Academic Year 2017-2018

Faculty Pharmaceutical, Biomedical and Veterinary Sciences

Biomedical Sciences

Investigating the role of the bed nucleus of the stria terminalis in cued fear

By:

Eline Princen

Master Thesis in partial fulfillment of the requirements for the degree

Master in Biomedical Sciences

Promoter: Dr. Laura Luyten

Co-promoter: Dr. Debby Van Dam

Supervisor: Dr. Kelly Luyck

Research Group Experimental Neurosurgery and Neuroanatomy

Campus Gasthuisberg

Onderwijs & Navorsing 1 (O&N I)

Herestraat 49 - box 1036

3000 Leuven

Acknowledgements

I would like to thank my supervisor Dr. Kelly Luyck, for her guidance during my master's thesis and for supporting me on every step along the way. She taught me how to perform all kinds of practical techniques and helped me in the writing process of this thesis. I especially want to thank you, Kelly, for your devotion towards my master's thesis, ensuring it to be of high quality.

I also want to thank my promoter Dr. Laura Luyten for giving me the opportunity to carry out my master's thesis in the lab of Experimental Neurochirurgy and Neuroanatomy. For my thesis, I wanted to work on a subject in the field of neuropsychology, and I am thankful for the chance to work on a related topic. Dr. Luyten was ever so kind and patient to bring me understanding on the subject, and to help me with all my questions.

Furthermore, I would like to thank my co-promotor Dr. Debby Van Dam to represent the UAntwerp counterpart of this thesis. Your pleasant and interesting classes have contributed to my passion for (behavioural) neuroscience, making this thesis a pleasant and not a laborious work.

I also want to thank Prof. Bart Nuttin, for occasionally taking the time to look at the progression of my thesis, and preparing me for my defence by asking me sharp questions.

In addition, I would like to thank Dr. Elisa Maes and Prof. Stuart Maudsley for taking the time to evaluate my thesis, and to be members of the jury.

Finally, many thanks to my parents (thank you mom, for listening to all my frustrations), my boyfriend, and the rest of my family for showing interest in my thesis – especially since I am sure at times they had no idea what I was talking about – and for standing by me throughout this period.

Samenvatting

Angststoornissen treffen tot 20% van de volwassen bevolking. Ze worden gekenmerkt door buitensporige vormen van angst- en vreesreacties, die onder normale omstandigheden als adaptief worden beschouwd, en noodzakelijk zijn in het dagelijks leven. Wanneer deze buitensporige reacties het dagelijks functioneren en welzijn grondig verstoren (bv. weigeren om het huis te verlaten), kan een angststoornis vastgesteld worden. Angststoornissen vormen een spectrum tussen pathologische vrees- en/of angstreacties. Vrees kan beschreven worden als een kortstondige, stimulus-gebonden reactie die snel weer verdwijnt eens de dreiging weggehaald wordt (zoals in arachnofobie). Angst is echter een meer langgerekte, aanhoudende respons die veroorzaakt wordt door diffuse stimuli, en kan er bijvoorbeeld voor zorgen dat men voortdurend zit te piekeren of zich buitensporig zorgen maakt over van alles (zoals in gegeneraliseerde angststoornis).

Zowel proefdier- als mensonderzoek heeft aangetoond dat de neuronale netwerken die angst en vrees moduleren voor het grootste deel overlappen. Volgens de algemene consensus speelt de bed nucleus van de stria terminalis (BST) een belangrijke rol in aanhoudende angst, maar niet in kortstondige vrees, die op zijn beurt hoofdzakelijk geregeld wordt door de (centrale) amygdala.

In deze masterthesis streven we ernaar de mogelijke rol van de BST in vreesreacties te evalueren o.b.v. enkele intrigerende resultaten eerder behaald door onze onderzoeksgroep. In een vorige studie met ratten werd aangetoond dat post-training elektrolytische BST-letsels de door vrees versterkte opschrikreflex (mate van opschrikken als gevolg van een luide knal) na een geconditioneerde toon, op significante wijze verminderden. Dit staat in contrast met de algemene visie op de rol van de BST in langdurige angst, maar niet in kortstondige vreesreacties. Wanneer we ons echter baseren op de hoge contextuele *freezing* (complete immobiliteit bij een dreiging) niveaus, veronderstellen we dat het protocol misschien te aversief was. Dit kan geleid hebben tot hoge algemene stressniveaus, die de significantie van de geconditioneerde toon tot op zekere hoogte ondermijnd kunnen hebben. Gezien de gekende rol van de BST in stressreacties, kunnen we niet uitsluiten dat BST-letsels geïnterfereerd kunnen hebben met algemene stress, eerder dan (rechtstreeks) de door vrees versterkte opschrikreflex te hebben beïnvloed.

In deze thesis trachtten we het effect van BST-letsels in vrees opnieuw te evalueren, deze keer d.m.v. een conditioneringsprotocol dat lagere algemene stressniveaus uitlokt. In de eerste twee experimenten doelden we erop het conditioneringsprotocol te optimaliseren en minder aversief te maken. Zo werden de luide knallen (zogenaamde *startles*) van het protocol verwijderd, aangezien ze van nature vrij aversief kunnen zijn. Hiernaast hebben we o.a. het aantal schokken en hun sterkte tijdens de trainingssessie aangepast. Nadat we een protocol verkregen dat zuivere, significante vrees opriep t.o.v. een geconditioneerde toon in naïeve dieren, gingen we verder met een letselexperiment. In dit experiment doorliepen de dieren het geoptimaliseerde protocol dat we bekwamen in Experiment 2, en kregen ze 27 h na de trainingssessie een BST of *sham* (nagebootste chirurgische interventie) letsel. In contrast met onze eerder gepubliceerde bevindingen, tonen onze resultaten aan dat BST-letsels de expressie van vrees t.o.v. de toon (verhoogde *freezing*) niet aantasten.

Deze resultaten liggen in lijn met de algemene visie omtrent de rol van de BST in angst, maar niet in kortstondige vrees. Onze data suggereren dat de eerder behaalde resultaten in ons lab beïnvloed waren door hoge niveaus van algemene stress, en ze veronderstellen een rol van de BST in stressreacties. Tenslotte benadrukt deze studie het belang van uitgebreide validatie van het gedragsprotocol, aangezien subtiele veranderingen in het paradigma de gedragsresultaten en de daaraan verbonden neurowetenschappelijke conclusies grondig kunnen beïnvloeden.

Abstract

Anxiety disorders affect up to 20% of the adult population, and are hallmarked by excessive forms of fear and anxiety, which are normally adaptive responses necessary for survival. When these exaggerated responses impair normal daily functioning (e.g. refusal to leave the house), an anxiety disorder might be diagnosed. Fear can be described as a phasic, stimulus-bound response which disappears rapidly once the threat is removed, e.g. as seen in arachnophobia. Anxiety however is a more lingering, sustained response that is evoked by diffuse stimuli, for example causing people to constantly worry and agonize about everything (e.g. as seen in generalized anxiety disorder).

Both studies in rodents and human participants have shown that the neuronal networks underlying fear and anxiety responses overlap for the greater part. The general consensus dictates that the bed nucleus of the stria terminalis (BST) plays an important role in sustained anxiety responses, but not in phasic fear, which is mainly regulated by the (central) amygdala.

In this master's thesis, we aim to evaluate the potential involvement of the BST in phasic fear responses, based on some intriguing results previously obtained by our research group. In this rat study, electrolytic post-training lesions of the BST were shown to significantly impair fear-potentiated startle toward a conditioning tone, in contrast with the general accepted view on the role of the BST in anxiety, but not in fear. However, based on high contextual freezing levels in this experiment, we hypothesised that the protocol might have been to aversive, leading to increased general stress, which may have overshadowed the significance of the conditioned tone to some extent. Given the predominant role of the BST in stress responses, we therefore hypothesised that BST lesions may have interfered with general stress rather than cued fear.

In this thesis, we aim to address this concern by adapting the conditioning protocol to minimalize general stress levels and to achieve optimal cued fear expression in naïve rats. In the first two experiments, we aimed to reduce the aversiveness of the conditioning protocol by adapting several parameters. For example, we removed the acoustic startle probes from the protocol, since they have been shown to be rather aversive by nature. In addition, we reduced the number of foot shocks presented during the training session. As the adaptations in the first experiment did not sufficiently reduce general stress levels, we continued to adjust parameters (e.g. by further reducing the number and strength of unconditioned stimuli presented during the training session) in the second experiment. After obtaining a protocol that elicited significant cued fear (and low general stress) in naïve animals, we proceeded with a lesion experiment. The results of this third experiment indicated that BST lesions do not affect the expression of cued fear, since both animals with sham and BST lesions displayed significant cued fear, as indexed by freezing measurements during tone vs. context presentations.

These results are in line with the majority of human and rodent research data and with the general accepted view on fear and anxiety neurocircuitry. Our data suggest that the previously obtained results in our lab might have been confounded by high levels of general stress, and imply a role of the BST in these stress responses. Finally, this study highlights the importance of extensive behavioural protocol validation, as subtle changes to the paradigm might severely influence behavioural outcome.

List of abbreviations

5-HT	5-hydroxytryptamine (serotonin)
AL	Anterolateral
ALIC	Anterior limbs of the internal capsule
AM	Anteromedial
ANOVA	Analysis of variance
AV	Anteroventral
BLA	Basolateral amygdala
BST	Bed nucleus of the stria terminalis
CBT	Cognitive-behavioural therapy
CeA	Central amygdala
CRF	Corticotropin-releasing factor
CS	Conditioned stimulus
DBS	Deep brain stimulation
DSM	Diagnostic and Statistical Manual of Mental Disorders
EPM	Elevated plus maze
fMRI	Functional magnetic resonance imaging
GABA	Gamma-aminobutyric acid
GAD	Generalised anxiety disorder
HPA	Hypothalamic-pituitary-adrenal
IQR	Interquartile range
ITI	Intertrial interval
MeA	Medial amygdala
MRN	Median raphe nucleus
MWU	Mann-Whitney U
OCD	Obsessive-compulsive disorder
OFT	Open field test
OTR	Oxytocin receptor
PET	Positron Emission Tomography
PTSD	Post-traumatic stress disorder
SD	Standard deviation
SEM	Standard error of the mean
SNRI	Selective serotonin norepinephrine reuptake inhibitor
SSRI	Selective serotonin reuptake inhibitor
US	Unconditioned stimulus
VMH	Ventromedial hypothalamic nucleus

Table of contents

I. Introduction

1. Fear and anxiety: What is the difference?

Every individual will experience fear or anxiety at some point in life. Both are adaptive responses that used to be, and still are, important to survive. An instinctive fear of, for example, fire, heights, deep water and potentially poisonous animals (e.g. spiders and snakes) have undoubtedly saved many lives. A dysregulation of these functions can however lead to anxiety disorders, which affect up to 1 out of 5 adults and are amongst the most common mental disorders [1].

But at what point are fear and anxiety considered to be pathological? When fear or anxiety get out of hand, they can lead to excessive responses; like avoiding dusty and dark places altogether out of fear of seeing a spider, or not daring to come close to a river because of the fear of falling in and drowning. When these exaggerated responses impair normal daily functioning (e.g. refusal to leave the house), an anxiety disorder might be diagnosed.

Anxiety disorders comprise multiple conditions which are hallmarked by pathological fear and/or anxiety responses. Although fear and anxiety result in similar behaviour, they have some different key features. According to the early work of Davis and Walker [2], fear is defined as a stimulus-bound reaction to an imminent threat, often referred to as 'phasic fear'. As soon as the stimulus is removed, fear will decline. Anxiety on the other hand is characterized by a long-lasting state of fear caused by more diffuse stimuli, and is sometimes referred to as 'sustained fear'. Note that according to this view, the distinction between fear and anxiety is predominantly determined by the duration of the response, i.e. phasic and sustained, respectively.

As mentioned before, both responses can become pathological when the well-being of the individual is significantly impaired. The Diagnostic and Statistical Manual of Mental Disorders (DSM) (5th ed.; DSM–5; American Psychiatric Association, 2013) [3] defines anxiety disorders as "disorders that share features of excessive fear and anxiety and related behavioural disturbances". According to the DSM, fear is described as the emotional response to an imminent threat (= phasic response), whereas anxiety is considered the chronic anticipation of future threats (= sustained response). These might result in flushes of autonomic arousal, thoughts of immediate danger, escape behaviours, muscle tension, hypervigilance (i.e. increased alertness/awareness for potential danger) and avoidance of possibly dangerous situations. A classic example of pathological fear entails arachnophobia, i.e. fear of spiders, whereas the anxiety end of the spectrum is represented by generalized anxiety disorder (GAD), an illness which is hallmarked by continuous worrying to the point that the individual's well-being is dramatically impaired [1].

While the theoretical distinction between fear and anxiety has led to some pivotal insights into the neurobiology of both responses (see *I.3 The extended amygdala: neuroanatomical basis of fear and anxiety*) [2], we note that clinical diagnosis is often far more complex than this seminal distinction. For instance, arachnophobia is a stimulus-bound condition, suggesting that, as soon as the threat is removed, in this case the spider, the fear response should disappear rapidly. However, these patients often show hypervigilance; they will scan the environment for spiders and show heightened awareness, and in extreme cases they will avoid risky environments altogether. These behaviours, hypervigilance and avoidance, are two important features of anxiety, showing that in practice, the distinction between fear and anxiety is rarely as clear-cut as in theory.

2. Rodent models of fear and anxiety

While human (imaging) research has recently made some major contributions to the field (see *I.5.2* and *I.6.2*), most knowledge on fear and anxiety has been derived from animal research. Intuitively, animal models allow for more invasive techniques, which is indispensable toward gaining more fundamental insights on brain functioning.

One of the most commonly used approaches to model fear and anxiety responses, both in rodents and humans, is through the use of conditioning paradigms. Conditioning is a form of associative learning, where a previously neutral stimulus gains significance through pairing with an unconditioned stimulus. The most well-known example of classic conditioning is Pavlov's dog. Here, a neutral stimulus (sound of a bell) which does not elicit a response in itself, is paired with food presentation (= unconditioned stimulus or US), which in itself results in salivation (= unconditioned response). Initially, only presentation of the food, but not the bell, will elicit a response. However, after presenting the dog with food and the bell at the same time, the bell will gain significance (= conditioned stimulus or CS) and will cause the dog to salivate even when no food is presented (= conditioned response).

Different conditioning paradigms have been used to investigate how fear and anxiety are wired in the brain. Cued fear conditioning – analogous to the example of Pavlov's dog, but with an aversive unconditioned stimulus, e.g. shock – models stimulus-bound, phasic fear responses and is therefore considered a relevant model for specific phobias. To model anxiety, which is a more long-lasting response, a different approach is required. To this end, context conditioning has been put forward, where unsignalled shocks are presented in a certain context, without the presence of a temporal predictor (such as a tone). Upon re-exposure to the context, the animals will be in chronic state of anxiety, as they continuously anticipate the presentation of a foot shock. Since this response is long-lasting and not bound to a specific stimulus (in contrast to cued fear conditioning), context conditioning is considered a valuable model to study anxiety [4, 5].

To quantify fear and anxiety responses in these models, two measurements are commonly used: freezing and startle. The startle reflex is the full-body response to a loud sound burst and is elevated when the subject is in a state of fear or anxiety. The startle response in rodents has many similarities with the eyeblink component of startle reflex in humans, which has face validity for studying fear states and anxiety disorders [2]. A second measurement is freezing, which is the complete absence of movement, except the movement necessary for breathing. This behaviour is typical for rodents after they detect potential danger (e.g. freezing to avoid being noticed by a predator).

In addition to conditioning protocols, other paradigms may be used to model innate (or unconditioned) fear and anxiety responses. For example, the elevated plus maze (EPM) and the open field test (OFT) are two of the most commonly used unconditioned fear/anxiety paradigms [6, 7]. The EPM triggers an innate fear of open space and heights, since it consists of a heightened platform with two open and two closed arms. Time spent in the closed versus the open arms forms an indication of the amount of innate fear/anxiety. The OFT on the other hand is a simple box without roof, which elicits an innate aversion of open spaces and bright light. Time spent near the edges/corners (also referred to as thigmotaxis) has been proposed as a measure of anxious behaviour. In addition, a simple bright light could also serve as an anxiogenic stimulus for nocturnal species such as rats, in any context or experimental set-up [8]. However, no consensus is reached yet on whether these above-mentioned innate responses model the fear or anxiety end of the spectrum [9]. Another downfall of these unconditioned paradigms is that they are less

reproducible over time and do not require any associative learning, which plays a major role in anxiety disorders (i.e. etiological validity) [10].

3. The extended amygdala: neuroanatomical basis of fear and anxiety

Multiple studies, using mostly conditioning paradigms, have suggested that neural pathways underlying fear and anxiety are largely overlapping, but nonetheless show some striking differences. Research on how fear and anxiety are wired in the brain has revealed an important role for the 'extended amygdala', a brain region which includes the central (CeA) and medial part (MeA) of the amygdala, and the bed nucleus of the stria terminalis (BST) [2]. The BST is a limbic forebrain structure which envelops the posterior part of the anterior commissure, and is strongly connected to the amygdala via columns of cells called the stria terminalis.

The amygdala and the BST are very similar structures in terms of input, output, neurotransmitters and cell types [11, 12]. In fact, it has been suggested that they were originally one and the same structure, but were segregated throughout evolution [13], which makes it even more intriguing that differences in functionality between these regions are noted.

Multiple lesion and inactivation studies have demonstrated the role of the CeA particularly in phasic fear, and that of the BST in sustained anxiety [2]. In an early study by Davis & Walker [8], sustained anxiety was evoked by an unconditioned bright light, which is considered aversive to rats, since they are nocturnal animals. In the same study, phasic fear was assessed using a cue that was previously paired with foot shocks. In both conditions, the acoustic startle response was measured. Intriguingly, BST inhibition reduced anxiety evoked by exposure to a bright light, while phasic fear was not affected. Reversely, CeA inhibition reduced cued fear expression, but did not affect light-enhanced startle.

Based on these and other data (see *I.5 Evidence supporting the early model of the extended amygdala*), Davis and colleagues developed a neurobehavioral model to account for the differential involvement of the CeA and the BST in fear and anxiety responses (Fig. 1) [2]. This early description of the extended amygdala by Davis and Walker is widely accepted and is considered as one of the most influential models in the field.

