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BETTER SAFE THAN SORRY? THE ROLE OF THE EXTENDED AMYGDALA CIRCUITRY IN FEAR GENERALIZATION AND ANXIETY Dewi Cornelia Elisabeth Maria van der Geugten

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Better Safe than Sorry?

The role of the Extended Amygdala Circuitry in Fear Generalization and Anxiety

Dewi C.E.M. van der Geugten

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The role of the extended amygdala circuitry in fear generalization and anxiety

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GENERAL INTRODUCTION

Generalization of Fear

Learning to fear situations or cues that are potentially harmful or dangerous is important for survival. However, when this fear becomes excessive and negatively interferes with one's daily functioning, the fear response can be considered dysfunctional and referred to as maladaptive. It is such maladaptive fear and anxiety that can lead to the development of anxiety- and stress-related disorders, such as Generalized Anxiety Disorder (GAD), Panic Disorder (PD), Posttraumatic Stress Disorder (PTSD), and Adjustment Disorder (AD) (Cooper et al., 2022; Dymond et al., 2015; Fraunfelter, Gerdes & Alpers, 2022; Lis et al., 2020; Morey et al., 2015). Characteristic for these disorders is the persistent and excessive worrying, generalization of fear to safe circumstances, and emotional distress, which can lead to functional impairment and a decreased quality of life.

Anxiety- and stress-related disorders are a widespread mental health problem, impacting daily life of millions of people worldwide. The prevalence of these disorders varies substantially depending on the population subgroup, country, and type of disorder. For instance, the National Institute of Mental Health (NIHM) estimates that 6.1-9.2% of adults will develop PTSD in their lifetime in the United States and Canada (Kessler et al., 2005; Van Ameringen et al., 2008; Goldstein et al., 2016), while the World Health Organization (WHO) found a lifetime prevalence of 2.1-2.3 % in upper-middle income and lower-middle income countries (Koenen et al., 2017). These disorders are frequently misdiagnosed, undertreated and underreported, making the actual prevalence higher. The economic costs of anxiety- and stress-related disorders worldwide are significant and include costs such as medical treatment, along with indirect costs such as productivity loss and decreased quality of life. Concerning only PTSD, the economic costs are already considerable, with an annual cost of $232 billion in 2018 within the United States (Davis et al., 2022). The societal costs include increased healthcare utilization and an impairment of social functioning.

A hallmark symptom of anxiety- and stress-related disorders like GAD and PTSD is the generalization of fear towards safe situations (Cooper et al., 2022; Dymond et al., 2015; Fraunfelter, Gerdes & Alpers, 2022; Lis et al., 2019; Morey et al., 2015). Whereas the generalization of fear to a certain degree might be adaptive to ensure recognition of threatening experiences across events, excessive generalization of fear to situations or cues

that do not warrant a fearful response can become restricting and harmful to one's wellbeing. Generalization of fear occurs both across contexts and stimuli (e.g. cues; tones, smells and lights). Constant, unnecessary heightened states of fear and arousal caused by excessive generalization responses, can lead to negative health consequences such as high blood pressure and sleep disturbances (Van Reeth et al., 2000; Brand, Hanson & Godaert, 2000). Additionally, overgeneralization of fear can lead to avoidance of behaviours that are deemed necessary (Wong & Pittig, 2020; Lommen et al., 2017), such as taking calculated risks or seeking medical treatments. Previous literature demonstrates that fear overgeneralization occurs due to changes in the way the brain processes and responds to emotionally charged stimuli (Dymond et al., 2015; Asok, Kandel & Rayman, 2018). Two brain regions that are believed to play a critical role in fear generalization behaviour are the amygdala and the bed nucleus stria terminalis (BNST) (Grosso et al., 2018; Ferrara et al., 2017), together known as the extended amygdala. The extended amygdala is a key structure involved in fear conditioning and the emotional response circuitry (Maren & Fanselow, 1996; LeDoux, 2003). In this thesis we will elaborate more on the role of the extended amygdala in fear generalization and anxiety- and stress-related disorders, and their possible risk factors.

Studying Fear and Anxiety

Fear is an emotional state elicited by acute, imminent threat, that is induced in the presence of acute sensory input indicative of this threat (Davis et al., 2010). As such, the fear response is thought to facilitate a coping strategy (typically active in nature) targeting the removal of this threat (Steimer, 2003). Anxiety (alternatively described as sustained fear), however, is defined as a future-oriented sustained state, that is induced by the anticipation of a potential, not-yet-encountered threat (Brinkmann et al., 2017), and is often linked to more passive coping strategies (e.g. passive avoidance). While both can be helpful in escaping from and avoiding danger, excessive or irrational fear and particularly anxiety can be debilitating. To better understand and measure these emotional states, various paradigms and tools have been developed to assess them independently. One of the most widely used paradigms for studying fear behaviour is fear conditioning, which has been used in both human and animal research to study the neuronal circuits underlying fear learning and anxiety-like behaviour (Kim & Jung, 2006), and to develop animal models for certain anxiety-

and stress-related disorders, e.g. PTSD (Yehuda & LeDoux, 2007; Zoladz et al., 2012). In classical fear conditioning, an initial neutral stimulus (i.e. the conditioned stimulus (CS)) is paired with the exposure to an aversive stimulus (the unconditioned stimulus (US)), such as (foot) shocks or malodour, to establish an association between the two. The conditioned response (CR) that is elicited by the CS in absence of the US is frequently used to measure this learned association.

Fear conditioning can occur to both specific contexts (i.e. contextual fear conditioning) and stimuli (cued fear conditioning). In contextual fear conditioning, the negative US is associated with a particular context such as a room, which often results in the avoidance of that particular context (Glotzbach et al., 2012), or when it is unavoidable, in freezing behaviour as an indication of fear. This type of fear conditioning is known to rely on the hippocampus and the amygdala (Corcoran & Maren, 2001; Goosens & Maren, 2001). In cued fear conditioning, an association is established between the US and a cue-stimulus, e.g. a sound or light. Whereas both paradigms can be used to study fear generalization (across contexts and cues), cued fear conditioning has the benefit that stimuli are more easily manipulated, and their differences quantified, compared to contexts. For instance, auditory stimuli can easily be adjusted in their frequency. As such, auditory cue fear conditioning has previously shown useful in studying stimulus generalization and discrimination (de Bundel et al., 2016; Duvarci et al., 2016; Dunsmoor et al., 2017; Norrholm et al., 2014). Particularly, previous research has used the paradigm of differential auditory fear conditioning (DAFC), in which 2 or more auditory stimuli are used, one of which is paired with the US while the others are not. The purpose of DAFC is to investigate how one distinguishes between similar stimuli of which one predicts an aversive outcome vs. others that do not, and it has been proven useful as a tool to investigate the neural and behavioural mechanisms underlying these responses (Duvarci, Bauer & Paré, 2009; De Bundel et al., 2016). The extent of generalization or discrimination of fear responses across stimuli is measured by assessing the fear response both towards the stimuli that were paired and not paired with the aversive outcome.

Studying anxiety in humans can be challenging, as often tools to measure anxiety rely on subjective report. For instance, self-report questionnaires, such as the State-Trait Anxiety Inventory (STAI) (Spielberger et al., 1983), ask the participants to rate the severity of their anxiety levels, which provides a measure of subjective anxiety. In order to evade this, more

objective forms of anxiety measurements, like physiological measurements (e.g. heart rate variability and skin conductance (Chalmers et al., 2014; Epstein & Roupenian, 1970)) in response to threat uncertainty can be utilized. In rodent research, anxiety-like behaviour is typically assessed by exposing rodents to novel, anxiogenic environments such as the elevated plus maze (EPM), the dark-light transfer test (DLT) and the open field (OF) (Harro, 2018; Lezak et al., 2022), which rely on the natural tendency of the animal to explore new environments but to avoid bright and lit spaces as these carry potential (thus non-encountered) danger.

Vulnerability Factors

Although the vast majority of individuals are exposed to stressful or traumatic life events during their lifetime, triggering fear responses, only a small portion of those individuals will develop anxiety- or stress-related psychopathology (De Vries & Olff, 2009; Alonso et al., 2004; Breslau et al., 2004), while others are resilient (Bonanno, 2004). Investigation of these inter-individual differences in susceptibility may generate important insights into the biological mechanisms contributing to resilience and health in the face of adversity. The complexity of the interaction of psychological, biological traits and the environment, combined with the diverse nature of psychiatric disorders, suggests that multiple risk factors play a role in the development of anxiety- and stress-related disorders (Uher and Zwicker, 2017).

Early Life Stress.

Childhood adversities, such as emotional and physical abuse and neglect, have been identified as risk factors for developing stress-related disorders like PTSD, PD and GAD (Famularo et al., 1992; Stein et al., 1996; Kessler al., 2010; Duits et al., 2015). This is supported by epidemiological studies indicating that 30% of mental health disorders can be explained by early life stress (ELS) exposure (Kessler et al., 2010). Further, animal studies have shown that ELS increases anxiety-like behaviour in adult rodents (Kalinichev et al., 2002; O'mahony et al., 2009; Ishikawa et al., 2019; Wei et al., 2010; Bolton et al., 2018; Berman et al., 2014). One of the more commonly used paradigms to induce ELS in rodents is the limited bedding and nesting paradigm (LBN). In this paradigm, dams and their pups are housed (from postnatal day 2-9) with limited bedding and nesting material, preventing

the dam from building a proper nest. This increases stress levels in the damn, and negatively impacts maternal care, which becomes unpredictable and fragmented, which subsequently affects the pups (Walker et al., 2017; Schmidt, 2019). LBN can have a long-lasting effect on the development of the brain and the behaviour of the pups. For instance, LBN has been shown to increase anxiety-like behaviour in the open field (Guadagno et al., 2018), elevated plus maze (Dalle Molle et al., 2012; Guadagno et al., 2018) and dark-light transfer test (Wang et al., 2011). Some papers even suggest that ELS in rats accelerates contextual fear generalization learning (Elliot & Richardson, 2019). ELS can also result in cognitive deficits (Masson et al., 2015; Geoffroy et al., 2016) and alter processing of negative emotional information (Pollak and Sinha, 2002; Pollak and Tolley-Schell, 2003; Pollak et al., 2009). Further, it has been shown that the hypothalamic–pituitary–adrenal axis (HPA-axis) and autonomic nervous system are affected by ELS (Alkon et al., 2014; Koss et al., 2017; Loman & Gunnar, 2010; Gluckman et al., 2007). These systems are pivotal for appropriate psychological and behavioural responses to the environment later in life (Lupien et al., 2009; Fagundes, Glaser & Kiecolt-Glaser, 2013; Gunnar, Doom & Esposito, 2015). Given that both ELS and fear overgeneralization have been identified as contributing factors in the development of anxiety disorders in adulthood, it has been suggested that ELS might play a role in the development of fear overgeneralization as well (Duits et al., 2015; Dymond et al., 2015).

Trait Anxiety.

Over the past decades, many predisposing traits that increase vulnerability to developing stress- and anxiety-related disorders have been identified (Mineka and Oehlberg, 2008; Sharma et al., 2016). Trait anxiety is considered to be one of these factors, and refers to a relatively stable tendency to experience anxiety across a variety of situations and time. It differs from state anxiety, which is a temporary emotional response to a specific event (Morrissette et al., 2007) and therefore more indicative of fear. Studies have shown that high trait anxiety is linked with impaired safety learning (Gazendam, Kamphuis, & Kindt, 2013; Haddad et al., 2012), and a characteristic of patients with PD (Muris, Merckelbach, & Rassin, 2000), GAD (Rapee, 1991), PTSD (Casada & Roache, 2005; Orsillo et al., 1996), and SAD (Amir, Beard, & Przeworski, 2005). However, trait anxiety differences have not been linked to a differential capacity to acquire differential fear conditioning (Joos et al., 2012;

Sehlmeyer et al., 2011; Torrents-Rodas et al., 2013), and the link between trait anxiety and fear generalization is not so apparent (Torrents-Rodas et al., 2013).

Neural Correlates: The Extended Amygdala

Human Studies.

The amygdala is located in the temporal lobe of the brain where it plays a crucial role in processing emotions, particularly fear and anxiety. It is well established using human neuroimaging paradigms, that the amygdala increases its activity in response to aversive stimuli. This is for example supported by the observation of the strongest amygdala responses towards fearful faces, compared to neutral, happy or angry faces (Whalen et al., 1998; Whalen et al., 2001). In classical fear conditioning paradigms, strong positive correlations between regional cerebral blood flow (rCBF) changes in the amygdala and the CS have been found using positron emission tomography (PET) (Fredrikson et al., 1995; Furmark et al., 1997). These findings further translate to neuroimaging studies in patients suffering from anxiety-related disorders, in which several functional magnetic resonance imaging (fMRI) and PET studies have shown a hyperresponsivity of the amygdala after symptom provocation or negative emotional processing (Birbaumer et al., 1998; Lorberbaum et al., 2004; Phan et al., 2006; Tillfors et al., 2001; Wik et al., 1993; Dilger et al., 2003; Straube et al., 2006; Veltman et al., 2004). Similarly, in patients with PTSD, increased amygdala activity was found in response to threat- or trauma-related stimuli (Liberzon et al., 1999; Rauch et al., 2000; Shin et al., 2004; William et al., 2006), although these results have not always been replicated (Britton et al., 2005; Phan et al., 2006).

While fMRI studies have provided valuable insights into amygdala function, recent research started focusing on the BNST. Distinct from the amygdala, which seems to be involved in immediate responses to acute, perceived threat (Davis et al., 1997; Davis et al., 2010; Fanselow, 1994; Hitchcock & Davis, 1986), the BNST is activated during the anticipation of threat or when the threat is (still) uncertain (Choi et al., 2012; Grupe et al., 2013; Klumpers et al., 2015). During threat exposure (e.g. electric shock) this response shifts towards the amygdala (Klumpers et al., 2017). Whether mediation of stress responses during the anticipation or uncertainty of threat exclusively recruits the BNST within the extended

amygdala is still unknown, as others have reported a role for the amygdala during anxious anticipation as well (Carlson et al., 2011; Nitschke et al., 2009).

There are several important pitfalls associated with studying the extended amygdala in human subjects that can limit the validity of the results. The amygdala and BNST are both relatively small and located deep in the brain and therefore difficult to study with available methods. In humans, researchers typically rely on indirect measures to assess their function, e.g. fMRI (Haller & Bartsch, 2009). These measurements are influenced by a range of factors that are not directly related to neuronal activity, such as blood flow and oxygen consumption (Logothethis et al., 2001). In addition, human neuroimaging methods have limited temporal and spatial resolution, meaning that they can only provide a rough estimate of course extended amygdala activity (Li et al., 1996; Salmon & Hustinx, 2015). Furthermore, the extended amygdala and the processing of fear and anxiety in human subjects do not work in isolation and the subjects' stress, mood, genetics (Hairi et al., 2002) and life experiences (Gee et al., 2013; Jedd et al., 2015; VanTieghem & Tottenham, 2018) interact in a complex way in modulating extended amygdala function, increasing experimental noise. It is therefore difficult to identify and isolate single determinants in human neuroimaging studies, which highlights the need for carefully controlled studies that can mitigate these variables. Moreover, the restricted spatial resolution impedes the study of the complex intra-regional subnuclei, which diverge in function (see following section). Animal models allow us to overcome these pitfalls, as they can provide improved spatial resolution to assess brain function, while offering control over a vast majority of extraneous variables. Animal models also allow for the manipulation of regional activity and connectivity in a controlled manner to show causality rather than studying mere associations between brain function and behaviour.

Animal Studies.

The tools and techniques used in animals research, in comparison to studies using human participants, allow for a more in-depth investigation of the extended amygdala. Grossly, the amygdala can be divided into four subregions. The lateral amygdala (LA) receives sensory input from the thalamus, and seems to mediate CS-US associations (Ghosh & Chatterji, 2015; Maren & Quirk, 2004c). Lesions of the LA have been shown to block the acquisition of a conditioned fear response (Nader et al., 2001); an effect mimicked by the chemogenetic

inhibition of LA pyramidal neurons (Tipps et al., 2018). The LA innervates the basolateral amygdala (BLA), which is situated ventrally to the LA, as well as the central amygdala (CeA), positioned more medially, in order to induce fear responses following conditioning (Paré & Smith, 1994; Pitkänen et al., 1995; Davis & Shi, 2000). The majority of the neurons in the BLA is glutamatergic, with approximately only 20% being GABAergic (Spampanato, Polepalli & Sah, 2011). The BLA is known for strengthening the consolidation of fear memory by projecting amongst others to the hippocampus, insula, caudate nucleus and the nucleus accumbens (McGaugh, 2004; Huff et al., 2013). Moreover, differential activation of the BLA allows for rapid switching between low and high fear states (Herry et al., 2008), while inactivation leads to a decrease of fear responses (Amano et al., 2011). Receiving both input from the LA and BLA (Paré & Smith, 1994; Savander et al., 1995), initially it was believed that the CeA was a passive relay station towards the brainstem and hypothalamic sites (Samson and Paré, 2005; Hopkins and Holstege, 1978; Kapp et al., 1979; Bellgowan & Helmstetter, 1996). However, the CeA has been shown to be necessary for both the acquisition and expression of conditioned fear (Goosens and Maren, 2003). Wilensky et al. (2006) showed that inactivation of the CeA with muscimol impairs fear learning and expression, and that blocking local protein synthesis prevents the consolidation of fear memory. Although many studies have investigated the CeA as a whole, the CeA is not a homogenous region and can be divided into a lateral (CeL) and medial (CeM) part. While the CeM is believed to mostly project outwards of the amygdala complex, e.g. brain stem and hypothalamus (Holzschneider & Mulert, 2011; LeDoux et al., 1988), the CeL primarly controls CeM output by dense GABAergic projections (Cassell, Freedman & Shi, 1999; McDonald & Augustine, 1993; Sun, Yi & Cassell, 1994). These anatomical differences result in different functions of the subregions as well. Ciocchi et al. (2010) showed that the CeL is necessary for the acquisition of fear, while the regulation and expression of fear following conditioning is controlled by the CeM. The amygdala subregions are highly interconnected, with these interconnections regulating subregion function. The BLA affects the CeA through glutamatergic projections (Duvarci & Pare, 2014), particularly to the CeL (Ciocchi et al., 2010). In turn, the CeL regulates the CeM by predominantly GABAergic projections (Ciocchi et al., 2010a), regulating freezing responses.

The BNST is implicated in mediating more sustained, anxiety-like responses (Davis et al., 2010; Münsterkötter et al., 2015; Walker et al., 2003). In support of this, lesions of the BNST

impede anxiety- like states and inhibit freezing responses towards contextual threat stimuli (Duvarci et al., 2009; Haufler, Nagy & Pare, 2013; Sullivan et al., 2004). However, lesions of the BNST do not affect the immediate expression of fear towards conditioned cues, which is considered to be regulated by the CeA (Duvarci et al., 2011; Haufler et al., 2013). The BNST can be divided into the anterior and posterior regions (Nguyen et al., 2016), with seemingly different roles. While activation of the posterior BNST (pBNST) following stress seems to reduce its subsequent anxiogenic effects (Henckens et al., 2016), the anterior BNST (aBNST) seems to contribute to sustained fear and anxiety (Greenwood et al., 2005; Wang et al., 2020). Yet, the main divisions of the aBNST, namely the anterolateral (AL) and anteromedial (AM) nuclei, have been suggested to mediate opposite fear outcomes (Gungor & Pare, 2013; Haufler et al., 2013), with the AM seemingly exciting the neural networks involved in fear behaviour, whereas AL has an inhibiting influence. These regional differences could result from the diverse amygdala input towards both regions, but can also be caused by distinct intrinsic BNST input (Haufler et al., 2013)(fig. 1).

Animal work has also illustrated that the BNST is comprised by a rich variety of neuronal subtypes (Allen et al., 1984; Daniel & Rainnie, 2016; Lange et al., 2017; Nguyen et al., 2016). The BNST mainly consists of inhibitory neurons (66-79% vs approx. 15-20% of glutamatergic neurons) (Daniel & Rainnie, 2016; Nguyen et al., 2016; Kim et al., 2013). However, in the ventral region of the BNST (BNSTV) the distribution appears to be more equal with 32% GABAergic and 29% glutamatergic neurons (Nguyen et al., 2016). Yet, these neuronal populations also co-express distinct neuromodulators. The most studied are the corticotrophin-releasing factor (CRF) neurons, which can be particularly found in the oval and fusiform nucleus (BNSTOV, BNSTFU) (Cummings et al., 1983; Morin et al., 1999; Nguyen et al., 2016). These CRF neurons project to other brain regions, e.g. hypothalamus, and regulate autonomic and endocrine stress responses (Giardino et al., 2018). Dysregulation of the CRF system in the BNST has been implicated in various animal models for psychiatric disorders, including PTSD and anxiety disorders (Binder & Nemeroff, 2010; Elharrar et al., 2013; Lebow et al., 2012). Another type of BNST neuron that seems mainly involved in reducing the expression of fear and anxiety responses, is the protein kinase C-delta-expressing (*PKC-delta+*) class. The *PKC-delta+* neurons are mostly found in the BNSTOV and CeA (Daniel & Rainnie, 2016; Haubensak et al., 2010). Within the CeA, the CeL *PKC-delta+* neurons exert an inhibitory control over *PKC-delta-* CeM neurons, reducing fearful behaviour (Haubensak

et al., 2010), making that CeA *PKC-delta+* neurons are considered to be fear-off entities within the CeA. Given the involvement of the *PKC-delta+* neurons in the BNST in anxiety-related responses (Davis, 2006), and its anatomical similarities to the amygdala (Haubensak et al., 2010), *PKC-delta+* neurons in the BNST have been proposed to represent anxiety-off neurons (Daniel & Rainnie, 2016). Further, *PKC-delta+* neurons in both the CeA and BNSTOV have been shown to reduce fear generalization behaviour (Botta et al., 2015; De Bundel et al., 2016), which suggests that increased *PKC-delta+* neuronal activity could reduce fear generalization and possibly contribute to a phenotype resilient to anxiety-like disorders.

Interestingly, the BNST is a brain region that has been shown to exhibit sexual dimorphism, displaying both structural and functional differences between males and females. In particular, studies comparing male and female BNST structure have shown that the BNST is larger in males (Allen & Gorski, 1990; Chung et al., 2002); a difference likely driven by differential exposure to hormonal factors such as testosterone during early development (Chung et al., 2000; del Abril et al., 1987; Murray et al., 2009). Furthermore, the BNST might play a role in the modulation of sexual behaviours, which are evidently different across sexes (Avery, Clauss & Blackford, 2016; Greenberg et al., 2014; Liu, Salamone & Sachs, 1997; Rigney et al., 2022).

The amygdala and BNST are interconnected via one direct, and two indirect pathways (Fox & Shackman, 2017; Avery, Clauss, & Blackford, 2016). The most prominent white fibre bundle connecting the structures is the stria terminalis, which bidirectionally connects the CeA and the BNST via the thalamus (Price & Amaral, 1981). The second indirect connection arises from the sublenticular extended amygdala, which connects the CeA with the BNSTAL (Oler et al., 2017; Fox & Shackman, 2017). The last connection is a direct one, the ventral amygdalofugal pathway, which connects the BLA and CeA to the dorsal BNST (Avery et al., 2016). Via these connections, the BNST is supplied with GABAergic input from the BLA via the CeA (Dong et al., 2001; Haufler et al., 2013), and direct glutamatergic input from the BLA (Vranjkovic et al., 2017; Haufler et al., 2013). In line with the strong structural connectivity, fMRI studies have revealed significant functional connectivity (approximated by correlations in activity patterns) between the regions during resting state (Oler et al., 2012, Oler et al., 2017, Avery et al., 2014, Torrisi et al., 2015). This strong interplay between

regions suggest related functional responses to threat (Walker et al., 2009, Fox et al., 2015, Gungor et al., 2015, Shackman and Fox, 2016, Oler et al., 2017), given that the CeA and BNST both activate the same downstream fear effectors (hypothalamic and brainstem structures) (Haufler et al., 2013).

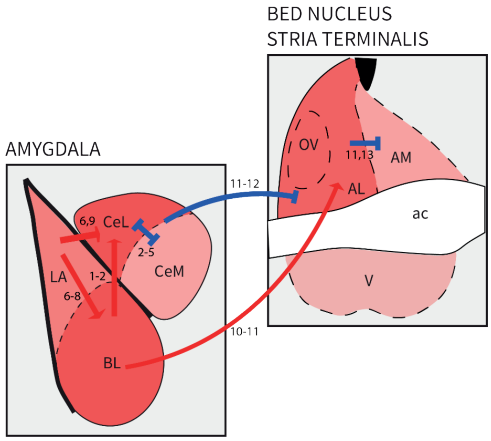

Figure 1. Schematic illustration of the extended amygdala subregions and its major pathways. 1: Duvarci & Paré, 2014. 2: Ciocchi et al., 2010. 3: Cassel, Freedman & Shi, 1999. 4: McDonald & Augustine, 1993. 5: Sun, Yi & Cassel, 1994. 6: Paré & Smith, 1994. 7: Pitkänen et al., 1995. 8: Davis & Shi, 2000. 9: Savander et al., 1995. 10: Vranjkovic et al., 2017. 11: Haufler et al., 2013. 12: Dong et al., 2001. 13: Gungor & Paré, 2013. Lateral amygdala (LA), basolateral amygdala (BLA), central lateral amygdala (CeL), central medial amygdala (CeM), oval nucleus (OV), anterolateral BNST (AL), anteromedial BNST (AM), ventral BNST (V), anterior commissure (ac).

Open Questions

Majority of work studying fear generalization has focused on the amygdala. The LA, and partly the CeA, mediates the CS-US association (Paré et al., 2004; Ghosh & Chatterji, 2015; Maren & Quirk, 2004c). The excitatory and inhibitory interplay of the neuronal populations of the LA has been associated with fear generalization when similarity of the CS's increases (Tovote et al., 2015; Grosso et al., 2018). Furthermore, CRF in the CeA has been shown to influence fear generalization behaviour when the strength of the association is weak (Sanford et al., 2017). Yet, lesions of the BNST have been shown to reduce fear generalization behaviour in rats (Duvarci et al., 2009), which implies that the BNST is involved as well. This was further corroborated by De Bundel et al. (2016), who showed that administration of a

dopamine D2R antagonist in the BNST or CeA is sufficient to increase the generalization of cued fear. These studies imply that there is an unidentified role for the BNST in fear generalization and discrimination. The role of the BNST in sustained anxiety, and the association between anxiety and fear overgeneralization, makes one question whether there are similar mechanisms within the extended amygdala at play in modulating both behavioural outcomes.

Another key question in studying the extended amygdala, is when deviations in the extended amygdala function as observed in patients arise since the majority of patient assessments are performed retrospectively. While previous animal models have provided valuable insight into the effects of stress exposure on the brain and behaviour, it is difficult to pinpoint the exact moment of alterations in the brain that can eventually cause pathology. Understanding the timing and extent of extended amygdala deviations in response to stress can provide valuable information on the underlying mechanisms of anxiety- and stress-related pathologies, and potentially lead to the development of effective interventions.

It has been suggested that risk factors, such as ELS, and their effect on stress response systems alter neuronal plasticity circuits that are crucial for stress regulation (Fareri & Tottenham, 2016). For instance, childhood adversities increase the rate of amygdala maturation (Moriceau et al., 2006, 2009; Ono et al., 2008), can heighten amygdala reactivity to emotional images (Herringa et al., 2016), and alter amygdala connectivity (Gee et al., 2019; VanTieghem & Tottenham, 2018; Jedd et al., 2019; Kim et al., 2019). Furthermore, the structure of the amygdala has been linked to trait anxiety (Etkin et al., 2004, Stein et al., 2007, Dickie and Armony, 2008). Increased amygdala volume has also been positively linked to one's capacity to discriminate threatening vs. safe cues (Winkelmann et al., 2016), while decreased amygdala volume has been observed in patients with spider phobia and PTSD (Fisler et al., 2013; Rogers et al., 2009; Woon & Hedges, 2008), although these volumetric differences could either be the cause or consequence of the disease. Interestingly, the activity of the BNST also correlates with trait anxiety (Avery, Clauss, & Blackford, 2016; Somerville, Whalen, & Kelley, 2010). Not only do the separate subcortical structures influence inter-individual susceptibility to developing stress-related disorders, the extended amygdala connectivity seems to modulate this as well. In rats, the interaction between the amygdala and BNST configures inter-individual differences in the expression of fear and

anxiety (Duvarci et al., 2009). This was supported by a study in humans, which suggested a relationship between extended amygdala connectivity and trait anxiety (Brinkmann et al., 2018). Given that vulnerability factors negatively affect the stress response systems, the question arises whether the extended amygdala circuitry is affected by these factors.

Aim of this Thesis

The overall aim of this thesis is to understand the role of the extended amygdala in mediating vulnerability to stress-related symptomatology (**chapter 2**) as well as fear generalization and subsequent anxiety-like behaviour (**chapter 3**). Moreover, I aimed at understanding how ELS constitutes risk for anxiety-like behaviour and tested whether fear generalization might be a moderating factor in this association (**chapter 4**).

In **chapter 2**, I start by exploring the understudied part of the extended amygdala, i.e. the BNST, and its contribution to susceptibility to develop PTSD-like behaviour following trauma exposure in mice. Recent advances in neurobiology have led to the development of a new method, called targeted recombination in active population (TRAP) (Guenthner et al., 2013), which allows for the permanent fluorescent labelling of activated neurons at a specific moment in time in living animals (without the need for immediate sacrificing of the animals). By utilizing this method, I was able to investigate neuronal activity within the BNST pre-, peri-, and post-trauma exposure to determine a potential role for aberrant BNST function in the susceptibility to the behavioural consequences of trauma exposure. By using a well-established PTSD-mouse model, I was able to characterize mice as either susceptible or resilient to developing PTSD following trauma, and link BNST neuronal activity with inter-individual differences in susceptibility to PTSD-like symptomatology. This approach allowed me to assess whether BNST activity mediates pre-existing risk, maladaptive trauma responding, or is implicated in pathology of PTSD-like behaviours following trauma.

In order to investigate the role of the extended amygdala circuit in fear generalization and subsequent anxiety-like symptomatology, in **chapter 3** I set up a differential auditory fear conditioning paradigm (DAFC) in mice that I used to assess fear generalization. DAFC was followed by a subset of anxiety tests to test for a potential link between the (over) generalization of fear and anxiety-like behaviour in general. Using this DAFC paradigm in

combination with TRAP (Guenthner et al., 2013) allowed me to investigate the extended amygdala neuronal circuitry during fear memory acquisition and early consolidation and link this with later anxiety-like behaviour. Lastly, to demonstrate the importance of the extended amygdala circuitry in fear- and anxiety-like behaviour, I manipulated one of the direct amygdala-BNST pathways with the purpose of modifying corresponding fear- and anxiety-like behaviour to show causality.

In **chapter 4**, I investigated whether ELS influences fear generalization and subsequent anxiety- like behaviour. Here, I used the LBN approach to elicit disruptive maternal behaviour causing stress in the male and female offspring. In adulthood, the offspring was tested on their fear generalization behaviour using the DAFC paradigm and anxiety-like behaviour using anxiety tests and behavioural outcomes of ELS offspring were compared to offspring that did not experience ELS.

In **chapter 5**, the main findings of my thesis are summarized and interpreted using the existing literature. Limitations of the studies are also discussed, and recommendations are made for future research.

2

THE ROLE OF THE ANTERIOR BED NUCLEUS STRIA TERMINALIS IN SUSCEPTIBILITY TO TRAUMA

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Abstract

Posttraumatic stress disorder (PTSD) is a debilitating stress-related disorder that one can develop following the exposure to a traumatic event. Although most individuals experience such an event during their lifetime, only a fraction of them actually develops PTSD. Investigation of the neural substrates underlying these differences in PTSD susceptibility, might provide unique insight into the mechanisms that should be targeted by improved treatment. Here, we used transgenic Targeted Recombination in Active Population (TRAP) mice to investigate the association between neuronal activity within the anterior bed nucleus stria terminalis (aBNST) pre-, peri-, and post-trauma and the development of PTSD-like symptomatology. Mice were subjected to a foot shock trauma paradigm, followed by a set of behavioural assays allowing for the identification of mice resilient or susceptible to PTSD-like behaviour. Three weeks following trauma, mice were re-exposed to either a novel context or a context related to the traumatic experience and their fear responses assessed as a proxy for trauma memory recall. Additionally, anxiety-like behaviour pre-trauma was assessed as potential risk factor for PTSD-like symptom development. Pre-trauma anxiety-like behaviour and basal aBNST activity did not predict later trauma resiliency or susceptibility. However, peri-trauma, susceptible mice exhibited overall lower levels of aBNST activity than resilient mice as well as atypical correlations between amygdala and aBNST neuronal activity, suggestive of aberrant functional connectivity within the extended amygdala. Post-trauma, no differences in basal aBNST activity were observed. Lastly, no group differences were observed in fear responses upon exposure to the trauma-related context. Yet, susceptible mice showed faster declines in their freezing rates over time during novel context exposure compared to resilient mice. No differences in aBNST activity in response to context (re)-exposure were observed. Together, our results indicate a role for aberrant aBNST signalling specifically during trauma processing in the later development of PTSD -like symptomatology.

Introduction

Posttraumatic stress disorder (PTSD) is a debilitating disorder that can occur in people that have experienced a traumatic event (American Psychiatric Association (APA), 2013). It belongs to the category of trauma- and stressor-related disorders (APA, 2013), and is characterized by a variety of symptoms including intrusive thoughts, avoidance of situations that may trigger aversive memories, alterations in negative cognition and mood, and increased arousal and reactivity (e.g. hypervigilance, reckless behaviour, and problems with sleeping and concentration). Although approximately 80% of people experience a traumatic event at least once in their lifetime (Frans et al., 2005), only around 15% of those people will eventually develop PTSD (Kessler et al., 2005; Hinton & Lewis-Fernández, 2011). However, we currently lack understanding of the inter-individual differences in trauma resiliency. Improved understanding of the neural processes leading to resilience could benefit prevention, early intervention and improved treatment of PTSD.

Whereas traditionally much emphasis has been placed on the amygdala in the investigation of fear and PTSD (Kirkpatrick & Heller, 2014; Pitman et al., 2012; Yehuda et al., 2015; Hughes & Shin, 2011; Feder, Nestler & Charney, 2009), recently this focus has expanded to other regions that are part of the extended amygdala, such as the Bed Nucleus Stria Terminalis (BNST), which has an important role in mediating and modulating states of anxiety. While increased anxiety is formally not part of PTSD-symptomatology, this particular aspect frequently co-occurs alongside the disorder (Rodriguez-Sierra et al., 2016; Pitman et al., 2012; Flook et al., 2020). Although both the amygdala and BNST may be responsible for the dysregulated stress response that is observed in PTSD patients (Rabellino et al., 2017; Brinkmann et al., 2017; Armony et al., 2005), significant differences exist between the two regions. Whereas the main output nucleus of the amygdala, the central amygdala (CeA), mediates fear responses to short discrete cues signalling imminent threat (Campeau and Davis, 1995; Walker and Davis, 1997), also known as ‘’phasic fear’’, the BNST has been implicated in conditioned fear to contexts or long and unpredictable threat cues (Davis et al., 2010; Sullivan et al., 2004), coined ‘’sustained fear or anxiety’’ (Davis et al., 2010, Sullivan et al., 2004; Duvarci et al., 2009). Lesions of the BNST also abolish the potentiation of startle responses by corticotrophin releasing factor (CRF), suggesting that unconditioned fear responses are also mediated by the BNST (Lee & Davis, 1997). Functional neuroimaging

studies in PTSD patients have indicated both an increase in phasic amygdala activation, as well as increased sustained activation of the BNST in response to unpredictable, aversive stimuli (Brinkmann et al., 2017). Moreover, patient studies have confirmed altered BNST-amygdala connectivity in anxiety pathophysiology (Resnik & Paz, 2015; Münsterkötter et al., 2015; Brinkmann et al., 2017) and linked both BNST activity (Buff et al., 2017) and amygdala-BNST connectivity (Somerville et al., 2010) to inter-individual differences in anxiety. As such, dysfunctional activity of the BNST and extended amygdala intrinsic connectivity could potentially relate to anxiety symptomatology in PTSD patients.

The BNST is a complex structure that can be divided into multiple heterogenous subnuclei, all of which have their own distinct connectivity and activity patterns, with their own functionalities. Firstly, the BNST is divided into an anterior and posterior section, with apparently opposing roles in modulating anxiety-like behaviour. The posterior BNST (pBNST) has been shown to inhibit the HPA-axis through its GABAergic projections to the paraventricular nucleus (Choi et al., 2007), and optogenetic activation of the pBNST has previously been reported to decrease anxiety, whereas its activation following trauma exposure reduced susceptibility to develop PTSD-like symptoms (Henckens et al., 2016). In contrast, the anterior BNST (aBNST) has been shown to have a nett activating contribution to the HPA-axis (Choi et al., 2007) and to contribute to sustained fear and anxiety with its heterogenous population of neurons (see review; Walker et al., 2009) (Greenwood et al., 2005; Wang et al., 2020). Yet, even within the aBNST, distinct functional subnuclei can be dissociated, roughly comprising five subregions. Firstly, the BNST anterolateral region (BNSTAL), which is mainly comprised of GABAergic neurons that are thought to suppress anxiety by inhibiting target regions such as the central amygdala (Haufler et al., 2013; Gungor & Pare, 2014; Sun and Cassell, 1993; Dong et al., 2001b). Yet, the BNSTAL also contains the oval nucleus (BNSTOV), rich in GABAergic neurons that co-express corticotrophin-releasing factor (CRF, Dabrowska et al., 2013a) and promote fear and anxiety by intrinsic projections within the BNST, as well as projections towards the ventral tegmental area (VTA), paraventricular nucleus and hypothalamus, among others (Dabrowska et al., 2011; Dong et al., 2001; Daniel & Rainnie, 2015). The anteromedial region (BNSTAM) seems to boost anxiety (Haufler et al., 2013; Gungor & Pare, 2014) and displays higher firing rates in high vs. low fear states, opposite to the BNSTAL (Haufler et al. 2013). Antagonistic roles for both subregions are further supported by inhibitory projections from the BNSTAL to

BNSTAM (Turesson et al., 2013). Lastly, there is the ventral BNST (BNSTV) that can be divided into the medial and lateral regions (BNSTMV, BNSTLV). The BNSTLV sends glutamatergic projections to the VTA that serve an anxiolytic role (Dumont & Williams, 2004).

