauge blunt-point needle. The AAV was injected at a rate of 100 nL/min. The needle was kept in place for 10 min after the completion of injection before being slowly raised and removed. Mice were sacrificed 2 weeks after injection to allow sufficient time for virus expression.

**Cholera toxin subunit B retrograde tracer**

Adult TetTag mice were stereotaxically injected with either 45nL (for ovBNST) or 150nL (for dBNST) of 0.5% Cholera Toxin Subunit B (CTB) Alexa Fluor 647 (Molecular Probes, C-34778) dissolved in sterile phosphate-buffered saline. The CTB solution was kept on ice and protected from light in between use. Bilateral injections were performed to target the dBNST (AP +0.65 mm, ML +/-1.1 mm, DV -4.25) using a 0.5 uL Neuros Syringe fitted with a 32 gauge blunt-point needle (Hamilton, 7000.5). The CTB solution was injected at a rate of 100 nL/min. The needle was kept in place for 10 min after the completion of injection before being slowly raised and removed. Mice were individually housed after the surgery and given a 10 day post-surgery recovery/isolation period before starting day 1 of behavioral experiments.
Cre-mediated knockout of BDNF

BDNF<sub>2L/2L</sub> mice were bilaterally injected with 300nL of Adeno-associated virus encoding either Cre recombinase and green fluorescent protein (AAV1/2-GFP-Cre) or only green fluorescent protein (AAV1/2-GFP). Bilateral injections were performed to target the basal amygdala (AP -1.35, ML +/-3.45, DV -5.2) using a 5 uL syringe (Hamilton, 75RN) fitted with a 33 gauge blunt-point needle. The AAV was injected at a rate of 100 nL/min. The needle was kept in place for 10 min after the completion of injection to allow for diffusion of the virus and prevent backflow before being slowly raised and removed. Mice were individually housed for 3 weeks post-surgery before starting behavioral experiments to allow sufficient time for virus expression and BDNF protein depletion. Accurate targeting of the virus was confirmed by analysis of GFP signal in both groups. Mice that did not have any GFP expression within the BA were excluded from the study. All GFP-expressing cells included in the perisomatic analysis were within the BA.

2.4 Tissue preparation and immunohistochemistry

Eighty to ninety minutes after the start of the retrieval session, mice were deeply anesthetized with a ketamine/xylazine solution and transcardially perfused with 0.1 M phosphate buffer (PB) for 1 min at a rate of 15 mL/min using an automatic perfusion pump. The PB was immediately followed by ice cold 4% paraformaldehyde (PFA) dissolved in 0.1 M PB for 5 min at a rate of 15 mL/min. Brains were extracted and post-fixed in 4% PFA for 24 hours at 4°C. Brains were then transferred to a 30% sucrose solution for 48-72 hours before being snap-frozen in -50°C isopentane for 3 min. The
frozen brains were stored in an airtight container at -20°C until sectioning. Brains were sectioned on a cryostat to a 20um section thickness, and sections were immediately placed in PBS 1X at room temperature once cut. Sections that were not used immediately for immunohistochemistry experiments were then stored in a glycerol-based cryoprotectant solution at -20°C and protected from light until use.

For immunohistochemistry, free-floating tissue sections were rinsed overnight in PBS1X to remove cryoprotectant residue. All rinses and incubations were done while protected from light with gentle agitation. Sections were rinsed three times for 15 min in PBS with 0.25% Triton X-100 (PBS-T), then transferred to a blocking solution of PBS-T with 10% normal goat serum (or 3% normal donkey serum for CB1R and SR2C antibodies) for 1 hr at room temperature. Sections were incubated in a primary antibody solution of rabbit anti-Zif268 (Santa Cruz SC-189, polyclonal; 1:3,000) alone or with either mouse anti-GAD67 (Millipore MAB5406; monoclonal; 1:10000), mouse anti-PV (Millipore MAB1572; monoclonal; 1:2000), mouse anti-CCK/Gastrin (kind gift of Dr. Gordon V. Ohning; monoclonal; 1:1000) or goat anti-CB1R (kind gift of Dr. K. Mackie, polyclonal; 1:2000). Additional primary antibodies used were rabbit anti-synapsinI (Thermo Scientific PA1-4673, polyclonal; 1:1,000), mouse anti-PSD95 (Pierce Antibodies MA1-045, monoclonal; 1:500), goat anti-SR2C (Santa Cruz sc-15081, polyclonal; 1:50), rabbit anti-CamKII (Abcam ab52476, monoclonal; 1:5000), rabbit anti-DRD1 (Bioss USA bs-1007R, polyclonal; 1:50), rabbit anti-c-fos N-terminal (Santa Cruz sc-52, polyclonal; 1:10,000), and rabbit anti-Rab3b (kind gift of Dr. T. Sudhof; polyclonal, 1:4,000). Primary antibodies were diluted in the blocking solution, incubated at 4°C for 72 hours, and rinsed 3 times for 15 minutes in PBS-T. Secondary antibodies
(Jackson Immuno Research; goat anti-rabbit 549 1:1500, goat anti-mouse 647 1:500, Donkey anti-rabbit 649 1:500, Donkey anti-goat 549 1:500) were diluted in the blocking solution and were then applied to the sections for 2 hours at room temperature followed by 3 rinses for 15 minutes in PBS-T. Sections were either mounted on slides and coverslipped using Prolong Gold Anti-fade Mounting Media with DAPI, or were additionally incubated in 500 uL of DAPI solution (1:20,000 in PBS 1X) at room temperature for 5 min followed by a 10 min rinse in PBS 1X before being mounted on slides and coverslipped with Prolong Gold Anti-fade Mounting Media. Slides were stored at 4°C and protected from light until imaging.

2.5 Microscopy

Widefield microscopy

Images for the BNST TetTag experiment (Fig. 3.2) were acquired on an inverted Zeiss Axio Observer epi-fluorescence widefield microscope using a 20X Plan-APOCHROMAT air objective (0.8 NA). The microscope was fitted with the TissueFAXS Whole Slide Scanning System, which allowed the automated and rapid scanning of specified regions of tissue across multiple slides. The source of fluorescent excitation was an X-CITE 120XL metal halide lamp and images were acquired with a PCO monochrome CCD camera 12 bit. Filter sets used included DAPI (Chroma 49000 ET), Alexa 488/Cy2 (Chroma 49002 ET), and Alexa 568/ Cy3 (Chroma 49008 ET). The scanning system used was located at The Ragon Institute of MGH, MIT, and Harvard.
Confocal microscopy

All other images were acquired on a Nikon A1R confocal laser scanning microscope using a 20X air, 40X oil, or 60X oil objective. The settings for PMT, laser power, gain and offset were identical between experimental groups. Excitation lines used were 405 nm, 488nm, 561 nm, and 639nm. Band-pass emission filters were used to capture the following regions of the spectrum: 425-475nm (DAPI), 500-550nm (GFP), 570-620nm (RFP), and 660-740nm (far red). 1024x1024 resolution images were acquired using sequential scanning and averaging of 2 line scans with a 1.1 pixel dwell time. The confocal pinhole size was set to 1 airy unit for the 647 nm wavelength channel.

For CTB experiments, detection of Zif, GFP, and CTB in the basal amygdala (BA) was assessed with images acquired at 20X with 2μm step sizes for a total of 8-10 Z sections per image field (minimum of 3 sections per mouse). The maximum intensity projection image was used for subsequent analysis.

For extinction experiments, detection of Zif in GFP neurons was assessed for the BA (minimum of 7 sections per mouse), hippocampal CA1 (dCA1 and vCA1, 4 sections per mouse) and the infralimbic prefrontal cortex (IL, 4 sections per mouse). For the detection of perisomatic GAD67 a 20x objective was used and image stacks were collected with a 2μm step. For the detection of the other perisomatic markers (PV, CCK and CB1) a 40x objective was used and image stacks were collected with a 1 μm step.
2.6 Image analysis

Histone-GFP fear neurons and immediate-early genes

For the BNST TetTag experiment, image analysis was performed manually by identifying and counting GFP+, Zif+, or GFP+Zif+ cells. Numbers of positive or colocalizing cells within a subdivision were quantified separately and normalized to the ROI area for each subdivision.

For all other TetTag experiments, image analysis was performed using ImageJ software. Quantification of GFP+ and Zif+ cells was done by thresholding the maximum intensity projection image to select positive cells above background fluorescence or basal protein expression. The thresholded image was then run through a particle filter to remove objects/particles that were too small to represent nuclei. The number of objects that passed through the particle size filter was automatically counted and recorded. GFP+ and Zif+ images were first quantified separately then overlaid to create a single image displaying the two thresholded channels. Cells that were positive for both GFP+ and Zif+ were manually counted and recorded. For CTB image analysis, masks of GFP+, Zif+, and colocalizing GFP+Zif+ cells were superimposed on the corresponding CTB image and GFP+Zif+CTB- and GFP+Zif+CTB+ cells were manually identified and counted for each section. Left and right BA were analyzed for each section, with 3-6 sections per mouse. Final percentages of cell types are averages per mouse as a percentage of large DAPI-labeled cells.
**Synaptic marker colocalization**

Synaptic colocalization was analyzed from confocal image stacks using Imaris 7.2 3D imaging software. Colocalizing synaptic markers were identified by creating “spots” based on SynapsinI puncta and filtering for spots that contained above-threshold intensity pixels for PSD-95 puncta within a specified radius. A three-dimensional isosurface mask of GFP+ axons was used to select for overlapping SynI/PSD95 spots. A fiber tract present in the same image field of the tissue section was completely void of above-threshold staining of synaptic markers.

**Perisomatic synapse quantification**

For the TetTag experiments: for each marker (GAD67, PV, CCK and CB1R), tagged fear neurons (GFP+Zif- and GFP+Zif+) were randomly selected in the BA (around 27 neurons per mouse), and confocal images were analyzed at the z-plane where the diameter of the soma was largest. A mask for each perisomatic marker was generated by thresholding the image of the perisomatic marker. An oval-shaped outline was drawn to include all of the pixels that were positive for the perisomatic marker around a single neuron. A standard threshold was determined and applied to the perisomatic marker channel. The number of positive pixels (above threshold) and positive clusters (groups of adjacent positive pixels, made up of 5 or more pixels) within the outline was counted using Image J. To normalize for variation in size of neurons, the numbers of pixels and clusters were divided by the outline diameter of the ROI. One mouse from the FC+EXT group was excluded from the perisomatic marker analysis, since no active fear neurons (GFP+Zif+) were found in the basal amygdala of this mouse.
For the BDNF experiment: GFP-expressing soma in the BA were randomly selected and confocal images were analyzed at the z-plane where the diameter of the soma was largest. A freeform outline was drawn around the GFP+ soma to include any immediately adjacent perisomatic synapses. A standard threshold was determined for the perisomatic marker and applied to all images. The number of positive pixels within the outline was counted using Image J. To normalize for variation in size of neurons, the numbers of pixels was divided by the ROI perimeter. In the event that the GFP+ soma was also positive for PV, the cell was excluded from analysis.