In short, sensory information related to fear stimuli (e.g. olfactory, visual and tactile stimuli) enters the basolateral amygdala (BLA) complex. The BLA projects to both the medial and lateral division of the CeA. The medial CeA mediates phasic fear responses by rapidly activating the periaqueductal grey, which causes freezing, and the caudal pontine reticular nucleus, which mediates the startle response. In addition, the hypothalamic-pituitary-adrenal (HPA) axis is activated, resulting in the release of stress hormones, increased blood pressure and tachycardia. In contrast to the rapid fear

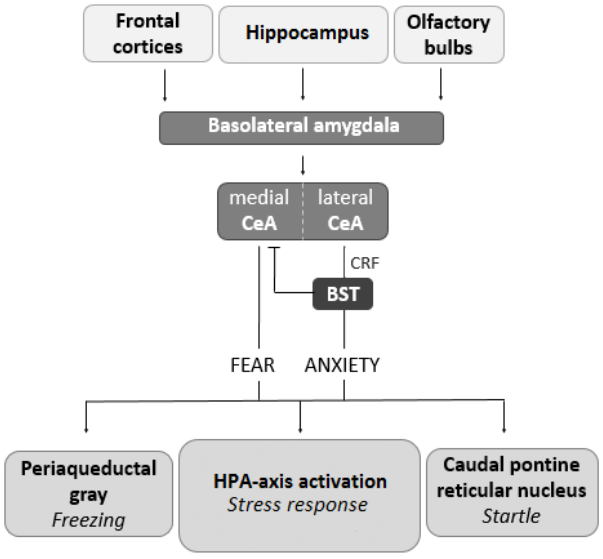


Figure 1: Neuroanatomic connections that presumably form the basis for fear and anxiety responses. CeA = central amygdala, BST = bed nucleus of the stria terminalis, CRF = corticotropin releasing factor, HPA-axis = hypothalamic-pituitary-adrenal axis. Adapted from Walker et al. (2002).

response evoked through the medial CeA, the lateral CeA will gradually activate the BST through release of corticotropin-releasing factor (CRF), which causes a sustained anxiety reaction. The BST itself can mediate anxiety responses by evoking stress responses, and anxiogenic effects in general (e.g. increased freezing and startle), through CRF release [14, 15]. Both BLA and BST projections to the ventromedial hypothalamic nucleus (VMH) mediate defensive and aggressive behaviours [16]. Transition from phasic fear to sustained anxiety may be mediated by inhibition of the medial CeA by the BST, since inhibition of the BST (through chemical or electrolytic lesions) lowers sustained anxiety [17, 18] but increases phasic fear [19].

4. BST anatomy and functionality

Within the small region that is the BST, about 12 anatomically different nuclei can be distinguished – although their exact number and location are still under discussion [16, 20]. Early research on fear conditioning focused primarily on the anterior part of the BST [21], since this region receives dense projections from the CeA, a key mediator of fear [22]. As the posterior BST is mainly involved in social functions, such as defensive and reproductive behaviour [23-25], we will only discuss the anterior subdivisions of the BST, which play a key role in anxiety [2]. According to Paxinos et al. [26], the anterior BST can be divided in three main subregions: the anteroventral (AV) BST, located ventral to the anterior commissure, and the anteromedial (AM) and anterolateral (AL) BST (Fig. 2), both dorsal to the anterior commissure [16].

Even though most BST neurons are GABAergic [27], the BST displays a multitude of neurotransmitters and -peptides that contribute to its functioning [28]. For example, serotonin is known to play a crucial role in BST signalling. Activation of 5-$HT_{(1A)}$ receptors causes inhibitory responses in the BST and reduces anxiety-like behaviour, while activation of 5-$HT_{(2A)}$, 5-$HT_{(2C)}$ and 5-$HT_{(7)}$ receptors lead to opposite responses [29, 30]. Of all serotonin receptor types, the 5-$HT_{(1A)}$ receptors are most frequently found in the BST [31], which explains why selective serotonin reuptake inhibitors (SSRI's) are often employed as anxiolytic drugs [32] (see *I.5.3 BST as a target for anxiety disorders*). In addition to serotonin, CRF neurons can be found both in the oval nucleus, a portion of the AL BST, and in the fusiform nucleus, a part of the AV BST (Fig. 2) [33-36].

Intriguingly, different subdivisions of the BST show distinct neurochemical profiles [28] and projections [37], sometimes leading to opposing functionalities [38, 39]. For example, the oval nucleus causes anxiogenic effects when activated by the CeA during stress, while the rest of the AL BST seems to be innervated by the BLA for the larger part, and predominantly has anxiolytic effects [16].

In summary, we state that the BST is a highly complex structure which displays asymmetry between its different subdivisions, in terms of (1) neurochemical content, (2) internal projections and (3) connections with other parts of the anxiety network.

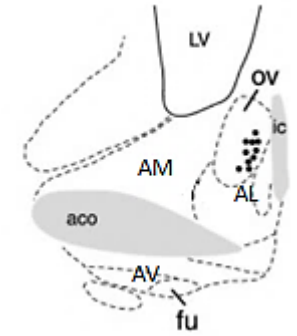


Figure 2: Structure of the BST. AL = anterolateral, AV = anteroventral, AM = anteromedial, LV = lateral ventricle, aco = anterior commissure; olfactory limb, ov = oval ventricle, ic = internal capsule, fu = fusiform ventricle. Adapted from Dong et al. (2001).

5. Evidence supporting the early model of the extended amygdala

After establishing that fear and anxiety are mediated by different neuroanatomical substrates, as originally described by the research group of Davis and colleagues [40], further efforts were made towards investigating the relative contributions of those structures to fear and anxiety. In what follows, some of the key findings which support the model of Davis et al. in both rodents and human participants are listed, followed by a brief overview of potential therapies for anxiety disorders in the light of this model.

5.1 Rodent studies

Using micro-Positron Emission Tomography (micro-PET) imaging, our lab previously investigated the neurocircuitry in awake rats that underwent context or cue conditioning, as models for anxiety and fear, respectively. Hypermetabolism was found in a cluster comprising the BST in rats expressing contextual anxiety, in comparison with both control rats and animals expressing cued fear. This provides additional evidence for the role of the BST in contextual anxiety, but not in cued fear [41]. In addition, we found that lesions of the BST significantly reduced contextual anxiety, as indexed by freezing and startle [17]. In line with our findings and the model of Davis et al., other research groups demonstrated that lesions of the BST significantly disrupted contextual anxiety, but not cued fear responses [18, 42, 43].

For instance, Sullivan et al. (2004) obtained similar results in a study where BST lesions reduced behavioural (freezing) and neuroendocrine (corticosterone) responses to a context conditioned; but not a cue-conditioned fear stimulus in rats, suggesting a role for the BST in contextual, but not cued fear. Lesions in the CeA, on the other hand, reduced anxiety responses to both a conditioned context and cue [18]. In addition, Zimmerman et al. (2011) showed that BST lesions or BST inactivation disrupt freezing to a conditioned context but not to a conditioned tone [42], again underlining the role of the BST in context conditioning but not in cue conditioning. Earlier, this research group also showed that the amygdala, more specifically the CeA, plays a role in the acquisition and expression of fear responses to both contextual and cue-conditioned stimuli [43].

5.2 Human imaging studies

The differential involvement of amygdala and the BST in fear and anxiety was also confirmed by multiple human fear conditioning experiments. In a study by Alvarez et al. (2011), participants were exposed to three different rooms using a computer paradigm. In one room, the participants received electric shocks preceded by a cue (= cued fear), while unsignalled shocks were given in a different room (= contextual anxiety). In a third condition, no shocks were given at all. Whole-brain functional magnetic resonance imaging (fMRI) showed sustained activity in the BST complex during anxiety (but not fear), while cued fear was associated with increased activity in the dorsal amygdala [44].

Another fMRI study found increased BST activation in arachnophobics versus healthy participants during anticipation of a phobia-relevant stimulus (i.e. picture of a spider) [45]. This suggests that the BST is involved in the anticipation of an aversive event and the accompanying state of hypervigilance. A more recent fMRI study revealed that phobic patients show stronger amygdala activation during phasic fear and higher BST activation during sustained fear in comparison with controls, which underlines the temporal activation pattern of the amygdala (phasic) and BST (sustained) [46]. The latter studies are particularly relevant as they both demonstrate BST activity during *pathological* anxiety, rather than *adaptive* anxiety as a result of (context) conditioning.

In general, these human fear conditioning studies indicate a role of BST activity in sustained anxiety and hypervigilance, but not in phasic fear responses.

5.3 BST as a target for anxiety disorders

The pivotal role of the BST in anxiety suggests its potential relevance as a therapeutic target for anxiety disorders. In this section, we provide a short summary on different therapies that have been used in patients suffering from anxiety disorders, some of them targeting the BST specifically.

Common therapies for anxiety disorders consist of cognitive-behavioural therapy (CBT) – a form of psychotherapy – and pharmacological treatment. SSRI's and benzodiazepines [47] are often used, which is interesting given the importance of serotonin and GABA transmission in the BST (see *I.4. BST anatomy and functionality*). However, current guidelines do not recommend benzodiazepines as first-line treatment anymore, because of potential side effects. SSRI's and selective serotonin norepinephrine reuptake inhibitors (SNRI's) are more routinely used nowadays [48, 49]. The combination of both CBT and pharmacological treatment can be particularly effective [50-52]. Unfortunately, more than 33% of patients with anxiety disorders do not respond sufficiently to pharmacological treatments [53].

For these severely affected, treatment-resistant patients, therapeutic brain lesions can serve as a last-resort treatment option [54-57]. More recently, deep brain stimulation (DBS) emerged as a valuable alternative. In contrast to therapeutic lesioning, high-frequency electrical stimulation through electrodes is adaptable: parameters can be altered until satisfactory therapeutic effects are obtained. In addition, permanent lesions could lead to unwanted side-effects, while effects of DBS are completely reversible (stimulation can be switched off at all times) [58].

Lesioning targets often became the new DBS targets. Patients suffering from severe, treatment-resistant obsessive-compulsive disorder (OCD) for example, can be treated with high-frequency stimulation in the anterior limbs of the internal capsule (ALIC) [59] and in the BST [60]. Stimulation in both regions has shown to reduce obsessions, compulsions, and associated anxiety and depressive symptoms. Since more patients benefited from stimulation in the BST, the BST might even form a better stimulation target compared with ALIC for alleviation of OCD symptoms [60, 61].

While DBS reduced dysfunctional and pathological fear, it did not cause patients to take unnecessary risks (e.g. jumping in front of a car), implying that functional, innate fear was still intact in treated patients. Mood and anxiety symptoms usually improved first, before a decrease in obsessions and compulsions was noted. This suggests that an anxiolytic effect in the BST could be responsible for the attenuation of OCD symptoms (i.e. obsessions, rituals). Interestingly, stimulation in the BST region was particularly effective in patients with OCD subtypes in which anxiety is more prominent [62]. In addition, our lab recently showed that electrical BST stimulation significantly reduced contextual freezing in a rat model of anxiety, which was unrelated to obsessions and compulsions [63]. Combined, these findings suggest that DBS in the BST could provide a safe, last-resort treatment option for severely affected anxiety patients.

6. Beyond the model of the extended amygdala

Even though the evidence described above is quite convincing, some recent findings do not completely fit in the model of the extended amygdala as described by Davis et al. (Fig. 1). While their model is still valuable and has provided a highly influential framework for research in this

field, it might need some extensions, since the functional dissociation between the BST and the CeA does not seem as strict as was thought before.

6.1 Rodent studies

Meloni et al. (2006) demonstrated the role of the BST in the expression of cued fear [19], by chemically inactivating the BST in rats with muscimol. BST inactivation led to increased fear-potentiated startle in response to a conditioned light that was previously coupled with a shock. In addition, Haufler et al. showed that about 25% of anterior BST neurons are responsive during presentation of a conditioned tone [64]. Another recording study implemented conditioned tones with variable duration to represent phasic and sustained fear components. Here, the phasic component was found to coincide with repression of two categories of anterolateral BST neurons [65], suggesting that the BST might be involved in cued fear responses. In addition, SSRI infusion in the BST, but not in the CeA, increased freezing to a conditioned tone [66]. This and other evidence in the literature indicates that the BST might be able to modulate the processing of discrete threatening cues after all [16].

As stated before, the model of Davis et al. suggests immediate CeA activation upon threat encounter, while BST activation requires a slower, gradual response (i.e. through CRF transmission). In contrast with this assumption, Hammack et al. (2015) demonstrated that the BST responds immediately to threats. In this study, rats underwent pre-training BST lesions. These lesions caused the rats to freeze less than sham animals in a conditioned aversive context, which is in line with the model of Davis et al. Intriguingly however, the difference in freezing between the two groups was constant over time. This suggests that BST activity in a conditioned aversive context does not change over time, in contrast to the early model stating that BST activity is delayed relative to medial CeA activation, which may thus not necessarily be the case [16, 67].

A study by Duvarci et al. (2009) also showed that BST activity affects the processing of short cues in rats with pre-training BST lesions. The rats were conditioned to both a 30-second auditory stimulus that was paired with a foot shock (CS+) and another one that was not (CS-). Rats with BST lesions acquired similar levels of freezing to the CS+ as sham animals, however, lesion animals froze less to the CS- [68].

Finally, a recent study by Moaddab et al. (2017) revealed BST involvement in the acquisition, but not consolidation, of conditioned fear to a distinct cue in rats. When injecting an oxytocin receptor (OTR) antagonist in the OTR rich centre of the BST, its dorsolateral part, formation of cued fear was impaired [69].

6.2 Human studies

In addition to the animal research described above, similar findings challenging the early model of the extended amygdala were established in human imaging studies. An imaging study by Mobbs et al. (2010) revealed BST-activation during the presentation of a short-lasting threat, a 4-second video of a tarantula shown to non-phobic participants [70]. Another imaging study demonstrated phasic BST responses during short-lasting threats as well [71]. Here, a short cue (0.75 s) predicted a foot shock that followed shortly after (within 0.75 s-5.75 s). During this short anticipation period, BST activity was heightened in comparison with the period after a safety cue that indicated that no shocks were following. Yet another fMRI study, by Grupe et al. (2013), revealed BST activity during a 2 to 8-second anticipatory period between a cue predicting an aversive event, and the aversive event itself [72]. Consistently, Klumpers et al. (2015) observed that BST activity was associated with the anticipation of threat, during the 8 to 12-second interval (11 to 13-second

interval in a second experiment) between the cue related to the threat (4 s in a first experiment, 6 to 12 s in a second experiment) and shock presentation [73].

To conclude, it seems that the role of the BST extends further than mediating aversive responses to diffuse threatening stimuli, and can also modulate responses to discrete threats. Concurrently, the central part of the amygdala might also play a role in regulating sustained anxiety. We refer the reader to excellent reviews by Gungor and Paré [16] and Shackman and Fox [13]; which discuss animal and human data, respectively, that are not in line with the early views of CeA and BST segregation. Although the early model of the extended amygdala should not be disregarded, the functional segregation between the BST and the CeA is not as black and white as thought before. Instead, it is likely that both regions work together to modulate phasic fear and sustained anxiety responses.

7. Electrolytic post-training BST lesions reduce cued fear

In line with the controversy regarding the model of Davis et al., our lab recently showed that the BST might play a role in the expression of cued fear [74].

The study by Luyck et al. (2018) entails three separate experiments that set out to evaluate the effect of BST lesions on cued fear expression (Fig. 3). On day 1, all animals underwent a habituation phase, during which they were presented with 30 acoustic startle stimuli in an experimental cage, referred to as the startle box. The next day, during the pre-test, animals were presented with 15 startle probes that were preceded by a 10-s tone (cued fear measurement) and 15 single startles probes (contextual anxiety measurement). The day after, the animals were conditioned to the tone by administration of foot shocks at the end of each tone. Following this training session, BST lesions were made. Finally, a post-test (identical to pre-test) was conducted, during which fear was quantified with respect to pre-test. In all experiments, freezing and startle measurements were combined as behavioural outcomes of fear and anxiety. In the first experiment, training and testing were conducted in the same context, and lesions were made 3 h after training. Surprisingly, the lesion group showed no significant cued fear expression during post-test. In the second experiment, training was conducted in a different context, to exclude contextual influences on the cued fear response, which may have confounded the findings within the first experiment. Despite this context modification, lesioned animals still did not display significant startle or freezing responses to the tone, in contrast to non-lesioned animals. In the third experiment, BST

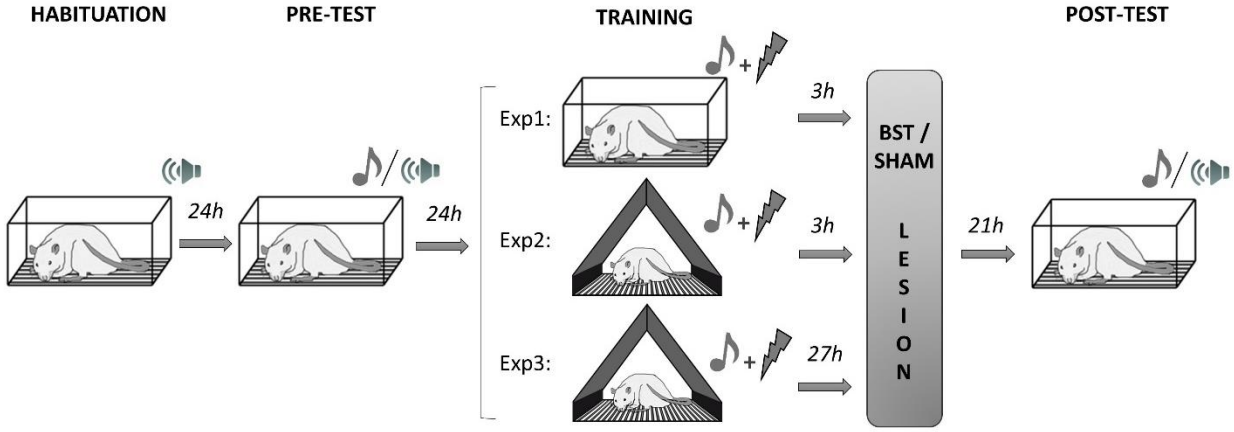


Figure 3: Experimental design of all conditioning protocols used in the study of Luyck et al. (2018). Exp = experiment.

lesions were applied 27 hours instead of 3 hours after the training session, to avoid potential influencing of the consolidation of the fear memory. Nonetheless, impairment of fear-potentiated startle was observed in all experiments [74].

These data suggest that BST lesions disrupt cued fear responses, which contradicts the findings described in *I.3 The extended amygdala: neuroanatomical basis of fear and anxiety*.

8. Aim of the study

In the current study, we further evaluated the results obtained by Luyck et al. [74], which were discussed above (*I.7 Electrolytic post-training BST lesions reduce cued fear*). The authors showed that electrolytic BST lesions significantly disrupted fear-potentiated startle in a cued fear procedure, which challenges early views on BST involvement in fear and anxiety responses [2].

We hypothesised that these surprising results could be attributed to elevated levels of general stress, that are not directly linked to the conditioned tone. This assumption was mainly based on the unexpectedly high freezing levels that were observed when the tone was not presented (=contextual freezing). Since the BST plays a key role in stress responses [28], the results of Luyck et al. might reflect a reduction of overall stress and contextual fear levels, rather than a direct impairment of cued fear. If the protocols used in these studies were too aversive for the animals (e.g. too many/strong foot shocks), this may have overshadowed the predictive value of the tone. In this sense, the animals would constantly be stressed or anxious, which might explain why BST lesions affected fear-potentiated startle.

Another explanation could be that a fully functioning BST is required for generalisation of cued fear memories, meaning that a fear-eliciting cue trained in one context should still be recognised as threatening in a different context. For instance, if you were bitten by a dog on the street, you should know to avoid that particular dog when you see it in the park. If the BST is required for generalising fear memories from one context to another, this may have confounded the results of Luyck et al.

The overall goal of the current study was to address the first hypothesis and to evaluate why BST lesions appeared to abolish cued fear expression in the experiments conducted by Luyck et al. Therefore, we adapted the behavioural protocol to make it less aversive and to evoke lower general stress levels, in order to obtain more refined cued fear results.

As a first step, we aimed to reduce the overall stress response in naïve rats. To this end, we completely removed startle probes from our behavioural paradigm. The loud white noise probes used for acoustic startle reflex measurements have been shown to be rather aversive by nature, and might elicit considerable physiological arousal [75]. In a retrospective analysis of one of the lesion experiments described by Luyck et al. [74], we showed that presentation of startle probes during habituation indeed elicited strong freezing, even though no conditioning had taken place at this time (see Appendix *VI.1* for a more detailed description). Although some freezing toward these novel and loud startle stimuli is normal (i.e. this is why a habituation session is included in the first place), unexpectedly high freezing levels with peaks up to 45% were observed. Based on these findings, we hypothesised that the exclusion of startle probes might reduce general stress. If, by removing startle probes from our behavioural design, BST lesions no longer affect the expression of cued fear (as indexed by freezing during tone vs. context presentations), the results obtained by Luyck et al. could most likely be due to a reduction of general stress levels.

