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Neuroimaging and psychophysiological markers of fear-related disorders

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Abstract

Fear-related disorders such as panic disorder, specific phobias, or post-traumatic stress disorder, are highly burdensome and prevalent, making research on their pathogenic mechanisms an imperative. One of the central etiologic models of fear-related disorders is that they arise and manifest based on differences in associative fear-learning mechanisms, presenting a unifying mechanism underlying currently distinct diagnoses. This suggests that capturing learning-related deficits indicates pathology and may thereby serve as an informative diagnostic biomarker. Importantly, no studies up to date have compared different psychophysiological readouts regarding their ability to index specific fear-conditioning subprocesses with regard to fear-related pathology. Consequently, this thesis aimed at uncovering learning-related psychophysiological markers of fear-related disorders.

In the first study of this thesis, patients with fear-related disorders and healthy controls showed similar learning trajectories and there was also no evidence for a dimensional association between the severity of the disorder and learning or memory-related markers. In the context of an increasing number of studies reporting similar null findings, our results therefore question the diagnostic validity of the associative fear learning model of fear-related disorders. In the second study of this thesis, patients with fear-related disorders and healthy controls were compared regarding their startle reflex, a basal measure of threat reactivity if probed in an aversive context. Patients with fear-related disorders showed a significantly faster initiation of the startle reflex. As startle under threat depends on modulatory inputs of the amygdala and as shorter startle latencies were associated with smaller amygdala volumes, patients with fear-related disorders may be characterized by an increased readiness, or hyperarousal, of amygdala-modulated startle circuits. The third study of this thesis investigated fear memory formation using an experimental trauma film paradigm in healthy participants, increasing the ecological validity of the classical fear conditioning setups used in the first two studies. There was no evidence for learning-related differences in predicting intrusion formation or loss of contextual memory, two core mnemonic symptoms of post-traumatic stress disorder. However, hippocampal deactivation towards highly aversive films predicted less severe intrusive memory formation. Such a distinct hippocampal stress-related signature may therefore be a correlate of adaptive stress signaling.

Together, the results of all three studies speak against learning-related differences as key characteristics of fear-related pathology and thereby against one of the dominant theories on the emergence and maintenance of fear-related disorders. Even if small differences in fear-conditioning existed, they would therefore be too small to be suited as a cross-diagnostic biomarker. Instead, results provide evidence for a sympathetic hyperarousal-account of fear-related disorders. Our findings therefore suggest a paradigm shift away from very specific fear-conditioning paradigms toward more basic fear-eliciting paradigms that prioritize the extraction of stable and reliable biological markers.

Contents

Abbreviations

BeCOME	Biological Classification of Mental Disorders
BLA	basolateral amygdala
BOLD	blood-oxygen-level-dependent
Ce	central amygdala
CR	conditioned response
CRF	corticotropin-releasing factor
CS	conditioned stimulus
EMG	electromyography
fMRI	functional magnetic resonance imaging
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal
ITC	intercalated cells
LC	locus coeruleus
NA	noradrenaline
PTSD	Posttraumatic Stress Disorder
RDoC	Research Domain Criteria
SAM	sympathetic–adrenal–medullary
US	unconditioned stimulus

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1 Introductory summary

1.1 Fear-related pathology

1.1.1 A brief outline on the etiology, prevalence and treatment of anxiety- and fear-related disorders

Anxiety and fear are emotions that arise in response to aversive situations and encompass cognitive, physiological and behavioral changes that help to confront, escape or avoid these situations. The distinction between anxiety and fear is not sharp, but many definitions agree that they are differentially elicited depending on the proximity of threat (Craske et al., 2011; Mobbs et al., 2019; Mobbs, 2018). While anxiety is a preparatory response to a future negative event, fear is an alerting response to an acute threat, leading to persisting memories of the threatening situation (Perusini & Fanselow, 2015). The abilities to vividly imagine future outcomes and to re-experience remembered events, are highly adaptive as they enable us to cope with challenges and ensure long-term survival (Pine, Wise, & Murray, 2021). For example, being involved in a car accident will lead to strong, vivid memories that help to stay alert in future traffic situations. Pathological anxiety evolves, however, when past, present or potential future situations elicit disproportionately strong fearful states, indicating a mismatch between a certain situation and the emotional response. This could for example be the case if someone who experienced an accident suffered from panic attacks anytime when leaving their house. Patients with anxiety disorders are therefore excessively fearful of perceived threats originating from within themselves, for example in the form of thoughts or unusual body sensations, or in their environment, for example of unknown places or social encounters (Pittig, Treanor, LeBeau, & Craske, 2018). Importantly, the etiology of anxiety disorders is highly multi-factorial, with pathology arising from interactions between the individual genetic background and environmental factors such as the experience of traumatic events or adverse political or societal circumstances (Matosin, Halldorsdottir, & Binder, 2018).

Human vulnerability to pathological anxiety has become very apparent during the Covid-19 pandemic with increased attention towards – and rising numbers of – anxiety disorders and associated comorbidities such as depression or sleeping disorders (Hajek et al., 2022; Pashazadeh Kan et al., 2021). At the same time, this example also shows that it is not easy to differentiate between proportional worry of an uncertain future, and prolonged, paralyzing anxiety leaving the individual incapable of dealing with unknown and straining situations. It also indicates that anxiety reflects a spectrum with a wide intermediate range that transitions into a severe pathological manifestation, making it difficult to define

a clear diagnostic threshold given the respective historical, political and sociological circumstances. Importantly, although the biological underpinnings of anxiety and fear are increasingly well understood, it is unclear which risk factors and mechanisms lead to specific symptoms and on which observation levels these pathogenic processes can be measured most efficiently (Elbau, Binder, & Spoormaker, 2019). Up to now, there are no reliable and replicable biomarkers indicating fear-or anxiety-related malfunctioning.

With a 12-month prevalence of about 15%, anxiety disorders are among the most frequent psychiatric disorders in Germany, leading to high individual burden and downstream societal challenges (Jacobi et al., 2014). Anxiety disorders affect women more often than men and their prevalence is correlated with socio-economic status, reflecting increased stress exposure and less access to treatment in low income groups. Anxiety disorders often recur in a waxing and waning pattern and may generalize to further stimuli, leading to high levels of chronicity and comorbidity rates (Kessler, Ruscio, Shear, & Wittchen, 2010). Based on large-scale epidemiologic and genetic studies, pathological anxiety can be roughly divided into anxious-misery-related disorders and fear-related disorders (Krueger, 1999). These types of disorders differ in their core symptomatology: Anxious-misery-related disorders are characterized by a sustained tonic anxiety and comprise major depressive disorder, dysthymia, and generalized anxiety disorder. Fear-related disorders are defined by an acute (phasic) fear response that is elicited by a specific object or situation (Davis, Walker, Miles, & Grillon, 2010) and comprise panic disorder, social anxiety disorder, and specific phobia (Slade & Watson, 2006; Vollebergh et al., 2001, see Hettema, Prescott, Myers, Neale, & Kendler, 2005; Kendler, Prescott, Myers, & Neale, 2003 for different results on panic disorder). Posttraumatic stress disorder (PTSD) encompasses symptoms of depression, but several of its core clusters show high overlap with other fear-related disorders such as hyperarousal or avoidance (American Psychiatric Association, 2014). In the following work with its focus on fear-related pathology, PTSD will therefore be conceptualized as a fear-related disorder.

Different types of treatment are available (Bandelow, Michaelis, & Wedekind, 2017): On the one hand, pharmacological agents mainly targeting the adrenergic, serotonergic or GABA-ergic systems have shown pre-to-post effectiveness in alleviating anxiety. However, medication-based approaches often solely target the symptom level and simplify the supposed pathogenic and therapeutic mechanisms. Importantly, they may cause severe and sometimes unbearable side effects. On the other hand, cognitive behavioral therapies (involving psychoeducation, cognitive restructuring and repetitive exposure to the feared stimuli) are effective treatments (Carpenter et al., 2018), although efficacy does not surpass the drug-placebo effect (Bandelow et al., 2015). Often, the combination of medication and psychotherapy yields the largest effects (Bandelow et al., 2015; Sigurvinsdóttir, Jensínudóttir, Baldvinsdóttir, Smárason, & Skarphedinsson, 2020) which has for example been attributed to early-medication induced shifts in emotional processing which later allow patients to learn novel emotion-relevant associations in a more positive context (Godlewska & Harmer, 2020). Critically, treatment selection is based on a trial

and error approach and all treatment options are far away from a precision medicine approach, i.e. that they are not sufficiently tailored to the individual symptom profile (Kessler & Luedtke, 2021; Schumann et al., 2014). This intolerable state is mainly rooted in the lack of data linking specific symptom profiles with treatment success.

1.1.2 The translational gap between basic neuroscientific findings and the diagnostics of fear-related disorders

The described difficulties in determining diagnostic categories for clinical use are also apparent in the two main current diagnostic systems, the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2014), and the 10th revision of the International Classification of Diseases (World Health Organization, 1993): First, both systems do not fully agree on their nosology of fear and anxiety related disorders. Second, even within each diagnostic system there is high symptom heterogeneity within the same diagnostic category (for example emotional numbing and hyperarousal within PTSD (Zoellner, Pruitt, Farach, & Jun, 2014)). Finally, there is a high conceptual overlap between distinct diagnostic categories (for example intrusive imagery as a symptom present in phobias, generalized anxiety disorder or PTSD (Brewin, Gregory, Lipton, & Burgess, 2010)). Together, these arguments contribute to the difficulties of finding meaningful boundaries between diagnostic labels. Rooted in this lack of diagnostic construct validity, basic findings in the genetics and neuroscience fields have not mapped well onto existing diagnostic categories and research techniques have been aimed at disorders that are inadequately conceptualized.

Dimensional approaches have tried to bridge this translational gap between basic and clinical science. One example for such a dimensional approach are the Research Domain Criteria (RDoC) that help to characterize patients within five research domains and their subconstructs (Insel et al., 2010). These RDoC should be assessed in different units of analysis (ranging from molecular to behavioral observation levels) and have the overall goal to uncover basic transdiagnostic pathogenic mechanisms and clinically meaningful endophenotypes. In the case of fear-related pathology, relevant research domains are the negative valence system, the cognitive system and the arousal/modulatory systems with respective exemplary subconstructs of acute threat, memory retention or difficulties in the sleep-wake regulation (Lonsdorf & Richter, 2017; Kelly, Killgore, & Haynes, 2016). Although the RDoC have been criticized, for example for being created in an expert-driven top-down fashion, the general concept of breaking down the biological mechanisms seems promising in (re-) mapping anxiety and fear-related disorders, ultimately contributing to the development of appropriate treatment options (Shankman & Gorka, 2015; Zoellner & Foa, 2016).

Following an endophenotype-based approach, one way to receive a more comprehensive picture of the biological underpinnings of fear-related pathology, is to compare patients

with the diagnosis of different types of fear-related disorders and healthy control participants regarding their physiological fear-response in an experimental setting. On the one hand, such an approach allows to dimensionally relate similar symptoms across diagnostic groups to specific physiological readouts. On the other hand, collecting such databases enables the identification of data-driven subgroups within the fear-response that could represent endophenotypes with a specific etiology or a specific drug response profile.

1.1.3 Physiological correlates of fear: Two distinct but overlapping stress response systems

As mentioned above, most definitions of fear as a human emotion converge on fear as a human reaction to threat and wellbeing (Mobbs et al., 2019; Craske et al., 2011). While fear certainly has subjective (the feeling of intense acute anxiety) and behavioral (trying to escape) aspects, an important characteristic are the physiological changes associated with fear (Mobbs, 2018; LeDoux, 2014). Especially this physiological component of fear is tightly related to the concept of stress, a condition in which " [...] environmental demands tax or exceed the adaptive capacity of an organism, resulting in psychological and biological changes [...]" (S. Cohen & Kessler, 1995).

The stress response depends highly on the nature and duration of the threat, as situations of acute versus sustained threat require differential and finetuned response mechanisms. During acute threat, for example when seeing a deer jumping on the street while driving a car, a very rapid mobilization of energy is needed, enabling the driver to quickly push the breaks and to stay alert in case of more deer passing the street. During a demanding seven-hour exam, on the other hand, a different coping mechanism is needed that enables the student to focus for a longer period of time. These different states of the stress-response are driven by two distinct but interlinked response systems – the sympatho–adrenal–medullary (SAM) arm of the autonomous nervous system and the hypothalamus-pituitary-adrenal (HPA) axis. Both stress systems support the organism in adaptively reacting to challenging situations (Ulrich-Lai & Herman, 2009). The involved neurotransmitters, peptides and steroids are stress mediators with specific, but partly overlapping acting sites and temporal niches, thereby enabling synergistic actions between them (figure 1.1, Joëls & Baram, 2009; Schwabe, Hermans, Joëls, & Roozendaal, 2022). Since fear elicits a bodily stress response, the dynamics of stress-related neurotransmitters and physiological changes can be used to measure the dynamics of the fear response:

Within milliseconds to minutes after stressor onset, the SAM initiates the first wave of the stress response by releasing monoamines such as adrenaline and noradrenaline (NA). The general increase in circulating levels of NA by sympathetic nerves as well as adrenaline from the adrenal medulla lead to various measurable activating effects in the organism such as increases in blood pressure and heart rate, pupil diameter, muscular

tone and sweating. Due to immediate reflex antagonistic effects by the parasympathetic nervous system, activation of the SAM decreases quickly and results in a short-lived response. Especially in highly threatening situations, the amygdala holds a key role in modulating responsiveness of the SAM. Its key output region, the central nucleus of the amygdala (Ce), receives input from the thalamus, the brain's main relay station of incoming sensory information, and can exacerbate threat reactivity through direct projections to the hypothalamus (Mcdonald, 1998; Tovote, Fadok, & Lüthi, 2015; Turner & Herkenham, 1991). The function of the amygdala as the central site in fear-memory (see 1.1.4) also enables a top-down initiation of the SAM, for example when remembering or anticipating aversive events (Herman et al., 2003). This points towards the role of previous experiences and individual differences in shaping the stress-response and causing elevated stress-levels.

Shortly after the initiation of the SAM, stress exposure also activates the hypothalamus, leading to a downstream hormonal cascade called the HPA-axis. Within the HPA-axis, the stress-induced hypothalamic release of corticotropin-releasing factor (CRF) triggers the production of the adrenocorticotropic hormone in the pituitary glands, eventually causing the secretion of cortisol by the adrenal glands. As this hormonal cascade takes time, this second wave of the stress response is slower than the SAM. It acts through glucocorticoid receptors (GR) and is responsible for prolonged stress responses such as the mobilization of stored energy or the consolidation of the information associated with stress. Eventually, it initiates a negative feedback loop, leading to an inhibition of further cortisol release. The hippocampus holds an important position in the regulation of the stress response. On the one hand, it expresses high levels of GR and is thereby a central part of handbrake of the HPA-axis (Herman et al., 2016). On the other hand, it has inhibitory connections to the hypothalamus and the amygdala, dampening their reactions to threat (Tovote et al., 2015).

To summarize, the stress response cannot only be elicited in a reactive, bottom-up manner, but can be modulated or even elicited by regions involved in emotional processing such as the amygdala or the hippocampus, for example when remembering or anticipating aversive events (Herman et al., 2003).

1.1.4 The conceptual relevance of fear-learning to understand fear-related pathology

The associative learning model of fear-related disorders

Patients with fear-related disorders suffer from excessive fear reactions that are out of proportion to the current setting, demonstrating that an acute fear-response to an actual or imagined situation is a core characteristic of fear-related disorders. There are a lot of incidental reports of phobias resulting from one or multiple aversive experiences with

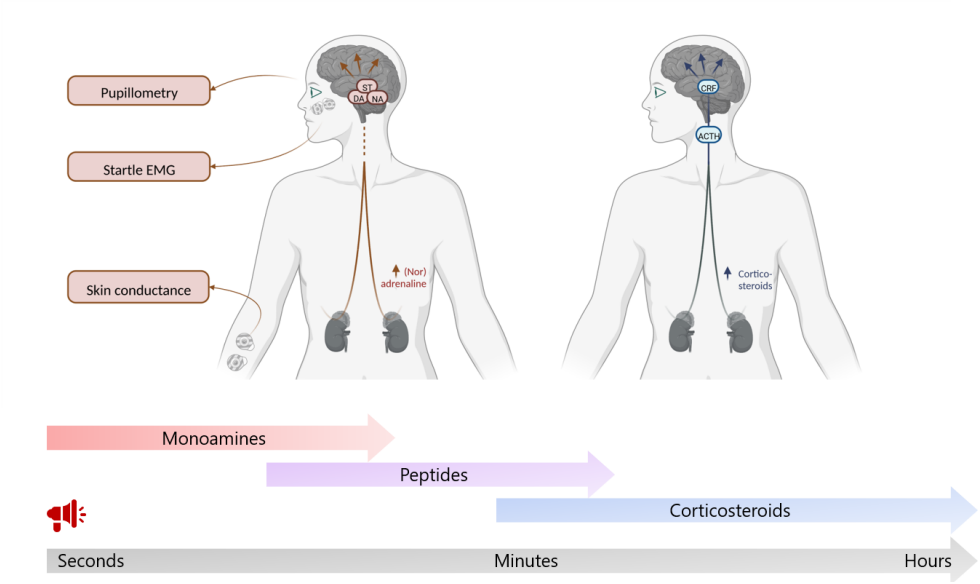

Figure 1.1: Monoamines, peptides and steroid stress mediators have specific, but overlapping temporal and spatial niches. This results in a fine-tuned "neuro-symphony of stress" (Joëls & Baram, 2009) that enables a nuanced response to the specific aspects of a stressful situation (left figure = SAM, right figure = HPA-axis). Increases in (nor)adrenergic tone initiated by the SAM lead to instantaneous bodily changes described as the fight-or-flight response (Scott-Solomon et al., 2021). These changes encompass wider pupils, higher muscular tone or increased sweating and can be quantified using psychophysiological methods such as pupillometry, startle electromyography and skin conductance measurements. Figure partly adapted from Schwabe et al. (2022) and created with BioRender.com. NA: noradrenaline, DA: dopamine, ST: serotonine, CRF: corticotropin-releasing factor, ACTH: adrenocorticotropic hormone.

the later feared situation or object (although more than 50% of cases report never having had such an encounter (King, Eleonora, & Ollendick, 1998)). In case of PTSD, witnessing or experiencing an extremely aversive situation is even a necessary diagnostic criterion (American Psychiatric Association, 2014). Memories of the trauma tend to involuntary arise in the form of flashbacks or intrusions. A past experience is therefore often the catalyst or underlying the development of a fear-related disorder, suggesting that the neural representation of a previous experience leads the affected person to suffer from a wide range of symptoms. Accordingly, one of the main theories regarding the etiology of fear-related disorders proposes that learning-related differences underlie the vulnerability towards pathological anxiety (Craske, Treanor, Conway, Zbozinek, & Vervliet, 2014; Duits et al., 2015; Kindt, 2014; Marin et al., 2017; Krypotos, Effting, Kindt, & Beckers, 2015; Mineka & Zinbarg, 2006; Pittig et al., 2018; Scheveneels, Boddez, Vervliet, & Hermans, 2016; Jovanovic et al., 2010).

The literature on fear-learning and the etiology of fear-related disorders had its beginnings more than a 100 years ago (Vervliet & Boddez, 2020) with Watson and colleagues re-

searching the development of the rich behavioral and emotional repertoire seen in adults. They hypothesized that adult emotional behavior is gradually formed by transferring the innate emotional reactions to specific stimuli seen during infancy to a wide range of other, related stimuli. Watson observed that "by the method of conditioned reflexes, emotional reactions can be called out by situations (stimuli) which do not at first call them out" (Watson & Morgan, 1917). Their studies provided evidence that a previously neutral object (a white rat) started to elicit a fear-response (by a boy called Albert) through its repeated pairing with a loud and frightening noise (Watson & Rayner, 1920). Albeit being criticized for insufficient ethical considerations, their seminal "Little Albert" study laid the foundation of (human) conditioning research and the conception that fear learning subserves pathological fear.

Fear-conditioning: One term, multiple meanings

What is the basic process in fear-conditioning? Pavlovian or classical conditioning as a procedure describes the repetitive pairing of an unconditioned stimulus (US, for example a noxious tactile stimulation) eliciting an autonomous physiological response with an initially neutral stimulus (for example a specific visual cue). Over repetition and time, the previously neutral stimulus becomes the conditioned stimulus (CS+) and elicits a conditioned response (CR). The strength of the CR is thought to reflect the associative memory formation between the CS+ and the US.

The term fear-conditioning has different connotations (Lonsdorf et al., 2017). First, it can be used interchangeably with aversive associative learning, a translational process due to the involvement of highly conserved fear circuits (Janak & Tye, 2015). Second, it is used as an umbrella term for an experimental paradigm encompassing fear-acquisition, as well as manipulations of the original CS-US association (Lonsdorf & Richter, 2017): Often in fear-conditioning paradigms, one or multiple CS+ are followed by aversive US, while another stimulus (CS-) is never paired with the US and thereby serves as a safety cue or control stimulus. Repetitive exposure to the CS+ without the associated US leads to a gradual decrease of the CR, called extinction learning. It is also common to probe the generalization of the CS+, investigating whether perceptually or conceptually related stimuli also elicit a fear response (Dunsmoor & Paz, 2015) or to measure the relative expression of fear versus extinction memory when confronting a participant with the extinguished CS+ at a remote time point (Milad et al., 2007). Third, the different procedures described above also function as models regarding hypothesized processes underlying pathological anxiety. For example, while fear-acquisition and extinction procedures model the acquisition and maintenance of fear-related disorders, spontaneous recovery serves as a model for the relapse of a disorder. While these models exhibit high face-validity (Vervliet & Raes, 2013) their external validity is controversially debated, since, for example, decreased safety learning does not serve as a reliable diagnostic marker for

anxiety disorders and difficulties in experimental extinction learning cannot sufficiently predict exposure therapy success (Davey, 2017; Scheveneels, Boddez, & Hermans, 2021; Scheveneels et al., 2016; Vervliet & Boddez, 2020, see Ball, Knapp, Paulus, & Stein, 2017; Roesmann et al., 2022; Waters & Pine, 2016; Forcadell et al., 2017; Lange et al., 2020 for indications that markers derived from fear-conditioning are associated with exposure therapy success).