In addition to these distinct subnuclei, each subnucleus contains several neuronal subclasses, both inhibitory and excitatory, which connect both intrinsically as well as extrinsically. GABAergic neurons prevail overall in the aBNST, of which neurons co-expressing CRF, somatostatin or protein kinase C-delta (*PKC-delta*) (Ye & Veinante, 2019; Magableh & Lundy, 2014) are the most prevalent subclasses (see review; Gungor & Pare, 2016). GABAergic neurons are more prevalent in the BNSTAL and BNSTOV, whereas the BNSTV also contains a considerable amount of glutamatergic neurons (Nguyen et al., 2016). Although the involvement of the BNST in PTSD pathophysiology seems apparent, the precise subregional and neuronal subtype contribution remains unclear, and dedicated animal studies allowing its detailed dissection are necessary for its elucidation.

Here, we set out to investigate the association between aBNST subregional activity and susceptibility to develop PTSD-like symptomatology following trauma exposure in mice. We used a transgenic mouse model that allowed for the permanent fluorescent labelling of active neurons either before, during or after trauma exposure without the need for immediate sacrifice (Guenthner et al., 2013). Using a well-established PTSD induction model (Lebow et al., 2012; Henckens et al., 2017), mice were exposed to a traumatic event and – using a variety of behavioural tests assessing PTSD-like symptomatology – later categorized as susceptible or resilient after which aBNST activity across groups was compared. Additionally, based on correlational data between the amygdala and the aBNST, we investigated a potential contribution of altered amygdala-aBNST crosstalk during the processing of trauma in mediating its long-term consequences. Lastly, we assessed contextual fear memory recall and found that susceptible mice displayed differential freezing rates over time when exposed to a novel context, but not trauma or trigger contexts, compared to resilient mice.

Material & Methods

Animals.

This study consisted of three separate experiments: cohort 1 (n = 48) to assess pre-trauma, cohort 2 (n = 44) to assess peri-trauma, and cohort 3 (n = 48) to assess post-trauma neuronal activity in the aBNST. Two founder mouse lines, ArcCreER^{T2} (*B6.129(Cg)-Arc$^{tm1.1(cre/ERT2)Luo}$/J*, strain 021881) and conditional *tdTomato* (*B6.Cg-Gt(ROSA)26Sor$^{tm9(CAG-tdTomato)Hze}$/J*, strain 007909), were purchased from The Jackson Laboratory and bred in house to generate heterozygote ArcCreER^{T2}xtdTomato offspring, referred to as ArcTRAP. The genetic background of the mice allows *Arc*-expressing (i.e. activated) neurons to be labeled by the fluorescent protein *tdTomato* in a 36 hour time window after the injection with the compound tamoxifen (Guenthner et al., 2013). Based on the fact that the implemented PTSD model (Lebow et al., 2012; Henckens et al., 2017) has only been validated in males, only male mice were used for this study. Mice were group housed (3-4 mice per cage) in individually ventilated cages on a reverse 12 h light/dark cycle (09:00 - 21:00 h) at the Central Animal Facility of the Radboud University Nijmegen, The Netherlands, according to institutional guidelines. Food and water were available *ad libitum*, and behavioural testing occurred at least 4 hours into the animals' active phase. The experimental protocols were in line with the international guidelines and approved by the Central Committee for Animal Experiments, Den Haag, The Netherlands.

Experimental Design.

The PTSD induction protocol was based on the PTSD mouse model as described by Lebow et al. (2012). Briefly, mice were exposed to a traumatic event (severe unpredictable foot shock) followed by a less severe trigger (mild, predictable foot shock) on the subsequent day. After a week of recovery, mice were subjected to a set of behavioural tests to assess their phenotype to categorize them as susceptible, intermediate, or resilient. PTSD-like behaviour was defined as increased risk taking behaviour (dark-light transfer test), hyperarousal (marble burying test), hypervigilance and impaired sensorimotor gaiting (acoustic startle and pre-pulse inhibition test), and insomnia (locomotion activity in the light phase) (APA, 2013; Lebow et al., 2012). On the final day of the experiment, mice were exposed to a novel context (cohort 1), the trigger context (cohort 2), or the trauma context (cohort 3) to induce contextual fear memory recall, after which they were sacrificed by perfusion-fixation (Fig. 1).

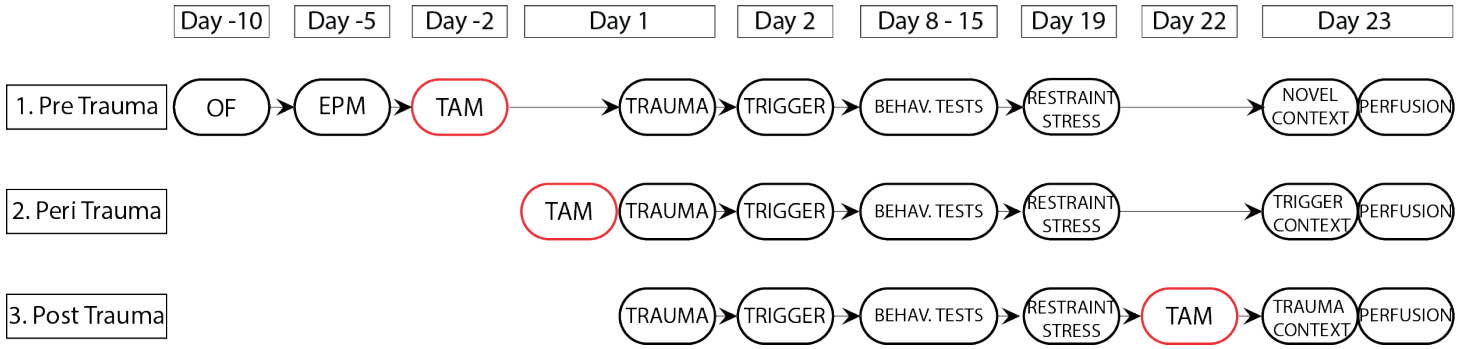


Figure 1. PTSD-paradigm timeline. Timeline of the 3 cohorts, indicating the tamoxifen injection (TAM) pre-, peri-, and post-trauma induction. OF, open field; EPM, elevated plus maze; TAM, tamoxifen; TRAUMA, trauma induction; TRIGGER, trigger induction; NOVEL CONTEXT, exposure to novel context; TRIGGER CONTEXT, exposure to trigger context; TRAUMA CONTEXT, exposure to trauma context.

Tamoxifen.

ArcTRAP mice received an intraperitoneal (i.p.) injection with a tamoxifen solution in the morning either pre- (day -2), peri- (day 1), or post- (day 22) trauma. Tamoxifen was dissolved in a 10% ethanol/90% corn oil solution at a concentration of 10 mg/mL by overnight sonication. The solution was stored at -20 °C. On the day of the injection, the solution was heated to body temperature and injected at 150 mg/kg dosage.

Behavioural Testing.

Open field. To assess pre-trauma basal anxiety in cohort 1, mice were tested in the open field (OF) test. They were placed in the corner of an open, white Plexiglas box (50x50x45 cm) that was lightened to 120 lux for a 10-min test session that was recorded by a camera above the apparatus. The time spent in the centre (inner 25x25 cm), number of visits to the centre, and total distance travelled were analysed using Ethovision software (Noldus, Wageningen, Netherlands).

Elevated plus maze. The second test to assess pre-trauma baseline anxiety in cohort 1 was the elevated plus maze (EPM). The EPM consisted of a centre part (5x5 cm), with attached to it two opposing open (30.5x5 cm) and two opposing closed arms (30.5x5x15 cm) that were elevated at 53.5 cm above the floor. The EPM was lightened with 6-9 lux. Mice were placed at the end of one of the closed arms, facing the centre, and recorded for 5 minutes using a camera above the apparatus. Time spent in the open arms, the number of visits to the open arms, and total distance travelled were measured and analysed using Ethovision software (Noldus, Wageningen, Netherlands).

PTSD induction. For the trauma session, mice were moved to the dark experimental room in groups of 2-4 in dark, carton boxes and placed individually in context A boxes that were connected to a shock generator (Bussey-Saksida, ABET II TOUCH). Context A consisted of a dark, triangular shaped Plexiglas box with a steel grid and metal tray. The boxes were sprayed with 1% acetic acid, and mice were subjected to 70 dB background noise and no illumination during the trauma induction. Here, they received 14 1 mA unpredictable shocks, each lasting 1 s, and spread over 85 min in variable intervals. On the second day, approximately 21 hours after the trauma induction, mice were moved to and from the experimental room in groups of 2-4 in see-through cages to start the trigger for which they

were individually placed in context B boxes. Context B consisted of curved white walls and a steel grid with a white tray underneath. The house light was turned on and the context was cleaned with 7 % ethanol. In context B, mice were subjected to 5 foot shocks of 0.7 mA, each lasting 1 s and presented over fixed (1 min) intervals. Videos of the trigger session were analysed for freezing behaviour, to test for potential differences in fear responding across phenotypic groups.

Dark-light transfer test. The dark-light transfer test was used to assess risk taking behaviour (Lebow et al., 2012). Mice were placed individually in the dark compartment of the dark-light apparatus (29 x 14 cm) that was connected to a brightly lit arena (~1000 lux, 29 x 29 cm) via a retractable door. The movement of the mouse was recorded by a camera mounted above the apparatus. Behaviour was scored automatically with Ethovision XT (Noldus). Time spent in the risk assessment area, a small area at the opening of the door of the light compartment (6 x 3 cm), was measured to calculate the percentage risk assessment as the amount of time spent in the risk assessment zone as a percentage of total time spent in the lit arena outside of that zone.

Marble burying test. The marble burying test was used to assess hyperarousal. The mouse was placed in a 10 lux illuminated black, open box (30 x 27 cm) which contained a layer of corn cops (5 cm) with 20 marbles centrally arranged (4 x 5) on top of it. The mouse was placed in the corner of the box to initiate the task. Mice were videotaped for 25 min, and videos were manually scored by assessing the amount of unburied marbled after 25 minutes.

Acoustic startle and pre-pulse inhibition. This test was based on the Acoustic Startle Response test of Lebow et al. (2012) and designed to measure hypervigilance and sensorimotor gaiting in mice. Mice were individually placed in small, see-through, Plexiglas constrainers, that were mounted on a vibration sensitive platform inside a ventilated cabinet that contained two high-frequency loudspeakers (SR-LAB, San Diego Instruments). The test started with an acclimatization period of 5 min during which a background noise of 70 dB was presented that lasted throughout the 30-min session. Thirty-two startle stimuli of 120 dB, 40 ms in duration and with a random varying inter-trial interval were presented with another 36 startle stimuli preceded by a 20 ms pre-pulse of randomly 75 dB, 80 dB or 85 dB.

Measurements used were the latency to peak startle amplitude and the average percentage pre-pulse inhibition; the percentage of startle inhibition observed for the different pre-pulse stimuli (%PPI = (1 - (mean pre-pulse startle response/mean startle response without pre-pulse)) x 100).

Home cage locomotion. Immediately after the pre-pulse inhibition test, mice were singly housed for 72 hours in Phenotyper cages (45 x 45 cm, Noldus) while their locomotion was being recorded by an infrared-based automated system (Ethovision XT (Noldus)). The first 24 hours was considered habituation time. Disturbances in sleeping behaviour in the mice were assessed by measuring the average locomotion during the two light phases.

Context (re-)exposure. At the final day of the experiment, mice of cohort 1 were placed in a new context C (similar to contexts A and B) that consisted of an illuminated Plexiglas chamber, with a white triangular box and a white plate underneath the metal grid for the duration of 10 minutes. The box was sprayed with 1 % lactic acid, and a continuous 80 dB 10 kHz tone was played. Mice of cohorts 2-3 were again placed in context B or A, respectively, for the duration of 10 minutes, following the exact same procedure as during the trigger or trauma session (for cohorts 2 and 3, respectively) to induce fear memory recall. No shocks were administered during this context re-exposure session. Behaviour was recorded and freezing behaviour was automatically scored using Ethovision XT (Noldus) for the pre-trauma cohort, while freezing behaviour was manually scored by an observer blinded to the experimental group (Noldus, the observer XT12) for the peri- and post-trauma cohort, since the quality of these videos did not reach the requirements for automatically scoring. Manual and automatic scoring were compared for a subset of videos and were highly correlated ($r(15) = .89$, $p = .012$).

Behavioural categorization.

In order to categorize mice as either susceptible or resilient, the top 20% of mice showing the most extreme behaviour per test were given a score. Specifically, mice with the lowest values for percentage risk assessment and latency to peak startle amplitude were attributed 3 points, and the lowest percentage pre-pulse inhibition was granted 2 points. Conversely, mice with the highest values for locomotion in the light phase and total marbles buried were attributed 1 point. A compound measure was generated by tallying the scores for all

tests together, and mice with a total score of four or higher (necessitating extreme behaviour in multiple tests) were considered to be susceptible. Mice with a total score of zero were considered resilient as they did not show any PTSD-like symptomatology.

Restraint Stress.

Mice were restraint for 25 minutes in plastic 50 mL tubes, and blood samples were taken from the tail at baseline (t = 0 min), immediately post stress (t = 25 min), and following stress recovery (t = 90 min). Blood samples were stored on ice until centrifuged (3500 rpm, 20 min, 4 °C) and plasma was extracted. Plasma samples were stored at -20 °C until assayed for corticosterone with the Corticosterone Double Antibody RIA Kit (MP Biomedicals, Orangeburg, NY, USA). The data from these assays are not part of this chapter.

Brain activity assessment.

Sacrifice. Animals were sacrificed by perfusion-fixation 90 minutes after the (re-)exposure session. Mice were first anesthetized with 4-5% isoflurane, after which they were i.p. injected with an overdose of pentobarbital. Then, animals were perfused with 1 x phosphate-buffered saline (PBS) following 4% paraformaldehyde (PFA), and their brains extracted and post-fixated in 4% PFA for 24 hours. Then, brain hemispheres were separated, and right hemispheres were submerged in 30% sucrose in 1 x PBS for > 48 h, and cut in 30 um thick slices by use of a freezing microtome.

Immunohistochemistry. BNST sections (0.38 - 0.14 mm from Bregma) were slide mounted on adhesion slides (Epredia™ SuperFrost plus™, 10149870, Thermo Fisher Scientific). Sections were washed 3 times 10 minutes in 1 x PBS. Sections were then blocked in PBS-BT (0.3% Triton X-100 / 1% Bovin Serum Albumin / 1 x PBS) for 30 minutes. Incubation of the primary antibodies in PBS-BT occurred overnight (cohorts 1-2; guinea pig anti-*cFos*, 1:750, #226004, Synaptic Systems; cohorts 1-3; mouse anti-*PKC-delta*, 1:500, #610398, BD Biosciences). Then, sections were washed 3 times 10 minutes in 1 x PBS, and incubated with the secondary antibodies in PBS-BT for 3 hours (cohorts 1-2; donkey anti-*cFos* Alexa 647, 1:400, #706-605-148, Jackson Immuno Research; cohorts 1-3; goat anti- mouse Alexa 488, 1:200, #15626746, Thermo Fisher Scientific). Sections were rinsed with 1 x PBS and incubated in DAPI for 10 minutes (1:1000, #62248, Thermo Fisher Scientific). Lastly, sections

were air dried and cover slipped (#345789, Merk Chemicals, Fluorsave). *PKC-delta*-expression was solely used as a marker for the BNSTOV region.

Image acquisition and cell counting. Images were captured through a light microscope (Axio Imager 2, Zeiss) using a 10x objective lens and a LED module (Colibri 2, Zeiss). For each animal, at least 4 sections of the BNST were analysed. Cells were manually counted per region in Fiji (Schindelin et al., 2012) by an experimenter blinded to the experimental groups (Fig. 2). Cell counts were normalized to the amount of DAPI cells per region to obtain standardized measures of BNST readouts corrected for differences in region size and cell density.

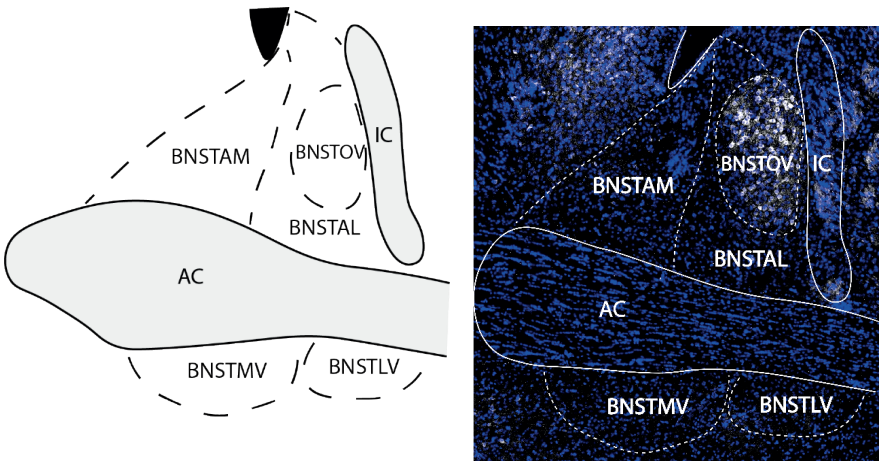


Figure 2. aBNST subregion boundaries for cell counting. Regions were divided into the oval nucleus (BNSTOV), anterolateral region (BNSTAL), anteromedial region (BNSTAM), and the medial and lateral ventral region (BNSTMV, BNSTLV). AC, anterior commissure.

Statistical analyses.

Data were analysed using IBM SPSS statistics 25 and 27. Data points deviating more than three interquartile ranges from the median were considered outliers and removed from further analyses. Group comparisons were made between the susceptible and resilient mice. The data were subjected to the Shapiro-Wilks test for normality and normally distributed data were tested with an independent samples two-tailed t-test (freezing behaviour) or one-tailed t-tests (PTSD-like behaviours), while non-parametric data were tested with Mann-Whitney U tests. Readouts that also included within-subject variables (e.g. aBNST subregions or time) were analysed using linear mixed modelling. For correlational

analyses, Pearson or Spearman correlations were carried out, depending on whether data distribution complied to normality. Correlations were statistically compared by running a Fisher r-to-z transformation and one-tailed tests for significance. A value of $p < .05$ was considered statistically significant. Data was visualized with Graphpad prism 9.

Results

Mice underwent a set of behavioural tests following PTSD induction in order to assess their susceptibility to the long-term behavioural consequences of trauma exposure. Susceptible mice ($n_{pre} = 12$, $n_{peri} = 10$, $n_{post} = 7$) and resilient ones ($n_{pre} = 12$, $n_{peri} = 12$, $n_{post} = 11$) significantly differed on their overall PTSD-like symptom score in all three cohorts (pre-trauma; $U(24) = 144$, $p < .001$, peri-trauma: $U(21) = 120$, $p < .001$), post-trauma: $U(19) = 88$, $p < .001$). See supplementary Data for the results of the separate behavioural tests per experimental cohort (Fig. S1).

Pre-trauma anxiety and anterior BNST activity do not predict susceptibility to PTSD-like symptoms

To test whether pre-trauma anxiety levels predicted susceptibility to PTSD-like symptomatology and whether this related to pre-trauma activity in the aBNST, we compared later coined resilient and susceptible mice on their behaviour in the open field and elevated plus maze tests. Groups did not differ in terms of the total distance moved ($t(21) < 1$, $p = .827$), duration spent in the centre ($U(24) = 78$, $p = .729$), or frequency visiting the centre ($U(24) = 74.5$, $p = .887$) in the OF. Similarly, groups did not differ in terms of distance travelled ($t(17.439) < 1$, $p = .430$), duration spent in open arms ($U(24) = 45$, $p = .128$), or frequency visiting the open arms ($t(22) = .980$, $p = .338$) in the EPM (Fig. 3a-b). As such, anxiety-like behaviour pre-trauma was not predictive of susceptibility to develop a PTSD-like behavioural phenotype following trauma exposure.

To determine whether susceptible and resilient mice differed in terms of pre-trauma basal aBNST activity, we compared the number of pre-trauma labelled *Arc*-expressing neurons across groups. Results revealed clear subregional differences in aBNST *Arc*-expressing neurons ($F(4,87) = 16.473$, $p < .001$; BNSTAL = BNSTOV > BNSTAM > BNSTLV = BNSTMV, all p's $< .004$). Yet, no differences in cell counts were observed across groups (main effect;

$F(1,87) = 1.418$, $p = .237$, group*subregion interaction; $F(4,87) = .767$, $p = .549$, Fig. 3c). Lastly, the number of *Arc*-expressing neurons in the aBNST activity did not significantly correlate with the animals' overall PTSD-like symptom score (Table S1), suggesting that – similar to pre-trauma anxiety-like behaviour - pre-trauma basal aBNST activity is not related to PTSD-like susceptibility. However, we did find a significant association between behaviour in the EPM and the number of *Arc*-expressing neurons in the BNSTOV; spending more time in the centre ($r(16) = .611$, $p = .012$) and moving greater total distance ($r(16) = .564$, $p = .023$) related to higher basal neuronal cell count in the BNSTOV (Fig. S2).

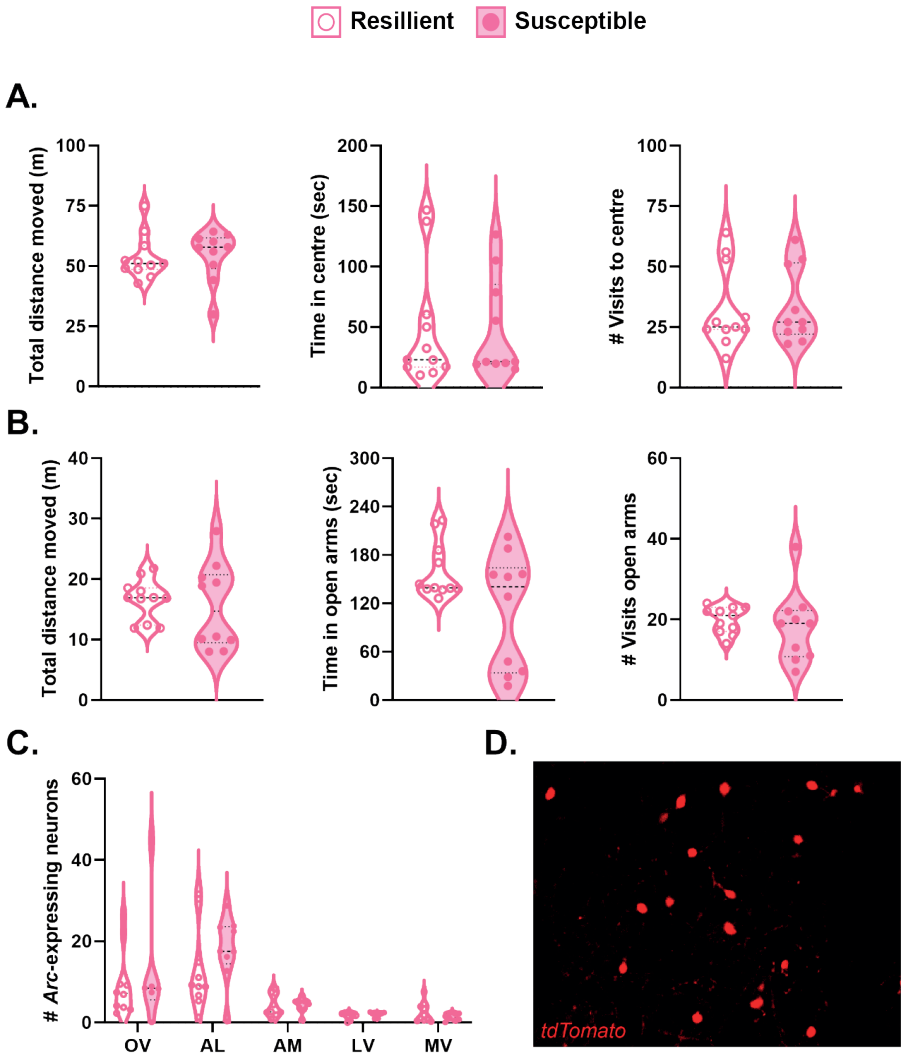


Figure 3. Pre-trauma assessment of anxiety-like behaviour in susceptible vs. resilient mice of cohort 1. Revealed no significant differences between groups in the open field (A) and elevated plus maze (B). No significant differences were found in number of Arc-expressing neurons in the aBNST under pre-trauma basal conditions (C). Arc-expressing neurons labelled by tdTomato in the BNST (D). OV: oval nucleus, AL: anterolateral BNST, AM: anteromedial BNST, LV: ventral lateral BNST, MV: ventral medial BNST.

On the final day of the protocol, mice of this experimental cohort were exposed to a novel context (similar to the trauma/trigger context in a number of features) in order to assess potential generalized recall of contextual fear. Here, susceptible and resilient mice did not show any differences in the total amount of freezing ($F(1,19) = 1.082$, $p = .311$), yet they did show a differential freezing pattern over time (group*time interaction: $F(1,171) = 2.542$, $p = .012$). While susceptible mice reduced their freezing behaviour as time passed ($F(9,81) = 4.970$, $p < .001$), resilient mice did not ($F(2.969, 29.693) = 1.94$, $p = .144$) (Fig. 4b). These data suggest that although both groups initially fear the novel context to a similar degree, the susceptible animals decrease their fear response faster in comparison to the resilient animals. To test whether differences in generalized fear recall between resilient and susceptible mice were also reflected in terms of activity differences in the aBNST, we analysed the aBNST for immediate early gene *cFos* expression as proxy for neuronal activity. The number of *cFos*- expressing neurons differed across aBNST subregions ($F(4,100) = 12.780$, $p < .001$) (BNSTAL = BNSTAM > BNSTOV = BNSTLV > BNSTMV, all p's < .004), but was not different across groups (main effect of group; $F(1,100) = .038$, $p = .845$, group*subregion interaction; $F(4,100) = .257$, $p = .905$, Fig. 4a). Apparently, the differential freezing pattern over time between resilient and susceptible mice was not reflected in differential aBNST activity.

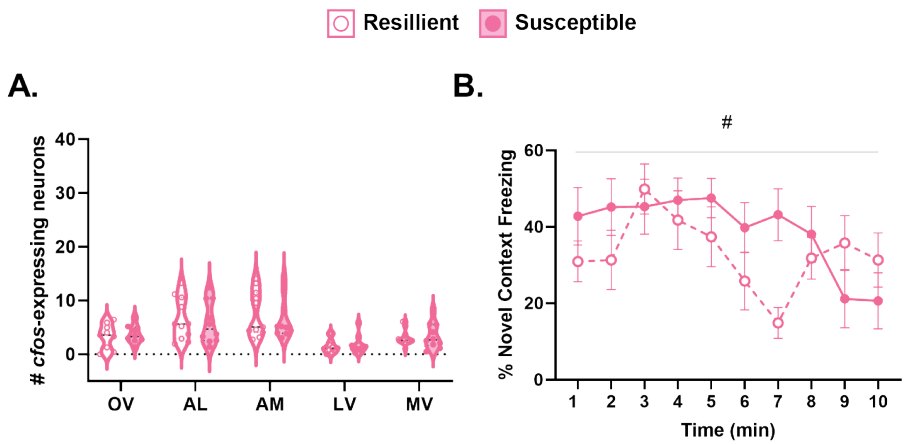


Figure 4. aBNST activity and freezing behaviour upon exposure to a novel context*. No significant differences were found in aBNST activity between the resilient and susceptible mice (A), but susceptible mice showed a differential freezing pattern over time (B). #: p < 0.05, group*time interaction. OV: oval nucleus, AL: anterolateral BNST, AM: anteromedial BNST, LV: ventral lateral BNST, MV: ventral medial BNST.*

Lower aBNST peri-trauma activity predicts PTSD-like symptom development

To test whether differences during trauma processing were predictive of later susceptibility to develop PTSD-like symptoms, behaviour during exposure to the trigger session was measured and compared between susceptible and resilient mice. Freezing during trigger ($t(14) = 1.775$, $p = .098$, Fig. 5a) exposure was similar between the two groups.

In terms of aBNST *Arc*-expressing neurons peri-trauma, we again found strong differences in cell counts across aBNST subregions ($F(4,75) = 19.846$, $p < .001$). *Post hoc* test revealed that all subregions differed significantly from each other (all p's < .002; BNSTAL > BNSTOV > BNSTAM > BNSTMV > BNSTLV). Importantly, we also observed a main effect of group ($F(1,75) = 4.191$, $p = .044$), in the absence of a significant group*subregion interaction ($F(4,83) = 11.536$, $p < .001$, see Fig. 5b). Contrary to our expectations, resilient mice showed higher levels of peri-trauma aBNST *Arc*-expressing cell counts than susceptible mice. Further correlational analysis revealed that peri-trauma counts in the BNSTAL ($r(13) = -.678$, $p = .011$) and BNSTOV ($r(13) = -.614$, $p = .026$) correlated negatively with overall freezing during the trigger session (see Fig. 5c-d), indicating a negative association between aBNST activity and fear acquisition/expression during contextual fear learning as well as PTSD-like symptomatology (Table S2).

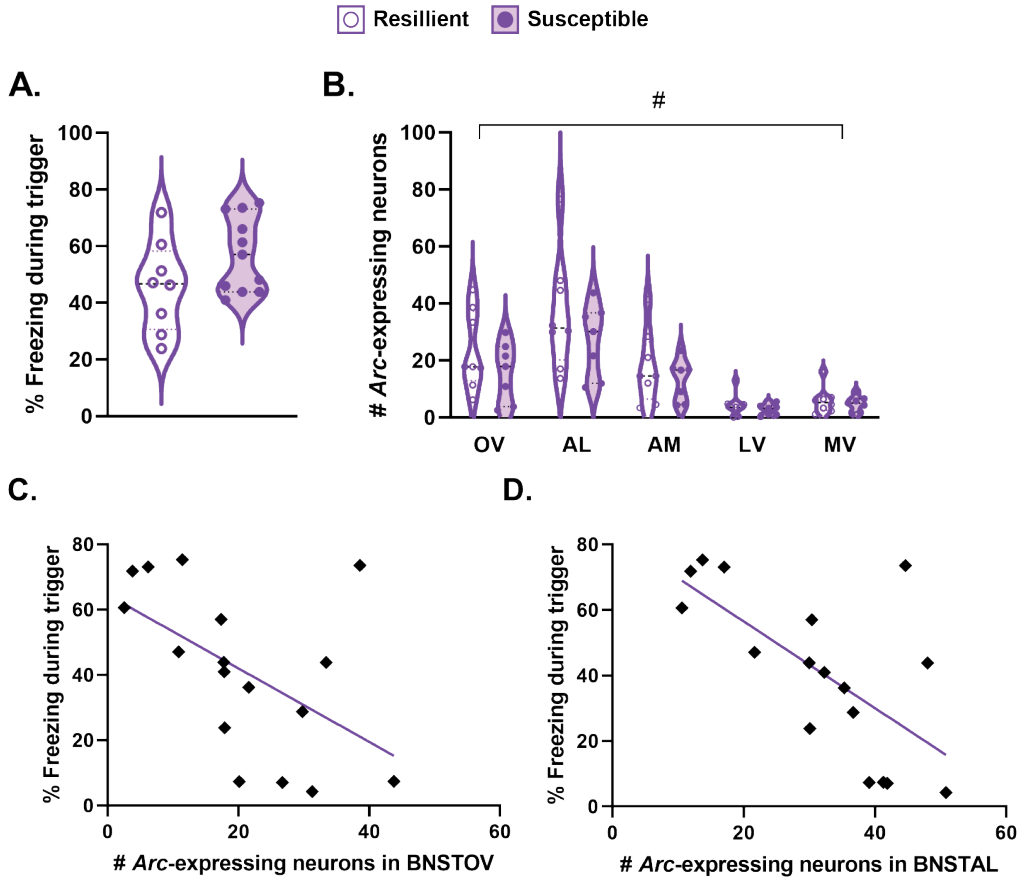


Figure 5. Exposure to trigger. Percentage freezing (A) during trigger exposure with corresponding aBNST activity (B). Negative correlational relationship between percentage freezing during trigger session and number of Arc- expressing neurons in BNSTOV (C) and BNSTAL (D). #: $p < .05$, main effect of group. OV: oval nucleus, AL: anterolateral BNST, AM: anteromedial BNST, LV: ventral lateral BNST, MV: ventral medial BNST.

To investigate the role for aBNST-amygdala crosstalk during the processing of trauma, peri-trauma cell counts in the aBNST subregions were correlated with previously published amygdala counts of *Arc*-expressing neurons peri-trauma in the same animals (Dirven et al. 2022) within each of the groups separately, as a proxy for their connectivity. Resilient animals displayed strong correlations in activity between subregions within the amygdala, as well as high intra-aBNST connectivity (Fig. 6). Moreover, resilient animals tended to show overall positive correlations in cell counts between the basolateral amygdala (BLA) and the aBNST, indicating that higher activity of the BLA during trauma processing correlated with higher aBNST activity. In contrast, susceptible mice did not show significant correlations in *Arc*-expressing neurons among amygdala subregions, and no clear association between BLA cell counts and those in the aBNST. Direct comparison of the groups revealed that resilient animals indeed displayed significantly increased correlations in active cell counts between the BLA and LA (z = 1.67, p = .047), BLA and BNSTOV (z = 1.69, p = .046), BLA and BNSTMV (z = 1.83, p = .034) and a tendency towards increased correlated cell counts between the BLA and BNSTAM (z = 1.41, p = .079). Susceptible animals did show high correlations in *Arc*-expressing neurons across aBNST subregions, similar to resilient animals. Moreover, susceptible mice displayed significant positive correlations in counts between the lateral amygdala (LA) and aBNST, which were absent in resilient animals. These differences just failed to reach significance in the BNSTAL (z = 1.62, p = .053), with resilient mice tending to show lower correlated *Arc*-expressing neurons with the LA compared to susceptible mice. Altogether, these data suggest that amygdala-aBNST crosstalk during the processing of the traumatic experience differs across susceptible and resilient mice.

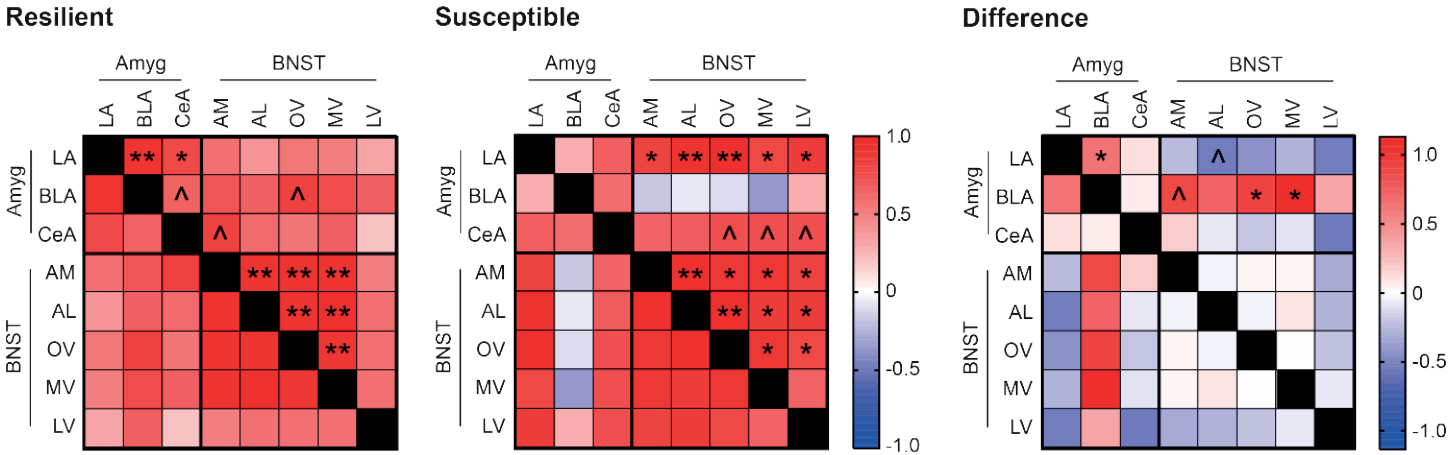


Figure 6. Correlation plots of number of activated (i.e., Arc-expressing) neurons peri-trauma in the amygdala and anterior bed nucleus stria terminalis of resilient and susceptible mice, as well as their difference. *Heatmaps indicate r-values. ^: p < .10, *: p < .05. **: p < .01. Amyg: amygdala, BNST: bed nucleus stria terminalis, AL: anterolateral BNST, AM: anteromedial BNST, BLA: basolateral amygdala, CeA: central amygdala, LA: lateral amygdala, LV: lateroventral BNST, MV: medioventral BNST, OV: oval nucleus of the BNST.*

On the final day of the protocol, mice in this experimental cohort were re-exposed to the trigger context to assess remote contextual fear recall. Freezing behaviour was not different between the two groups in terms of overall levels ($F(1,163.690) = .150$, $p = .699$) or the reduction in freezing behaviour over time ($F(9,167.649) = 4.194$, $p < .001$, group*time interaction: $F(9,167.649) = .602$, $p = .794$, Fig. 7a). In line with this, recall-induced aBNST neuronal activity revealed a main effect of subregion ($F(4,83) = 11.536$, $p < .001$), without effects of group ($F(1,83) = 1.439$, $p = .234$) or a group*subregion interaction ($F(4,83) = .145$, $p = .965$, Fig. 7b). *Post hoc* tests revealed that the BNSTAL and BNSTAM differed significantly from BNSTOV, BNSTMV and BNSTLV (BNSTAL = BNSTAM > BNSTOV= BNSTMV = BNSTLV, all p's < .001). Analyses of the neurons that were both activated during contextual fear memory encoding (by *tdTomato*) and remote recall (by *cFos*) allowed us to assess reactivated neurons, as potentially part of the contextual fear memory engram. No main effects of subregion ($F(4,70) = 2.142$, $p = .085$), group ($F(1,70) = .281$, $p = .598$) or group*subregion interaction were found ($F(4,70) = .690$, $p = .601$) in terms of number of reactivated cells (Fig. 7c-d). Yet, correlational analyses revealed a positive relationship between BNSTAL reactivation and PTSD-like symptom score ($r(14) = .715$, $p = .004$).