### 2.7 Statistical analysis

All data were analyzed using GraphPad Prism 6 software. Data are presented as means +/- SEM and used ANOVAs repeated-measures and two-tailed t-tests (paired or unpaired) for normally distributed variables to evaluate statistical significance with p<0.05 as the level of statistical significance. Significance of column means compared to chance levels were evaluated using a one sample t-test of the average minus chance level against a hypothetical mean of zero.
Chapter 3:

Activation of a BA-BNST pathway in contextual fear
3.1 The dorsal BNST activation in contextual fear

The bed nucleus of the stria terminalis (BNST) has been reported to play a role in the expression of contextual fear, however the specific contributions and functions of the individual subdivisions are poorly understood (Zimmerman & Maren 2011; Sullivan et al. 2004; Gonzalez-Pardo et al. 2011; Gomes et al. 2010). We used a c-fos based reporter mouse, the TetTag transgenic mouse (Reijmers et al. 2007; Trouche et al., 2013), to image neurons in the dBNST that were activated during contextual fear conditioning and to determine their potential role in the retrieval of the fear memory (Fig. 3.1A). The TetTag mouse allowed us to tag neurons that were activated during the period of fear conditioning, while an immediate-early gene marker, egr-1/zif268 (Zif), was used to identify neurons that were active during retrieval of the fear memory (Fig. 3.1B). Combined, the GFP and Zif markers provide two temporally defined activation profiles for the same set of cells within the BNST, allowing us to distinguish cells that were activated during fear conditioning alone, retrieval alone, or during both. One group of mice was subjected to a contextual fear conditioning paradigm (FC group), while the other group remained in their home cage as controls (HC group). The FC group was assessed for contextual fear memory three days later in a retrieval session and perfused shortly after to allow for peak Zif expression in cells activated during memory retrieval (Fig. 3.1B).
**Figure 3.1**

**Tagging active neurons in the TetTag mouse.** (A) Schematic of the double transgenic TetTag mouse line. Tetracycline transcription activator (tTA) is expressed under control of the c-fos promoter. In the absence of doxycycline (DOX), tTA binds to the tet operator (tetO) in the second transgene and drives expression of histone2B-GFP (His-GFP). His-GFP is long-lasting and localized to the nucleus of cells activated during the off-DOX period. (B) Experimental outline for FC (n=7) versus HC (n=7) groups. Lower panel shows the corresponding expression timeline of the His-GFP (green) and immediate early gene Zif268 (Zif) (red) proteins, and overlapping expression (yellow). Zif expression is detected with immunohistochemistry.

All mice in the FC group showed increasing levels of freezing compared to baseline (before shocks in session 1) during fear conditioning trials and maintained freezing when re-exposed to the fear context during the retrieval session three days later (Fig. 3.2A), displaying retention of the contextual fear memory. As specific subdivisions of the dBNST were reported to play differential roles in anxiety (Kim et al. 2013), we quantified the numbers of GFP+ fear neurons in the oval BNST (ovBNST) and
anterodorsal BNST (adBNST) subnuclei separately (Fig. 3.2B). In the ovBNST, we found that the FC group had a significant increase in GFP+ neurons when compared to the HC group, indicating activation of ovBNST neurons during contextual fear conditioning (Fig. 3.2C). In contrast, contextual fear conditioning had no significant effect on the number of GFP+ neurons in the adBNST (Fig. 3.2C). Neither the ovBNST nor the adBNST showed differences in Zif+ cells between the FC and HC groups (Fig. 3.2D). Additionally, no differences were seen in the number of reactivated GFP+Zif+ cells in the ovBNST and adBNST in either HC or FC groups (Fig. 3.2E), and none of the groups were significantly above chance level (Fig. 3.2F). These data suggest that, though contextual fear conditioning activates the ovBNST, neither the ovBNST nor the adBNST are activated during expression of contextual fear when the fear memory is retrieved.
Activation of the ovBNST during contextual fear conditioning. (A) The FC group showed typical levels of freezing during fear conditioning sessions (S1, S2, S3) and a retrieval session (RET) three days later. Freezing percentages are an average of the second and third minutes of each session. (B) His-GFP expression in the dBNST after fear conditioning. Scale bar = 200um. (C) Fear conditioning increased the number of GFP+ neurons in the ovBNST (p = 0.011) but not the adBNST (p = 0.54) in comparison to HC group. (D) There was not a significant difference in the number of Zif+ cells after retrieval in the FC group in either the ovBNST (p = 0.55) or adBNST (p = 0.47). (E) There was not a significant difference in the number of GFP+Zif+ cells in the ovBNST (p = 0.19) or adBNST (p = 0.88) in the FC group compared to the HC group. (F) The number of GFP+Zif+ cells minus chance level was not significantly above zero in ovBNST or adBNST for either HC or FC groups. Chance level is defined as the product

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Figure 3.2

Activation of the ovBNST during contextual fear conditioning. (A) The FC group showed typical levels of freezing during fear conditioning sessions (S1, S2, S3) and a retrieval session (RET) three days later. Freezing percentages are an average of the second and third minutes of each session. (B) His-GFP expression in the dBNST after fear conditioning. Scale bar = 200um. (C) Fear conditioning increased the number of GFP+ neurons in the ovBNST (p = 0.011) but not the adBNST (p = 0.54) in comparison to HC group. (D) There was not a significant difference in the number of Zif+ cells after retrieval in the FC group in either the ovBNST (p = 0.55) or adBNST (p = 0.47). (E) There was not a significant difference in the number of GFP+Zif+ cells in the ovBNST (p = 0.19) or adBNST (p = 0.88) in the FC group compared to the HC group. (F) The number of GFP+Zif+ cells minus chance level was not significantly above zero in ovBNST or adBNST for either HC or FC groups. Chance level is defined as the product
of GFP+ and Zif+ percentages for each group. Graphs show means ± SEM. (HC group n=7, FC group n=7)
Figure 3.3

**Retrograde tracer in ovBNST identifies projection cells in the basal amygdala. (A)**

Retrograde tracer CTB-647 restricted to the injection site in the ovBNST (n=1). **(B-D)**

Retrogradely labeled CTB+ soma in the anterior BA (aBA), posterior BA (pBA), central amygdala (CeA), and lateral amygdala (LA). Scale bars for A-D = 200um.

Taking into consideration the strong evidence supporting a role for the BA in contextual fear (Goosens and Maren 2001; Onishi and Xavier 2010), we decided to focus on this BA-ovBNST pathway. To further characterize the BA-ovBNST pathway, we performed an anterograde tracing experiment to determine whether BA projection neurons send axons that synapse within the ovBNST. We injected an AAV2-GFP tracer virus to label axonal projections originating from cells in the BA. We imaged the BNST from a mouse in which the virus injection was restricted to the BA (Fig. 3.4A and B). We observed axonal processes in the adBNST, as expected, but in addition we also observed processes in the ovBNST (Fig. 3.4C). To confirm synaptic contact of the BA-originating axons in the ovBNST, we performed a colocalization study with pre- and post-synaptic markers, SynapsinI and PSD-95, respectively. We found multiple sites of
SynapsinI/PSD-95 colocalization along GFP-labeled axons in the ovBNST, indicating that BA projection neurons send axons that make excitatory synaptic contacts within the ovBNST (Fig. 3.4D and E).

Figure 3.4

**Basal amygdala cells project to and make synaptic contact within ovBNST. (A)**
Anterograde tracer AAV-GFP expression in the soma of BA neurons near injection site. (n=2) (B) GFP+ axonal projections from the BA coursing around the CeA through the stria terminalis. (C) GFP+ axonal projections from the BA in the ovBNST and adBNST subdivisions. Scale bar = 200um. (D) 3-dimensional rendering of IHC colocalization of synapsin I and PSD95 at sites along GFP+ axons in the ovBNST (purple dots indicated by white arrows show areas of colocalization of the synaptic markers). Yellow box in Fig. 3.4C indicates approximate image location. Scale bar = 10um. (E) Confocal image
3.3 Anterior basal amygdala neurons reactivated in contextual fear memory retrieval do not project to the dorsal BNST

After we identified BA projection neurons that monosynaptically project to the ovBNST, we hypothesized that a BA → BNST pathway might contribute to the activation of the ovBNST during contextual fear conditioning. We used CTB to retrogradely label BA → dorsal BNST (dBNST) projection neurons in TetTag mice, and divided the mice into FC and HC groups (Fig. 3.5A). All mice in the FC group showed increasing levels of freezing compared to baseline during fear conditioning sessions and maintained freezing when re-exposed to the fear context during the retrieval session three days later (Fig. 3.5B), displaying retention of the contextual fear memory. Initial attempts of the CTB injections were targeted to the ovBNST to selectively isolate the BA-ovBNST pathway and determine its specific activation. However, due to spread of the tracer, the CTB injections targeted the general dBNST, which included both the adBNST and the ovBNST (Fig. 3.5C). Mice in which the tracer spread outside of the dBNST were excluded from the study.
Figure 3.5

Tagging activity of the BA-BNST pathway during contextual fear. (A) Experimental schema for CTB-injected fear conditioned group (FC; n=9) versus home cage group (HC; n=8). CTB was injected bilaterally in the dBNST for all mice in both groups. Lower panel shows the corresponding expression timeline of the CTB, His-GFP, Zif. (B) The FC group showed typical levels of freezing during fear conditioning sessions (S1, S2, S3) and retrieval session (RET) three days later. Freezing percentages are an average of the second and third minutes of each session. (C) CTB-647 expression near the injection site in the dBNST. Scale bar = 200um.

The BA can further be divided into the anterior subdivision (aBA) and posterior subdivision (pBA). During our preliminary analysis, we observed a notable difference in CTB intensity and pattern between the anterior (aBA) and posterior (pBA) subdivisions of the BA (Fig. 3.6A). This led us to quantify the data separately for these two subdivisions, as they might contain parallel BA- dBNST pathways with different properties. The average percentage of CTB-labeled cells was similar across groups for
both BA subdivisions (Fig. 3.6B). We first analyzed the activation levels of the non-dBNST-projecting fear neurons (CTB-). We found a significant increase in GFP+CTB-neurons in the aBA, but not the pBA, in the FC group versus HC group, revealing activation of non-dBNST-projecting neurons in the aBA during contextual fear conditioning (Fig. 3.6C). We also observed a significant difference in Zif+CTB- neurons in the aBA, but not the pBA, between the FC and HC groups (Fig. 3.6D). In agreement with earlier studies (Reijmers et al. 2007; Trouche et al. 2013; Tayler et al. 2013), the fear neurons in the aBA of the FC group were reactivated during the retrieval session (Fig. 3.6E). This population was the only group that was reactivated significantly above chance level (Fig. 3.6F). These GFP+Zif+CTB- cells in the aBA likely participate in a circuit between the BA and one or more downstream structures that support the storage of the fear memory (Reijmers et al. 2007).
Non-dBNST-projecting neurons in the anterior basal amygdala are reactivated in contextual fear memory retrieval. (A) CTB positive cells (CTB+, grayscale) in the BA with Zif+ mask (red), GFP+ mask (green), and colocalizing GFP+Zif+ cells (yellow). Scale bar = 100um. (B) Percentage of CTB+ cells among DAPI cells in the aBA and pBA were similar between FC and HC groups. (C) Fear conditioning increased the percentage of GFP+CTB- fear neurons among CTB negative cells (CTB-) in the aBA (p = 0.0043) but not the pBA (p = 0.24). (D) Fear conditioning increased the percentage of Zif+CTB- neurons among CTB- cells in the aBA (p = 0.048) but not the pBA (p = 0.57). (E) Fear conditioning increased the percentage of GFP+Zif+CTB- reactivated fear neurons among CTB- cells in the aBA (p = 0.0002). There was no significant difference in the pBA (p = 0.14). (F) The number of GFP+Zif+ cells minus chance level was significantly above
zero in the aBA of the FC group, indicating reactivation of fear neurons during retrieval. Graphs show means ± SEM. (HC group n=8, FC group n=9).