The neural basis of fear learning

Although fear-conditioning and associated behaviors require the interplay of large brain networks (Fullana et al., 2016, 2018; Tovote et al., 2015), rodent research has converged on a key fear and extinction circuitry centered around the amygdala and the hippocampus (two structures within the limbic system) as well as the prefrontal cortex (Izquierdo, Furini, & Myskiw, 2016; Milad & Quirk, 2012; Tovote et al., 2015) (see figure 1.2):

Two subnuclei of the amygdala, the basolateral amygdala (BLA) and the Ce take over key functions in fear memory acquisition. Sensory inputs of all modalities project to the BLA, carrying for example information on a visual cue and a noxious tactile stimulus. Exhibiting activity-dependent plasticity, the neuronal ensembles activated in response to the fearful event then form the substrate underlying the formation, storage and recall of this memory (Tovote et al., 2015). Lesions of the BLA completely abolish fear learning in rodents, demonstrating its unique role in establishing fear memories (Fanselow & Ledoux, 1999; Johansen, Cain, Ostroff, & Ledoux, 2011). The Ce is the core output region of the amygdala. Its projections to the locus coeruleus (LC) in the brainstem, the brain's main noradrenergic output center, as well as the hypothalamus result in an autonomic fear response (see top-down sympathetic component of the fear response described in 1.1.3) while projections to the periaqueductal grey are responsible for freezing behavior (LeDoux, Iwata, Cicchetti, & Reis, 1988).

Next to sensory inputs to the amygdala, the hippocampus provides contextual information present during the fear eliciting situation (Fanselow & Ledoux, 1999; Kheirbek et al., 2013; Lesuis et al., 2021; Pape & Pare, 2010; Phillips & LeDoux, 1992; Walker & Davis, 2002). The term contextual describes internal and external circumstances or contingencies that can either be directly associated with a US but can also embed specific cues (CS+) that become conditioned to the US (Maren, Phan, & Liberzon, 2013).

Apart from the amygdala and the hippocampus, the medial prefrontal cortex is another essential structure in regulating fear. On the one hand, its prelimbic subregion supports fear expression by targeting the BLA. On the other hand, its infralimbic subregion dampens fear expression by targeting areas reducing Ce output such as the lateral division of the Ce and the intercalated cells (ITC) (Milad & Quirk, 2012).

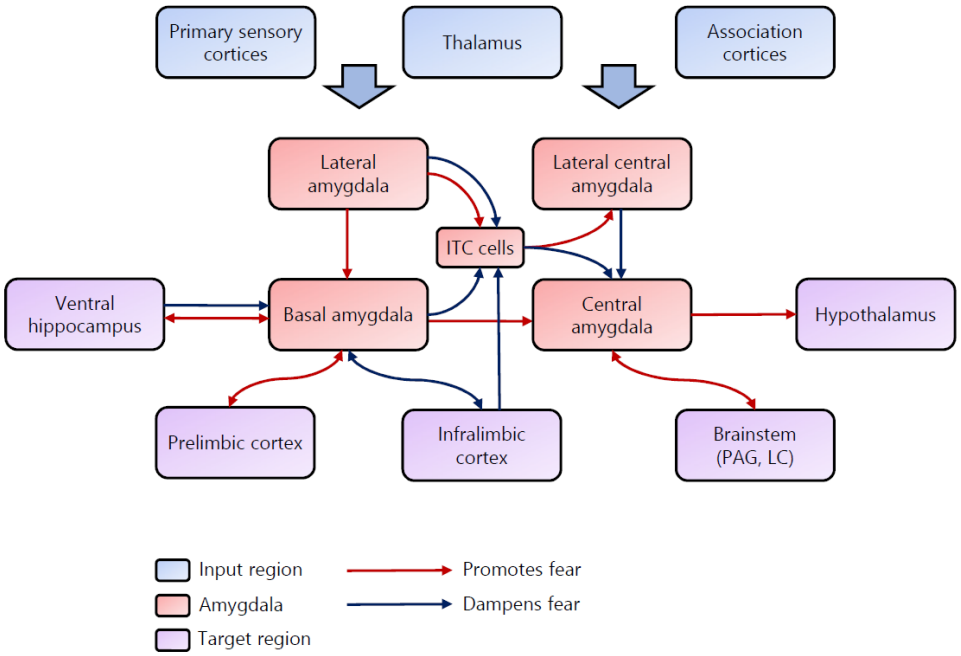

Figure 1.2: Fear learning is achieved by fear-related neuronal plasticity in the amygdala and its sensory input regions such as the thalamus or primary sensory cortices. Importantly, this plasticity is modulated by connections between the basal amygdala, the ventral hippocampus and the prelimbic cortex. As its main output nucleus, the central nucleus of the amygdala projects to the brainstem centers and the hypothalamus, promoting fear expression. Fear extinction is achieved by the same structures but mediated by different circuit elements. Here, projections from the infralimbic cortex to ITC cells and the basal amygdala is essential to dampen fear expression. Figure partly adapted from Tovote et al. (2015). PAG: periaqueductal grey, LC: locus coeruleus, ITC cells: intercalated cells

Once a fear memory has been formed, repeated exposure of the CS+ without the US leads to a gradual decrease of the CR. However, these CRs may return spontaneously at a later timepoint, when confronted with an unsignaled US, or upon exposure to the CS outside the extinction context (Goode & Maren, 2014). Observations of this return of fear led to the conclusion that extinction does not erase the original CS-US association, but forms a novel context-dependent memory trace, inhibiting the CR (Bouton & Bolles, 1979). As summarized in figure 1.2, amygdala and hippocampus are therefore not only central in the acquisition of fear, but also regulate the expression of fear versus extinction memory. Differential amygdala circuits comprise fear and extinction neurons whose firing patterns are strongly associated with fear expression while the hippocampus serves as a contextual gate, favoring the reinstatement of fear versus extinction engrams (Giustino & Maren, 2015). It is assumed that the hippocampus can perform this task because of its ability to perform pattern completion and pattern separation in distinct subfields. Pattern completion describes the process enabling the retrieval of memory patterns from

degraded and partial representations, facilitating generalization between similar stimuli. Pattern separation, in turn, describes the process of converting overlapping input signals into distinct, non-overlapping output signals (Hunsaker & Kesner, 2013).

Measuring fear-conditioning using psychophysiological measures

As it is not possible to investigate learning related processes at the level of the synapse in humans, human research focuses on correlational brain activity at the systems level as well as on measurable peripheral physiological activity to be able to draw conclusions on latent learning processes.

Human functional magnetic resonance imaging (fMRI) studies have partly confirmed the role of the rodent fear circuitry in human fear learning but have also directed attention to large-scale networks underlying fear acquisition and extinction (Fullana et al., 2016, 2018). Contrasting CS+ to CS- related brain activity yields robust fear-related blood-oxygen-level-dependent (BOLD) responses in cortical, subcortical and brainstem regions involved in the perception and reaction to as well as the encoding of salient stimuli (such as the insula or the dorsal anterior cingulate cortex, the human equivalent to the prelimbic cortex (Milad & Quirk, 2012)), as well as the striatum and the lateral prefrontal cortex. In comparison, contrasting CS- to CS+ elicits safety-related activity in regions summarized as the safety network (such as the hippocampus and the ventromedial prefrontal cortex, the human equivalent to the infralimbic cortex (Milad & Quirk, 2012)). Interestingly, none of the meta-analytic contrasts revealed fear-related activity in the amygdala. The lack of robust threat-related amygdala activation has been attributed to the quick habituation of its BOLD signal and technical constraints of fMRI, but has also initiated a discussion on the role of the amygdala in human threat processing and fear-conditioning. Potentially, the lack of amygdala activation could also indicate that the unconditioned stimuli applied in human research are only perceived as aversive, but are not strong enough to elicit fear (Fullana et al., 2018; Hennings, Cooper, Lewis-Peacock, & Dunsmoor, 2022; Visser, Bathelt, Scholte, & Kindt, 2021).

The quantification of fear induced bodily changes, such as increases in sweating, pupil size or muscular tone, can also serve as a surrogate marker for associative fear learning (Lonsdorf & Richter, 2017). Here, the core assumption is that the strength of the CS-US association is reflected in the magnitude of the downstream fear-response. Frequently used psychophysiological methods other than fMRI include the quantification of the skin conductance response, pupillometry or startle electromyography (EMG) (Leuchs, Schneider, & Spoormaker, 2018). Importantly, the different output organs of the SAM and the HPA-axis and their respective readout measures have distinct characteristics and capture different aspects of the fear-response. Skin conductance increases in response to arousing stimuli, independent of their valence and habituates quickly (Hamm & Stark,

1993). Probing the eyeblink component of the startle reflex informs about valence processing, as startle reactions are potentiated in negative conditions and alleviated under positive conditions (Cook, Hawk, Davis, & Stevenson, 1991; Lang, Bradley, & Cuthbert, 1990). Additionally, the startle circuitry is highly conserved and very well described with startle under threat involving a modulation of the reflex by the amygdala (Kuhn et al., 2020). Tracking pupil size under constant light conditions can further inform about underlying LC-modulated noradrenergic arousal (Finke, Roesmann, Stalder, & Klucken, 2021; Strauch, Wang, Einhäuser, Van der Stigchel, & Naber, 2022). Importantly, when probing these readouts in fear-conditioning studies, trial-by-trial correlations are only low (Leuchs et al., 2018), demonstrating that all measures have a distinct profile and therefore also have the potential to differentially inform fear-related pathology.

1.1.5 Fear conditioning in fear-related disorders

The core assumption of the associative fear learning model of fear-related disorders is that deficits in fear learning underlie their emergence and maintenance (Craske et al., 2014; Duits et al., 2015; Kindt, 2014; Marin et al., 2017; Krypotos et al., 2015; Mineka & Zinbarg, 2006; Pittig et al., 2018; Scheveneels et al., 2016; Jovanovic et al., 2010). Diagnostic validity of this model would therefore entail that patients with fear-related disorders exhibit distinct physiological response patterns in fear-conditioning procedures. Before the start of this work, this model had received considerable support from meta-analyses, showing that patients with fear-related disorders display impaired safety learning to the CS- during fear acquisition and impaired extinction learning to the CS+ during fear extinction (Duits et al., 2015; Lissek et al., 2005). Furthermore, multiple studies demonstrated that patients with fear-related disorders show a tendency to recall the fear rather than the safety memory under ambiguous situations or in return of fear manipulations (Marin et al., 2017; McLaughlin et al., 2015; Milad et al., 2009).

However, overall effect sizes were small and many studies had low sample sizes, potentially causing a file-drawer effect, exacerbated by artificial group comparisons of individuals with one specific anxiety disorder. Moreover, no studies up to date have compared different psychophysiological readouts regarding their ability to index specific fear-conditioning subprocesses in anxiety populations. Finally, many studies have not controlled for the medication status of their patient populations and most importantly, there is no definite response-profile allowing to inform diagnostics. The aim of the first project of this thesis was therefore to compare fear learning in healthy control participants to unmedicated patients exhibiting different types and severity levels of fear-related pathology, aiming at the discovery of specific psychophysiological endophenotypes.

1.2 Assessing fear-learning markers in patients with fear-related disorders

Rationale and hypotheses. In order to aid the discovery of psychophysiological endophenotypes in fear-related disorders, project 1 compared healthy controls who had never had a diagnosis of a fear-related disorder to thoroughly clinically characterized and unmedicated patients fulfilling diagnostic criteria of a fear-related disorder or PTSD. We were specifically interested in quantifying learning-related differences between patients with different types and severities of fear-related disorders. Participants underwent a classical fear-conditioning task with differently colored shapes as CS and aversive airblasts as well as electrical stimulations to the wrist as US. Importantly, we continuously measured changes in pupil dilations, skin conductance level and startle reactivity throughout fear acquisition and subsequent extinction on the first day as well as fear recall on the following day. We expected that these psychophysiological readouts would differ regarding their ability to discriminate between patients and controls, potentially in a task-phase dependent manner. Our ultimate goal was to discover subgroups within the physiological response profiles, hypothesizing that distinct data-driven classes would represent meaningful fear-related endophenotypes. The sample included 35 carefully screened control participants, who had never had a psychiatric diagnosis, 73 participants fulfilling diagnostic criteria for a specific phobia, social phobia, agoraphobia or panic disorder and 21 patients with PTSD. Data were acquired as part of an ongoing inhouse study - the Biological Classification of Mental Disorders study (BeCOME) (Brückl et al., 2020) that is designed to identify biologically based subtypes of stress-related psychiatric disorders. The study comprises healthy participants and patients with affective and anxiety disorders and quantifies their physiological and behavioral phenotypes in validated psychological tasks designed to probe basic systems in human functioning. Participants undergo thorough clinical assessment as well as comprehensive baseline and task-evoked omics-assessment such as genotyping or measuring stress-evoked cortisol responding.

Summary and discussion of findings. Bayesian statistics indicated successful fear acquisition, extinction and return of fear. Although there was anecdotal evidence for the PTSD group exhibiting an overall increased startle response during fear extinction, we did not observe robust learning-related differences between response patterns of patients with fear-related disorders and healthy controls in either measure or task phase. In fact, there was even anecdotal evidence against an association between differential response strength in all readouts and the impact or the severity of the fear-related disorder. The statistical methods used in Pöhlchen et al. (2020) aimed at discovering which pre-defined factors were most likely to have caused the observed physiological response profiles. Hypothesizing that this pre-definition of groups could have concealed relevant trends in the data, a follow-up project used latent-class growth curve models, an unsupervised clustering method aimed at detecting latent subgroups in time-dependent data. Applying this technique resulted in unstable cluster solutions with subgroups being non-informative regarding pathology. These analyses therefore also add to evidence against the existence

of pathology-relevant subgroups characterized by distinct fear-learning patterns. From a meta-research perspective, sharing and publishing such null findings is essential to quantify the full range of observed effect sizes and to aid replication as well as re-analysis of data.

How can our null-findings be reconciled with meta-analytic findings of patient-control differences during fear-acquisition and extinction (Duits et al., 2015; Lissek et al., 2005)? Taking a closer look at the studies driving these meta-analytic differences revealed that effect sizes for studies employing disorder-specific stimuli (e.g. pictures of spiders in the case of spider phobia, Schweckendiek et al. (2011)), were very high, but around zero for studies using electrical shocks. This demonstrates that learning-related deficits are not generic, but likely arise in conditioning and extinction procedures probing already established fear memories. Importantly, more recent and larger scale studies have also reported a lack of learning-related differences in patients with anxiety- and fear-related disorders (Abend et al., 2020; Acheson et al., 2015; Duits et al., 2021; Hennings, McClay, Lewis-Peacock, & Dunsmoor, 2020; Wake, van Reekum, & Dodd, 2021; Schenker et al., 2022; Adolph, Teismann, Wannemüller, & Margraf, 2022), calling for a further update of the meta-analysis of classical fear conditioning in the anxiety disorders that also incorporates the US type as a moderator.

Implications of project 1: Based on these null findings, we argue that the effect size of generic learning related differences between patients with fear-related disorders and control participants has been overestimated. Dimensionally relating the strength of fear-related pathology and fear conditioning markers provided anecdotal evidence against a correlation between the severity of a fear-related disorder and generic deficits in fear-conditioning. From a theoretical perspective, our results therefore call for a critical re-evaluation of one of the main theories in the field, speaking against an even small manifestation of generic differences in associative fear learning in fear-related pathology, (Craske et al., 2014; Duits et al., 2015; Kindt, 2014; Marin et al., 2017; Krypotos et al., 2015; Mineka & Zinbarg, 2006; Pittig et al., 2018; Scheveneels et al., 2016; Jovanovic et al., 2010). From a clinical perspective, even if small differences between patients and controls existed, they would not qualify as diagnostic markers with fear-learning paradigms incapable of reliably capturing individual variability linked to psychopathology. Importantly, the clinical utility of a test is not only defined by its diagnostic validity, but also by its ability to treatment response (Davey, 2017; Vervliet & Raes, 2013). A recent study showed that even in the absence of generic learning differences between healthy controls and patients with anxiety disorders, individual extinction learning abilities were still predictive of exposure therapy success (Adolph et al., 2022). In summary, although classical fear conditioning remains a highly valuable, translational paradigm with large experimental effects, it has stayed behind in its explanatory power with regard to fear-related pathology and diagnostic utility (Fullana et al., 2020).

The fact that patient-control differences in the meta-analysis by Duits et al. (2015) were driven by disorder-specific stimuli shows that previous aversive experiences, and hence processes involved in long-term episodic memory formation, play an important role in the etiology of fear-related disorders. Potential ways forward would therefore be to improve the ecological validity of fear-conditioning paradigms, moving away from simplistic geometric stimuli to more complex and relatable experimental situations as well as prolonging the experimental timeframe to more than one or two days. Therefore, we set up a study to investigate the evolution of fear-memory over the timeframe of one week (see chapter 1.4). Based on the lack of reliable amygdala activation in classical human fear conditioning studies (Fullana et al., 2016), indicating low levels of fear, as well as our associated criticism of the lack of ecological validity of these paradigms, we applied the conditioned-intrusion paradigm (Brueckner, Lass-Hennemann, Wilhelm, Ferreira de Sá, & Michael, 2019; Kunze, Arntz, & Kindt, 2015; Miedl et al., 2020; Rattel et al., 2019; Wegerer, Blechert, Kerschbaum, & Wilhelm, 2013), a more ecologically valid setup comprising aversive movie scenes as US that model a traumatic experience (reviewed in James et al., 2016).

Together with existing literature on elevated startle responding in psychiatric disorders (Boecker & Pauli, 2019; Duits et al., 2017; Jovanovic et al., 2010; Norrholm et al., 2011; Stevens, Lieberman, Funkhouser, Correa, & Shankman, 2019), our anecdotal evidence on generically increased startle in PTSD during extinction suggested that hyperexcitability in amygdala-modulated fear pathways could serve as a marker of fear-related pathology. Potentially, genetic influences and experience-based modulations of neural processing in the limbic system could lead to increased reactivity in situations requiring modulatory inputs of the amygdala. Accordingly, one follow-up project involved a deep-dive into startle responding under threat. Existing human fear-conditioning literature focuses on the amplitude on the startle reflex. In the Schizophrenia field, however, startle latency is an informative readout, indicating prolonged neural processing speed in patient populations (Greenwood et al., 2020; Hasenkamp et al., 2010; Geyer & Braff, 1982; Ludewig, Geyer, Etzensberger, & Vollenweider, 2002; Storozheva, Kirenskaya, Novototsky-Vlasov, Telesheva, & Pletnikov, 2016). Combining rationales from both, the Schizophrenia and the anxiety fields, we therefore extracted amplitude as well as latency components of the startle response during the fear-conditioning task and related it to fear pathology and amygdala morphology (see chapter 3).

1.3 Startle latency as a marker for amygdala-mediated hyperarousal

Rationale and hypotheses. The aim of the second project was to investigate similarities and differences of startle amplitude and latency features derived from the fear-acquisition phase of a classical fear conditioning task. We hypothesized that amplitude and latency of the startle reflex are direct readouts of amygdala processing and that patients with fear-related disorders display increased reactivity in circuits modulated by the amygdala. The

hypotheses were operationalized by (re-) analyzing startle data from the fear-conditioning task of the BeCOME study (see chapter 1.2). We ran a principal component analysis on a variety of startle features derived from 206 participants and investigated the associations between startle amplitude and latency features with amygdala volume and patient-control status.

Summary and discussion of findings. The principle component analysis revealed that amplitude and latency of the startle reflex are two largely uncorrelated measures. Similar to startle amplitude, startle latency showed a fear-dependent task modulation with shorter startle latencies indicating more fearful states. Different to startle amplitude, however, patients displayed shorter startle latencies throughout the fear learning task. Importantly, the difference between patients and controls was not learning-related, i.e. the difference did not evolve over the course of fear acquisition or extinction in a stimulus-dependent manner but indicated a more basic physiological, non-associative mechanism resulting in an increase in startle reactivity. Shorter startle latencies during fear-conditioning may therefore indicate increased threat reactivity and potentially serve as a marker of individuals at risk– or patients already fulfilling the diagnostic criteria of a fear-related disorder.

A further difference between amplitude and latency was shown with regard to amygdala morphology. While startle amplitude was not related to amygdala volume, startle latency showed a unique sex-specific association with gray matter volume of the amygdala, especially its basolateral nucleus. Here, shorter latencies in male participants were associated with smaller bilateral amygdalae. This is in accordance with sex-specific associations between brain structure and fear-related pathology. While early life stress in men has been shown to involve grey matter loss in the limbic system, it may lead to an overactive and possibly enlarged amygdala in females (Irle et al., 2010; Warnell, Pecukonis, & Redcay, 2018; Lawson et al., 2017).

Based on the fact that latencies were decreased in a stimulus-unspecific manner across the complete fear-acquisition phase, we assume that the exact order or timing of the stimuli as well as the experimental setup of our fear-conditioning task is not decisive in observing differences between patients and control participants. The important two aspects seem to be presenting repetitive startle sounds, leading to a better estimation of mean startle latency and presenting startle sounds within an additional stressor condition, for example in fear-conditioning or unpredictable threat paradigms, leading to a higher utility of the marker.

Implications of project 2. As the extraction of startle latency features is not common in the current fear-conditioning and anxiety disorder research literature, replication of its associations with amygdala volume and fear-related pathology are urgently needed. For that purpose, existing studies collecting startle responses from healthy or patient populations could be readily re-analyzed. Given successful replication, we assume that shortened startle latencies constitute an endophenotype of fear-related pathology that

represents increased processing speed of amygdala neuronal ensembles under threat. Compared to startle amplitudes, startle latencies may therefore be better suited to capture individual variability informative for fear-related pathology. Further, the fact that the association between startle latency and amygdala volume was specific to males is another example for the need to not only control for, but to also investigate gender and sex as a biological variable in medical research (Bale & Epperson, 2017).

1.4 Hippocampal contributions to pathological memory formation

Rationale and hypotheses. First and foremost, this project was initiated to increase the ecological validity in human fear research by implementing a paradigm inducing more relatable fear experiences and fear memories. To achieve this goal, we turned to the conditioned-intrusion paradigm (Wegerer et al., 2013): Two highly aversive and two neutral movie scenes served as US and four picture frames taken from the respective movies served as CS. The movies are played directly after the picture presentation, leading to a subjective and physiological conditioned fear-response towards the CS (Franke et al., 2021). Previous studies using the trauma film paradigm in different forms have reliably shown that watching highly aversive movie scenes elicits stressful intrusive memories in the following days (Ney, Schenker, & Lipp, 2022) and elicits amygdala activity (Rattel et al., 2019). Consequently, the core advantage of this paradigm is that the US resembles a negative experience and therefore creates a meaningful episodic memory trace while the conditioning of the CS allows to measure learning-induced changes in its neural representation and physiological correlates.

Second, we aimed at investigating more remote time-points of fear-recall, hypothesizing that the relevant time-scales to investigate pathological fear memory formation would exceed the normal one- or two-day paradigms. Bridging the gap to classical episodic memory research (Dunsmoor & Paz, 2015), we assumed that differences in systems consolidation could underlie vulnerability of fear-related disorders. The concept of systems consolidation entails that the brain sites supporting memory encoding and consolidation are organized in different systems whose contributions to storing and recalling memories change over time, repetition and sleep (Cowansage, 2018; Frankland & Bontempi, 2005). One of the core regions involved in the initial encoding and indexing of memories is the hippocampus with neocortical sites gaining importance at more remote time points (McClelland, McNaughton, & O'Reilly, 1995; Moscovitch, Cabeza, Winocur, & Nadel, 2016). Interestingly, the wheres and whens of systems consolidation have recently been shown to be more variable than previously assumed (commented on in Pöhlchen & Schönauer, 2020) and first studies have begun to explore the concept that individual differences in systems consolidation may underlie the formation of pathological memory symptoms, such as flashbacks or trauma-induced amnesia (Krenz, Sommer, Alink, Roozendaal, & Schwabe, 2021). We therefore planned to investigate the physiological and

neural correlates of fear memory encoding and consolidation in the longer term, i.e. over the course of 7 days.