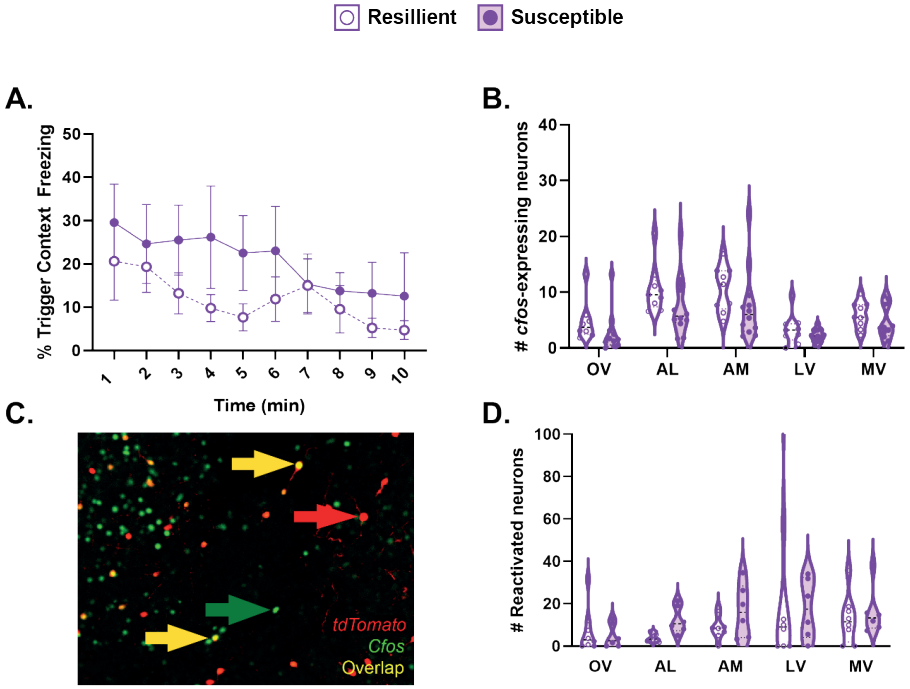


Figure 7. Freezing and aBNST cfos-expressing neurons during re-exposure to the trigger context*. No significant differences were found in percentage freezing towards trigger context re-exposure between susceptible and resilient mice (A), nor did we find any difference in re-exposure-induced aBNST activity (B). Lastly, we did not find any differences in reactivation rate of aBNST neurons between the two groups (C-D). OV: oval nucleus, AL: anterolateral BNST, AM: anteromedial BNST, LV: ventral lateral BNST, MV: ventral medial BNST.*

PTSD-like symptomatology is not associated with differential aBNST activity post-trauma

To determine whether PTSD-like symptomatology is related to alterations in aBNST activity, we also labelled active neurons (quantified by the number of *Arc*-expressing neurons) under basal conditions post-trauma. Again, we observed a significant main effect of subregion ($F(4,90) = 44.764$, $p < .001$), in the absence of a main effect of group ($F(1,90) = .158$, $p = .692$) or group*subregion interaction ($F(4,90) = .073$, $p = .990$, Fig. 8a). *Post hoc* analyses revealed significant differences between the majority of subregions (BNSTAL > BNSTOV > BNSTAM > BNSTLV = BNSTMV, all *p*'s < .001). Further, correlational analyses between basal subregional *Arc*-expressing neurons and PTSD-like symptom score did not reveal any significant correlations, suggesting that PTSD-like symptomatology is not associated with altered post-trauma basal aBNST activity (Table S1).

On the final day of testing, animals in this experimental cohort were re-exposed to the trauma context. Susceptible mice showed similar levels of freezing as resilient mice ($F(1,16) = .052$, $p = .824$). Freezing rates changed over time ($F(9,144) = .3210$, $p = .001$), but not differently between groups ($F(9,144) = .623$, $p = .776$, Fig. 8b). As such, both groups clearly expressed a contextual fear memory towards the trauma context, but this expression did not differ between the groups.

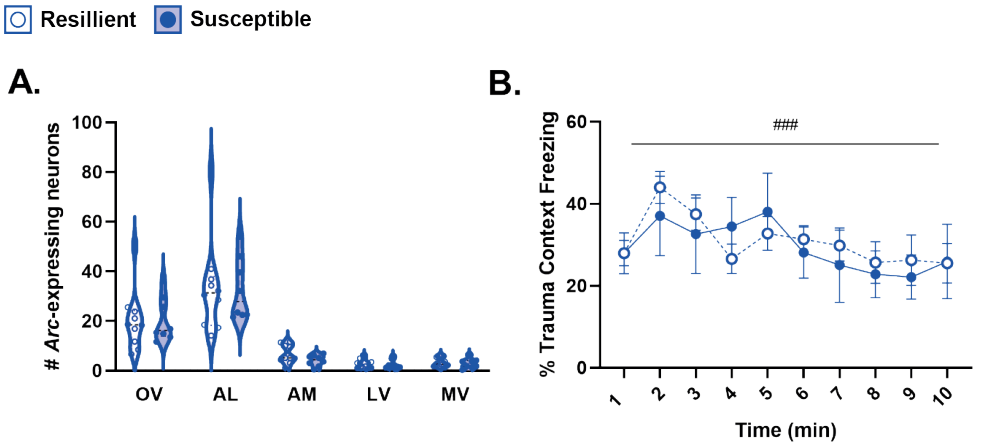


Figure 8. Basal post-trauma aBNST Arc-expressing neurons (A) and freezing behaviour during re-exposure to the trauma context (B)*. No significant differences were found in basal aBNST counts between susceptible and resilient mice. No differences in percentage freezing were found between the two groups, however we did find a main effect of time. ###: p < 0.001, main effect of time. OV: oval nucleus, AL: anterolateral BNST, AM: anteromedial BNST, LV: ventral lateral BNST, MV: ventral medial BNST.*

Discussion

Here, we set out to determine the role of aBNST activity in conferring susceptibility to developing PTSD- like behavioural symptoms following exposure to a PTSD induction method in mice. Anxiety-like behaviour and aBNST activity under basal conditions preceding trauma exposure did not predict later resiliency or susceptibility to PTSD-like symptoms. Yet, susceptible mice displayed lower levels of overall aBNST activated cell counts peri-trauma in comparison to resilient mice and aBNST activated cell counts negatively related to freezing behaviour during fear memory acquisition. Interestingly, we also observed distinct correlations between the number of *Arc*-expressing neurons in amygdala subregions and those in the aBNST, suggesting aberrant amygdala-aBNST functional connectivity in susceptible mice. No differences in aBNST *Arc*-expressing neurons were observed under basal conditions post-trauma. In response to contextual fear memory recall, susceptible mice showed quicker declines in their freezing rates over time when exposed to a novel context similar to prior conditioned contexts, compared to resilient mice. No behavioural differences were observed between groups upon exposure to the actual trauma and trigger contexts, nor were there any differences in aBNST activity in response to context (re)- exposure.

Patient studies into the contribution of the BNST to psychopathology are clearly limited in their spatial resolution, preventing the detailed study of the functionally distinct BNST subregions. Moreover, BNST responses to actual trauma exposure in patients are for obvious ethical reasons inaccessible. Here, we used an animal model for PTSD that was earlier found to be associated with aberrant function of the posterior BNST (Lebow et al., 2012, Henckens et al., 2017) to investigate the role of the anterior BNST in the development of PTSD-like symptomatology following trauma. In contrast to traditional animal studies investigating the effects of trauma by comparing trauma-exposed to non-exposed controls, we distinguished between adaptive vs maladaptive trauma coping and the development of PTSD-like symptoms as a consequence of it, by exposing all animals to a traumatic event and assessing behavioural phenotypes afterwards. We used a compound score of multiple behavioural PTSD-like symptoms to categorize the animals as either susceptible or resilient (Lebow et al., 2012; Henckens et al., 2017). Mice were selected based on PTSD-like behaviours reflecting the hyperarousal and reactivity symptom cluster of PTSD in the DSM-V

(e.g. impaired risk assessment, increased anxiety and hypervigilance, reduced pre-pulse inhibition and disturbed sleeping behaviour) (APA, 2013). This approach more closely resembles the patients' situation in which a combination of various symptoms can lead to PTSD diagnosis (APA, 2013). In line with large symptom heterogeneity in patients (Bonano & Mancini, 2012; Lanius et al., 2006), we observed high intra- and inter-cohort variability in the exact behavioural traits of susceptible mice. However, whereas individual symptom profiles differed, susceptible mice showed significantly more PTSD-like symptoms than resilient mice throughout all cohorts.

Resilient and susceptible mice showed no differences in basal anxiety and the number of aBNST *Arc*- expressing neurons pre-trauma. This is in contrast to previous research in humans suggesting that heightened levels of (trait) anxiety negatively affect trauma processing and consequently increases the risk for PTSD development and symptom severity (Schweizer et al., 2017; Christiansen & Elklit, 2008; La Greca, Silverman & Wasserstein, 1998; Larsson, Backstrom & Johanson, 2008; Brunet et al., 2013; Jaksic et al., 2012). Yet, other work has suggested that the association between pre-trauma anxiety and trauma susceptibility only becomes apparent under conditions of mild threat (Sullivan et al., 2004; Nalloor et al., 2011). As such, associations between anxiety pre-trauma and trauma susceptibility may only emerge under more challenging conditions than the ones assessed here. A similar scenario might hold for aBNST activity pre-trauma. In our study, aBNST activity pre-trauma and post-trauma was labelled under home cage conditions and activity was therefore assessed in the absence of any threatening cues or contexts that would activate the aBNST differently between groups. The absence of differences in the number of aBNST *Arc*-expressing neurons post-trauma corresponds to findings of no differences in BNST activity in PTSD patients during resting state compared to controls (Sheynin et al., 2020; Yin et al., 2011), whereas patients did display a heightened BNST response to threatening cues opposed to neutral ones (Liberzon et al., 1999; Brinkmann et al., 2017). Future work should investigate whether the introduction of a mild challenge would elicit a stronger anxiogenic response in susceptible mice pre- and post-trauma, as well as differently recruit the aBNST, to fully investigate the association between aBNST function and trauma susceptibility.

Peri-trauma activity of the aBNST as assessed by the number of *Arc*-expressing neurons was however shown to be lower in susceptible mice in comparison to resilient mice. This is in contrast to our hypothesis and literature indicating heightened levels of BNST activity in PTSD patients, although these comparisons are often made against non-traumatised controls (Binkmann et al., 2017; Awashti et al., 2020) and consider the BNST as a single, homogenous unity, not dissociating the anterior from posterior BNST (Feola et al., 2023; Brinkmann et al., 2017; Awashti et al., 2020). However, it is important to note that the labelled *Arc*-expressing neurons in our study only reflect activated glutamatergic neurons, as the immediate early gene *Arc* is hardly expressed in GABAergic cells (Bramham et al., 2008). This excludes an important fraction of aBNST neurons, which are mostly GABAergic (Haubensak et al., 2010; Daniel & Rainnie, 2016; Partridge et al., 2016). In the dorsal-anterior regions, around 66-79% of neurons are GABAergic (Daniel & Rainnie, 2016; Nguyen et al., 2016; Kim et al., 2013), while the ventral and posterior regions have a more equal distribution (Nguyen et al., 2016; Poulin et al., 2009). Yet, we preferred using the transgenic ArcTRAP line over the alternative FosTRAP line, because of its enhanced labelling sensitivity in several other brain regions (Guenther et al., 2013). As a result, our current study is limited to solely making inferences about the role of the glutamatergic neuronal population of the aBNST. This could potentially explain some of the discrepancies between our results, particularly the lower levels of peri-trauma aBNST *Arc*-expressing neurons in susceptible mice compared to resilient ones, but also the negative association between aBNST activated cell counts and freezing behaviour, and reports of a mainly anxiogenic role for the aBNST. Interestingly, decreases in excitatory/inhibitory balance within the anteroventral BNST have been related to increased fear expression (Bartsch et al., 2021), matching our findings. Also, noradrenaline – known to be related to hyperarousal and found to be increased in PTSD patients (Ronzoni et al., 2016; O'Donnell, Hegadoren & Coupland, 2004) – has been reported to trigger GABAergic inhibition of excitatory neurons within the BNSTLV (Dumont and Williams, 2004), which matches the observation of lower activity of these neurons in susceptible mice. Yet, there is other work reporting on anxiogenic role for BNST glutamatergic neurons as well (Jennings et al., 2013; Luskin et al., 2021), suggesting regional and projection-site specific roles for these neurons similarly to what is observed for GABAergic cell populations in the BNST.

Interestingly, we also observed differential correlations in activity patterns between the subregions of the extended amygdala (Dirven et al., 2022) and the aBNST in susceptible mice. Of particular interest seems to be the reduced positive crosstalk between the BLA and aBNST in susceptible mice. BLA projections to the BNSTOV have previously been implicated in the encoding of contextual fear conditioning (Russell et al., 2020), whereas BLA projections to the BNSTAL in general were found to contribute to the sustained fear response to unpredictable threat, and shown to be modulated by endocannabinoid signalling (Lange et al., 2017), known to be affected in PTSD (Sloan et al., 2019; Hill et al., 2013). Inhibition of BLA-aBNST projection neurons was moreover found to suppress conditioned sustained freezing during recall (Vantrease et al., 2022). As the BLA projection neurons were found to target *GAD67*-negative neurons within the BNSTAL (Lange et al., 2017), increased BLA activity during contextual fear conditioning is expected to increase aBNST glutamatergic activity. We only observe such positive correlation in activity within resilient mice, making it tempting to speculate that this connectivity contributes to a healthy encoding of contextual fear that seems to be distorted in susceptible mice. Yet, future studies should investigate this speculation.

We did not find any clear deviations in fear recall between susceptible and resilient mice, neither in behavioural fear responses nor in terms of aBNST activity. Noteworthy, our model, focusing on impaired risk assessment, high anxiety, hypervigilance, attention disturbances and insomnia, is strongly based on the 'trauma-related arousal and reactivity' symptom cluster of PTSD in the DSM-V (APA, 2013). While assessing multiple symptoms, it likely does not capture the full, complex human PTSD-symptomatology, and might thus lack the maladaptive trauma memory trace as observed in PTSD patients. Yet, prior observations on deviations in recall-induced neuronal activity in the hippocampus (Dirven et al., under revision) and amygdala (Dirven et al., 2022) within this mouse model would suggest memory abnormalities in susceptible mice. However, these prior findings have been interpreted in terms of aberrant fear memory quality in susceptible mice, which might not be readily captured by re-exposing them to the exact same environment (in case of the trauma and trigger context), or to salient cues present in that environment (the shock grid in the novel context). Future studies should further investigate this hypothesis and test whether potential behavioural manifestations of a maladaptive fear memory are associated with aberrant aBNST activity.

Some limitations to this work should be mentioned. Firstly, we examined solely aBNST activity through the use of immediate early genes (IEG) such as *cFos* and *Arc* while ignoring the cell type diversity within the BNST (Bota et al., 2012; Moffitt et al., 2018; Welch et al., 2019). We attempted several immunohistochemical stainings to identify these cell populations (e.g., CRF, GAD67, somatostatin), but failed to achieve reliable stainings of the aBNST. Likely this is why most other studies make use of specific types of transgenic mouse lines to investigate their target cell type in the aBNST (Partridge et al., 2016; Bruzsik et al., 2021; de Bundel et al., 2016). This was not a possibility in the current study, as we already utilized the TRAP mouse line. However, in future work the PTSD-mouse model could be combined with another type of transgenic line to investigate specific cell types. Secondly, despite women being more likely to develop PTSD in their lifetime (Breslin et al., 1997; Brewin, Andrews & Valentine, 2000) and the sexually dimorphic nature of the BNST (Lebow et al., 2016), only male mice were used in our study as our PTSD-mouse line was validated solely with male mice (Lebow et al., 2012). For future purposes, investigating susceptibility to PTSD-like symptomatology in female mice would be greatly beneficial, although the current PTSD mouse model should be altered specifically for female rodents, as females tend to display different coping behaviour in response to stress as well as differential behavioural consequences in comparison to males (Gruene et al., 2015; Genn et al., 2003; Johnston & File, 1991; Stack et al., 2010, see **chapter 4** of this thesis). Thirdly, in this study we have only performed immunohistochemistry on the right hemisphere of the mouse brains following the PTSD paradigm. The left hemisphere was processed via a relatively new whole brain clearing technique, called iDISCO+ (Renier et al., 2014), that allowed us to investigate pre-, peri-, and post-trauma brain activity in a hypothesis free manner. Lastly, due to technical difficulties, we were unable to analyze brain activity during the exposure to the trauma context in the final cohort 3, preventing us from comparing aBNST activity between trauma context with the trigger and a novel context.

Concluding, we observed no associations between pre-trauma basal anxiety and pre- and post- trauma basal aBNST activity and susceptibility to PTSD-like symptomatology in mice. Yet, our results indicate that lower glutamatergic activity within the aBNST during trauma processing is associated with later development of PTSD-like symptoms. Further, the observed differences in amygdala-aBNST correlations in activity counts suggest a differential functional connectivity of the extended amygdala in susceptible compared to resilient mice.

Future studies should follow up on these initial results by investigating activity in different neuronal subclasses and under conditions of mild threat pre- and posttrauma, to evoke aBNST activation.

Supplementary Data

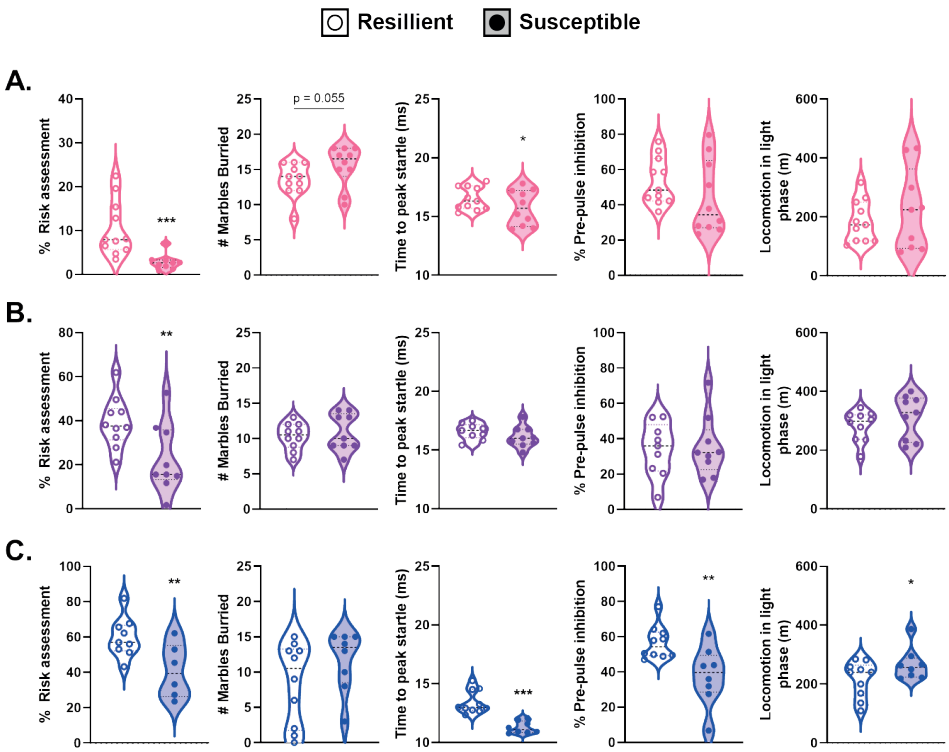


Figure S1. PTSD-like symptoms in mice. *A. Pre-trauma cohort 1. Susceptible animals showed significantly less risk assessment behaviour (U= 0, p < .001), reduced pre-pulse inhibition (U = 41, p = .039), as well as trend-level significant higher marble burying behaviour (t(22) = 1.667, p = .055). B. Peri-trauma cohort 2. Susceptible mice displayed strongly reduced risk assessment behaviour (t(20) = 3.221, p = .002), but no significant differences on other behavioural tests (all p's > .05). C. Post-trauma cohort 3. Susceptible mice showed lower risk assessment behaviour (t(17) = 3.261, p = .003), a shorter reaction time to peak startle (t(14.604) = 5.901, p < .001), reduced pre-pulse inhibition (t(17) = 2.811, p = .006), as well as higher locomotor activity in the light phase (t(17) = 2.067, p = .027) compared to resilient ones. ***: p < 0.001, **: p < 0.01, *: p < 0.05.*

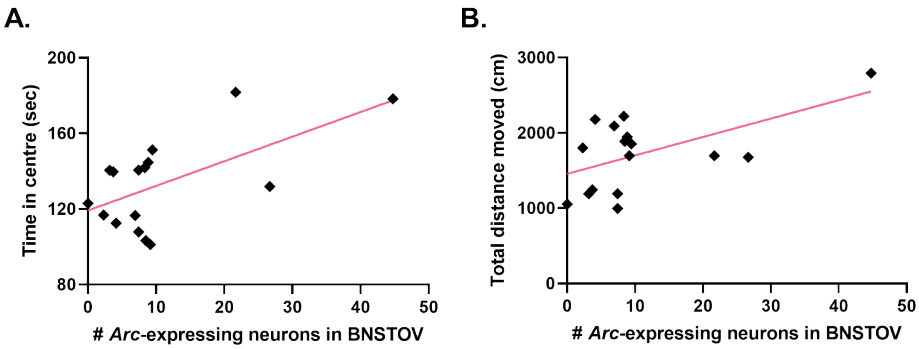


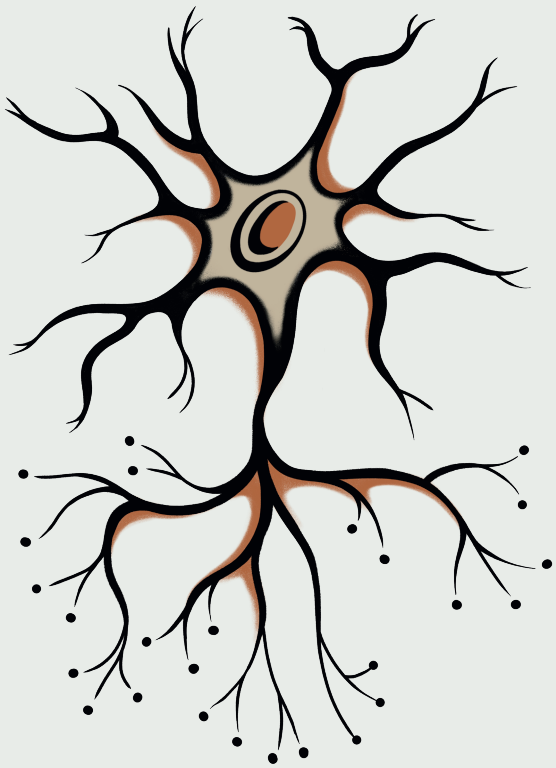
Figure S2. Positive correlational relationships between anxiety-like behavior in the elevated plus maze and pre-trauma basal aBNST activity*. Time spent in the centre (r(16) = .611, p = .012) (A), and moving greater distance (r(16) = .564, p = .023) (B), related to higher basal activated cell count in the BNSTOV.*

aBNST subregion	Pre-trauma	Peri-trauma	Post-trauma
aBNST	*r*(14) = .139, *p* = .635	*r*(15) = -.176, *p* = .530	*r*(20) = .019, *p* = .953
aBNSTAL	*r*(19) = .240, *p* = .323	*r*(15) = -.178, *p* = .526	*rho*(20) = .086, *p* = .720
aBNSTOV	*rho*(16) = .015, *p* = .995	*r*(15) = -.232, *p* = .406	*rho*(20) = -0.24, *p* = .919
aBNSTAM	*r*(17) = -.032, *p* = .902	*r*(15) = -.115, *p* = .682	*r*(20) = -.356, *p* = .124
aBNSTLV	*rho*(17) = .285, *p* = .267	*rho*(15) = -.055, *p* = .845	*rho*(20) = -.032, *p* = .892
aBNSTMV	*r*(18) = -.330, *p* = .181	*rho*(15) = -.059, *p* = .834	*r*(20) = -.115, *p* = .630

Table S1. PTSD-symptom score correlations with pre-trauma, peri-trauma and post-trauma aBNST activity. No significant correlations were found between aBNST subregion activity and PTSD-symptom score.

aBNST subregion	Percentage freezing during trigger session
aBNSTAL	*r*(13) = -.678, *p* = .011*
aBNSTOV	*r*(13) = -.614, *p* = .026*
aBNSTAM	*r*(13) = - .476, *p* = .100
aBNSTLV	*rho*(13) = -.541, *p* = .056
aBNSTMV	*rho*(13) = -.333, *p* = .266

Table S2. Correlations between the percentage freezing during the trigger session with peri-trauma aBNST activity*. Freezing behaviour during the trigger session correlated negatively with aBNSTAL and aBNSTOV peri-trauma activity.*



3

EXTENDED AMYGDALA ACTIVITY AND CONNECTIVITY IN FEAR GENERALIZATION AND ANXIETY IN MICE

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*: equal contributions

Abstract

Generalizing fear for threatening situations to similar experiences is essential for survival. Yet, excessive generalization can become harmful for one's well-being and result in heightened fear and anxiety in completely safe situations. The extended amygdala circuitry has proven essential in mediating both fear and anxiety behaviours, yet its exact role in the generalization of fear is yet unclear. Here, we aimed to study the role of the extended amygdala circuitry in fear generalization and subsequent anxiety. In a series of mouse experiments, we mapped the recruitment of the amygdala and anterior bed nucleus stria terminalis (aBNST), as well as the correlations in their activity during the exposure to a differential auditory fear conditioning paradigm in which one auditory stimulus (CS+) was linked to foot shock delivery, whereas another auditory stimulus (CS-) was not. Different shock intensities were used to modulate the degree of fear generalization towards the CS- during subsequent fear memory recall, as well as subsequent display of anxiety-like behaviour. Moreover, we identified a subpopulation of neurons in the basolateral amygdala (BLA) projecting to the anterolateral BNST that is recruited during fear acquisition and manipulated these neurons using chemogenetics to assess their contribution to fear generalization and anxiety. Contrary to our expectations, heighted foot shock intensity increased overall freezing behavior in mice, but did not affect their generalization of fear towards the CS-. Neurally, we observed that higher shock intensity increased amygdala and aBNST activity during fear acquisition, while overall exposure to foot shocks, independent of their intensity, increased aBNST activity during subsequent exposure to an anxiogenic situation. Interestingly however, BLA activity during fear acquisition predicted later fear generalization, whereas BLA activity in an anxiogenic situation correlated negatively with aBNST activity, posing a critical role for the BLA in these behaviours. Activity-dependent tracing experiments revealed that BLA neurons recruited during fear acquisition primarily projected to the anterolateral BNST (BNSTAL). Activating these direct BLA-BNSTAL projections during fear acquisition reduced the expression of fear during learning as well as fear behaviour upon subsequent CS re-exposure. However, it did not seem to affect fear generalization, nor produce consistent effects on subsequent anxiety-related behaviour. Activating BLA-BNSTAL projections did not have an immediate effect on anxiety-like behaviour in the open field test. These data reveal a new circuitry within the extended amygdala that is critical for the acquisition and expression of cued fear, inviting future research to further dissect its contribution to generalized fear recall.

Introduction

Evolutionarily, it is essential to generalize adverse, threatening experiences across similar stimuli and events, considering the unlikely re-encounter of these events in the exact same form or way. As such, generalization of fear to alike experiences benefits survival and is highly adaptive. Yet, if fear generalization becomes excessive and extends to very dissimilar exposures and anticipatory anxiety, it can become harmful to one's wellbeing, restricting one's movements and deteriorating quality of life. This phenomenon is defined as maladaptive overgeneralization of fear (Johnson et al., 1992; McEwen, 1998) and is considered a common denominator in many stress- and anxiety-related disorders, including generalized anxiety disorder (GAD), panic disorders, phobias, and posttraumatic stress disorder (PTSD) (Craske et al., 2009; Davis et al., 2010; Lissek et al., 2010; Lissek, 2012; Lissek et al., 2014; McTeague et al., 2009; Morey et al., 2015).

While fear and anxiety serve a similar purpose, i.e. to prevent exposure to the source of danger by applying either active or passive coping mechanisms (Steimer, 2002), they have dissimilar characteristics. Fear (also referred to as phasic fear) can be described as an emotional state elicited by imminent threat, and is only expressed upon actual, acute sensory input indicative of this threat (Davis et al., 2010). As such, it typically dissipates once the threat is gone. Anxiety (also described as sustained fear), is defined as a future-oriented sustained state associated with the anticipation of potential, yet not-encountered threats (Barlow, 1988; Brinkmann et al., 2017). Since in case of fear overgeneralization a distant ambiguous cue or context is sufficient to trigger a previously acquired fear response, it might connect both phenomena. This is supported by computational methods suggesting that an ineffective discrimination between events (and thus generalization across them) increases overall threat perception and contributes to higher state anxiety (Raymond et al., 2017).

Fear and anxiety seem to be governed by similar, highly inter-connected, yet dissociable, neural circuits (Davis et al., 2010; Münsterkötter et al., 2015). The extended amygdala, a circuit that includes the Bed Nucleus of Stria Terminalis (BNST) and the amygdala, has been widely implicated in both fear and anxiety states (Münsterkötter et al., 2015; Shackman & Fox, 2016). The BNST and the amygdala are anatomically quite similar in terms of inputs,

outputs and cellular content (Davis et al., 2010) and are inter-connected (Brinkmann et al., 2018; Mobbs et al., 2010; Weis et al., 2019), yet seem to serve slightly different functions. States of fear, typically studied by fear conditioning, mainly recruit a local circuit within the amygdala. This involves the lateral (LA), basolateral (BLA) and centrolateral (CeL) and centromedial (CeM) subregions of the amygdala, with the CeM mediating fear output. In contrast, anxiety states have been primarily linked to recruitment of the BNST.

Prior work also implicated a role for the extended amygdala in fear generalization. Amygdala (hyper)activity has been associated with increased fear (Ciocchi et al., 2010), fear generalization (Ghosh & Chattarji, 2015; Rajbhandari et al., 2016), and anxiety (Botta et al., 2015; Etkin et al., 2004; Sajdyk et al. 1999; Truitt et al., 2009). Likewise, the BNST was found to be essential for fear generalization (Duvarci et al., 2010), hyperactive in generalized anxiety disorder (Buff et al., 2017), and linked to susceptibility for anxiety disorders (Avery et al., 2015; Figel et al., 2019; Münsterkötter et al., 2015). These observations suggest that the extended amygdala circuitry is essential for adequate fear discrimination vs. generalization (De Bundel et al., 2016; Kim et al., 2013). However, the exact amygdala-BNST circuits involved in fear generalization behaviour remain to be eluded, as well as the association between these circuits and anxiety symptomatology. Detailed understanding of these circuits is crucial for developing more optimized treatments and targeting the prevention of fear overgeneralization and anxiety symptomatology in a wide range of stress- and anxiety-related disorders.

Here, we set out to investigate the role of the extended amygdala circuitry in fear generalization and anxiety-like behaviour. We did so using transgenic mice, allowing for the detailed, longitudinal dissection of activity of the distinct subnuclei within both the amygdala and aBNST, their exact connections and their manipulation to assess causality. We used a differential auditory fear conditioning (DAFC) paradigm during which mice were exposed to two distinct auditory cues, that were either predictive (CS+) or non-predictive (CS-) of foot shock delivery. Subsequent fear responses to the (non-reinforced) CS+, as well as the generalization of the fear response to the CS- were assessed. Moreover, general anxiety levels were assessed using a set of tests for anxiety-like behaviour in order to investigate a potential association between the extent of fear generalization and subsequent anxiety. In four separate experiments, we studied how fear generalization relates to both

the activity and connectivity patterns of the amygdala and aBNST, to ultimately manipulate amygdala-aBNST connectivity to test for a causal relationship.

Materials and methods

Animals.

This study consisted of four separate experiments: Experiment 1 (n = 56) set out to assess neuronal activity in amygdala and aBNST subnuclei related to the acquisition of specific vs. generalized fear and anxiety-like behaviour by using a differential auditory fear conditioning paradigm (DAFC) in which different foot shock intensities were used to modulate levels of fear generalization and anxiety. Experiment 2 (n = 24) investigated the amygdala projections to the aBNST that are recruited during fear acquisition by activity-dependent anterograde viral tracing. Experiment 3 (n = 13) set out to show our ability to modulate activity of amygdala-aBNST projection neurons by chemogenetic manipulation. Experiment 4 (n = 39) aimed at providing causal evidence for the involvement of these projection neurons in the regulation of generalized fear and anxiety by means of chemogenetic manipulations (see Fig. 1 for experimental timelines). For experiments 1 and 2, male *FosTRAP2xtdTomato* offspring, referred to as *FosTRAP2* (Guenthner et al., 2013; Allen et al., 2017) were used; the offspring of *FosTRAP2* females (*Fos*$^{2A\text{-}iCreERT2}$, the Jackson Laboratory, #0030323, bred in-house) crossed with *tdTomato* males (*Ai9*, The Jackson Laboratory, #030323, bred in-house). In these mice, *cFos*-expressing (i.e. activated) neurons can be permanently labelled by the fluorescent protein *tdTomato*, by the injection with the compound 4-hydroxytamoxifen (4-OHT). For experiment 3 and 4, *C57BL/6* males were purchased from Charles River Laboratories (France). Due to the sexually dimorphic function of the BNST (Allen & Gorski, 1990; Shah et al., 2004; Whylings et al., 2020), as well as sex differences in fear acquisition, expression and anxiety (Merz, Kinner & Wolf, 2018; Inslicht et al., 2013; Kring & Gordon, 1998; Fenton et al., 2016; Johnston & File, 1991), only male mice were used for these experiments. Two weeks before the start of the DAFC, 10-12 week old mice were single housed in conventional Mouse Eurostandard type IIL cages (Techniplast) on a reverse light/dark cycle (lights on 09:00-21:00 h) at the Central Animal Facility of the Radboud University Nijmegen, The Netherlands, according to institutional guidelines. Food and water were available *ad libitum*, and behavioural testing occurred at least 3 h into the active

phase. The experimental protocols were in line with the international guidelines and approved by the Central Committee for Animal Experiments, Den Haag, The Netherlands.

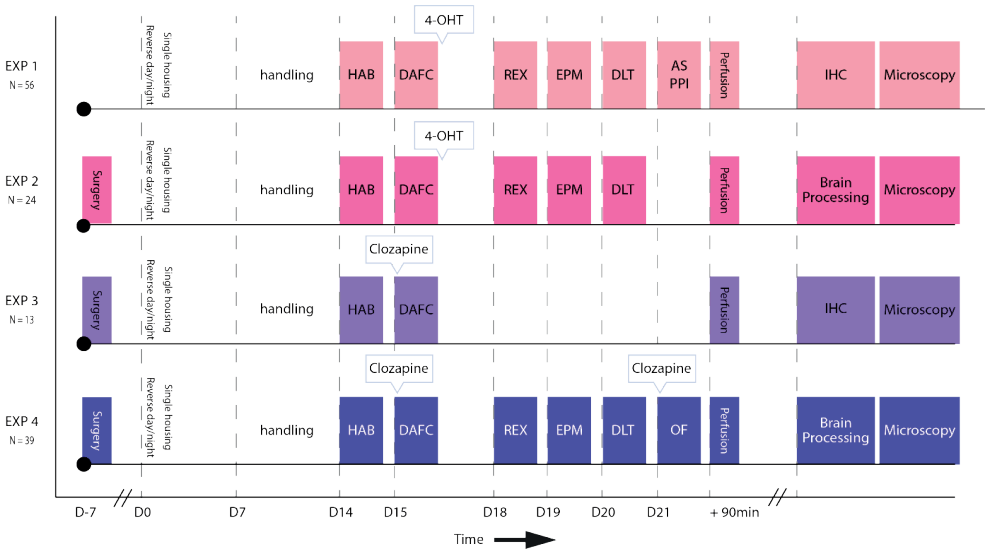


Figure 1. Overview of the experimental timelines*. Animals were exposed to the differential auditory fear conditioning (DAFC) paradigm, and tested on their fear generalization and anxiety behaviour. 90 min after the final test, animals were sacrificed and brain tissue was collected for further processing. D, day; HAB, habituation; DAFC, differential fear conditioning; REX, auditory cue re-exposure; EPM, elevated plus maze; DLT, dark-light transfer test; AS, acoustic startle test; PPI, pre-pulse inhibition test; OF, open field; IHC, immunohistochemistry; 4-OHT, 4-hydroxytamoxifen*

Surgery.