3.4 dBNST-projecting neurons in the posterior basal amygdala are activated during contextual fear conditioning

We next analyzed the activation patterns of the dBNST-projecting neurons in the BA to determine whether contextual fear conditioning activated the BA-dBNST circuit. We found that the dBNST-projecting neurons originating in the pBA, but not the aBA, were activated during contextual fear conditioning (Fig. 3.7A). We did not observe a difference in Zif+CTB+ cells in either subdivision (Fig. 3.7B). In addition, the dBNST-projecting fear neurons in the pBA were not reactivated upon retrieval of the fear memory as indicated by the analysis of the GFP+Zif+CTB+ neurons compared to the HC group (Fig. 3.7C). The percentage of reactivated fear neurons did not differ significantly from chance level in the aBA and pBA of either group (Fig. 3.7D). Our data therefore suggest that the BA-dBNST projection is not part of the fear memory engram. Notably, the activation patterns of the dBNST-projecting neurons in the pBA (Fig. 3.7A-D) mirrored the activation patterns of the ovBNST neurons (Fig. 3.2C-F), with both being activated during contextual fear conditioning without being reactivated during retrieval.
dBNST-projecting neurons in the posterior basal amygdala are activated in contextual fear. (A) Fear conditioning increased the percentage of GFP+CTB+ fear neurons among CTB+ cells in the pBA (p = 0.007), but not the aBA (p = 0.15). (B) There was no significant difference in the percentage of Zif+CTB+ cells among CTB+ cells in the aBA (p = 0.65) or pBA (p = 0.47). (C) There was no significant difference in the percentage of GFP+Zif+CTB+ cells among CTB+ cells in the aBA (p = 0.16) or pBA (p = 0.80). (D) In none of the groups was the number of GFP+Zif+ cells minus chance level significantly above zero. Graphs show means ± SEM. (HC group n=8, FC group n=9).

3.5 Characterization of His-GFP+ cells in the dorsal BNST

Given the vast heterogeneity of cell type and function in the BNST, it would be very informative to know the cell type characterization of the GFP+ fear neurons. Knowing whether the fear neurons are comprised of a specific cell type, and if so, which neurotransmitters and peptides it might express, would give us insight into the downstream function of the activated cells in contextual fear. While the TetTag system primarily tags glutamatergic neurons in the amygdala and hippocampus (Trouche et al.,
2013, Reijmers lab unpublished observations), the cell-specific expression of the double transgene in the BNST is unknown. To begin to address this question, we used tissue immunohistochemistry of select cell markers to characterize the GFP+ neurons in the dBNST of a fear conditioned TetTag mouse. The BNST receives rich dopaminergic and serotonergic input (Phelix et al. 1992), which could modulate the activity of the targeted cells. To determine if the GFP+ fear neurons in the BNST might preferentially represent a cell type enriched in one of these receptors, we immunohistochemically labeled for serotonin receptor 2C (SR2C) and dopamine receptor D1 (DRD1) in the BNST of TetTag tissue. Our preliminary and qualitative analysis showed that the GFP+ neurons seemed to be excluded from cells that somatically expressed high levels of SR2C (Fig. 3.8A), while preferentially overlapping with cells that were enriched for DRD1 (Fig. 3.8B). Further quantitative analysis and characterization of the GFP+ cells with other neurotransmitter and neuropeptides will be needed in determining the function of these cells in contextual fear.
Figure 3.8

Characterization of GFP+ fear neurons in the ovBNST. (A) Single plane confocal image of ovBNST with His-GFP direct fluorescence and serotonin receptor 2C (SR2C) in a fear conditioned TetTag mouse shows little to no colocalization between the cell populations. Top panel scale bar = 25um, bottom panel scale bar = 5um. (B) Single plane
confocal image of ovBNST with His-GFP direct fluorescence and dopamine receptor D1 (DRD1) in a fear conditioned TetTag mouse. Most of the GFP+ neurons show an enrichment of DRD1 localization in the soma and/or membrane. Top panel scale bar = 25um, bottom panel scale bar = 5um.

3.6 Activation of neurons caused by exposure to novel context

Placing mice in a novel context is not sufficient to induce expression of the tetO reporter protein in the BA of the LacZ-TetTag mouse line (Reijmers et al. 2007). However, we wanted to further evaluate the effect of novel context exposure on the activation of BA and DBNST subregions in the His-GFP TetTag mouse line. Since the TetTag reporter system is driven by the cfos promoter, we first analyzed the expression of endogenous cfos in the DBNST of C57Bl6 mice that were exposed to context only. We divided C57Bl6 mice into three groups: no shock group (NS group), in which mice were transferred to a novel context but did not receive foot shocks, fear conditioned group (FC group), in which mice were transferred to a novel context and received foot shocks as an aversive conditioned stimulus, and a home caged group (HC group), in which mice remained in their home cage for the duration of the experiment (Fig. 3.9A). The FC group displayed an increasing percentage of freezing as they received shocks during the training session, whereas the NS group showed little to no baseline freezing while in the novel context throughout the length of the session (Fig. 3.9B). We found that both the FC and NS groups had a significant increase in cfos expression in comparison to the HC group in both the aBA and pBA. Interestingly, while the FC group showed slightly more cfos expression than the NS group, the difference was slight and not
significant (Fig. 3.9C). These results indicate that the transfer of the mice to a novel context is alone sufficient to induce endogenous cfos expression in the BA in similar levels to mice that are fear conditioned to the context, after a single session. Next, we analyzed percentages of cfos expression in the DBNST subregions, including the ovBNST, adBNST, and mediodorsal BNST (mdBNST). We did not observe any significant differences in the ovBNST between the three groups, however the FC group was trending towards an increase (Fig. 3.9D). Both the FC and NS groups had a significant increase in cfos expression in comparison to the HC group in both the adBNST and mdBNST. In the adBNST, NS and FC groups showed similar levels of cfos. In the mdBNST, the FC group showed an increase in cfos compared to the NS group (Fig. 3.9D). These results in the dBNST indicate that one training session is not necessarily sufficient to cause activation of the ovBNST, however it is sufficient to induce cfos in the adBNST and mdBNST subdivisions. When compared to data from the TetTag experiments, these results emphasize the differences that can arise from variances in the activity reporter and experimental protocol.
Exposure to novel context induces cfos expression in BA and BNST subdivisions.

(A) Schema of experimental procedure for the three groups: home caged (HC, n=8), no shock (NS, n=8), and fear conditioned (FC, n=8). (B) The percentage of freezing over the 8 min session for FC and NS groups. Red bolts indicate times at which the FC group received foot shocks in the context. The FC group displayed increasing levels of freezing from baseline following shocks, while the NS group remained at baseline levels with an absence of freezing throughout the session. (C) Both FC and NS groups showed a significant increase in cfos expression as a percentage of DAPI nuclei in comparison with HC group in the aBA (p<0.0001 FCvsHC, p<0.0001 NSvsHC) and pBA (p<0.0005 FCvsHC, p<0.0001 NSvsHC) subdivisions. There was no significant difference of cfos expression between NS and FC groups in either the aBA or pBA. (D) There was no significant difference in the percentage of cfos expression in the ovBNST between the
three groups. Both FC and NS groups showed a significant increase in comparison with the HC group in the adBNST and mdBNST subdivisions. There was no significant difference of cfos expression between NS and FC groups in the adBNST. The percentage of cfos expression in the mdBNST was significantly higher in the FC group compared to the NS group.
Chapter 4:
Extinction-induced remodeling of perisomatic synapses in the basal amygdala
Content in sections 4.1 and 4.2 of this chapter have previously been published in *Neuron*, 2013; 80(4):1054-65 as “Fear extinction causes target-specific remodeling of perisomatic inhibitory synapses.” Authors on the manuscript were Stephanie Trouche, Jennifer Sasaki, Tiffany Tu, and Leon Reijmers. Figures and text are republished here with copyright permission from Elsevier. My specific contributions included immunohistochemistry, confocal imaging, and analysis of CamKII colocalization with GFP+ neurons in the basal amygdala (BA) (data used to address reviewer comments and filter cell-specific data shown in 4.1, 4.3, 4.4), supplemental immunohistochemistry and confocal imaging of perisomatic parvalbumin around GFP+Zif+ neurons in the BA (figure 4.5 C-H), and optimization of synaptic marker immunohistochemistry and colocalization (figure 4.6 E, supplementary figures). I additionally contributed to scientific discussion regarding the manuscript, as well as figure preparation (all figures) and editing of the manuscript before submission and during the revision.

**4.1 Extinction-induced silencing of fear neurons**

*Fear extinction silences the basal amygdala fear memory circuit*

Prior electrophysiological studies have revealed that fear extinction can decrease the firing of basal amygdala (BA) fear neurons (Amano et al. 2011; Herry et al. 2008; Livneh & Paz 2012), but the underlying mechanism is unclear. We used a c-fos-based reporter mouse, the TetTag mouse (Reijmers et al. 2007), to image the effect of contextual fear extinction on BA fear neuron activation. The TetTag mouse expresses long-lasting nuclear GFP under control of a c-fos promoter (Fig. 4.1A), which enabled us
to tag excitatory neurons activated during fear conditioning (i.e. fear neurons, Fig. 4.1B). The expression of the immediate-early gene Zif268/Egr1 (Zif) served as a marker for neurons activated during a later retrieval test (Okuno 2011; Reijmers et al. 2007) (Fig. 4.1C). Two groups of TetTag mice (FC, n=15; FC+EXT, n=17) were subjected to contextual fear conditioning (Figs. 4.1C and D). As expected, similar numbers of BA fear neurons were tagged with GFP in both groups (Fig. 4.1E). The next 2 days, only one group (FC+EXT) was subjected to extinction learning, while the other group (FC) remained in the home cage. Extinction caused the loss of fear expression as indicated by the total suppression of freezing at the end of the extinction procedure (Fig. 4.1F), and during retrieval on day 4 (Fig. 4.1G). Extinction had no significant effect on the activation of BA neurons that were not tagged during fear conditioning (GFP-Zif+, Fig. 4.1H). We analyzed the BA fear memory circuit by defining 2 types of fear neurons (i.e. tagged during fear conditioning): silent fear neurons (GFP+Zif-, fear neurons not reactivated during retrieval) and active fear neurons (GFP+Zif+, fear neurons reactivated during retrieval) (Fig. 4.1I). We found that fear extinction caused a 2.3-fold decrease in the number of active BA fear neurons, with a subgroup of fear neurons remaining active after extinction (GFP+Zif+; Fig. 4.1J). This extinction-induced silencing of the BA fear memory circuit, caused by contextual fear extinction, is similar to that previously found in electrophysiological studies on tone fear extinction (Amano et al. 2011; Livneh & Paz 2012; Herry et al. 2008). In addition, the observed concomitant reduction in active BA fear neurons and freezing replicates previous studies (Herry et al. 2008; Reijmers et al. 2007), and reflects the causative role of the basal amygdala in the behavioral expression of contextual fear (Maren 1998). Therefore, our results provide further support for a
model where extinction decreases fear by silencing the fear memory circuit within the BA.