Third, and relatedly, we wanted to investigate how hippocampal processing abilities shape encoding and consolidation of fear memories, exploring and contrasting the hippocampal functions of stress regulation (see 1.1.3) and forming distinct episodic memory traces (see 1.1.4). Cross-sectional findings on structural and functional hippocampal processing impairments in PTSD (Logue et al., 2018; Serra-Blasco et al., 2021; Lambert & McLaughlin, 2019) raise the questions whether hippocampal deficits are a pre-trauma risk factor shaping susceptibility to PTSD and whether one specific hippocampal mechanism leads to the observed memory deficits observed in PTSD. Pinpointing such a candidate mechanism, Lecei and van Winkel (2020) proposed that deficient hippocampal pattern separation may be one of the key processes impaired in PTSD. Pattern separation refers to the process of transforming similar inputs into distinct, non-overlapping representations. Thereby, it could support adaptive mnemonic processes such as the correct disambiguation of safety and threat and binding of the trauma memory to its respective temporal and spatial context (Anacker & Hen, 2017; Lambert & McLaughlin, 2019; Leal & Yassa, 2018; Liberzon & Abelson, 2016).

We therefore investigated whether hippocampal pattern separation abilities shape risk or resilience for pathological memory formation in 60 healthy women in an experimental PTSD model. To that end, we compared one group with low lure discrimination abilities (a behavioral proxy for hippocampal pattern separation (Stark, Kirwan, & Stark, 2019)) to another group with high lure discrimination abilities while they were undergoing the conditioned trauma-film paradigm. We probed hippocampal and pupillometric correlates of fear memory formation and collected information on intrusion distress and contextual memory in the week following the trauma-films.

Summary and discussion of findings. Participants reported high levels of arousal and emotional involvement when watching the aversive films, especially during the first presentations of the movies. In the following week, participants reported transient intrusions of the movie content. This demonstrates that the paradigm induced a fear-memory which remained stressful for a couple of days and that the paradigm succeeded in increasing the ecological validity compared to our classical fear-conditioning task (see project 1, chapter 1.2).

Importantly, as fear-related disorders do not arise over night but develop over time, we sought to prolong common experimental time frames from one or two days to one week, intending to compare hippocampal and pupillometric signaling during encoding compared to remote recall and relating differential hippocampal activity and pupil dilations to pathological memory formation. However, contrasting BOLD activity towards the CS+ and the CS- during the conditioning did not reveal any neural activity (in contrast to, for example Rattel et al., 2019) and differential pupil dilations also did not indicate successful conditioning. For this reason, analyses on CS responding during encoding and remote

fear recall were suspended and the focus was put on neural and pupil responses towards the movies during conditioning.

Focusing on the role of hippocampal processing in shaping pathological memory formation, we compared the two groups with low and high lure discrimination abilities with regard to intrusion distress and contextual memory loss. Contrary to our core hypothesis, both groups showed similar levels of intrusion distress and memory performance, speaking against pattern separation abilities as a generic risk factor in pathological memory formation. This null-finding argues against this specific cognitive hippocampal candidate mechanism (Lecei & van Winkel, 2020) underlying differences in hippocampus-based associative learning in PTSD. Importantly, hippocampal functioning still proved to be associated with intrusion distress. Independent of hippocampal pattern separation abilities, hippocampal reactivity predicted intrusion distress in the following week. More specifically, participants with a blunted stress-related signal to aversive compared to neutral movies reported more stressful memories of the aversive movies.

Apart from its cognitive function, the hippocampus is also a key structure in up – and downregulating the hypothalamus-pituitary-adrenal-gland (HPA)-mediated stress response (Herman et al., 2016). Differences in hippocampal signaling can therefore also be interpreted in light of hippocampal engagement in HPA axis regulation. How could the observed blunted hippocampal signal in response to aversive movie fragments contribute to the formation of a stressful memory? From a mechanistic perspective, blunted glucocorticoid signaling could underlie stronger fear memories by prolonging noradrenergic effects on memory consolidation, leading to strong and vivid memories of the aversive events (H. Cohen & Zohar, 2018). Although we did not measure concentrations or metabolites of noradrenaline during the conditioned-intrusion paradigm, we measured pupil size, serving as an indirect readout of LC-signaling and thus, noradrenaline fluctuations in the brain (Strauch et al., 2022). While tonic LC firing influences baseline pupil size, phasic LC signals, signaling saliency, lead to an additional dilation during threat. In our study, participants who displayed smaller pupil dilations on top of already heightened baseline pupil diameters towards the aversive compared to the neutral movies were also more likely to display a blunted hippocampal stress signal. Our findings of blunted hippocampal signaling and decreased phasic pupil dilations are therefore in line with findings of hypocortisolism (Meewisse, Reitsma, De Vries, Gersons, & Olff, 2007; M. C. Morris, Compas, & Garber, 2012; Schumacher et al., 2019; Speer, Semple, Naumovski, D'Cunha, & McKune, 2019) and heightened central noradrenaline levels in PTSD (Pan, Kaminga, Wen, & Liu, 2018; Geracioti et al., 2001; Naegeli et al., 2018) and suggest that pathological memory formation arises due to differences in the reactivity and most likely also in the interaction of the two main stress systems (see section 1.1.3).

Implications of project 3. In healthy women, intrusion distress was predicted by blunted hippocampal signaling, underlining the notion that individual differences in stress regulatory mechanisms underlie vulnerability to develop fear- or trauma-related disorders.

Importantly, hippocampal pattern separation abilities were not protective of intrusion distress or contextual memory loss.

One of the rationales for applying the conditioned-intrusion paradigm was to increase ecological validity in research of pathological fear. However, ecological validity is often orthogonal to experimental control as for example shown by the trade-off in measuring pupil dilations to complex visual movie scenes compared to simple geometric shapes. It will therefore be essential to pursue experimental environments that aim at keeping experimental control and ecological validity high, as for example done in virtual reality fear research (Binder, Pöhlchen, Zwanzger, & Spoormaker, 2022). To better understand the influence of the SAM on intrusion formation, it future studies should relate pupil time courses to noradrenergic signaling for example by measuring salivary alpha-amylase as a proxy for central noradrenaline (Ali & Nater, 2020) signaling before, during and after stressful experiences and measuring their interactions with the hippocampal BOLD signal under more carefully controlled experimental lighting conditions.

1.5 Sympathetic hyperarousal as a risk factor underlying fear-related psychopathology

The associative learning model of fear-related disorders implies that differences in fear-inhibition and extinction learning underlie the emergence and maintenance of fear-related pathology, comprising diagnoses such as specific phobia, panic disorder or PTSD (Craske et al., 2014; Duits et al., 2015; Kindt, 2014; Marin et al., 2017; Krypotos et al., 2015; Mineka & Zinbarg, 2006; Pittig et al., 2018; Scheveneels et al., 2016). This suggests that capturing learning-related deficits signifies pathology and may thereby serve as an informative diagnostic biomarker (Jovanovic et al., 2010). In order to measure learning-related deficits, the human fear-conditioning field quantifies associative fear learning by tracking the waxing and waning of fear responses under different experimental conditions (Lonsdorf & Merz, 2017).

In the first study of this thesis (Pöhlchen et al., 2020), however, patients with fear-related disorders and healthy controls showed similar response patterns over time and there was also no evidence for a dimensional association between the severity of the disorder and differences in fear acquisition, extinction or recall. In the context of an increasing number of studies reporting similar null findings, our results therefore question the diagnostic validity of the associative fear learning model of fear-related disorders (Abend et al., 2020; Acheson et al., 2015; Duits et al., 2021; Hennings et al., 2020; Wake et al., 2021; Schenker et al., 2022; Adolph et al., 2022). In contrast to a lack of learning related differences, the PTSD subgroup showed generically heightened startle responding during the extinction phase suggesting that a decrease in startle habituation may be more informative in explaining fear-related pathology than differences in associative fear learning.

In the second study of this thesis (Pöhlchen, Fietz, Czisch, Sämann, & Spoormaker, 2022), we pursued this idea and specifically investigated whether distinct characteristics of the

startle response differ between patients and control participants. While the amplitude and habituation of the startle reflex was similar in both groups, participants with a current diagnosis of a fear-related disorder including PTSD showed a significantly faster initiation of the startle reflex across all stimulus types. As startle under threat depends on modulatory inputs of the amygdala (Hitchcock & Davis, 1986; Kuhn et al., 2020) and as shorter startle latencies were associated with smaller amygdala volumes in our male study participants, we assume that patients with fear-related disorders exhibit an increased readiness, or hyperarousal, of amygdala-modulated startle circuits.

The third study of this thesis (Pöhlchen et al., in preparation) investigated fear memory formation using an experimental trauma paradigm, increasing the ecological validity of the classical fear conditioning setups used in the first two studies. Also in this study, there was no evidence for associative learning-related differences in predicting pathological fear memory. Instead, hippocampal reactivity during aversive movies predicted intrusion distress, a core mnemonic symptom of PTSD. Additionally, differential hippocampal signaling was associated with differential pupil dilations, a psychophysiological proxy measure for sympathetic output. Although highly speculative, these associations could indicate an interactive role of the HPA-axis and the sympathetic nervous system in shaping risk for the development of pathological fear memory.

Together, the results of all three studies speak against associative learning-related differences as key characteristics of fear-related pathology and thereby against one of the dominant theories on the emergence and maintenance of fear-related disorders. Different lines of research had previously indicated that generically disturbed associative learning mechanisms lie at the core of fear-related disorders and that malfunctioning of the neural circuits promoting fear and extinction learning characterizes fear-related pathology (Craske et al., 2014; Duits et al., 2015; Kindt, 2014; Marin et al., 2017; Krypotos et al., 2015; Mineka & Zinbarg, 2006; Pittig et al., 2018; Scheveneels et al., 2016; Jovanovic et al., 2010). The work of this thesis, however, suggests that fear-conditioning is not the best model to study the development and maintenance of pathological fear.

Instead, we propose that more basal physiological hyperarousal underlies fear-related pathology as revealed by shorter startle latencies and dampened hippocampal deactivation to aversive film fragments. In order to foster the discovery of reliable and practical biomarkers, translational fear research should incorporate basic threat reactivity paradigms, focusing on non-associative in addition to associative mechanisms.

What may be the mechanism underlying increased threat reactivity? Animal work has shown that enhanced noradrenergic activity in the amygdala leads to increased behavioral hyperarousal (Ronzoni, del Arco, Mora, & Segovia, 2016) and a hyperactive amygdala has also been described as a human hallmark of fear-related disorders, especially in PTSD (Badura-Brack et al., 2018). The core site of NA-output and NA-regulation in the brain is the LC, a structure in the brainstem comprising about 3000 cells. Importantly, the LC is strongly connected to the pupil, activating the sympathetic dilator muscle

in the iris through alpha-1 receptors and indirectly influencing the parasympathetic sphincter muscle in the iris (Samuels & Szabadi, 2008). Thereby, LC-NA activity is strongly connected to dilations and constrictions of the pupil (Aston-Jones & Cohen, 2005), allowing us to infer on sympathetic signaling by continuously measuring the pupil size (Strauch et al., 2022). Despite its small size, the LC has significant influences over a variety of cognitive processes. Against common assumptions, the LC is neither structurally nor functionally homogeneous. Through different firing modes and action sites with their specific noradrenaline receptor profile it increases global arousal but can also exert local, tailored effects (Poe et al., 2020). Under stress, the LC firing mode changes from low tonic to high tonic activity, resulting in an increase in alertness and hypervigilance at the cost of focused, exploratory behavior (Aston-Jones & Cohen, 2005; Arnsten, 2000). Importantly, a recent study in rodents demonstrated that a pathway from the LC to the Ce underlies defensive behavior under threat. Further, studies in humans showed that perceived stress levels increase LC – amygdala connectivity (Gu et al., 2020) and that PTSD patients displayed higher LC - amygdala connectivity under threat (Steuwe et al., 2015), indicating a role of LC-amygdala noradrenergic signaling in fear-related pathology. Compared to trauma-exposed controls, patients exhibit larger NA concentrations in their cerebrospinal fluid (Geracioti et al., 2001), display stronger LC activation to loud sounds (Naegeli et al., 2018) and fearful stimuli (Morey et al., 2015) and LC size was shown to be associated with anxious arousal across diagnostic boundaries (L. S. Morris, McCall, Charney, & Murrough, 2020). Although human studies on LC-activity have to be interpreted with caution due to the difficulties in measuring such a small area in vivo, these findings suggest that threat-induced LC (and LC-amygdala as well as LC-hippocampus) signaling is a prime candidate in explaining hyperarousal symptoms. Accordingly, L. S. Morris et al. (2020) argue that disturbances in LC functioning underlie clinical symptoms in fear-related disorders, for example shown by sympathetic nervous system arousal in PD or PTSD. Further demonstrating the role of noradrenergic signaling in fear-related disorders, pharmacological agents interacting with adreno receptors such as alpha-1 blockers or beta-blockers are employed to reduce fear-related symptoms (Koola, Varghese, & Fawcett, 2013; Steenen et al., 2016) and nightmares (Kung, Espinel, & Lapid, 2012). Both receptor types have been shown to promote fight-or-flight behaviors (Arnsten, 2000). Importantly, these medications are not effective or tolerable in all patients, suggesting the existence of subgroups with regard to noradrenergic malfunctioning (Hendrickson & Raskind, 2016). Another open question is whether LC-NA mediated effects on fear-related symptoms arise based on generic baseline differences in LC output and connectivity or whether they are driven by specific LC action modes and circuits, a view that would be supported by the work of this thesis showing that startle reactivity under threat was associated with fear-related pathology. The former assumption would indicate that measures such as resting state fMRI, startle responsivity under resting conditions or the baseline pupillary light reflex should be informative with regard to fear-related pathology. The latter would stress the importance of probing the LC-NA system or its proposed associated whole-brain

network "salience network" comprising the dorsal anterior cingulate and bilateral insula under stressful or even threatening conditions.

Circling back to the associative learning model of fear-related disorders, two further points are important to make. First, our focus on LC-mediated hyperarousal as a prime candidate in explaining fear-related pathology does not speak against the existence of subtle learning related differences in fear-related disorders per se. Increased noradrenergic signaling leads to stronger encoding and consolidation of emotional memories (Schwabe et al., 2022) and may also be causing the effects of disorder-specific differences in fear and extinction learning as reported in Duits et al. (2015). However, we argue that such differences may be driven by differences in LC-NA signaling in the first place, favoring more direct measures of hyperarousal under threat over very specific experimental fear-conditioning setups. Second, our critique of the diagnostic validity of this model has no implications on the efficacy of exposure-based treatments. Exposure therapy is effective in alleviating fear and corrective experiences are central in inhibiting already established fear memories(Carpenter et al., 2018; Bandelow et al., 2015). However, under the assumption that increased signaling in LC-amygdala pathways underlies fear-related pathology, different therapeutic methods and pharmacological interventions targeting this circuit and downregulating the amygdala should result in an an improvement of symptoms. Such approaches could, for example, involve an increase in prefrontal control over the amygdala through cognitive behavioral therapy (Brooks & Stein, 2015) or transcranial magnetic stimulation (Cirillo et al., 2019), a downregulation of amygdala activity via working memory interventions (de Voogd et al., 2022) or pharmacological interventions targeting specific LC subsystems (Poe et al., 2020).

The role of the amygdala in shaping physiological fear-responses was also stressed by the correlation between amygdala size and startle latency in Pöhlchen et al. (2022). Importantly the protective pattern of a larger amygdala volume relative to total intracranial volume was manifest only in males, pointing to a sex-specific association between brain structure and physiology and, more generally, sex-specific circuits underlying fear-related psychopathology. This finding could be explained by distinct effects of stress exposure on amygdala morphology. While early stress exposure in females may lead to a possibly enlarged and overactive amygdala, early life adversity in males may involve grey matter loss in the limbic system (Irle et al., 2010; Warnell et al., 2018; Lawson et al., 2017). Additionally, sex differences have also been described regarding LC activity and HPA-axis stress regulation (Bangasser, Wiersielis, & Khantsis, 2016). Poe et al. (2020) propose that rodent work showing increased LC sensitivity to CRF in females (Bangasser et al., 2010) could translate to a greater LC-NA response to stress, potentially underlying the higher prevalence of stress-related psychiatric disorders in females. Future studies should therefore focus on the interactions of biological sex, cycling hormones, and the main stress systems in shaping pathological fear.

Finally, next to the project-specific limitations mentioned in the respective articles, general limitations should be kept in mind in the overall interpretation of the work performed in this thesis. First, all studies reported on involved either patients with light to moderate impairments or healthy controls, precluding conclusions regarding severely affected individuals. This selection bias is rooted in the fact that severely impaired patients often undergo psychopharmacological treatment that strongly influences neural and psychophysiological responding, making it difficult to attribute effects to the disorder and not the medication. Second, despite the efforts to increase ecological validity, it is quite a leap to draw on experimental models with relatively mild stressors in order to conclude on pathology resulting from highly stressful experiences or life-time adversities based.

Taken together, the current state of knowledge and the open questions arising from the work in this thesis indicate that the connection between symptom profiles and disturbed LC-NA signaling are not understood well enough. It would therefore be of interest, to quantify the impact of maladaptive noradrenergic signaling as well as its influence on LC-Amygdala pathway on fear-related pathology (Hendrickson & Raskind, 2016). One potential way forward would be to apply longitudinal pupillometric and startle measurements in a transdiagnostic sample exhibiting fear-related symptoms. Both methods exhibit comparably high test-retest reliablity and are suited as surrogate markers of LC and Amygdala signaling if probed under baseline and threatening conditions (Kuhn et al., 2020; Strauch et al., 2022). Similarly, the associated whole-brain effects and salience network activity could be helpful to study. Given recent advances in functional and structural neuroimaging in humans (Suárez-Pereira et al., 2022), it may even be possible to directly relate fear-related physiology to LC activity and morphology. Combining psychophysiological and neuroimaging measurements with continuous sampling and experimental manipulations of noradrenergic agents could therefore not only inform about underlying pathogenic mechanisms, but potentially also inform treatment selection and treatment monitoring.

1.6 Conclusion

Fear-related disorders such as panic disorder, specific phobias, or PTSD, are highly straining and among the most prevalent psychiatric disorders, making fundamental research on their pathogenic mechanisms an important priority. Due to the heterogeneity within the same diagnostic label, however, it is difficult, or even impossible, to determine comprehensive biological underpinnings. Instead, it seems more promising to investigate pathology at a domain level, targeting the idea of clearly circumscribed biological endophenotypes that could be relevant for pathological subgroups within and across diagnostic boundaries (Insel et al., 2010). As a consequence of an increased fundamental understanding of the neurobiological underpinnings of specific symptoms, targeted treatment options can be developed (Arns, van Dijk, Luykx, van Wingen, & Olbrich, 2022; Kessler & Luedtke, 2021). Ultimately, the hope is to move away from a purely symptom-based diagnostic system to one that incorporates a process-view of psychiatric pathology (Elbau et al., 2019).

One of the most promising process-level views of fear-related disorders is that they arise and manifest based on differences in associate fear-learning mechanisms, presenting a unifying mechanism underlying currently distinct diagnoses (Graham & Milad, 2011). This thesis therefore aimed at uncovering learning-related imaging and psychophysiological endophenotypes of fear-related disorders. Critically, all articles presented in the context of this thesis demonstrate that fear-related disorders are not characterized by distinct fear-learning profiles (Pöhlchen et al., 2020, 2022, Pöhlchen et al, in preparation). Even if small differences in fear-conditioning existed, they would therefore be too small to be suited as a cross-diagnostic biomarker. Instead, findings provide evidence for a hyperarousal-account of fear-related disorders which is likely mediated by effects of LC signaling on limbic circuits. Our results therefore speak for shifting from highly elaborate fear-conditioning paradigms toward more basic fear-eliciting paradigms that focus on the extraction of stable and reliable physiological responses.

Circling back to the very beginning, it is essential to stress that the etiology of psychiatric disorders is highly multi-factorial and arises based on interactions between the individual genetic background and environmental factors. In my opinion, this gene by environment model also suggests that fundamental, neuroscientific research aiming at a biological understanding of psychiatric disorders is just one part of the endeavor to support affected persons. While this thesis focuses on individual mechanisms, it is also important to keep in mind, that the largest lever for improving mental health is to create a societal and political system that gives room to the variance in human experience but also accepts its responsibility in supporting mental health.

2 Project 1 | Assessing fear-learning markers in patients with fear-related disorders

2.1 Contributions and reference

The study "No robust differences in fear conditioning between patients with fear-related disorders and healthy controls" was published in *Behaviour Research and Therapy* in 2020.

DP and LL collected the data. DP and VIS conceived the method. DP, FPB, BB, TN, PT and VIS performed the data analysis. DP wrote the manuscript under supervision of VIS. VIS, TB and the BeCOME working group were responsible for the concept and design of the BeCOME study. All authors contributed to the interpretation of findings, provided critical revision of the manuscript for important intellectual content, and approved the final version for publication.

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No robust differences in fear conditioning between patients with fear-related disorders and healthy controls

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ABSTRACT

Fear conditioning and extinction serve as a dominant model for the development and maintenance of pathological anxiety, particularly for phasic fear to specific stimuli or situations. The validity of this model would be supported by differences in the physiological or subjective fear response between patients with fear-related disorders and healthy controls, whereas the model's validity would be questioned by a lack of such differences.

We derived pupillometry, skin conductance response and startle electromyography as well as unconditioned stimulus expectancy in a two-day fear acquisition, immediate extinction and recall task and compared an unmedicated group of patients (n = 73) with phobias or panic disorder and a group of patients with posttraumatic stress disorder (PTSD, n = 21) to a group of carefully screened healthy controls (n = 35).

Bayesian statistics showed no convincing evidence for a difference in physiological and subjective responses between the groups during fear acquisition, extinction learning or recall. Only the PTSD subgroup had altered startle reactions during extinction learning.

Our data do not provide evidence for general differences in associative fear or extinction learning in fear-related pathologies and thereby question the diagnostic validity of the associative fear learning model of these disorders.