Aside from the removal of startle probes, we also modified other parameters of the behavioural paradigm described by Luyck et al, including the number of foot shocks and CS presentations. In

addition, we decided to exclude the pre-test to reduce potential latent inhibition, and to include a second post-test for exploratory purposes.

After obtaining a protocol that elicited significant cued fear (and low general stress) in naïve animals, we proceeded with a lesion experiment. Using this modified and improved protocol, we applied post-training BST lesions as described by Luyck et al., to evaluate whether BST lesions still disrupt cued fear.

II. Materials & Methods

All experiments conducted in this master's thesis are covered by project number P185/2016, which was approved by the KU Leuven ethics committee for laboratory animal experimentation. This project is in accordance with the Belgian and European laws, guidelines and policies for animal experimentation, housing and care (Belgian Royal Decree of 29 May 2013 and European Directive 2010/63/EU on the protection of animals used for scientific purposes of 20 October 2010). All experiments were preregistered on the Open Science Framework.

1. Experiment 1

This experiment follows up on the most recent publication of the lab [74], where electrolytic BST lesions were shown to disrupt cued fear. As described in the literature section of this thesis, these results contradict much of the existing literature (see *I.7 Electrolytic post-training BST lesions reduce cued fear*) and might be due to high stress levels in our protocol (see *I.8 Aim of the study*). In the current experiment, we evaluate the use of an adapted conditioning protocol in order to reduce overall stress levels in naïve Wistar rats.

1.1 Subjects

Sixteen male Wistar rats (± 270-300 g) were used, based on previous studies performed in the lab [74]. All animals were housed in pairs with water and food available ad libitum. Animals were kept on a light-dark cycle of 14/10 hours, with lights on at 7:00 am, and with a room temperature of approximately 19°C. A plastic cage divider was used to avoid direct contact between the animals, while still allowing for social interaction.

1.2 Equipment

Two different contexts were used for the behavioural tests, Context A and Context B. The contexts were located on opposite sides of the same room in ventilated, sound-attenuating Med Associates (Fairfax, VT, USA) boxes.

Context A consists of a small animal cage (inner dimensions: 9.4 cm height, 8.2 cm width, and 16.5 cm length) and has a grid floor with six 5 mm-diameter stainless-steel rods. Startle Reflex software (version 5.95; Med Associates) was used for the presentation and sequencing of acoustic stimuli. A video camera (DCR-SR55E Super NightShot Plus, Sony Corporation, Minato, Tokyo, Japan) was positioned in front of the cage to record freezing behaviour of the animals. For this purpose, a dim red light was continuously on in the cage. One of two loudspeakers, both located 7 cm behind the cage, was used to deliver a continuous white background noise (55 dB), the other to deliver tone stimuli (4000 Hz, 75 dB, 10 s, 5 ms rise/fall). The loudspeakers were calibrated before each experiment. Between rats, the cage was cleaned with 70% ethanol.

Context B consists of a larger cage (21 cm height, 24.1 cm width, 30.5 cm length), with a grid floor with 19 rods (4.8 mm diameter, spaced 16 mm centre to centre) and a black triangular ceiling. The cage was cleaned with a scented cleaning product between rats, and the chamber was dimly-lit with a white light of 50 lux. Behaviour was recorded with a video camera (DCR-SR55E Super NightShot Plus, Sony Corporation), and presentation of tones and shocks was controlled by software (Video Freeze, Med Associates).

1.3 Behavioural fear conditioning protocol

In all experiments (*II.1.3*, *II.2.3*, *II.3.4*), rats were transferred to the testing room approximately 2-3 minutes before each behavioural session. Between the sessions, the animals were returned to their home cages. ExpTimer software [76] was used in all experiments to ensure that all rats were tested at the exact same time on every test day. Freezing was scored manually by a blinded observer.

The behavioural fear conditioning protocol consisted of 4 test days, which are described below (Fig. 4). Note that the training session and test 1 were separated by a 48-hour interval, whereas other tests took place on consecutive days. This 48-hour interval was implemented to allow for the insertion of a lesioning session in a later experiment (see *II.3 Experiment 3*).

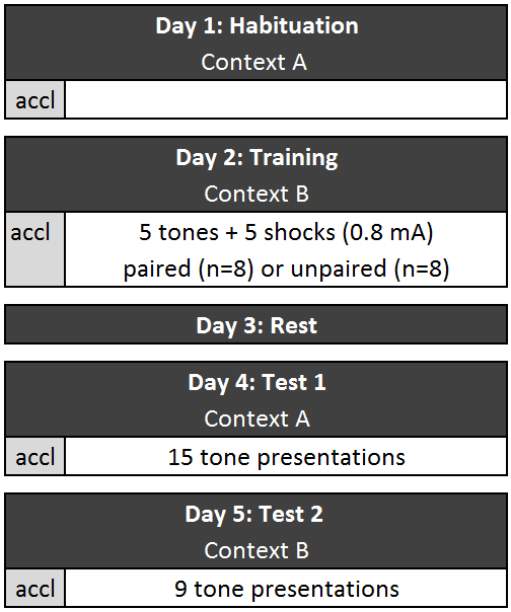


Figure 4: Behavioural fear conditioning protocol of Experiment 1. Accl = acclimation period.

1.3.1 Habituation

On day 1, all rats were placed in Context A for 23 minutes, where a continuous background noise of 55 dB was presented. Note that a background noise was administered during all test sessions that took place in Context A (habituation and test 1).

1.3.2 Training

On day 2, all rats were randomly assigned to two groups. The Paired group received 5 CS-US pairings, whereas the Unpaired group received unpaired presentations of 5 CSs and 5 USs. Training took place in Context B. The CS consisted of a 4000 Hz, 75 dB tone for a duration of 10 s. The US was a 0.8 mA foot shock for a duration of 500 ms. There was an acclimation period of 5 minutes, and the total duration of the training session was 23 min. The onset between CSs varied between 3-4 minutes for the Paired group, and the interval between US offsets and CS onsets varied between 1.5-2 minutes for the Unpaired group. In the Unpaired group, USs were delivered at the exact same time points as in the Paired group, and CSs were presented in between. Freezing was measured during each CS (10 s, measure of cued fear) and during the 10-second interval preceding each CS presentation (measure of contextual anxiety).

1.3.3 Test 1

On day 4, all animals were placed in Context A for a duration of 23 min. After a 5-min acclimation phase, 15 CSs were presented with an onset interval varying between 40-70 seconds. Freezing was measured during the 5-minute acclimation period, during each (10-s) CS ('tone'), and during the intertrial intervals (ITI's): 10-s measurements at fixed time points, both in between CSs ('between tones') and immediately before each CS ('before tone'). Freezing during the 'tone' served as a measurement of cued fear, while freezing during acclimation, 'between tones' and 'before tone' were used to quantify contextual anxiety.

1.3.4 Test 2

On day 5, all animals were placed in Context B (= training context) for a duration of 16 min. After a 5-min acclimation period, 9 CSs were presented with an onset interval that varied between 40-70 seconds. Freezing was measured as described for test 1 (see *II.1.3.3*).

1.4 Statistical analysis

Graphpad Prism (version 7.04, GraphPad Software, San Diego, CA, USA) was used for statistical analysis and the generation of graphs. Significance levels were set at $p<0.05$. Freezing scores during acclimation in both test 1 and test 2 were analysed using unpaired t-tests. Difference scores for freezing responses after acclimation (i.e. after 5 minutes) were analysed by means of a 3-way repeated measures ANOVA (RM-ANOVA), using factors 'Trial type' ('tone' versus 'before tone'), 'Group' (Paired vs. Unpaired) and 'CS' (considers every CS separately). Similarly, a 2-way ANOVA (RM-ANOVA) was performed to consider 'between tones' and 'before tone' measurements (factor 'Trial type'), in animals undergoing a Paired or Unpaired protocol (factor 'Group'). The 'between tones' measurement (*II.1.3 Behavioural fear conditioning protocol*) was solely included for exploratory purposes, to allow for a better comparison with Luyck et al. (2018) [74] (see Appendix *VI.2*), and will be discussed separately. Bonferroni's post-hoc test was used to specify group differences. Note that we used non-parametric alternatives to the tests described above, when criteria of normality and/or equal variances were not met, such as a Mann-Whitney U test (MWU). A Grubb's test was performed for outlier detection. Freezing data are shown as means ± SEM (in case of parametric testing) or medians ± IQR (in case of non-parametric testing).

2. Experiment 2

In the second experiment, the behavioural protocol described in Experiment 1 was slightly adapted with the aim of (further) reducing general stress levels.

2.1 Subjects

During the second experiment, 16 male Wistar rats (± 270-300 g) were used. The applied housing conditions and environmental parameters were the same as described for Experiment 1 (see *II.1.1 Subjects*).

2.2 Equipment

As in Experiment 1, Context A and B (see *II.1.2 Equipment*) were used for the behavioural fear conditioning protocol (Context A: habituation and test 1; Context B: training and test 2).

2.3 Behavioural fear conditioning protocol

Similar to Experiment 1, there were 4 test days. Training and test 1 were separated by a 48-h interval, whereas other tests took place on consecutive days (Fig. 5).

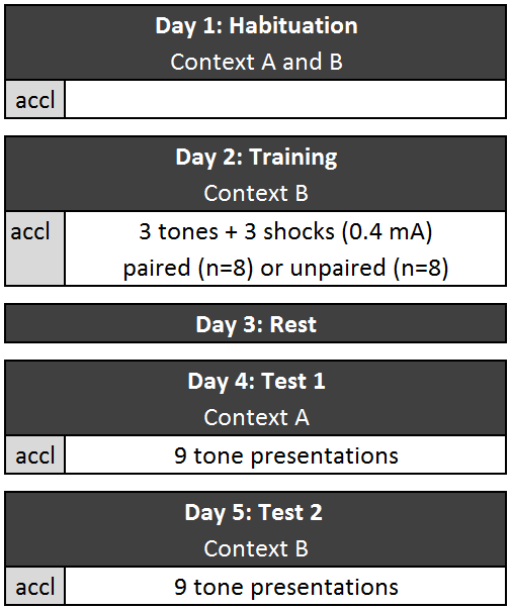


Figure 5: Behavioural fear conditioning protocol of Experiment 2. Accl = acclimation period.

2.3.1 Habituation

On the first day of the experiment, all animals underwent two habituation sessions of 23 minutes each, in both Context A and Context B. The order of the animals was counterbalanced, so that each session was separated by 4-4.5 hours.

2.3.2 Training

On day 2, the animals were randomly divided in two groups (Paired and Unpaired; n=8 per group). After a 5-min acclimation period, the Paired group received 3 CS-US pairings, the CS being a 4000 Hz, 75 dB tone of 10 s, the US being a 0.4 mA foot shock of 500 ms. The Unpaired group received 3 USs and 3 CSs in an unpaired manner. In the Paired group, the intervals between CS onsets were 7 min 20 s (CS1-CS2) and 6 min 40 s (CS2-CS3). In the Unpaired group, the interval between US offsets and CS onsets varied between 2 min 40 s and 3 min 40 s. The training took place in Context B and lasted for 23 min. Freezing was measured during each CS (10 s) and the 10-second interval preceding each CS presentation.

2.3.3 Test 1

On day 4, all animals were tested in Context A for a total duration of 23 min. After a 5-min acclimation period, they were presented with 9 CSs, with an onset interval that ranged between 100-130 s. Freezing was measured during the 5-minute acclimation period, during each (10-s) CS ('tone'), and during the intertrial intervals (ITI's): 10-s measurements at fixed time points, both in between CSs ('between tones') and immediately before each CS ('before tone'). Freezing during the 'tone' served as a measurement of cued fear, while freezing during acclimation, 'between tones' and 'before tone' were used to quantify contextual anxiety.

2.3.4 Test 2

On the fifth day, all animals were tested for 23 minutes in Context B. The behavioural protocol and freezing measurements were identical to that described for test 1, with the exception of the context that was used (test 1: Context A; test 2: Context B).

2.4 Statistical analysis

The statistical analysis was performed as described for Experiment 1 (see *II.1.4 Statistical analysis*).

3. Experiment 3

The protocol developed in Experiment 2, in which we adapted several potential stressors, resulted in satisfactory cued fear conditioning. The next step was to employ this protocol in a final experiment in which BST (or sham) lesions were applied in conditioned animals.

3.1 Subjects

Twenty-four male Wistar rats (± 250 g at the time of surgery) were included in the final experiment. The applied housing conditions and environmental parameters were the same as described for Experiment 1 and 2 (see *II.1.1 Subjects*). Note that the use of a plastic cage divider was particularly relevant in this experiment, to prevent damage to the surgical wounds by cage mates.

3.2 Surgical procedure

Stainless steel cannulas (23-gauge guide cannula C317G/5 mm and dummy stylet C317DC/5 mm, PlasticsOne, Roanoke, VA, USA) were implanted and placed on the dura of the rats directed toward the BST (anterior-posterior axis: 0.0 mm, medio-lateral: ± 3.4 mm, 20° angle to the sagittal plane). General anaesthesia (ketamine hydrochloride (22.5 mg/kg, Anesketin, Eurovet nv/sa, Heusden-Zolder, Belgium) and 0.15 mg/kg medetomine HCL (Kela, Sint-Niklaas, Belgium)) was used to anaesthetise the animals. The rats were placed in a stereotactic frame, after which a craniotomy was performed, using a drill to access the dura. Two drill holes were preserved for cannula placement, and four smaller ones for the insertion of stainless steel screws (Fine Science Tools, Heidelberg, Germany). The fixation screws were covered and connected with the cannulas using dental cement (Tetric® EvoFlow, Ivoclar Vivadent Inc., Mississauga, Ontario, Canada), after which the wound was sutured. The animals' body temperature was continuously monitored through the insertion of an anal probe and was kept constant by a feed-back controlled heating pad (Harvard Apparatus, Holliston, MA, USA). After surgery, post-operative pain treatment (Metacam, 1mg/kg, Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany) was administered. Animals were allowed to recover for a period of 6-7 days before the start of the experiment.

3.3 Equipment

As in Experiment 1 and 2, Context A and B (see *II.1.2 Equipment*) were used for the behavioural fear conditioning protocol (Context A: habituation and test 1; Context B: training and test 2).

3.4 Behavioural fear conditioning protocol

In Experiment 3 (Fig. 6), all rats were conditioned to a 10-s tone, using the protocol described for the Paired group of Experiment 2 (see *II.2.3 Behavioural fear conditioning protocol*). Rats were assigned to the Lesion (n=13) or Sham (n=11) group, based on their freezing values during the last CS during training. Note that group sizes were not equal to correct for possible exclusion of Lesion animals based on histological analysis, i.e. off-target lesions (see *II.3.6 Histology*). Lesions ('Lesion') or sham lesions ('Sham') were applied 27 h after the training session (see *II.3.5 Lesion procedure*). This 27-h interval was chosen to interfere with the expression (or retrieval) of fear, rather than with the consolidation of the cued fear memory [74]. Test 1 took place 21 h after the induction of (sham) lesions.

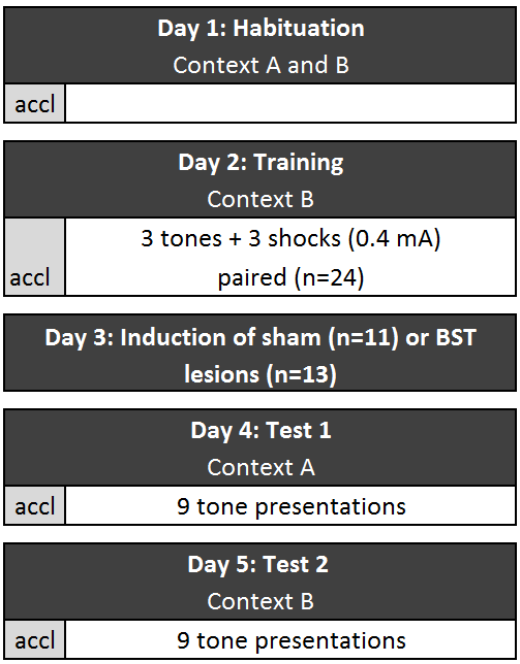


Figure 6: Behavioural fear conditioning protocol of Experiment 3. Accl = acclimation period.

3.5 Lesion procedure

The lesioning procedure was performed as previously described by Luyten et al. [17]. Rats were briefly anaesthetised with isoflurane (5% for induction and 2% for maintenance in 1.5–2.0 l/min oxygen), before a stainless-steel acupuncture needle (Acupro P20-3210, Medichin, Hasselt, Belgium) was inserted through the cannula to puncture the dura. Subsequently, custom-made insulated stainless-steel electrodes (200 µm in diameter) (008SW/30S, PlasticsOne) with a

transversally cut tip were inserted into the cannulas and lowered 6.3 mm below the dural surface, thereby bilaterally targeting the medial division of the anterior BST. The electrodes were connected to a stimulator (DS8000 and DLS100, World Precision Instruments, Stevenage, UK) through which an anodal direct current pulse of 1 mA (Lesion group) or 0 mA (Sham group) was applied for 15 s. Electrodes were removed after 1 minute. After electrode removal, anaesthesia was ended and the animals woke up a few minutes later. The whole procedure took about 10–15 min.

3.6 Histology

All animals were euthanised through an intraperitoneal injection of pentobarbital (2 ml; Nembutal, CEVA Santé Animale, Brussels, Belgium), approximately one week after testing. Perfusion of the rats was performed with a 10% sucrose solution (D(+)-Saccharose solution, VWR international bvba, Leuven, Belgium), and subsequently with a 4% formaldehyde solution (37% dissolved in water, stabilised with 5-15% methanol, Acros organics, Geel, Belgium, 10x diluted in DI water). The rat brains were dissected and stored in 4% formaldehyde, after which they were processed (Excelsior AS Tissue Processor, Thermo Fisher Scientific Inc., Waltham, MA, USA) and embedded in paraffin (HistoStar Embedding Workstation, Thermo Fisher Scientific Inc.). Coronal slices of 5 µm thick were collected with the microtome (Leica Biosystems GmbH, Nussloch, Germany), and subsequently stained with Cresyl Violet (0.5% cresyl violet acetate in dH2O, Merck KGaA, Darmstadt, Germany). The location of the electrode tips and the lesions was determined by microscopic analysis and transferred to a Paxinos coronal plate (Paxinos and Watson, 2005). When the largest diameter of the lesion comprised the anterior BST (within a 500 µm radius from bregma) and when clear damage (including necrosis and oedema) was visible on the bregma slice, Lesion animals were included in the experimental data. Sham animals were included when no damage in the BST (apart from electrode tracks) or surrounding areas was observed.