Figure 4.1

**Fear extinction silences the basal amygdala fear memory circuit.** (A) A double transgenic TetTag mouse line was used that expresses the tetracycline transcriptional activator (tTA) under control of the activity-regulated c-fos promoter. In the absence of doxycycline (−DOX), tTA binds to the tet operator (tetO) in the second transgene and
induces expression of a long-lasting histone2B-GFP (His-GFP) fusion protein. (B) Image of an excitatory basal amygdala (BA) neuron tagged with His-GFP (green) and positive for calcium/calmodulin-dependent kinase II (CamKIIa) (red). Scale bar, 10 μm. (C) Schema of the experimental procedure. “−DOX” opened a time window for tagging fear conditioning-activated neurons (GFP+; i.e., fear neurons) in two experimental groups (FC, n = 15; FC+EXT, n = 17). Zif expression during retrieval (Zif+) was used to detect the reactivation of tagged fear neurons (GFP+Zif+; i.e., active fear neurons). The absence of Zif in GFP+ neurons was used to identify silent fear neurons (GFP+Zif−). (D) As training progressed, FC and FC+EXT mice showed an increase in their level of freezing (between sessions: **p < 0.001 for FC and ***p < 0.001 for FC+EXT). (E) FC and FC+EXT groups had similar percentages of GFP+ neurons (sum of GFP+Zif− and GFP+Zif+) in the BA (p = 0.49). (F) The FC+EXT group showed a significant decrease in freezing during the extinction sessions on days 2 and 3 (**p < 0.001 E1 versus others and ***p < 0.001 E2 versus others). (G) Freezing during retrieval on day 4 in the FC+EXT group was absent and was significantly lower compared to the FC group (p = 0.00014). (H) There was no significant difference in the percentage of Zif+ among GFP− neurons between the two groups (p = 0.064). (I) Representative image of GFP-positive (GFP+, green) and Zif-positive (Zif+, red) nuclei in the basal amygdala (BA) of an FC (left) and an FC+EXT mouse (right). Arrows indicate active fear neurons (GFP+Zif+) and arrowheads indicate silent fear neurons (GFP+Zif−). Scale bar, 50 μm. (J) Extinction decreased the number of active BA fear neurons, as indicated by FC+EXT mice having less GFP+Zif+ neurons than FC mice (p = 0.00023). In both groups, the percentage of GFP+ZIF+ neurons was higher than chance level (FC: p = 0.0000098; FC+EXT p =
Activation of brain regions upstream of the basal amygdala is not altered after fear extinction

We next explored where extinction acted to cause a silencing of the BA fear memory circuit. We first addressed the possibility that contextual fear extinction might act on brain regions upstream of the BA, and thereby indirectly silencing fear neurons in the BA. The BA receives inputs from the hippocampus and infralimbic prefrontal cortex, brain regions that have been implicated in fear extinction (Hartley & Phelps 2010; Orsini & Maren 2012). We therefore tested if fear extinction altered the activation of excitatory neurons in the CA1 region of the hippocampus (both dorsal and ventral: dCA1 and vCA1), and in the infralimbic prefrontal cortex (IL). We analyzed the same brains that were used for the BA analysis, since the TetTag mouse tags neurons recruited by fear conditioning throughout the whole brain (Garner et al. 2012; Liu et al. 2012; Deng et al. 2013; Matsuo et al. 2008; Reijmers et al. 2007; Tayler et al. 2013). As expected, the FC and FC+EXT groups had similar percentages of neurons tagged in these brain regions during contextual fear conditioning (Fig. 4.2A). Contextual fear extinction did not alter the activation of non-tagged dCA1 and vCA1 neurons during retrieval (Fig. 4.2B), nor the reactivation of tagged dCA1 and vCA1 neurons during retrieval (Fig. 4.2C and D). These results are consistent with a previous study reporting that contextual fear extinction acts on a population of CA1 neurons that is segregated from the CA1 neurons recruited
during fear conditioning (Tronson et al. 2009), and indicate that the memory of the context is retained after contextual fear extinction (Rudy et al. 2004). Similar to the hippocampus, the activation of non-tagged IL neurons (Fig. 4.2B) and the reactivation of tagged IL neurons (Fig. 4.2C and D) were not affected by contextual fear extinction. Overall, we did not detect extinction-induced functional changes in two important brain structures upstream of the BA. We therefore shifted our focus to potential local changes within the BA that might have caused the silencing of the BA fear memory circuit.
Figure 4.2

Activation of brain regions upstream of the basal amygdala is not altered by fear extinction. (A) The FC and FC+EXT groups had similar percentages of GFP+ neurons in the dorsal CA1 (dCA1), ventral CA1 (vCA1), and infralimbic cortex (IL) (dCA1: \( p = \ldots \))
0.44; vCA1: p = 0.50; IL: p = 0.17). (B) Representative image of GFP+ neurons (green) and Zif+ neurons (red) in the dCA1 (top), vCA1 (middle), and IL (bottom) of an FC mouse (left) and an FC+EXT mouse (right). Blue, DAPI. Arrows indicate active fear neurons (GFP+Zif+). Scale bar, 50 μm. (C) No significant differences in the percentage of Zif+ among GFP− neurons were found (dCA1: p = 0.25; vCA1: p = 0.21; IL: p = 0.056). (D) Extinction had no effect on reactivation of dCA1, vCA1, and IL. The number of GFP+Zif+ neurons was similar between the two groups (dCA1: p = 0.26; vCA1: p = 0.61; IL: p = 0.27). The number of GFP+Zif+ neurons was above chance level in the dCA1, vCA1, and IL of both groups (dCA1: FC versus chance, p = 0.00017; FC-EXT versus chance, p = 0.000017; vCA1: FC versus chance, p = 0.0027; FC-EXT versus chance, p = 0.000062; IL: FC versus chance, p = 0.0072; FC-EXT versus chance, p = 0.0026). Chance level was determined by using the percentage of Zif+ among GFP− neurons as shown in (C). Graphs show means ± SEM. n.s., not significant; *p < 0.05, **p < 0.01, ***p < 0.001.
4.2 Target-specific remodeling of inhibitory synapses in the basal amygdala

_Fear extinction causes target-specific remodeling of perisomatic inhibitory synapses in the basal amygdala_

Around 85% of the neuronal cell population within the BA consists of excitatory projection neurons, whereas the remaining 15% are local interneurons that make inhibitory synapses onto the projection neurons (McDonald 1992). Because BA inhibitory interneurons have been implicated in fear extinction (Ehrlich et al. 2009; Heldt & Ressler 2007), we addressed the possibility that structural changes involving inhibitory circuits in the BA might have caused the extinction-induced silencing of BA fear neurons by increasing local inhibition. We first examined the expression of 67-kDa glutamic acid decarboxylase (GAD67), a key enzyme in GABA synthesis (Fig. 4.3A). We did not find evidence for increased GAD67 expression in either the complete BA or in the soma of BA interneurons (Fig. 4.3B and C), consistent with a recent study (Sangha et al. 2012). We hypothesized that fear extinction might act on a synaptic site where local interneurons interface with the BA fear neurons. We tested this hypothesis by imaging a special type of inhibitory synapses called perisomatic synapses. Perisomatic inhibitory synapses are a plausible candidate for silencing BA fear neurons, since they are well-positioned to modulate the functional activation of excitatory neurons (Miles et al. 1996). Consistent with our hypothesis, we found that silent fear neurons had increased GAD67 around their soma after extinction (Fig. 4.3D). Interestingly, this increase in perisomatic GAD67 was not observed around active fear neurons (Fig. 4.3E). The selective increase in perisomatic GAD67 around silent fear neurons seemed to be caused by a selective increase in the number of inhibitory synapses. Thus, our data reveal that extinction can
cause the target-specific remodeling of perisomatic inhibitory synapses in the BA, with extinction-induced changes in perisomatic GAD67 matching the activation states of the postsynaptic fear neurons.
Figure 4.3

Fear extinction causes target-specific remodeling of perisomatic inhibitory synapses in the basal amygdala. (A) Image of glutamic acid decarboxylase-67 (GAD67) immunolabeling of the basal amygdala (BA; scale bar, 100 μm). Arrows indicate GAD67-positive (GAD67+) soma in the BA. The inset shows a representative image of a GAD67+ interneuron soma (scale bar, 10 μm). (B) Extinction had no effect on GAD67 expression in the total BA (p = 0.30). (C) Extinction had no effect on somatic expression of GAD67 in the BA (p = 0.16). (D and E) Left: representative images of perisomatic GAD67 immunolabeling around silent BA fear neurons (D, GFP+Zif−) and active BA fear neurons (E, GFP+Zif+). Three images are shown for each fear neuron (top: GFP in green, GAD67 in gray; middle: Zif in red, GAD67 in gray; bottom: GAD67 mask in black, value for that neuron obtained by dividing black pixels by perimeter of yellow outline). Scale bars, 10 μm. Right: extinction increased perisomatic GAD67 around silent fear neurons (D, p = 0.047) but not around active fear neurons (E, p = 0.46). Graphs show means ± SEM. *p < 0.05.

Fear extinction increases perisomatic parvalbumin around silent BA fear neurons

We decided to further investigate the nature of the extinction-induced remodeling of perisomatic inhibitory synapses in the BA. Parvalbumin-positive interneurons (PV+)
are the predominant interneuron type in the BA, and form perisomatic synapses around BA excitatory projection neurons (McDonald & Betette 2001). Extinction did not change the expression of PV in the soma of BA interneurons (Fig. 4.4A and B). Next, we analyzed the presence of PV around the soma of BA fear neurons. Consistent with the extinction-induced increase in perisomatic GAD67, extinction also increased perisomatic PV around the silent fear neurons (Fig. 4.4C). Again, there was no significant increase around the active fear neurons (Fig. 4.4D). Importantly, the increase in perisomatic PV that we detected with image analysis is similar to that reported to increase perisomatic inhibition using electrophysiological analysis (Gittis et al. 2011; Kohara et al. 2007). Thus, our data provide strong support for an extinction-induced increase in perisomatic inhibition as the causative factor behind the decreased number of active BA fear neurons and the resulting silencing of the fear memory circuit. This reveals a direct connection between extinction-induced structural and functional changes in the BA.
Figure 4.4

Fear extinction increases perisomatic parvalbumin around silent BA fear neurons.

(A) Representative image of a parvalbumin-positive (PV+) interneuron soma in the basal amygdala (BA; scale bar, 10 μm). (B) Extinction had no effect on somatic expression of PV in the BA (p = 0.064). (C and D) Left: representative images of perisomatic PV immunolabeling around silent BA fear neurons (C, GFP+Zif−) and active BA fear neurons (D, GFP+Zif+). Three images are shown for each fear neuron (top: GFP in
green, PV in gray; middle: Zif in red, PV in gray; bottom: PV mask in black, value for that neuron obtained by dividing black pixels by perimeter of yellow outline). Scale bars, 10 μm. Right: extinction increased perisomatic PV around silent fear neurons (C, \( p = 0.0061 \)) but not around active fear neurons (D, \( p = 0.18 \)). Graphs show means ± SEM. **\( p < 0.01 \).