1. Introduction

About a 100 years ago, Watson and his colleagues were among the first to experimentally research emotional learning. They were investigating putative processes underlying the development of the rich emotional and behavioural repertoire seen in adults. In their observations, children showed only fundamental modes of emotional response (love, rage, and fear) that were elicited by very few and specific situations. One of their hypotheses was that "by the method of conditioned reflexes, emotional reactions can be called out by situations (stimuli) which do not at first call them out" (Watson & Morgan, 1917). Their most famous experiment (Watson & Rayner, 1920), known as the "Little Albert" study, provided experimental evidence that fear could be conditioned to a previously neutral white rat (conditioned stimulus, CS) by pairing it with a loud noise (unconditioned stimulus, US). Although Watson and Rayner (1920) did not include an explicit extinction phase, they noted that little Albert's fear response diminished after the passage of time and had to be freshened up by presenting the white rat again together with the loud noise. Due to limited time, they could not finish their research on experimental procedures to again remove little Albert's conditioned emotional responses. However, they already suggested repeatedly confronting Albert with the CS, possibly resulting in a fatigue of the original reflex and thereby provided an experimental design to induce extinction learning.

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Since then, research on human fear acquisition and extinction learning has extended its focus to investigating the development of pathological fear (Duits et al., 2015; Lissek et al., 2005). Fear acquisition and extinction learning serve as the main experimental models for the acquisition and maintenance of anxiety and fear-related disorders, offering explanatory pathways through which anxiety and fear develop and persist (Milad & Quirk, 2012; Norrholm & Jovanovic, 2018; Pittig, Treanor, LeBeau, & Craske, 2018). Notably, exposure therapy is grounded in the principles of extinction training and is the treatment of choice in most anxiety disorders (Barlow, 2018; Bouton, 2004; Marin et al., 2017; Milad & Quirk, 2012; Tolin, 2010).

During fear acquisition, a formerly neutral stimulus becomes a danger cue (CS+) by pairing it with an aversive event (the US). This leads to the acquisition of a conditioned fear response towards the CS +. In differential fear conditioning, another safe stimulus is presented (CS-) that is never followed by a US. During extinction learning, the CS + is repeatedly presented without the US, leading to a decrease in the conditioned fear response. Upon re-exposure to the CS + after extinction during a recall session, a return of the conditioned fear response is frequently observed (Vervliet, Baeyens, Van den Bergh, & Hermans, 2013). The strength of human fear and extinction learning is quantified by comparing the response to the CS + to the response to the CS- across fear acquisition, extinction, and recall sessions (Lonsdorf et al., 2017). Physiological responses, such as an increase in pupil dilations, skin conductance response (SCR), or startle electromyography (EMG) are recorded as readouts for a differential fear response. Ratings of US expectancy are typically employed as subjective measure of fear learning (Norrholm et al., 2006).

Theories on pathological fear include abnormalities in conditioned fear as a key etiological feature (Barlow, 2018; Mineka & Oehlberg, 2008; Norrholm et al., 2015). Importantly, fear acquisition and fear extinction are thought to be independent learning processes relying on partly distinct neural structures (Bouton, 2004; Quirk & Mueller, 2008). In differential fear conditioning procedures, pathological fear might therefore be reflected in an increased acquisition of the fear response to the CS+, a generalized fear response to the CS-, reduced inhibitory safety learning during extinction, or a combination thereof (Lissek et al., 2005).

To investigate whether patients with anxiety disorders indeed show differences in fear acquisition or extinction and to summarize the large number of studies on this subject, Duits et al. (2015) conducted a meta-analysis that expanded upon a previous meta-analysis by Lissek et al. (Lissek et al., 2005). In short, patients with anxiety disorders (mostly PTSD) showed a weak-to-moderate increase in fear responding to the conditioned safety cue ($d \sim 0.4$). During fear extinction, patients responded more strongly to the unreinforced CS + than control participants, indicating impaired extinction learning ($d \sim 0.35$). Although the authors reported no significant moderators of these effects, the effect size for extinction (CS + minus CS-) for studies employing electric shock as the US was around zero. The effect size for other US types was about 0.2, which was driven by three studies using disorder-specific US (e.g., images of phobic objects or situations), that revealed a large effect of $d \sim 0.7$ (Lissek et al., 2008; Schweckendiek et al., 2011; Wessa & Flor, 2007). In line with such subtle group differences in differential fear learning, another recent meta-analysis comparing anxious and non-anxious youth also did not detect differences in differential fear acquisition or extinction learning (Dvir, Horovitz, Aderka, & Shechner, 2019). This places doubt on the notion that pathological anxiety is a consequence of a general impairment in extinction learning.

Here we report a comparison of an unmedicated and clinically thoroughly characterized group of patients with acute fear-related disorders without PTSD, a group of PTSD patients and a group of healthy controls, who have never had a fear-related disorder, in a two-day classical fear acquisition, extinction and recall paradigm. We hypothesized that the diagnosis of a fear-related disorder or PTSD is reflected in altered fear- and extinction learning as well as in a different responding during recall. We were specifically interested in various complementary psychophysiological readouts, including pupillometry, the SCR, and startle EMG, allowing us to draw conclusions on the specific mechanisms underlying possible group differences.

2. Methods

2.1. Participants

The sample was recruited as part of the Biological Classification of Mental Disorders (BeCOME) study at the Max Planck Institute of Psychiatry (Brückl et al., 2019 submitted manuscript, registered on ClinicalTrials.gov: NCT03984084). The BeCOME study aims to characterize participants with a broad spectrum of affective, anxiety, and stress-related mental disorders as well as unaffected individuals along basic systems of human functioning (e.g. acute threat response or reward processing). The goal of BeCOME is to help identify biologically informed subtypes of mental disorders. To do so, participants undergo a thorough diagnostic and psychometric evaluation, omics assessments and in-depth phenotyping procedures. One of the applied paradigms is a fear conditioning task. Analyses on the first batch of control participants in this task (n = 51) have been published in Leuchs, Schneider, and Spoormaker (2018). The analyses presented here are based on an enrolled sample size with fear conditioning data of 226 participants. The study protocol was in accordance with the Declaration of Helsinki and approved by a local ethics committee (reference number: 350-14). All participants provided their written informed consent after the study protocol had been fully explained and were reimbursed for their participation.

To asses diagnostic information and thereby build our groups of interest, participants underwent a computer-based slightly modified version of the Munich-Composite International Diagnostic Interview (DIAX/M-CIDI, Wittchen & Pfister, 1997). This interview assessed symptoms, syndromes and diagnoses of the following mental disorders according to DSM-IV and ICD-10 criteria: nicotine use and dependence, anxiety disorders, depressive episodes and dysthymia, mania and bipolar disorders, psychoses, alcohol use, obsessive-compulsive disorders, illegal substance use and PTSD. The interview was conducted face-to-face by trained study assistants. For every participant, information on onset, duration and severity of these disorders was collected. In our analysis we differentiated between diagnoses that were present within the past year (defined as a current diagnosis) and those which were present in the past but did not occur within the past 12 months (defined as past lifetime diagnosis).

Of the overall sample (n = 226), 16 participants were excluded because they met BeCOME exclusion criteria of a current organic affective disorder or substance abuse. Strict criteria were used to define the control group: only participants without any current or past lifetime, full or subthreshold mental diagnoses were included, resulting in a control sample size of n = 35 (M_{age} = 32.7, SD_{age} = 10.1, 14 male). Every participant with a current specific phobia, social phobia, agoraphobia without or with panic attacks, or PD, but not current PTSD was included in the fear-related disorder group of n = 73 (M_{age} = 33.6, SD_{age} = 11.3, 18 male), regardless of other comorbid disorders. Patients with current PTSD were included in the PTSD group of n = 21 (M_{age} = 35.7 SD_{age} = 12.2, 11 male). Groups did not differ in age (BF_{10} = 0.125) or sex (BF_{10} = 0.756). The remaining participants meeting at least subthreshold lifetime criteria for a mental diagnosis other than a current fear-related disorder were not included in the analysis. The comorbidity pattern in the combined fear-related and PTSD group is presented in Table 1.

2.2. Psychometrics and calculation of impact and severity scores

Next to a battery of other psychometric questionnaires, participants filled out the Beck-Depression-Inventory (BDI-ll; Beck, Steer, & Brown,

Table 1
Comorbidity table on the sample of fear-related disorders including PTSD.

	Frequency of comorbid DSM-IV diagnoses in the group with fear-related disorders including PTSD (N = 94)			
	12-month		Past lifetime (but no 12-month diagnosis)	
	n	%	n	%
Specific phobia	54	57.5	9	9.6
Social phobia	30	32.0	5	5.1
Agoraphobia	9	9.6	1	1.1
Panic disorder with agoraphobia	21	22.3	1	1.1
Panic disorder without agoraphobia	10	10.6	1	1.1
PTSD	21	22.3	8	8.5
MDD	53	56.4	6	6.4
Dysthymia	36	38.3	0	0.0
Generalized Anxiety Disorder	27	28.7	0	0.0

1996) to assess depressive symptom severity within the past two weeks. To investigate the impact of fear-related disorders, we extracted responses from the CIDI item asking how fear and avoidance behaviour impact the daily life. This question was asked for every anxiety disorder. The question was scaled between 1 ("did not impair me at all") and 4 ("did impair me very much") and for each participant we took the highest value across the different fear-related disorders. Furthermore, symptom severity was estimated based on combined disorder specific questions. For PD this was simply the number of panic attacks in the last four weeks. For the phobias the presence of symptoms, scored from 1 ("almost never present") to 3 ("almost always present") was used as a proxy for severity. These scores were z-transformed within each category. If a participant had multiple fear-related disorders, we again chose only the highest value across categories.

2.3. Stimuli (CS and US)

We used three CS consisting of a square, a circle, and a rhombus in three different colours of equal brightness and surface. Stimulus assignments were sequentially randomly allocated, resulting in three assignment groups. One of the CS served as CS- and two served as CS+. The stimulus appeared for 4–4.8 s on a black background. US were presented at 4 s after CS onset. Stimuli were separated by a white fixation cross and intertrial intervals (ITIs) were jittered between 10 and 16 s.

Each CS+ was reinforced by one of two US with a 75% reinforcement rate. One US was a mild electric shock delivered to the wrist of the right hand (20 ms duration). Shock strength was individually set to be very unpleasant but not painful via a staircase procedure (0.5 mA steps, starting from 0.5 mA). Shocks were delivered with a Linear Isolated Stimulator (Stimsola, BIOPAC Systems, Inc., Goleta, CA, USA). The other US was a 250 ms 9 bar air blast to the larynx from approximately 1–2 cm (see Jovanovic et al., 2005). In addition to the US, we applied startle probes during 75% of CS presentations and during all 16 s ITIs. Startle probes consisted of 40 ms white noise at 108 dB with near-instantaneous rise time and were presented 3 s after CS onset, and 5.33 s before ITI offset, respectively.

2.4. Procedure

Participants underwent non-instructed fear acquisition followed by extinction on day 1 and recall on day 2 (see Fig. 1). Results from the subsequent reinstatement phase are not presented here. Participants arrived between 10 and 11 a.m. in our lab without windows that was optimized for constant light conditions. After electrode placement and adjusting the device to deliver the air blasts, participants were situated to a head-rest in front of a computer screen. Participants were informed that their physiological responses were recorded to measure bodily reactions to stress and were instructed to find out how the electric shock and the air blast were associated with the different stimuli. The experimental procedure started with a habituation phase, in which the startle sound was presented four times in a row interspersed with ITIs of 10, 12, 13 and 15 s. This was followed by three presentations of each CS and two mock ratings, in which participants could familiarize themselves with the computer-based rating procedure (pre-rating). During the following fear acquisition run, each CS was presented 12 times in a pseudorandomized order with no more than two consecutive presentations of the same stimulus type. In nine out of 12 CS + trials, the stimulus-specific US (mild shock or air blast) were presented at 4 s after CS + onset. Fear acquisition was divided into three blocks of four trials per CS. Participants were asked to rate their shock and air blast expectancy towards all CS after every block (fear acquisition ratings 1–3) by adjusting a visual slider ranging from 0% to 100% in steps of 10%. Participants could first indicate the probability of the occurrence on an air blast and second of the electrical shock. Directly after the last rating of the fear acquisition block, the non-instructed extinction block began with CS- and CS + shock presented 10 times each without the US. The CS + air was not shown during this phase. Again, participants were asked to rate their US expectancy after each block (2 blocks of 5 trials per CS, extinction ratings 1–2). After the last extinction block, participants also indicated their discomfort with the shock, the air blast, and the startle noise.

On the following day, participants returned at 9 a.m. The experimental set-up was identical to the previous day and participants were informed that their task was again to report the contingencies between the electric shock, the air blast and the different stimuli. After the pre-rating, all three CS were presented eight times each without reinforcement. Following ratings were presented after blocks of four trials per CS (ratings 1–2). Startle probes were applied during all experimental phases on both days. In our analysis we do not include trials of the CS followed by the air puff (but see Fig. S1 for startle reactions to the CSair).

2.5. Physiological recordings and readouts

Within each measurement category, valid responses (see below) were z-transformed over trials within participants across both test days to ensure comparability between participants. Responses were averaged across blocks. Detailed information on recording and preprocessing are presented in Leuchs et al. (2018) and in the methodological overview paper of the BeCOME-study (Brückl et al., 2019 submitted manuscript).

Pupil dilation. Pupil size and gaze coordinates were recorded after a standard nine-point calibration using the EyeLink 1000Plus desktop system (SR Research Ltd., Ottawa, Canada) with a sampling rate of 250 Hz. We tracked only the right eye, as the area under the left eye was occupied with EMG electrodes to measure startle reactivity. Eye tracking data was processed and analysed in MATLAB (version R2018a, MathWorks, Natick, USA). Missing data resulting from blinks were linearly interpolated between the last saccade before blink onset and the last saccade after blink offset. Saccade markers were provided by EyeLink software (SR research Ltd). Interpolated data were smoothed with a sliding window of 400 ms. Trial wise pupil dilations were calculated by subtracting the maximum pupil dilation within 2–4 s after stimulus onset from the average pupil size during a 500 ms baseline period preceding stimulus onset. Pupil size and gaze coordinates were segmented around trials (500 ms before CS onset to 4 s after CS onset) and automatically inspected for the following three different types of artefacts as grounds for exclusion. First, individual trials were excluded when over 50% of data points within one segment had to be interpolated (1.9 ± 6.5%, M ± SD, of trials during day 1, 1.9 ± 6.6% of trials during day 2). Three subjects were excluded due to excessive

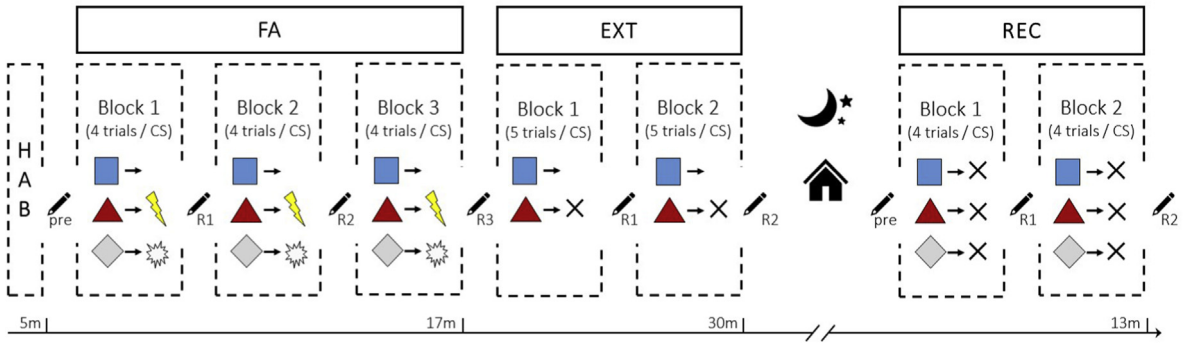

Fig. 1. Task. Participants underwent a differential fear acquisition, extinction and recall procedure. During three blocks of fear acquisition, one CS+ (red triangle) was followed by a shock and another CS+ (grey route) was followed by an air blast. One CS- (blue square) was never followed by any US. During two blocks of fear extinction, only the CS + followed by shocks was extinguished. During the recall session, all CS were presented again. Both CS+ were not reinforced during recall. Day 1 and day 2 were spaced apart by ~24 h. CS colours and shapes are chosen for illustrative purposes. HAB = habituation, FA = fear acquisition, EXT = fear extinction, REC = recall session, R = subjective rating. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

blinking (resulting in over 30% missing trials on each test day). Second, we disregarded trials with sudden jumps in pupil size that are likely caused by recording artefact. To do so, we split each trial into 5 sub-segments (500 ms baseline and four 1 s segments covering the stimulus presentation) and calculated the standard deviations for these sub-segments across all trials per participant. Trials deviating by more than 3.3 SD from the participant's average deviation in any of the sub-segments were excluded (1.2 ± 1.8% of trials during day 1, 8.8 ± 20.4% of trials during day 2). Third, we excluded trials when the gaze was not directed at the centre of the screen for more than 500 ms (14.1 ± 19.4% of trials during day 1, 13.5 ± 18.8% of trials during day 2). For this purpose, we defined a cut-off window around the participant's median gaze position across all trials. The limits of this window were informed by the mean gaze deviation across all participants on day 1. There was overlap between participants excluded for each criterion, resulting in 16.3 ± 19.8% of trials excluded on day 1 and 21.3 ± 25.4% on day 2. Pupil specific exclusion criteria or missing data on one of the test days led to a (block-wise) exclusion of 14 participants (five fear-related and three PTSD patients) from analyses of fear acquisition, 13 participants (four fear-related and three PTSD patients) from analyses of fear extinction and 26 participants (13 fear-related and four PTSD patients) from analyses of recall.

SCR. Skin conductance was recorded in μS via Ag/AgCl electrodes on the left palm at a sampling rate of 2000 Hz. The signal was amplified and transmitted using a wireless system and recorded in AcqKnowledge (BioNomadix amplifiers and receivers transmitting to a MPI150 monitoring system, all BIOPAC Systems, Inc.). Data were low-pass filtered at 1 Hz in AcqKnowledge. All further preprocessing and analysis steps were performed in MATLAB. After downsampling to 1000 Hz, SCR was segmented around trials (500 ms before CS onset to 4 s after CS onset). To quantify the SCR, the peak skin conductance between 2 and 4 s after CS onset was identified. To define the amplitude, the segment preceding the peak was searched for a minimum between 1 s after CS onset and the time of the peak. Trials where no peak could be detected were scored as zero and included in the analysis (23.7 ± 19.5% of trials during day 1, 22.9 ± 17.2% of trials during day 2). Three subjects were excluded for having over 67% of zero responses. When the standard deviation of a segment deviated by more than 3.3 SD from the mean SD across all trials, it qualified as an outlier and was excluded (4.4 ± 3.0% of trials during day 1, 4.5 ± 3.1% trials during day 2). Overall, SCR specific exclusion criteria or missing data on one of the test days led to an exclusion of five participants (two fear-related and two PTSD patients) from fear acquisition and extinction and six participants (four fear-related and one PTSD patient) from analyses of recall.

Startle EMG. Startle EMG was tracked with electrodes placed on the left orbicularis oculi muscle and a reference electrode behind the left ear using a wireless system (BioNomadix amplifiers and receivers transmitting to a MPI150 monitoring system, BIOPAC Systems, Inc.). Data was band-pass filtered between 28 and 400 Hz, rectified and low-pass filtered at 40 Hz (Blumenthal et al., 2005) in AcqKnowledge. All further steps were done in MATLAB. Startle data was downsampled to 1000 Hz and segmented around startle probes (50 ms baseline before startle onset and 200 ms after startle offset). Startle response was quantified as the amplitude between the baseline and the maximum response in a window of interest from 20 to 120 ms after the startle probe. Trials were excluded if the SD or maximum during the baseline exceeded the SD or maximum in the window of interest (9.4 ± 12.7% of trials during day 1, 9.0 ± 12.4% of trials during day 2). If participants had over 30% outlier trials within a day, they were excluded from startle analysis (14 participants from day 1, 10 participants from day 2). Overall, startle specific exclusion criteria or missing data on one of the test days led to an exclusion of 24 participants (11 fear-related and five PTSD patients) from fear acquisition, 21 participants (10 fear-related and five PTSD patients) from fear extinction and 20 participants (10 fear-related and six PTSD patients) from analyses of recall.

2.6. Statistical analyses

Group comparison analyses were Bayesian analyses as implemented in the software packages JASP 0.11.1. (https://jasp-stats.org/). We performed Bayesian repeated measures (rm) ANOVAs with group (fear-related vs. PTSD vs. healthy controls) as a *between* subject factor and stimulus (CS + shock, CS-) and time (blocks per task phase) as the *within* subjects factors. Stimulus assignment type was added as a covariate of no interest to the null model. For the startle analyses, the response to the ITI was added as an additional level to the stimulus factor.

In a Bayesian rmANOVA, the different models are compared on their likelihood given the data. Models include the null model (including the subject factor and stimulus assignment type), the models with the single factors, the models with two factors, the models with two factors plus any two way interaction with any or both of these factors, and the models with three factors as well as three factors and any two – or three-way interaction with either factor, resulting in a comparison of 19 possible models. The prior probability is equally distributed over all options (0.053) and the probability of the model given the data, P(M|data), provides the most relevant output. The different models (e.g. the null model, the model including group as a main factor and the model including group an interacting factor etc.) are compared against each other and receive different portions of posterior evidence based on their fit to the data. We refer to the model receiving the highest proportion of posterior evidence as the “winning model”.

When comparing two hypotheses, e.g. in a t-test or a correlation, the

posterior evidence for a hypothesis (alternative hypothesis, 1) compared to the null hypothesis (0) can be expressed as the Bayes Factor$_{10}$ (BF_{10}). While a BF_{10} around 1 provides no evidence for either the presence or the absence of an effect, a BF_{10} between 1 and 3 can be interpreted as anecdotal evidence in favour of the alternative hypothesis. A BF_{10} above 3 is seen as moderate evidence for the presence of an effect, while a BF_{10} above 10 provides already strong evidence for the presence of an effect. Conversely, a BF10 lower than 1/3 can be interpreted as moderate evidence in favour of the null hypothesis (Wagenmakers et al., 2018).

We further assessed all group effects and interactions of the various phases with additional frequentist statistics for comparability with previous work. This comes at the cost of an increased false positive rate due to the amount of tests (48 effects and interactions in 12 rmANOVAs: one group effect and three interactions with group per rmANOVA). To test the robustness of possible group effects or interactions with group, outliers were defined based on a deviation of the individual mean from the group's SD x 3.3. Next, the rmANOVAS were repeated without these outliers.

To investigate, whether the effect of symptom severity and impact – determined by the M-CIDI interview – had an effect on our results, differential scores between CS+ and CS- were calculated for all measures (pupil dilations, SCR, startle EMG and ratings) per block of interest (last fear acquisition block, last extinction block, first recall block). Possible associations of these readouts were tested using Bayesian correlations (stretched beta prior width = 1, which corresponds to a beta(1,1) distribution transformed to cover [-1,1], see Wagenmakers et al., 2018).