3.7 Statistics

Similar to Experiment 1, Graphpad Prism (version 7.04, GraphPad Software) was used for statistical analysis and the generation of graphs. Significance levels were set at $p<0.05$. Freezing scores during acclimation in both test 1 and test 2 were analysed using unpaired t-tests. Difference scores for freezing responses after acclimation (i.e. after 5 minutes) were analysed by means of a 3-way repeated measures ANOVA (RM-ANOVA), using factors 'Trial type' ('tone' versus 'before tone'), 'Group' (Lesion vs. Sham) and 'CS' (considers every CS separately). Similarly, a 2-way ANOVA (RM-ANOVA) was performed to consider 'between tones' and 'before tone' measurements (factor 'Trial type'), in animals undergoing BST (Lesion) or sham (Sham) lesions (factor 'Group'). The 'between tones' measurement (*II.3.4 Behavioural fear conditioning protocol*) was solely included for exploratory purposes, to allow for a better comparison with Luyck et al. (2018) [74] (see Appendix *VI.2*), and will be discussed separately. Bonferroni's post-hoc test was used to specify group differences. Planned contrasts included a comparison of freezing 'before tone' and during 'tone' on test 1 and test 2 in the Sham and Lesion groups separately, to allow for an assessment of cued fear in each of these groups. Note that we used non-parametric alternatives to the tests described above, when criteria of normality and/or equal variances were not met. A Grubb's test was performed for outlier detection. Freezing data are shown as means ± SEM (in case of parametric testing) or medians ± IQR (in case of non-parametric testing).

III. Results

As discussed in the Materials & Methods section above, we included two measurements for contextual anxiety at several time points during the test sessions in each experiment, 'before tone' and 'between tones'. Based on a retrospective analysis of the experiment of Luyck et al. [74] (see Appendix *VI.2*), we decided to use the 'before tone' measurement to quantify contextual anxiety in our main results. In addition, we conducted a direct comparison between both contextual anxiety measurements to allow for a better comparison with Luyck et al. [74]. These data are described in a separate section following the main results.

As stated in the Methods section (see *II.1.4, II.2.4, II.3.7*), freezing after acclimation was analysed using a 3-way ANOVA with factors 'Group', 'Trial type' and 'CS'. Note that, while we will describe all significant main effects and interactions, we will only perform post-hoc analysis on significant effects regarding the factors 'Trial type' and 'Group', not 'CS'. We will not further discuss significant effects regarding 'CS', neither will these effects be noted in figures.

1. Main results

1.1 Experiment 1

1.1.1 Training

Relatively high freezing values were obtained in both the Paired (tone: 52.5% ± 13.9%, mean ± SD, before tone: 48.0% ± 12.8%) and Unpaired group (tone: 57.1% ± 17.9%, before tone: 64.0% ± 15.3%) (Fig. 7). No significant effects of 'Group' or 'Trial type' were found. We did detect a main effect of 'CS' ($F_{(4,56)}$=12.39; p<0.0001), and interactions of 'CS' and 'Group' ($F_{(4,56)}$=6.89; p=0.0001), 'CS' and 'Trial type' ($F_{(4,56)}$=3.00; p<0.05) and 'CS', 'Trial type' and 'Group' combined ($F_{(4,56)}$=4.01; p<0.01).

1.1.2 Test 1

Since criteria for normality were not met for freezing during acclimation values, a Mann-Whitney U test was used for analysis. Freezing during acclimation was low both in the Paired (median, [IQR]: 5.7%, [0 to 17.5%]) and the Unpaired (1.7%, [0.8 to 6.9%]) group and did not differ significantly (MWU=27.5; p=0.66) (Fig. 8A).

Three-way ANOVA of freezing after the acclimation period revealed a main effect of 'Trial type' ($F_{(1,14)}$=12.15; p<0.01) and 'CS' ($F_{(14,196)}$=4.55; p<0.0001), but not of 'Group' nor any interactions.

1.1.3 Test 2

Freezing levels during acclimation in test 2 were high in both the Paired (44.9% ± 19.5%) and Unpaired (57.5% ± 28.4%) group and did not differ significantly ($t_{(14)}$=1.04; p=0.32) (Fig. 8B).

A 3-way ANOVA of freezing after the acclimation period revealed a significant interaction between 'Trial type' and 'Group' ($F_{(1,14)}$=5.62; p<0.05), but no main effect of 'Group' or 'Trial type'. Post-hoc analysis showed that rats of the Unpaired group froze more 'before tone' than during 'tone' (p<0.05). In addition, we found a main effect of 'CS' ($F_{(8,112)}$=3.74; p<0.001), and interactions of 'CS' and 'Group' ($F_{(8,112)}$=2.08; p=0.04), and 'CS', 'Trial type' and 'Group' combined ($F_{(8,112)}$=3.26; p<0.01).

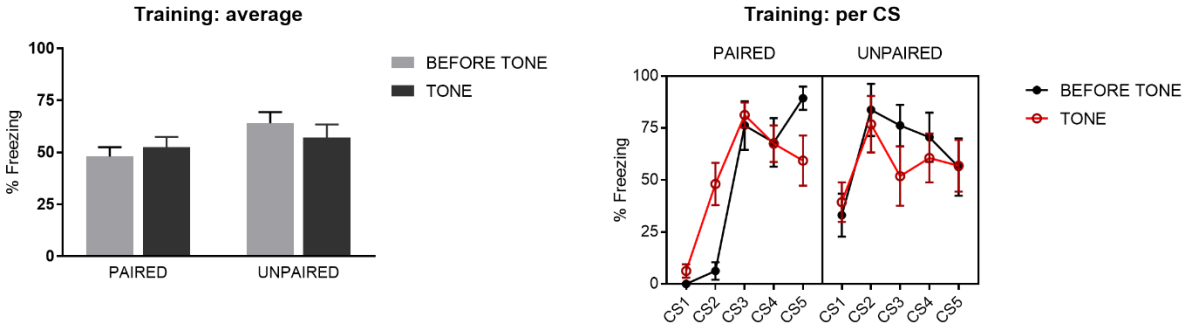


Figure 7: Percentage freezing during the training session of Experiment 1. Average freezing levels are shown in the left panel, the right panel shows freezing levels during each CS. Data are shown as means ± SEM (n=8 for each group).

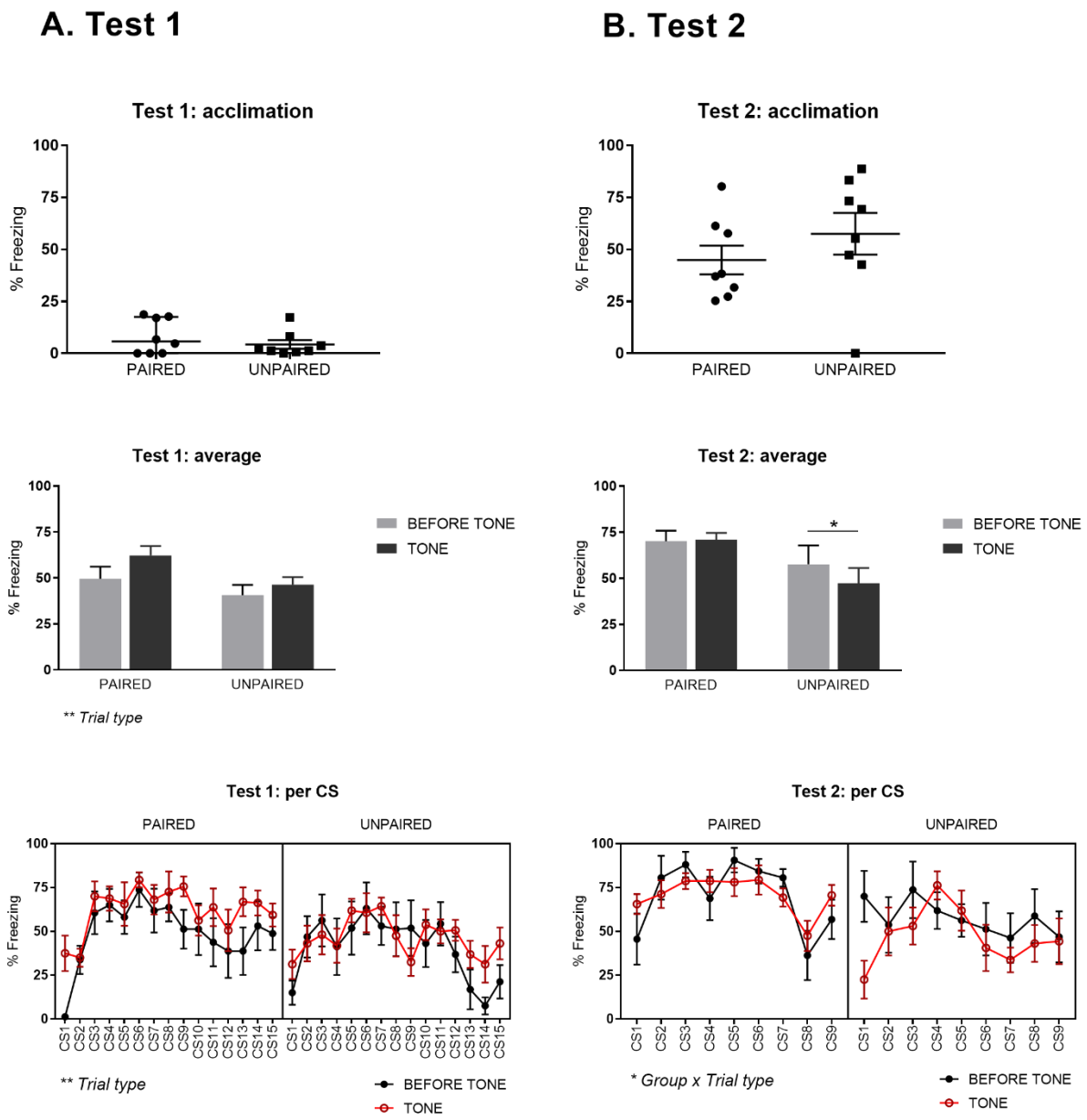


Figure 8: Percentage freezing during acclimation (upper two panels) and after acclimation (lower four panels) in test 1 and test 2 of Experiment 1. A. The left column describes results from test 1. B. In the right column, data of test 2 are shown. Data are shown as means ± SEM (n=8 for each group), with the exception of freezing during test 1: acclimation (median ± IQR). *: p≤0.05, **: p≤0.01.

1.2 Experiment 2

1.2.1 Training

Average freezing values during training 'before tone' (Paired group: 15.0% ± 13.4%, mean ± SD, Unpaired group: 34.4% ± 19.4%) were lower than during 'tone' (Paired group: 54.0% ± 11.0%, Unpaired group: 45.0% ± 9.9%).

Three-way ANOVA of freezing levels during the training session revealed a significant effect of 'Trial type' ($F_{(1,14)}$=26.28; p<0.001), of 'CS' ($F_{(2,28)}$=16.72; p<0.0001), and an interaction between 'Trial type' and 'Group' ($F_{(1,14)}$=8.58; p=0.01) but not of 'Group' (Fig. 9). Post-hoc analysis showed that animals of the Paired group show higher freezing values during 'tone' than 'before tone' (p<0.001).

1.2.2 Test 1

Freezing during acclimation did not meet criteria for parametric analysis. Freezing was low both in the Paired (median, [IQR]: 0.3%, [0 to 0.7%]) and the Unpaired (0%, [0 to 0.9%]) group and did not differ significantly (MWU=29.5; p=0.80) (Fig. 10A).

Three-way ANOVA revealed a main effect of 'Trial type' ($F_{(1,14)}$=99.10; p<0.0001), 'Group' ($F_{(1,14)}$=14.33; p<0.01), and an interaction between both ($F_{(1,14)}$=15.44; p<0.01). Post-hoc analysis showed that the rats freeze more during the 'tone' than 'before tone', both in the Paired (p<0.0001) and the Unpaired group (p<0.01). In addition, animals of the Paired group froze significantly more during tone presentation, compared to the Unpaired group (p<0.001).

1.2.3 Test 2

Freezing during acclimation did not meet criteria for parametric analysis. Freezing levels did not differ between Paired (11.8%, [3.0 to 48.8%]) and Unpaired (10.2%, [2.2 to 19.3%]) groups (MWU=28.5; p=0.75) (Fig. 10B).

After the acclimation period, 3-way ANOVA of freezing levels unveiled a significant effect of 'Trial type' ($F_{(1,14)}$=54.74; p<0.0001), 'Group' ($F_{(1,14)}$=19.06; p<0.001), 'CS' ($F_{(8,112)}$=3.09; p<0.01) and an interaction between 'Trial type' and 'Group' ($F_{(1,14)}$=11.29; p<0.01). Post-hoc Bonferroni analysis showed that animals in the Paired group freeze more during 'tone' than 'before tone' (p<0.0001). Furthermore, rats of the Paired group show higher freezing levels during the 'tone' than rats of the Unpaired group (p<0.001).

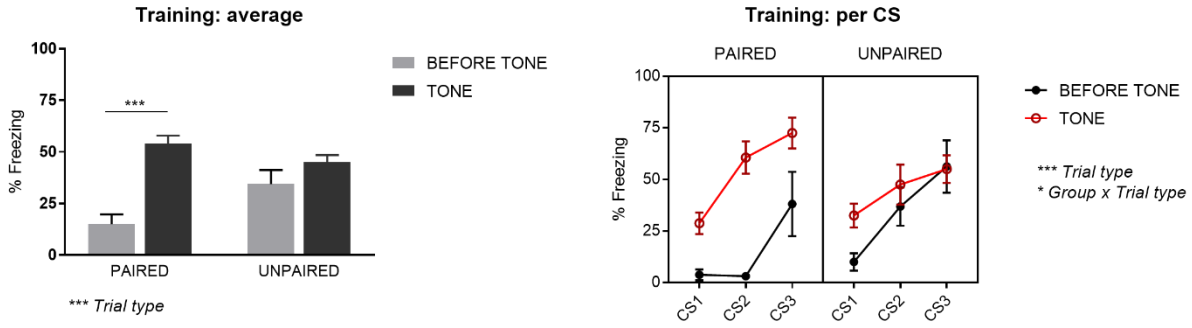


Figure 9: Percentage freezing during the training session of Experiment 2. Average freezing levels are shown in the left panel, the right panel shows freezing levels during each CS. Data are shown as means ± SEM (n=8 for each group). *: p≤0.05, ***: p≤0.001.

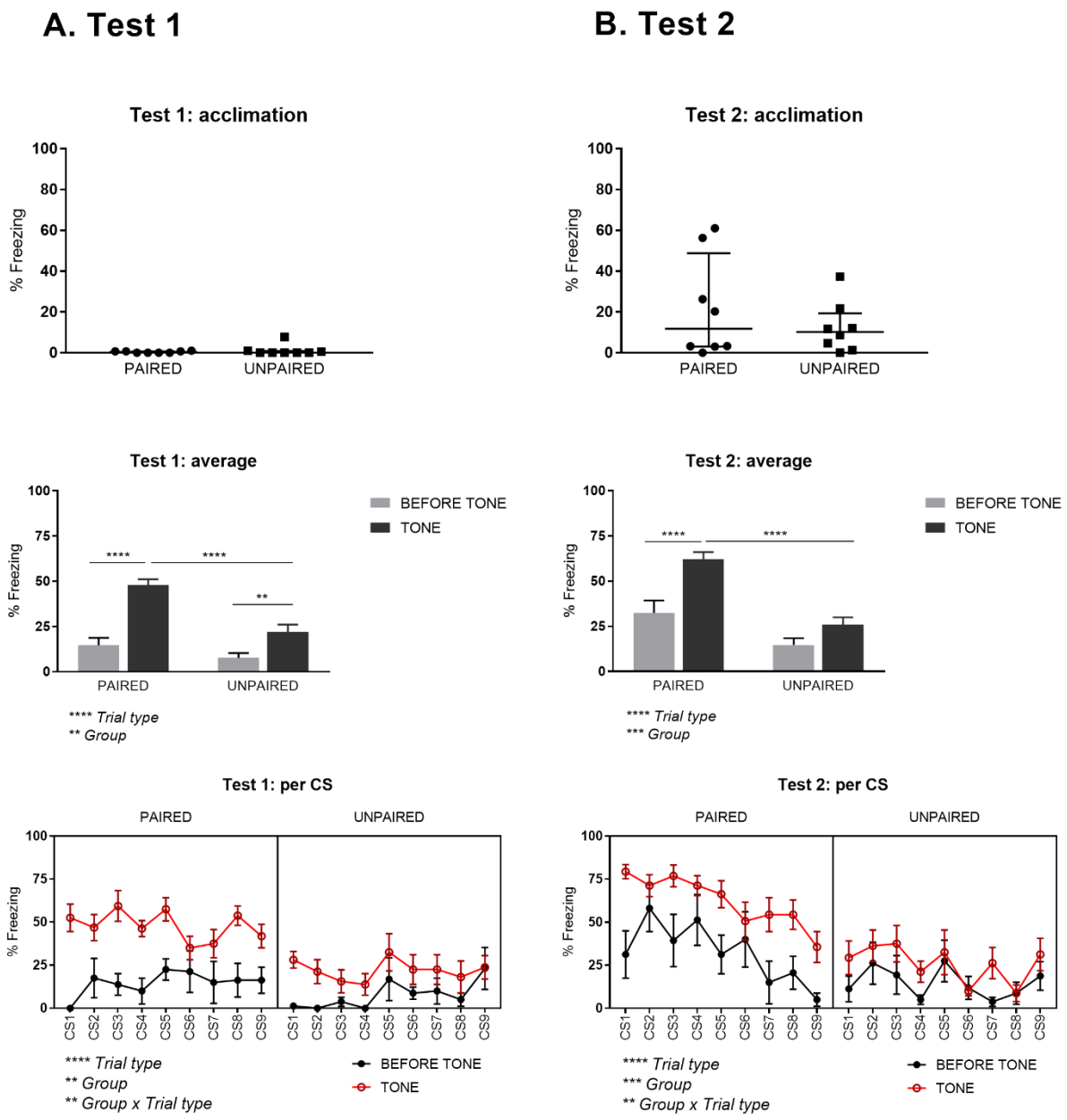


Figure 10: Percentage freezing during acclimation (upper two panels) and after acclimation (lower four panels) in test 1 and test 2 of Experiment 2. A. The left column displays results from test 1. B. The right column shows results from test 2. Data are shown as means ± SEM (n=8 for each group), with the exception of freezing during acclimation (median ± IQR). *: p≤0.05, **: p≤0.01, ***: p≤0.001, ****: p≤0.0001.

1.3 Experiment 3

Four animals were excluded from the lesion group, due to insufficient damage to the bilateral medial BST (see *II.3.6 Histology*). In total, 11 rats were included in the Sham group and 9 animals in the Lesion group (Fig. 11).

1.3.1 Training

Average freezing values during training 'before tone' (Lesion group: 20.4% ± 21.1%, mean ± SD, Sham group: 22.9% ± 12.1%) were lower than during 'tone' (Lesion group: 51.9% ± 12.6%, Sham group: 47.7% ± 15.0%).

Analysis of freezing levels during the training session using a 3-way ANOVA showed a significant effect of 'Trial type' ($F_{(1,18)}$=68.97; p<0.0001) and of 'CS' ($F_{(2,36)}$=32.80; p<0.0001) but not of 'Group'. In addition, we found an interaction between 'CS' and 'Trial type' ($F_{(2,36)}$=4.18; p<0.05) (Fig. 12).

1.3.2 Test 1

Since criteria for normality were not met for freezing during acclimation values, a Mann-Whitney U test was used for analysis. Freezing during acclimation was low both in the Sham (median, [IQR]: 1.3%, [0.3 to 6.0%]) and the Lesion (1.0%, [0 to 3.0%]) group and did not differ significantly (MWU=36.5; p=0.34) (Fig. 13A).