*The extinction-induced increase in perisomatic parvalbumin reflects new learning*

We asked whether the extinction-induced increase in perisomatic PV might have reversed any fear conditioning-induced changes in those synapses, which would indicate that BA perisomatic inhibitory synapses were part of the original fear circuit. To address this question, we performed a separate experiment where we compared a fear conditioned group (FC) with a home cage group (HC) (Fig. 4.5A and B). Consistent with our previous study (Reijmers et al. 2007), BA neurons activated during fear conditioning were tagged with long-lasting expression of GFP (Fig. 4.5C). During retrieval on day 4, the FC group showed significant freezing (Fig. 4.5D). The retrieval of contextual fear caused activation of both untagged (GFP-Zif+; Fig. 5E) and tagged (GFP+Zif+; Fig. 5F) neurons in the BA, with a preferential reactivation of the tagged BA fear neurons (Fig. 4.5E and F). Importantly, we did not find fear conditioning-induced changes in perisomatic PV around silent or active fear neurons (Fig. 4.5G and H). These data strongly suggest that the extinction-induced changes in PV+ perisomatic synapses constituted a new form of learning that occurred within the extinction circuit.
Figure 4.5

The extinction-induced increase in perisomatic parvalbumin reflects new learning.

(A) Design of an experiment with a home cage (HC, n = 8) and a fear-conditioned group (FC, n = 8). (B) Freezing during contextual fear conditioning on day 1. As training progressed, FC mice showed an increase in their level of freezing (p = 0.0006). (C) The FC group had a larger number of GFP+ neurons than the HC group in the basal amygdala (BA, p = 0.0049). (D) FC mice showed significant freezing during retrieval on day 4 (p =
The FC group had a larger number of GFP−Zif+ neurons than the HC group (p = 0.036). (F) The FC group had a larger number of GFP+Zif+ neurons than the HC group (p = 0.0043). Only in the FC group was the percentage of GFP+ZIF+ neurons higher than chance level, confirming that a subset of BA fear neurons was reactivated during retrieval (HC: p = 0.23; FC: p = 0.016). Chance level was determined by using the percentage of Zif+ among GFP− neurons as shown in (E). (G and H) Fear conditioning had no effect on perisomatic PV around either type of tagged BA neuron. (G) GFP+Zif−, p = 0.10. (H) GFP+Zif+, p = 0.49. Graphs show means ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

_Fear extinction increases perisomatic CB1R around active BA fear neurons_

In addition to PV+ perisomatic synapses, the BA also contains perisomatic inhibitory synapses that originate from cholecystokinin (CCK) interneurons (Yoshida et al. 2011). We therefore examined if fear extinction also affected perisomatic CCK+ synapses. Extinction did not change the expression of CCK in the soma of BA interneurons (Fig. 4.6A and B). In addition, perisomatic CCK around fear neurons, either silent or active, was not altered by fear extinction (Fig. 6C and D). We next examined if extinction might have affected CCK+ perisomatic synapses without changing CCK levels. Recently, it was discovered that BA perisomatic synapses positive for CCK, but not PV, contain a unique enrichment of proteins involved in endocannabinoid signaling, including cannabinoid receptor type 1 (CB1R) (Yoshida et al. 2011). Since CB1R in the BA have been implicated in fear extinction (Marsicano et al. 2002), we examined
whether perisomatic CB1R presence was modulated by extinction and whether this modulation was target-specific. We first confirmed the presence of CB1R in CCK+, but not PV+, perisomatic synapses (Fig. 4.6E). We did not observe labeling of CB1R in the soma of BA interneurons, so somatic expression of CB1R was not quantified. Perisomatic CB1R presence around the silent fear neurons was similar in the FC and FC+EXT groups (Fig. 4.6F). Intriguingly, extinction increased perisomatic CB1R around the active fear neurons (Fig. 4.6G). Since CB1R inhibit the release of γ-aminobutyric acid (GABA) (Katona et al. 2001), these results suggest that the extinction-induced upregulation of perisomatic CB1R facilitated the persistence of a small subset of active BA fear neurons in the extinction group (Fig. 4.1J).
Figure 4.6

Fear extinction increases perisomatic CB1R around active BA fear neurons. (A)

Representative image of cholecystokinin-positive (CCK+) interneuron soma in the basal
amygdala (BA; scale bar, 10 μm). (B) Extinction had no effect on somatic expression of CCK in the BA (p = 0.25). (C and D) Extinction had no effect on perisomatic CCK around silent (C, GFP+Zif−, p = 0.25) or active BA fear neurons (D, GFP+Zif+, p = 0.42). (E) Top: colocalization of perisomatic CB1R (red) and perisomatic CCK (gray) in the BA. Bottom: no colocalization of perisomatic CB1R (red) and perisomatic PV (gray) was detected in the BA. Scale bars, 10 μm. (F and G) Left: representative images of perisomatic CB1R immunolabeling around silent BA fear neurons (F, GFP+Zif−) and active BA fear neurons (G, GFP+Zif+). Three images are shown for each fear neuron (top: GFP in green, CB1R in gray; middle: Zif in red, CB1R in gray; bottom: CB1R mask in black, value for that neuron obtained by dividing black pixels by perimeter of yellow outline). Scale bars, 10 μm. Right: extinction increased perisomatic CB1R expressed in CCK terminals around active fear neurons (G, p = 0.036) but not around silent fear neurons (F, p = 0.19). Graphs show means ± SEM. *p < 0.05.

4.3 Investigation of BDNF-regulated perisomatic parvalbumin modulation

Studies have shown that brain-derived neurotrophic factor (BDNF) signaling in the BA can support fear extinction, and that postsynaptic release of BDNF promotes the formation of perisomatic PV synapses in the cortex (Hong et al. 2008; Huang et al. 1999; Kohara et al. 2007). We decided to investigate whether BDNF signaling in the BA was an underlying mechanism of fear extinction which functioned by upregulating perisomatic PV around fear neurons, thus increasing perisomatic inhibition. To probe the effect of BDNF on perisomatic PV in the BA and extinction behavior, we used
BDNF<sub>2L/2L</sub> mutant mice to selectively target BDNF depletion in the BA before mice were subjected to fear conditioning and extinction trials. In a pilot experiment, bilateral injections of an AAV-CreGFP virus was used to achieve BDNF knockout in the BA (CreGFP Group, n=4), while an AAV-GFP virus was injected in a second group as a control (GFP group, n=4). Prior testing of both AAV viruses in Rosa LacZ mice showed effective Cre recombination in GFP-expressing cells in mice that were injected with AAV-CreGFP, as well as proper localization in the BA (Fig. 4.7A-B).

![Fig. 4.7](image)

**Figure 4.7**

**Reporter protein is selectively driven by AAV-CreGFP virus expression.** (A)

Expression of GFP and LacZ labeling in the BA of a Rosa LacZ mouse injected with
AAV-GFP. LacZ labeling is absent. (B) Expression of GFP and LacZ labeling in the BA of a Rosa LacZ mouse injected with AAV-CreGFP. LacZ labeling is present in all GFP-expressing cells.

The BDNF<sub>2L/2L</sub> mice were stereotaxically injected with either AAV-GFP-Cre or AAV-GFP and submitted to contextual fear conditioning, followed by two days of extinction training, and a retrieval test (Fig. 4.8A). The AAV-GFP-Cre group did not show any significant differences in the learning or extinction of the fear memory (Fig. 4.8B-D). However, it should be noted that the lack of a behavioral effect could be due to the small group sizes in this preliminary experiment, as well as variability in viral expression in the BA between mice. Interestingly, one mouse in the AAV-GFP-Cre group that had the fullest expression of the virus in the BA also seemed to have slightly impaired extinction as evidenced by the higher freezing level during the retrieval test (Fig. 4.8D). Since we were interested in looking at the effect of the BDNF knockout on perisomatic synapses in the BA, we analyzed levels of PV that were surrounding the GFP-expressing neurons in both groups (Fig. 4.8E). We found a significant decrease of PV in the AAV-GFP-Cre group compared to the AAV-GFP control (Fig. 4.8F), suggesting that the knockout of BDNF in these cells leads to a decrease in perisomatic PV. We additionally quantified expression of PV in the hippocampal CA1 region in the same tissue sections to see if the difference was selective to the area of viral induction. We did not see any difference in PV between the two groups in the CA1 (Fig. 4.8G-H), indicating a specific manipulation of PV in the BA where BDNF was knocked down.
**Figure 4.8**

**BDNF knockout in the basal amygdala leads to decreased levels of parvalbumin in perisomatic synapses.** (A) Schema of the experimental procedure for the two groups, AAV-GFP-Cre (n=3) and AAV-GFP (n=4), depending on what virus was delivered to the BA. Fear conditioning was performed 3 weeks after the bilateral injection to allow time for viral expression and knockout of the BDNF protein. (B) As contextual fear conditioning progressed, both groups displayed an increase in their level of freezing in between sessions. (C) Both groups showed similar levels of freezing during extinction and (D) the retrieval session. (E) Representative image of a GFP+ cell (green) in the BA in both groups, with immunofluorescently-labeled PV (red). Outline displayed shows ROI used to determine which perisomatic PV puncta to include in the quantification. (F) The fluorescent mean intensity of PV around GFP+ cells was significantly decreased in the AAV-GFP-Cre group. (p = 0.0262) (G) Representative image of PV labeling in the hippocampal CA1 region in both groups. Green channel shows an absence of virus-expressing GFP+ cells. (H) The fluorescent mean intensity of PV in the CA1 regions is the same between the two groups. Graphs show means ± SEM. *p < 0.05.
Chapter 5:
Discussion
5.1 Overview

The studies performed and discussed here provide new insight into the circuitry underlying contextual fear learning and the mechanisms of contextual fear extinction. Chapter 3 of this thesis describes studies identifying a novel BA-BNST pathway that may contribute to the acquisition and/or consolidation during contextual fear conditioning. Through the combination of a cfos-based transgenic mouse and anatomical tracers, we investigated the activation profiles of projection-specific neurons in the BA during contextual fear conditioning and retrieval. We discovered that the population of dBNST-projecting neurons was only activated during the fear conditioning window, while a separate, non-BNST-projecting population participated in the retrieval of the memory. Furthermore, we present evidence of synaptic contact between the BA and a subregion of the BNST that is preferentially activated during fear conditioning. Taken together, our data are in agreement with a model in which a BA-ovBNST projection participates in the acquisition/consolidation of contextual fear, while an alternate BA output circuit that does not project to the dBNST is responsible for the storage of the fear memory. These findings advance our knowledge of the neural fear circuitry by further defining the roles of projection-specific fear neurons in the BA and BNST during the different stages of contextual fear learning and memory.