3. Results

Pupil dilation. For pupil dilation (Fig. 2) the best model for fear acquisition contained the time and stimulus effect P(M|data) = 0.71 (Table S1). For fear extinction, the best model was the model including time with P(M|data) = 0.40 (Table S2). In the recall session, the model containing time and stimulus and their interaction received most evidence P(M|data) = 0.67 (Table S3). There was no evidence for models including either a group effect or an interaction with group (stimulus x group, time x group, time x stimulus x group) during any task phase.

SCR. Regarding the SCRs (Fig. 3), Bayesian repeated measures ANOVAs for fear acquisition showed similar effects as in pupillometry. Here, the best model contained the time and stimulus effects P(M|data) = 0.89 (Table S4). For fear extinction, the best model contained only the stimulus effect P(M|data) = 0.68 (Table S5). In the recall session, the model containing time and stimulus and their interaction received most evidence P(M|data) = 0.85 (Table S6). Again, there was no evidence for a model containing a group effect in any task phase.

Startle EMG. In startle EMG (Fig. 4), the best model in fear acquisition contained stimulus, time and their interaction P(M|data) = 0.55

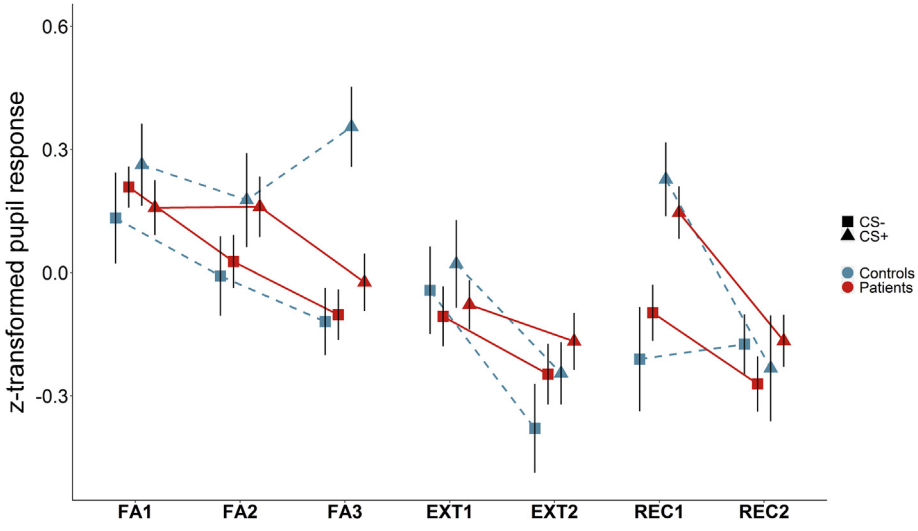

Fig. 2. Z-transformed pupil response during fear acquisition (FA), extinction (EXT) and recall (REC) for healthy controls and patients with fear-related disorders. Dashed lines represent responses from healthy controls, straight lines represent responses from the fear-related group. For plots including the PTSD group, please refer to Fig. S2. Black bars represent standard error of the mean (SEM).

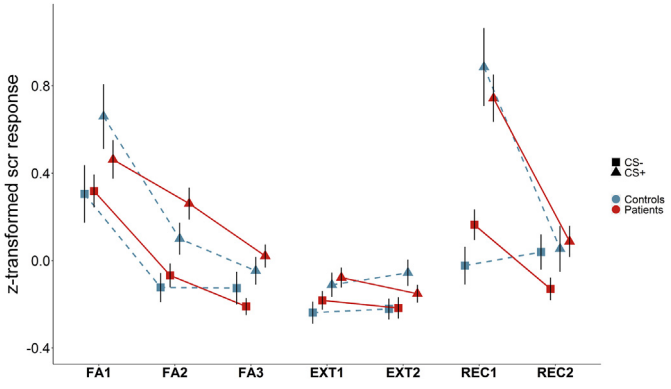

Fig. 3. Z-transformed SCR during fear acquisition (FA), extinction (EXT) and recall (REC) for healthy controls and patients with fear-related disorders. Dashed lines represent responses from healthy controls, straight lines represent responses from the fear-related group. For plots including the PTSD group, please refer to Fig. S3. Black bars represent SEM.

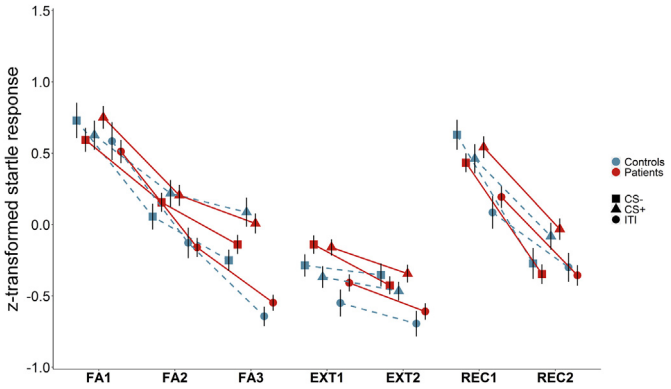

Fig. 4. Z-transformed startle EMG during fear acquisition (FA), extinction (EXT) and recall (REC) for healthy controls and patients with fear-related disorders. Dashed lines represent responses from healthy controls, straight lines represent responses from the fear-related group. For plots including the PTSD group, please refer to Fig. S4. Black bars represent SEM.

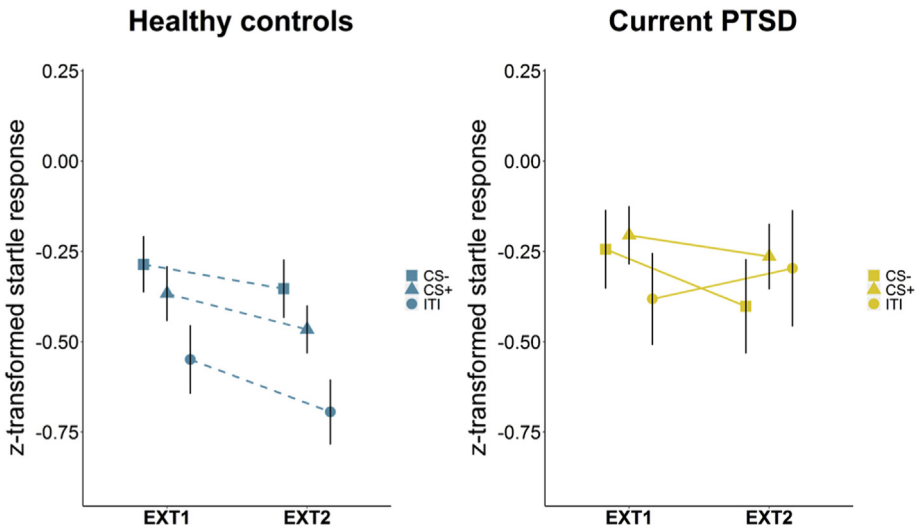

Fig. 5. Z-transformed startle EMG during fear extinction (EXT) for healthy controls and patients with a current diagnosis of PTSD. Dashed lines represent responses from healthy controls, straight lines represent responses from the PTSD group. Black bars represent SEM.

(Table S7). For fear extinction, the model receiving most evidence included time and stimulus P(M|data) = 0.66 (Table S8). For the recall session, the best model again contained stimulus, time and their interaction P(M|data) = 0.85 (Table S9). In startle EMG, there was no substantial evidence for a model containing a group effect in any task phase.

Based on previous findings in PTSD patients and startle responses specifically, we further compared patients with a current PTSD diagnosis (n with valid startle data = 16) to healthy controls (Fig. 5). During fear acquisition, the results were comparable to the analysis comparing fear-related disorders and PTSD to healthy controls (the model including time and stimulus received the largest posterior probability P(M|data) 0.73, Table S10). During fear extinction, however, the winning model contained the group effect P(M|data) = 0.20, and all models including group received a combined P(M|data) ~ 0.63, providing anecdotal evidence for a group effect at extinction (Table S11). There were no outliers in this analysis.

3.1. Ratings

For the ratings (Fig. 6) the model with stimulus, time and their interaction received most evidence P(M|data) = 0.95 (Table S12). During extinction, the best model contained time and stimulus P (M|data) = 0.49 (Table S13). During recall, evidence favored the model with stimulus, time and their interaction, P(M|data) = 0.97 (Table S14). As in the physiological measures, analyses on the ratings did not yield evidence supporting a model containing the group effect in any task phase.

3.2. Control analyses

In short, most frequentist rmANOVAS did not result in any significant group effects or interactions with group regarding the comparison between fear-related disorders, PTSD and healthy controls. Only for SCR during extinction, there was a nominally significant stimulus x group interaction effect, driven by higher responding to the CS + in the control group, $F(1,120) = 3.36$, $p = 0.039$, partial $\eta 2 = 0.053$. This effect disappeared after removal of all outliers, $F(1,114) = 1.57$, $p = 0.211$, partial $\eta 2 = 0.027$.

Additional control analyses comparing patients with fear-related disorders to healthy controls in Bayesian ANOVAs revealed similar results to the comparison across three groups (fear-related vs. PTSD vs. healthy controls, Table S18 – S29). In the analogous frequentist analyses, again only the analysis on SCR during extinction, and additionally during recall, revealed group effects that could be referred to as nominally significant. During extinction, there was a nominally significant

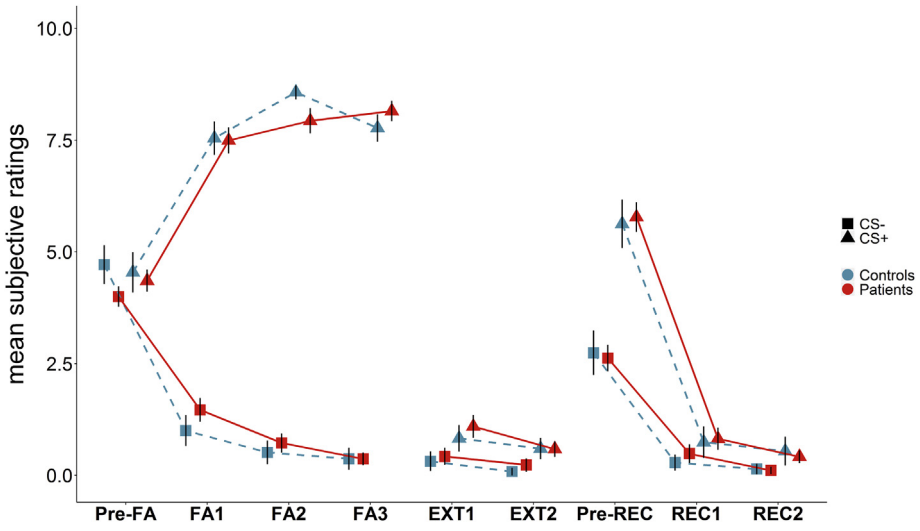

Fig. 6. Shock expectancy during fear acquisition (FA), extinction (EXT) and recall (REC) for healthy controls and patients with fear-related disorders. Dashed lines represent responses from healthy controls, straight lines represent responses from the fear-related group. For plots including the PTSD group, please refer to Fig. S5. Black bars represent SEM.

stimulus x time x group interaction, driven by higher responding to the CS+ in the control group, $F(1,102) = 4.53$, $p = 0.036$, partial $\eta 2 = 0.043$. This effect disappeared after removal of all outliers, $F(1,97) = 0.94$, $p = 0.334$, partial $\eta 2 = 0.010$. During recall, there was a nominally significant stimulus x time x group interaction $F(1,100) = 4.18$, $p = 0.043$, partial $\eta 2 = 0.040$, characterized by less differential responding in the fear-related disorder group. This effect was still nominally significant after removal of all outliers, $F(1,95) = 5.43$, $p = 0.022$, partial $\eta 2 = 0.054$, but would not survive correction for the amount of relevant tests.

Six subjects could be considered non-learners as they rated the CS- as equally or more likely to be followed by shocks than the CS + at the end of fear acquisition. Excluding them from the analyses did not change the results.

Next, we investigated whether symptom severity and impact were associated with differential responding in any measure and block of interest (last fear acquisition block, last extinction block, first recall block). Surprisingly, there was anecdotal to moderate evidence against an effect of symptom severity or impact of the disorder on the differential physiological or subjective responding ($0.11 < BF_{10} < 0.84$, Tables S15 and S16).

Furthermore, due to a high comorbidity between fear-related disorders and depression in our sample the possibility of depression severity masking underlying group differences was addressed. Again, there was anecdotal to moderate evidence against a correlation between any differential responses and the BDI scores ($0.09 < BF_{10} < 1.37$, see Table S17), except for anecdotal evidence for a positive correlation between BDI and differential ratings after the last block of extinction ($r = 0.2$ $BF_{10} = 4.2$). As our sample comprises 41 patients with a fear-related disorder (including PTSD), but without comorbid depression, we also compared conditioned responses in this group compared to healthy controls. Bayesian ANOVAS followed the same pattern as in the comparison of fear-related disorders (including patients with comorbid depression) and healthy controls (Table S30 – S40). These results indicate that it is unlikely that the lack of physiological differences between groups is caused by reduced physiological arousal in patients with comorbid depression.

4. Discussion

In the present study, we compared patients with acute fear-related disorders, PTSD and healthy controls who had never been affected by a psychiatric condition in a classical fear acquisition, immediate extinction and next day recall paradigm. We did not observe any robust effects in any of the physiological and subjective readouts during fear acquisition, extinction and recall. We only observed anecdotal evidence for an effect of group in a specific post-hoc analysis, which revealed generally increased startle EMG responses in the extinction session for the group with PTSD.

In line with Duits et al. (2015) and Dvir et al. (2019), there was no evidence for heightened fear acquisition towards the CS+ in anxiety disorders in any measure. Contrary to Duits et al. (2015), however, we also did not observe evidence for increased responses to CS- during fear acquisition in the fear-related disorder group in any dependent measure. Additionally, no measure revealed evidence for increased responses to the CS+ during extinction in the whole group of patients with fear-related disorders. In their meta-analysis, Duits et al. (2015) reported a small effect for increased differential physiological responding during extinction ($d \sim 0.2$). However, this effect was around 0 for studies using shocks as US compared to ~0.2 for other US types (e.g. air blasts). It was particularly high for disorder-specific US ($d \sim 0.7$) and again around 0 for studies using electric shock as US. This effect was not significant in the analysis of moderators of the meta-analysis, probably due to the low number of studies addressing it. As we also used electric shocks as generic US, we believe our results are roughly in line with these meta-analytic findings.

Nonetheless, previous work has repeatedly shown increased startle responses during fear extinction in specific groups, such as PTSD patients (Glover et al., 2011; Jovanovic et al., 2010; Zuj & Norrholm, 2019). We specifically aimed to replicate this effect by comparing the PTSD group separately to the healthy controls. We only observed an anecdotal group effect during extinction in the startle EMG, representing increased scores to the three unreinforced stimuli that were presented (unreinforced CS+, CS- and ITI). We see several plausible explanations for such an effect: First, it might reflect reduced habituation in the PTSD group, pointing towards hyperarousal as a non-associative learning effect. Second, it is possible that PTSD patients condition to the experimental context, leading to a generalized fear response to the ITI and subsequent difficulties in extinction of this generalized fear response. Third, the patterns might reflect a failure of the subject to recognize the switch from the fear acquisition to the extinction session. Hypothetically, healthy controls might more easily recognize the new (temporal) context in which the CS + stopped signalling threat and inhibit their fear response accordingly (Dunsmoor et al., 2018; Gershman & Hartley, 2015). PTSD patients, in turn, might have difficulties in contextualizing this experience and therefore show less inhibition in their fear responses. As PTSD is caused by a traumatic experience (the US), it has face validity to assume that altered fear conditioning to peri-traumatic stimuli or the traumatic context are part of its aetiology. Possibly, increased startle reactivity in PTSD patients is rooted in a particularly vulnerable fear circuitry (Andrewes & Jenkins, 2019; Norrholm & Jovanovic, 2018), e.g. exaggerated amygdala activity (Rauch, Shin, & Phelps, 2006; Stevens et al., 2017) and decreased medial prefrontal cortex activity (Liberzon & Sripada, 2008; Milad et al., 2009). However, our results have to be interpreted with caution due to the low sample size in the PTSD group and the inconclusiveness of the Bayes Factor: our data simply suggest that we cannot exclude the possibility of a weak-to-moderate effect.

Although Watson (1916) already had an interest in establishing the change in pupil diameter as a conditioned response, pupil dilation towards the CS is an under-represented readout in fear conditioning paradigms with promising between stimulus effects (Korn, Staib, Tzovara, Castegnetti, & Bach, 2017; Leuchs, Schneider, Czisch, & Spoormaker, 2017; Reinhard, Lachnit, & Koenig, 2006; Visser, Scholte, Beemsterboer, & Kindt, 2013). In previous studies on healthy subjects, pupil dilation has been shown to reflect online updating of US predictions (Koenig, Uengoer, & Lachnit, 2018) and threat expectancy (Leuchs et al., 2018). During fear acquisition and reward learning, pupil dilation to the CS + has been robustly related to activity in the salience-network on a trial-by-trial level, indicating that it reflects valence-nonspecific emotional arousal (Leuchs et al., 2017; Schneider, Leuchs, Czisch, Sämann, & Spoormaker, 2018). In our study, pupil dilation towards the CS did not differ in patients with fear-related disorders and healthy controls. It must be added that the task was not optimized for pupillometry, as pupil dilation peaks in the moments before US at stimulus offset. Due to the presentation of a startle sound in the last second before stimulus offset, that also elicits a pupillary response (Leuchs et al., 2018), the stimulus effect in the pupil may be distorted.

In addition to the psychophysiological recordings, we asked participants to estimate US expectancies for all CS. These ratings indexed how shock expectancy changed throughout the task. Some studies have argued that US expectancy is higher in patients with anxiety disorders, especially during extinction (e.g. Michael, Blechert, Vriends, Margraf, & Wilhelm, 2007; Rabinak, Mori, Lyons, Milad, & Phan, 2017). Also in this measure, however, we only observed anecdotal evidence in favour of a positive correlation between differential ratings and BDI values.

If patients with fear-related disorders exhibited substantial differences in associative fear learning, models containing the respective group interaction should receive considerable evidence. However, most articles report post-hoc t-tests of different variables, which leaves room for a-posteriori analysis definitions and comes with the disadvantages of multiple testing (Krypotos, Klugkist, & Engelhard, 2017; Lonsdorf

et al., 2017; Nieuwenhuis, Forstmann, & Wagenmakers, 2011). In our case, we ran 12 rmANOVAs with 48 effects of- or interactions with group. If we had performed t-tests, this would have led to seven t-tests (one per block) per readout or 28 tests in total for the main group comparison only. It seems obvious that such a procedure may occasionally lead to false-positive findings. Conclusions drawn from these diverse effect quantifications often do not replicate when applied to standardized metrics (Haaker, Golkar, Hermans, & Lonsdorf, 2014; Lonsdorf, Merz, & Fullana, 2019). Furthermore, some psychophysiological measures come with their specific pitfalls. SCR, for example, are skewed and prone to outliers, which can affect test values considerably. These arguments can be illustrated by our own SCR data comparing patients with fear-related disorders and healthy controls. In the Bayesian analyses, there was neither evidence for a main effect, nor evidence for an interaction with group in any task phase. In the frequentist rmANOVAs, there were nominally significant interactions with groups during extinction and recall. During extinction, this effect disappeared when controlling for outliers. The interaction with group during the recall session was robust to outliers, and appears in line with previous work (Marin et al., 2017; McLaughlin et al., 2015; Milad et al., 2009). However, it does not survive multiple test correction in a frequentist approach. We think this underlines the importance of focusing on moderate-to-large effects within a robust analysis framework, given the instability of the 'significance' of smaller effects and the resulting difficulty in differentiating between true effects and statistical noise. This particularly applies to medium samples like ours.

Our sample of fear-related disorders consisted of patients with acute social phobia, specific phobias, agoraphobia with or without panic attacks, PD or PTSD. Comorbidities were present in the large majority of the sample, and sample sizes for the specific disorders were rather small, making it difficult to compare the disorders. Our patient groups exhibited large rates of comorbid depression, reflecting the upper end of established comorbidity rates (Lamers et al., 2011; Spinhoven, Penninx, van Hemert, de Rooij, & Elzinga, 2014) and thereby making it an ecologically valid sample. It may be argued that depression influences fear and extinction learning or recall, although, to our knowledge, no study so far has directly compared fear and extinction learning in patients with or without comorbid depression (Pittig et al., 2018). To address if depression had such a masking effect in our sample, we computed correlations between BDI scores and differential scores in acquisition, extinction and recall. Most evidence was in favour of no correlation, indicating that a masking effect of comorbid depression on these variables is unlikely (see also Dibbets, van den Broek, & Evers, 2015).

Furthermore, since severity of fear-related disorders has been shown to drive group effects in studies comparing anxiety disorders to healthy controls (Marin et al., 2017; Norrholm et al., 2011), we also examined the impact and the severity of the fear-related disorders derived from the CIDI interview. There was anecdotal evidence against a correlation between impact or severity and the differential scores in all measures and blocks of interest. Although the quantification of impact and severity is debatable as it was not based on any validated measure, it still supports the notion that fear acquisition, extinction or recall did not change in a continuous manner.

Our study design comes with some limitations. First, the presentation of a startle sound within the stimulus interval might have influenced fear-acquisition as well as conditioned responding during later stages. Second, we paired two CS+ with two different US. We included an extinction phase where only one CS was extinguished and a recall session that was designed to compare recall of an extinguished compared to an unextinguished stimulus, increasing overall uncertainty. Third, despite a large initial sample, clinical and measurement related exclusions led to a considerable cutdown in sample size. Fourth, due to the passive nature of the task, engagement of participants was rather low, attenuating fear-inducing properties of the applied US. All these factors could have interacted with another, possibly reducing group effects.

Nonetheless, we are not the first study revealing no differences in generic fear conditioning and extinction between anxiety disorder patients and healthy controls. In the meta-analysis by Duits et al. (2015), small to moderate group-differences in extinction appeared to be driven by paradigms that included disorder-specific stimuli, with effect sizes for generic fear conditioning and extinction tasks being around zero. Out of approximately 15 medium-to-large size studies published on the topic since that meta-analysis, most did not find any differences in physiological measures during extinction. The question surfacing is at which state of the evidence will the field start accepting the possibility that there are no substantial differences between anxiety disorder patients and healthy controls in generic fear conditioning and extinction? Our data are in line with the literature and show that it is rather probable to find nominal significance for some of the tests at some point in the analyses, yielding little consistency across studies. We are also not the first to observe the lack of medium-to-large effects: Fullana et al. (2019) stated in a recent review that studies in controlled laboratory settings have to date not been able to reproduce patient-specific alterations in fear learning measures. In our opinion, this leaves us with two directions:

First, even if small effects exist, the diagnostic validity of generic fear conditioning and extinction will be very low. Although the clinical utility of a test is not only defined by its diagnostic validity, but also by its ability to predict disorder development or treatment response (Davey, 2017; Vervliet & Raes, 2013), the argument of diagnostic validity is essential if fear conditioning and extinction learning should serve as generic models for clinical anxiety, ultimately aiding standard symptom based diagnostics. Even if small effects exist, much larger studies will be needed to establish reliable differences between patients and controls, leaving the paradigm unsuitable to capture individual variability linked to psychopathology. This does not necessarily mean that there is no causal pathway from abnormalities in fear conditioning and/or extinction to pathological fear, as fear learning abnormalities might solely lead to fear-related disorders in conjunction with other factors (Mackie 1965). Even though this particular causal pathway seems hard to verify or falsify in our case, small effect sizes could still exist and be scientifically relevant. Second, we might want to think about ways to increase the US intensity by evoking the disorder-specific memories, e.g. by using specific and relevant stimuli. Ways to achieve this would be to include ecologically valid CS and US in trauma-film paradigms or to apply fear conditioning tasks in a virtual environment with disorder-specific stimuli (Huff, Hernandez, Blanding, & LaBar, 2009; Kunze, Arntz, & Kindt, 2015; Reichenberger, Porsch, Wittmann, Zimmermann, & Shiban, 2017; Wegerer, Blechert, Kerschbaum, & Wilhelm, 2013). Here, one advantage is that some of these paradigms are also helpful to study fear or extinction memory consolidation and could provide more conclusive evidence on the relevance of this process for the development and maintenance of pathological fear.