Mice in experiments 2, 3 and 4 first underwent intracranial brain surgery. Prior to all surgeries (48-24 h), mice received carprofen in their drinking water (1:1000) until 72 h post-operative (unless longer was necessary in case of a loss of bodyweight). For the surgery, mice were anesthetised using isoflurane inhalation (4-5% for induction, 1.5-3% maintenance), and received a local injection under the scalp (0.1 uL), which was a mix of 10 mg lidocaine and 5 mg bupivacaine with saline (1:1:2). Mice were allowed to recover for at least 2 weeks before the start of the behavioural experiments, both for the animals' health and to allow viral transfection to occur prior to recombination. For all viral injections a 26 gauge syringe was used to inject the virus at 100 nL/min. The syringe was left in place for 10 min following injection to ensure diffusion and reduce backflow.

For experiment 2, 0.4 uL *AAV5-pAAV-hSyn-DIO-EGFP* (Addgene, #50457-AAV5) was bilaterally injected into the BLA (ML: +3.20/-3.20; AP: -1.1; DV; -4.65 mm relative to Bregma). Targeting was successful in 11 out of 13 animals. In 5 of these animals, both hemispheres were correctly targeted, while in the other 6 only 1 hemisphere was targeted correctly. All hemispheres with a correctly targeted BLA were included for analyses. aBNST subregions were considered to have anterograde labelling when clear signal intensity was found within the subregion.

For experiment 3 and 4, 0.4 uL of either *AAV9-hSYN-DIO-mCherry* (control) (Addgene, #50459-AAV9), *AAV9-hSYN-DIO-hM3D(Gq)-mCherry* (Addgene, #44361-AAV9, experiment 3 and 4) or *AAV9-hSYN-DIO-hM4D((Gi)-mCherry* (Addgene, #44362-AAV9, experiment 3) was bilaterally injected into the BLA (ML: +3.20/-3.20; AP: -1.1; DV; -4.5 mm relative to Bregma). These mice received an additional bilateral injection of 0.4 uL *EEN.AAV.hSYN.HI.eGFP-Cre.WPRE.SV40* (Addgene, #105530-AAVrg) into the aBNST (ML: +0.85/-0.85; AP: +0.03; DV: -4.5 mm from Bregma), ensuring viral recombination and *mCherry* expression in aBNST-projecting BLA neurons specifically.

Handling.

To reduce anxiety induced by human handling, all mice were handled starting one week before the start of the experiment. Mice were handled 5 times for several minutes, distributed over the course of 7 days. On handling sessions 1 and 2, mice were tail grabbed and placed on the experimenter's lab coat sleeve. Mice were able to explore the experimenter's lower arm for 2 min, while the researcher held the tail without exerting force or limiting the freedom of movement. On handling sessions 3-5, mice were lifted by cupping and allowed to freely explore the open hands of the researcher for 2 min. After each session, mice were placed back in the home cage and left undisturbed until the next session. Mice were cupped from then onwards.

Habituation to context.

On the first day of the behavioural experiment, mice were habituated to two contexts (Campden boxes, Bussey-Saksida touch screen chambers, model 80614) in order to familiarize them to the contexts prior to cued conditioning. Context A consisted of a triangular shaped box with black walls, a metal grid floor, no lighting, and was sprayed with

1% acetic acid. After 2 min of habituation to this context, animals were exposed to 8 repetitions of two auditory tones (CS-; 10 kHz, 10 s, 85 dB, CS+; 5 kHz, 10 s, 85 dB), in a semi-random order with variable intervals (ITI: 40-120 s). Context B consisted of a box with white round walls, a white PVC floor, 45 Lux light and had no distinguished smell. During habituation to this context, no auditory tones were presented. Animals were exposed to both contexts for 30 min long, with 3 h in between the first and second session. Exposure to the contexts was counter-balanced across mice.

Clozapine.

For experiment 3 and 4, clozapine (Sigma-Aldrich, #C6305) was first dissolved in 1 M HCl and 1 x PBS, to generate a stock solution with a concentration of 0.2% clozapine / 5% 1 M HCL / 95% 1 x PBS. Then, the stock solution was added to 1 x PBS to create a final working solution concentration of 0.0006% clozapine/ 0.015% 1 M HCL / 100% 1 x PBS. pH was set to 7.2-7.4. 30-45 min before the DAFC (experiment 3 and 4) and the open field test (OF) (experiment 4), animals received a intraperitoneal (i.p.) injection of 0.05 mg/kg clozapine by a trained experimenter blinded to the experimental group of the animal.

Differential auditory fear conditioning (DAFC).

One day after the habituation sessions, mice were placed in context A boxes and exposed to 10 repetitions of CS+ (10 s) and 10 of CS- (10 s) in a fixed semi-random order and at variable intervals (ITI: 40-120 s) for a total duration of 29 min. Coinciding with the last second of each CS+, animals received a 1 s foot shock, whereas the CS- was never followed by a foot shock. For experiment 1, control animals received no foot shock, weak shock animals received a foot shock of 0.2 mA, and strong shock animals received a foot shock of 1.2 mA. For experiments 2, 3 and 4, all animals received a foot shock of 0.3 mA. Foot shock intensities were based on own pilot data and previous literature suggesting that the intensity of the foot shock modulates the extent to which fear generalizes towards the CS- (specific fear vs. generalized fear phenotype) (De Bundel et al., 2016; Ghosh & Chatterji, 2015).

4-Hydroxytamoxifen.

4-Hydroxytamoxifen (4-OHT) (Sigma-Aldrich, #H6278) was first dissolved in ethanol, and later corn oil, by heating (max 55 °C) and sonicating the solution to receive a final concentration of 10 mg/mL 4-OHT / 10% ethanol / 90% corn oil. The final solution was

stored in the dark at 4 °C. Shortly before injection, the solution was sonicated again and brought to room temperature. For experiment 1, mice received a 50 mg/kg 4-OHT i.p. injection directly following DAFC. Each animal was placed back in its home cage directly following the injection, and the cages were covered with filter tops to prevent the spread of airborne 4-OHT. Animals were left undisturbed in their cages for 72 h following injection (until re-exposure), and cages were cleaned immediately following the re-exposure session.

Auditory cue re-exposure.

Approximately 72 h following DAFC and 4-OHT administration, mice were again exposed to the two auditory tones in 2 separate sessions (4 10 s tone presentations each, 40-120 s interval, context B) with a 2 h delay between sessions. The order in which these sessions were presented was counterbalanced across animals. Behaviour was recorded by a built-in video camera and freezing (immobility with the exception of breathing) was scored off-line by a rater blinded to the experimental condition (The Observer, Noldus, Wageningen, Netherlands). Time spent freezing during the first 2 min of the first re-exposure session was used to assess context-induced fear, and 10 s before and during each tone presentation, to assess tone-induced freezing as a proxy for fear memory strength and generalization. To assess fear generalization across auditory cues, relative freezing scores were calculated by dividing CS- induced freezing by CS+ induced freezing (Ghosh et al., 2014; Bender et al., 2018) .

Elevated plus maze.

The day following cue re-exposure, mice were tested on the elevated plus maze (EPM). The EPM is a plus-shaped apparatus, 53.5 cm above the floor, with a central square and two open and two closed arms opposite of each other (total length of the opposite arms spanning 90 cm). Each arm was 5 cm wide, and open arms were lined with an edge of 3 mm high whereas closed arms were lined by 15 cm high, black walls. The apparatus was illuminated with 40 Lux. At the start of the test, the mouse was placed at the end of one of the enclosed arms, facing the centre. The animal was free to explore the maze for 10 min while an overhead camera recorded the animals' behaviour. Time spent on the enclosed and open arms, indicative of anxiety-like vs exploratory behaviour respectively, was measured by Ethovision software (Noldus, Wageningen, Netherlands). In addition, the distance moved, latency to enter and frequency to visit the open arms was assessed. Only

the first 5 min of the tests were used for data-analysis conform to standard assessments (Walf & Frye, 2007) and to ensure that solely anxiety behaviour, opposed to habituation behaviour to a novel context, was assessed. Yet, analysis of the full 10 min exposure did not change the general conclusions.

Dark-light transfer test.

The subsequent day, mice were subjected to the dark-light transfer test (DLT). The DLT setup is a box (42 x 21 x 30 cm) consisting of a dark compartment (one third) and a light compartment (two thirds, illuminated by 970-1250 Lux) separated by a dividing wall. At the start of the test, mice were placed in the dark compartment and allowed to explore the setup for 10 min. They could travel between compartments through a small opening in the dividing wall (7 x 7 cm) and their behaviour recorded by an overhead camera. A risk assessment zone, a small region (6 x 3 cm) in front of the opening in the light compartment, was defined to measure total risk assessment behaviour, calculated by the time spent in the risk assessment area as percentage of the total time spent in the remainder of the light compartment outside of the risk compartment. Furthermore, time spent, distance moved, and frequency and latency to enter into the light compartment was analysed for the first 5 min by Ethovision software (Noldus, Wageningen, Netherlands), for similar reasons as mentioned for the EPM. Analysis of the full 10 min exposure did not change conclusions.

Acoustic startle and pre-pulse inhibition test.

For experiment 1, on the last day of the experiment, the mice underwent the acoustic startle and pre-pulse inhibition test. The mice were individually placed in plexiglass tubes that were mounted on the top of a vibration-sensitive platform. This platform was placed inside a ventilated box that contained two high-frequency speakers and a sensor for detection of movement (SR-LAB, San Diego instruments). The acoustic startle session started with a 5 min acclimatization period to background white noise (70 dB), which was maintained throughout the whole session of 35 min. After the acclimatization, 32 startle stimuli of 120 dB with a duration of 40 ms were presented. The presentation of these stimuli was interspersed by the presentation of 36 additional startle stimuli that were preceded 100 ms earlier by 20 ms pre-pulses of either 75, 80 or 85 dB with randomly varying inter-trial intervals of 12-30 s. Maximum startle response and latency to peak startle was measured for the individually presented acoustic startle stimuli and also for the startle stimuli that

were preceded by pre-pulses. Unfortunately, technical issues with the setup resulted in an insufficient sensitivity to reliably record the startle responses, making that the data were not considered for further analyses.

Open field test.

For experiment 3, on the final day of the experiment, the mice underwent the open field (OF). Mice were placed in the corner of a brightly lit, white, square plexiglass box (50 x 50 x 40 cm, 120 lux), which they could explore for 5 min. Behaviour of the mice was recorded and time spent and distance travelled in the centre (defined as the middle quadrant), as well as total distance travelled were analysed by Ethovision software (Noldus, Wageningen, Netherlands).

Brain collection and processing.

For all experiments, 90 min after the final behavioural test, mice were anesthetized by inhalation isoflurane and overdosed by i.p. injection with pentobarbital (120 mg/kg). Then, mice were perfused with 1 x PBS and 4% paraformaldehyde (PFA). Brains were extracted and post-fixated for 24 h in 4% PFA at 4 °C. Afterwards, brains were temporarily stored in 1 x PBS until brain processing commenced. All brains were emerged in 30% sucrose for 48 h before slicing. Brains were sliced in 30 um thickness using a freezing sliding microtome. The slices were stored in 1 x PBS + 0.01% sodium azide at 4 °C until further processing.

For experiment 1, 4-6 slices of the amygdala (Bregma -1.46:-2.06 mm) and 4 slices of the aBNST (Bregma 0.26:0.14 mm) were selected. For experiment 3, 6 slices of the amygdala (Bregma -1.46:-2.06 mm) were selected. Slices were mounted on adhesion slides (EprediaTM SuperFrost plusTM, Thermo Fisher Scientific, #10149870) and dried in the dark, after which each section was circled with a PAP pen (Sigma Aldrich, #Z672548-1EA). First, the sections were blocked with PBS-BT (1 x PBS, 1% BSA (Thermo Fisher, #37525) and 0.3% Triton X-100 (Sigma-Aldrich, #T8787)) for 30 min in the dark at room temperature (RT). After this, incubation with the primary antibodies (Experiments 1 and 3: 50-75 uL per section, guinea pig anti-*cFos*, 1:750, Synaptic Systems, #226004; Experiment 1: mouse anti-*PKCdelta,* 1:500, BD Bioscience, #610398) diluted in PBS-BT took place overnight in the dark at RT. After incubation for 17 h, the sections were washed 3 times for 10 min in 1 x PBS. Following this, sections were incubated with secondary antibodies (experiment 1 and 3: 50-75 uL per

section, Alexa 647-conjugated donkey anti-guinea pig, 1:200, Jackson ImmunoResearch, #706605148; experiment 1: Alexa 488-conjugated goat anti-mouse, 1:200, Invitrogen, #A32723) diluted in PBS-BT for 3 h in the dark at RT. Then, the secondary antibodies were rinsed off once with 1 x PBS, and the sections were incubated with DAPI 1:1000 (Thermo Fisher, 62248) in 1 x PBS for 15 min. The slices were again washed 3 times for 10 min in 1 x PBS, and finally the PAP pen was carefully removed with 70% ethanol and slices were left to dry in the dark. Then, slices were embedded in Fluorsave (EMD Millipore, 345789) and left to dry overnight in the dark at RT. Slices were stored at 4 °C.

For experiment 2 and 4, 8 slices of the amygdala (Bregma -1.46:-2.06 mm) were selected and mounted on adhesion slides (EprediaTM SuperFrost plusTM, 10149870, Thermo Fisher Scientific) and left to dry in the dark at RT. Then, the slices were embedded in Fluorsave (EMD Millipore, 345789) and left to dry overnight in the dark at RT. Slices were stored at 4 °C.

Image acquisition and analyses.

Images were captured with a light microscope (Axio Imager 2, Zeiss) using a LED module (Colibri 2, Zeiss) and a 10x objective (experiment 1, 3 and 4) or a 20x objective (experiment 2) (see supplementary Table S1). Cell counting (experiments 1 and 3) in the amygdala and aBNST was performed in the Fiji programme ImageJ by an experimenter blinded to the experimental condition. Cell counts were normalized by correcting for the total DAPI cell counts per subregion (experiment 1) or by total area size (experiment 3). The amygdala was divided into the following regions; lateral amygdala (LA), basolateral amygdala (BLA), central lateral amygdala (CeL), central medial amygdala (CeM). The aBNST was divided as follows: anterolateral (BNSTAL), oval (BNSTOV), anteromedial (BNSTAM), ventromedial (BNSTMV) and ventrolateral (BNSTLV). In experiment 1, *PKC-delta* labelled cells were used to identify the CeL and BNSTOV (see supplementary Fig. S1).

Statistical analyses.

Data was analysed using IBM® SPSS® Statistics 25. For all analyses, data points deviating >3 interquartile ranges (IQR) from the median were considered outliers and removed from further analyses.

Behavioural data. General linear mixed models were performed for cued-induced freezing during re-exposure, with either shock exposure (shock vs. non-shock), group (experiment 1: weak vs. strong; experiment 4: control vs. DREADD), and testing session (CS+ first vs. CS- first) as between-subject factor, and time (tone 1 vs. 2 vs. 3 vs. 4) and CS type (CS+ vs. CS-) as within-subject factors to assess their effects on fear behaviour. For context-induced freezing, we performed one-way ANOVAs with either shock exposure (shock vs. non-shock) or group (experiment 1; weak vs. strong shock, experiment 4; DREADD vs. control) as variables of interest. To assess fear generalization behaviour, we performed one-way ANOVAs on relative freezing (CS-/CS+) with testing order (CS+ or CS- tested first) as a factor. Anxiety parameters were analysed by either independent samples t-tests or Mann-Whitney U tests, depending on whether the data followed a normal distribution as assessed by the Shapiro-Wilk test for normality. For *post hoc* testing, independent samples t-tests or paired t-tests were used. For experiment 4, the percentage freezing during DAFC was analysed using a repeated measures ANOVA, with group (control vs. DREADD) as a between-subject factor, and time and CS type (CS+ vs. CS-) as within subject factors.

Neuronal activity. Linear mixed model analyses were performed on cell counts, with either shock exposure (shock vs. non-shock) or group (experiment 1: weak vs. strong; experiment 3: control vs. DREADD) as between-subject factor and subregion (LA/BLA/CeL/CeM or BNSTAL/BNSTAM/BNSTOV/BNSTMV/BNSTLV) as within-subject factor. For *post hoc* testing, independent samples t-tests or paired t-tests were used.

Figures (GraphPad Prism) show average ± standard of the mean (SEM) in case of normally distributed data, and median ± IQRs in case of deviation from normal distribution.

Results

1. Neuronal activity patterns in the extended amygdala during fear encoding and anxiety-like behaviour

First, we set out to assess the neuronal activity patterns in the extended amygdala associated with the acquisition of fear memory and its subsequent (potentially generalized) expression, as well as later anxiety-like behaviour. To induce distinct fear behaviour across experimental groups, mice were exposed to varying shock intensities; either 0 mA (control group), 0.3 mA

(weak shock group) or 1.2 mA (strong shock group), an approach previously shown to induce differential levels of fear generalization (De Bundel et al., 2016). We hypothesized that amygdala recruitment would differ between fear phenotypes, with stronger foot shock increasing regional activity as well as the recruitment of the aBNST.

1.1 Fear recall.

To assess levels of fear generalization, we measured both context- and cue-induced freezing. Context-induced freezing (i.e. freezing prior to the presentation of the first auditory cue) was higher in previously shocked animals compared to non-shocked animals ($F(1,49) = 5.869$, $p = .019$). Yet, within the shocked groups no significant differences in context-induced freezing levels were observed depending on shock intensity ($F(1,37) = 1.064$, $p = .309$, Fig. 2).

As expected, shocked animals also showed significantly higher levels of freezing behaviour towards the tones ($F(1,103.536) = 110.873$, $p < .001$) compared to non-shocked animals. Furthermore, we found a shock exposure*CS type interaction ($F(1,103.536) = 3.984$, $p = .049$), which was caused by the fact that shocked animals froze relatively more towards the CS+ than CS- ($t(37) = 4.080$, $p < .001$), whereas non-shocked animals did not differentiate between the two tones ($t(11) = 1.474$, $p = .169$). No main effects for test session, time or CS type were observed, nor any other significant interactions between these factors (all p's > 0.275, see supplementary Fig. S2 for freezing levels over time).

Among the mice that received foot shocks of varying intensity during DAFC, the strong shock group showed higher levels of freezing towards both CSs than the weak shock group ($F(1,78.557) = 37.056$, $p < .001$). Further, a main effect of CS type was observed ($F(1,78.557) = 11.587$, $p = .001$), caused by higher levels of freezing towards the CS+ than CS-, suggesting that the mice differentiated between the CS+ and CS-. Yet, they still showed strong freezing responses to the CS-, as freezing levels during CS- presentation were much higher than pre-tone freezing levels ($t(36) = 11.241$, $p < .001$). No effects of time were seen (all p's > .071), indicating stable freezing levels upon repeated tone re-exposure. Lastly, a CS type*test session interaction was found ($F(1,78.557) = 4.798$, $p = .031$), which appeared driven by higher CS+ freezing when the CS+ tone was presented in the second session following the CS- ($t(37) = 2.112$, $p = .042$), as opposed to the first session, while freezing towards the CS- did not change depending on session number ($t(36) = 0.457$, $p = .651$). No significant main

effect of test session was observed, suggesting that in itself no extinction took place over the two sessions ($F(1,78.557) = 2.106$, $p = .151$).

To assess fear generalization irrespective of the strength of the fear response, relative freezing scores were calculated by normalizing CS- induced freezing to CS+ induced freezing. Here, we also did not find significant differences between the weak and strong shock group ($F(1,33) = 0.439$, $p = .512$), indicating that both groups showed a similar extent of fear generalization towards the CS-. Relative freezing outcomes were not affected by the order in which the CSs were presented (main effect order: $F(1,33) = 0.381$, $p = .541$; group*order interaction: $F(1,33) = 1.809$, $p = .188$). As already suggested by the main effect of CS type on absolute freezing levels, both groups did differentiate between the CS+ and CS-, with the average relative freezing differing significantly from 1 (weak shock: $t(24) = 3.545$, $p = .002$; strong shock: $t(11) = 2.806$, $p = .017$). Yet, mean freezing rates to the CS- relative to the CS+ were high (M ± SD: 39.59 ± 14.58% and 59.97 ± 16.23% in the weak and strong shock group, respectively), the actual extent to which the mice differentiated between the CS+ and CS- was relatively low.

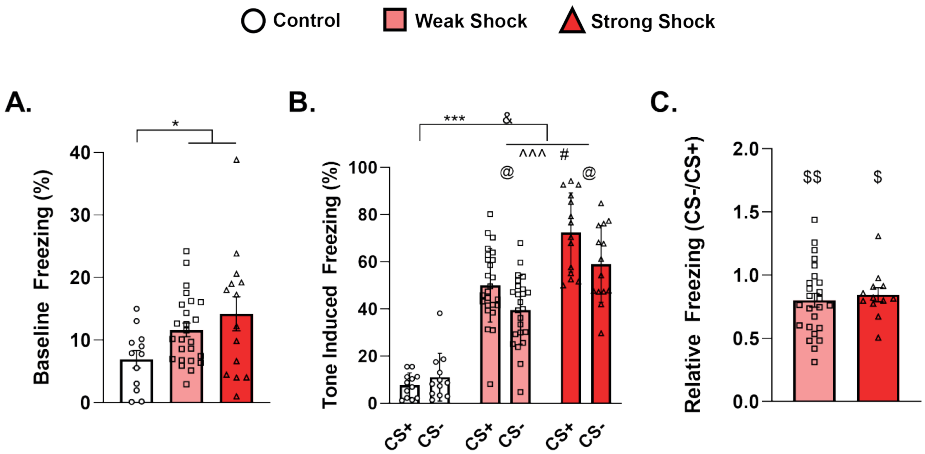


Figure 2. Freezing responses upon context and tone re-exposure. Mice receiving foot shock during prior differential auditory fear conditioning showed higher levels of context-related freezing before tone-onset (A), higher tone-induced freezing (B), and froze relatively more towards the CS+ than CS- compared to the non-shocked group. Mice receiving strong shocks showed higher levels of freezing than mice receiving weak shocks. Relative freezing rates towards the CS- were however similar for both the animals receiving weak and strong shocks, with the animals differentiating between the tones, but still showing significant CS- freezing (C). ***: $p < 0.001$, *: $p < 0.05$, main effect of shock exposure. &: $p < 0.05$, shock exposure*CS type interaction. ^^^: $p < 0.001$, main effect of shock intensity. #: $p < 0.05$, test session*CS type interaction. @: $p < 0.05$, main effect of CS type. $: $p < 0.05$, $$: $p < 0.01$, effect of tone differentiation.

1.2 Anxiety-like behaviour.

To assess the effect of prior fear conditioning on anxiety-like behaviour, we next compared the shocked vs. non-shocked animals in their behaviour on the EPM. Shocked animals spent significantly more time in the closed arms of the maze ($t(49) = 2.511$, $p = .015$) and displayed a longer latency to first enter the open arms of the maze ($U = 338$, $p = .012$) compared to non-shocked animals, indicative of increased anxiety levels (Fig. 3). Yet, shock exposure did not modulate the time spent on the open arms of the maze ($t(49) = 0.942$, $p = .351$). We did not find any differences in the total distance moved on the maze, or the distance moved on either the open or closed arms (all p's > .188). Anxiety-like behaviour in the EPM was not dependent on the shock intensity, as no significant differences were observed between animals being conditioning with weak vs. strong foot shocks in any of the behavioural readouts (all p's > .268).

The next day, mice were tested in the dark-light transfer test (DLT). Mice that received foot shocks displayed a lower latency to enter the light area ($U = 119.5$, $p = .039$), as well as a higher percentage of risk assessment behaviour ($U = 284$, $p = .009$), compared to non-shocked animals. However, no significant differences between shocked vs. non-shocked animals were observed in the distance moved in the light zone, time spent in the light zone, or frequency of visiting the light zone (all p's > .124). Behaviour in the DLT was not dependent on shock intensity (all p's > .591).

On the final day, mice were tested in the acoustic startle and pre-pulse inhibition test. Unfortunately, acoustic startle responses and pre-pulse inhibitions levels could not be assessed due to technical limitations. However, corresponding neuronal activity levels were assessed.

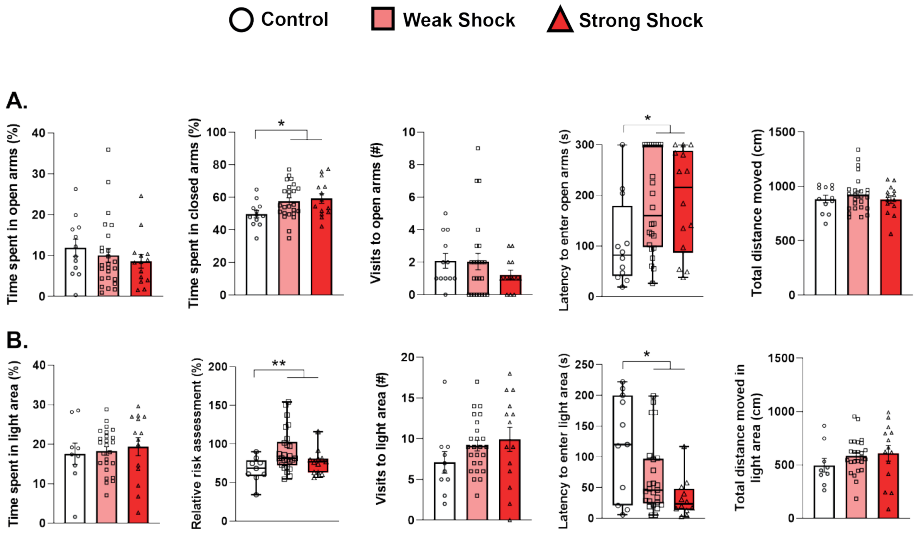


Figure 3. Anxiety-like behaviour as assessed by the elevated plus maze (A) and dark-light transfer test (B). Mice that received foot shocks during prior conditioning spent significantly more time in the closed arms, and displayed longer latencies to first enter the open arms on the elevated plus maze compared to non-shocked mice. In the dark-light transfer test, mice that received foot shocks displayed higher risk assessment, but showed shorter latencies to enter the light area. None of the anxiety-like behaviours was different between mice that received weak vs. strong foot shocks. **: $p < 0.01$, *: $p < 0.01$, main effect of shock exposure.

1.3 Extended amygdala neuronal activity during fear acquisition.

To assess extended amygdala activity during fear acquisition (and early memory consolidation), we labelled activated (i.e. *cFos* expressing) neurons during this period by injecting 4-OHT immediately after the DAFC paradigm in *FosTRAP2* mice, which induced the permanent expression of the fluorescent marker *tdTomato*. Comparison of the number of *tdTomato*-expressing cells in the amygdala across animals that received shocks during conditioning vs. animals that did not, revealed a trend-level difference across groups ($F(1,193) = 3.186$, $p = .076$, Fig. 4A), with shocked animals tending to show higher numbers compared to non-shocked animals. Furthermore, we observed a significant effect of amygdala subregion ($F(3,192) = 10.855$, $p < .001$), without any shock exposure*subregion interaction ($F(1,192) = 0.706$, $p = .550$), indicating that although the subnuclei show different numbers of *tdTomato*-expressing cells, their number was not differentially affected by shock exposure. Exploratory *post hoc* tests comparing subregions directly, revealed a significant effect of shock exposure in the LA ($t(45) = 2.872$, $p = .006$), but not the BLA, CeL or CeM (all

p's > .389). When comparing the mice receiving weak shocks vs. strong shocks, we found that animals that received strong foot shocks showed overall higher numbers of *tdTomato*-expressing cells in the amygdala than animals that received weak shocks ($F(1,145) = 7.938$, $p = .006$). Again, cell numbers depended on the subregion ($F(3, 145) = 8.067$, $p < .001$), without showing a group*subregion interaction ($F(3,145) = 0.173$, $p = .914$). Further exploratory *post hoc* analyses revealed a significant effect of shock intensity in the BLA ($t(34) = 2.741$, $p = .008$), but not the LA, CeL or CeM (all *p*'s > .05).

A linear mixed model revealed no main effect of shock exposure ($F(1,249) = .309$, $p = .579$) on the number of *tdTomato*-expressing cells in the aBNST during fear acquisition, but a significant shock exposure*subregion interaction effect ($F(4, 249) = 5.381$, $p < .001$) together with a main effect of subregion ($F(4,249) = 10.541$, $p < .001$, Fig. 4B). The interaction effect appeared driven by effects of shock exposure on activity in the BNSTOV ($p = .020$), BNSTMV ($p = .015$) and BNSTLV ($p = .025$), but not BNSTAL ($p = .523$) and BNSTAM ($p = .335$).

When comparing the mice receiving weak vs. strong shocks, we observed a main effect of group ($F(1,190) = 37.772$, $p < .001$), as well as a main effect of subregion ($F(3,190) = 7.194$, $p < .001$), but no significant group*subregion interaction ($F(4,190) = 2.049$, $p = 0.089$). The main effect was caused by higher numbers of *tdTomato*-expressing cells in the strong shock group, independent og subregion. Exploratory *post hoc* analyses comparing subregions directly, revealed a significant effect of shock intensity on the BNSTAL ($t(36) = 2.675$. $p = .011$), BNSTAM ($t(36) = 5.053$, $p < .001$) and BNSTMV ($t(36) = 2.915$, $p = .006$), but not BNSTOV and BNSTLV (all *p*'s > .097).

1.4 Extended amygdala neuronal activity during anxiety-related conditions.

To investigate a potential link between the neural circuitry supporting the acquisition of fear and that involved in the expression of subsequent anxiety-like behaviour, mice were sacrificed after the acoustic startle assessment and immunohistochemistry was performed to label *cFos*-expressing neurons. Prior foot shock exposure did not affect the number of *cFos*-expressing cells in the amygdala (main effect of group: $F(1,196) = .494$, $p = .483$; shock exposure*subregion interaction: $F(3,196) = .885$, $p = .450$, Fig. 4C), whereas activity differed across amygdala subregions ($F(3,196) = 4.074$, $p = .008$), with the number of *cFos* cells being smallest in the CeM (LA/BLA/CeL > CeM (LA > CeM; $t(48) = 5.604$, $p < .001$. BLA > CeM; $t(48)$

= 5.256, $p < .001$. CeL > CeM; $t(48) = 3.200$, $p = .002$). The number of *cFos*-expressing cells in the amygdala did also not depend on shock intensity (main effect of group: $F(1,148) = .290$, $p = .591$; group*subregion interaction: $F(3,148) = .099$, $p = .960$), but again differed across subregions ($F(3,148) = 6.276$, $p < .001$)(LA/BLA/CeL > CeM. LA > CeM; ($t(36) = 5.631$, $p < .001$. BLA > CeM; ($t(36) = 5.248$, $p < .001$. CeL > CeM; ($t(36) = 3.723$, $p = .001$).

Interestingly, aBNST *cFos*-expression in response to the anxiety test was modulated by a main effect of shock exposure ($F(1,244) = 8.607$, $p = .004$), a main effect of subregion ($F(4,244) = 14.313$, $p < .001$, all p's < .001, except OV/AL ($p = .055$), OV/LV ($p = .077$), AM/MV ($p = .872$)) but there was no shock exposure*subregion interaction ($F(4,244) = 1.600$, $p = .175$). Remarkably, animals that previously received foot shocks displayed overall *lower* numbers of *cFos*-expressing cells in the aBNST following the anxiety test, regardless of the subregion. No effect of shock intensity was observed on aBNST activity (main effect of group: $F(1,185) = 1.182$, $p = .278$; group*subregion interaction: $F(4,185) = .737$, $p = .568$; main effect of subregion: $F(4,185) = 11.862$, $p < .001$, Fig. 4D).

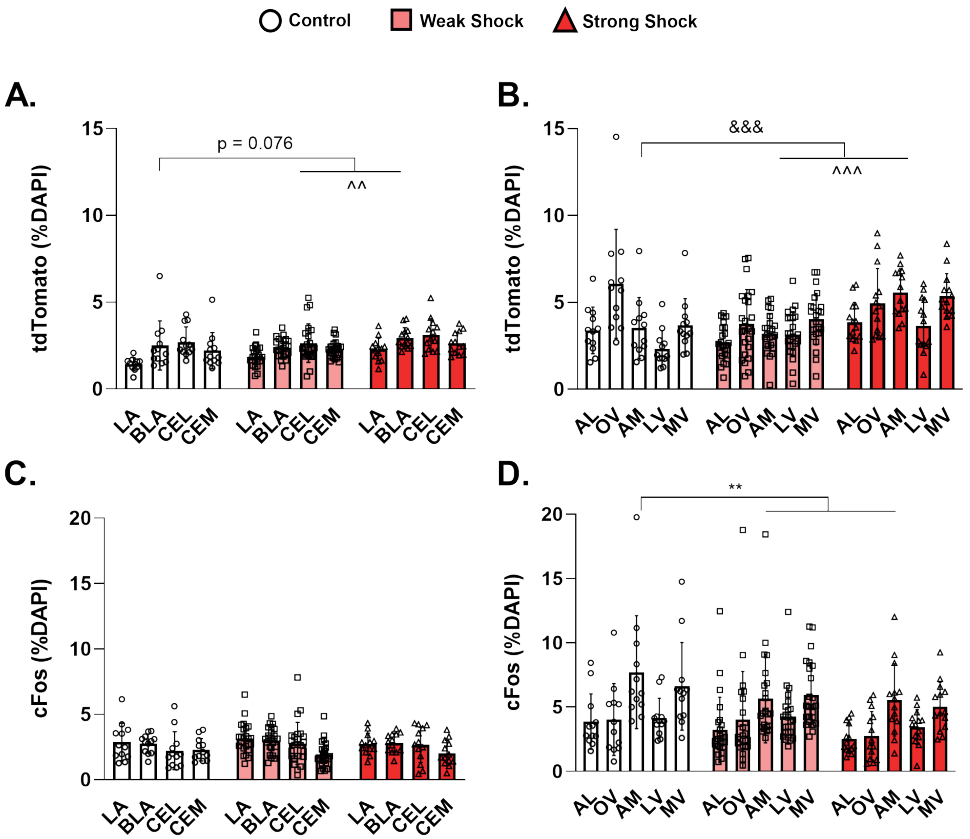


Figure 4. Neuronal activity patterns in the extended amygdala in response to fear acquisition and anxiogenic situations, assessed by the number of tdTomato- and cFos-expressing cells respectively. *Compared to non-shocked animals, shocked animals showed trend-level higher numbers of tdTomato-expressing cells in the amygdala, and a shock exposure*subregion interaction in the aBNST, which was driven by increased numbers of activated neurons in the BNSTAM and BNSTMV subregions in shocked compared to non-shocked mice (A, B). Furthermore, mice that received strong foot shocks showed overall higher tdTomato-expressing cells in both the amygdala (A) and aBNST (B) compared to mice that received weak shocks. In contrast, shocked animals showed lower numbers of cFos-expressing cells in the aBNST compared to non-shocked mice (D), with no significant differences in cFos expression in the amygdala (C). **: $p < 0.01$, main effect of shock exposure. &&&: $p < 0.001$, shock exposure*subregion interaction. ^^: $p < 0.01$ or ^^^: $p < 0.001$, main effect of shock intensity. La: lateral amygdala; BLA: basolateral amygdala; CEL: central lateral amygdala; CEM: central medial amygdala; AL: anterolateral aBNST; OV: oval nucleus; AM: anteromedial aBNST; LV: ventral lateral aBNST; MV: ventral medial aBNST.*

1.6 Relationship between extended amygdala neuronal activity and behaviour.

In order to more directly link neuronal activity in the extended amygdala to the behavioural measures of fear and anxiety, correlational analyses were performed. Strong positive correlations were found between the number of *tdTomato*-expressing cells in the amygdala and aBNST, in line with prior literature suggesting a strong connectivity between these regions (Fig. 5). Moreover, the number of *tdTomato*-expressing cells in the BLA positively correlated with freezing behaviour during tone re-exposure (CS+: $r(36) = .408$, $p = .013$; CS-: $r(36) = .482$, $p = .003$), as well as with relative freezing rates ($r(34) = .506$, $p = .002$), indicating that BLA activity during fear acquisition predicts both the strength of fear memory and fear generalization behaviour (Fig. 5B). No strong correlations were found between the number of *cFos*-expressing cells and anxiety-related behaviour. Noteworthy, these behavioural outcomes were also assessed at earlier timepoints than *cFos* expression. However, a trend-level negative correlation between the number of *cFos*-expressing cells in the BLA and BNSTAL was found under anxiety-inducing conditions ($r(36) = -.321$, $p = .057$), suggesting that the BLA might negatively regulate BNSTAL activity under anxiogenic situations (or vice versa, Fig. 5C).