Chapter 4 of this thesis details studies investigating the inhibitory synaptic mechanisms contributing to the suppression of BA fear neurons during extinction training. We found extinction-induced remodeling of parvalbumin and cholecystokinin perisomatic synapses preferentially around BA fear neurons. Specifically, there was an increase in parvalbumin perisomatic inhibitory synapses around silenced fear neurons,
providing a mechanism of suppression of fear after extinction. Furthermore, there was an increase in cannabinoid 1 receptors, which suppress inhibition originating from cholecystokinin synapses, around fear neurons that remained active after extinction training. We also present preliminary data demonstrating a possible mechanistic link between BDNF and parvalbumin perisomatic synapses that may underlie the extinction-induced changes seen in the BA. These data significantly contribute to our understanding of the mechanisms by which the extinction circuit can directly modulate the fear circuit to suppress fear expression.
5.2 BA and BNST circuits in contextual fear

The BA-dBNST pathway in contextual fear

The association of a previously neutral context with an aversive stimulus, and the persistence of that association, relies on plasticity and signals communicated between many distinct brain regions. While the BA is central to the contextual fear circuit, its efferent and afferent projections are integral in the modulation and gating of the fear memory (Senn et al. 2014; Tye et al. 2011; Pape & Pare 2010). The projection target of neurons in the BA is often closely tied to the function as well. Our analysis of the dBNST-projecting neurons in the BA during contextual fear conditioning and retrieval revealed a function-specific participation of the BA → dBNST pathway. We found this particular subset of BA fear neurons was not preferentially reactivated during the fear retrieval session, in contrast to other BA fear neurons. The mice displayed similar levels of freezing to the context between the last fear conditioning session and the retrieval session, indicating that the fear memory was still intact at the time of testing. Taken together, these results suggest that the BA → dBNST pathway plays a role in fear conditioning, such as during the acquisition or consolidation of the fear memory, but that the specific population of neurons is not essential for the storage of the fear memory. However, although this pathway is active during contextual fear, it remains to be seen whether the pathway is necessary for the learning of the fear memory. It is possible that the disruption of the BA-dBNST pathway in lesion studies was the cause of, or contributed to, the impairment in contextual fear (Sullivan et al. 2004; Onishi & Xavier 2010; Walker & Davis 1997), but it is also likely that other affected amygdala and BNST circuits contributed to the behavioral phenotype.
The activation profile of the dBNST-projecting fear neurons in the BA is in contrast to non-dBNST-projecting fear neurons, which we found to be reactivated during contextual fear retrieval. This population of reactivated neurons has previously been shown to integrate into the memory engram that supports the lasting fear memory (Reijmers et al. 2007), however their projection target was unknown. Research indicating the activity of circuits from the BA to the PL (Senn et al. 2014; Rozeske et al. 2014) and the CeA (Sullivan et al. 2004; LeDoux 2000; Goosens & Maren 2001) in contextual fear provide plausible alternative downstream targets of the reactivated BA fear neurons. Additional experiments combining tracers with the TetTag mouse system can provide further insight into the projection-specific character of these reactivated cells.

*Differential activation of BNST subdivisions*

The BNST supports contextual fear (Sullivan et al. 2004; Luyten et al. 2011), with a heterogeneity of functions among its many subdivisions (Rodriguez-Sierra et al. 2013; Kim et al. 2013; Ali et al. 2012; Haufler et al. 2013). We present evidence that within the lateral region of the dorsal BNST, a population of neurons in the ovBNST subdivision is specifically involved in the contextual fear conditioning process. In contrast, we did not see an increased activation of the adBNST subdivision. A recent study found that the ovBNST is an anxiogenic structure and the BA-adBNST circuit is anxiolytic (Kim et al. 2013). Our finding that the ovBNST, but not the adBNST, is activated by contextual fear conditioning appears in agreement with the reported opposite functions of these subdivisions in anxiety.
Activation of the mdBNST, another subdivision of the dBNST, has been implicated in fear research (Haufler et al. 2013; Ali et al. 2012). We did not include the mdBNST in our original analysis due to a striking absence of GFP-tagged neurons in the region in both the HC and FC groups. Expression of the GFP neuronal tag could be variable between brain regions and dependent on the particular site of integration of the transgene. Therefore, absence of tagging in a particular brain region does not necessarily rule out the possibility of cellular activation during the off-dox window. Another reason for the absence of GFP labeling in the mdBNST could be that the subdivision is not activated after strong or multiple fear conditioning sessions, but only during the retrieval of the memory. In this case, we would not detect an increase in GFP cells, which are tagged during the fear conditioning process, but only an increase in the IEG marker, which is specific for activation during the retrieval session. This hypothesis is supported by a recent study using a Fos-Tau-LazZ transgenic mouse that indicated the activation of the mdBNST only after the retrieval of the contextual fear memory (Ali et al. 2012).

Based on our findings that suggest the selective participation of the ovBNST in contextual fear, we propose that the majority of dBNST-projecting neurons in the BA that are activated during contextual fear conditioning project to the ovBNST, and not the adBNST. Although we could not directly assess the activity of the BA-ovBNST circuit due to tracer spread within the BNST, we believe that the BA-ovBNST pathway is recruited by contextual fear conditioning for two reasons. First, the activation pattern of the dBNST-projecting BA neurons, i.e. activation during conditioning without reactivation during retrieval, was mirrored by the ovBNST fear neurons, but not by the adBNST neurons. Combined with the observed direct excitatory projection from the BA
to ovBNST, this suggests that the ovBNST neurons are a direct downstream target of contextual fear conditioning-activated BA neurons. Second, as described previously, stimulation of the BA-adBNST pathway has been shown to produce an anxiolytic phenotype (Kim et al. 2013). This would suggest that the adBNST-projecting neurons in the BA are not the activated population observed after fear conditioning. Thus, there would be two distinct and opposing BA-BNST circuits, specific to the subdivision of the BNST that the BA neurons project to. Future research would be needed to determine whether the two anatomically parallel circuits directly interface with each other through the micro-circuitry of the BNST.

Figure 5.1

**Model of the BA-dBNST pathway in contextual fear.** Neurons located in the pBA are activated by contextual fear conditioning, but not reactivated during retrieval. These non-reactivated fear neurons in the pBA participate in the acquisition and/or consolidation of
contextual fear through a monosynaptic excitatory projection to neurons located in the ovBNST. The reactivated fear neurons in the aBA participate in the storage and expression of the fear memory by projecting to one or more brain regions that are located outside of the dBNST (for example the central amygdala).

It is not immediately clear why the BA-ovBNST pathway would be active during contextual fear conditioning, without being incorporated into a lasting fear memory trace as indicated by its inactivity during retrieval. We hypothesize that the BA-ovBNST pathway plays a role in the acquisition or consolidation of the contextual fear memory by exerting a modulatory effect on the fear memory engram. The ovBNST might exert such a modulatory effect through one or more of its downstream brain structures (Dong, Petrovich, Watts, et al. 2001). Two recent studies found a monosynaptic projection from ovBNST neurons to serotonin neurons located in the dorsal raphe nucleus (DRN) (Weissbourd et al. 2014; Pollak Dorocic et al. 2014). This projection may be relevant given the involvement of serotonin in contextual fear memory (Bauer 2014; Rainnie 1999). Specifically, the ovBNST neurons could modulate the learning of contextual fear memory by stimulating the release of serotonin from the DRN to other brain regions.

The ovBNST is also reciprocally connected with the CeA (Dong, Petrovich, Watts, et al. 2001; Dong, Petrovich & Swanson 2001). The ovBNST might therefore directly modulate the formation of the contextual fear memory trace within the CeA (Goosens & Maren 2001; Pitts et al. 2009; Zimmerman et al. 2007; Koo et al. 2004). Future studies aimed at determining where contextual fear conditioning-activated
ovBNST fear neurons project to might elucidate how activation of a BA-ovBNST pathway can modulate contextual fear memory storage.

Gating of fear through the ovBNST

Serotonin

The neurotransmitter and peptidergic characterization of the activated fear neurons in the BNST would provide insight into their function, modulation, and downstream connections. 5-hydroxytryptamine, or serotonin, is a monoamine neurotransmitter that can act on serotonin receptors to increase or decrease neuronal activity by regulating intracellular ion concentrations. The precise effect of serotonin on a cell primarily depends on the subtype of the receptor(s) expressed (Barnes & Sharp 1999). The BNST, along with the BLA, mPFC, and hippocampus, contains a dense concentration of serotonin receptors (Corne-Hébert et al. 1999), and serotonin has been shown to be involved in the modulation of contextual fear memories (Bauer 2014). The anterolateral BNST displays a variety of serotonin receptor subtypes, including moderate to high levels of SR1A, SR2C, and SR7. These subtypes are differentially expressed in BNST neurons depending on the characteristic physiological properties of the cell (Guo et al. 2009). Interestingly, serotonin reuptake inhibitors (SSRIs) used as anti-depressant drugs are often associated with an acute and transient increase in anxiety-like behavior after initial administration, which can be reversed by serotonin receptor antagonists (Bagdy et al. 2002). Fluoxetine, a common SSRI, induced enhanced auditory fear learning concomitant with increased levels of Arc in the CeA and BNST (Ravinder et al.
Additionally, infusion of fluoxetine directly into the BNST, but not the CeA, generated similar effects on fear behavior. The BNST infusion was also sufficient to increase levels of Arc in the CeA, suggesting a BNST $\rightarrow$ CeA pathway that contributes to fear learning and is modulated by levels of serotonin. Conversely, BNST infusion of cannabidiol, an anxiolytic that produces its effects through the activation of SR1A, led to an impairment in contextual fear (Gomes et al. 2010). The effect of serotonin in the BNST on contextual fear appears to be dependent on the subtype of serotonin receptor activated, and a bi-directional modulation of BNST neurons by different serotonin receptors has been observed (Guo et al. 2009). Our finding that the ovBNST fear neurons do not largely overlap with soma that stain for SR2C does not exclude the possibility that these fear neurons are a site of serotonergic modulation, but rather begins to narrow the possible receptor subtypes that may interact with the fear circuit through the ovBNST. Future studies investigating the localization of other serotonin receptor subtypes will provide insight into the role of serotonin on the activity of the fear neurons in the BNST.

*Dopamine*

Dopamine is a neurotransmitter that has also been implicated in the process of contextual fear learning and memory (Heath et al. 2015; Pezze & Feldon 2004). Optogenetic stimulation of dopamine receptor 1A (Drd1a)-expressing ovBNST neurons that project to the adBNST resulted in a net inhibition of the adBNST and produced an anxiogenic phenotype (Kim et al. 2013). Furthermore, striatal Drd1 has been shown to be essential for contextual fear conditioning (Ikegami et al. 2014), and expression of the receptor in the dentate gyrus contributes to the formation of the contextual representation that is necessary for fear learning (Sariñana et al. 2014), demonstrating the modulatory
influence of dopamine on the contextual fear circuit. The ovBNST contains a high density of dopaminergic fiber terminals, and neurons display both major subtypes of dopamine receptors, Drd1 and Drd2 (Freedman & Cassell 1994; Krawczyk et al. 2014). Within the ovBNST, dopamine activity may contribute to the pathology of addiction observed in rodent models via modulation of NMDAR signaling (Krawczyk et al. 2014). We show that the ovBNST fear neurons express Drd1 somatically, which may be significant for two reasons. First, this population of ovBNST fear neurons may be the same as the adBNST-projecting ovBNST neurons that were selectively targeted using a Drd1a::Cre transgenic mouse by Kim et al. Since activation of this pathway was anxiogenic, it is possible that this same pathway plays a role in the generation of contextual fear similarly through the inhibition of the adBNST. Second, the expression of Drd1 in the fear neuron population provides insight into potential regulation of the fear circuit through dopamine signaling in the ovBNST, possibly by modulating the activity of the BA-ovBNST pathway during contextual fear acquisition or consolidation.