To conclude, we observed no robust differences in associative fear learning, extinction training and recall between patients with fear-related disorders and healthy controls. These results strengthen the evidence against the notion that the diagnosis of a fear-related disorder should be reflected in increased conditionability or impaired extinction learning.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brat.2020.103610.

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3 Project 2 | Startle latency as a marker for amygdala-mediated hyperarousal in fear-related disorders

3.1 Contributions and reference

The study "Startle Latency as a Potential Marker for Amygdala-Mediated Hyperarousal" was published in *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging* in 2022.

DP, JF, VIS, and PGS conceived the method and performed the data analysis. DP wrote the first draft of the manuscript, and VIS and PGS contributed to the writing. VIS, PGS, MC, and the BeCOME working group were responsible for the BeCOME study concept and design. All authors contributed to the interpretation of findings, provided critical revision of the manuscript for important intellectual content, and approved the final version for publication.

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Biological Psychiatry: CNNI

Archival Report

Startle Latency as a Potential Marker for Amygdala-Mediated Hyperarousal

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ABSTRACT

BACKGROUND: Fear-related disorders are characterized by hyperexcitability in reflexive circuits and maladaptive associative learning mechanisms. The startle reflex is suited to investigate both processes, either by probing it under baseline conditions or by deriving it in fear conditioning studies. In anxiety research, the amplitude of the fear-potentiated startle has been shown to be influenced by amygdalar circuits and has typically been the readout of interest. In schizophrenia research, prolonged startle peak latency under neutral conditions is an established readout, thought to reflect impaired processing speed. We therefore explored whether startle latency is an informative readout for human anxiety research.

METHODS: We investigated potential similarities and differences of startle peak latency and amplitude derived from a classical fear conditioning task in a sample of 206 participants with varying severity levels of anxiety disorders and healthy control subjects. We first reduced startle response to stable components and regressed individual amygdala gray matter volumes onto the resulting startle measures. We then probed time, stimulus, and group effects of startle latency.

RESULTS: We showed that startle latency and startle amplitude were 2 largely uncorrelated measures; startle latency, but not amplitude, showed a sex-specific association with gray matter volume of the amygdala; startle latencies showed a fear-dependent task modulation; and patients with fear-related disorders displayed shorter startle latencies throughout the fear learning task.

CONCLUSIONS: These data provide support for the notion that probing startle latencies under threat may engage amygdala-modulated threat processing, making them a complementary marker for human anxiety research.

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Reacting to threat and adapting behavior to learned signals of threat and safety is essential for survival of an organism. While protective reflexes ensure quick responses to generic threatening stimuli, associative learning mechanisms enable the modulation of these reflexes based on experience. Fear-related disorders may evolve if generic hyperexcitability acts alone or in combination with abnormalities in associative fear learning (1–3). Accordingly, these disorders are characterized by behavioral and physiological signs of hyperarousal, such as heightened threat responsivity, changes in heart rate variability, or electroencephalographic signs of increased hyperarousal (4–6). At the same time, impaired safety learning has been proposed as a core mechanism of pathogenesis and maintenance of these disorders (7).

The acoustic startle reflex is a highly conserved brainstem reflex that is elicited by sudden, unexpected stimuli and serves to protect the organism from acute threat (8). The reflex has an eyeblink component that can be measured using electromyography of the orbicularis oculi muscle (9). The amplitudes and latencies of these eyeblink reactions to startling stimuli depend on the underlying arousal level, but are additionally modulated by valence, implicating that the reflex is potentiated in negative, as opposed to positive, equally arousing states (10). These characteristics make the startle response an interesting readout of physiological hyperarousal as well as emotional learning—2 putative mechanisms underlying pathological anxiety. Consequently, startle reactions are probed in fear conditioning studies when a startling sound is presented during aversively conditioned stimuli (CS+) and neutral conditioned stimuli (CS−), and the respective response magnitudes are quantified and compared between conditions (11,12).

Our understanding of the neurobiological underpinnings of the fear-potentiated startle reflex largely stems from rodent studies and converges on a pivotal role of the central amygdala that modulates the primary acoustic startle pathway (cochlear root neurons–nucleus reticularis pontis caudalis–motor effectors) (13,14). Further key regions that influence either the central amygdala or the nucleus reticularis pontis caudalis include the periaqueductal gray, the basolateral amygdala, and the bed nucleus of the stria terminalis (15,16). Demonstrating that the amygdala is directly involved in the modulation of the startle response in humans, Kuhn *et al.* (17) reported not only higher amygdala activity in fear-potentiated compared with baseline startle trials, but also a correlation

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between the amplitude of the startle reflex and the amygdalar response on a trial-by-trial basis. An investigation of the effect of structural amygdala variations on startle measures in adults, however, is lacking. Such an analysis would be informative, as interindividual differences in the structure of the amygdala are a candidate biological mechanism underlying variation in startle response and could potentially inform endophenotype research of fear-related disorders. Structural magnetic resonance imaging (MRI) studies have reported that the volume of limbic structures differs between males and females (18), with larger, more recent studies narrowing down such claims to very subtle cross-sectional sex differences in relative amygdala volume (19–21). Preclinical evidence further supports an interplay of sex hormones and the cellular composition and structure of the amygdala potentially driving sex-specific response to and coping with anxiogenic stimuli and tasks (22). As the amygdala is one of the key areas in fear processing (23), and given the higher prevalence of fear-related disorders in women (24), sexually differentiating neurodevelopmental mechanisms may partly contribute to the pathogenesis of fear-related disorders, making sex differences a factor of interest when examining associations between amygdala structure and startle response. Importantly, the high test-retest reliability of structural MRI measures (25) allows the examination of meaningful individual differences.

Despite a large body of literature relating fear-potentiated startle amplitude to psychopathology (26–29), recent fear learning studies comparing startle amplitudes across conditioning and extinction have failed to show robust learning-related differences between healthy control subjects and patients with anxiety disorders (30–33). At the same time, there is growing interest in startle amplitudes as general markers of psychological functioning (5,34). By contrast, in schizophrenia research, startle peak latency under neutral conditions is a more established readout used as a proxy for neural processing speed. Startle peak latency—defined as time until the maximum startle response is reached—shows heritability estimates between 29% and 68% (35,36). Most studies report that latency is prolonged in patients with schizophrenia and their first-degree relatives (35–39), rendering it a candidate endophenotype for schizophrenia-related disorders. There is scant research that addresses startle latency within a (pathological) fear context, but early studies in human participants have shown that high arousal levels as well as anticipatory anxiety not only increase the startle amplitude, but also reduce the startle onset latency (40,41). Accordingly, in response to yohimbine, an anxiogenic α_2-adrenoreceptor antagonist that increases the noradrenergic tone (42), startle onset latency is reduced (43), while benzodiazepines prolong it (44–46).

In the present study, we examined both startle amplitude and peak latency features in a fear learning task in a sample of healthy control subjects and individuals with varying severity and types of anxiety and affective disorders. First, we sought to understand whether the amplitude and latency of the startle reflex carry distinct information. To disentangle amplitude and latency features in the whole sample, we performed a principal component analysis (PCA) and evaluated the pattern and robustness of the resulting components. If the many interrelated amplitude and latency variables across the task phases (early, middle, and late fear conditioning) would load onto orthogonal components, this would be a strong argument for the notion that startle latency contains different information from startle amplitude. If the components had mixed loadings of amplitude and latency variables, for instance, per task phase, this would be a strong argument against these values carrying different information. We assumed that the different startle features would load onto different components but had no specific hypothesis on the type or number of components. Second, we evaluated whether the resulting startle components would be associated with amygdala morphology as obtained with structural MRI. We hypothesized that amygdala volume would correlate with the startle response and wanted to explore whether such effects would occur in a sex-dependent manner. Third, we were interested to see whether startle latency is a relevant readout for fear conditioning tasks, hypothesizing that startle latencies to aversively conditioned stimuli are different from startle latencies to safety stimuli. Fourth, we hypothesized that healthy control subjects and patients with fear-related disorders would show differences in startle latency in the fear conditioning task. This would be especially relevant given that we failed to find group differences in startle amplitude in our previous study (31).

METHODS AND MATERIALS

Participants

Data for the presented analyses come from the ongoing BeCOME (Biological Classification of Mental Disorders) study at the Max Planck Institute of Psychiatry in Munich, Germany (registered on ClinicalTrials.gov: NCT03984084) (47). The study is designed to identify biology-based classes of stress-related disorders and recruits healthy control subjects and patients with stress-related disorders with different degrees of severity. The study protocol is in accordance with the Declaration of Helsinki and was approved by the local ethics committee. All participants provided written informed consent after the study protocol had been fully explained and were reimbursed for their participation. Participants underwent neuropsychological testing, neuroimaging, omics-based assessment, and a computer-based slightly modified version of the Munich-Composite International Diagnostic Interview (DIAX/M-CIDI) (48). Participants of the BeCOME study pass a psychometric test battery and undergo a number of validated psychological tasks to stimulate responses in basic systems of human functioning. Among the tasks is a 2-day classical fear learning, extinction, recall, and return of fear paradigm that continuously assesses physiological response (Figure 1). To compare startle latency during fear learning between groups, participants who had no current (last symptom occurrence $\leq$1 year) psychiatric diagnosis and additionally no lifetime (no symptom occurrence $>$1 year) diagnosis of a fear-related disorder (49) or posttraumatic stress disorder (PTSD) were assigned to the control group, and participants fulfilling the diagnostic criteria for a current fear-related disorder or PTSD were assigned to the fear-related disorder group (Table 1). Previous analyses of the fear conditioning data have been reported by Leuchs *et al.* (50) and Pöhlchen *et al.* (31) addressing task effects and potential group differences in classical fear learning readouts (unconditioned stimulus [US]

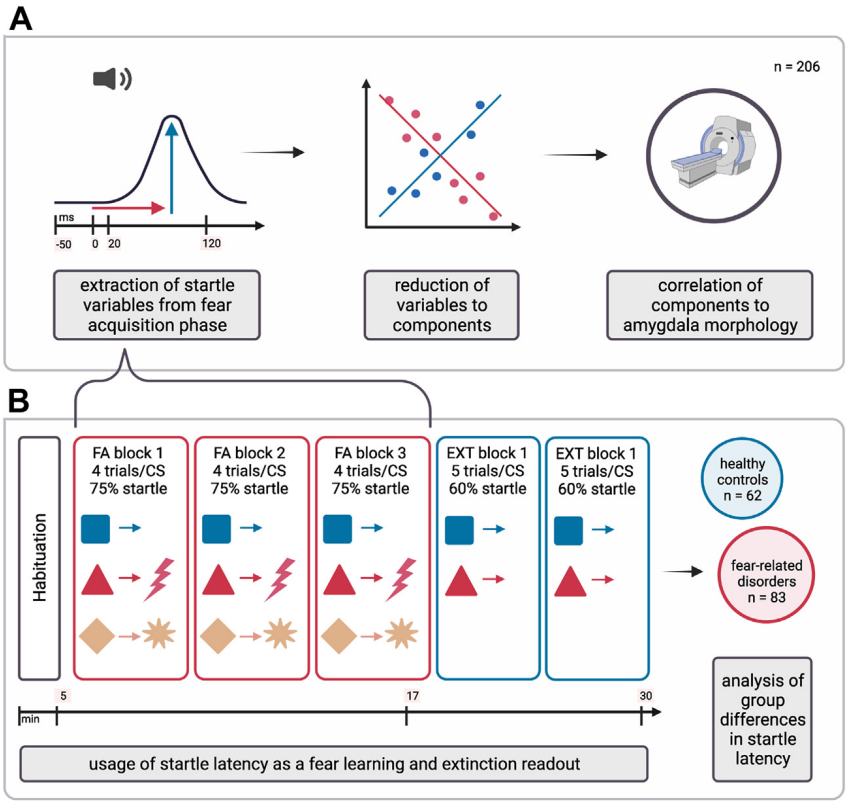

Figure 1. Procedure and research questions. **(A)** First, startle amplitude and latency measures were extracted from the fear acquisition phase of the fear conditioning task. Second, these features served as inputs to a principal component analysis. Third, the resulting component scores were used for correlations with amygdala gray matter. **(B)** Fourth, startle latency features were used as fear learning and extinction readouts. Participants underwent a habituation phase, 3 blocks of fear acquisition (FA), and 2 blocks of extinction (EXT) on the same day. During FA, one colored shape (blue square, CS−) was never followed by an aversive stimulus. Another colored shape (red triangle, CS+shock) was followed by an electric shock to the wrist. A third colored shape (yellow rhombus, CS+air) was followed by an air blast to the throat. During extinction, only CS− and CS+shock were presented. No electric shocks or air blasts were delivered. All blocks were separated by unconditioned stimulus expectancy ratings. All analyses up to here were performed on a sample of 206 participants. Fifth, time- and stimulus-dependent latency values were compared between healthy control subjects (n = 62) and individuals with a diagnosis of a fear-related disorder (n = 83). Colors and shapes were chosen for illustration purposes only. CS, conditioned stimulus. [Created with BioRender (https://biorender.com/).]

expectancy ratings, startle amplitude, skin conductance responses, and pupil dilations).

Structural MRI and Volumetry

Data for structural MRI analyses were high-resolution T1-weighted images acquired on a 3T scanner (Discovery MR750; GE Healthcare) (sequence: sagittal fast spoiled gradient-echo three-dimensional BRAVO, echo time 2.3 ms, repetition time 6.2 ms, inversion time 450 ms, flip angle 12°, field of view 25.6 × 25.6 cm^2, matrix 256 × 256 × 200, resulting voxel size 1 × 1 × 1 mm^3). We used FreeSurfer, version 7.0 (https://surfer.nmr.mgh.harvard.edu/) to perform an automated cortical and subcortical segmentation, followed by a quality control of the resulting parcellation (ENIGMA; http://enigma.ini.usc.edu/protocols/imaging-protocols), which resulted in the exclusion of 1 subject. Volumes of the brainstem and the midbrain were extracted for control analyses (51). Then, we applied the combined hippocampal subfield and amygdala subnuclei segmentation. Of the 9 amygdala subnuclei, only 4 (accessory basal nucleus, basal nucleus, corticoamygdaloid transition area, lateral nucleus) were included into the next steps. The exclusion of the 5 remaining subnuclei was based on their volume of <200 µL (Figure S3), which was determined as a cutoff for sufficient reliability in an article comparing measurement reliability of amygdala subnuclei (52). Results on the remaining subnuclei are reported in the Supplement. To measure local gray matter (GM) volume, binary versions of the respective subnuclei at a 1 × 1 × 1 mm^3 resolution were multiplied with the (native space) GM probability map, and volumes (in microliters) of both hemispheres were summed. As a global correction variable, we calculated the intracranial volume (see Supplement for further details on structural MRI processing).

Fear Conditioning Task

The uninstructed classical fear conditioning task comprised a habituation, an acquisition, and a subsequent extinction phase on the same day (Figure 1B). Fear acquisition and extinction were divided into 5 blocks separated by US expectancy ratings. The fear acquisition phase consisted of 3 blocks during which participants learned to associate 3 distinct CS with no aversive outcome, an air blast, or an electric shock. Startle probes consisted of 40 ms of white noise at 108 dB with near-instantaneous rise time. In each block, all 3 stimulus types (CS−, CS+air, and CS+shock) were presented 4 times in a pseudorandomized order. Reinforcement rates for both CS+ were 75%, and startle probes occurred in 75% of trials and in 40% of intertrial intervals (ITIs). The extinction phase directly followed upon acquisition and comprised 2 blocks. Here, CS+air was not presented anymore, and CS+shock and CS− were shown 5 times each per block. No US occurred during extinction, but startle probes were delivered in 60% of trials.

Startle Response Quantification

Startle amplitude was quantified as the difference between the average baseline and the maximum muscular response in a

Table 1. Sample Descriptive Statistics

Participant Characteristics	*n* (%) [*n* Female]	Age, Years, Mean (SD)
Undergoing Fear Conditioning	276	
With Valid Blockwise Fear Acquisition Data	241	
Not Fulfilling Outlier Criteria	226	
Combined Morphometry and Physiological Data	206 [134]	33.67 (11.58)
Case-Control Assignment		
Not assigned to any group	61	
Assigned to control group	62 [37]	33.11 (11.10)
Assigned to fear-related disorder group	83 [54]	32.41 (11.00)
Patients With Fear-Related Disorders[a] Fulfilling Diagnostic Criteria for		
Specific phobia	48 (58%) [33]	
Panic disorder	31 (37%) [19]	
PTSD	19 (23%) [8]	
Patients With Fear-Related Disorders Fulfilling Comorbid Diagnostic Criteria for		
Major depressive disorder	49 (59%) [31]	
Dysthymia	31 (37%) [16]	
Bipolar disorder	3 (4%) [1]	

Of the 276 participants in the fear conditioning task, 241 had complete blockwise fear acquisition data, meaning that there was at least one valid startle trial for each stimulus type and task phase. There were 226 participants who did not meet outlier criteria for the principal component data (individual component scores >3 SD away from the mean component scores). Of these 226 participants, 206 had undergone structural magnetic resonance imaging and passed visual inspection of gray matter and amygdala subnuclei maps. This pool of participants available for the analyses comprised participants with different recencies and severity levels of affective and anxiety disorders and healthy participants. For the analysis on case-control group differences, participants fulfilling the diagnostic criteria for a fear-related disorder or PTSD (last symptom occurrence ≤1 year) were assigned to the patient group (n = 83). Participants without a current psychiatric diagnosis and no lifetime (no symptom occurrence >1 year) diagnosis of a fear-related disorder (phobias or panic disorder) or PTSD were assigned to the control group (n = 62). The remaining participants were not assigned to any group and were therefore not included in any group comparisons (n = 61).

PTSD, posttraumatic stress disorder.

[a]Numbers do not add up to the total number of the fear-related disorder group (n = 83) as participants were required to fulfill diagnostic criteria for at least 1 fear-related disorder, but were allowed to fulfill diagnostic criteria for more than 1 fear-related disorder.

window of interest from 20 to 120 ms after the startle probe. Startle peak latency was defined as the time from sound onset to the identified peak (Figure 1; Figure S1). We refrained from analyzing startle onset latency, as automatic detection of the onset of the startle reflex resulted in many misclassified onsets (Figure S2). Individual trials were excluded if the standard deviation of the 50-ms preceding baseline exceeded the standard deviation of the 100-ms startle segment (mean ± SD = 5.4 ± 6.6% excluded trials).

Preparation for PCA

A PCA was performed to disentangle generic amplitude and latency features and to reduce the number of variables for the following analyses (Figure 1A). A total of 32 features from the fear acquisition phase served as input variables to the PCA. We averaged the startle responses for the 4 stimulus types across the 3 fear acquisition blocks, resulting in 12 amplitude and 12 startle latency variables for fear acquisition. We additionally calculated 4 startle decrease indices, separately for amplitude and latency. First, startle decrease was calculated by dividing the response strength in the last 2 trials by the response strength in the first 2 trials during conditioning. Second, startle decrease during the third and second to last trials was divided by startle response during the second and third trials. Startle decrease values represent percentage of response retention over time (startle end/startle beginning) × 100. Lastly, we fitted a linear model to the complete startle time series. From this analysis, we gained an intercept and a slope (Table 2).

Statistics

To extract stable startle components and reduce the number of variables, we used PCA and the varimax rotation in IBM SPSS, version 25 (IBM Corp.), entering the 32 startle features from the habituation and fear acquisition phase. Components with an eigenvalue >1 were extracted. The PCA was run again after row-wise outlier removal with individual component scores >3 standard deviations away from the mean component scores.

To investigate associations between these startle components and amygdala morphology, we first fitted generalized linear regression models in MATLAB (The MathWorks, Inc.). These regression models contained the nuisance variables intracranial volume, sex, age, and sex-by-age interaction and the variables of interest amygdala volume and sex-by-amygdala volume interaction. Males serve as the reference category. We adjusted our results for all explorative multiple testing by Bonferroni correction for the 4 components and 2 amygdala terms (8 tests, $\alpha = 0.05/8 = 0.00625$). Complementary analyses including the predictors age-by-amygdala and age-by-sex-by-amygdala were run to control for a potential further modulation of the effects by age. We then assessed possible subregional differences of the amygdala by replacing the total amygdala volume by subnuclei volumes in the models, again with an interaction term sex-by-volume. Only

Table 2. Results From a PCA on Startle Latency and Startle Amplitude Variables Derived From the Fear Acquisition Phase of the Fear Conditioning Experiment (*N* = 206)

Variables	PC1: Mean Amplitude	PC2: Mean Latency	PC3: Latency Decrease	PC4: Amplitude Decrease
Latency Decrease 1			0.776	
Latency Decrease 2			0.781	
Latency Decrease Intercept		0.825[a]	−0.530	
Latency Decrease Slope			0.942[a]	
Latency CS− FA1		0.673		
Latency CS− FA2		0.695		
Latency CS− FA3		0.591		
Latency CS+shock FA1		0.656	−0.421	
Latency CS+shock FA2		0.676		
Latency CS+shock FA3		0.737		
Latency CS+air FA1		0.661		
Latency CS+air FA2		0.713		
Latency CS+air FA3		0.583		
Latency ITI FA1		0.648		
Latency ITI FA2		0.658		
Latency ITI FA3		0.530	0.399	
Amplitude Decrease 1				0.814[a]
Amplitude Decrease 2				0.788
Amplitude Decrease Intercept	0.961[a]			
Amplitude Decrease Slope	−0.495			0.743
Amplitude CS− FA1	0.916			
Amplitude CS− FA2	0.920			
Amplitude CS− FA3	0.892			
Amplitude CS+shock FA1	0.922			
Amplitude CS+shock FA2	0.928			
Amplitude CS+shock FA3	0.910			
Amplitude CS+air FA1	0.911			
Amplitude CS+air FA2	0.914			
Amplitude CS+air FA3	0.904			
Amplitude ITI FA1	0.908			
Amplitude ITI FA2	0.891			
Amplitude ITI FA3	0.846			

FA1/FA2/FA3 = mean response during block 1/2/3 of fear acquisition; decrease 1 = percent change from the first 2 trials to the last 2 trials; decrease 2 = percent change from the second and third trials to the second to last two trials; time series intercept/slope = intercept/slope from fitting a linear model to the startle time series.