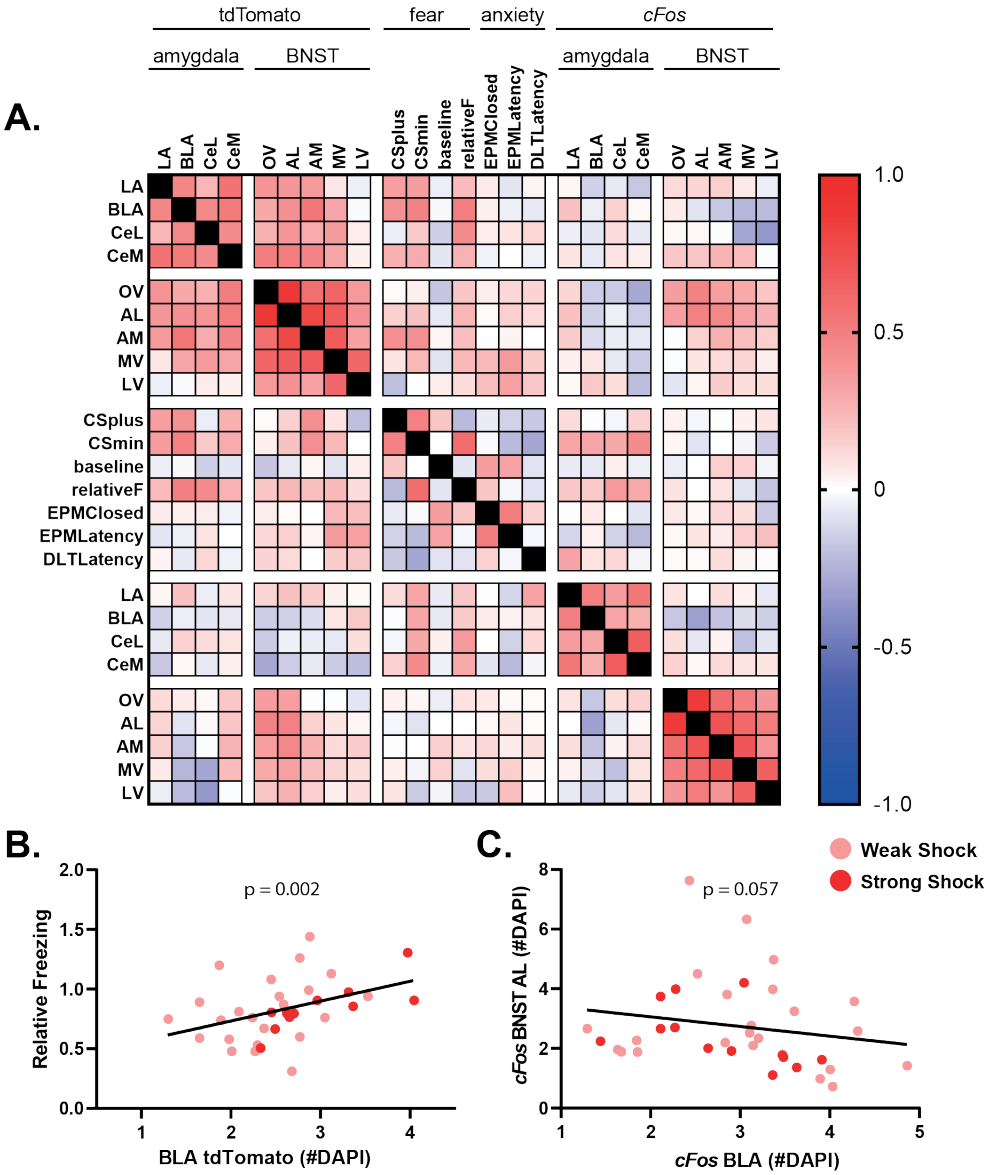
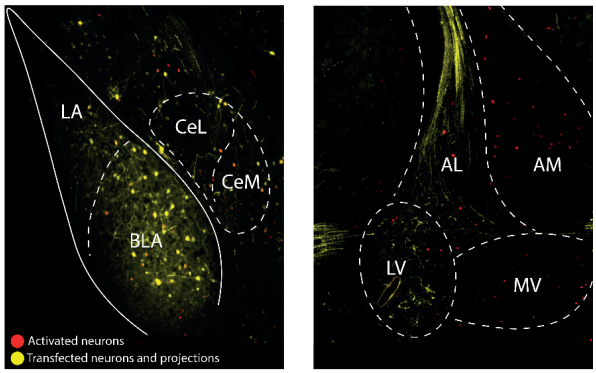


Figure 5. Correlations between fear- and anxiety-like behaviour and amygdala and aBNSTneuornal activity (assessed by the number of tdTomato- and cFos-expressing cells respectively) of mice exposed to foot shock during conditioning (A). *We observed a strong positive correlation between the number of tdTomato-expressing cells in the BLA and relative freezing (B), and a trend level negative correlation between the number of cFos-expressing cells in the BLA and BNSTAL in response to the anxiety test (C).*

2. Recruitment of amygdala-aBNST projection neurons during differential auditory fear conditioning

The results from experiment 1 imply that the generalized fear and anxiety phenotype is not dependent on foot shock intensity, which is why we continued our study with a single intensity. Shock exposure overall did affect neuronal activity in the extended amygdala and was related with strong crosstalk between the amygdala and aBNST during fear acquisition in particular, making that we further investigated the main projections between the amygdala and the aBNST that could mediate the development of generalized fear and increased anxiety. Considering the strong association between BLA activity during fear acquisition and later behavioural fear generalization, as well its suggested connectivity with the BNSTAL, we narrowed our scope towards BLA projection neurons specifically. Therefore, we next labelled activated BLA projection neurons by injecting an anterograde AAV (expressing the fluorescent protein *GFP* in an activity-dependent manner, i.e. depending on the expression of *cFos*, AAV5-pAAV-hSyn-DIO-EGFP) in *FosTRAP2* mice. As expected, *GFP*-expression was restricted to amygdala neurons that also expressed *tdTomato*. Whereas our primary target was the BLA subregion of the amygdala, we observed some heterogeneity in the specificity of the transfection, which we used to our benefit to map activated amygdala subregion – aBNST subregion connectivity more generally. Animals in which the BLA was targeted in a specific manner were found to display particularly strong projections in the BNSTAL (Fig. 6), indicative of the activation of a direct BLA-BNSTAL connection during fear acquisition. Similar observations were made for the CeA and BNSTAL. We barely observed any amygdala projections in the BNSTAM, indicating an absence of direct amygdala-BNSTAM signalling during fear acquisition. Lastly, there was a strong relationship between the extent of CeA transfection and projections observed in the BNSTV, which was in line with earlier findings that the CeA mainly projects to both the BNSTAL and BNSTV (Gungor, Yamamoto & Paré, 2015).

A.



B.

	BNSTAL	BNSTAM	BNSTV
LA	-	- -	+
BLA	++	- -	+
CeL	++	- -	++
CeM	++	- -	++

Figure 6. aBNST projections from amygdala neurons activated during fear acquisition (A). Activated neurons (i.e., those expressing cFos and thereby tdTomato in FosTRAP2 mice) are seen in red, whereas transfected neurons (expressing Cre-dependent GFP) are labelled in yellow. Direct projections from the BLA subregion were mostly found in the BNSTAL and BNSTV subregion of the aBNST (B). LA: lateral amygdala; BLA: basolateral amygdala; CEL: central lateral amygdala; CEM: central medial amygdala; AL: anterolateral aBNST; AM: anteromedial aBNST; LV: ventral lateral aBNST; MV: ventral medial aBNST.

3. Chemogenetic manipulation of BLA-BNSTAL projection neurons during differential fear acquisition

We next wanted to investigate the exact role of BLA projection neurons to the BNSTAL in mediating fear generalization and anxiety-like behaviour. As we observed a negative association between neuronal activity in the BLA and that in the BNSTAL, we hypothesized a fear and anxiety regulatory role for these projection neurons, which would be in line with prior literature showing anxiolytic effects as a result of the direct BLA-BNSTAL pathway (Dong et al., 2001a; Krettek & Price, 1978a). First, we needed to establish that we were able to manipulate the activity of these projection neurons by means of chemogenetic manipulation. Therefore, *C57BL/6* mice were intracranially injected with either a Cre-dependent control virus (*AAV9-hSYN-DIO-mCherry*), or viruses coding the excitatory DREADD receptor (*AAV9-hSYN-DIO-hM3D(Gq)-mCherry*), or inhibitory DREADD receptor

(*AAV9-hSYN-DIO-hM4D(Gi)-mCherry*) virus in the BLA, and a retrograde Cre-expressing virus (*EEN.AAV.hSYN.HI.eGFP-Cre.WPRE.SV40*) in the aBNST. To activate the DREADD receptors, mice were injected with low dose clozapine 30 min prior to DAFC and sacrificed 90 min post-DAFC to assess the activity of the projection neurons by means of immunohistochemistry for *cFos*.

Linear mixed models revealed a main effect of treatment group ($F(2,53) = 70.206$, $p < .001$, Fig. 7A) on the percentage of transfected BLA-aBNST projection neurons (labelled by mCherry) that were activated (i.e. expressing *cFos*). Further *post hoc* testing revealed that this effect was driven by the excitatory DREADD condition, with a larger fraction of BLA-aBNST projections neurons being activated in this group compared to the control ($t(5.136) = .874$, $p < .001$) and inhibitory DREADD condition ($t(8) = 6.725$, $p < .001$). In contrast, the inhibition of BLA-aBNST projection neurons in the inhibitory DREADD group did not result in a significantly lower percentage of activated transfected cells in comparison to controls ($t(7) = .756$, $p = .474$). As can be expected for the effects of systemically administered clozapine, the effect of DREADD receptor activation was similar across amygdala subregions, indicated by the absence of a main effect of amygdala subregion ($F(2,53) = .448$, $p = .641$), and group*subregion interaction ($F(4,53) = .227$, $p = .922$) on activation rates. Yet, we found a strong main effect of amygdala subregion on the total number of transfected BLA projection neurons ($F(2,55) = 26.68$, $p < .001$, Fig. 7B), where the number of transfected cells was highest in the BLA > LA > CeA (BLA > LA; $t(17) = 4.909$, $p < .001$. BLA > CeA; $t(16) = 4.898$, $p < .001$. LA > CeA; $t(17) = 3.144$, $p = .006$), indicating that our viral manipulation mostly affected BLA projection neurons. Although the total number of transfected BLA-aBNST projection neurons also slightly differed between treatment groups ($F(2, 55) = 3.636$, $p = .033$), *post hoc* testing revealed that this effect was driven by the difference between the inhibitory and excitatory DREADD groups ($t(9) = 2.781$, $p = .021$).

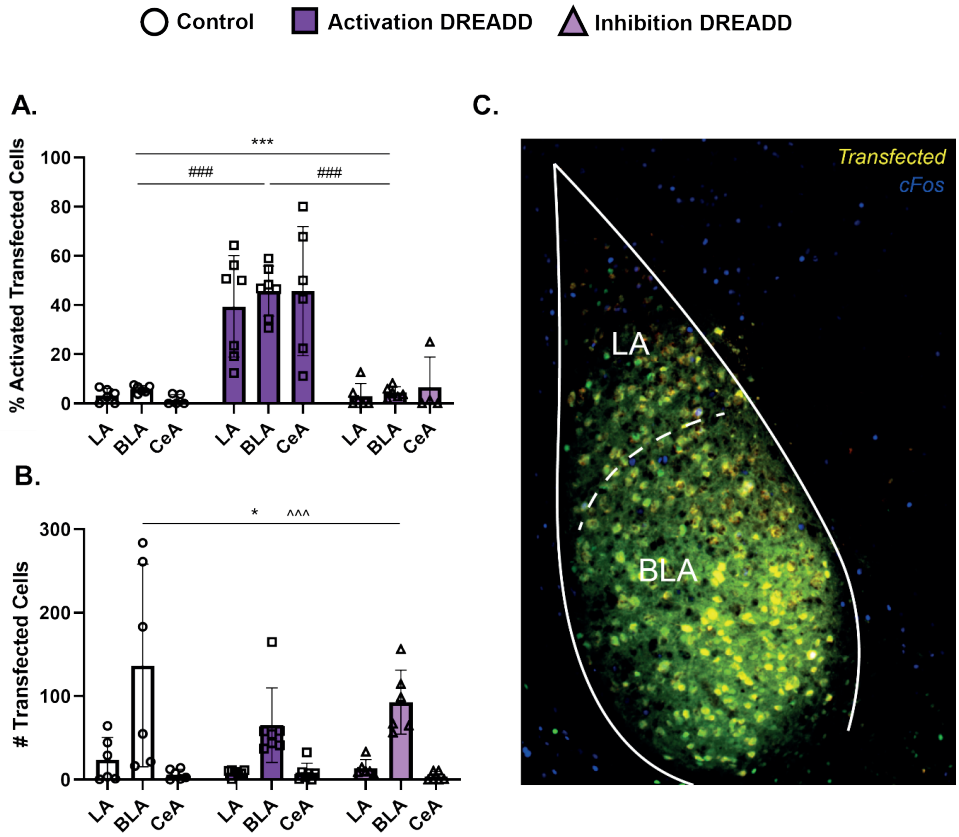


Figure 7. Percentage activated transfected amygdala-aBNST projection neurons (A) and the number of transfected amygdala-aBNST projection neurons (B) following clozapine administration and differential auditory fear conditioning*. Post hoc testing revealed a significantly higher percentage of activated transfected projection neurons in the excitatory DREADD group in comparison to the control and inhibitory DREADD groups (A). The number of transfected BLA-aBNST projection neurons depended both on the treatment group and amygdala subregion, with most projection neurons being detected in the BLA (B). ***: p < 0.001, *:p < 0.05, main effect of group. ^^^: p < 0.001, main effect of subregion. ###: p < 0.001, post hoc group comparison. LA: lateral amygdala; BLA: basolateral amygdala; CEA: central amygdala.*

4. Behavioural effects of chemogenetic activation of BLA-aBNST-projection neurons during differential fear acquisition and the subsequent recall of fear and expression of anxiety

We finally wanted to investigate the role of these aBNST-projecting BLA neurons in mediating the long-term behavioural consequences of fear memory acquisition. Based on their hypothesized anxiolytic role and the outcome of experiment 3, we chose to chemogenetically

activate these neurons during differential fear acquisition (and early consolidation) and assess the effects of this manipulation on fear recall, fear generalization and anxiety-like behaviour. Additionally, to investigate whether these projection neurons also have an immediate modulatory role in the expression of anxiety-like behaviour, we activated them during the open field test (OF).

4.1 Behavioural effects of activating BLA-aBNST-projection neurons during differential fear acquisition on fear acquisition and recall.

To activate the BLA-aBNST projection neurons, *C57BL/6* mice were first intracranially injected with either a *AAV9-hSYN-DIO-mCherry* (control) or *AAV9-hSYN-DIO-hM-3D(Gq)-mCherry* (activator) virus in the BLA, and an *EEN.AAV.hSYN.HI.eGFP-Cre.WPRE.SV40* virus in the aBNST. Following viral incubation and recovery from surgery, mice received an injection with low dose clozapine 30 min prior to DAFC, identical to experiment 3.

We first tested whether this had an immediate effect on fear learning and expression, by analysing freezing levels over the course of conditioning. Here, we found that over time, the percentage freezing in response to the tones increased for both groups (main effect of time: $F(2.637, 79.101) = 64.289$, $p < .001$, Fig. 8A), as was expected due to the repeated exposure to foot shocks upon CS+ exposure. Furthermore, control mice froze significantly more during the tones in comparison to the excitatory DREADD group (main effect of group; $F(1, 30) = 17.960$, $p < .001$), and this difference increased over time (time*group interaction: $F(2.637, 79.101) = 21.197$, $p < .001$). When dissecting freezing responses during the CS+ and CS- separately, we found that on average mice froze more during the presentation of the CS+ compared to the CS- (main effect of CS type: ($F(1, 16) = 6.383$, $p = .022$, Fig. 8B). Yet, control mice learned the association between the CS+ and the foot shock better in comparison to the excitatory DREADD mice (CS type*group interaction: $F(1, 16) = 6.555$, $p = .021$), as they tended to show higher percentages of freezing towards the CS+, but show equal percentages of freezing towards the CS-, in comparison to the DREADD group).

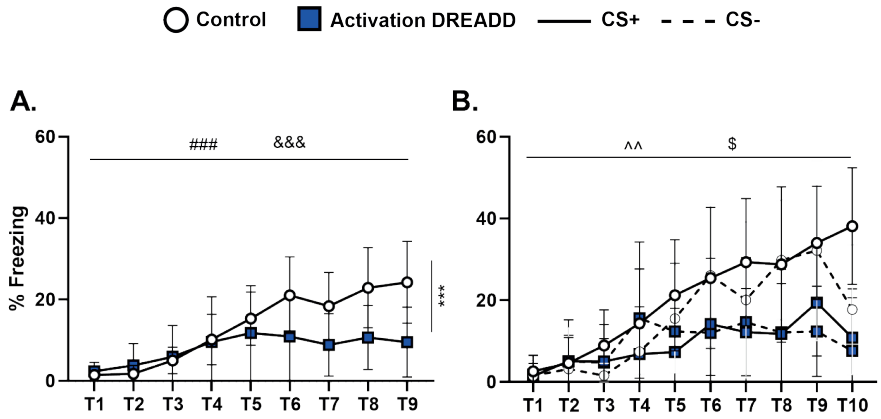


Figure 8. Percentage freezing during the DAFC paradigm*. Overall freezing levels of controls vs. DREADD treatment mice (A), and freezing levels dissected per CS type (CS+ vs CS-) for these groups (control vs. excitatory DREADD). ***: p < 0.001, main effect of group. ###: p < 0.001, main effect of time. &&&: p < 0.001, group*time interaction. ^^: p < 0.01, main effect of CS type. $: p < 0.05, CS type*group interaction.*

Next, we assessed the effects of prior chemogenetic activation on the subsequent recall of fear by measuring both context- and cue-induced freezing upon re-exposure. No significant differences were found between the excitatory DREADD vs. control groups in terms of context-induced freezing ($t(33) = 1.705$, $p = .098$, Fig. 9A). Yet, mice of the excitatory DREADD group showed significantly lower levels of freezing during the presentation of both tones in comparison to control animals (main effect of treatment: $F(1, 279) = 366.012$, $p < .001$), suggesting that the activation of BLA-aBNST projection neurons did not only suppress the expression of fear during DAFC, but also memory formation. Treatment did however not affect fear generalization across CS types (treatment*CS type interaction: $F(1, 279) = 0.042$, $p = .838$, Fig. 9B). Overall, all mice froze more towards the CS+ than CS- ($F(1,279) = 17.094$, $p < .001$). Although we did not find a significant difference between groups on relative freezing rates towards the CS- ($t(32) = 1.228$, $p = .228$), relative freezing rates differed significantly from 1 in the excitatory DREADD group ($t(16) = 2.366$, $p = .031$), indicating the mice successfully dissociated the CS+ from CS-, but not the control group ($t(16) = 1.588$, $p = .132$, Fig. 9C). This suggests that increased activity of BLA-aBNST projection neurons during fear acquisition not only reduces the strength of the fear response upon memory recall, but also contributes to a certain degree to its specificity. In contrast to earlier experiments, we also observed main effects of time (M ± SD; T1 = 38.656 ± 2.715%; T2 = 33.910 ± 2.728%; T3

= 32.127 ± 2.964%; T4 = 30.767 ± 2.758%) ($F(3,279) = 3.484$, $p = .016$), and test session (Session 1 = 31.881 ± 1.880%; Session2 = 35.849 ±2.069%) ($F(1,279) = 4.215$, $p = .041$). These effects were caused by within-session reductions in freezing levels upon repeated exposure to the CSs, and increased freezing in the second session. No further interactions between treatment group, time, test session and CS type were found (all p's > .063).

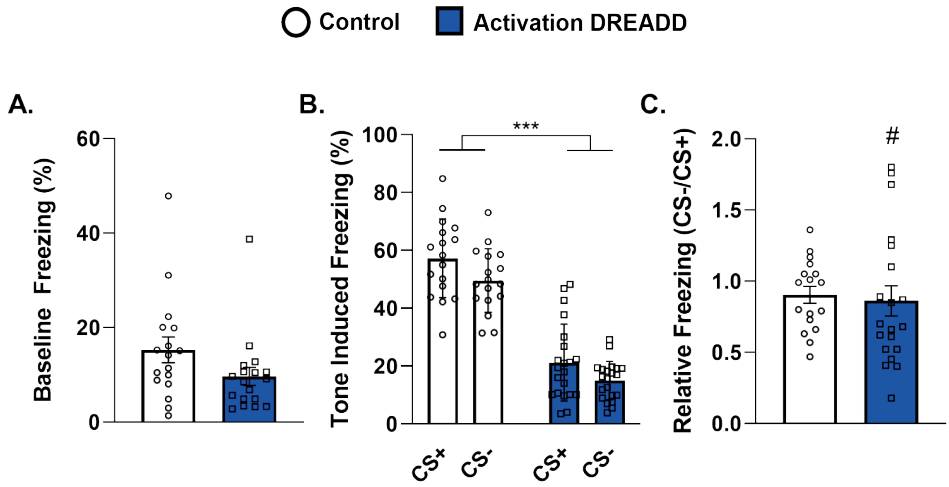


Figure 9. Context- (A), tone-induced (B) and relative freezing levels (C) in mice following chemogenetic manipulaton of the activity of BLA-aBNST projection neurons during differential auditory fear conditioning*. The excitatory DREADD group showed lower tone-induced freezing compared to the control group (B), as well as significant differentiation between the CS- and CS+. ***: p < 0.001, main effect of group. #: p < 0.05, different from 1.*

4.2 Behavioural effects of activating BLA aBNST-projection neurons during differential fear acquisition on subsequent anxiety-like behaviour.

Next, we compared the behaviour of the excitatory DREADD and control groups on anxiety-like behaviour on the EPM. We did not find any group differences in time spent in the open arms, time spent in the closed arms, the frequency to enter the open arms, or the total distance travelled on the maze (all p's > .135, Fig. 10A). Excitatory DREADD mice however showed a significantly longer latency to enter the open arms of the EPM, in comparison to controls ($t(32) = 2.796$, $p = .009$). This was opposite to our expectations, as this would suggest an anxiogenic effect of projection neuron activation.

The next day, mice were tested in the DLT. No significant differences between the excitatory DREADD vs control groups were observed in any of the behavioural readouts (distance moved in the light zone, time spent in the light zone, frequency and latency visiting the light zone, and relative risk assessment; all *p*'s > .407, Fig. 10B).

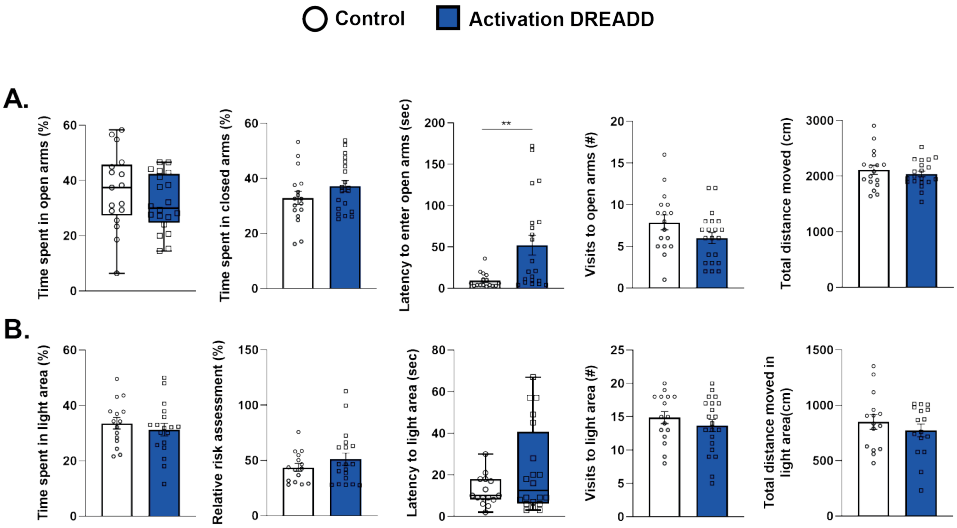


Figure 10. Anxiety-like behaviour as assessed on the elevated plus maze (EPM, A) and dark-light transfer test (DLT, B). *Mice in which BLA-aBNST projection neurons were chemogenetically activated during prior differential fear conditioning displayed a longer latency to enter the open arms of the EPM compared to the control group (A). No significant differences were found between controls and excitatory DREADD animals on the DLT (B). **: p < 0.01.*

4.3 Immediate behavioural effects of activating BLA aBNST-projection neurons on anxiety-like behaviour.

To assess whether the activation of BLA-aBNST-projection neurons directly affects the expression of anxiety-like behaviour, we compared behaviour in the open field test (OF) across groups following the i.p. injection of clozapine. We did not find any significant differences between the excitatory DREADD vs. control groups on the total distance moved, time spent in the centre, frequency and latency to enter centre area of the OF (all *p*'s > .189, Fig. 11A-D). As such, BLA-aBNST projection neurons do not seem to modulate the direct expression of anxiety-like behaviour.

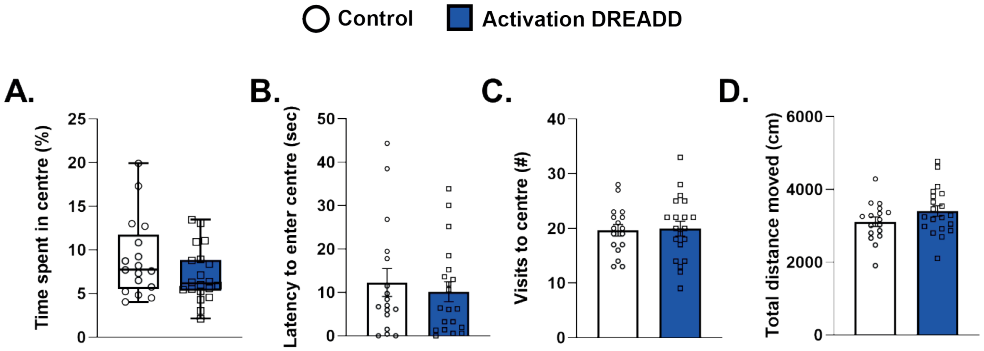


Figure 11. Anxiety-like behaviour as assessed in the open field (A-D) upon immediate activation of BLA aBNST-projection neurons. No significant effects of treatment were observed on anxiety-like behaviour.

Discussion

In a series of experiments, we investigated the role of the amygdala-aBNST circuitry during the acquisition of generalized fear and subsequent anxiety-like behaviour following a differential auditory fear conditioning paradigm (DAFC). We mapped subregion recruitment and correlations in subregion activity, characterized the exact connections involved and finally manipulated them to test for causality to behaviour. We found that activation of basolateral amygdala (BLA) neurons projecting to the anterolateral bed nucleus of the stria terminalis (aBNSTAL) during the acquisition of DAFC hampers fear learning and subsequent recall upon tone re-exposure. In a first experiment we intended to modulate cue-specific fear and a fear generalization and anxiety phenotypes by conditioning mice with different foot shock intensities; yet the sole difference between the mice receiving weak vs. strong foot shocks were the overall cue-induced freezing levels. No prominent, long-lasting group differences were observed on a variety of anxiety-read outs between groups. Higher shock intensity increased amygdala and aBNST activity during fear acquisition, but did not modulate amygdala or aBNST activity during anxiogenic conditions thereafter. Compared to the control group, both shock exposed groups displayed an increase in anxiety-like behaviour on several of the anxiety-read outs of the EPM and DLT, which was associated with lower aBNST activity in these groups. Intriguingly, BLA activity during fear acquisition predicted later fear generalization behaviour, and BLA activity during an anxiety-test tended to negatively correlate with activity in the BNSTAL. Using activity-dependent viral tracing, we

confirmed that BLA neurons projecting to the BNSTAL are recruited during the acquisition of DAFC. Activation of these direct projections during DAFC impairs fear learning and subsequent fear recall upon tone re-exposure. No immediate effects of activating BLA-BNSTAL projections were observed on anxiety-like behaviour in the OF test.

Modulating shock intensity in the DAFC paradigm affected the strength of the fear memory/expression, but did not result in the modulation of fear generalization phenotypes. Mice still dissociated between the CS+ and CS- with a very strong shock intensity (1.2 mA), whereas a very low shock intensity (0.2 mA) still resulted in relatively high freezing towards the CS-. Previous studies successfully utilized DAFC paradigms to investigate fear generalization (Duvarci et al., 2009; De Bundel et al., 2016), and used different foot shock intensities to modulate the extent of generalization (De Bundel et al., 2016). There are a few experimental variables that could explain these discrepancies. Firstly, in contrast to De Bundel et al. (2016), our re-exposure session following DAFC took place 3 days after DAFC (instead of the following day) to ensure the expression of the *tdTomato* following 4-OHT administration in *FosTRAP2* mice in earlier experiments (Guenthner et al., 2013). Secondly, we used perceptually more similar CSs (5 kHz vs. 10 kHz, whereas De Bundel used 2.5 kHz vs. 10 kHz), since we initially attempted to include an extra CS to examine the levels of fear generalization behaviour to both safe stimuli experienced within and beyond the fear conditioning context. We intended to compensate for this effect and facilitate the dissociation between the CS types by implementing a stronger habituation session of 10 tone-presentations per CS, instead of 5 (De Bundel et al., 2016). There are also other experimental parameters that could have influenced the extent to which animals condition to certain cues and contexts (Baldi, Lorenzini, Bucherelli, 2004; Rudy et al., 1996; Ghirlanda & Enquist, 2003; Vervliet et al., 2011; Jenkins and Harrison, 1960) such as the rodent's age, species and strain (Rudy et al., 1996; Stiedel et al., 1999). Although all shock-exposed mice dissociated the CS+ from CS-, considerable fear generalization took place as a consequence of shock exposure. Firstly, shock exposure induced significant increases in context-induced freezing. Secondly, freezing rates towards the CS- were much higher than pre-tone freezing rates, indicating that surely, the mice did not interpret the CS- as safety signal.

We have also found an absence of the effect of foot shock intensity in terms of anxiety-like behaviour. Mice previously exposed to foot shocks showed anxiogenic behaviour in the

EPM (spending more time in the closed arms and longer latencies to enter the open arms), but this effect was independent of foot shock intensity. Previous literature has shown that foot shock exposure increases subsequent anxiety-like behaviour, and these effects can last up to a month following exposure (Korte, Bauws & Bohus, 1999; Lemoine et al., 1990; Kinn Rød et al., 2012; Dijken et al., 1992), and associations between fear generalization and anxiety-like behaviour in the EPM have been reported before (Duvarci et al., 2009). Both processes have been linked to aBNST activity, as lesions of the aBNST abolished anxiety-like behaviour and induced high discrimination abilities during fear learning (Duvarci et al., 2009). Yet, we did not observe any significant correlations between fear generalization readouts and anxiety-like behaviour. Our findings are however in line with prior research in humans showing that individual variation in trait anxiety was not associated with fear generalization, only to low or high fear behaviour towards the CSs (Torrents-Rodas et al., 2013). However, in these participants trait anxiety was assessed prior to fear learning, testing it is a risk factor rather than consequence of generalized fear. Regardless, our behavioural observations imply that we were successful in generating generalized fear and an anxiety-like phenotype by shock exposure, yet this phenotype was not dependent on foot shock intensity, which is why we continued our studies with a single intensity.

At the neural level, we observed potentiating effects of greater shock intensity on neuronal activity in both the amygdala and aBNST, matching the behavioural effects on overall increased fear memory strength. The role of the amygdala in fear acquisition has been well established (LaBar et al., 1998; Buchel et al., 1998; Wilensky et al., 1999; Muller et al., 1997; Helmstetter & Bellgowan, 1994), and its increased activation upon learning has also previously been shown to predict fear memory strength (Frick et al., 2022; Crimmins et al., 2023). Here, we additionally showed a positive correlation between DAFC-induced BLA activity and subsequent fear generalization, which further contributes to the existing literature about the role of the BLA in modulating fear generalization and discrimination (Rajbhandari et al., 2016; Likhtik et al., 2014; Resnik & Paz, 2015). The role of the aBNST in contextual and cued fear conditioning has long been the subject of discourse, as it was initially believed to be only involved in contextual fear learning and anxiety (Zimmerman & Maren, 2011; Sullivan et al., 2004; Avery et al., 2016). Yet, later studies also implicated the aBNST in cued fear conditioning (Bruzsik et al., 2021; Radke et al., 2009; Duvarci et al., 2009). The aBNST has been implicated most strongly in dealing with unpredictable threat

signals (either long CSs (Waddel et al., 2006; Hammack et al., 2015) or CSs followed by shock at unpredictable latencies (Daldrup et al., 2016; Lange et al., 2017), as opposed to predictable ones such as the case in typical cued fear conditioning (Goode & Maren, 2017; Ressler et al., 2020). We observed no main effect of shock exposure on aBNST activity, but an increased activity in mice receiving strong foot shocks compared to those receiving weaker ones. Yet, one could argue that during our fear conditioning paradigm, mice were not well capable of predicting the onset of foot shock, as evidenced by considerable freezing during fear acquisition towards the CS- as well (experiment 4). This was later displayed by their fear responses both upon context and CS- exposure. Future analyses of the freezing behaviour of the mice during DAFC (in experiment 1) could test whether the mice's ability to discriminate between the CS types during fear acquisition correlated with the involvement of the aBNST.

The neuronal activity patterns observed under anxiogenic situations did not match those during fear learning in a straightforward manner. Amygdala activity was not different between the different shock intensity groups. This could be explained by the fact that there was no concrete threat present during these tests, and thus no discrete fear responses initiated. Yet, many studies have shown amygdala involvement in anxiety-like behaviour (Kalin et al., 2004; Lessher et al., 2008; Etkin et al., 2009; Lyons & Thiele, 2010), but one should note that we did not observe major behavioural differences across groups in terms of anxiety-like behaviour. More strikingly, we observed diminished neuronal activity in the aBNST in the shocked group compared to the non-shock group. Interestingly, previous work has also reported on a negative association between the activity of BNSTAL neurons and (acoustic) startle behaviour (Meloni et al., 2006; Grungor and Pare, 2014; Sink et al., 2011), suggesting that our shocked animals could have been more startled than non-shocked animals. However, due to technical complications, we were unable to assess this association.

Our correlational analyses revealed a trend towards a negative relationship between BNSTAL and BLA activity during anxiogenic situations. Moreover, we observed that BLA neurons directly projecting towards the BNSTAL were recruited during DAFC. These findings suggest a modulatory role in fear and anxiety for these BLA-BNSTAL projection neurons. Although we did not further classify these projection neurons, most BLA-BNSTAL projections are considered to be glutamatergic (Crowley et al., 2016; Dong et al., 2001a). Their exact

target cell type in the BNSTAL is yet unknown, however the vast majority of BNSTAL neurons are GABAergic (Day et al., 1999; Poulin et al., 2009; Kudo et al., 2012), and have been shown to exert anxiolytic influences (Dunn, 1987; Henke, 1984; Haufler, Nagy, & Pare, 2013; Cullinan et al., 1993; McDonnald et al., 1999; Dong et al., 2001a), among others through the modulating CeA output (Sun and Cassell, 1993; Dong et al., 2001b). This suggests that BLA-BNSTAL neurons are glutamatergic and that their activation enhances the activity of local GABAergic neurons that subsequently regulate fear behaviour.

Finally, we intended to reduce fear generalization and anxiety-like behaviour by activating the BLA neurons projecting to the aBNST(AL). This manipulation induced a strong reduction in overall freezing behaviour during both fear acquisition and later recall, however the discrimination between the CSs was not obviously improved. Other work focussing on circuit manipulations has shown that the opposite manipulation of ours, i.e. inhibition of BLA-aBNST projection neurons, during the fear conditioning towards a prolonged tone did not affect either fear acquisition or subsequent recall (Vantrease et al., 2022). This apparent inconsistency could be explained by our observation (in experiment 4) that BLA-aBNST projections are only recruited to a limited extent during fear conditioning, making their suppression rather ineffective.

We did not find clear effects of activating BLA-aBNST projection neurons during DAFC on subsequent anxiety-like behaviour, nor of their direct activation during the actual assessment of anxiety-like behaviour. This suggests that the BLA-aBNST circuitry manipulated might potentially play a distinct role in the mediation of fear memory processing vs. the expression of anxiety-like behaviour. In contrast to our findings, optogenetic inhibition of these neurons was previously found to increase anxiety-like behaviour on the EPM and OF, as well as respiratory rate (Kim et al., 2013; Crowley et al., 2016). However, others did not observe such an effect on behaviour in the OF using chemogenetic inhibition (Vantrease et al., 2022). Noteworthy, these manipulations were in the opposite direction of ours (inhibition vs. excitation).

In future work, we still intend to assess the effect of activation of amygdala- aBNST projection neurons on neuronal activity within the aBNST subregions themselves, to further understand how the behavioural effects are established. The CeA is strongly implicated in the regulation

of freezing behaviour, proposing it as a potential target of the BNSTAL. The BNSTAL has previously been shown to exert a GABAergic influence on cued fear via a direct projection to the CeA (Gungor, Yamamoto & Paré, 2015), further supporting this possibility. Lastly, here we have looked into extended amygdala subregion activity, its correlations and causality during fear acquisition and later anxiety-like behaviour. Assessment of circuit recruitment and regional activity during fear recall might be an interesting target for future investigation.