**CRF**

Corticotropin-releasing factor (CRF) acts as a peptide neurotransmitter in the BNST, where it can enhance GABAergic inhibitory transmission through the activation of CRF receptor 1 (Kash & Winder 2006). Globally, CRF plays a well-known role in the HPA-axis regulated stress response, and stress induction is associated with a concomitant increase in CRF. The function of CRF in contextual fear is less established, though chronic CRF-overexpression in the BNST after fear conditioning using a fear-potentiated startle paradigm strengthened the conditioned fear memory (Sink et al. 2012), and CRF-infusion in the BNST leads to an increase in sustained-fear response (Walker et al. 2009).
Together, these findings suggest a distinct role for CRF in the fear circuit through activity in the BNST. CRF terminals and receptors are found in the dBNST, with a particularly dense enrichment of both in the ovBNST (Cummings et al. 1983). The ovBNST receives CRF-containing processes from the CeA (Sakanaka et al. 1986), and recent evidence suggests that dopaminergic terminals from the PAG preferentially synapse onto and influence the activity of CRF neurons in the BNST (Meloni et al. 2006). Thus, there are multiple potential mechanisms in place that may contribute to the activity modulation of ovBNST CRF neurons through other structures in the fear circuit.

Building off of the model that a BA-ovBNST pathway is involved in the acquisition or consolidation of contextual fear, peptidergic signaling acting on the target neurons of this pathway may be an integral point of regulation of the downstream action. As described here, the diversity of neurotransmitters and neuropeptides represented in the ovBNST are well-placed to interact with the ovBNST fear neurons and modulate their output. While a more detailed characterization of the ovBNST fear neurons is necessary to understand which systems may contribute most significantly to their activity, our preliminary immunohistochemical results combined with the TetTag system provide a simple and straightforward method of visualizing the cell type and potential function of neurons that participate in the fear circuit.

*The temporal specificity of activation of the BA-BNST pathway*

While the TetTag system gives us the advantage of visualizing neuronal activation during two separate time windows, many different mechanisms underlying learning occur rapidly and cannot be resolved with the temporal resolution provided by
the system. All mice are given high dox food immediately following the conclusion of fear conditioning, however when and how much of the chow is eaten over the course of the following night is variable between mice. This makes it challenging to pinpoint the exact time at which dox is at sufficient levels in the brain to block further driving of the TetO promoter in c-fos activated cells. However, one night on high dox chow, following five days off dox chow, is sufficient to block further tagging of activated neurons in the TetTag mouse (Reijmers et al. 2007). Thus, the increase of tagged neurons after fear conditioning may represent cells that were activated not only by fear conditioning itself, but also by other memory processes that occur within the following 18 hours. The distinct stages that contribute to the learning of a fear memory include the acquisition phase and the consolidation phase. Acquisition refers to the learning of the association between the context and the aversive stimulus, and occurs immediately during the conditioning period. Consolidation is dependent on the synthesis of new proteins in response to cellular activation, and occurs within the 24 hours following fear conditioning (Dudai 2004). For this reason, we can conclude that the BA-dBNST pathway studied here is activated during the learning of the contextual fear memory, but we cannot distinguish whether its activation specifically occurs during the acquisition, consolidation, or both. Further experiments in a more temporally precise system would be necessary in dissecting the functional role of the dBNST-projecting population in the BA.

The differences we observed between the endogenous c-fos labeling in C57Bl6 mice and the GFP neurons in TetTag mice during fear conditioning may also be attributed to the varying time windows of activation and/or tagging. While the GFP neurons represent a summation of cellular activity over a longer multi-hour time window,
as described previously, the cfos labeling from the C57Bl6 mice represents cellular activity on a much more discrete timescale within minutes of the fear conditioning. Another explanation for the observed differential activation is that the TetTag mice underwent a much stronger fear conditioning paradigm, with a total of 12 foot shocks over the course of three sessions. In comparison, the C57Bl6 mice were submitted to 4 foot shocks over the course of one session. The difference in number, strength, and time of the conditioning sessions could possibly lead to the recruitment of distinct neural circuit activation. Despite these differences, our findings, together with previous BNST research, supports a role for the BNST in contextual fear, though further dissection of the temporal activity of the BNST subdivisions is needed.

_activation of BA and BNST subregions by context alone_

It has previously been demonstrated that TetTag mice show no difference in the number of tagged cells in the BA between the HC group and a no-shock (NS) control group, indicating that exploration of the novel context alone does not significantly induce activity-regulated tagging of cells in the basal amygdala (Reijmers et al. 2007). Furthermore, a relatively strong conditioning paradigm was required to induce significant activation of the TetTag reporter, with a single conditioning session insufficient to detect an increase in tagged cells in the FC verus HC groups (L. Reijmers, unpublished observation). Based on these previous results, we believe that the dBNST-projecting BA neurons analyzed here represent cells that were activated by the contextual fear conditioning and not the novel context or handling alone. However, it is still possible that the expression of the His-GFP tag in the BNST is more readily inducible than in the BA. In this case, handling or novel context exploration could potentially contribute to
increase in ovBNST GFP neurons in the FC group. To determine if the activation of the ovBNST in the TetTag mouse is specifically due to the contextual fear paradigm, a no-shock group (NS group) would need to be utilized as an additional control. The NS group would be subjected to the same handling and contextual presentation as the FC group, but would not be exposed to the aversive stimulus. In the event that the NS group does display an increase in tagged neurons in the BNST compared to the HC group, it would be useful to further characterize the cells to determine if they are a distinct population from neurons tagged in the FC group.

Summary of the BA-BNST

In summary, our data point to a BA-ovBNST pathway that participates in contextual fear, particularly during the acquisition and/or consolidation of contextual fear memories. Our findings can inform future optogenetic and pharmacogenetic studies of the BA-ovBNST pathway that are aimed at establishing its necessity and sufficiency within the contextual fear circuit. We also observed that the role of BA projection neurons in contextual fear memory differentiates across BA subdivisions (posterior versus anterior BA) and across downstream projection targets (dBNST versus non-dBNST). Additionally, our study highlights the utility of combining the TetTag mouse with tracer injections for assigning function to precisely defined anatomical circuits. Further experiments are necessary in determining the specificity of the ovBNST activity in contextual fear versus exposure to a novel context, and the downstream function of the BA-dBNST pathway. This knowledge contributes to the overall understanding of the contextual fear circuit, including the role of the dorsal BNST in contextual fear and a BA-BNST pathway that may contribute to the initial learning of the fear memory.
5.2 Interface between fear and extinction circuits via perisomatic synapses

Inhibition and synaptic plasticity in the amygdala are necessary for the extinction of contextual fear (Herry et al. 2010; Ehrlich et al. 2009). The process of extinction relies at least partially on new learning, presumably leading to changes in a neural circuit that interacts with and gates the fear circuit (Bouton & Bolles 1979; Herry et al. 2010; Orsini & Maren 2012). We show that the BA is a site of such interaction, as there is a decrease in reactivated fear neurons that corresponds with the suppressed levels of fear expression after extinction. Such changes were not observed in hippocampus or mPFC, indicating a unique function of the amygdala as a site of plasticity and regulation within the extinction circuit. However, both the hippocampus and mPFC have been shown to support extinction memory, and contribute to the regulation of fear through distinct mechanisms (Orsini et al. 2011; Senn et al. 2014). Our findings further support the idea that extinction is a form of learning rather than erasure, as the extinction-induced remodeling of perisomatic synapses was not simply a reversion of changes made after fear conditioning. This is in agreement with the fact that conditioned fear responses can reappear over time, even after apparent successful extinction training, indicating the fear memory is still intact (Courtin, Karalis, et al. 2013). The perisomatic remodeling was observed to be target-specific and correlated with the silent or active state of the fear neuron after extinction.

PV perisomatic synapses

The majority of PV interneuronal contact with pyramidal neurons in the BA occurs via perisomatic synapses (Pitkänen & Amaral 1993), providing an ideal
opportunity for inhibiting the activity of target neurons. Our findings show that modulation of PV perisomatic inhibitory synapses correlates with the silent or active state of fear neurons after extinction. This provides a straightforward and efficient mechanism for regulating the neurons that support fear memory in the amygdala. The increase in perisomatic PV was observed specifically around silenced fear neurons, illustrating a targeted inhibition of the neurons that contribute to fear expression. We propose that this increased perisomatic inhibition suppresses the activity of a subset of fear neurons and brings them into a silent state during extinction. As the number of active fear neurons decreases in the BA, the generation of a fear behavior in response to the context also decreases. Notably, not all of the fear neurons are silenced, as a population of neurons is reactivated during the retrieval test even after extinction. These remaining active fear neurons did not generate a fear response, as the extinguished mice did not freeze when re-exposed to the conditioned context.

**CB1R modulation of CCK perisomatic synapses**

Endocannabinoids and cannabinoid receptor 1 (CB1R) expression have been shown to support the extinction of conditioned fear (Marsicano et al. 2002; Chhatwal et al. 2005; Chhatwal et al. 2009). The extinction effect seems to be mediated at least in part by CB1R located in the BA (Roche et al. 2007). CB1R is enriched in presynaptic terminals of CCK interneurons in the BA and contributes to the suppression of inhibitory transmission (Katona et al. 2001). Our finding that CB1R expression was preferentially increased around reactivated neurons after extinction training suggests a role for CB1R in keeping these fear neurons active. This was surprising, since perpetuating the reactivation of fear neurons seems counterintuitive to the generation of extinction behavior. Although
the reason for this is currently unclear, it is possible that these neurons underlie the perpetuation of the fear memory after extinction and trigger spontaneous recovery by reawakening the fear circuit. However, this does not explain how CB1R supports fear extinction, as evidenced by impairment in extinction after CB1R deletion in rodents (Marsicano et al. 2002; Roche et al. 2007). It is possible that there is another function of CB1R that contributes to extinction, distinct from its role in keeping a select number of fear neurons active. In agreement with this scenario, we additionally saw an increase in perisomatic CB1R around BA neurons that were active during the retrieval testing, but had not been tagged as a fear neuron during fear conditioning. These active neurons could be a separate population of BA neurons that specifically respond to extinction learning (Herry et al. 2008). CB1R localized to CCK synapses can give rise to both short- and long-term plasticity, with distinct signaling mechanisms and timing properties associated with each (Freund & Katona 2007). It is perhaps this ability of the CB1R to contribute dual functions at the synapse that may differentiate its role in supporting extinction versus keeping fear neurons active. Further experiments with a focus on the timing and electrophysiological effects of CB1R in the BA after extinction would be useful in identifying and distinguishing these possible roles.