CS, conditioned stimulus; FA, fear acquisition; ITI, intertrial interval; PCA, principal component analysis.

[a]Variable with the highest loading onto the respective principal component.

the startle latency component was forwarded to this stage. Again, a Bonferroni correction for testing 4 subnuclei represented by 2 terms was applied (8 tests, $\alpha = 0.05/8 = 0.00625$).

To investigate whether startle latency also serves as an informative readout in classical fear conditioning analyses, we ran 4 repeated-measures analyses of variance (rmANOVAs) in JASP, Version 0.14.1 (https://jasp-stats.org/). The first 2 rmANOVAs contained the complete sample ($N = 206$) and quantified stimulus and time effects during fear acquisition and extinction, respectively. Two separate analyses were performed because the CS+shock was excluded from extinction. We repeated these 2 rmANOVAs on participants with a diagnosis of a current fear-related disorder or PTSD ($n = 83$) and patients without any current psychiatric diagnosis ($n = 62$) and explored additional group and all group interaction effects. All rmANOVAs included a stimulus factor (with 4 levels during fear acquisition [CS−, CS+shock, CS+air, and ITI] and 3 levels during extinction [CS−, CS+shock, ITI]), a time factor (with 3 blocks in fear acquisition and 2 blocks in fear extinction), and their interaction. When Mauchly's test of sphericity indicated that the assumption of sphericity was violated, a Greenhouse-Geisser (GG) correction was applied (p_{GG}). Partial η^2 served as an effect size measure. In case of significant main effects, Bonferroni-corrected post hoc tests were performed, and Cohen's *d* is reported.

RESULTS

Startle Amplitude and Latency Carry Distinct Information

Startle peak latency and amplitude during fear acquisition were not correlated ($\rho = 0.13$, $p = .063$) (Figure 2). This was

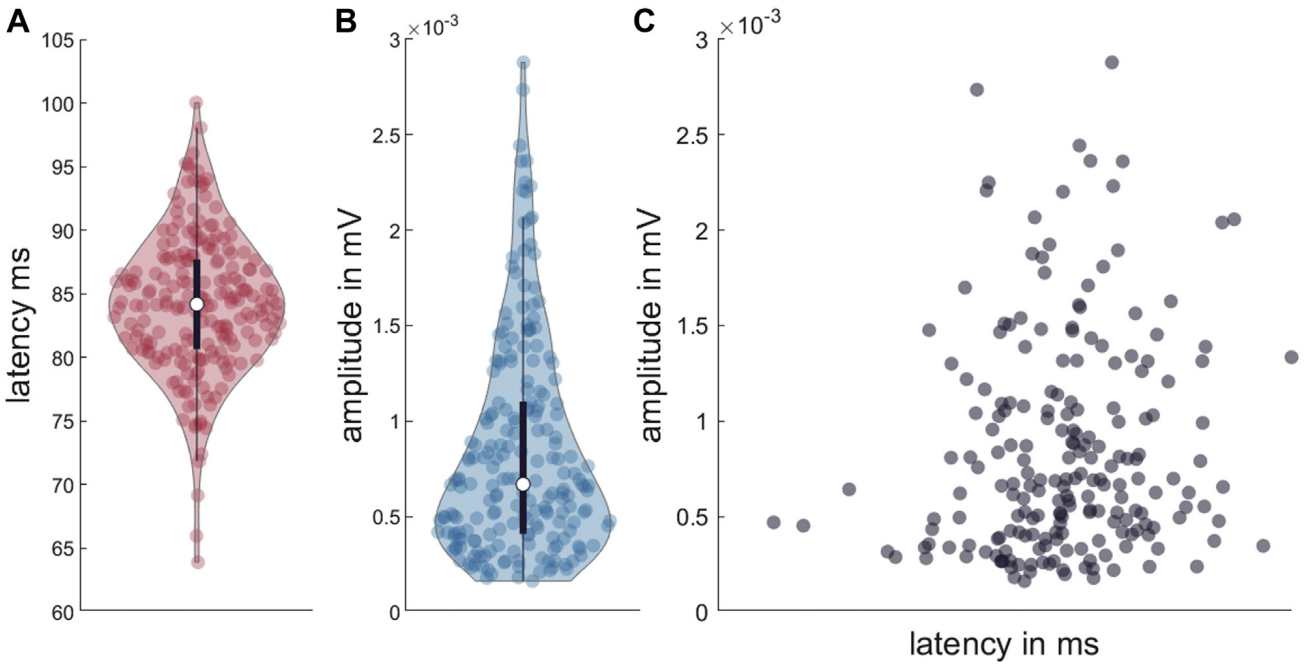

Figure 2. Raw startle latency and amplitude values. **(A)** Distribution of mean startle latency during fear acquisition. **(B)** Distribution of mean startle amplitude during fear acquisition. **(C)** Spearman rank correlation between mean latency **(A)** and amplitude **(B)** during fear acquisition was 0.13 (p = .063; N = 206).

confirmed by the PCA with startle latency– and startle amplitude–related features loading onto separate components (Figure 3). In total, the PCA approach resulted in 4 components representing mean amplitude (PC1), mean latency (PC2), latency decrease (PC3), and amplitude decrease (PC4) of the startle reflex (Table 2).

Startle Latency Shows a Sex-Specific Association With Amygdala GM Volume

When predicting these 4 startle components by amygdala GM volume and the sex-by-amygdala GM volume interaction across the complete sample, only startle latency showed a significant association (Tables 3 and 4). The positive relationship between amygdala volume and startle latency was strongly driven by male participants ($p_{amygdala}$ = .772, $p_{sex\text{-}by\text{-}amygdala}$ = .003, adjusted R = 0.158) (Table 3 and Figure 4). To probe the regional specificity of this effect, the same analysis was repeated using total hippocampal, midbrain, and brainstem GM volume and showed no significant associations with startle latency (Table S2).

We then explored whether the 4 reliably segmented subnuclei of the amygdala show a specific contribution. All subnuclei showed a significant sex-by-subnucleus volume interaction and confirmed the stronger correlation in men (Table S1 and Figure S4) with GM volume of the basal nucleus ($p_{basal\ nucleus}$ = .454, $p_{sex\text{-}by\text{-}basal\ nucleus}$ = .003, R^2_{adj} = 0.169) showing the strongest contribution. When adding the additional predictors age-by-amygdala and the 3-way interaction age-by-sex-by-amygdala, the sex-by-amygdala interaction lost its significance, but the 3-way interaction was significant ($p_{age\text{-}by\text{-}amygdala}$ = .04, $p_{sex\text{-}by\text{-}age\text{-}by\text{-}amygdala}$ = .03, R_{adj} = 0.173) (Table S3). The same positive association between amygdala GM volume and startle latency could be seen in males when visualized by age (Figure S5).

Startle Latencies Across Conditioning and Extinction Are Shorter in Patients With Fear-Related Disorders

To investigate whether startle latency is modulated by the context in which the startle sound is presented, we performed rmANOVAs for fear conditioning and extinction across the

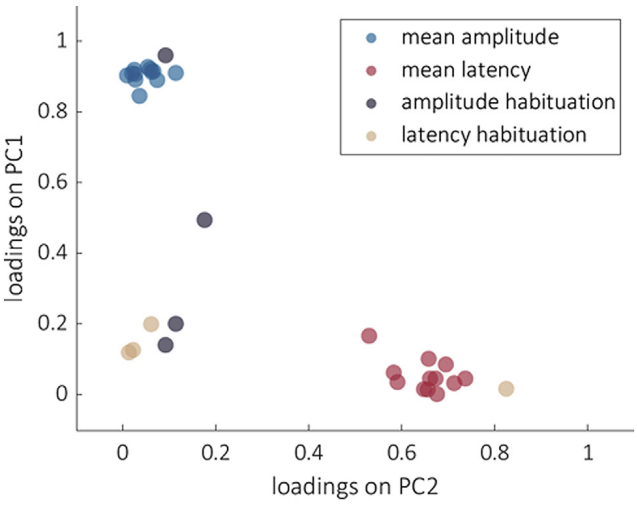

Figure 3. Loadings of the 32 startle features on PC1 and PC2. Amplitude-related features (light blue) are separable from latency-related features (red). PC, principal component.

Table 3. Unstandardized β Coefficients and *p* Values From 4 Regression Models

β and *p* Values	PC1: Mean Amplitude	PC2: Mean Latency	PC3: Latency Decrease	PC4: Amplitude Decrease
β_{main}	<0.001	<0.001	<0.001	<0.001
p_{main}	.628	.772	.331	.808
$\beta_{interaction}$	<0.001	0.001	<0.001	0.001
$p_{interaction}$	.802	.003[a]	.246	.250
R^2_{adj}[b]	0.003	0.158	0.009	0.033

Intracranial volume, sex, age, amygdala volume, and the sex-by-amygdala interaction were regressed on the startle components (N = 206, df = 199).

[a]Robust to Bonferroni correction.

[b]Explained variance for the whole model including all nuisance variables.

complete sample (N = 206). During conditioning, startle latencies decreased over time and were modulated by the stimulus context in which startle sounds were presented (time: $F_{2,410} = 51.5$, $p < .001$, $\eta^2_p = 0.20$; stimulus: $F_{3,615} = 7.87$, $p_{GG} < .001$, $\eta^2_p = 0.04$; interaction: $F_{6,1230} = 2.42$, $p_{GG} < .030$, $\eta^2_p = 0.01$) (Figure 5A). Post hoc tests on stimulus type indicated that mean latency in CS+air trials was significantly shorter compared with the mean latency in ITI trials ($p_{Bonferroni} \leq .001$, $d = 0.331$), CS+shock trials ($p_{Bonferroni} = .036$, $d = 0.192$), and CS− trials ($p_{Bonferroni} = .007$, $d = 0.228$). Post hoc tests on time indicated significant differences between all time points (all $p_{Bonferroni} < .001$, all $d > 0.3$). During extinction, startle latency did not differ between time points and was not modulated by stimulus type (all $p > .05$, all $\eta^2_p < 0.009$).

Finally, we repeated these analyses to specifically compare startle latency between healthy control subjects and participants with fear-related disorders (Table 1). The fear-related disorder group showed shortened startle latencies across stimuli and time points during both conditioning and extinction (group effects during conditioning: $F_{1,143} = 4.71$, $p = .032$, $\eta^2_p = 0.03$; extinction: $F_{1,138} = 5.13$, $p = .025$, $\eta^2_p = 0.04$) (Figure 5B). There were no significant interactions with group and stimulus or time (all $p > .06$, all $\eta^2_p < 0.019$). All post hoc results as well as additional analyses including sex, age, and their respective interactions as covariates are reported in the Supplement. For complementary analyses on startle amplitude and US expectancy ratings, see Pöhlchen *et al.* (31).

DISCUSSION

In the present study, we investigated amplitude and latency features of the startle response acquired during a fear learning task in a sample of healthy control subjects and individuals with anxiety disorders. First, we observed that peak amplitude and peak latency of the startle reflex projected on orthogonal components of a PCA performed on all startle features, demonstrating that startle latency provides information independently of startle amplitude. Second, in contrast to startle amplitude, startle latency was associated with amygdala GM volume. Third, startle latencies habituated over time and showed a fear-dependent modulation with shorter latencies in more fearful states, making them a relevant readout of fear learning. Finally, patients with fear-related disorders and PTSD showed generally reduced startle latencies. This was independent of task phase, suggesting that startle peak latency might be a marker with utility in shorter versions or related tasks with a threat context. Taken together, our findings suggest that startle latency may reflect a basic physiological process with relevance for human anxiety research.

Table 4. Prediction of Startle Latency Component (PC2) by Amygdala Gray Matter Volume

Variables	β	SE	*t* Value	*p* Value
ICV	<0.001	<0.001	−0.799	.425
Sex	−3.767	1.648	−2.285	.023
Age	0.030	0.007	4.560	<.001
Age × Sex	−0.010	0.012	−0.849	.397
Amygdala × Sex	0.001	<0.001	2.964	.003
Amygdala	<0.001	<0.001	0.290	.772

ICV, sex, age, age-by-sex interaction, amygdala volume, and sex-by-amygdala interaction were regressed on PC2 (N = 206, df = 199).

ICV, intracranial volume; PC, principal component.

Across the complete sample, startle latencies showed a time-dependent decrease and fear potentiation during fear acquisition. Mean startle latencies during the presentation of CS+air were shortest compared with all other stimulus types, demonstrating not only the expected differences between CS+ and CS−, but also differences between the 2 types of CS+ applied in our study. When comparing patients with fear-related disorders and PTSD with healthy control subjects, startle latencies indicated similar learning patterns across groups. Importantly, patients displayed shorter latencies throughout fear acquisition and extinction that were independent of stimulus and time. This general modulation of startle latency by diagnostic status and the increasingly shorter startle latencies during conditioning converge with previous work reporting that startle onset latency decreases with increasing levels of arousal (40) and anticipatory anxiety (41). The absence of group effects on specific stimuli or task phases indicates that differences between patients and healthy control subjects may not be related to learning, but rather grounded in more basic physiological, nonassociative mechanisms that could lead to a potentiation of anticipatory response (30).

Startle latency is being discussed as an endophenotype indexing impaired neural processing speed in schizophrenia and related disorders (36), with many studies observing increased startle peak latency in participants with schizophrenia compared with control subjects [(31–34), but see (53–55)]. How can this be reconciled with the significantly shorter startle latencies in patients with fear-related disorders or PTSD? The main reason seems that startle responses in our

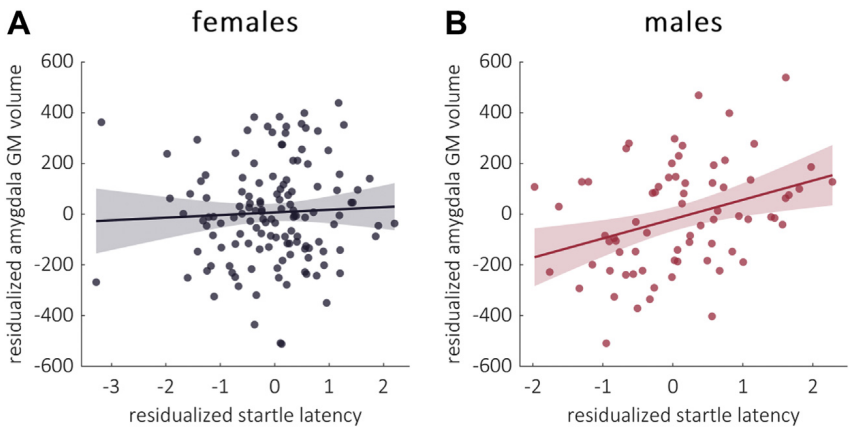

Figure 4. Association between the startle latency component and the gray matter (GM) volume of the amygdala in female **(A)** and male **(B)** participants. For illustration purposes, age and intracranial volume were regressed out of amygdala volume and startle latency.

study protocol were not probed under neutral conditions but during a classical fear conditioning task, which has been shown to extend the primary acoustic startle pathway by the involvement of the amygdala (15,16). It is therefore likely that differences in amygdala-modulated threat processing, i.e., neuronal processing speed under threat, underlie these group differences.

In line with this idea, startle latency and amygdala volume were correlated: more specifically, larger volumes predicted longer latencies. In general, however, the literature on amygdala morphology in anxiety disorders and related subclinical traits is quite heterogeneous. On one hand, previous work has identified increased amygdala volumes in patients with generalized anxiety disorder and positive correlations between anxiety symptoms and amygdala volume in children and adolescents (56–58) as well as between amygdala volume and social anxiety in healthy women (59). On the other hand, studies have shown lower amygdala volumes in panic disorder (60–62) in mixed samples, in spider phobia in a female-only sample (63), and in generalized social phobia, where GM loss was more pronounced in male subjects compared with female subjects (64). One study observed a negative correlation of amygdala size with psychological distress in early adolescence (65), and a further two studies observed such a correlation with anxiety ratings in adults (66,67). In agreement with reduced amygdala volumes in fear-related disorders, a meta-analysis on subcortical volume differences found a small effect of reduced amygdala volume in patients with PTSD (Cohen's $d = -0.11$) compared with trauma-exposed control subjects (68). Our results revealed a similar protective pattern of larger amygdala volume and longer peak latencies, although this relationship was manifest only in males and additionally

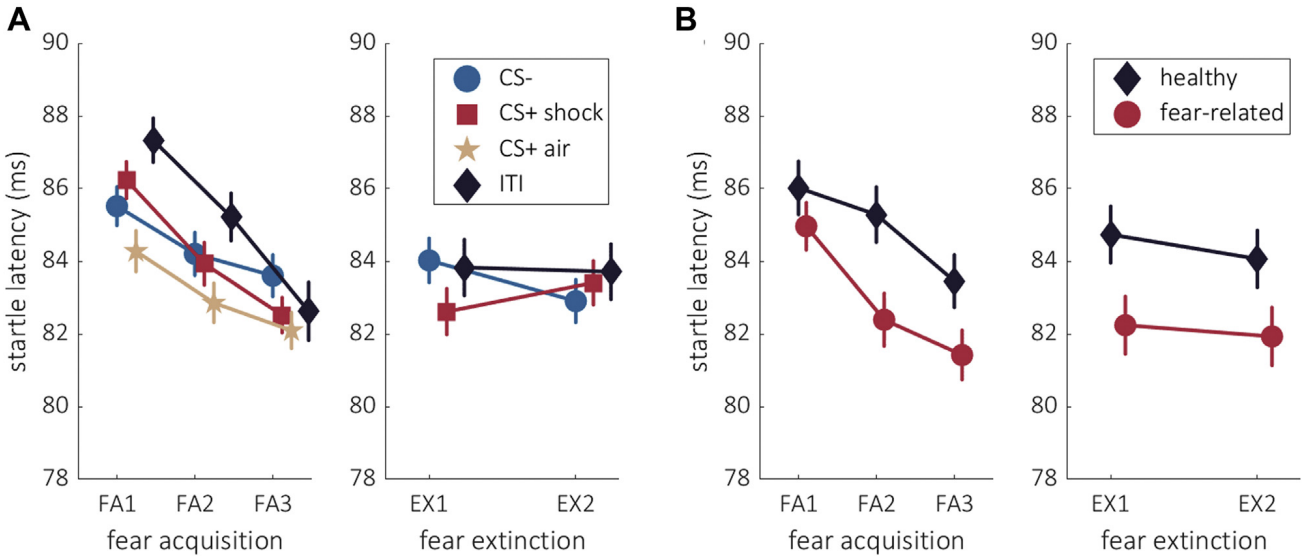

Figure 5. Startle latency across fear acquisition and fear extinction. **(A)** Startle latency is depicted in milliseconds across 3 blocks of fear acquisition (FA1, FA2, FA3) and 2 blocks of fear extinction (EX1, EX2). The different stimulus types (CS−, CS+shock, CS+air, ITI) are marked in different colors ($N = 206$). **(B)** Mean startle latency in healthy participants ($n = 62$) and participants with a fear-related disorder ($n = 83$). Error bars are SEM. CS, conditioned stimulus; ITI, intertrial interval.

interacted with age. The amygdala subnuclei analyses revealed the same effect direction, with the basal nucleus showing the descriptively largest results. This points toward sex-specific associations between brain structure and physiology and, more generally, sex-specific circuits underlying trauma- and fear-related psychopathology. Such dichotomy is not unprecedented: while early stress exposure in females may lead to an overactive and possibly enlarged amygdala, anxiety and early life stress in males have been shown to involve GM loss in the limbic system (64,69,70). Importantly, our study adds to the clear indication to investigate sex as a biological variable (71).

Several limitations should be mentioned. First, our study design is correlational, prohibiting inference on causal associations between amygdala volume, startle latency, and fear-related psychopathology. Studying (epi-)genetic as well as environmental factors longitudinally might help to create more specific causal models. Reported overlaps between genetic risk variants for anxiety disorders and variants that predict a lower amygdala volume (72) are encouraging to connect our findings to specific molecular data. Second, the accuracy of the automated amygdala (subnuclei) segmentation depends strongly on the size of the respective regions. Therefore, we cannot rule out that minor differences in the association between the subnuclei and startle latency are driven by measurement reliability. Third, as we did not present CS+air during extinction, we could not analyze whether the shortened startle latencies during fear acquisition would be prolonged again during extinction. Fourth, given the very low effect sizes and complex interactions between age, sex, and case-control status, it will be essential to replicate these findings and to further pinpoint the suitability of startle peak latency as a complementary readout in fear-conditioning studies.

A few previous studies have reported anxiety-related increases in startle amplitude and decreases of startle onset latency (40,43–46) or found strong inverse correlations of startle amplitude and startle onset latency (41), suggesting that both measures are not independent readouts. For our analyses, we refrained from analyzing startle onset latencies owing to the lack of clear guidelines on how to define and detect the onset of the startle response (Figure S2). Based on our findings that patients with fear-related disorders or PTSD displayed shorter startle latencies independent of task phase and stimulus type, we hypothesize shorter latencies to be independent of our specific fear-conditioning setup. If startle latency was a marker reflecting a basic physiological mechanism that is robustly altered in patients with anxiety disorders, it would be important to show that higher anxiety levels are also associated with shorter startle latencies in other startle paradigms, e.g., an affective startle modulation paradigm or a shorter version of our fear-conditioning task. Theoretically, with resonance to the evolution of fear circuits, startle latency might represent a marker of faster processing speed of the amygdala, which is critical in environments that require a fast defense or flight reactions. With startle electromyography being relatively easy to assess and given the large number of already existing fear-conditioning datasets, startle latency should be further analyzed to assess its utility as an endophenotype beyond the schizophrenia spectrum and generally place it within anxiety disorders research.

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VIS, PGS, MC, and the BeCOME working group were responsible for the study concept and design. DP, JF, VIS, and PGS conceived the method and performed the data analysis. DP wrote the first draft of the manuscript, and VIS and PGS contributed to the writing. All authors contributed to the interpretation of findings, provided critical revision of the manuscript for important intellectual content, and approved the final version for publication.

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ARTICLE INFORMATION

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4 Project 3 | Hippocampal contributions to pathological memory formation in an experimental PTSD model (manuscript in prepraration)

4.1 Contributions and reference

The study "The relationship between hippocampal reactivity to aversive movies and intrusive memory formation is not mediated by hippocampal pattern separation" has not yet been published and the manuscript is currently prepared for submission

DP and JG collected the data, DP, JG, MP, and AB collected piloting data, DP, FW, MS, and VS conceived the method, DP and JG performed the data analysis. DP wrote the the manuscript under supervision of VS. All authors contributed to the interpretation of findings, provided critical revision of the manuscript for important intellectual content, and approved the current version of the manuscript.