Three-way ANOVA of freezing after the acclimation period revealed a main effect of 'Trial type' ($F_{(1,18)}$=79.09; p<0.0001) and of 'CS' ($F_{(8,144)}$=2.53; p=0.01), but not of 'Group'. A significant interaction was found between 'CS' and 'Group' ($F_{(8,144)}$=4.62; p<0.0001) and between 'CS' and 'Trial type' ($F_{(8,144)}$=2.16; p<0.05). Planned comparisons (corrected for multiple testing, alpha=0.025) showed significantly higher freezing during 'tone' compared to 'before tone' in both the Sham ($t_{(10)}$=7.86; p<0.0001) and the Lesion group ($t_{(8)}$=8.03; p<0.0001).

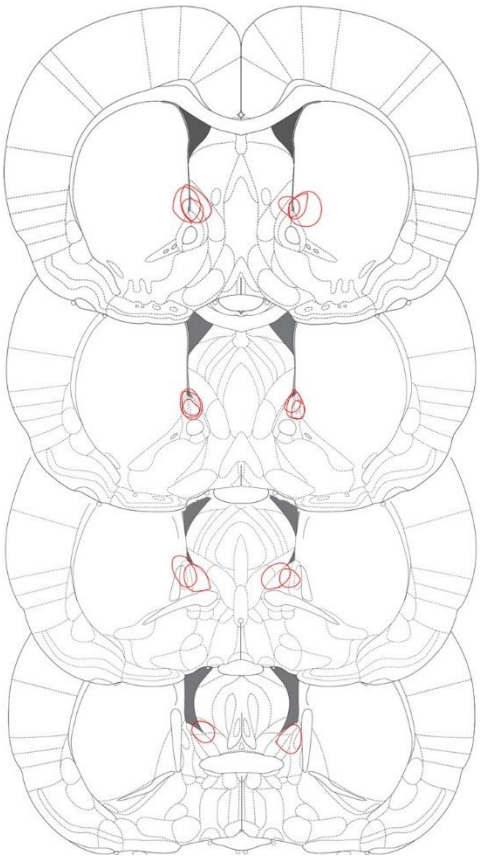


Figure 11: Reconstruction of electrolytic lesions in the BST. The maximal diameter of each lesion is shown. Coronal slices shown from top to bottom are +0.48 mm, +0.24 mm, 0.00 mm and -0.24 mm with respect to bregma. Adapted from Paxinos and Watson (2005).

1.3.3 Test 2

One animal of the Sham group was excluded from test 2 based on outlier analysis (Z=2.88 during acclimation, Z=2.73 'before tone', Z=2.42 'between tones'; p<0.05, critical value of Z=2.35, see Appendix *VI.3*).

Freezing during acclimation did not meet criteria for parametric testing. Freezing was low both in the Sham (5.2%, [2.9 to 7.2%]) and the Lesion (4.3% [2.8 to 10.3%]) group and did not differ significantly (MWU=44; p=0.95) (Fig. 13B).

Three-way ANOVA of freezing after the acclimation period revealed a main effect of 'Trial type' ($F_{(1,17)}$=338.1; p<0.0001) but not of 'Group' or 'CS'. A significant interaction was found between 'CS' and 'Trial type' ($F_{(8,136)}$=6.98; p<0.0001). Planned comparisons (corrected for multiple testing, alpha=0.025) showed significantly higher freezing during 'tone' compared to 'before tone' in both the Sham ($t_{(9)}$=12.96; p<0.0001) and the Lesion group ($t_{(8)}$=13.43; p<0.0001).

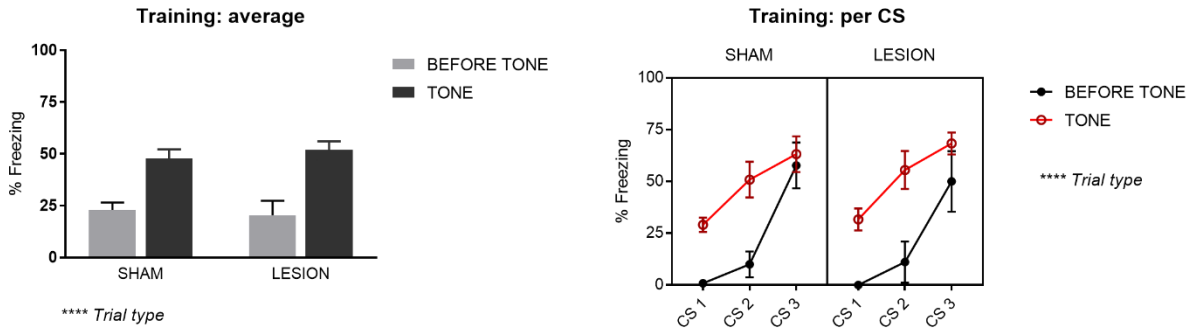


Figure 12: Percentage freezing during the training session of Experiment 3. Average freezing levels are shown in the left panel, the right panel shows freezing levels during each CS. Data are shown as means ± SEM (n=11 for the Sham group, n=9 for the Lesion group). ****: $p \leq 0.0001$.

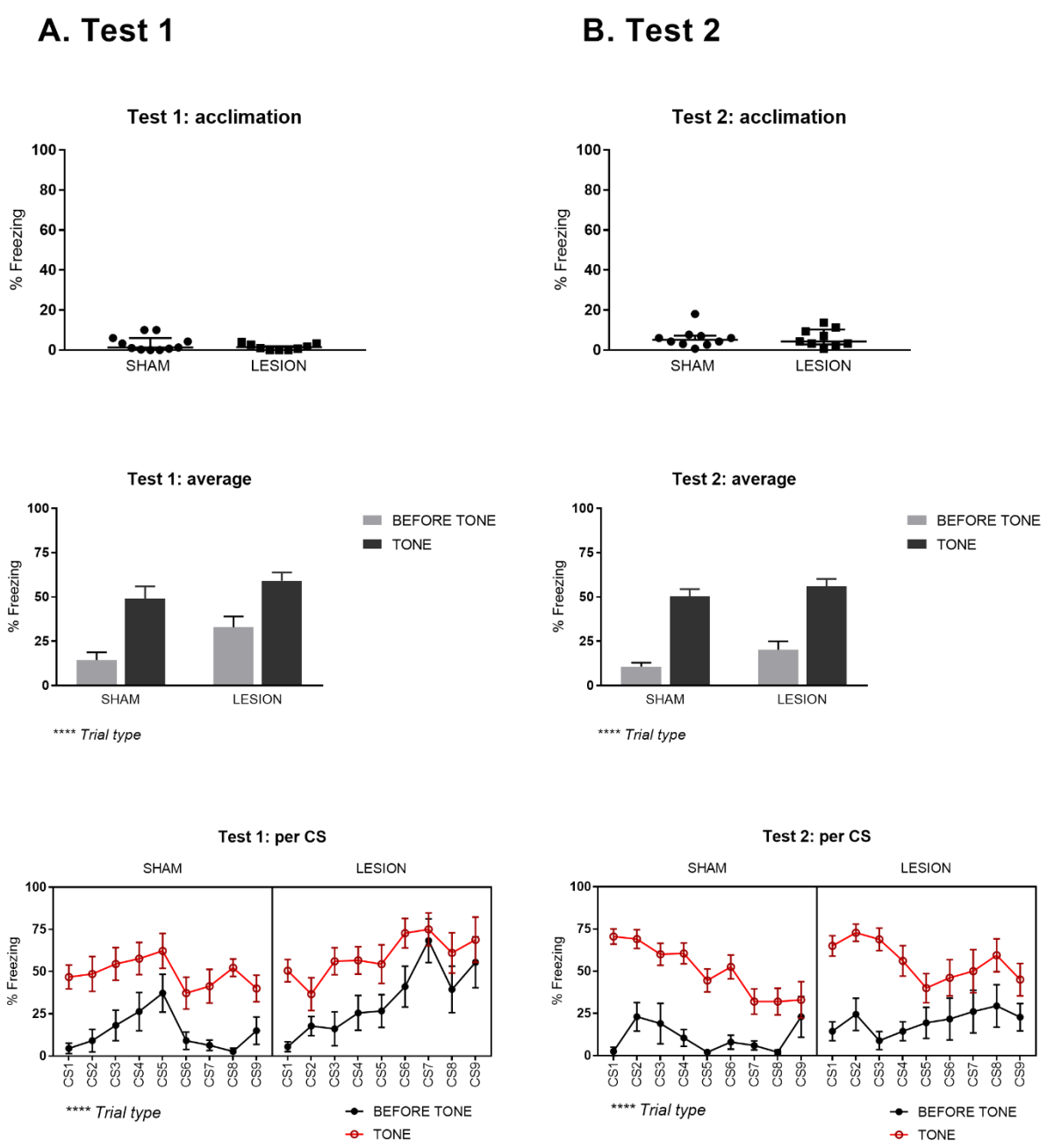


Figure 13: Percentage freezing during acclimation (upper two panels) and after acclimation (lower four panels) in test 1 and test 2 of Experiment 3. A. In the left column, data from test 1 are shown. B. The right column shows the results of test 2. Data are shown as means ± SEM (n=9 for the Lesion group, n=11 for the Sham group in test 1, n=10 for the Sham group in test 2), with the exception of freezing during acclimation (median ± IQR). ****: p≤0.0001.

2. Context comparison

In this section, we briefly compare both measurements of contextual anxiety which were collected after the first 5 minutes of acclimation throughout the test sessions, i.e. 'before tone' and 'between tones'. We hypothesised that 'before tone' measurements would yield lower freezing scores, as they are presumably less influenced by the preceding tone (see Appendix *VI.2*).

2.1 Experiment 1

2.1.1 Test 1

Freezing scores after the acclimation period were slightly different 'before tone' compared to 'between tones', both for the Paired (before tone: 49.6% ± 18.6%, mean ± SD, between tones: 54.8% ± 18.4%) and the Unpaired (before tone: 40.2% ± 15.6%, between tones: 39.7% ± 14.4%) group. Despite these nominal differences, a 2-way ANOVA did not reveal a significant effect of 'Trial type', nor of 'Group' nor of an interaction between both factors (Fig. 14A, left panel).

2.1.2 Test 2

Freezing scores of 'before tone' and 'between tones' after the acclimation period were very similar, both for the Paired (before tone: 70.2% ± 16.0%, between tones: 72.0% ± 22.1%) and the Unpaired (before tone: 57.6% ± 28.9%, between tones: 62.8% ± 22.8%) group. Similar to test 1, no significant effects were observed (Fig 14A, right panel).

2.2 Experiment 2

2.2.1 Test 1

Freezing scores after the acclimation period were nominally lower 'before tone' compared to 'between tones', both for the Paired (before tone: 14.7% ± 11.2%, mean ± SD, between tones: 21.7% ± 16.2%) and the Unpaired (before tone: 7.6% ± 7.5%, between tones: 9.2% ± 7.5%) group. However, 2-way ANOVA of 'before tone' and 'between tones' freezing levels after the acclimation period revealed no significant effect of 'Trial type', nor of 'Group' nor of an interaction between 'Group' and 'Trial type' (Fig. 14B, left panel).

2.2.2 Test 2

Similar to test 1, freezing scores were slightly lower 'before tone' compared to 'between tones', both for the Paired (before tone: 32.4% ± 19.5%, between tones: 34.9% ± 18.1%) and the Unpaired (before tone: 14.7% ± 10.3%, between tones: 16.8% ± 13.1%) group. A 2-way ANOVA showed a significant effect of 'Group' ($F_{(1,14)}=5.60$; $p<0.05$), but not of 'Trial type' nor of an interaction between both (Fig. 14B, right panel).

2.3 Experiment 3

2.3.1 Test 1

Freezing scores were nominally lower 'before tone' compared to 'between tones' for the Sham (before tone: 14.3% ± 14.7%, mean ± SD, between tones: 19.7% ± 14.9%) group. For the Lesion group, these values were very similar (before tone: 32.9% ± 18.1%, between tones: 32.8% ± 17.9%). A 2-way ANOVA of 'before tone' and 'between tones' freezing levels after the

acclimation period revealed a significant effect of 'Group' ($F_{(1,18)}$=5.01; $p<0.05$) (Fig. 14C, left panel), but not of 'Trial type'.

2.3.2 Test 2

Freezing scores after the acclimation period were nominally lower 'before tone' compared to 'between tones', both for the Sham (before tone: 10.7% ± 7.0%, between tones: 17.9% ± 10.1%) and the Lesion group (before tone: 20.2% ± 14.4%, between tones: 24.6% ± 17.2%). This was corroborated by a 2-way ANOVA, which showed a significant effect of 'Trial type' ($F_{(1,17)}$=14.56; p=0.001) (Fig. 14C, right panel).

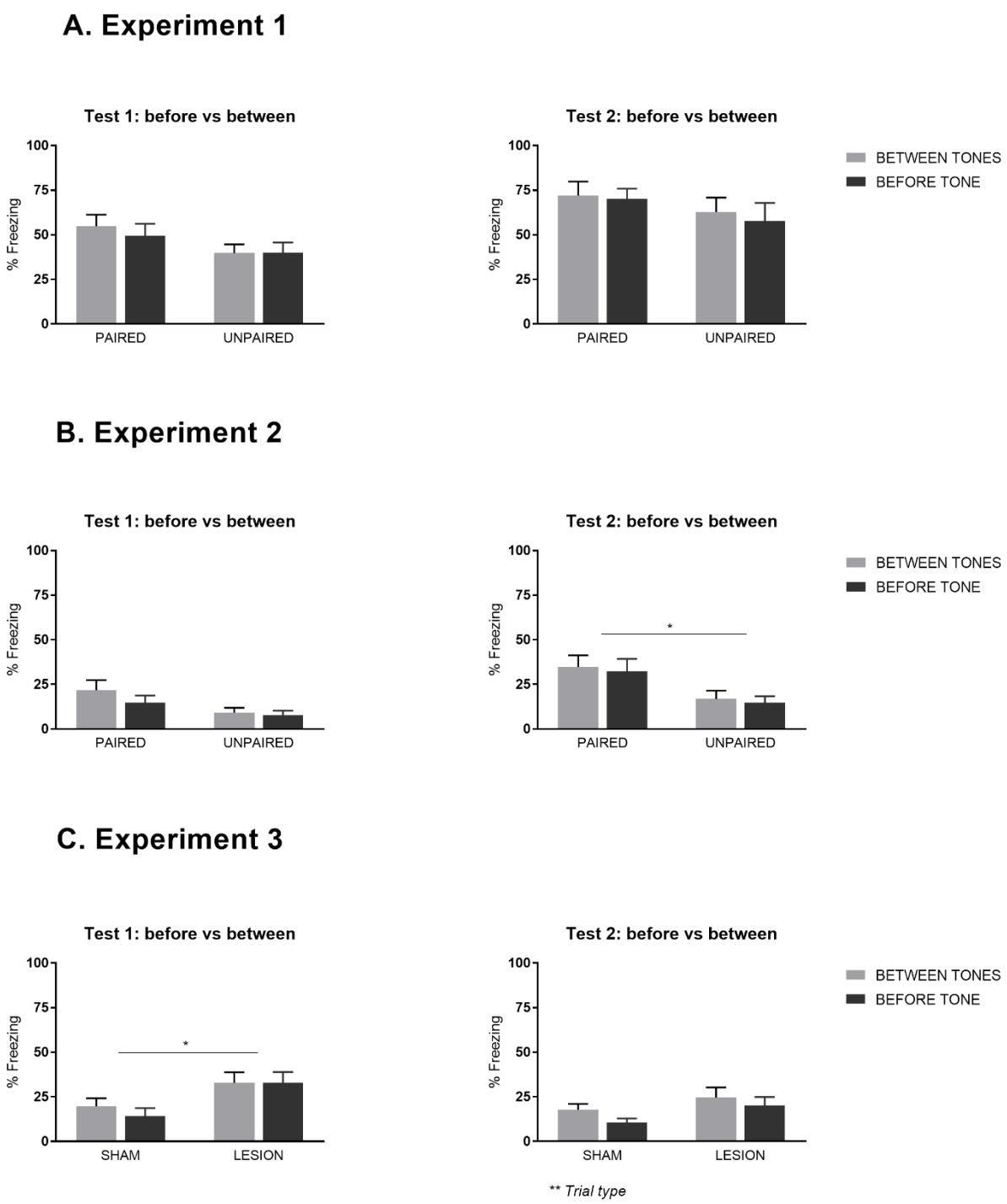


Figure 14: Percentage freezing after acclimation during 'between tones' and 'before tone' context presentations in Experiment 1 (A.), 2 (B.) and 3 (C.). Data are shown as means ± SEM (Experiment 1 and 2: n=8 for each group, Experiment 3: n=9 for the Lesion group, n=11 for the Sham group in test 1, n=10 for the Sham group in test 2). *: $p \leq 0.05$, **: $p \leq 0.01$.

IV. Discussion

The generally accepted view on fear and anxiety neurocircuits dictates that phasic fear is mediated mainly by the CeA, while the BST mediates sustained anxiety responses [2, 40] (see *I. Introduction*). We have discussed various data from human and rodent research that are in line with this model. However, some findings challenge this strict functional dissociation between the BST and the CeA. In this respect, a study previously conducted in our lab by Luyck et al. [74] found remarkable results, in the sense that post-training electrolytic BST lesions blocked fear-potentiated startle in rats. However, as demonstrated in Appendix *VI.2*, the animals failed to discriminate between tone and tone-free intervals in terms of freezing behaviour. In addition, additional retrospective analyses of this published experiment showed remarkably high freezing values upon startle presentation during habituation, e.g. before any conditioning took place (see Appendix *VI.1*). This suggests that general stress levels might have been too high and that the protocols used during these experiments may have been experienced as rather aversive by the rats. If the animals were continuously anxious or under stress, this may have overshadowed the predictive value of the conditioned tone. Since the BST is a key mediator of stress responses [28]; what was interpreted as in impairment of cued fear might in reality have been a reduction of contextual anxiety and overall stress levels.

To address this hypothesis, we aimed to optimise our conditioning protocol to obtain purer cued fear responses, by reducing general stress in multiple ways. Once the protocol was optimised in naïve rats, we continued with an experiment in which we applied BST lesions, similar to Luyck et al. [74]. For a simplified overview of the conducted experiments, see Fig. 15.

	Experiment 1	**Experiment 2**	**Experiment 3**
Day 1: Habituation			
Day 2: Training	5x ♪ ϟ (0.8 mA) Paired/Unpaired	3x ♪ ϟ (0.4 mA) Paired/Unpaired	3x ♪ ϟ (0.4 mA) Paired
Day 3: Rest			BST/sham lesion
Day 4: Test 1	15x ♪	9x ♪	9x ♪
Day 5: Test 2	9x ♪	9x ♪	9x ♪

Figure 15: Overview of the protocols used in the three different experiments described in this thesis. Experiments 1 and 2 were conducted in naïve animals, while Experiment 3 included surgical interventions (required for cannula implantation). Colour code: Context A (light grey), Context B (dark grey). The "♪" symbol represents a tone, the "ϟ" symbol represents a foot shock. In Experiment 1, habituation only took place in Context A. Five tones each co-terminated with a 0.8 mA foot shock during the training in the Paired group (n=8), while the tones and shocks were not paired in the Unpaired group (n=8). During test 1, 15 tones were presented, and in test 2, 9 tones were presented. In Experiment 2 and 3, habituation took place both in Context A and B. During the training, only 3 shocks and tones were given in a paired (Experiment 2: n=8) or unpaired (Experiment 2: n=8) fashion, and the shocks were lower in amplitude (0.4 mA). In test 1, the number of presented tones was lowered to 9 instead of 15. In Experiment 3, all rats underwent the paired cued fear protocol of Experiment 2 and underwent BST (n=13) or sham (n=11) lesions 27 h after the training session.