In conclusion, we here identified BLA-aBNST projection neurons as a clear modulator of cued fear conditioning. These findings uncover previously unknown connections in the extended amygdala that play a vital role in acquisition and expression of cued fear. These results encourage future investigations to delve deeper into understanding how this circuitry influences generalized fear recall.

Supplementary Material

	350	488	555	647
EXP1	100 ms / DAPI	600 ms / Pkcdelta	800 ms / tdTomato	1000 ms / cFos
EXP2	-	1000 ms / GFP	800 ms / tdTomato	
EXP3		1000 ms / mCherry	800 ms / GFP	1000 ms / cFos
EXP4		1000 ms / mCherry	800 ms / GFP	

Table S1. Exposure times used for channels 350, 488, 555, 647 on the Axio Imager 2 (Zeiss) to visualize fluorescent signals.

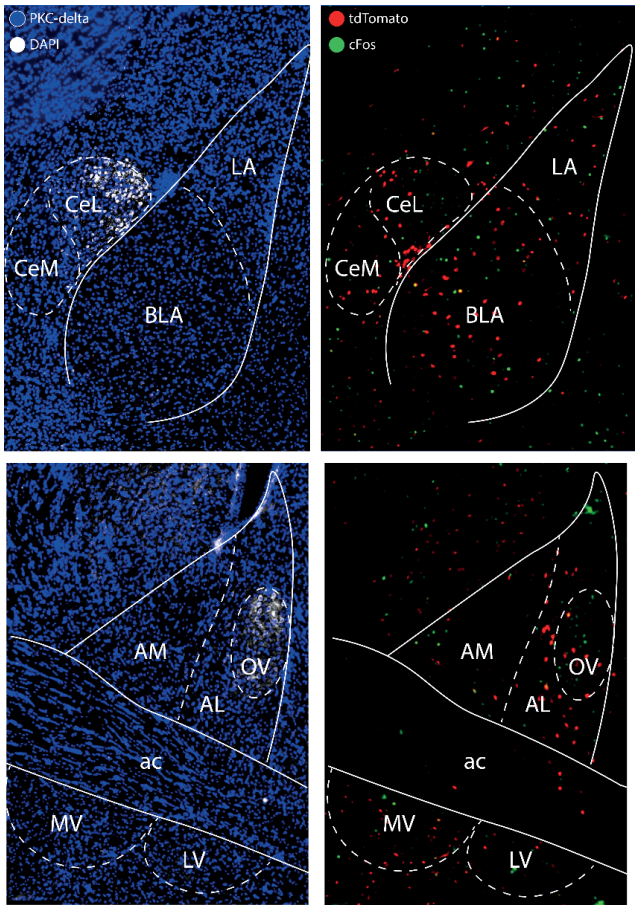


Figure S1. **Anatomically defined subregions in the extended amygdala**. The amygdala was divided into the following regions; lateral amygdala (LA), basolateral amygdala (BLA), central lateral amygdala (CeL), central medial amygdala (CeM). The aBNST was divided as follows: anterolateral (BNSTAL), oval BNST (BNSTOV), anteromedial (BNSTAM), ventromedial (BNSTMV) and ventrolateral (BNSTLV). DAPI and PKC-delta were used to define the CeL and BNSTOV subregion, while the anterior commissure (ac) was used as the natural border between the dorsal and ventral aBNST subregions.

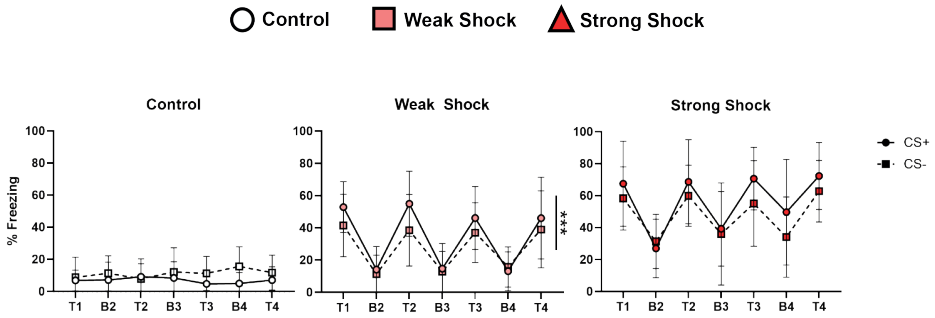


Figure S2. Percentage freezing during and just prior to tone re-exposure (experiment 1). Percentage freezing prior to the tone, or during tone presentation, did not change over time within the groups (all p's > .161), nor were there interactions between time and CS-type (CS+ or CS-) (all p's > .164). In the weak shock group, the percentage freezing was higher towards the CS+ tone in comparison to the CS- ($F(1,23) = 11.161$, $p = .003$). B2-4, baseline freezing prior to the tones (10 s); T1-4, freezing during the tone presentations (10 s).

4

FEAR GENERALIZATION AND ANXIETY-LIKE BEHAVIOURS IN MICE: THE ROLE OF EARLY LIFE STRESS AND SEX DIFFERENCES

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Abstract

Exposure to early life stress (ELS) is a major risk factor for mood and anxiety disorders later in life. Here, we aimed to study fear generalization as potential key mediator in the development of anxiety-like behaviours after ELS exposure in mice. To this end, we subjected mouse dams to limited bedding and nesting, previously shown to evoke unpredictable and fragmented maternal care, inducing stress in the offspring. In adulthood, male and female ELS and control offspring were subjected to a differential auditory fear conditioning paradigm (DAFC), in which two auditory cues were presented of which one always ended with the administration of a foot shock (conditioned stimulus, CS+), and the other cue was never followed by foot shock (CS-). Three days later, mice were subjected to a memory recall test in which both auditory cues were presented and freezing behaviour was recorded to assess generalization of tone-related fear. Subsequent anxiety-like behaviour was assessed in the elevated plus maze (EPM) and dark-light transfer test (DLT). Offspring's bodyweight was negatively affected during and slightly after ELS, but normalized at weaning. Further results revealed no effects of ELS on fear generalization towards the CS- or fear recall in general, but females displayed more context-induced fear than males. Additionally, we found a significant sex-dependent effect of ELS in the EPM, in which ELS females entered the open arms faster and more often than control females; an effect that was not observed for males. No effects of ELS or sex were observed in the DLT. Finally, based on prior observations of the female menstrual cycle modulating stress and fear behaviours, we explored whether oestrous cycle phase in females during DAFC affected subsequent fear and anxiety-like behaviour, and observed that females conditioned in the dioestrous/metestrus phase tended to show more specific fear towards the CS+. Overall, our results suggest that ELS exposure has no profound effects on tone fear generalization, but rendered females more resistant to subsequent anxiety-like behaviour. Lastly, oestrous cycle phase seems to influence fear generalization, emphasizing the need for further dedicated study.

Introduction

Early life stress (ELS), i.e. the stress inflicted by adverse childhood experiences (ACE) such as abuse, neglect, poverty, exposure to war and starvation (Agorastos et al., 2019; Blair & Raver, 2016), is known to increase risk for mood and anxiety disorders in adulthood (U.S. Department of Health and Human Services (HHS), 2022; Famularo et al., 1992; Lähdepuro et al., 2019; Agid et al., 1999). 4-9 % of children exposed to one or more ACEs show anxiety-like behaviour (Elmore & Crouch, 2020; Porche et al., 2016), while 10-50 % of ACE-exposed adolescents are experiencing some type of mild to severe anxiety (Gibb et al., 2007; Lee et al. 2020). A growing body of evidence acknowledges the high incidence of ACEs in countries around the globe and across income groups, and general consensus is that more than half of all adults has experienced at least one ACE, whereas approximately 12 % of American adults even experienced more than four ACEs (HHS, 2022; Dong et al., 2004; Kessler et al., 1997; Green et al., 2010; Benjet et al., 2009; Kessler et al., 2010). The economic impact of ACE-related illness is monumental, with annual healthcare costs estimated at US$ 581 billion in Europe and US$ 748 billion in North America (Bellis et al., 2019). It is believed that ELS influences neurodevelopment and thereby predisposes individuals to a broad array of physiological and neural changes, leading to altered function of the brain circuitry that governs emotion, memory, and fear responses (Smith et al., 2019; Koss & Gunnar, 2018; Pechtel & Pizzagalli, 2011), increasing risk of psychopathology (Enoch, 2011). To aid prevention, early intervention and treatment of psychopathology following ELS exposure it is essential to enhance our understanding of the impact of ELS on the brain.

One finding that may be a key feature in the development of mood and anxiety disorders is the observation that patients characteristically present fear overgeneralization (Cooper et al., 20220). Whereas fear generalization is an adaptive response that promotes survival in the face of a stimulus that resembles a conditioned harmful stimulus, fear overgeneralization is maladaptive with fear occurring in response to stimuli that bear only minimal resemblance to the threatening stimulus (Steimer, 2022; Lissek, 2012). Fear overgeneralization can be a great burden as it allows past aversive experiences to greatly limit one's capabilities in daily life, as severe distress may be experienced even during situations unrelated to the original adversity (Dymond et al., 2015). Since fear overgeneralization may proliferate the fear

response to a growing repertoire of cues and contexts, it has been proposed as key mechanism for the development of mood and anxiety disorders (Lissek et al., 2014).

Therefore, we hypothesized that ELS may increase risk on psychopathology by promoting fear generalization. The present research aims to test this hypothesis and elucidate the effect of ELS on fear generalization and anxiety-like behaviour in mice. Based on the presence of clear sex differences in the prevalence of stress-related psychological disorders (Mclean et al., 2011; Beesdo et al., 2010; Gum et al., 2009), we investigated both male and female offspring. Since the female reproductive hormones are thought to play a critical role in mediating these differences (Altshuler et al., 1998; Yonkers & Ellison, 1996), and fluctuate over the course of the oestrous cycle, oestrous cycle phase was monitored in female mice. Mice were subjected to either ELS or a control condition. To induce ELS, the limited bedding and nesting (LBN) model was utilized. These conditions promote fragmented, unpredictable and abusive maternal behaviour, leading to chronic stress in pups (Walker et al., 2017). Offspring was housed on LBN material from postnatal day (PND) 2-9, a period that constitutes part of the rodent stress-hyporesponsive period (Schmidt, 2019), and is characterized by hypothalamic pituitary adrenal axis (HPA-axis) desensitization. This period corresponds to the developing brain of humans during the first 3 months of life (van Bodegom et al., 2017), and governs a neuroprotective mechanism that shields the brain from deleterious effects of excessive corticosteroid signalling in the face of stress. However, corticosteroid exposure is not eliminated entirely, allowing severe stressors such as LBN to overrule this mechanism and thereby permanently alter stress physiology (Dymond et al., 2015; van Bodegom et al., 2017). In adulthood, mice were tested in a differential auditory fear conditioning (DAFC) paradigm in which they were taught to fear one auditory cue predictive of a foot shock (the conditioned stimulus; CS+), but not another auditory cue that is never followed by a foot shock (CS-). Fear generalization was assessed by measuring fear (i.e. freezing) responses to either auditory cue three days later. Subsequently, general anxiety levels were tested using the elevated plus maze and dark-light transfer test. Given the vast body of epidemiological evidence (Reiser et al., 2014; Gardner et al., 2019; Whitaker et al., 2021; Gibb et al., 2007; Lee et al., 2020; Zare et al., 2018), we hypothesized that ELS promotes fear generalization and increases anxiety-like behaviour in mice, potentially in a sex- and oestrous-cycle phase specific manner (Barker & Galea, 2010; Gupta et al., 2001; McDermott, Liu & Schrader, 2012). Here, we have found sex-dependent effects of ELS on contextual freezing and

anxiety-like behaviour, despite not finding ELS effects on tone-induced fear generalization behaviour.

Materials & Methods

Mice and early-life procedures.

Two founder mouse lines, *Fos*$^{2A\text{-}iCreERT2}$ females (The Jackson Laboratory, #030323, bred in-house) and conditional *tdTomato* males (*Ai9*, The Jackson Laboratory, #007909, bred in-house) were bred to generate heterozygous male and female *FosTRAP2xtdTomato* offspring, referred to as FosTRAP2. This genetic construct allows for immediate labelling of *Fos*-expressing (i.e. activated) neurons by the fluorescent protein *tdTomato* after injections with 4-hydroxytamoxifen (4-OHT). For the purpose of reproduction, females were housed together with a male for one week, after which they were separated and housed in conventional Mouse Eurostandard type IIL cages (Tecniplast) on a reverse day/night cycle (lights on between 8.00-20.00 h). Cages were checked daily for pups. To standardize litter size, only litters with 6 or more pups were included in the study, and litters with more than 6 pups were culled to 6 pups on PND2, striving for equal sex distribution. Litters wherein more than 2 pups were eaten by the dam after ELS exposure were excluded from this study (n = 1). At PND2, dams and pups were weighed, after which they were randomly assigned to either ELS or control (CTRL) conditions. All experimental procedures were in compliance with European Union Directive 010/63/EU and were approved by the Central Authority for Scientific Procedures on Animals (CCD), Den Haag, The Netherlands. All efforts were made to minimize animal suffering and to reduce the number of animals.

Early Life Stress Procedures.

ELS was induced by limited bedding and nesting (LBN). In LBN conditions, the floor was covered with limited sawdust bedding and a fine-gauge stainless steel mesh was inserted 1 cm above the cage floor. A square piece of cotton nesting material was placed on top of the mesh (2.5 x 5 cm). CTRL cages were equipped with 100 g of sawdust bedding and nesting material (5 x 5 cm). Food and water were available *ad libitum* and cages were covered with filter tops to minimize disturbances of the animals due to external scents and sounds. Pups were weighed prior to entering the LBN/CTRL housing conditions on PND2 (see Fig. 1 for experimental timeline). From PND2 to PND9, animals were left undisturbed. From PND9

onwards, all animals were housed in cages equipped with a standard amount of nesting and bedding materials (similar to CTRL conditions) without filtertops. Weighing occurred on PND9, PND15 and PND21, after which the litters were weaned. At weaning, pups were ear punched for identification purposes and males and females were housed separately in different housing rooms (2-5 animals per cage). During this time, animals from different litters, but similar experimental group, were housed together. Two weeks before the start of the behavioural experiments, animals (7-8 weeks old) were single housed in conventional Mouse Eurostandard type IIL cages (Tecniplast) with 100 g saw dust bedding and cotton nesting material (5 x 5 cm).

General testing procedures.

Offspring was first habituated to human handling for 5 days. In the morning of each testing day, mice were moved from their housing rooms to dark temporary housing rooms situated next to the experimental room. Mice were subsequently left undisturbed for at least 2 hours to allow for acclimation. Males and females were placed in separate rooms to prevent mice of opposite sexes smelling each other. During the behavioural tests, the researcher exited the room so that the animal was not disturbed during the test. After each test, the researcher came back into the experimental room, put the mouse back in its home cage and placed the cage back in the temporary housing room. After every session, the test apparatus was disinfected with Incidin™ OxyWipe (Ecolab) in order to start the next session.

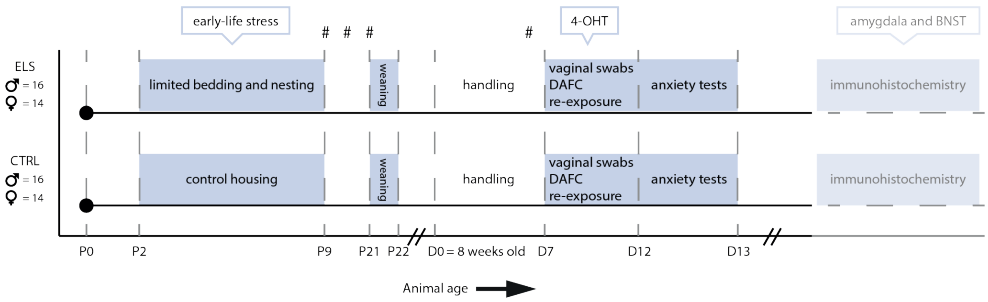

Figure. 1. Overview of experimental timeline. Animals were exposed to LBN or CTRL conditions from P2-P9 and were subsequently submitted to several behavioural tests and finally sacrificed. Immunohistochemistry on brain tissue (in gray) will be performed at a later stage. *#: weighing of animals on P9, P15, P21 and last day of handling.*

Handling.

To habituate mice to human handling, mice were handled by cupping for several minutes, 5 times over the course of 7 days before the start of the first behavioural test (see Supplementary Materials, Table 1). On some occasions, mice remained too stressed to be handled by cupping (n = 3; 2 CTRL females, 1 ELS female). These animals were instead tail grabbed throughout the behavioural experiments to prevent an increase in stress and possible injury of the animals. On the last handling session, mice were weighed and this weight was used to determine injection doses of 4-OHT and as a baseline for potential weight loss due to behavioural testing.

Differential Auditory Fear Conditioning (DAFC) procedure.

Habituation. On the first day of the behavioural experiment, animals were habituated to two contexts in order to reduce novelty stress at the time of the actual testing. Context A consisted of a triangular shaped box with black walls, a metal grid floor and was sprayed with 1 % acetic acid. After 2 minutes of habituation to this context, animals were exposed to two auditory tones (CS-; 10 kHz, 10 s, 85 dB. CS+; 5 kHz, 10 s, 85 dB), in a semi-random order of 8 repetitions each with variable intervals (ITI: 40-120 s). Context B consisted of a round shaped box with white walls and a white PVC floor, and had no distinguished smell. During habituation to this context, no auditory tones were presented to the animals. Animals were exposed to both contexts, each 30 minutes long, with 3 hours in between the first and second session. Exposure to the contexts was counter-balanced across animals (Fig. 2).

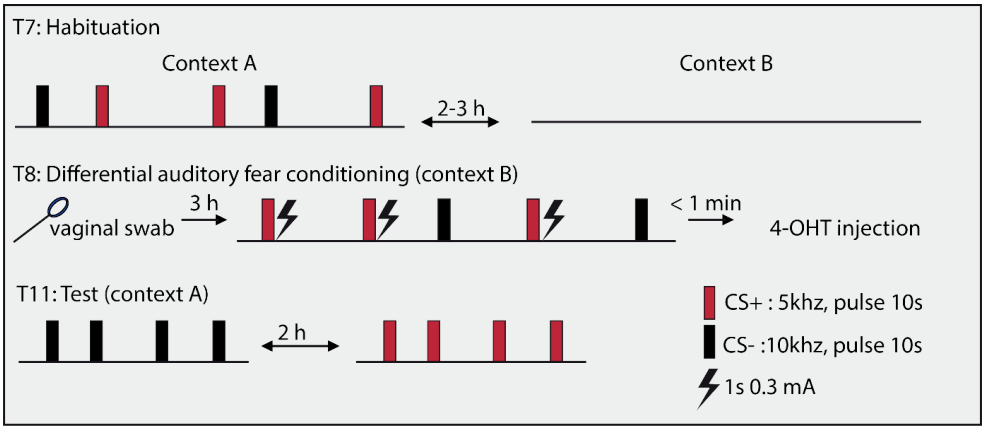

Figure 2. Differential auditory fear conditioning paradigm. Animals were exposed to the two contexts at T5. The next day, the female mice received a vaginal swab, and three hours later all mice were exposed to the DAFC. This was immediately followed with an i.p. injection with 4-OHT. Three days later, mice were again exposed to the CS+ and CS- in two separate sessions to assess fear memory recall.

Oestrous Cycle Determination. The next day (at least 3 hours prior to DAFC), the oestrous cycle of all female mice was determined. Vaginal swabs were taken *in duplo* using an inoculation loop. The swabs were smeared on glass slides and air dried. To stain the cells, slides were submerged completely for 10 minutes in buffered Giemsa's solution (119 mL 50 mM Tris-HCL (pH 7.0-7.4) (Roche-10812846001, Sigma-Aldrich) and 8.75 mL Giemsa's solution (1.09204, Sigma-Aldrich). Next, slides were inspected by use of light microscopy. Histological feature, including cell nuclei and morphology, were used to determine the oestrous phase of the mouse (Fig. 3).

Training. At least 3 hours after oestrous cycle determination, mice were placed in context A for 29 minutes and subjected to DAFC. After 2 minutes of habituation to context A, mice were exposed to 2x10 presentations of the CS+ and CS- tones in a semi-random order (ITI = 40-120 s, protocol identical for all mice). The final second of the CS+ always coincided with a 1 s 0.3 mA foot shock, while the CS- never coincided with any foot shock. Immediately after the DAFC session, mice were i.p. injected with 4-OHT (50-60 mg/kg) and left undisturbed in their home cages for 72 hours. Filter tops were placed on top of the home cages, to prevent the spread of airborne 4-OHT.

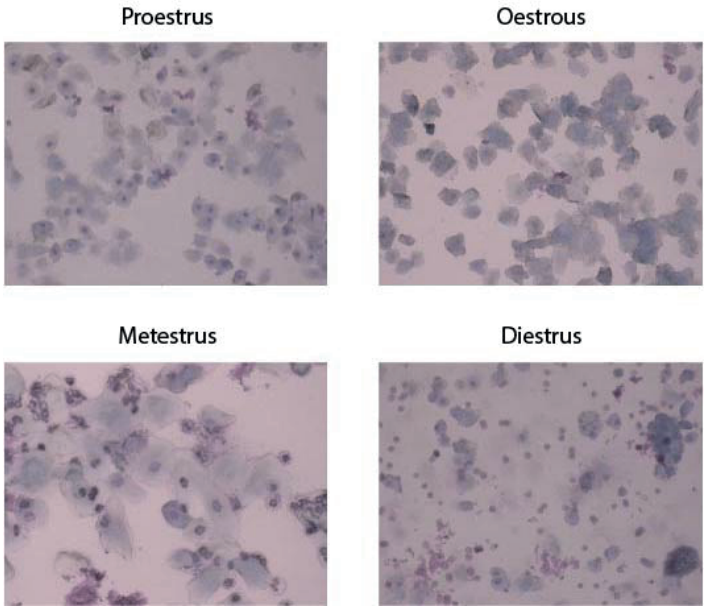

Figure 3. Histology of vaginal swabs to assess oestrous cycle in female mice. Given their histological and hormonal similarities, the proestrus and oestrus stage (high oestrogen and progesterone), and the metestrus and dioestrus phase (low oestrogen and progesterone) were combined in data analyses to optimize statistical power.

Fear memory recall. Three days after DAFC, conditioned mice were submitted to a fear recall test by re-exposing them to the auditory cues in context B, where their fear memory was measured by assessing their freezing behaviour. Fear recall for both cues was tested in separate sessions, with one tone re-exposure session to the CS- and one tone re-exposure session to the CS+, in counter-balanced order across mice and split by 2 hours between sessions. Each session lasted 380 s and took place in context B. During each session, 4 tones (4x CS- or 4x CS+) with a duration of 10 seconds each were played at variable intervals (delay to first tone = 120 s, ITI = 40-120 s, 85 dB). No foot shocks were administered during the recall tests. Videos of these sessions were recorded. Following this test, mice were individually placed in clean cages and returned to the temporary housing room.

4-hydroxytamoxifen (4-OHT). 4-OHT was dissolved in ethanol and sonicated in a water bath of 45-55 °C. Second, the mixture of 4-OHT and ethanol was dissolved in corn oil. The final solution consisted of 1 % 4-OHT, 10 % ethanol and 90 % corn oil, resulting in a 4-OHT concentration of 10 mg/mL. The solution was sonicated at room temperature (RT) for a few

hours, until the solution fully dissolved. The solution was stored in -20 °C and sonicated for at least one hour before use.

Assessment of Anxiety-like behaviour.

Elevated plus maze. The day after fear recall, animals were tested on the elevated plus maze (EPM). The plus-shaped apparatus was elevated 50 cm above the ground and spanned 90 cm from end to end. Each arm was 5 cm wide, open arms were lined by an edge of 3 mm to prevent falling of the mice, and the closed arms were lined by 15 cm high, black walls. The apparatus was illuminated to 35 Lux and situated in a dedicated testing room in which no other mice or people were present. At the start of the session, mice were placed at the end of the enclosed arm, facing the centre of the maze. Each mouse underwent the EPM individually and was recorded by an overhead camera for subsequent data analysis. The animal was then free to explore the area for 10 minutes, with the first 5 minutes being used for data analysis. The parameters assessed were distance travelled, time spent in open arms, latency to first enter the open arm and frequency visiting the open arms.

Dark/Light Transfer Test. The subsequent day, mice were tested in the dark/light transfer test (DLT). The testing setup was a box (42 x 21 x 30 cm) consisting of a small, dark compartment (one third) and a large, light compartment (two thirds, 975-1250 lux) separated by a dividing wall. After being placed in the dark compartment, animals could freely roam both compartments of the apparatus for 10 minutes (with only the first 5 minutes used in data analysis) through an opening (7 x 7 cm) in the dividing wall. Time spent in the risk assessment zone, a small area (7 x 3 cm) in front of the opening on the light side, was measured to calculate the risk assessment time as a percentage of total time spent in the rest of the light compartment. Moreover, time, distance, frequency of entry and latency to first entry into the light compartment was monitored.

Sacrifice and Brain Tissue Collection. 90 minutes after the DLT, mice were anesthetized by inhalation isoflurane and overdosed by i.p. injection with pentobarbital (200 uL). Next, they were perfused with 1 x PBS and 4 % paraformaldehyde (PFA). Brains were extracted and subjected to 24 hours post-fixation in PFA at 4 °C. Lastly, brains were stored in 1 x PBS at 4 °C until immunohistological analysis.

Data analysis. Behaviour of mice was recorded during fear recall, the EPM and the DLT by an overhead camera. Video data of the fear recall session was scored manually by an observer blinded to the experimental group using Observer XT14 (Noldus), during which the duration of freezing to the CS- and CS+ was assessed, as well as freezing behaviour observed during the first 2 minutes before the onset of the first tone to assess context-induced fear. Video data of the EPM and DLT was scored automatically with Ethovision XT15 (Noldus) to determine the time spent and distance travelled in each zone. In addition, EPM and DLT data were scored manually to assess frequency of and latency to first entry into the open arms of the EPM and the light zone of the DLT by an observer blinded to the experimental group.

Statistical analysis. Using IBM® SPSS® Statistics 27, 2-Way ANOVAs were performed, with sex (male vs. female), treatment (CTRL vs. ELS) and (for freezing readouts) testing order (CS+ first vs. CS- first) and CS-type (CS+ vs. CS-) as factors to assess their effects on fear generalization and anxiety readouts. A paired samples t-test was used to explore whether animals displayed CS discrimination (CS+/CS- vs. 1). In addition, 2-Way ANOVAs were performed in females only with treatment (CTRL vs. ELS), oestrous cycle ((pro)oestrous vs. dioestrus-metestrus) and (for freezing readouts) testing order (CS+ first vs. CS- first) as factors to assess the effect of oestrous cycle phase during DAFC on the same readouts. To optimize statistical power, cycle stages were grouped into proestrus-oestrous and dioestrus-metestrus based on circulating hormone levels. Moreover, this division increased reliable group assignment as the stages within these groups have comparable histological features (Fig. 3). For all 2-Way ANOVAs, Tukey HSD *post hoc* tests were performed if the assumption of equal variance was met as determined by a non-significant ($p > 0.05$) Levene's Test. In the case of unequal variance ($p < 0.05$), Games-Howell tests were performed. Moreover, repeated measures ANOVAs were performed to assess body weight development over time (PND2, PND9, PND15) with time as within-subject factor (3 levels) and treatment and sex as between-subjects factor. Since animal identification only happened at PND21, two separate statistical models were used. The repeated measures ANOVA for PND2, PND9 and PND15 were performed on average body weights per sex per litter. A 2-way ANOVA for PND21 was performed on individual animal's body weights. If sphericity was assumed by means of Mauchly's test for sphericity ($p > 0.05$), no correction was applied. If Mauchly's test indicated a violation of the sphericity assumption ($p < 0.05$), either a Greenhouse-Geisser

correction (ε < 0.75) or Huyn-Feldt correction (ε > 0.75) was applied. For the repeated measures ANOVA, independent samples t-tests were performed for *post hoc* analyses. Data are represented in violin plots showing the median and interquartile ranges and plotted in GraphPad prism (v9).

Results

Body weight of the mice was analysed to assess the effect of ELS induced by the LBN model (Fig. 4). Average body weight per litter per sex as assessed at PND2, PND9 and PND15 revealed a significant main effect of time (F(1.342, 21.480) = 3413.954, p < .001), treatment (F(1, 16) = 6.964, p = .018), and a trend level effect of treatment*time interaction (F(1.342, 21.480) = 3.793, p = .054), in the absence of any effects of sex (all p's > .566). Subsequent *post hoc* analyses indicated that the interaction effect was attributable to a reduced body weight of ELS animals as compared to control animals at PND9 (M_{ELS} = 5.36 g, SD_{ELS} = .33 and M_{CTRL} = 5.79 g, SD_{CTRL} = .39, t(56) = 21.081, p < .001) and PND15 (M_{ELS} = 8.30 g, SD_{ELS} = .42 and M_{CTRL} = 8.69 g, SD_{CTRL} = .53, t(56) = 21.081, p = .003), without any body weight differences across groups at PND2 (M_{ELS} = 1.828 g, SD_{ELS} = .154 and M_{CTRL} = 1.867 g, SD_{CTRL} = .200, t(18) = .495, p < .626). At PND21, individual body weights of identified animals did not reveal a main effect of sex (F(1,43) = .015, p = .902), treatment (F(1,43) = 1.039, p = .314) or treatment*sex interaction (F(1,43) = 1.116, p = .297). These findings suggest that body weight is negatively affected during ELS exposure and slightly after, but that these differences disappeared at weaning.

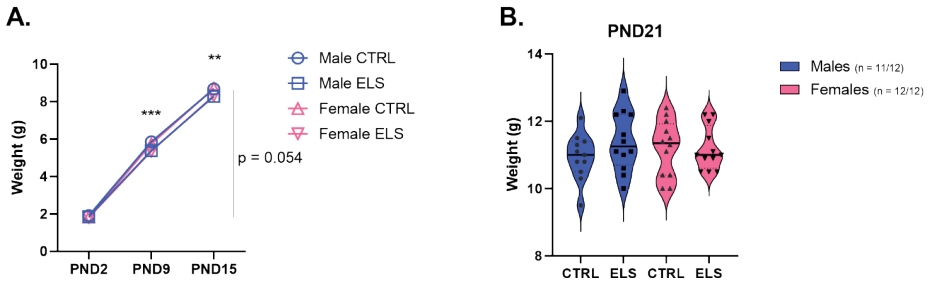

Figure 4. Body weights of ELS and CTRL offspring. Animals were weighed at PND2, PND9, PND15 and PND21. (A) Body weights at PND2, 9 and 15 were analysed as litter averages per sex, whereas body weights at PND21 (B) were of individual animals. ELS animals weighed significantly less at PND9 and PND15 as compared to CTRL animals. At PND21, differences had disappeared. ***: p < .01, ***: p < .001, p = .054 trend effect time* treatment interaction.*

To assess levels of fear generalization, we measured both context- and cue-induced freezing. Context-induced freezing, defined as time spent freezing upon exposure to context B (i.e. the non-conditioned context), was significantly higher in females (M_F = 25.57 s, SD_F = 12.83) than in males (M_M = 11 s, SD_M = 8.83, $F(1, 50) = 19.959$, $p < .001$), with no effect of treatment ($F(1, 50) = .012$, $p = .914$) or treatment*sex interaction ($F(1, 50) = 2.415$, $p = .120$, Fig. 5A).

Similar sex differences were observed in freezing upon cue exposure (M_F = 62.2 s, SD_F = 23.0 and M_M = 48.2 s, SD_M = 18.3, $F(1, 59.811) = 9.420$, $p = .003$), but again without treatment effect ($F(1, 59.811) = 1.419$, $p = .238$) or treatment*sex interaction ($F(1, 59.811) = .319$, $p = .574$). Further, there was a significant effect of CS type ($F(1, 57.992) = 11.995$, $p = .001$) indicating that freezing levels were higher towards the CS+ than the CS-. Moreover, average freezing towards the CSs was consistently higher than context-induced freezing (all *p*'s < .004). Yet, we did not find any interactions with CS type, sex or treatment (all *p*'s > .05), nor did we find any order effects of interactions with the order in which the CS types were presented during fear recall (all *p*'s > .05).

To assess specifically fear generalization across the auditory cues, relative freezing scores were calculated by dividing CS- induced freezing by CS+ induced freezing (Ghosh et al., 2014; Bender et al., 2018) (Fig. 5B). These scores did not reveal a significant main effect of treatment ($F(1, 48) = 1.541$, $p = .221$), sex ($F(1, 48) = .251$, $p = .134$), or treatment*sex interaction ($F(1, 48) = .013$, $p = .911$). Although again we did not find an effect of testing

order on relative freezing (F(1, 48) = .858, p = .359), we did observe a sex*testing order interaction (F(1, 48) = 7.386, p = .009). Both males and females showed similar relative freezing when presented with the CS+ first (M_F = .90 , SD_F = .28 and M_M = .79, SD_M = .18, t(27) = -1.317, p = .199), but males displayed higher levels of relative freezing than females when the CS- was presented first (M_F = .73, SD_F = .29 and M_M = 1.12, SD_M = .49, t(25) = 2.521, p = .018), but this was irrespective of treatment condition. Lastly, exploratory analyses revealed that relative freezing was significantly lower than 1 – indicative of distinct freezing responses towards the CS+ vs CS- - only in ELS males (t(14) = 2.190, two-sided p = .046) and females (t(12) = 3.724, two-sided p = .003), but not in CTRL males (t(12) = .180, two-sided p = .860) and females (t(14) = 1.497, two-sided p = .157).

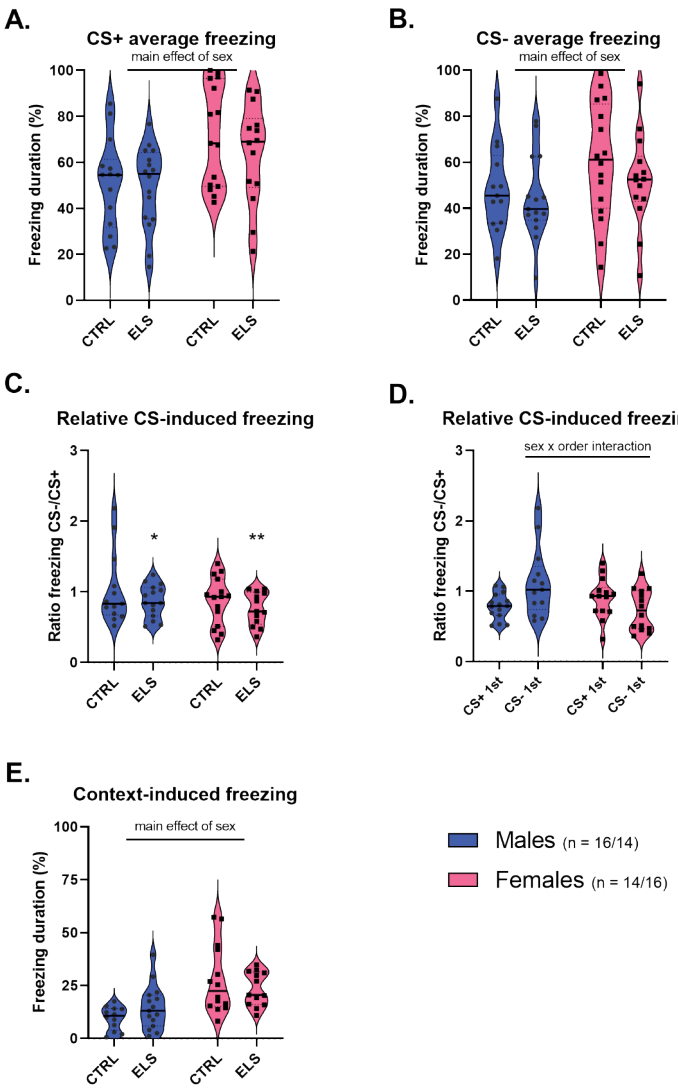

Figure 5. Freezing behaviour during fear recall tests. Females exhibited significantly higher CS+ induced (A), CS- induced (B), and context-induced freezing (E) than males, but no overall sex differences were observed in relative CS-freezing rates (C). No significant treatment effects were observed for any of the readouts, yet ELS males and females significantly distinguished between CS+ and CS-, whereas control groups did not (C). Relative CS-freezing rates were modulated by a sex*testing order interaction (D), where males displayed higher levels of relative freezing compared to females when the CS- was presented first. **: p < .05, **: p < .01, relative freezing is different in comparison to 1.*

To assess anxiety-like behaviour, mice were subsequently tested in the EPM. No significant effects of treatment, sex, or treatment*sex interactions were observed for total distance moved, distance moved in open arms, and time spent in open arms of the EPM (all *p*'s > .331. Fig. 6A-C). Moreover, relative risk assessment was not affected by treatment, sex, or a treatment*sex interaction (all *p*'s > .269). In contrast, we found significant sex*treatment interaction effects for both the latency to the first open arm entry ($F(54, 1) = 7.401$, $p = .009$, Fig. 6D) and the frequency of open arms entry ($F(1, 54) = 6.693$, $p = .012$, Fig. 6E). *Post hoc* tests indicated that exposure to ELS in females significantly decreased their latency to enter the open arms ($M_{F, ELS} = 156.71$ s, $SD_{F, ELS} = 110.17$) in comparison to control females ($M_{F, CTRL} = 275.71$ s, $SD_{F, CTRL} = 47.63$, $t(26) = 3.710$, $p = .001$), and this effect was not observed in males ($M_{M, ELS} = 216.56$ s, $SD_{M, ELS} = 113.25$ and $M_{M, CTRL} = 191.00$ s, $SD_{M, CTRL} = 114.78$, $t(28) = .631$, $p = .545$). Similarly, ELS increased frequency of open arm entry in females compared to control conditions ($M_{F, ELS} = 1.29$, $SD_{F, ELS} = .99$ and $M_{F, CTRL} = .43$, $SD_{F, CTRL} = .76$, $t(26) = 2.567$, $p = .016$), and this finding did not extend to males ($M_{M, ELS} = .75$, $SD_{M, ELS} = 1.06$ and $M_{M, CTRL} = 1.36$, $SD_{M, CTRL} = 1.39$, $t(28) = 1.351$, $p = .187$).