Together, the extinction-induced remodeling observed in perisomatic synapses surrounding BA fear neurons lends itself to a model in which these changes work together to shift the balance of silent to active fear neurons after extinction (Fig. 5.2). This change in ratio ultimately contributes to the suppression of activity in the fear network and a decrease in the freezing behavior in extinguished mice.
Figure 5.2

Model of extinction-induced functional and structural changes in the BA that contribute to fear behavior response. (A) Extinction increases the ratio of silent versus active fear neurons in the BA, thereby causing the elimination of behavioral expression of fear as indicated by the absence of freezing during the retrieval session. In this model, the increased number of silent fear neurons is a result of increased perisomatic inhibition by PV interneurons. The increase in CB1R in CCK perisomatic synapses occurs around a select subset of BA fear neurons that remain active due to increased CB1R-mediated disinhibition.
**BDNF signaling in PV perisomatic synapses**

Research strongly suggests that brain-derived neurotrophic factor (BDNF) is an important modulator of glutamatergic and GABAergic synapses (Gottmann et al. 2009). In the cortex, disruption of BDNF expression leads to the formation of fewer inhibitory synapses, although the total number of inhibitory cells remains unaltered (Kohara et al. 2007; Hong et al. 2008). The release of BDNF is gated by the postsynaptic cell, and can specifically promote the presynaptic formation of PV+ inhibitory perisomatic synapses (Jiao et al. 2011). We present evidence that BDNF in glutamatergic neurons in the BA can modulate the surrounding PV+ perisomatic synapses, potentially acting as a signaling mechanism after extinction to suppress the activity of fear neurons. Given the critical role of inhibitory synaptic plasticity in the BA during the extinction of fear, as described previously, a role for BDNF in extinction seems likely. Indeed, adult heterozygous BDNF-knockout mice, which express endogenous BDNF at half the normal levels, were observed to suffer from an impairment in fear extinction (Psotta et al. 2013).

Additionally, a single nucleotide polymorphism in the BDNF gene is associated with a selective impairment in fear extinction in both mice and humans, having no effect during fear conditioning (Soliman et al. 2010). Blockade of the tyrosine kinase B receptor (TrkB), the receptor for BDNF, in the BA led to a disruption in BDNF signaling and an impairment of the retention of extinction memory (Chhatwal et al. 2006). Notably, the within-session extinction rates were not affected by the TrkB disruption in this study, suggesting that BDNF signaling participates in the mechanism underlying consolidation, not acquisition, of the extinction memory. Recently, it has been shown that conditional knockout of TrkB, the BDNF signaling receptor, in PV+ interneurons leads to
impairment in the consolidation of auditory fear extinction in male mice. Fear consolidation was not affected by the deletion of TrkB (Lucas et al. 2014). This study provides compelling evidence in support of a BDNF-PV signaling pathway supporting the extinction of fear. However, since the TrkB deletion was not specific to the amygdala, it still remains to be seen whether the PV+ interneurons in the BA contributed to or are necessary for this behavioral effect. Taken together, these data suggest a role for BDNF in the formation of inhibitory synapses that gate fear extinction within the BA.

BDNF is produced and secreted almost exclusively by glutamatergic cells while its receptor, tyrosine receptor kinase B (TrkB), is present in both glutamatergic and GABAergic cells (Swanwick et al. 2004; Cellerino et al. 1996). Expression of the Cre virus in PV+ cells or other interneurons would have little to no affect, considering they do not normally express BDNF. Therefore, the action of BDNF-regulated changes on perisomatic PV+ synapses described here is likely exerted in a cell-autonomous manner. It is possible that BDNF secreted by surrounding cells could also influence the plasticity of the synapses surrounding the target cell. Further analysis of non-Cre-expressing cells in the BA would be necessary in determining this.

Summary of extinction-induced perisomatic modulation

In summary, our findings reveal a mechanism by which the extinction circuit can directly interact with and modulate the activity of the fear circuit. Through perisomatic inhibition in the BA, neurons that are part of the contextual fear memory engram are likely directly targeted and suppressed after extinction, providing a controlled manner of fear regulation after extinction training. Another population of fear neurons remains
active after extinction, with a separate target-specific modulation of perisomatic CB1R that seems to support its active state. These active fear neurons do not induce the fear response to the conditioned context, and their function is currently unknown. While extinction-induced perisomatic modulation appears to support extinction learning overall, it remains to be seen whether this change is necessary or sufficient for the extinction of conditioned fear.
5.4 Future perspectives and concluding remarks

Long-range projection targets of the silenced BA fear neurons

In our circuit study of the BA-BNST pathway described in Chapter 3, we identified a subpopulation of non-dBNST-projecting neurons in the aBA that were preferentially reactivated by fear retrieval. It can further be extrapolated that within these reactivated neurons there exists a subset that are then silenced after extinction, as seen in Chapter 4. It would be interesting and informative to bridge the findings between these two lines of research, through combining both circuit tracing and perisomatic labeling in extinguished TetTag mice, to determine the long-range projection targets of the subset of neurons that are silenced after extinction. This method would further be useful in determining if the silent versus active fear neurons after extinction, along with their correlative perisomatic modulations, project to different downstream brain regions. By further segregating the fear neurons by projection target, we may be able to distinguish finer effects of extinction on the perisomatic synapses. Given the roles of the BA-hippocampus and BA-mPFC circuits in extinction, these would be likely projection populations to initially investigate with this method.

Stability of extinction-induced changes in perisomatic synapses

Do the extinction-induced perisomatic changes described here endure over time, in tandem with the extinction memory? Alternatively, is the extinction memory only initially supported by the inhibition provided by these synapses and later moved to a different, higher-order system for the persistence of the memory? These questions have important implications for the targeting of therapeutic intervention, as the mechanisms
underlying extinction memory are likely to change over time. While fear conditioned subjects undergo extinction learning at a similar rate, the persistence of the extinction memory or the occurrence of spontaneous recovery is highly variable and thought to be dependent on a mixture of underlying processes (Courtin, Karalis, et al. 2013). To address whether the targeted perisomatic inhibition around silent and active fear neurons supports the extinction memory in a lasting manner, it would be necessary to investigate whether the observed changes are stable in extinguished animals after a longer time-lapse between the extinction training and retrieval test. Furthermore, this would allow us to analyze rates of spontaneous recovery in the mice, and whether the occurrence and severity of spontaneous recovery coincides with reversals in the perisomatic changes.

**Sex-specific differences in the fear circuit**

Historically, detailed research on the fear circuit has largely centered on males, with less than 2% of studies exploring fear and extinction memory focusing on the female brain (Lebron-Milad & Milad 2012). In addition to an increased prevalence of fear and anxiety disorders in women (Breslau 1997), there is mounting evidence that female sex hormones, such as estradiol, may specifically modulate the fear and extinction circuits through interacting with signaling pathways and plasticity (Cover et al. 2014). Because of this discrepancy, we may be seeing an unbalanced view of the mechanisms underlying fear, ultimately obscuring progress in therapy targeted for the general patient population. While sex differences were not within the scope of the experiments detailed in this thesis, it is an interesting and important factor that should be incorporated into future studies.
The effects of estrogen may also modulate the molecular mechanisms of fear extinction. The amygdala contains relatively high levels of estrogen receptors, and estrogen has been shown to stimulate activity and plasticity in amygdalar neurons (Lebron-Milad & Milad 2012; Walf & Frye 2006). Interestingly, estrogen has been linked with the activity of BDNF signaling in the hippocampus of females (Solum & Handa 2002; Scharfman & Maclusky 2005; Scharfman & MacLusky 2014). Given BDNF’s likely role in supporting fear extinction (Chhatwal et al. 2006; Soliman et al. 2010) together with our findings, it remains to be seen whether estrogen in the amygdala could interfere with the formation of extinction-induced perisomatic inhibitory synapses via BDNF signaling. Although we did not see significant differences between males and females in our research presented here, a more extensive analytical comparison between the two sexes may be necessary to determine if a sex-specific effect of extinction on inhibitory perisomatic synapses exists, and whether the difference is due to estrogen-BDNF signaling in the BA.

*Advantage of the TetTag system*

The combination of the TetTag mouse with a retrograde tracer, as employed in the studies described here, provides a versatile method for studying the functional properties of anatomically defined projection neurons. Previous studies have combined retrograde tracers with the imaging of endogenous immediate-early gene expression. Though these studies have provided important functional insights into specific components of the fear circuit (Majak & Pitkanen 2003; Orsini et al. 2011; Senn et al. 2014), they were limited to determining functional properties at a single time-point, for example during either conditioning or retrieval only. In contrast, the TetTag mouse enables the assessment of
functional properties at two time-points, and can therefore be used to identify neurons that are active during both conditioning and retrieval (Reijmers et al., 2007). Here we demonstrate the advantage of this feature by identifying two pathways that are both activated by contextual fear conditioning (BA-dBNST, BA-non-dBNST), with only one of these pathways being reactivated during retrieval. Combining the TetTag mouse with tracer injections therefore provides a unique tool for the identification of defined anatomical connections that are stable components of a memory engram.

The TetTag optical toolbox can further be expanded to investigate activity at the synaptic level of projection neurons, or to selectively drive or inhibit circuits in a function-specific manner. TetO-regulated viruses combined with the TetTag mouse would present an elegant method of selectively labeling circuits with spatial, temporal, and functional control. Viral expression of a fluorescently-tagged synaptic protein, such as synaptophysin, under the TetO promoter will allow the visualization of synapses originating from activated cells in the injection site. This can be used to identify synaptic changes, either in size or number, in a specified neural circuit, conferring a greater understanding of the synaptic plasticity that occurs downstream of the cellular activation.

Prior to our work with the CTB tracer, we attempted to use this method to explore the activation of the BA-BNST pathway. However, we found the leakiness of the promoter to be problematic, leading to difficulties in limiting expression to activated neurons in the BA. Despite this technical challenge, we believe the general method would be straightforward to implement and provide a great amount of information on distinct functional circuits.
**Conclusion**

Research over the past century has laid the foundation for understanding the generation of fear in health and disease, but the recent development of new methods and tools for dissecting the neural circuit has revealed a deeper and more intricate view of the mechanisms that underlie fear learning and memory. The work we present here contributes to the framework of the fear circuit by describing a novel BA-BNST pathway that may support contextual fear learning through activation during acquisition and/or consolidation of fear. Knowledge of this pathway sheds light on the function of the BNST in contextual fear conditioning and provides an additional point of potential modulation in the fear circuit. Further experiments are necessary to determine the extent of the contribution of this pathway to the acquisition and/or consolidation of the contextual fear memory, and whether its potential dysregulation could lead to further fear-related pathologies. We also provide evidence of an extinction-induced inhibition of the fear circuit through modulation of perisomatic synapses in the basal amygdala. Our model describes a straightforward mechanism through which the extinction circuit can directly interact with and suppress the fear circuit, leading to the decrease in fear expression after extinction. Furthermore, our research shows that the methods described here can be more widely applied to dissect functional neural circuitry and cellular activation in a non-invasive and physiologically-relevant manner. Together, these findings have important implications for the treatment of fear disorders by providing potential targets in the brain for future pharmaceutical and behavioral therapies, as well as novel techniques for the use of scientific investigation into the realm of learning and memory.
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