1. Experiment 1

To reduce the aversiveness of the protocol used by Luyck et al. (2018), we decided to omit all startle probes, since they have been shown to elicit considerable physiological arousal [75] and might contribute to heightened general stress levels. In a retrospective analysis of the experiments of Luyck et al. (see Appendix *VI.1*) we came to a similar conclusion: the introduction of startle probes during the habituation session elicited unexpectedly high levels of freezing that also influenced other sessions of the behavioural conditioning protocol. In addition, in the current experiment, the number of foot shocks and CS presentations during the training session were reduced and the pre-test was excluded to reduce latent inhibition. The latter refers to the observation that a familiar stimulus takes longer to acquire meaning than a new stimulus [77, 78].

For a thorough evaluation of cued fear, we chose for a comparison with a control group receiving shocks in an unpaired manner, since unpaired CS-US presentations form an optimal contrast with cue conditioning (explicitly paired CS-US presentations) [79]. By comparing freezing levels during tone presentations of the Paired and the Unpaired group, the value assigned to the tone as temporal predictor of an aversive event could be evaluated. Ideally, freezing during the tone should be higher in the Paired than the Unpaired group, since the tone should form a good temporal predictor in the paired protocol, but not in the unpaired protocol. In addition, our design allowed for a direct comparison of contextual freezing between the two groups, which has rarely been considered by other researchers. Given the value of the tone as a temporal predictor in the paired protocol, we expected freezing levels during context presentations to be low in the Paired group. Due to context conditioning, we expected these values to be rather high in the Unpaired group, and freezing to tone and context presentations to be similar.

We conducted two test sessions on consecutive days, the first one in a different context than the one used for training, the second one in the same context. This additional test day allows to evaluate contextual influences in the cued fear paradigm.

In contrast with our hypotheses, animals from the Paired group did not show significantly higher freezing during the tone, compared to context presentations in neither test session, nor during training. Contextual freezing during training was rather high, which suggests that the rats were not able to return to a state of rest in between US and CS presentations and general stress levels were too high. In line with this, we did not observe differences between the Paired and the Unpaired group in the training session, suggesting that the value of the tone was overshadowed by high general stress. In addition, the Unpaired group showed strong freezing responses during contextual anxiety measurements in test 1, even though the animals were exposed to a different context than the one used for training (training: Context B; test 1: Context A). This indicates that these elevated freezing levels were not specific to the training context (i.e. due to context conditioning), but generalized to the modified test context [80].

When animals were re-exposed to the training context (test 2), freezing levels were even higher than those of test 1 (especially during acclimation) in both groups. We expected to see this elevation in the Unpaired group, since the animals were context conditioned and the tone had no predictive value. In contrast with our expectations, animals in the Paired group also showed very high freezing levels, suggesting they were too stressed to be able to distinguish between tone and context presentations. As this was already observed during test 1, it came as no surprise that placing them in their training context would elicit even higher freezing levels.

2. Experiment 2

In Experiment 2, we attempted to further reduce general stress levels by making the protocol even less aversive. To this end, rats were trained with 3 CS-US pairings instead of 5, and the foot shock amplitude was lowered from 0.8 mA to 0.4 mA. In addition, the number of tones presented in test 1 were reduced from 15 to 9. A multitude of tones may work aversive after a while, since this succession of tones may lead to anticipation of more tones and thus to development of general anxiety. In addition, a longer ITI would mean less influence of cued fear responses on contextual anxiety measurements.

Using this adapted protocol, we observed that Paired animals froze significantly more during tone compared to context measurements during training and both tests, which is an indication of successful cued fear conditioning. In addition, it showed that 'clean' cued fear responses can be obtained in the same context as the one the animals were trained in (Context B). Freezing levels in the Paired group were significantly lower in test 1 than in test 2. This indicates that contextual anxiety might have partly contributed to cued fear measurements, but it formed no problem, since a clear distinction between tone and context presentations remained intact.

Freezing levels of the Unpaired group were remarkably lower than those of the Paired group, in both tests. In theory, we might have expected higher freezing in test 2, since the Unpaired animals previously received unsignalled shocks in this context. However, we hypothesise that for context conditioning, the protocol was not strong enough anymore. This could explain the remarkably low freezing levels during both tests in comparison with the Paired group, as well as why we observed no noteworthy elevation of freezing levels from test 1 to test 2 in the Unpaired group.

Surprisingly, the animals of the Unpaired group showed higher freezing levels during tone than context presentations in test 1. A possible explanation may be that the animals considered the tone to be a unique feature of their training context, causing them to generalise their contextual anxiety to this new context.

In general, freezing levels were lower in Experiment 2 than in Experiment 1, which suggests that we successfully lowered general stress levels by modifying our protocol.

3. Experiment 3

With the optimised protocol obtained in Experiment 2, we conducted a final experiment in which we applied BST or sham lesions 27 h after training. In all animals, freezing levels were significantly higher during 'tone' than 'before tone' during the training session, which indicated that cue conditioning was successful. After training, rats were assigned to 'Sham' or 'Lesion' groups so that freezing levels during training were comparable.

In test 1, freezing levels were low during the acclimation phase in both groups. We indeed expected low contextual fear levels since the rats were trained in a perceptually different context. After acclimation, both the Lesion and the Sham group clearly distinguished between tone and context presentations, which indicates successful cued fear conditioning. Freezing responses to tone and context presentations were similar in Lesion and Sham rats, suggesting that BST lesions did not affect the cued fear response. However, the nominal freezing values of the lesioned animals were higher in general compared to those of the Sham rats, but this difference was not expected and was not statistically significant. A possible explanation for this phenomenon may be that some Lesion animals appeared to be sleeping towards the end of the test session. While these rats were active and grooming in the beginning of test 1, they became more and more relaxed towards the end. High

freezing scores may thus have been wrongly allocated to animals that were sleeping instead. In Fig. 13A 'test 1: per CS', this translates to rising freezing levels towards the end of the test session. When the freezing values of the rats that were sleeping (according to an observer blinded to group allocation) were corrected and set to zero, this apparent difference in freezing between Lesion and Sham rats disappeared.

Low freezing scores during acclimation in test 2 indicate low contextual anxiety and general stress. Since test 2 took place in the same context as training, these low values may indicate that conditioning to the tone was practically unconfounded by context conditioning, since the context itself was not experienced as aversive. As in test 1, results indicate a good discrimination between tone and context presentations in both the Sham and Lesion group.

In a separate result section, we directly compared two behavioural measurements of contextual freezing, either 'between tones' or immediately 'before tone'. In addition, in Appendix *VI.2*, we discuss a retrospective analysis of contextual anxiety measurements applied to the experiments of Luyck et al. [74]. We hypothesised that 'before tone' would be less influenced by previous tone presentation and would therefore reveal lower values. In line with this hypothesis, nominal freezing values of 'before tone' were indeed lower than those of 'between tones'. Interestingly, in Experiment 3 of this thesis, 'before tone' values were significantly lower than 'between tones' freezing levels in test 2 ($p=0.001$), suggesting that the 'before tone' measurement is indeed a contextual anxiety measurement that is less influenced by cued fear responses.

Finally, we note that one rat was excluded from test 2 due to it being a significant outlier (see Appendix *VI.3*). Also in prior studies [74], sham rats are sometimes identified as significant outliers due to high freezing levels, while this is not the case in animals with BST lesions. We hypothesise that some sham rats are not able to discriminate well between the tone and the context, leading to high levels of contextual anxiety upon re-exposure to the training context [68, 81]. It is noteworthy that this particular rat was not an outlier for test 1, meaning that the animal did attach predictive value to the tone in a context different than the one used for training.

4. General discussion

In this master's thesis, we continued to investigate the role of the BST, following up on previous experiments in our lab that suggested a role of the BST in cued fear [74], in contrast to much of the existing literature [2, 18, 40-42, 44, 46]. In this published study, electrolytic BST lesions significantly disrupted fear-potentiated startle in rats conditioned to a 10-s tone. In a retrospective analysis, we noticed that the presentation of startle probes during the habituation phase elicited rather high freezing levels, that continued to be heightened during the other test sessions of the behavioural conditioning protocol (see Appendix *VI.1*). In addition, contextual freezing levels during post-test were exceptionally high – even in the Sham group the difference with freezing to the conditioned tone was not significant. Even though in terms of startle responses, Sham rats could distinguish between tone and context presentations, these high freezing levels suggest increased general stress levels, which might interfere with cued fear measurements. This hypothesis becomes even more relevant as the BST is known to play a key role in general stress responses [28]. Therefore, we hypothesised that the cued fear responses in Lesion animals (in terms of startle response) were not disrupted because the BST would play a role in cued fear per se, but because BST lesions lowered levels of general stress that overshadowed the cued fear response (see Appendix *VI.2*).

To evaluate this hypothesis, we adapted the protocol of Luyck et al. to make it less aversive and to elicit less general stress. To this end, we performed two protocol optimisation experiments in naïve animals, in which we e.g. removed all acoustic startle probes as well as the pre-test session, and reduced the number and amplitude of foot shocks presented during the training session. As the protocol of the second experiment elicited significant cued fear results (and low general stress), we proceeded with a lesion experiment. The results of this third and last experiment indicated no effect of BST lesions on the expression of cued fear, in line with the majority of the existing literature.

In what follows, we address some key findings and concerns related to the experiments conducted in this master's thesis.

Freezing vs. startle

First, we address the remarkable difference between startle and freezing outcomes in the experiment by Luyck et al., which formed the basis for the current master's thesis. Based on startle responses, Sham animals showed intact cued fear responses, but Lesion rats did not. In terms of freezing however, neither Sham nor Lesions animals showed cued fear responses (no significant difference with contextual anxiety measures).

First of all, these data show that more information can be derived from combining startle and freezing indices, than from one single measurement. If Luyck et al. only evaluated startle measurements, they could have concluded that cued fear conditioning was very effective and pure. Had they only looked at freezing, no cued fear responses would have been detected. In addition, freezing and startle represent opposite motor responses, thereby excluding possible effects on motor behaviour. Whereas freezing suppresses movement, usually over longer time periods (e.g. to avoid being noticed by a predator), startle reflects an almost instantaneous motor response. Moreover, freezing responses may be more sensitive to the strength of aversive conditioning, due to it being a 'passive' response, in comparison with the 'active' startle response, and may therefore require a lower threshold of fear [79, 82, 83]. This could explain why startle responses rendered pure cued fear results, while freezing levels may have been too high to allow for a distinction between tone and context.

In previous studies as well, discrepancies between startle and freezing responses have been found. In a study by Luyten et al. (2011) for example, contextual conditioning with different numbers of unpaired CS-US presentations all resulted in a significant increase in contextual freezing (on both test days), but not in startle responses [84]. Another study applied median raphe nucleus (MRN) electrolytic lesions or injections of 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino) tetralin, a 5-HT_{1A} receptor agonist) one day after training. While lesions or injections did not affect an increase in fear-potentiated startle after training with paired CS-US presentations, they did reduce freezing responses [85]. Besides the conclusion of Silva et al. on the involvement of the MRN in these behavioural responses, it is interesting to notice that freezing and startle seem to be recruited by dissociable systems. Earlier, Walker et al. (2003) noted that the neuronal pathways mediating freezing and startle responses overlap largely, but ultimately diverge to two different brain stem regions [40]. For more information the startle reflex neurocircuitry, we refer the reader to a study by Davis et al. 1982 [86].

Importance of general stress in cued fear paradigms

As stated before, the current data appear to contradict the results previously obtained in our lab by Luyck et al. Nevertheless, in this publication, it was already hypothesised that the disruption of cued fear responses in lesioned animals might not be due to a (direct) role of the BST in cued fear,

but rather to its role in general stress. Since all rats showed rather high contextual freezing levels in the experiment of Luyck et al., we suggested that this general stress overshadowed cued fear responses and that BST lesions disrupted these stress responses, instead of the cued fear responses (see Appendix *VI.2*). In this master's thesis, we specifically developed a protocol for cued fear conditioning without causing excessive arousal, to ensure that our cued fear data would not be influenced by general stress, and to obtain untainted phasic fear responses, in terms of freezing. Together, the retrospective analyses of the experiment of Luyck et al. (see Appendix *VI.1* and *VI.2*) and the results of the third experiment conducted in this master's thesis, suggest that indeed the results previously obtained by Luyck et al. were most likely due to heightened stress levels.

In this light, our results demonstrate the importance of general stress levels when conducting cued fear experiments. Many researchers that carry out cued fear experiments do not check contextual anxiety or general stress levels in between their cued fear measurements. We would recommend optimising cued fear protocols to reduce general stress levels, before drawing any conclusions on e.g. the effect of certain interventions (such as brain lesions) on cued fear levels. This is especially of importance when the distinction between phasic fear and sustained anxiety is being investigated, which is highly pertinent in the field of BST research. In summary, the results of this thesis emphasise the relevance of extensive protocol validation, since slight adjustments to the conditioning paradigm could considerably influence behavioural outcome.

The role of the BST in general stress and (pathological) anxiety

Furthermore, our results are in line with the important role of the BST in general stress responses. As mentioned before, the BST plays an important role in the regulation of the HPA-axis during stress [28, 87, 88]. The BST can be described as an important extrahypothalamic centre that relays and integrates limbic and autonomic information related to stress responses [87]. It plays a key physiological role in autonomic and neuroendocrine adjustments elicited by aversive threat and physical exercise, as well as in the integration of cardiovascular responses [28]. In fact, different divisions of the BST differentially regulate HPA-axis activity [89]. The anterior BST (which was our lesion target) promotes, and the posterior BST inhibits HPA-axis activity, respectively. The central role of the BST in brain stress networks allows it to have such a modular function.

In addition to its involvement in general, adaptive stress, BST modulation may also mediate the development of pathological stress and anxiety responses. In a study by Bruijnzeel et al. (1999), the BST showed altered reactivity to stressful challenges, that may play a role in long-term sensitisation of neuroendocrine and autonomic responses [90]. Another study showed that chronic psychosocial stress in mice caused chronic activation of regions known to regulate depressive and anxiety-like behaviour, among which the BST. Chronic psychosocial stress is a well-established risk factor for neuropsychiatric diseases, and chronic activation of its underlying neuronal circuits may relate to persistent brain activity modifications or abnormalities. These modifications have a potential relevance for the development of anxiety and depression in humans [91]. Similarly, a study by Daniel et al. (2016) posed that stress or drug abuse could alter BST activity and shift it towards a pathological state [92].

Two other studies pose exaggerated threat perception as the central problem of anxiety disorders, rather than the capacity to inhibit fear, causing defensive behaviours to perturb the activities of normal daily life [9, 93]. Given the role of the BST in threat anticipation, this hypothesis indirectly points out that a dysregulation of the BST may be the cause of multiple anxiety disorders. In line with this, study by Forray et al. (2004) poses that dysregulation of BST functionality may underlie

the pathophysiology of stress-related psychiatric diseases, such as PTSD, addiction and melancholic depression, which are all disorders associated with abnormal responses to stress [87].

In conclusion, these studies pose altered BST activity or BST dysregulation (whether or not triggered by chronic stress exposure) at the centre of stress- or anxiety-related disorders, revealing an entwinement of general stress and anxiety, and a role of the BST in both.

The role of the BST: beyond the conventional model of the extended amygdala

As stated abundantly, the BST plays an essential role in defensive responses to anticipated or actual threats. As defined by Davis and Walker in the early model of the extended amygdala (see *I.3*), the BST is essential in processing complex threats of long duration (=sustained anxiety responses), but not in responses to concrete, short-lasting stimuli (=phasic fear responses).

However, Goode et al. suggested that the BST may mediate defensive responses based on the *temporal unpredictability* of an aversive outcome of an antecedent threat, regardless of the modality or duration of this stimulus [94]. They speculate that the BST is involved in organising fear responses to stimuli with a low temporal predictability, i.e. stimuli that poorly predict *when* danger will occur, no matter the duration, modality, or complexity of those threats. Indeed, in the experiments conducted in this thesis, (contextual) anxiety was modelled by unpaired CS-US presentations, resulting in low temporal predictability of the aversive event (i.e. shock), whereas (cued) fear was modelled by explicitly paired CS-US presentations, leading to a high temporal predictability of aversive events following tone presentation.

Another characteristic of the BST as described in the early model of the extended amygdala, is its *delayed activity* (i.e. slow recruitment) in response to *long-duration threats* (defined as sustained anxiety responses, usually through context conditioning), and not in short-lasting threats (defined as phasic fear responses, e.g. through cue conditioning). In this light, a human imaging study by Choi et al. (2012) showed heightened BST activity during a 0.75-s cue that predicted a shock [71]. Other earlier discussed research (see *I.6 Beyond the model of the extended amygdala*), showed evidence of BST activity immediately in response to a threat [67], in processing longer-duration cues [68] and during the anticipation period between a cue and its coupled unconditioned stimulus [72, 73]. In line with this, a study by Waddell et al. (2006) has shown that cue conditioning with longer-duration cues depends on the BST [95]. Furthermore, the BST seems to be critical in the acquisition of fear to long-duration cues, which seems to indicate some aspects of cognitive processing when long-duration fear cues are used [9]. In summary, these studies show that BST recruitment does not require context conditioning protocols, but that the BST is also involved in the (nearly immediate) processing of relatively short threats, such as tones. We hypothesise that the BST is only involved in processing relatively short cues if these cues (or the following anticipation periods) are long enough to cause temporal unpredictability of the following aversive events. Both in this master's thesis and in the study of Luyck et al. [74], we worked with relatively short tones (10 s). In the study of Luyck et al., BST lesions seemed to disrupt cued fear in animals that showed exceptionally high freezing levels, which we interpreted as high levels of general stress. These continuously high levels of stress may have caused the animals to lose the predictive value of the conditioned tone out of sight and consequently, to chronically anticipate aversive events (=a key feature of anxiety disorders [10]). The involvement of the BST in the disruption of cued fear responses may thus be due to the unpredictability of aversive events (even though the animals underwent a paired protocol), independently of the duration of the conditioned tone.

Another issue with the role of the BST in sustained anxiety, is that sustained anxiety commonly is described as being caused by *diffuse or vague stimuli*. However, this idea is in contrast with the

fact that contextual anxiety depends on the recognition of a multitude of specific features of the conditioned context [9]. In addition, multiple studies described in the previous paragraph show that the BST is also entrained upon presentation of a specific cue [68, 71-73, 95], not just during exposure to a conditioned context. On a side note, we state that that, even though all freezing levels of the Unpaired group were fairly low in both test sessions in Experiment 2, the animals froze more during tone than during context presentations. We hypothesised that the tone was a particular feature of the training context (=Context B) the animals remembered and used to generalise their contextual anxiety to Context A.

In summary, we suggest that the generally accepted view on the role of the BST in sustained anxiety responses, describing a (delayed) role of the BST in vague, long-duration threats, might need some modifications. We suggest an (immediate) role of the BST during anticipation of unpredictable threats, rather than in responses to diffuse and long-duration threats. Hence, in addition to responses to threats of longer duration, the BST may also be activated in reactions to short, discrete (but unpredictable) threats. This nuance on the role of the BST may explain certain research results not in line with the conventional early model of the extended amygdala (e.g., see [67, 68, 71-73]).

Future perspectives

The results obtained in this master's thesis contradict our previous findings regarding a role of the BST in cued fear, and underscore its role in general stress responses. Towards future experiments, we especially emphasise the importance of extensive protocol validation in cued fear conditioning.