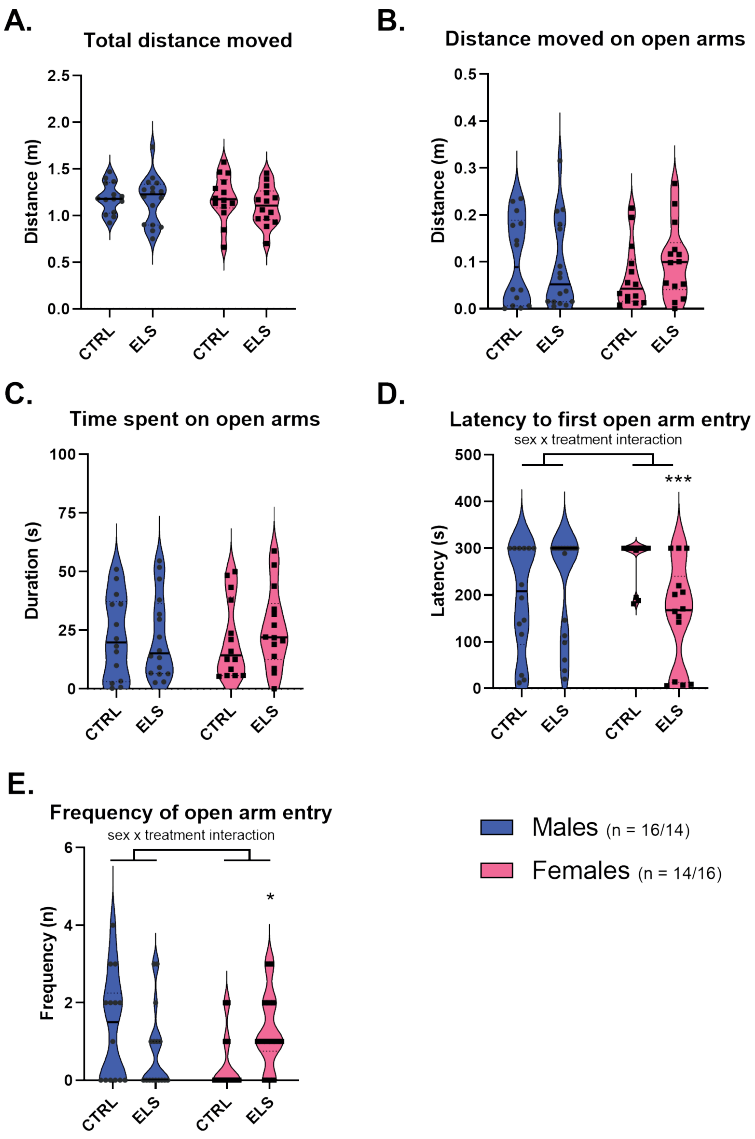

Figure 6. Anxiety-like behaviour in the Elevated Plus Maze. No significant effects of treatment or sex were observed in the total distance moved (A), distance moved on the open arms (B), and time spent on the open arms (C). However, ELS females entered the open arms significantly more often (D) and more quickly (E) than CTRL females. This effect was not found in males. ****: p < .001 or *:p < .05, ELS compared to respective control.*

In the DLT, no significant differences were observed between experimental groups for distance moved in the light zone, time spent in the light zone, time spent in the risk assessment zone and relative risk assessment (computed by dividing time spent in risk assessment by time spent in light zone) (all *p*'s > .05, Fig. 7A-D). In contrast to EPM data, the frequency of light zone entry and the latency to first light zone entry did also not differ between experimental groups (all *p*'s > .05, Fig. 7E-F).

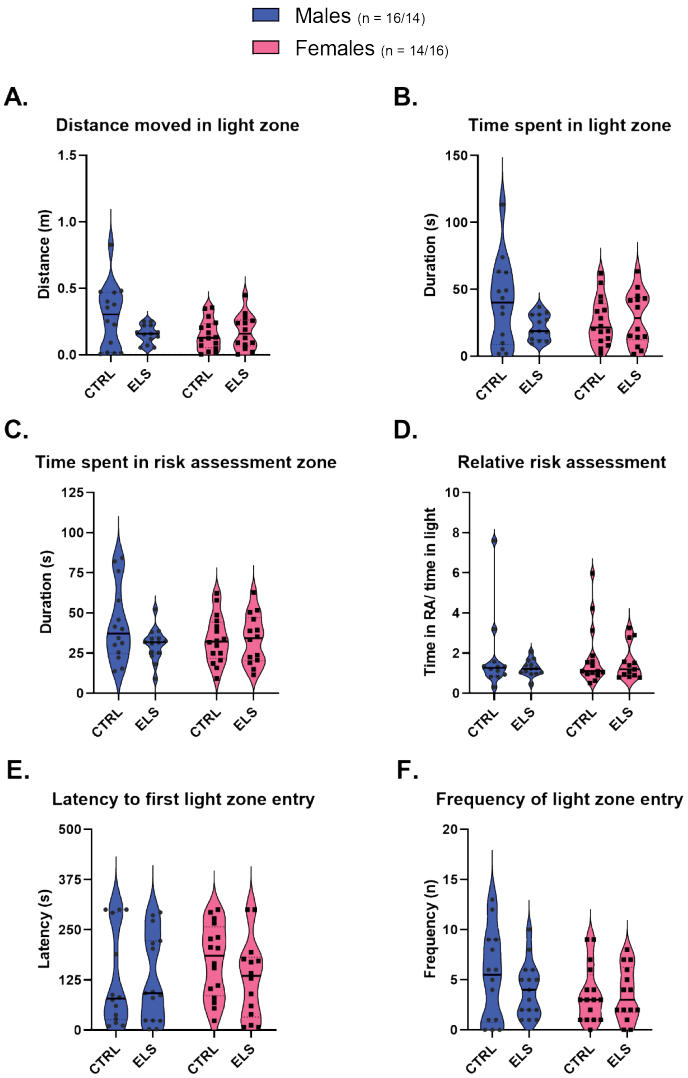

Figure 7. Anxiety-like behaviour in the Dark-Light Transfer Test. No significant effects of treatment or sex were observed in the distance moved in the light zone (A), time spent in the light zone (B), time spent in risk assessment zone (C) and relative risk assessment (D). Moreover, no significant differences were found in the latency to first light zone entry (E) and frequency of light zone entry (F).

Finally, we assessed the effect of the oestrous cycle phase on fear generalization and anxiety-like behaviour between treatment groups in females. We observed no significant effects of treatment or oestrous cycle phase on context-, and cue-induced freezing (all *p*'s > .05, Fig. 8A, 8C-D). We did however find an overall effect of CS that indicated that freezing towards CS+ was generally higher than CS- in females ($F(1, 29.528) = 4.984$, $p = .033$, $M_{CS+} = 6.78$, SD = 2.19, $M_{CS-} = 5.65$, SD = 2.31, data not shown). Further, we found a trend level effect of the oestrous cycle phase on relative CS-freezing levels ($F(1, 21) = 4.186$, $p = .053$, Fig. 8B). This effect suggests that females that were in their dioestrous-metoestrous phase during DAFC ($M = .77$, $SD = .29$) discriminated the CS- and CS+ better than females that were in the (pro)oestrous phase during DAFC ($M = 1.08$, $SD = .58$). No significant treatment effect ($F(1, 21) = .005$, $p = .943$), testing order effect ($F(1,21) = .105$, $p = .750$) or any interaction effects were (all *p's* > .05) observed for relative CS-freezing levels. Cycle phase did not modulate behaviour in the EPM and DLT (all *p*'s > .05, data not shown).

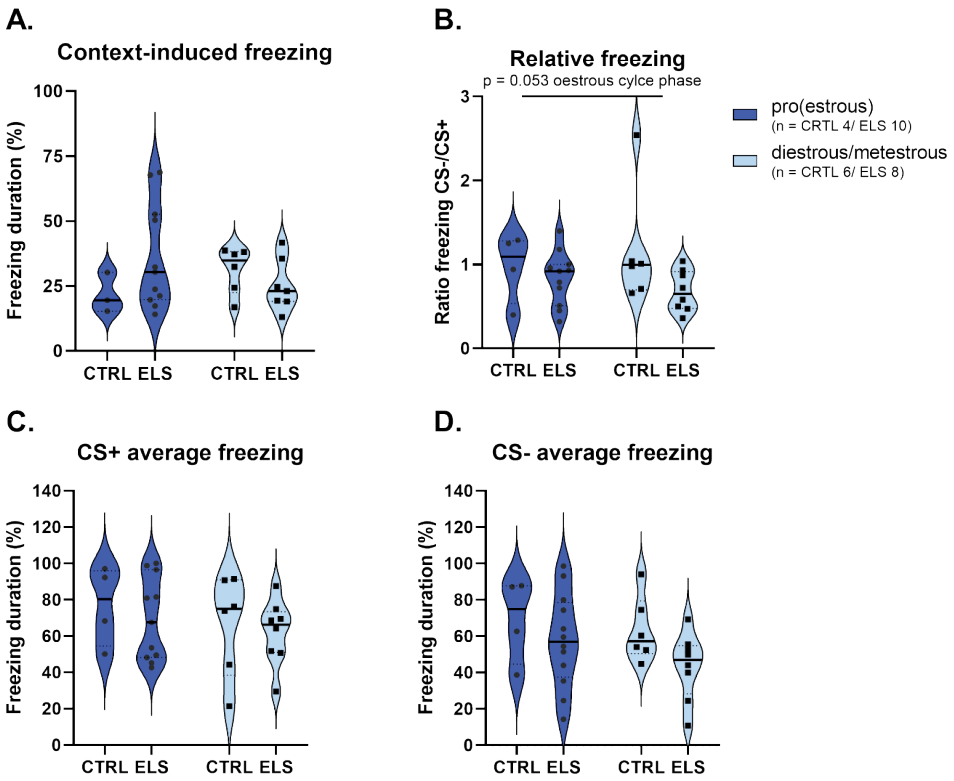

Figure 8. Freezing behaviour during auditory cue re-exposure in females modelling oestrous cycle phase as factor of interest. Although no significant differences across oestrous cycle groups and/or treatment conditions were found in context-induced freezing (A) and CS-induced freezing (C-D), a trend level effect of oestrous cycle phase was observed for relative CS-freezing rates (B), driven by better discrimination by females conditioned in their dioestrous-metoestrous phase than females conditioned in their (pro)estrous phase.

Discussion

The present study investigated the effects of early life stress (ELS) on fear generalization and resulting anxiety-like behaviour using a limited bedding and nesting (LBN) mouse model. Contrary to our hypothesis, we found that ELS induced by LBN did not have any effect on fear generalization, and specifically affected female offspring by reducing anxiety-like behaviour. ELS-exposed females were quicker to enter the open arms of the EPM and entered them more frequently as compared to control females. In contrast, strong differences between sexes regardless of treatment group were apparent in freezing behaviour. In addition, the data suggests that an effect of the oestrous cycle on fear discrimination in females may exist, wherein being in the (pro)estrous stage during fear learning could predispose females to more generalized fear as compared to being in the diestrous-metestrous phase. However, the current study was not designed for this purpose, making that we lacked statistical power to achieve significance for this effect of the oestrous cycle phase.

LBN is a commonly used model for ELS in rodents, which has been shown to result in many lasting effects on the behavioural level, including alterations in sleep-wake behaviour, anhedonia and impaired cognitive function (Walker et al., 2017; Wang et al., 2022; Ivy et al., 2008). The LBN paradigm exerts its effects by manipulating the maternal behaviour of the dams (i.e. inducing fragmented, unpredictable or adverse maternal care) while requiring minimal researcher intervention as compared to other models such as maternal separation (Walker et al., 2017; Sanchez et al., 2017). Exposure to LBN elevates offspring's corticosterone plasma levels during early development and in adulthood (Rice et al., 2008), in addition to adrenal hypertrophy in the final stages of LBN (Avishai-Eliner et al., 2001; Brunson et al., 2005; Gilles et al., 1996; Ivy et al., 2008) and increased glutamatergic innervation of corticotropin releasing hormone (CRH)-expressing hypothalamic neurons (Gunn et al., 2013). Thus, LBN exposure induces HPA-axis changes during the neonatal period which could contribute to long-lasting behavioural effects. Here, corticosterone levels measurements were not done as an objective and quantitative indictor of stress. Possibly, LBN was not perceived as severe adversity by the mice, but rather as a mild stressor which the animals recover from within several days. Alternatively, LBN may lack consistency to induce severe stress in all animals. Since LBN is mediated by behaviour of the dam that

alters her maternal care, which can be different between both dams and individual pups, variability in the model and its effects have been observed before. For instance, some dams show no change in overall durations of maternal care behaviours (Ivy et al., 2008), whereas other dams engage in kicking and other abusive behaviours, ultimately accounting for inconsistent health outcomes in the offspring (Walker et al., 2017; Gallo et al., 2019; Moriceau et al., 2009; Raineki et al., 2008). In this study, maternal care was not monitored, so we have no indication whether intact maternal care was provided to the litter, or to make distinctions between litters based on maternal type behaviour. All things considered, it is plausible that LBN only temporarily inflicted stress that did not result in longer lasting neurobehavioural changes.

In our model, we have dissociated male mice from female mice in investigating the effects of ELS, since human data has shown that the prevalence of anxiety disorders is much higher in females than in males (McLean et al., 2011), thereby hypothesizing that early life adversity may particularly affect women. Correspondingly, LBN has shown to induce different behavioural effects in male and female mice. For instance, male mice show a deficiency in cue-discrimination abilities and contextual and spatial memory formation following LBN, while female mice do not (Arp et al., 2016; Kanatsou et al., 2016). However, female mice show increased anxiety-like behaviour in early life and somewhat reduced memory formation following ELS (Kanatsou et al., 2016). Furthermore, rodent studies have shown that female mice exhibit more generalized fear than male mice, albeit in contextual fear generalization (Keiser et al., 2017). In our study, we have observed more freezing by female than male mice, both in context-induced and auditory cue-induced freezing, irrespective of ELS history. A logical explanation could be that females experience inherently higher novelty stress, resulting in more freezing behaviour, although the literature is inconclusive in that regard. Some studies have shown that females indeed show more freezing during cued conditioning, as well as during extinction training (Borkar et al., 2020), while other studies show that overall freezing levels are similar across sexes (Tryon et al., 2021; Day et al., 2020) and argue that females simply show less fear discrimination and therefore only appear to display higher stress (Day et al., 2020). The effect may also reside in differences in fear learning between the sexes. A previous study has shown that female rats are more sensitive to electrical shocks as measured by flinching, shuffling, and jumping after foot shocks of varying amperage (Beatty et al., 2004). It follows that fear may be consolidated more

strongly in females, which could manifest as more extensive freezing during fear recall, much like our results show (Baldi et al., 2004). Future additional analysis of the pre-DAFC freezing data acquired during habituation, as well as freezing levels during the DAFC itself, might resolve this issue. Lastly, it is a possibility that males and females express their fear in a different manner. Several studies have shown that a broad repertoire of behaviours may signal fear, including darting, which resembles an active attempt to escape, and defecating, and that these behaviours may be sexually dimorphic (Gruene et al., 2015; Russo & Parsons, 2021).

Although implementation of LBN in rodent studies has been shown to have a lasting impact on many different factors, its effect on anxiety-like behaviour has not always been conclusive. Some studies reported higher anxiety-like behaviour after LBN on the open field, EPM or DLT test (Dalle Molle et al., 2012; Guadagno et al., 2018; Wang et al., 2011), while others did not find these differences (Molet et al., 2016; Naninck et al., 2015). Here, we observed significantly lower bodyweights on PND9 and PND15 in ELS exposed mice in comparison to controls, which is in line with previous literature (van der Kooij et al., 2015; Moussaoui et al., 2016). Body weights normalized at weaning. Furthermore, we expected to find an effect of ELS on fear. However, we observed minimal effects of ELS on fear generalization measured by freezing, as we found that fear generalization in ELS animals was significantly lower than 1, whereas this was not the case for CTRL animals. In addition, although overall we did not find an effect of treatment on both the EPM and DLT, we did observe a reduction in anxiety-like behaviour in female ELS mice in comparison to female CRTL mice. As such, one could speculate that these effects of ELS reflect stress-inoculation, in which intermittent exposure to a mild stressor improves stress coping in later life; a proven effective approach to reduce state anxiety in humans (Saunders et al., 1996). Given that increased stressor severity (foot shock intensity) leads to fear generalization (Baldi et al., 2004), one could speculate that stress-inoculation induced reductions in experienced stress levels during DAFC may facilitate more discriminative fear learning. This finding contrasts prior reports on contextual fear generalization due to ELS (Elliott & Richardson, 2019). However, cued and contextual fear generalization are supported by different neural circuitries during fear learning, consolidation, and recall, which can potentially explain these discrepancies. The bed nucleus of the stria terminalis (BNST), a major output pathway of the amygdala, is known to be sexually dimorphic (Lebow et al., 2016), and has been shown to be involved in

contextual fear recall in males, but not females (Urien & Bauer, 2022). Yet, during auditory fear recall to a CS- in the same experiment, the BNST was similarly recruited in both sexes. Intriguingly, the BNST has also been associated with ELS, anxiety and fear generalization (Hu et al., 2020; Duvarci et al., 2009). To further explore this potential role of the BNST in ELS, fear learning, and fear generalization, we will at a later stage immunohistochemically analyse (amygdala and) BNST activity in the brains of all animals, as labelled by 4-OHT administration during DAFC. It would be of particular interest to compare BNST activity in ELS females to both control females and ELS males to investigate potentially sexually dimorphic stress inoculation effects.

Finally, oestrous cycle phase seemed to modulate cue fear generalization. Although this effect only reached trend-level significance, speculating about the potential underpinnings of this effect may prove useful to set up future experiments that can elucidate the influence of the oestrous cycle on fear learning and discrimination in females. Our data suggests that females conditioned in the diestrous-metestrous phase store their fear memory in a more specific manner than females conditioned in the (pro)oestrous phase. This is in line with expectations, as decreased levels of ovarian steroids in the diestrous and metestrous phase have been shown to facilitate cued fear discrimination in rats (Trask et al., 2020). Additionally, oestrogen has been proven to contribute to contextual fear generalization (Lynch et al., 2013). Yet, progesterone has a role in counteracting the effect of oestrogen (Hiroi & Neumaier, 2006). As such, the exact moment within a phase of the oestrous cycle may very well determine the dominant hormonal effect since different hormone levels rise and fall at slightly distinct moments in the cycle (Scharfman & NacLusky, 2006). As such, future dedicated studies should carefully monitor the oestrous cycle in females and dissociate prestrous and estrous phases. Also, given the fluctuating hormone levels, the effects of the oestrous cycle on readouts may be different on training days than testing days, further confounding data interpretation. In conclusion, despite the difficulties with accurately capturing the cycle phase of female mice within this study, our observations suggest a possible influence of the oestrous cycle in facilitating cued fear generalization.

Some limitations to this work should be mentioned as well. Firstly, all experimental groups displayed high levels of freezing in response to the CS-, indicating high fear generalization in the control condition already. It is worth investigating whether splitting the DAFC training

session into one CS- session and one CS+ session leads to better tone discrimination in CTRL animals (as observed before (Rescorla, 1976)) as this would enhance sensitivity for the detection of fear generalization. Secondly, especially when investigating sex differences caused by ELS on fear and anxiety-behaviour, implementing different types of behavioural tests that both capture male and female natural anxiety-related behavioural tendencies could increase the scientific validity and value of the research. Most rodent anxiety tests have been heavily standardized to males, although it has been shown that males and females implement different strategic mechanisms to potentially threatening stimuli (Gruene et al., 2015; Roman & Arborelius, 2009). Although testing anxiety-like behaviour related to novelty seeking and locomotion is evolutionary more relevant for males than females (Palanza, 2001), the validity of anxiety research would benefit from the inclusion of more social anxiety-based tests, proven more sensitive to capture anxiety-like behaviour in females (Genn et al., 2003; Johnston & File, 1991; Stack et al., 2010). Lastly, studies dedicated to unravelling the effects of the oestrous cycle on fear generalization and anxiety, must incorporate multiple cycle measurements, or even implement near continuous measuring. This will facilitate accurate data interpretation despite the complex hormonal effects that the cycle exerts on behaviour.

In conclusion, ELS did not significantly affect cued fear generalization, although relative freezing in ELS animals was significantly different from 1 and relative freezing in CTRL animals was not. However, ELS had a strong sex-dependent effect on explorative behaviour in the EPM, wherein ELS females entered open arms more quickly and frequently than control females, a phenomenon that might be explained by stress inoculation. Moreover, we observed strong sex differences in context-induced and CS-induced freezing, in which females froze consistently more than males, possibly indicating that females process fear conditioning differently than males which ultimately leads to more fear expression. Lastly, our findings suggest that females in the diestrous-metestrous phase may show better cued fear discrimination, but future dedicated studies should further investigate this association.

Supplementary materials

Handling session	Procedures
1	Mice were tail grabbed and placed on the experimenter's lab coat sleeve. Animals were allowed to explore the experimenter's lower arm for 2 minutes, while the researcher held the tail without exerting force or limiting freedom of movement. After 2 minutes, the mice were placed back in their cages.
2	Same as handling day 1
3	Mice were grabbed by cupping and elevated approximately 50 cm above the cage while sitting on the open hands of the researcher without restraint. Animals were allowed to explore for 2 minutes and were not held by their tails. After 2 minutes, the mice were placed back in their cages.
4	Same as handling day 3
5	Same as handling day 3 + weighing

Table S1: Handling schedule of the mice.

5

GENERAL DISCUSSION

Anxiety- and stress-related disorders constitute an enormous economic and societal impact on the worldwide population, and both their prevention and treatment is still subeffective. As such, the identification of the mechanistic underpinnings of these disorders remains crucial. In this thesis, I set out to contribute to the understanding of fear and anxiety symptoms by studying the role of the extended amygdala, a key player in susceptibility to developing stress-related symptomatology. To study its intricacies, mice were utilized, in which one can study its relationship with fear and anxiety in a more thorough and controlled way compared to what is possible in humans using (f)MRI. I have tested several behavioural paradigms known to modulate susceptibility to stress-related symptomatology and used a variety of techniques to investigate the role of the extended amygdala in the corresponding behaviours. First, I have used an established mouse model for posttraumatic stress disorder (PTSD) that allowed for the differentiation between trauma susceptible and resilient mice and the investigation of the role of bed nucleus stria terminalis (BNST) neuronal activity in mediating these differences. Then, I set up a differential auditory fear conditioning (DAFC) paradigm in order to modulate fear generalization behaviour in a controlled setting while investigating extended amygdala activity and connectivity, as well as manipulating this connectivity to investigate its causal contribution. Lastly, I have paired this paradigm with an early life stress (ELS) manipulation in order to identify potential mechanisms by which early life adversities form a risk factor for maladaptive fear generalization and anxiety-like behaviour later in life.

In **chapter 2**, my objective was to investigate the contribution of neuronal activity in the anterior BNST (aBNST) to the development of PTSD-like symptoms after trauma exposure in a longitudinal fashion. Anxiety-like behaviour and aBNST activity prior to trauma exposure appeared not predictive of susceptibility to or resilience against later PTSD-like symptoms. However, susceptible mice exhibited lower levels of aBNST activity in the period surrounding the trauma exposure. I also observed differential correlations between amygdala subregion activity and aBNST activity across phenotypic groups, implicating abnormal peri-trauma amygdala-aBNST functional connectivity in trauma susceptibility. No differences in aBNST activity were observed under basal conditions after trauma exposure. In terms of fear expression in response to contexts triggering fear memory recall, susceptible mice displayed faster declines in their fear responses (freezing rates) when exposed to a novel context similar to previously aversive contexts compared to resilient mice. There were no behavioural

differences between the groups during exposure to the actual trauma and trigger contexts, and no differences in aBNST activity were observed in response to context (re)-exposure. These findings collectively suggest that the aberrant signalling in the aBNST during the processing of trauma contributes to the emergence of PTSD-like symptomatology at a later stage.

In **chapter 3**, I aimed to investigate the contribution of the amygdala-BNST circuitry to generalized fear memory and subsequent anxiety-like behaviour. My approach involved the mapping of extended amygdala subregion recruitment and connectivity during fear learning (DAFC), characterizing the main connection of interest in the circuitry, and manipulating this connection to determine its contribution to a behavioural phenotype. I set out to modulate fear generalization and anxiety-like phenotypes by conditioning mice using different foot shock intensities. Exposure to foot shocks increased fear and anxiety-like behaviour, as well as decreased later fear recall aBNST activity levels, although exposure to foot shocks per se was not related to differences in amygdala or aBNST activity during fear acquisition. Modulating foot shock intensity appeared only successful in modulating the extend of the fear response, rather than its specificity, without exerting differential effects on anxiety-related behaviours. Further, increased foot shock intensity was related to higher activity levels in both the amygdala and aBNST during fear acquisition. In the next experiments, I confirmed the recruitment of direct projections from the basolateral amygdala (BLA) to the anterolateral BNST (BNSTAL) projections during the acquisition of fear memory and demonstrated that the activation of this connection during DAFC contributed to a reduction of fear responses both upon initial fear learning and later recall upon cue re-exposure. No effects of activation were observed for the acute expression of anxiety-like behaviour. In summary, I have identified projection neurons from the BLA to the BNSTAL as a prominent regulator of cued fear conditioning. These results reveal novel connections within the extended amygdala that are crucial for both acquiring and expressing fear responses to specific cues.

In **chapter 4**, I examined the impact of ELS, induced by limited bedding and nesting (LBN), on later life fear generalization and subsequent anxiety-like behaviour. Surprisingly, my results showed that ELS induced by LBN did not affect fear generalization, but decreased later anxiety-like behaviour in female offspring specifically. ELS-exposed females

demonstrated an increased frequency and reduced latency of entering the open arms of the elevated plus maze (EPM) compared to control females. Moreover, I observed significant differences in freezing behaviour upon fear memory recall between sexes irrespective of their early life condition. Moreover, the data suggested an effect of the oestrous cycle on fear discrimination in females. It appeared that female mice in the (pro)oestrous stage during fear learning showed a greater generalization of fear than mice that were in the dioestrous-metoestrous phase during fear learning. These differences only reached trend-level significance, as the study was not primarily designed to explore the effects of oestrous cycle, making the comparisons underpowered. In summary, my findings indicate that ELS exposure does not have a significant impact on the generalization of fear responses to tones. However, it does seem make females more resilient to later anxiety-like behaviours. Additionally, the phase of the oestrous cycle appears to influence fear generalization, underscoring the importance of conducting more specific and in-depth research in this area.

The Extended Amygdala in Trauma Susceptibility vs Resiliency: A Working Model

I have started this thesis with introducing the concept of inter-individual differences and how investigating these differences might generate important insight into biological mechanisms contributing to resilience and health. As discussed in **chapter 2**, although many individuals experience trauma during their lifetime, only a small portion of them will actually develop posttraumatic stress disorder (PTSD). The extended amygdala was proposed to be a key factor in this susceptibility to developing PTSD, with reduced amygdala volume being implicated with PTSD susceptibility (Rogers et al., 2009; Woon & Hedges, 2008). Conversely, increased amygdala volume has been linked to improved cue discrimination (Winkelmann et al., 2016), arguing for a potential role for impaired discriminative fear in pathology. Previously, it was found that exaggerated activity in specifically the BLA peri-trauma predicted susceptibility to later PTSD-like symptoms in mice (Dirven et al., 2022). This is not surprising, given the critical role of the BLA in the acquisition of fear conditioning (Davis et al., 1997; Davis et al., 2010; Fanselow, 1994; Hitchcock & Davis, 1986) and in strengthening the consolidation of fear memories (Huff et al., 2013). In **chapter 2**, we intended to extend to these findings by investigating the role of the aBNST in susceptibility to the long-term

behavioural consequences of trauma exposure. The BNST seems to be more involved in the regulation of sustained anxiety and threat anticipation in comparison to the core amygdala regions that seem to be more relevant in responding to immediate threat. As PTSD is characterized by an increased readiness for potential threat and PTSD-symptomatology has been shown to correlate with BNST activity (Somerville, Whalen & Kelley, 2010; Awashti et al., 2020), we postulated that the BNST might be affected. Contrary to expectations, I observed that increased BNST activity peri-trauma was associated with resilience to developing PTSD-like symptoms in mice. Important to note is that our activity measurements only allowed the assessment of activated glutamatergic neurons, as the immediate early gene marker used for this study (i.e. *Arc*) is hardly expressed in GABAergic neurons (Bramham et al., 2008). Subsequent correlational analyses to assess both intra- and inter-subregion functional connectivity in the extended amygdala peri-trauma, further added to the evidence that these subregions might be communicating differently as a function of trauma susceptibility. Most apparent was the observation of an increased correlation between activity in the BLA and the anxiogenic subregions of the aBNST (i.e. BNSTOV, BNSTAM, BNSTMV) in the resilient group compared to the susceptible group. By integrating the correlational relationships between glutamatergic amygdala and aBNST activity, combined with current literature investigating extended amygdala connectivity, I have designed a theoretical model that might explain how the amygdala-BNST circuit works during trauma processing in animals susceptible vs resilient to developing PTSD-like symptoms (Fig. 1).

Resilience.

In resilient mice, strong correlations in glutamatergic activity across the amygdala subregions were observed, as well as the strong correlations of glutamatergic activity across the subregions of the aBNST, in the absence of an association between activity in the lateral amygdala (LA) and the aBNST subregions. As such, I speculate that the low anxiety-like profile in resilient mice might relate to the activation of LA glutamatergic neurons that project to the BLA, which in turn modulates the aBNST through both the direct and indirect pathway. The indirect pathway includes a glutamatergic projection to the centromedial amygdala (CeM), where the activation of somatostatin neuron population could reduce the anxiogenic influence of the centrolateral amygdala (CeL) on the oval nucleus of the BNST (BNSTOV) (Ciocchi et al., 2010; Ahrens et al., 2018; Haubensak et al., 2010). Furthermore,

the BLA projects directly to the anterodorsal BNST (anterolateral BNST (BNSTAL): Krettek and Price, 1978a; Dong et al., 2001a. anteromedial BNST (BNSTAM): Kim et al., 2013), and this direct, glutamatergic BLA-BNSTAL pathway increases anxiety (Dong et al., 2001; Krettek & Price, 1978a), presumably via the strong BNSTAL inhibitory projections towards the BNSTAM (Turresson et al., 2013). Lastly, I speculate that the BNSTAL utilizes its GABAergic BNSTAL-CeA pathway to further reduces the anxiogenic influence of the CeA on the BNST (Sun and Cassell, 1993; Dong et al., 2001b) in resilient mice (Fig. 1).

Susceptibility.

Analysis of the correlations in glutamatergic activity peri-trauma in the susceptible mice revealed a strong positive correlation between activity in the LA and the aBNST, whereas intra-amygdala correlations in activity (that were eminent in resilient mice) were less prominent. The LA is known to mediate the associations between conditioned (CS) and unconditioned (US) stimuli during fear conditioning (Ghosh & Chatterji, 2015; Maren & Quirk, 2004c), and affect the oval (BNSTOV) and BNSTAL indirectly via its projections to the CeA. In contrast to the other amygdala subnuclei, a substantial part of the CeA neurons is GABAergic (Ye & Veinante, 2019) and the CeA-aBNST pathway is mostly modulated by GABAergic projection neurons that regulate the consolidation of contextual fear memories (Pitts et al., 2009) and anxiety-like behaviour (Moreira et al., 2007). Most prominent projections from the CeA to the aBNST (e.g. the BNSTAL) originate from the CeL (Weller and Smith, 1982; Sun et al., 1991), with less contributions from the CeM (Sun and Cassell, 1993; Bienkowski and Rinaman, 2013). Yet, the CeM directly modulates CeL output (Ye & Veinante, 2019). I propose that in susceptible mice, the BNSTOV GABAergic population receives strong inhibitory GABAergic input from the CeL (e.g. CeL SOM neurons (Ahrens et al., 2018)) during trauma processing, leading to an eventual disinhibition of the glutamatergic population residing in the BNSTOV (Ahrens et al., 2018; Ye & Veinante, 2019). This would increase the firing of the BNSTOV and its modulatory output to the other anxiogenic subregions of the BNST, such as the anteromedial medial ventral (BNSTMV) and lateral ventral BNST (BNSTLV) (Dong et al., 2001; Asok et al., 2018). I further hypothesize that this anxiogenic effect is additionally supported by increased recruitment of the direct GABAergic CeL-BNSTAL pathway (Weller & Smith, 1982; Sun et al., 1991), that suppresses the inhibitory influence the BNSTAL exerts on the BNSTAM (Turresson et al., 2013). The exact contribution of the BNSTV on fear learning and consolidation has been relatively understudied, although

evidence for an anxiogenic role of glutamatergic ventral BNST efferents is accumulating (Jennings et al., 2013; Crane et al., 2003; Spencer et al., 2005; Choi et al., 2007; Dumont & Williams, 2004).

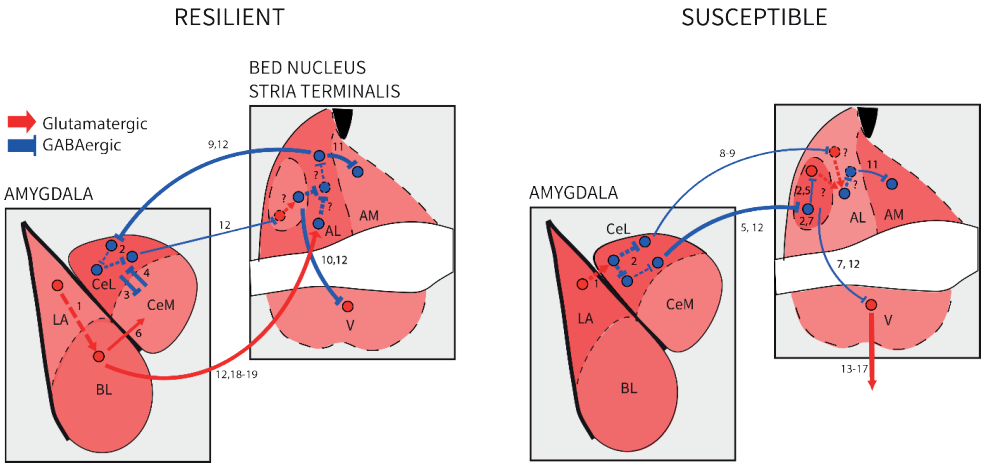

Figure 1. Working model of the extended amygdala in susceptible and resilient mice. This model is based on the correlations in glutamatergic activity as observed in my study, as well as prior literature on both the structural connections across the extended amygdala subregions and their contribution to fear and anxiety-like behaviour. 1: John et al., 2013. 2: Ye & Veinante, 2019. 3: Haubensak et al., 2010. 4: Ciocchi et al., 2010. 5: Ahrens et al., 2018. 6: Paré, Smith & Paré, 1995. 7: Asok et al., 2018. 8: Weller & Smith, 1982. 9: Sun & Cassel, 1993. 10: Dabrowska et al., 2016. 11: Turresson et al., 2013. 12: Dong et al., 2001. 13: Jennings et al., 2013. 14: Crane et al., 2003. 15: Spencer et al., 2005. 16: Choi et al., 2007. 17: Dumont & Williams, 2004. 18: Krettek & Price, 1978. 19: Chapter 3, experiment 4.

The Extended Amygdala in Fear Generalization

Besides exploring the extended amygdala's potential role in both trauma resiliency and susceptibility, it is important to consider the relevance of the extended amygdala in mediating a hallmark symptom of the disorder: fear overgeneralization. Fear overgeneralization is not unique to PTSD, as it is a feature in many anxiety- and stress-related disorders, such as generalized anxiety disorder, phobias, panic disorders (Cooper et al., 2022; Dymond et al., 2015; Fraunfelter, Gerdes & Alpers, 2022; Lis et al., 2020; Morey at al., 2015). In this context, understanding how the extended amygdala contributes to the pathophysiology of fear overgeneralization may further provide valuable insights into the maladaptive development and maintenance of anxiety- and stress-related pathology.

In **chapter 3**, I report increased amygdala and aBNST overall activity during fear acquisition following high vs. low shock intensity. Further, in line with current literature (Krettek and Price, 1978a; Dong et al., 2001a), I confirmed the activation of direct BLA-BNSTAL projections during fear acquisition, and showed that activation of these projection neurons suppressed the acquisition of a fear response and later fear memory recall. These results are in accordance with our previously postulated working model of the protective effect of recruitment of direct BLA-BNSTAL projections peri-trauma in mice resilient to developing PTSD-like symptomatology as a consequence of trauma exposure. The fear suppressing effect is most likely caused by the strong inhibitory influence the BNSTAL exerts on its adjacent aBNST regions involved in producing anxiogenic behaviour (Dedic et al., 2018; Gungor & Paré, 2014; Dunn et al., 1987), as well as inhibiting the CeL (Gungor et al., 2016; Ye & Veinante, 2019).