While we only used freezing in our validated protocol, we appreciate that other researchers may prefer startle as a behavioural outcome, or would wish to combine startle and freezing measurements. By combining both measurements, more information can be obtained on the state of fear or anxiety compared to single measurements. In addition, it rules out the possibility that motor deficits are at the basis of the measured behavioural indices, as freezing and startle reflect contrasting motor responses. A possible approach to combining both startle and freezing measurements is to re-introduce acoustic startles in the protocol, while making them less stressful and aversive, e.g. by reducing the number or loudness of startle presentations. We once again stress the importance of keeping an eye on general stress levels during the development of an adapted protocol. Another interesting option could be to make use of air puffs instead of startle probes [96-98]. Since air puffs have been proven to be less aversive than acoustic startles [75], they might serve as a valuable alternative to evoke a startle response, as they will trigger less general stress.

Future experiments may also focus on the exact role of the BST in (pathological) stress and anxiety responses, e.g. by further investigating the coherence between well-established role of the BST in general stress responses, in anxiety, and in stress- and anxiety-related disorders. While we note that it is difficult to fully untangle stress and fear/anxiety responses, the protocol modifications described in this thesis are a first step in the right direction.

It may also be interesting to revise the theoretical definition of fear and anxiety described by Davis and Walker, in the light of recent findings. Rather than focussing on a distinction between fear and anxiety based on the duration of the response (phasic and sustained, respectively), future research could focus on the role of the BST in processing unpredictable threats, that may be short-lasting and with discrete features, rather than in vague long-lasting threats by definition.

V. Conclusion

In conclusion, the results discussed in this master's thesis indicate that the BST is not involved in cued fear, as electrolytic post-training lesions did not disrupt freezing to a cue conditioned stimulus in rats. This result is in line with a multitude of research and the generally accepted view on the role of the BST in anxiety, rather than in fear. It is however not consistent with previous data obtained in our lab, where electrolytic post-training lesions of the BST were shown to block fear-potentiated startle. As freezing levels were exceptionally high in this experiment, this master's thesis set out to optimise the cued fear protocol used in this study, to elicit as little general stress as possible and thus obtain optimal cued fear expression. By implementing such an improved protocol, BST lesions no longer disrupted cued fear responses. Therefore, we suggest that our previous findings can be largely attributed to a role of the BST in general stress, rather than in cued fear. This thesis demonstrates the importance of thoroughly examining behavioural conditioning protocols before drawing conclusions, since subtle chances in the protocol may lead to major changes in behavioural outcome.

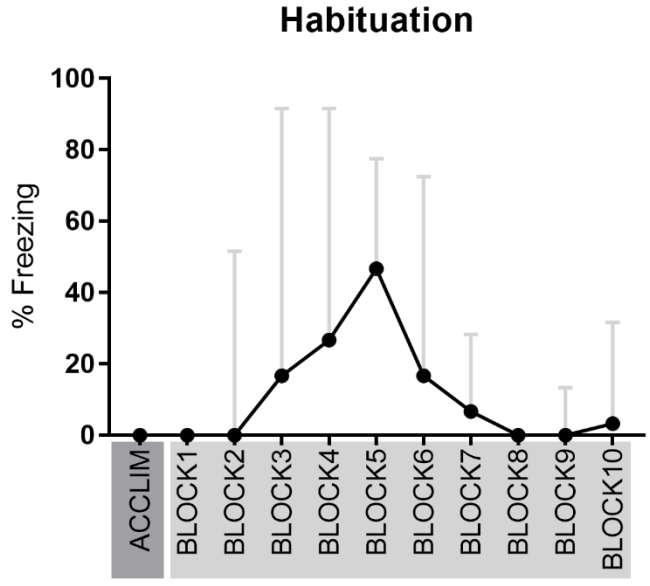
VI. Appendix

1. Acoustic startle probes: a source of general stress?

As described by Lissek et al., acoustic startle probes can be rather aversive by nature, and might elicit considerable physiological arousal [75]. To evaluate how aversive the acoustic startle probes used in our previous lesion experiments are, we decided to review freezing behaviour in a subset of animals of Experiment 3 in the paper by Luyck et al. (2018) [74] (see Fig. 3 for an overview of the experimental design or *I.7* for a description of the experiment). This novel analysis brought to light that the acoustic startle probes elicit unexpectedly high freezing responses during habituation, i.e. even before the start of the conditioning procedure.

In the experiments described by Luyck et al., the behavioural conditioning paradigm started with a habituation session of 20 min, during which acoustic startles were introduced after a 5 min-acclimation phase, to habituate the animals to the startle probes. Freezing was measured during acclimation (5 min) and during the 10-s intervals preceding each startle probe. No freezing was detected during the acclimation phase (Suppl. Fig. 1). However, a strong increase in freezing was observed after presentations of the startle probes started, with peaks up to 46.7% (median). A paired t-test showed that freezing levels were significantly higher when startle probes are administered (with an average of 22.8% ± 21.0%, mean ± SD), than during acclimation (0.0% ± 0.0%) (t_8=3.26; p=0.01). As discussed in *I.8 Aim of the study*, some response to these novel, rather intrusive stimuli is expected, which is why the habituation phase was included in the first place. However, an increase in freezing to this extent was rather surprising.

Toward the end of the habituation session, freezing seemed to decrease. Even then, freezing levels remained high in some rats, that seemed to be more sensitive to the startles than others. Animals that showed strong overall freezing upon startle presentation during habituation, also showed higher freezing levels during pre-test, as soon as startle probes were administered, but not during pre-test acclimation. These data suggest that the startles itself are aversive, to the extent that they could significantly influence the general stress response. In this sense, it is possible that the results of Luyck et al. can be (partly) attributed to a reduction of the general stress response, rather than a direct disruption of cued fear.



Supplementary figure 1: Percentage freezing during the habituation phase of Experiment 3 by Luyck et al. (2018). Data are represented as medial values + the upper limits of the interquartile range (n=9). The different blocks represent average freezing values in the 10-s intervals preceding three consecutive startle probes. Acclim = acclimation.

2. Measuring contextual anxiety in a cued fear paradigm

In this appendix, we performed a retrospective analysis of Experiment 3 described by Luyck et al. (2018) [74] (see *I.7* for a description of the experiments). The aim of this analysis was to directly compare two measurements of contextual anxiety: 'between tones' (cf. Luyck et al.) or 'before tone'.

As described earlier (see *I.7*), the behavioural protocol of the cued fear experiment by Luyck et al. consisted of a habituation phase, a pre-test, a training session, and a post-test. During pre- and post-test, rats were presented with 30 acoustic startle probes. Startle amplitude during half of the probes served as a measurement of contextual anxiety, while the other 15 probes were preceded by the tone to which the animals were conditioned, and served as a measurement of cued fear. Freezing during the 10-s interval preceding the startle probes was used as an additional measurement of cued fear (during the tone preceding half of the startle probes) or of contextual anxiety (during the tone-free interval preceding the other 15 startle probes, = 'between tones' measurement).

Ideally, presentation of a (conditioned) tone should influence the measurements of contextual fear as little as possible. This can be achieved by increasing the interval between the tone and the following contextual fear measurement to the fullest, i.e. by measuring freezing immediately before presentation of the next tone (= 'before tone' measurement). We hypothesised that the latter measurement would be less confounded by previous tone presentation and therefore reveal lower freezing levels. In this retrospective analysis, we therefore measured contextual freezing during 10-s intervals preceding each tone (= 'before tone' measurement), to make a comparison between 'before tone' and 'between tones' possible. Note that freezing 'between tones' and during 'tone' for this new analysis were both scored by the same blinded observer.

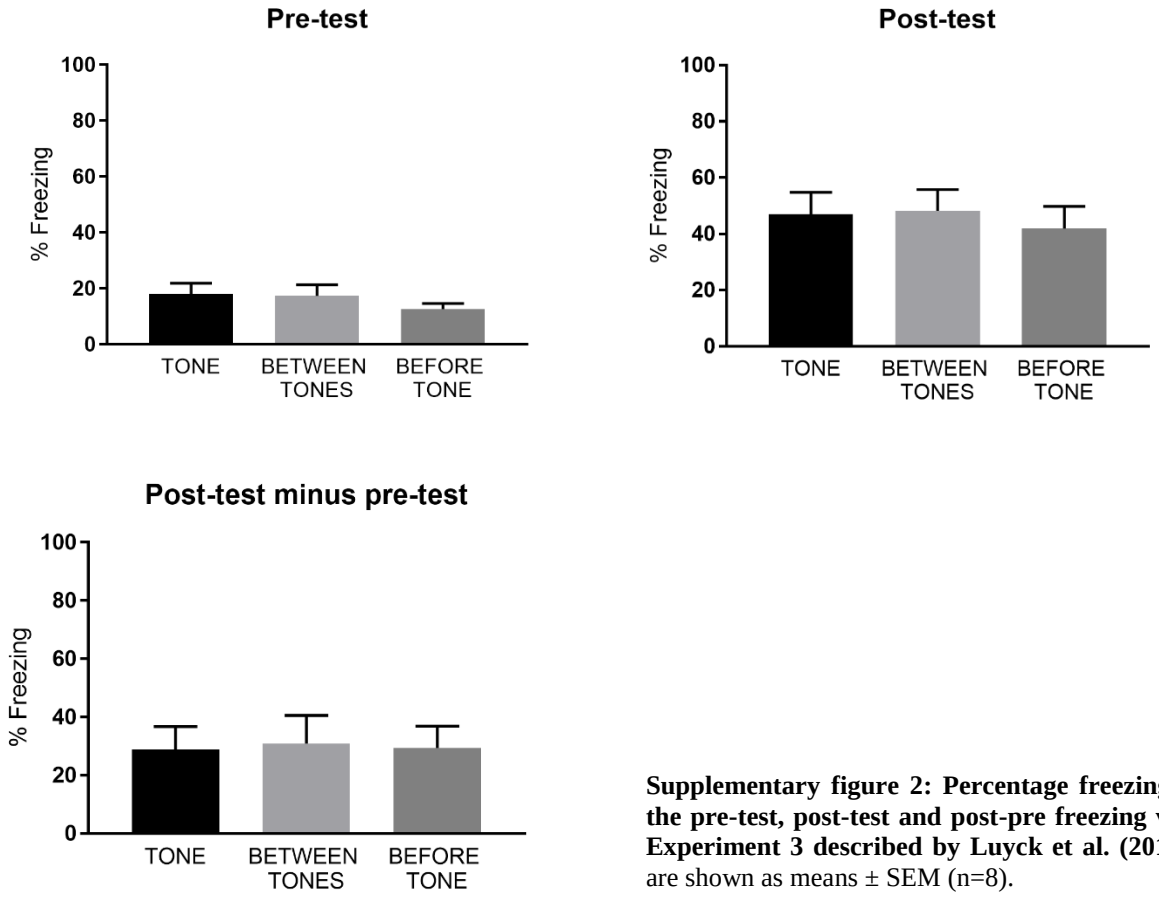
In Experiment 2 and 3 by Luyck et al., Sham animals displayed strong cued fear in terms of startle response, but not in terms of freezing. This may be due to high overall freezing levels, so that no significant difference between 'between tones' and during 'tone' could be made. By using 'before tone' as a measurement of contextual anxiety instead of 'between tones', lower freezing levels during context presentation could lead to the detection of cued fear in terms of freezing (revealing a significant difference between 'before tone' and during 'tone'), in line with the startle results.

A parametric one-way ANOVA was performed on pre-test, post-test and on difference scores between pre- and post-test (from here on referred to as 'post-pre' values) of Experiment 3 (Suppl. Fig. 2). The factors used were 'before tone', 'between tone', and 'tone'. Note that the 'tone' measurement was included to evaluate cued fear with respect to both contextual measurements. One animal was a significant outlier in the pre-test (Z-value=2.52; $p<0.05$, critical Z-value=2.22) and was therefore excluded from all analyses (see Appendix *VI.3*).

While nominal freezing values post-pre appeared lower 'before tone' (29.3% ± 21.4%, mean ± SD) compared to 'between tones' (30.9% ± 27.4%), no significant differences were found ($F_{(1.72, 12.04)}=0.08$; $p=0.90$). Similar results were obtained for separate analyses of pre-test ($F_{(1.73, 12.12)}=1.04$; $p=0.37$) and post-test ($F_{(1.30, 9.14)}=1.20$; $p=0.32$).

We hypothesised that 'before tone' measurements would be less influenced by previous tone presentation than 'between tones' measurements. Although we did not obtain significant differences between both contextual measurements, nominal values of 'before tone' intervals were lower compared to those of 'between tones', which is in line with our hypothesis. Therefore, we chose to include the 'before tone' interval as our main measurement of contextual anxiety in this thesis. In addition, a separate section of the results was devoted to a direct comparison between both context measurements for each experiment, to allow for a better analogy with Luyck et al.

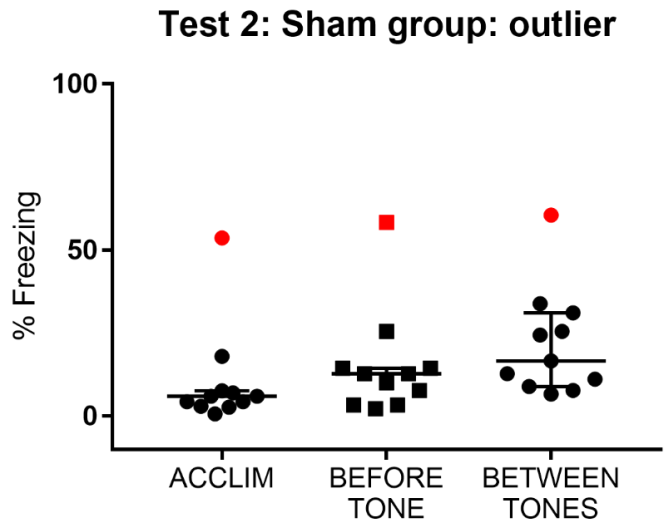
Finally, we once again emphasize the fact that in Luyck et al. (2018) the animals failed to discriminate between tone and (both) tone-free intervals, in terms of freezing behaviour. This suggests that general stress levels were high and might have overshadowed the cued fear measurements in this study, thereby highlighting the relevance of the protocol used for conditioning, and thus of the experiments conducted in this thesis.



Supplementary figure 2: Percentage freezing during the pre-test, post-test and post-pre freezing values of Experiment 3 described by Luyck et al. (2018). Data are shown as means ± SEM (n=8).

3. Visual depiction of outlier analysis in Experiment 3

We analysed all experimental data that did not pass the normality test on outliers using a Grubbs test. In Experiment 3, one Sham rat formed a significant outlier in all measurements of test 2 (Suppl. Fig. 3), and was therefore excluded from this test ($Z=2.88$ during acclimation, $Z=2.73$ 'before tone', $Z=2.42$ 'between tones'; $p<0.05$, critical Z-value=2.35). These data indicate that this excluded rat showed excessive contextual freezing responses. Since test 2 took place in the same context in which the rats were trained, it seems that this rat attached too much importance to the experimental context, while the tone was in fact the most important predictor of foot shocks.



Supplementary figure 3: Indication of an outlier during different phases of test 2 in Experiment 3. One rat of the Sham group was identified as a significant outlier for all contextual freezing measurements in test 2. All data points related to this animal are indicated in red. Data are shown as median ± IQR (n=11). Acclim = acclimation.

VII. References

1. Munir, S. and J. Hughes, *Anxiety, Generalized Anxiety Disorder (GAD)*, in *StatPearls*. 2017: Treasure Island (FL).
2. Davis, M., D.L. Walker, L. Miles, and C. Grillon, *Phasic vs sustained fear in rats and humans: role of the extended amygdala in fear vs anxiety.* Neuropsychopharmacology, 2010. **35**(1): p. 105-35.
3. American Psychiatric Association, *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. 2013, Washington, DC.
4. Luyten, L., D. Vansteenwegen, K. van Kuyck, L. Gabriels, and B. Nuttin, *Contextual conditioning in rats as an animal model for generalized anxiety disorder.* Cogn Affect Behav Neurosci, 2011. **11**(2): p. 228-44.
5. Grillon, C., J.M. Baas, B. Cornwell, and L. Johnson, *Context conditioning and behavioral avoidance in a virtual reality environment: effect of predictability.* Biol Psychiatry, 2006. **60**(7): p. 752-9.
6. Korte, S.M. and S.F. De Boer, *A robust animal model of state anxiety: fear-potentiated behaviour in the elevated plus-maze.* Eur J Pharmacol, 2003. **463**(1-3): p. 163-75.
7. Carola, V., F. D'Olimpio, E. Brunamonti, F. Mangia, and P. Renzi, *Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice.* Behav Brain Res, 2002. **134**(1-2): p. 49-57.
8. Walker, D.L. and M. Davis, *Double dissociation between the involvement of the bed nucleus of the stria terminalis and the central nucleus of the amygdala in startle increases produced by conditioned versus unconditioned fear.* J Neurosci, 1997. **17**(23): p. 9375-83.
9. Perusini, J.N. and M.S. Fanselow, *Neurobehavioral perspectives on the distinction between fear and anxiety.* Learn Mem, 2015. **22**(9): p. 417-25.
10. Mineka, S. and R. Zinbarg, *A contemporary learning theory perspective on the etiology of anxiety disorders: it's not what you thought it was.* Am Psychol, 2006. **61**(1): p. 10-26.
11. Alheid, G.F., C.A. Beltramino, J.S. De Olmos, M.S. Forbes, D.J. Swanson, and L. Heimer, *The neuronal organization of the supracapsular part of the stria terminalis in the rat: the dorsal component of the extended amygdala.* Neuroscience, 1998. **84**(4): p. 967-96.
12. Alheid, G.F. and L. Heimer, *New perspectives in basal forebrain organization of special relevance for neuropsychiatric disorders: the striatopallidal, amygdaloid, and corticopetal components of substantia innominata.* Neuroscience, 1988. **27**(1): p. 1-39.
13. Shackman, A.J. and A.S. Fox, *Contributions of the Central Extended Amygdala to Fear and Anxiety.* J Neurosci, 2016. **36**(31): p. 8050-63.
14. Lee, Y. and M. Davis, *Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex.* J Neurosci, 1997. **17**(16): p. 6434-46.
15. Lee, Y. and M. Davis, *Role of the septum in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex.* J Neurosci, 1997. **17**(16): p. 6424-33.
16. Gungor, N.Z. and D. Pare, *Functional Heterogeneity in the Bed Nucleus of the Stria Terminalis.* J Neurosci, 2016. **36**(31): p. 8038-49.
17. Luyten, L., K. van Kuyck, D. Vansteenwegen, and B. Nuttin, *Electrolytic lesions of the bed nucleus of the stria terminalis disrupt freezing and startle potentiation in a conditioned context.* Behav Brain Res, 2011. **222**(2): p. 357-62.