My current model of the extended amygdala's role in fear acquisition and consolidation is based on both my own results and literature and provides a framework for further investigation. However, it is important to note that this model is primarily based on the results of **chapter 2** and previous work in my lab (Dirven et al., 2022), which solely investigated glutamatergic neurons in the amygdala and BNST. **Chapter 3** contributed to this model by showing a causal relationship between the BLA-BNSTAL pathway during fear memory acquisition and consolidation and later fear recall and anxiety-like behaviour. However, it remains unclear what the exact underpinnings are of the different neuronal populations residing in the amygdala and aBNST and how they connect with one another. Currently, the neuronal properties (i.e., their glutamatergic or GABAergic nature) of the amygdala and aBNST during fear acquisition and consolidation of **chapter 3** are further investigated in our lab in order to provide more detail and improve our current model.

Vulnerability Factors

In-depth analysis of inter-individual variations in stress susceptibility could provide valuable insight into the underlying biological mechanisms that contribute to resilience to anxiety and stress and promote well-being in the presence of challenging circumstances (Uher and Zwicker, 2017). Exploring multiple risk factors that contribute to the onset of such disorders would be necessary to fully comprehend the complexity of these conditions. In **chapter 2**, I

aimed to investigate the biological underpinnings of inter-individual differences, by categorizing mice as resilient vs susceptible to developing PTSD-like behaviour based on their PTSD-like symptom score. By using the PTSD-mouse model, I was able to assess pre-trauma risk factors indicative of susceptibility to PTSD-like behaviour following trauma exposure.

Trait Anxiety.

One risk factor, trait anxiety, is defined by a relatively stable tendency to experience anxiety throughout time and has been linked to numerous anxiety- and stress-related disorders. High trait anxiety has been found predictive of susceptibility to PTSD-symptom severity (Christiansen & Elklit, 2008; Jaksic et al., 2012) as well as anxiety disorders (Mundy et al., 2015; Butler & Rapee, 1991). In **chapter 2** I investigated whether differences in pre-trauma trait anxiety predicted trauma susceptibility by including behavioural tests targeting anxiety, i.e. the EPM and OF. Earlier research in rodents has shown that high trait anxiety is linked to anxiogenic behaviour on these tests (Muigg et al., 2009; Liebsch et al., 1998b). However, in our study no differences were observed in pre-trauma anxiety behaviour in these behavioural assays between resilient and susceptible to PTSD-like behaviour, thereby implicating that differential trait anxiety pre-trauma was not a key determinant factor in this particular setting. This observation is in line with prior reports showing that in animal work specifically, pre-existing susceptibility solely becomes apparent as elevated anxiety-like behaviour following exposure to a stressor (i.e. under challenging conditions), not at baseline (Nalloor, Bunting & Vazdarjanva, 2011). Related to this absence of an association with pre-trauma anxiety-like behaviour, prior research observed no associations between baseline BLA activity or in the BLA response to a novel context (Nalloor, Bunting & Vazdarjanva, 2011) and later susceptibility to stress (Huang et al., 2021), much similar to our findings in the aBNST.

Early Life Stress.

ELS is a risk factor for developing anxiety- and stress-related disorders like panic disorder, generalized anxiety disorder, and PTSD (Famularo et al., 1992; Stein et al., 1996; Kessler al., 2010; Duits et al., 2015). It is believed that ELS interacts with other factors like genetics and later life adversities to affect stress responsiveness and vulnerability to developing psychiatric disorders. In **chapter 4**, I hypothesized that ELS may increase risk on psychopathology by promoting fear (over)generalization. This hypothesis was corroborated

by the cumulative stress or multiple hit hypothesis, stating that an increase in stressful events during early stages of life increases vulnerability upon new stress exposure later in life (McEwen, 2003). The idea of this model is founded in the principle that the cumulative impact of adverse environments can contribute to an elevated allostatic load, ultimately raising the likelihood of developing mental health conditions. I investigated whether ELS might exert risk by modulating fear generalization behaviour, but obtained no evidence for this. ELS offspring and offspring raised under standard conditions displayed similar levels of fear responding to both the stimulus signalling danger and that signalling safety, and did not differ in their proportion of these responses. Moreover, I did not observe any anxiogenic effects of ELS on later behaviour in anxiety paradigms. According to the cumulative or multiple hit hypothesis, ELS should have increased stress responsiveness, e.g. by inducing increased levels of freezing and anxiety-like behaviour, which is not supported by my results.

More recently, different hypotheses have been formulated to explain the effect of ELS on risk on later psychopathology. The match/mismatch hypothesis states that the early-life environment plays a crucial role in shaping or priming an individual's coping mechanisms, equipping them to better handle similarly adverse environments later in life (Nederhof & Schmidt, 2012; Belsky and Pluess, 2009; Ricon et al., 2012; Schmidt, 2011). This conceptual framework can also explain instances where stress exposed individuals display similar behaviour as individuals that were not exposed to stress, when encountered with an environment similar to the environment they grew up with (Champagne et al., 2008; Oomen et al., 2010). Similarly, the stress-inoculation theory proposes that intermittent exposure to a mild stressor improves later stress coping behaviour (Saunders et al., 1996). In my study, one could speculate that stress inoculation decreases the level of stress experienced during DAFC, which would facilitate more discriminative fear learning. Both these models are plausible from an ethological and evolutionary standpoint, given that individuals who grow up in stressful environments may encounter similar situations in adulthood and develop effective coping strategies. An increasing amount of research is supporting the match/mismatch hypothesis, which suggests that rodents are less impacted by negative juvenile and adult conditions as a result of ELS (Buwalda et al., 2013; Daskalakis et al., 2012; Raftogianni et al., 2012; Ricon et al., 2012; Zalosnik et al., 2014). In humans, there is also evidence to suggest that lifetime adversity may alleviate the response to acute stress in adulthood (Elzinga et al., 2008). In **chapter 4**, I observed that ELS animals do not show any

differential stress coping response (i.e. their fear recall freezing behaviour) in comparison to control animals, whereas the match/mismatch theory would suggest reduced fear responses (or differential stress coping behaviour in general) in ELS vs. control mice. A possible explanation for the absence of differences between the ELS and control group is that the nature of the DAFC environment did not match particularly well with the early life environment within either group. Limited nesting and bedding and thereby a presumed fragmentation and unpredictability of maternal care for an extended time during early life development, nor the low stress early life environment that was experienced by the control group, might be particularly comparable to a short-lived fear conditioning paradigm later in life.

However, one aspect where we can take into account sensitivity differences towards stress is sex, as it has been shown that males and females are inherently differently affected by stress (Bangasser & Valentino, 2014; Verma, Balhara & Gupta, 2011; Kelly et al., 2008). In **chapter 4**, I did not find an overall effect of ELS on fear and anxiety parameters, but did observe a significant interaction effect between sex and ELS, wherein ELS females showed lower levels of anxiety-like behaviour than control females on the EPM, whereas no such effect was observed in males. This fits both the match/mismatch hypothesis and stress inoculation theory, where a mild ELS may have primed female mice for later anxiety-inducing situations making them less impacted by the novel, generally anxiety-inducing, environment of the EPM. The origin of the increased impact of ELS on female offspring could be explained by the quality of maternal care given by the dam to the female pups, as research has shown that in ELS conditions, female pups received more adverse care than males (Keller, Nowak & Roth, 2019). While I did not measure the quality of the maternal care, it is clear from the results of **chapter 4** that ELS differently affected the female mice in comparison to the males, which resulted in a better adapted behavioural response on the anxiety-inducing test in females. The role of the extended amygdala in mediating these effects is currently under investigation in our lab.

In conclusion, I did not find support for the cumulative or multiple-hit hypothesis since the ELS group was not significantly more affected by later life stress exposure that the control group. My results seem to be more in line with the match/mismatch hypothesis and the stress inoculation theory, although alterations to the study design are necessary in order to

compare the nature of the ELS environment and that with stressors in later life to make further statements about the hypothesis with regards to both sexes. Lastly, in this study I have not linked the effect of ELS on the extended-amygdala yet, as currently the brain material is still being analysed.

Sex differences

Men and women are physiologically and behaviourally differently affected by stressors (Bangasser & Valentino, 2014; Verma, Balhara & Gupta, 2011; Kelly et al., 2008). It is therefore not surprising that the prevalence of anxiety- and stress-related psychiatric disorders differs between men and women. For instance, women are more likely to develop PTSD and are 2-3 times more likely to develop generalized anxiety disorder (GAD) during their lifetime than men (Breslau et al., 1997; Brewin, Andrews & Valentine, 2000; Mclean et al., 2011; Beesdo et al., 2010; Gum et al., 2009). In **chapter 4**, I observed higher levels of freezing behaviour, both in a familiar context and towards the auditory cues in females compared to males, irrespective of ELS history. These differential freezing levels could have several causes. Firstly, it could be that females experience similar stress levels upon context- and cue- re-exposure as males, but express their fear differently (Shanazz et al., 2002; Hawley et al., 2012). Secondly, it could be that the females experience the foot shock administration during DAFC as more intense because of their lower body weight (Beatty et al., 2004). Previous studies have not always consistently shown that freezing levels are different between sexes (Tryon et al., 2012; Day et al., 2020). Other factors, such as differential fear discrimination learning (Day et al., 2020), stronger fear consolidation (Baldi et al., 2004) or divergent fear expression behaviour (Gruene et al., 2015; Russo & Parsons, 2021) might be at play as well. In addition, the female reproductive hormones are thought to play a critical role in mediating these differences in fear responding (Altshuler et al., 1998). Oestrogen modulates stress responses by regulating the expression of the corticotropic releasing factor (CRF) gene (Vamvakopoulos & Chrousos, 1993), which orchestrates the hypothalamic-pituitary-adrenal (HPA) axis and is expressed in multiple brain regions involved in mediating and modulating stress responses and anxiety-like behaviour (Gray & Bingaman, 1996; Dunn & Berridge, 1990; Shekhar et al., 2005). In **chapter 4**, I found a trend-level significance in the effect of oestrus cycle phase and cued fear generalization behaviour, suggesting that females conditioned in the dioestrus-metestrus

phase (characterized by reduced oestrogen levels) store their fear memory in a more specific manner than females in the (pro)oestrus phase. Similarly, Trask et al. (2020) have shown that decreased levels of ovarian steroids in the dioestrus and metetrus phase facilitate cued fear discrimination, and Lynch at al. (2013) found that oestrogen contributes to contextual fear generalization. Whereas I included the assessment of the oestrous cycle phase at a single time point to correct for this potentially confounding factor in my readouts, my findings indicate that the hormonal status of the females deserves further investigation in future dedicated studies.

Limitations and Future Directions

Differential Auditory Fear Conditioning Paradigm.

This thesis was set out to investigate the neuronal substrate of fear generalization by inducing different levels of fear generalization across experimental groups (**chapter 3** and **4**). Moreover, I aimed to investigate whether increased rates of fear generalization related to elevated anxiety-like behaviour and as such had a shared neuronal substrate. Initially, I intended to induce two distinct degrees of fear generalization by modulating the intensity of the foot shocks administered during the DAFC paradigm. Prior literature has indicated that the generalization of fear responses is more pronounced in case of higher stress levels (de Bundel et al., 2016; Duvarci et al., 2009). Despite extensive pilots of training conditions, I did not succeed in creating conditions in which the safety cue (CS-) was indeed interpreted as safe by the mice. Contrary to my study, previous studies utilizing DAFC were successful in creating experimental groups that displayed either cue-discrimination or cue-generalization by modulating the strength of the foot shock (de Bundel et al. 2016; Duvarci et al., 2009). Yet, in none of these studies the CS- was fully considered as safe, with freezing levels always exceeding those of baseline levels. Yet, the other studies succeeded in modulating the degree to which rodents dissociated between the cues. Specifically, I based my experimental settings on a study by de Bundel et al. (2016), in which foot shock intensity was modulated to generate different fear generalization phenotypes. Foot shock intensity in my study did not similarly impact the phenotypical behaviours. Strong shock animals showed similar discrimination levels towards the CS's in comparison to the weak shock group, albeit their overall levels of freezing were higher in comparison. My experiment utilized a 5 kHz and 10 kHz tone, instead of the 2.5 kHz and 7.5 kHz that was employed in de Bundel et al. (2016).

While designing the current DAFC in the preceding pilots, the rationale behind deviating from the auditory cues was based on the possibility of modulating the auditory cues such that equal perceptual generalization was possible. In my initial design, a CS novel (CS*) was included that was presented during the re-exposure session but not during the initial fear conditioning. The CS* would have been different from both the CS+ and CS-, but equal in perceptual difference from the CS+ as the CS-, and would have allowed us to look at the differences in fear generalization within a single adverse episode (being exposed to foot shocks in a setting where the CS- was present) and towards perceptually related, but episodically separated cues (the CS*). However, due to the lack of significant behavioural differences in fear generalization towards the CS- and CS*, the presence of testing order effects during re-exposure when using 3, but not 2, auditory cues, and the substantial increase in mice needed to correct for the extra testing variable, I decided to continue without the CS*. Due to time and resource constraints, I continued the experiment in the present design instead of repiloting the DAFC with dissimilar tone-stimuli.

Due to the logarithmic nature of hearing perception, although the physical difference in frequency as implemented here is the same as in the Bundel et al. (i.e. 5 kHz), the perceptual experience for mice is different due to the location of the two ranges on different parts of the hearing range (Hefner & Hefner, 2007). The region between 5 kHz-10 kHz is located more towards the higher end of the hearing range, compared to the 2.5 kHz-7.5 kHz, and is therefore less sensitive for cue-discrimination. This could suggest that the dissimilarity between perceptual properties of the auditory stimuli might have impacted my results, which should perceptually be different enough to result in the desired effect (Ghirlanda and Enquist, 2003; Vervliet et al., 2011; Jenkins and Harrison, 1960), although fear generalization is not fully explained by perceptual discrimination failure (Guttman & Kalish, 1956; Shepard, 1987). Moreover, noteworthy, even the experimental group receiving strong foot shocks successfully discriminated between the CS+ and CS-, arguing against the fact that they were incapable of perceptually differentiating them.

The effect of the DAFC paradigm further extended into the results from the subset of anxiety tests in **chapter 3** and **chapter 4**. Here, only minor anxiety-like behavioural differences were found between the compared groups within each chapter. Based on previous literature I had expected to see an extension of the effects of the fear conditioning paradigm into

anxiety-like behaviour (Dunsmoor & Paz, 2015; Lissek et al., 2014). Firstly, I only found little effect of shock exposure upon anxiety behaviour. A likely explanation can be found in the practical set-up of the DAFC paradigm. Here, in order to generate a differential fear response towards the CS+ and CS- in at least a subset of mice, mice were conditioned for rather short durations with relatively low shock intensities in a single session. In addition, the period in between the fear conditioning session and the initial anxiety tests was several days to allow for the endogenous fluorescence labelling to become expressed in the brain following the 4-OHT injection before start of the re-exposure test session. The effect of fear conditioning was previously shown to quickly dissipate over time, ranging from one day to a couple of weeks depending on the anxiety test that follows (Korte, de Boer & Bohuis, 1999; Korte & de Boer, 2003). It has therefore been suggested to re-expose the animals to the fearful context shortly before the anxiety test, in order to reinstate the state anxiety that was induced by the fear conditioning paradigm (Korte, de Boer & Bohuis, 1999).

Secondly, I did not find any differences in anxiety behaviour between the weak shock and strong shock group. It is likely that the absence of a differential fear generalization phenotype between the two groups extends towards their anxiety-like behaviour. For future research, it would be beneficial to redesign the current DAFC paradigm in order to modulate more convincingly fear generalization phenotypes. For example, one could consider extending the fear conditioning into longer or multiple sessions of conditioning which would likely contribute to a stronger and potentially less generalized fear memory, and re-expose the mice to the fear conditioning context or cues in order to re-establish their state anxiety shortly before the start of the anxiety tests. Implementing these modifications could lead to more defined fear and anxiety phenotypes overall, which would greatly benefit the research into the extended amygdala and its role in anxiety and stress-related disorders.

The Neuronal Underpinnings of the Extended Amygdala.

In my thesis I used the targeted recombination in activate populations (TRAP) to label neurons depending on the expression of a certain immediate early gene (IEG). Similar IEG-based techniques targeting activated neurons are used quite abundantly in literature (Hoffmann, Smith & Verbalis, 1993; Renier et al., 2016; Ploski et al., 2008; Krukoff, 1999; Brudzynski & Wang, 1996). Although this new technique allowed me to label active neurons during specific time-points without the necessity for the immediate sacrificing of the

animals, there are still some limitations to using this technique. In **chapter 2**, I utilized the ArcTRAP mouse line (Guenthner et al., 2013) in order to investigate neuronal activity pre-, peri-, and post-trauma in the extended amygdala. The line was selected based on its superior labelling sensitivity compared to the FosTRAP line, but *Arc* is primarily expressed in glutamatergic neuronal populations (Bramham et al., 2008). This restricts the scope and impact of my findings, given that the majority of BNST neurons is GABAergic. In **chapter 3**, I utilized a newly developed FosTRAP2 mouse line (Allen et al., 2017), which removed some of the drawbacks of the previous FosTRAP and ArcTRAP models (i.e. superior labelling sensitivity and selectivity) (Guenthner et al., 2013). One major advantage of the FosTRAP2 line, is that it allows for the investigation of both glutamatergic and GABAergic neurons, as both express the IEG *cFos*. However, in our current studies I did not differentiate between these neuronal subpopulations, restricted by the poor labelling quality of several tested antibodies for potentially interesting targets in the BNST. Establishing the neuronal populations involved will further contribute to an improved working model on the extended amygdala circuit in case of susceptibility and resilience.

Besides simply differentiating between glutamatergic and GABAergic populations, it would be beneficial to identify the exact molecular characteristics of the populations residing in the extended amygdala. The BNST contains many cell types (Bota et al., 2012) and mapping the different spatial areas of the BNST with their specific molecular cell types might help clarify how each subregion regulates fear and anxiety behaviour. So far, up to 37 distinct neuronal subtypes have been found in the BNST (Moffitt et al., 2018; Welch et al., 2019). An extensive discussion of all these cell types is beyond the scope of this thesis, but one of the most prominent subtypes are the CRF expressing cells. CRF neurons within the BNST mostly reside in the BNSTOV (Nguyen et al., 2016; Cummings et al., 1983; Morin et al., 1999) and local CRF release is partially determined by CeA activity (Vranjkovic et al., 2017; Daniel & Rainnie, 2016). Optogenetic activation of CRFR2-expressing neurons in the pBNST has been shown to decrease anxiety and impaired fear memory for stressful events, while inhibition has yielded opposite results (Henckens et al., 2017), yet these findings are not always consistent over the whole BNST region (Bruzsik et al., 2021). Further, it was shown that the activation of these cells following trauma exposure reduced susceptibility to PTSD-like behaviour (Henckens et al., 2017). Another prominent cell subtype is the SOM expressing cells, that mostly resides in the BNSTOV (Bruzsik et al., 2021). Optogenetic activation of

BNSTOV SOM+ neurons was found to induce anxiety-like behaviour and enhance fear memory consolidation, while their suppression reduced place avoidance and increased exploration behaviour (Asok et al., 2018). Given these apparent opposing roles for different cell types within the BNST in modulating stress and anxiety behaviour, understanding their role in fear and anxiety in **chapter 2** and **chapter 3** would greatly benefit our understanding of the exact underpinnings of the contribution of the extended amygdala to anxiety- and stress-related disorders. Although I have utilized a marker for *PKC-delta* and attempted multiple cell markers, e.g. SOM and CRF, the quality of the antibody stainings within the aBNST was insufficient to warrant further analyses. For future experiments, use of Cre-expressing mouse lines in these specific cell populations could be utilized in combination with our behavioural paradigms in order to investigate the role of each of these populations in mediating anxiety- and stress-related behaviour.

Differentiation between the Sexes.

Despite research indicating the physiological and behavioural differences between men and women in stress responsiveness as well as its consequences (Bangasser & Valentino, 2014; Verma, Balhara & Gupta, 2011; Kelly et al., 2008), I used male mice only in the majority of experiments (**chapter 2** and **3**). The original PTSD-paradigm was validated only in male mice (Lebow et al., 2012), and proven unsuccessful to induce a similar phenotype in females (personal correspondence, Alon Chen lab). This might relate to the fact that female mice display naturally different coping mechanisms in response to stress in comparison to males (Genn et al., 2003; Gruene et al., 2015; Stack et al., 2010; Johnston & File, 1991). Even despite of these behavioural differences, the sexually dimorphic nature of the BNST (Lebow et al., 2016) would have necessitated the additional inclusion of female mice, increasing the sample size by two. With the current ArcTRAP and TRAP2 model, it was initially uncertain whether the use of 4-OHT would affect males and females differently, given that 4-OHT is an oestrogen receptor antagonist (Sakamoto et al., 2002). In preparation of **chapter 4**, my pilot studies indicated that 4-OHT injections did not differently induce neuronal labelling or affect behaviour between males and females. This was further supported by literature suggesting that tamoxifen has no long-lasting effects in the brain (Chucair-Elliott et al., 2019), nor on behaviour (Rotheneichner et al., 2017) in both sexes. This has led us to include both females and males in our study investigating the effect of ELS on fear and anxiety behaviour. Here, I did find some behavioural differences. Specifically, I found that females

showed higher levels of freezing during fear memory recall compared to males. However, these differences were not reflected in differential anxiety levels. The battery of anxiety-related tests that were used in these studies were heavily standardized to male rodents that display lack of locomotion in response to stressors, while females have been reported to display more active coping behaviour in order to escape from adversity (Gruene et al., 2015). In order to improve our insight into the role of the extended amygdala in fear and anxiety-like behaviour in rodents, it would be worthwhile to extend my current model to females in order to better represent the stress coping mechanisms used by female rodents. For this it would for instance be interesting to include more social anxiety-based tests as these were proven more sensitive to capture anxiety-like behaviour in females (Genn et al., 2003; Johnston & File, 1991; Stack et al., 2010).

Concluding remarks

In this thesis, I set out to characterize the role of the extended amygdala circuitry in fear generalization and anxiety-like behaviour. To this end, I investigated aBNST neuronal activity pre-, peri-, and post-trauma in trauma susceptible vs. resilient mice and found support for a protective role for glutamatergic activity within the aBNST during trauma processing against the development of later PTSD-like symptomatology. Further, I mapped extended amygdala subregion recruitment during fear acquisition, characterized the recruitment of a specific BLA-BNST connection during this process, and demonstrated that activation of this connection during fear learning results in reduced fear expression upon acquisition and recall. Lastly, I explored the effect of ELS as a risk factor for developing maladaptive fear generalization and anxiety-like behaviour. I did not observe differences in fear generalization, yet found an anxiolytic effect of ELS in females. Analyses of the extended-amygdala following ELS vs. controls should provide us further insight in the mechanistic underpinnings of these behavioural effects. As an improved understanding of the involvement of the extended amygdala circuitry seems crucial to advance the prevention and treatment strategies of anxiety- and stress-related psychopathology, future research should build on these findings.

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485.


MUCH·LOVE

Appendices

NEDERLANDSE SAMENVATTING

ACKNOWLEDGEMENTS

CURRICULUM VITAE

RESEARCH DATA MANAGEMENT

DONDERS GRADUATE SCHOOL FOR COGNITIVE NEUROSCIENCE

Nederlandse samenvatting

Het is belangrijk om situaties die potentieel schadelijk of gevaarlijk zijn te herkennen zodat je veilige beslissingen kan maken. Echter, wanneer de angst om dergelijke situaties tegen te komen buitensporig wordt en een negatieve invloed heeft op je dagelijks functioneren, dan spreken we van disfunctioneel en onaangepast angstgedrag. Het is deze mate van onaangepaste angst en bezorgdheid die kunnen leiden tot de ontwikkeling van angst- en stress-gerelateerde stoornissen, zoals bijvoorbeeld post-traumatische stressstoornis (PTSS). Kenmerkend voor dit soort stoornissen is de generalisatie van de angst naar veilige omstandigheden en het emotioneel lijden dat daarmee gepaard gaat, wat kan leiden tot beperkingen in het dagelijks leven evenals een verminderde kwaliteit van leven. Twee hersengebieden die een cruciale rol lijken te spelen bij de generalisatie van angst zijn de amygdala en de bed nucleus stria terminalis (BNST). Samen vormen ze een belangrijk hersencircuit dat betrokken is bij zowel het aanleren van angst alsmede de emotionele reactie die daarop volgt. Het doel van dit proefschrift was om de rol van het amygdala-BNST circuit bij angst generalisatie en angst- en stress-gerelateerde stoornissen te onderzoeken.

Om dit te onderzoeken heb ik gebruik gemaakt van angstconditionering in muizen, door middel van het toedienen van elektrische voetschokken die dienden te worden geassocieerd met een specifieke context (box; hoofdstuk 2) of bepaald geluid (hoofdstuk 3 en 4). Vervolgens heb ik de mate van angst ten gevolge van deze conditionering getest met verschillende gedragstesten. Om dit angstgedrag vervolgens te koppelen aan het amygdala-BNST circuit, heb ik de activiteit in deze gebieden op verschillende momenten in mijn gedragsexperimenten gemeten en na afloop van het experiment bekeken en gekwantificeerd.

In hoofdstuk 2 heb ik de associatie onderzocht tussen neuronale activiteit in de BNST voor, tijdens en na een traumatische ervaring en hoe dit gelinkt is aan de ontwikkeling van PTSS-achtige symptomen. Muizen werden onderworpen aan onvoorspelbare en oncontroleerbare voetschokken, gevolgd door een set van gedragstesten waarmee muizen als ongevoelig of vatbaar voor PTSS-achtige gedraging gecategoriseerd konden worden. Drie weken na het trauma werden de muizen opnieuw blootgesteld aan een nieuwe context die leek op de context waarin ze schokken hadden gekregen, of een context die verband

hield met de traumatische ervaring, en hun angstreacties (d.w.z. de mate waarin ze 'bevroren' tijdens de blootstelling aan de context) werden beoordeeld als een indicatie voor het ophalen van de herinnering aan het trauma. Daarnaast werd angstig gedrag vóór het trauma gemeten als mogelijke risicofactor voor de ontwikkeling van PTSS-achtige symptomen. Angstig gedrag vóór het trauma en de bijbehorende BNST activiteit bleken geen voorspellende waarde te hebben voor latere gevoeligheid voor het trauma. Echter, tijdens het trauma vertoonden vatbare muizen lagere BNST activiteit in vergelijking met ongevoelige muizen, evenals afwijkende verbanden tussen amygdala en BNST neuronale activiteit wat kan wijzen op een verstoorde functionele connectiviteit tussen de twee hersenstructuren. Ik nam geen verschillen in BNST activiteit na het trauma waar tussen de vatbare en ongevoelige muizen. Ten slotte werden er geen groepsverschillen waargenomen in angstreacties bij blootstelling aan de trauma-gerelateerde contexten, noch werden er verschillen ontdekt in BNST-activiteit bij (her)blootstelling aan de context. Toch vertoonden vatbare muizen snellere afnames in hun bevriezingspercentages in de loop van de tijd tijdens blootstelling aan de nieuwe context in vergelijking met ongevoelige muizen. Samengevat suggereren deze resultaten een rol voor verstoorde BNST-signalering en communicatie met de amygdala tijdens de verwerking van het trauma in de latere ontwikkeling van PTSS-achtige symptomen.

In hoofdstuk 3 heb ik de rol van het amygdala-BNST circuit in angst (generalisatie) onderzocht. In een reeks experimenten heb ik de rekrutering van de amygdala en BNST in kaart gebracht, evenals het verband tussen gedrag en neuronale activiteit. Hierbij heb ik gebruik gemaakt van een differentieel auditief angst conditionering (differential auditory fear conditioning, DAFC) paradigma, waarbij ik gebruik heb gemaakt van twee auditieve stimuli, waarvan er één gekoppeld was aan de toediening van een elektrische voetschok en de andere stimulus niet. Verschillende schokintensiteiten werden gebruikt om de mate van angstgeneralisatie naar de stimuli te beïnvloeden, en dit gedrag te koppelen aan amygdala-BNST neuronale activiteit. Vervolgens heb ik een subpopulatie van neuronen in een subgebied van de amygdala, de basolaterale amygdala (BLA), geïdentificeerd die projecteert naar de BNST en die gerekruteerd wordt tijdens het aanleren van angst. Vervolgens heb ik de activiteit van deze projectieneuronen gemanipuleerd om hun bijdrage aan angst (generalisatie) causaal te testen. Hier bleek dat een hogere schokintensiteit de activiteit van de amygdala en BNST verhoogde tijdens het aanleren van angst, terwijl

algemene blootstelling aan voetschokken alleen BNST activiteit verhoogde tijdens blootstelling aan angstige situaties. BLA activiteit tijdens het aanleren van angst voorspelde de mate van angstgeneralisatie, terwijl BLA activiteit tijdens angstopwekkende situaties negatief leek te correleren met BNST activiteit. Het activeren van BLA-BNST projecties tijdens het aanleren van angst verminderde de uiting van de angst. Het leek echter geen invloed te hebben op de generalisatie van angst. Samengevat onthullen deze resultaten een nieuw amygdala-BNST circuit dat essentieel is voor het aanleren en uiten van angst.

In hoofdstuk 4 heb ik gekeken naar de invloed van stress vroeg tijdens in het leven (early life stress, ELS) als risicofactor op later angstgedrag. In dit onderzoek kregen moedermuizen slechts beperkt nestmateriaal in de kooi vlak na de geboorte van hun jongen; een manipulatie waarvan eerder aangetoond is dat deze leidt tot onvoorspelbare en inconsistente zorg van de moeder voor haar jongen en daardoor stress in het nageslacht. Vervolgens werd het nageslacht op volwassen leeftijd blootgesteld aan het DAFC paradigma. Daarna werd angstgeneralisatie en angstgerelateerd gedrag beoordeeld door middel van verscheidene gedragstesten. Het lichaamsgewicht van het nageslacht werd negatief beïnvloed tijdens en kort na ELS, maar normaliseerde daarna. Verdere resultaten toonden geen effecten van ELS op angstgeneralisatie of angstherinnering in het algemeen, maar het vrouwelijk nageslacht vertoonde meer door de angst voor zowel een nieuwe context en beide stimuli dan het mannelijk nageslacht. Bovendien vond ik dat ELS vrouwtjes verminderd angstgedrag vertoonden in vergelijking met ELS mannetjes, en dat de oestrogeencyclus bij vrouwtjes tijdens de DAFC van invloed was op het daaropvolgende angstgeneralisatie gedrag. Deze resultaten suggereren dat blootstelling aan ELS geen vergaande effecten heeft op angstgeneralisatie naar de stimuli gerelateerd aan gevaar, maar dat vrouwtjes meer weerstand vertonen tegen daaropvolgend angstgerelateerd gedrag. Tot slot lijkt de fase van de oestrogeencyclus invloed te hebben op angstgeneralisatie, maar meer onderzoek is nodig om deze effecten verder te bevestigen.

Dit proefschrift had als doel de rol van het amygdala-BNST circuit te karakteriseren bij angst (generalisatie). Aangezien een beter begrip van de betrokkenheid van het amygdala-BNST circuit in de ontwikkeling van angst cruciaal lijkt voor het bevorderen van preventie- en behandelstrategieën van angst- en stress-gerelateerde psychopathologie, is het cruciaal als toekomstig onderzoek voortbouwt op deze bevindingen.

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Ik wil ook graag mijn girlies bedanken voor alle steun en toeverlaat in de afgelopen jaren. Puck, je bent er al bij sinds het begin en we hebben samen al enorm veel levensfasen meegemaakt. Het is fijn om iemand in mijn leven te hebben die er altijd is en die me als geen ander kent. Heel erg bedankt voor alle steun en de altijd onwijs goede gesprekken. Maartje, een van de liefste mensen die ik ken, en altijd in voor een feestje of een activiteit om de sleur van de dag te doorbreken! Ik ben ontzettend dankbaar dat we elkaar toevallig 11 jaar geleden zijn tegengekomen, en ondanks de afstand elkaar nog steeds regelmatig zien! Britt, laten we maar zeggen dat alle mooie dingen het waard zijn want voor jou heb ik m'n best moeten doen, maar hoe blij ben jij wel niet dat ik dat heb gedaan hé (en ik ook wel een beetje). Dank dat je altijd dicht bij jezelf blijft, de waarheid spreekt en mijn reality check op deze wereld bent. Jonelle, ik heb je pas het laatste jaar van m'n PhD leren kennen en tijd doorbrengen met jou is altijd outrageously entertaining. Het is fijn om iemand dichtbij te hebben die in is voor letterlijk elk gek idee of activiteit, en dat heb ik zeker het laatste jaar enorm nodig gehad. Bedankt! Vera, jij kwam als een bonuskadootje mee toen ik Maxime leerde kennen, en ik ben je enorm dankbaar dat je Maxime hebt aangemoedigd deze meid een kans te geven, want daardoor heb ik 2 fantastische mensen in mijn leven gekregen (en straks drie!) die ik binnenkort zelfs mijn buufjes mag noemen. Bedankt lieverd! Robin Lausjah, niet bepaald een girlie maar wel iemand die ik in het bijzonder nog wil benoemen. Bedankt dat je er al jaren bent voor Max en mij! Jij kan als geen ander mijn knoppen indrukken, tot groot vermaak van jou zelf en Max, maar wanneer ik advies nodig heb van een van de slimste life guru's die ik ken, dan ben je er!

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Curriculum Vitae

Dewi was born in Geldrop on February 25, 1994. After finishing her pre-university secondary education at the Sint-Joris Topsport Talentschool, where her elective subject was dance, she moved to Amsterdam and completed her Bachelor's degree in Psychology with a specialization in Brain and Cognition at the University of Amsterdam between 2012 and 2014. Subsequently, Dewi pursued the Plasticity and Memory track at the Research Master of Cognitive Neuroscience at Radboud University, Nijmegen, in 2016. During her master's program, she conducted research on the neural correlates of susceptibility to Posttraumatic Stress Disorder (PTSD) using a PTSD-mouse model under the supervision of dr. Marloes Henckens in the lab of prof. dr. Judith Homberg. In 2018, Dewi was awarded the RadboudUMC Top Talent Grant, facilitating her PhD research on the extended amygdala in fear generalization at the Donders Centre for Medical Neuroscience. She completed her PhD in 2023 under the guidance of dr. Marloes Henckens, prof. dr. Judith Homberg, and prof. dr. Hans van Bokhoven.

Research Data Management

Ethics

This research adheres to the applicable laws and ethical guidelines. Chapters 2, 3, and 4 were performed according to the Dutch federal regulations for animal protection and welfare, and were approved by the Central Committee on Animal Experiments (Centrale Commissie Dierproeven [CCD], The Hague, The Netherlands, approval number AVD1030020186565). All efforts were made to minimize animal suffering and to reduce number of animals used. All performed animal experiments and protocols are logged in IVentionLES and accessible to the local Animal Welfare Body (AWB).

FAIR

All data acquired during my PhD at the Radboudumc and Donders Institute for Brain, Cognition and Behaviour are archived according to the Findable, Accessible, Interoperable, and Re-usable (FAIR) principles.

For chapters 2, 3, and 4, (raw) data obtained from animal experiments have been stored on the Donders project folder and were accessible to all members involved in the project. All performed laboratory experiments and protocols were stored in Labguru and were accessible to all members of the department of Cognitive Neurosciences.

Data were made reusable by adding sufficient documentation (research protocol and a readme file) and by using preferred and sustainable data formats. The laboratory journals, protocols and data can be obtained upon request by contacting the department of Cognitive Neurosciences at the Radboudumc and Donders Institute for Brain, Cognition and Behaviour in Nijmegen, the Netherlands. The data not suitable for reuse will be archived for 10 years after termination of the study.

Donders Graduate School for Cognitive Neuroscience

For a successful research Institute, it is vital to train the next generation of young scientists. To achieve this goal, the Donders Institute for Brain, Cognition and Behaviour established the Donders Graduate School for Cognitive Neuroscience (DGCN), which was officially recognised as a national graduate school in 2009. The Graduate School covers training at both Master's and PhD level and provides an excellent educational context fully aligned with the research programme of the Donders Institute.

The school successfully attracts highly talented national and international students in biology, physics, psycholinguistics, psychology, behavioral science, medicine and related disciplines. Selective admission and assessment centers guarantee the enrolment of the best and most motivated students.

The DGCN tracks the career of PhD graduates carefully. More than 50% of PhD alumni show a continuation in academia with postdoc positions at top institutes worldwide, e.g. Stanford University, University of Oxford, University of Cambridge, UCL London, MPI Leipzig, Hanyang University in South Korea, NTNU Norway, University of Illinois, North Western University, Northeastern University in Boston, ETH Zürich, University of Vienna etc.. Positions outside academia spread among the following sectors: specialists in a medical environment, mainly in genetics, geriatrics, psychiatry and neurology. Specialists in a psychological environment, e.g. as specialist in neuropsychology, psychological diagnostics or therapy. Positions in higher education as coordinators or lecturers. A smaller percentage enters business as research consultants, analysts or head of research and development. Fewer graduates stay in a research environment as lab coordinators, technical support or policy advisors. Upcoming possibilities are positions in the IT sector and management position in pharmaceutical industry. In general, the PhDs graduates almost invariably continue with high-quality positions that play an important role in our knowledge economy.

For more information on the DGCN as well as past and upcoming defenses please visit: http://www.ru.nl/donders/graduate-school/phd/