18. Sullivan, G.M., J. Apergis, D.E. Bush, L.R. Johnson, M. Hou, and J.E. Ledoux, *Lesions in the bed nucleus of the stria terminalis disrupt corticosterone and freezing responses elicited by a contextual but not by a specific cue-conditioned fear stimulus.* Neuroscience, 2004. **128**(1): p. 7-14.
19. Meloni, E.G., A. Jackson, L.P. Gerety, B.M. Cohen, and W.A. Carlezon, Jr., *Role of the bed nucleus of the stria terminalis (BST) in the expression of conditioned fear.* Ann N Y Acad Sci, 2006. **1071**: p. 538-41.
20. Dumont É, C., *What is the bed nucleus of the stria terminalis?* Prog Neuropsychopharmacol Biol Psychiatry, 2009. **33**(8): p. 1289-90.
21. LeDoux, J.E., J. Iwata, P. Cicchetti, and D.J. Reis, *Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear.* J Neurosci, 1988. **8**(7): p. 2517-29.
22. Krettek, J.E. and J.L. Price, *Amygdaloid projections to subcortical structures within the basal forebrain and brainstem in the rat and cat.* J Comp Neurol, 1978. **178**(2): p. 225-54.
23. Dong, H.W. and L.W. Swanson, *Projections from bed nuclei of the stria terminalis, posterior division: implications for cerebral hemisphere regulation of defensive and reproductive behaviors.* J Comp Neurol, 2004. **471**(4): p. 396-433.
24. Newman, S.W., *The medial extended amygdala in male reproductive behavior. A node in the mammalian social behavior network.* Ann N Y Acad Sci, 1999. **877**: p. 242-57.
25. Simerly, R.B., *Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain.* Annu Rev Neurosci, 2002. **25**: p. 507-36.
26. Paxinos, G. and C. Watson, *The rat brain in stereotaxic coordinates*. 5th ed. 2005, Amsterdam: Elsevier Academic.
27. Sun, N. and M.D. Cassell, *Intrinsic GABAergic neurons in the rat central extended amygdala.* J Comp Neurol, 1993. **330**(3): p. 381-404.
28. Crestani, C.C., F.H. Alves, F.V. Gomes, L.B. Resstel, F.M. Correa, and J.P. Herman, *Mechanisms in the bed nucleus of the stria terminalis involved in control of autonomic and neuroendocrine functions: a review.* Curr Neuropharmacol, 2013. **11**(2): p. 141-59.
29. Levita, L., S.E. Hammack, I. Mania, X.Y. Li, M. Davis, and D.G. Rainnie, *5-hydroxytryptamine1A-like receptor activation in the bed nucleus of the stria terminalis: electrophysiological and behavioral studies.* Neuroscience, 2004. **128**(3): p. 583-96.
30. Rainnie, D.G., *Neurons of the bed nucleus of the stria terminalis (BNST). Electrophysiological properties and their response to serotonin.* Ann N Y Acad Sci, 1999. **877**: p. 695-9.
31. Guo, J.D., S.E. Hammack, R. Hazra, L. Levita, and D.G. Rainnie, *Bi-directional modulation of bed nucleus of stria terminalis neurons by 5-HT: molecular expression and functional properties of excitatory 5-HT receptor subtypes.* Neuroscience, 2009. **164**(4): p. 1776-93.
32. Hammack, S.E., J.D. Guo, R. Hazra, J. Dabrowska, K.M. Myers, and D.G. Rainnie, *The response of neurons in the bed nucleus of the stria terminalis to serotonin: implications for anxiety.* Prog Neuropsychopharmacol Biol Psychiatry, 2009. **33**(8): p. 1309-20.
33. Dabrowska, J., R. Hazra, J.D. Guo, S. Dewitt, and D.G. Rainnie, *Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis.* Front Neurosci, 2013. **7**: p. 156.

34. Dong, H.W., G.D. Petrovich, A.G. Watts, and L.W. Swanson, *Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain.* J Comp Neurol, 2001. **436**(4): p. 430-55.
35. Phelix, C.F. and W.K. Paull, *Demonstration of distinct corticotropin releasing factor--containing neuron populations in the bed nucleus of the stria terminalis. A light and electron microscopic immunocytochemical study in the rat.* Histochemistry, 1990. **94**(4): p. 345-64.
36. Cummings, S., R. Elde, J. Ells, and A. Lindall, *Corticotropin-releasing factor immunoreactivity is widely distributed within the central nervous system of the rat: an immunohistochemical study.* J Neurosci, 1983. **3**(7): p. 1355-68.
37. Turesson, H.K., O.E. Rodriguez-Sierra, and D. Pare, *Intrinsic connections in the anterior part of the bed nucleus of the stria terminalis.* J Neurophysiol, 2013. **109**(10): p. 2438-50.
38. Kim, S.Y., A. Adhikari, S.Y. Lee, J.H. Marshel, C.K. Kim, C.S. Mallory, M. Lo, S. Pak, J. Mattis, B.K. Lim, R.C. Malenka, M.R. Warden, R. Neve, K.M. Tye, and K. Deisseroth, *Diverging neural pathways assemble a behavioural state from separable features in anxiety.* Nature, 2013. **496**(7444): p. 219-23.
39. Jennings, J.H., D.R. Sparta, A.M. Stamatakis, R.L. Ung, K.E. Pleil, T.L. Kash, and G.D. Stuber, *Distinct extended amygdala circuits for divergent motivational states.* Nature, 2013. **496**(7444): p. 224-8.
40. Walker, D.L., D.J. Toufexis, and M. Davis, *Role of the bed nucleus of the stria terminalis versus the amygdala in fear, stress, and anxiety.* Eur J Pharmacol, 2003. **463**(1-3): p. 199-216.
41. Luyten, L., C. Casteels, D. Vansteenwegen, K. van Kuyck, M. Koole, K. Van Laere, and B. Nuttin, *Micro-positron emission tomography imaging of rat brain metabolism during expression of contextual conditioning.* J Neurosci, 2012. **32**(1): p. 254-63.
42. Zimmerman, J.M. and S. Maren, *The bed nucleus of the stria terminalis is required for the expression of contextual but not auditory freezing in rats with basolateral amygdala lesions.* Neurobiol Learn Mem, 2011. **95**(2): p. 199-205.
43. Zimmerman, J.M., C.A. Rabinak, I.G. McLachlan, and S. Maren, *The central nucleus of the amygdala is essential for acquiring and expressing conditional fear after overtraining.* Learn Mem, 2007. **14**(9): p. 634-44.
44. Alvarez, R.P., G. Chen, J. Bodurka, R. Kaplan, and C. Grillon, *Phasic and sustained fear in humans elicits distinct patterns of brain activity.* Neuroimage, 2011. **55**(1): p. 389-400.
45. Straube, T., H.J. Mentzel, and W.H. Miltner, *Waiting for spiders: brain activation during anticipatory anxiety in spider phobics.* Neuroimage, 2007. **37**(4): p. 1427-36.
46. Munsterkotter, A.L., S. Notzon, R. Redlich, D. Grotegerd, K. Dohm, V. Arolt, H. Kugel, P. Zwanzger, and U. Dannlowski, *Spider or No Spider? Neural Correlates of Sustained and Phasic Fear in Spider Phobia.* Depress Anxiety, 2015. **32**(9): p. 656-63.
47. Bounds, C.G. and V.L. Nelson, *Benzodiazepines*, in *StatPearls*. 2018, StatPearls Publishing LLC.: Treasure Island (FL).
48. Wang, Z., S.P.H. Whiteside, L. Sim, W. Farah, A.S. Morrow, M. Alsawas, P. Barrionuevo, M. Tello, N. Asi, B. Beuschel, L. Daraz, J. Almasri, F. Zaiem, L. Larrea-Mantilla, O.J. Ponce, A. LeBlanc, L.J. Prokop, and M.H. Murad, *Comparative Effectiveness and Safety of Cognitive Behavioral Therapy and Pharmacotherapy for Childhood Anxiety Disorders: A Systematic Review and Meta-analysis.* JAMA Pediatr, 2017. **171**(11): p. 1049-1056.
49. Bandelow, B., S. Michaelis, and D. Wedekind, *Treatment of anxiety disorders.* Dialogues Clin Neurosci, 2017. **19**(2): p. 93-107.

50. Thibaut, F., *Anxiety disorders: a review of current literature.* Dialogues Clin Neurosci, 2017. **19**(2): p. 87-88.
51. Patel, D.R., C. Feucht, K. Brown, and J. Ramsay, *Pharmacological treatment of anxiety disorders in children and adolescents: a review for practitioners.* Transl Pediatr, 2018. **7**(1): p. 23-35.
52. Tolin, D.F., *Can Cognitive Behavioral Therapy for Anxiety and Depression Be Improved with Pharmacotherapy? A Meta-analysis.* Psychiatr Clin North Am, 2017. **40**(4): p. 715-738.
53. Maron, E. and D. Nutt, *Biological predictors of pharmacological therapy in anxiety disorders.* Dialogues Clin Neurosci, 2015. **17**(3): p. 305-17.
54. Pedrosa-Sanchez, M. and R.G. Sola, *[Modern day psychosurgery: a new approach to neurosurgery in psychiatric disease]*. Rev Neurol, 2003. **36**(9): p. 887-97.
55. Harat, M. and M. Rudas, *[Surgical treatment of mental disorders]*. Psychiatr Pol, 2002. **36**(5): p. 793-804.
56. Harat, M., M. Rudas, and J. Rybakowski, *Psychosurgery: the past and present of ablation procedures.* Neuro Endocrinol Lett, 2008. **29 Suppl 1**: p. 105-22.
57. Leiphart, J.W. and F.H. Valone, 3rd, *Stereotactic lesions for the treatment of psychiatric disorders.* J Neurosurg, 2010. **113**(6): p. 1204-11.
58. Benabid, A.L. and N. Torres, *New targets for DBS.* Parkinsonism Relat Disord, 2012. **18 Suppl 1**: p. S21-3.
59. Nuttin, B., P. Cosyns, H. Demeulemeester, J. Gybels, and B. Meyerson, *Electrical stimulation in anterior limbs of internal capsules in patients with obsessive-compulsive disorder.* Lancet, 1999. **354**(9189): p. 1526.
60. Luyten, L., S. Hendrickx, S. Raymaekers, L. Gabriels, and B. Nuttin, *Electrical stimulation in the bed nucleus of the stria terminalis alleviates severe obsessive-compulsive disorder.* Mol Psychiatry, 2016. **21**(9): p. 1272-80.
61. Greenberg, B.D., L.A. Gabriels, D.A. Malone, Jr., A.R. Rezai, G.M. Friehs, M.S. Okun, N.A. Shapira, K.D. Foote, P.R. Cosyns, C.S. Kubu, P.F. Malloy, S.P. Salloway, J.E. Giftakis, M.T. Rise, A.G. Machado, K.B. Baker, P.H. Stypulkowski, W.K. Goodman, S.A. Rasmussen, and B.J. Nuttin, *Deep brain stimulation of the ventral internal capsule/ventral striatum for obsessive-compulsive disorder: worldwide experience.* Mol Psychiatry, 2010. **15**(1): p. 64-79.
62. Denys, D., M. Mantione, M. Figee, P. van den Munckhof, F. Koerselman, H. Westenberg, A. Bosch, and R. Schuurman, *Deep brain stimulation of the nucleus accumbens for treatment-refractory obsessive-compulsive disorder.* Arch Gen Psychiatry, 2010. **67**(10): p. 1061-8.
63. Luyck, K., T. Tambuyzer, M. Deprez, J. Rangarajan, B. Nuttin, and L. Luyten, *Electrical stimulation of the bed nucleus of the stria terminalis reduces anxiety in a rat model.* Transl Psychiatry, 2017. **7**(2): p. e1033.
64. Haufler, D., F.Z. Nagy, and D. Pare, *Neuronal correlates of fear conditioning in the bed nucleus of the stria terminalis.* Learn Mem, 2013. **20**(11): p. 633-41.
65. Daldrup, T., J. Lesting, P. Meuth, T. Seidenbecher, and H.C. Pape, *Neuronal correlates of sustained fear in the anterolateral part of the bed nucleus of stria terminalis.* Neurobiol Learn Mem, 2016. **131**: p. 137-46.
66. Ravinder, S., N.S. Burghardt, R. Brodsky, E.P. Bauer, and S. Chattarji, *A role for the extended amygdala in the fear-enhancing effects of acute selective serotonin reuptake inhibitor treatment.* Transl Psychiatry, 2013. **3**: p. e209.

67. Hammack, S.E., T.P. Todd, M. Kocho-Schellenberg, and M.E. Bouton, *Role of the bed nucleus of the stria terminalis in the acquisition of contextual fear at long or short context-shock intervals.* Behav Neurosci, 2015. **129**(5): p. 673-8.
68. Duvarci, S., E.P. Bauer, and D. Pare, *The bed nucleus of the stria terminalis mediates inter-individual variations in anxiety and fear.* J Neurosci, 2009. **29**(33): p. 10357-61.
69. Moaddab, M. and J. Dabrowska, *Oxytocin receptor neurotransmission in the dorsolateral bed nucleus of the stria terminalis facilitates the acquisition of cued fear in the fear-potentiated startle paradigm in rats.* Neuropharmacology, 2017. **121**: p. 130-139.
70. Mobbs, D., R. Yu, J.B. Rowe, H. Eich, O. FeldmanHall, and T. Dalgleish, *Neural activity associated with monitoring the oscillating threat value of a tarantula.* Proc Natl Acad Sci U S A, 2010. **107**(47): p. 20582-6.
71. Choi, J.M., S. Padmala, and L. Pessoa, *Impact of state anxiety on the interaction between threat monitoring and cognition.* Neuroimage, 2012. **59**(2): p. 1912-23.
72. Grupe, D.W., D.J. Oathes, and J.B. Nitschke, *Dissecting the anticipation of aversion reveals dissociable neural networks.* Cereb Cortex, 2013. **23**(8): p. 1874-83.
73. Klumpers, F., M.C. Kroes, I. Heitland, D. Everaerd, S.E. Akkermans, R.S. Oosting, G. van Wingen, B. Franke, J.L. Kenemans, G. Fernandez, and J.M. Baas, *Dorsomedial Prefrontal Cortex Mediates the Impact of Serotonin Transporter Linked Polymorphic Region Genotype on Anticipatory Threat Reactions.* Biol Psychiatry, 2015. **78**(8): p. 582-9.
74. Luyck, K., B. Nuttin, and L. Luyten, *Electrolytic post-training lesions of the bed nucleus of the stria terminalis block startle potentiation in a cued fear conditioning procedure.* Brain Structure and Function, 2018.
75. Lissek, S., J.M. Baas, D.S. Pine, K. Orme, S. Dvir, M. Nugent, E. Rosenberger, E. Rawson, and C. Grillon, *Airpuff startle probes: an efficacious and less aversive alternative to white-noise.* Biol Psychol, 2005. **68**(3): p. 283-97.
76. Luyten, L. and F. Van Cappellen, *ExpTimer: timer software to facilitate complex, multi-step procedures.* Journal of Open Research Software, 2013. **1**(1): p. e2.
77. Quinn, V.F. and B. Colagiuri, *Using Learning Strategies to Inhibit the Nocebo Effect.* Int Rev Neurobiol, 2018. **138**: p. 307-327.
78. Lingawi, N.W., R.F. Westbrook, and V. Laurent, *Extinction and Latent Inhibition Involve a Similar Form of Inhibitory Learning that is Stored in and Retrieved from the Infralimbic Cortex.* Cereb Cortex, 2017. **27**(12): p. 5547-5556.
79. Luyten, L., D. Vansteenwegen, K. van Kuyck, D. Deckers, and B. Nuttin, *Optimization of a contextual conditioning protocol for rats using combined measurements of startle amplitude and freezing: the effects of shock intensity and different types of conditioning.* J Neurosci Methods, 2011. **194**(2): p. 305-11.
80. Luyten, L., N. Schroyens, K. Luyck, M.S. Fanselow, and T. Beckers, *No effect of glucose administration in a novel contextual fear generalization protocol in rats.* Transl Psychiatry, 2016. **6**(9): p. e903.
81. Baas, J.M., *Individual differences in predicting aversive events and modulating contextual anxiety in a context and cue conditioning paradigm.* Biol Psychol, 2013. **92**(1): p. 17-25.
82. McNish, K.A., J.C. Gewirtz, and M. Davis, *Evidence of contextual fear after lesions of the hippocampus: a disruption of freezing but not fear-potentiated startle.* J Neurosci, 1997. **17**(23): p. 9353-60.
83. Richardson, R. and G.P. McNally, *Effects of an odor paired with illness on startle, freezing, and analgesia in rats.* Physiol Behav, 2003. **78**(2): p. 213-9.

84. Luyten, L., D. Vansteenwegen, K. Kuyck, and B. Nuttin, *Towards chronic contextual conditioning in rats: the effects of different numbers of unpaired tone-shock presentations on freezing time and startle.* Acta Neurobiol Exp (Wars), 2011. **71**(3): p. 331-8.
85. Silva, R.C., A.C. Gargaro, and M.L. Brandao, *Differential regulation of the expression of contextual freezing and fear-potentiated startle by 5-HT mechanisms of the median raphe nucleus.* Behav Brain Res, 2004. **151**(1-2): p. 93-101.
86. Davis, M., D. Gendelman, M. Tischler, and P. Gendelman, *A primary acoustic startle circuit: lesion and stimulation studies.* The Journal of Neuroscience, 1982. **2**(6): p. 791-805.
87. Forray, M.I. and K. Gysling, *Role of noradrenergic projections to the bed nucleus of the stria terminalis in the regulation of the hypothalamic-pituitary-adrenal axis.* Brain Res Brain Res Rev, 2004. **47**(1-3): p. 145-60.
88. Myers, B., C. Mark Dolgas, J. Kasckow, W.E. Cullinan, and J.P. Herman, *Central stress-integrative circuits: forebrain glutamatergic and GABAergic projections to the dorsomedial hypothalamus, medial preoptic area, and bed nucleus of the stria terminalis.* Brain Struct Funct, 2014. **219**(4): p. 1287-303.
89. Ch'ng, S., J. Fu, R.M. Brown, S.J. McDougall, and A.J. Lawrence, *The intersection of stress and reward: BNST modulation of aversive and appetitive states.* Prog Neuropsychopharmacol Biol Psychiatry, 2018.
90. Bruijnzeel, A.W., R. Stam, J.C. Compaan, G. Croiset, L.M. Akkermans, B. Olivier, and V.M. Wiegant, *Long-term sensitization of Fos-responsivity in the rat central nervous system after a single stressful experience.* Brain Res, 1999. **819**(1-2): p. 15-22.
91. Laine, M.A., E. Sokolowska, M. Dudek, S.A. Callan, P. Hyytia, and I. Hovatta, *Brain activation induced by chronic psychosocial stress in mice.* Sci Rep, 2017. **7**(1): p. 15061.
92. Daniel, S.E. and D.G. Rainnie, *Stress Modulation of Opposing Circuits in the Bed Nucleus of the Stria Terminalis.* Neuropsychopharmacology, 2016. **41**(1): p. 103-25.
93. Grillon, C., *Startle reactivity and anxiety disorders: aversive conditioning, context, and neurobiology.* Biological Psychiatry, 2002. **52**(10): p. 958-975.
94. Goode, T.D. and S. Maren, *Role of the bed nucleus of the stria terminalis in aversive learning and memory.* Learn Mem, 2017. **24**(9): p. 480-491.
95. Waddell, J., R.W. Morris, and M.E. Bouton, *Effects of bed nucleus of the stria terminalis lesions on conditioned anxiety: aversive conditioning with long-duration conditional stimuli and reinstatement of extinguished fear.* Behav Neurosci, 2006. **120**(2): p. 324-36.
96. Brown, K.L. and D.S. Woodruff-Pak, *Eyeblink Conditioning in Animal Models and Humans*, in *Animal Models of Behavioral Analysis*, J. Raber, Editor. 2011, Humana Press: Totowa, NJ. p. 1-27.
97. A. Schmajuk, N. and B. A. Christiansen, *Eyeblink conditioning in rats*. Vol. 48. 1990. 755-8.
98. Curzon, P., N.R. Rustay, and K.E. Browman, *Cued and Contextual Fear Conditioning for Rodents*, in *Methods of Behavior Analysis in Neuroscience*, J.J. Buccafusco, Editor. 2009, CRC Press/Taylor & Francis: Boca Raton (FL).