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The relationship between hippocampal reactivity to aversive movies and intrusive memory formation is not mediated by hippocampal pattern separation

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Abstract

PTSD can be conceptualized as a memory disorder with symptoms of memory loss concerning contextual, peritraumatic experiences as well as intrusive, vivid memories of salient aspects of the trauma. Deficits in hippocampal processing are assumed to play a role in pathological memory formation, due to the hippocampal role in encoding and storing episodic experiences. It is unclear, however, which hippocampal mechanism contributes to the above-mentioned symptoms. Hippocampal pattern separation abilities promote adaptive mnemonic processes such as the correct disambiguation of safety and threat and binding of the trauma memory to its respective temporal and spatial context. Deficits in hippocampal pattern separation could thereby underly pathological memory formation in PTSD.

To investigate the role of hippocampal processing in predicting contextual memory loss and intrusive memory formation in the aftermath of aversive experiences, we probed neural and physiological correlates of the conditioned-intrusion paradigm, a fear-conditioning task that uses short aversive movie fragments as unconditioned stimuli and pictures taken from these movies as conditioned stimuli. To assess the potentially mediating effect of pattern separation abilities, we compared one group of healthy female participants with very low lure discrimination abilities (a behavioral proxy for hippocampal pattern separation) to another group with very high lure discrimination abilities with regard to hippocampal activity patterns and collected information on intrusions and memory performance in the week following the aversive movie fragments.

Hippocampal signaling during movie watching was associated with intrusion formation with a weaker hippocampal deactivation during aversive movies predicting stronger intrusion distress. Critically, neither behavioral lure discrimination abilities, nor neural pattern separation signals in hippocampal subfields were associated with the observed individual differences in hippocampal processing. Instead, pupil dilations during movie watching were correlated with hippocampal reactivity.

Our study provides evidence for a hippocampal contribution to intrusion formation. However, this was not driven by the hypothesized cognitive mechanism of pattern separation but more likely by basal stress reactivity.

Introduction

How large are human individual differences in encoding, consolidating and retrieving memories from aversive experiences? Post-traumatic stress disorder (PTSD) has a strong mnemonic symptom profile with a rigid association of the perceptual and autonomic event markers leading to involuntary recall of the event in the form of flashbacks and intrusions. This hypermnesia coincides with a memory deficit for peritraumatic hippocampus-dependent contextual information, impairing the capacity of the traumatized person to restrict fear to the correct environment (Brewin, 2011; van Marle, 2015). Individual differences in the structure and function of brain regions transforming perception into lasting memories may therefore underlie vulnerability to develop PTSD in the aftermath of highly aversive events. One of the core regions involved in the initial encoding and indexing of memories is the hippocampus (McClelland et al., 1995; Moscovitch et al., 2016).

To date, there is robust evidence for deficits in hippocampal volume and function in PTSD. Large-scale structural correlational meta-analyses show reduced hippocampal and temporal lobe volume in PTSD patients (Logue et al., 2018; Serra-Blasco et al., 2021) with twin – and prospective studies specifying that reduced hippocampus volume is not only a consequence of stress effects on hippocampal structure but may also be a pre-existing risk factor (Gilbertson et al., 2002, 2007; Pitman et al., 2006). Complementing the structural hippocampal deficits was a recent meta-analysis that reported medium effect sizes of reduced hippocampus-dependent associative learning in PTSD patients compared to healthy and trauma-exposed control (Lambert & McLaughlin, 2019). Patients displayed reduced associative learning independent of valence and sensory modality with the comparatively largest deficits in the processing of spatial locations of cues in context (Smith et al., 2015; Tempesta et al., 2012).

This points to one of the dominant etiological models of PTSD, aiming to explain the onset and persistence of the disorder through associative fear learning processes (Mineka & Oehlberg, 2008; Pittig et al., 2018). Within this model, neutral stimuli and hippocampus-dependent contextual information present during traumatic events become associated with severe threat and elicit conditioned fear, a process that is dependent on plasticity in the amygdala (Fanselow & Ledoux, 1999; Johansen et al., 2011). Fear-extinction describes the establishment of hippocampus-dependent contextual safety memories. Extinction memories help to inhibit the original fear memory (Milad & Quirk, 2012). Their establishment is an assumed core process in the natural recovery from highly aversive experiences and the explanatory mechanism behind exposure therapy (Craske et al., 2008, 2014). The associative fear learning model of PTSD proposes that increased threat reactivity in the amygdala and deficits in hippocampus-dependent safety learning are core etiological features of PTSD. However, the mild electric shocks commonly used as unconditioned stimuli (US) in human "fear" conditioning tasks do not leave a personally meaningful episodic memory trace. In order to help bridge the gap between classical fear conditioning and episodic memory research (Dunsmoor & Kroes, 2019), we therefore turned to the trauma film paradigm (reviewed in James et al., 2016) which induces a lively fear

experience and elicits aversive memories by utilizing aversive movie fragments containing violent and emotionally charged scenes (Brueckner et al., 2019; Kunze et al., 2015). In our adapted version, the conditioned intrusion paradigm (Miedl et al., 2020a; Rattel et al., 2019; Wegerer et al., 2013), initially neutral pictures representing objects or locations from the movies serve as conditioned stimuli (CS). During the conditioning session, some of these pictures (CS+) are followed by short aversive movie clips (US) whereas others (CS-) are followed by short neutral movie clips (Franke et al., 2022). We subsequently asked participants to report their intrusive thoughts of the aversive film clips in the following days through smartwatch-based ecological momentary assessments. We hypothesized that the strength of hippocampal responding to conditioned and intrinsically aversive stimuli would be associated with intrusion distress and contextual memory impairments.

Moreover, to simultaneously examine a candidate mechanism subserving these possible individual differences in hippocampal functioning, we focused on hippocampal pattern separation that has been proposed as a key mechanism in PTSD (Lecei and van Winkel, 2020). Pattern separation refers to the process of transforming similar inputs into distinct, non-overlapping representations. Thereby, it could support adaptive mnemonic processes such as the correct disambiguation of safety and threat and binding of the trauma memory to its respective temporal and spatial context (Anacker & Hen, 2017; Lambert & McLaughlin, 2019; Leal & Yassa, 2018; Liberzon & Abelson, 2016). Work in animals has demonstrated that pattern separation is performed mainly by the dentate gyrus (DG) with the CA3 also showing pattern separation signals depending on the input (Hainmueller & Bartos, 2020; Lacy et al., 2011; Leutgeb et al., 2007; Neunuebel & Knierim, 2014). Work in humans has confirmed the role of these hippocampal subfields in pattern separation (Baker et al., 2016; Berron et al., 2016; Lacy et al., 2011). Exploring the idea of pattern separation as a mechanism linking deficient hippocampal processing to an increased risk of mood and anxiety disorders, Grupe and colleagues (2021) showed worse lure discrimination abilities, a behavioral proxy of hippocampal pattern separation, was associated with stress reactivity. Importantly, however, experimental evidence for an effect of deficient functional DG processing, or its behavioral counterpart lure discrimination, in pathological memory formation is scarce.

To investigate the interactions of lure discrimination abilities and hippocampal activity in pathological memory formation, we tracked the encoding and consolidation of aversive experiences in individuals with high and low pattern separation abilities. We adopted a quasi-experimental design where healthy female participants were divided into two groups with high – and low lure discrimination abilities. With this approach, we could examine whether lure discrimination abilities serve as a pre-trauma risk factor for PTSD, testing the hypothesis of individual differences in pattern separation as a candidate mechanism underlying the hypothesized relationship between hippocampal functioning and intrusion-related distress and loss of contextual detail. Therefore, our second hypothesis was that the group with low lure discrimination abilities would show differences in hippocampal activity during fear-conditioning and exhibit higher intrusion distress and lower context memory. To investigate pattern

separation not only at the behavioral, but also at the neural level, we measured the specificity of hippocampal signaling to the presented stimuli. Our third hypothesis was that less distinguishable DG activation patterns during fear-conditioning would be associated with hippocampal activity during fear-conditioning, intrusion-related distress and loss of contextual detail.

Material and methods

Participants and procedure

Sixty women between 19 and 35 years of age (M =24.73, SD =4.13) participated in the study that was approved by the local ethics committee of the medical faculty of the LMU Munich (project number: 20-561). To be eligible for study participation, potential participants underwent an online version of the mnemonic similarity task and had to perform either above (1 SD above the mean LDI, M =0.3, SD =0.18) or below (1 SD below mean LDI) average. Further inclusion criteria were an age between 18 and 35 years, no current or past psychiatric or neurological illness (questionnaire-based), no current or past use of medication (other than contraceptives), no regular consumption of violent films or games (more than twice per month) and absence of MRI-contraindications (foreign metal objects, pregnancy, left-handedness). Exclusion criteria comprised a PCL-5 score above 33, indicating PTSD. All participants gave written informed consent after the study protocol had been explained to them in detail and received financial compensation for their participation. One participant dropped out of the study before the first visit. During their first visit (**Fig. 1A**), participants provided basic demographic information and completed a series of computerized questionnaires including the German version of the Childhood Trauma Questionnaire (CTQ, Bader et al., 2009), the Life Event Checklist for DSM-5 (LEC-5, (Gray et al., 2004), the PTSD checklist for DSM-V (PCL-5, Blevins et al., 2015) as well as the brief version of the Patient Health Questionnaire (PHQ-9, Kroenke et al., 2001) and provided some demographic information. Three participants fulfilled diagnostic criteria for PTSD and were excluded, resulting in a final sample size of 56. Towards the end of their first visit, participants were explained how to use a smartwatch for ecological momentary assessments (SAMSUNG Galaxy Watch 46mm) via an inhouse app and instructed to wear a portable headband electroencephalogram (EEG) (Dreem 2, Dreem, Paris) for at-home sleep recordings to control for potential differences in sleep fragmentation. Between two and five days after their first visit, participants underwent their first MRI procedure, comprising structural scans and a functional scan while being subjected to a modified version of the conditioned-intrusion paradigm. One week after conditioning, participants first underwent the recall session in the scanner. Next, they answered questions on details and contextual information from the previously presented movies and underwent the emotional MST while being scanned again. Finally, smartwatch and headbands were collected and participants were debriefed.

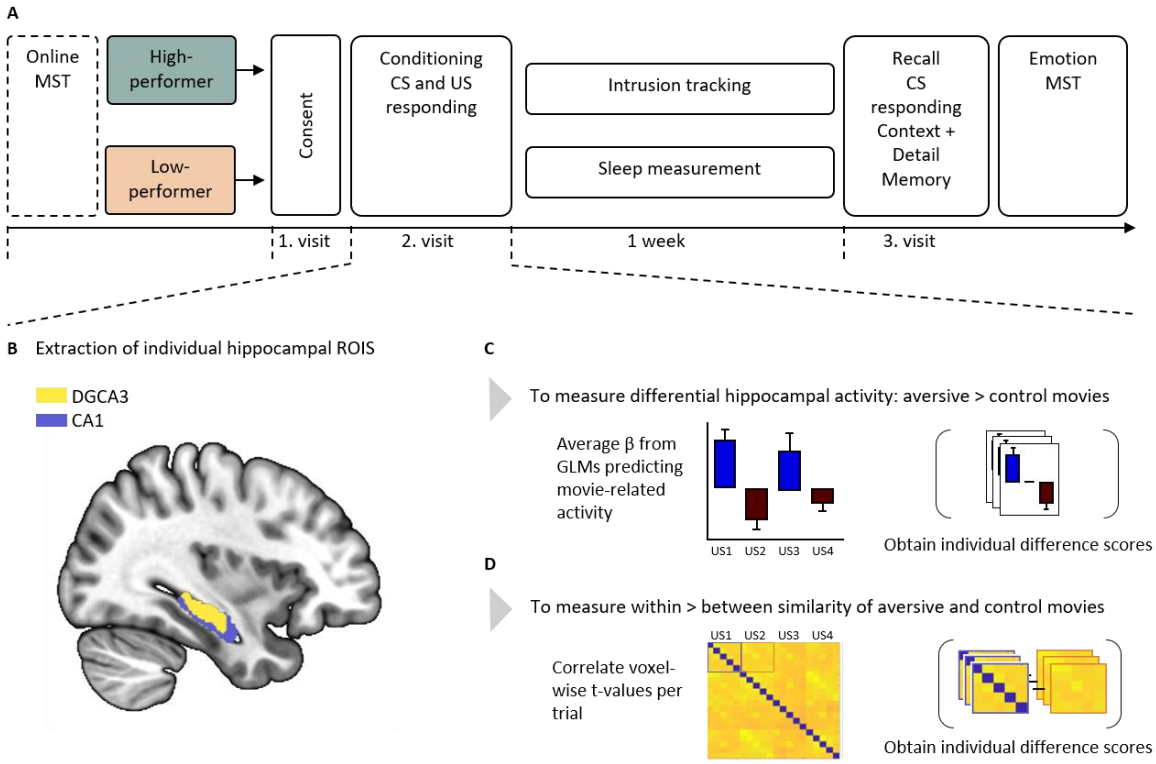

Figure 1. A: Study procedure. After completing the online version of the MST, participants were assigned to the high – or low performance groups and invited for three separate visits at the institute (Day 0, Day 1 and Day 8). B: Exemplary extraction of individual hippocampal ROIS based on the Freesurfer pipeline. C: Differential hippocampal activity is derived as a marker of hippocampal response strength to aversive and control movies. D: Representational similarity within and between movies is derived as a marker for stimulus specific spatial representations in the hippocampus.

Conditioned-intrusion paradigm: Conditioning and Recall procedure

In our adapted version of the conditioned-intrusion paradigm, four short 16 s film clips served as US (two aversive and two neutral movies) and four 4 s neutral images extracted from the film clips served as CS. Two of these images were followed by an aversive movie and the remaining two images were followed by the respective neutral movies. Aversive movies consisted of severe interpersonal violence (one rape scene and a homicide scene involving a fire-extinguisher, both taken from the movie "Irreversible" (Noé, 2002)). The neutral movies consisted of interpersonal interactions, once showing the dance scene from the movie "Pulp Fiction" (Tarantino, 1994) and once showing a basketball game from the movie "Coach Carter" (Carter, 2005). After a short habituation phase with two presentations per CS, the CS were presented 12 times each, using a movie-reinforcement rate of 50%. Trials of consecutive CS-US presentations were separated by inter-trial-intervals jittered between 8 and 14 s. After habituation as well as after trial six and trial 12, participants indicated their general wellbeing and arousal levels as well as CS-specific valence. Presentation orders were counterbalanced across participants. During recall, participants saw eight presentations of each CS and were asked to perform their subjective ratings before, during and after the recall procedure. Between the recall procedure and the emotional MST, participants were remained in the scanner and were asked to answer questions

regarding the order of events in the movies. The individual rank orders of events per movie were correlated with the correct movie rank order. The correlation value was then summed for the negative movies leading to possible contextual memory scores between 0 and 2.

Mnemonic similarity task and lure discrimination performance

Participants underwent two versions of the mnemonic similarity task. During recruitment, participants were directed to an online version of the regular mnemonic similarity task (MST). This task provides a lure discrimination index (LDI) that has been shown to depend on hippocampal pattern separation abilities (Stark et al., 2019). It consists of an encoding phase where participants are presented with objects of everyday life and have to indicate whether the objects are usually located indoors or outdoors. In the testing phase, participants are presented with pictures they had already seen in the encoding phase (targets), pictures that are similar, but not identical to previously seen pictures (lures) and entirely novel pictures (foils). For each picture, participants are asked to indicate whether they are "old", "similar", or "new". Subtracting the ratio of "similar" responses to foils from the ratio of "similar" response to lures results in the LDI, a behavioral proxy for hippocampal pattern separation (Stark et al., 2019). Stimulus material for the online version of the MST was freely available under https://github.com/celstark/MST. 60 out of 1000 + participants were selected based on their performance in the online MST and grouped into the high or low LDI groups. During the last visit at the institute, participants also underwent an emotional variant of the MST while in the scanner. The emotional MST differs from the original MST in that pictures of emotionally charged and neutral scenes are used instead of pictures of everyday objects, thereby testing the effect of emotion on pattern separation and allowing to derive a valence-dependent LDI. Stimulus material for the emotional version of the emotional MST was kindly provided by Stephanie Leal (Leal et al., 2014).

Ambulatory assessment of intrusions, wellbeing and headband EEG

Participants were instructed to report intrusive memories of the film clips experienced on the day of film viewing and on the following seven days in an event-based manner through a smartwatch with an in-house developed app. Intrusions were defined to participants as every images or thoughts of the movies that came to their mind. Additionally, the smartwatch app reminded participants to fill out a sleep quality questionnaire every morning and a general wellbeing questionnaire every evening. Intrusion distress, calculated by summing the average intrusion-related distress from the two negative movie fragments, was taken as the main intrusion-related variable as it relates to the burden experienced by the individual participants and was distributed in a bimodal fashion.

Psychophysiological recordings and preprocessing

Pupillometry, pulse plethysmography (PPG) and skin conductance responses (SCR) were acquired throughout all fMRI sessions but due to a technical error, SCR could only be analyzed for half of the participants. Pupil size and gaze coordinates were recorded with a sampling rate of 250 Hz with an MR-compatible eye tracker (EyeLink 1000 Plus; SR Research Ltd., Ottawa, Canada). After a

standard nine-point calibration procedure, we continuously measured the right eye. Preprocessing was done in MATLAB (version 2019a, MathWorks, Natick, USA) and consisted of a linear interpolation between the last saccade before blink onset and last saccade after blink offset (markers provided by the EyeLink software, SR research Ltd.), smoothing with a sliding window of 400 ms and a z-transformation of the complete pupil timeseries. This timeseries was segmented around CS and US trials with a 0.5 s baseline and 4 s and 16 s trial length, respectively. CS segments were subjected to an automated artifact correction comprising three steps: First, gaze had to be directed towards the center of the screen which was defined by drawing a cut-off window around the individual's median gaze position across trials and informing the limits of this window by the mean gaze deviation across all participants. If the gaze was not directed at this window for ≥0.5 s, the trial was discarded. Second, a general quality criterion was given to each datapoint in the pupil timeseries which was based on the number of adjacent missing pupil values due to blinks. If the summed quality of all datapoints per trial exceeded a certain threshold corresponding roughly to ≥50% interpolation, the trial was excluded. Third, sudden gaze shifts were determined by splitting each trial into the 0.5 s baseline segment and four 1 s segments covering the CS period and calculating the SD for all segments across all trials on an individual level. Trials that deviated from the individual's average deviation in any of the segments were excluded. In sum, 38.8 ± 33.2 % of trials were excluded. Trial-wise pupil readouts for the valid CS segments were derived by subtracting the maximum pupil dilation within the last second before CS offset and the coinciding US onset from a meaned 0.5 s baseline period before stimulus onset. To investigate conditioning effects, we averaged across each two trials during habituation, each six trials during the first block of fear acquisition and each six trials during the second block of fear acquisition. Trial-wise pupil readouts for the US segments were derived by averaging across the whole stimulus interval and all repetitions. Additionally, we derived baseline pupil dilations by averaging across the first eight seconds during fixation cross presentation. For three out of the 56 participants, pupillometric data could not be acquired due to technical reasons. Based on the high exclusion rates for the CS analyses, and the fact that valid pupillometry data had to be present for all blocks (habituation, fear acquisition 1 and fear acquisition 2) and stimuli, only 35 participants entered the conditioning analyses. For the comparison of aversive and neutral movies, data of 40 participants could be used.

Structural MRI acquisition, volumetry and region of interest extraction

Neuroimaging data were acquired on a GE 3 Tesla scanner (General Electric, Discovery MR750, Milwaukee, USA) with a 32-channel head coil located at the Max Planck Institute of Psychiatry in Munich. Before the encoding session (**Fig. 1A**), structural high-resolution T1- weighted images were acquired (sagittal fast spoiled gradient-echo three-dimensional BRAVO, echo time 2.3 ms, repetition time 6.2 ms, inversion time 450 ms, flip angle 12 °, field of view 25.6mm^2, 43 slices, resulting voxel size 1mm^3). FreeSurfer (version 7.0, https://surfer.nmr.mgh.harvard.edu/) was used to perform an automated cortical and subcortical segmentation. This was followed by the combined segmentation of

hippocampal subfields and amygdala subnuclei (Iglesias et al., 2015) as well as a quality check of the resulting parcellation (Sämann et al., 2022). To create individualized maps of the hippocampal subfields relevant to this study, six (CA3 head, CA3 body, CA4 head, CA4 body, DG granule cell molecular layer head, DG granule cell molecular layer body) of the seventeen hippocampal subfields were combined into a DG-CA3 ROI and two (CA1 head, CA1 body) into a CA1 ROI (**Fig. 1B**). The subfield ROI masks, next to the whole hippocampus, were normalized using the deformation information resulting from the fast diffeomorphic registration algorithm normalization (DARTEL, Ashburner, 2007) and resliced to $2mm^3$.

Functional MRI acquisition and pre-processing

For the encoding and recall fMRI sessions, a T2*-weighted echo-planar-imaging sequence (EPI, was acquired (TR =2500 ms, TE =20 ms, FA, matrix, FOV, 42 slices, resulting voxel size 1.9x1.9x3.5 mm^3). In all analyses on functional fMRI data, the first four volumes were discarded due to possible non-steady state effects. Before each functional run, a single spin-echo EPI T2-weighted image with almost identical settings as the fMRI sequence was acquired. It only differed in its TR (10000ms) and TE (37 ms), therefore showing the same geometric distortions as the fMRI series but displaying a higher signal to noise ratio. This image was used for segmentation and spatial normalization. FMRI data were pre-processed using the statistical parametric mapping software (SPM12, https://www.fil.ion.ucl.ac.uk/spm/software/spm12/) in MATLAB. Functional images were registered to the first volume and slice-time corrected. Functional images and the structural EPI images were then co-registered to the participants mean functional image, followed by a segmentation of the structural EPI image into grey matter, white matter and CSF. DARTEL (Ashburner, 2007) was applied to normalize functional images to MNI space. Warped functional images were resliced to $2mm^3$ and spatially smoothed using an isotropic Gaussian kernel (FWHM 6x6x6 mm^3). The 24 denoising parameters included the first three components from a principal component analysis on the time courses of white matter and cerebrospinal fluid and their derivatives as well the six z-transformed motion parameters and their derivatives. The threshold for exclusion based on excessive movement was set at 2 mm translation, resulting in the exclusion of two participants. Further four participants were excluded due to technical errors, resulting in a final imaging sample of 50.

Two different first level analyses were run with both analyses incorporating all nuisance variables and excluding the first four volumes to avoid non-steady-state effects. The first GLM modelled each picture and movie type and their time modulation into separate regressors using a high pass filter of 500 Hz and resulting in 16 regressors (4 picture regressors and their time modulation and 4 movie regressors and their time modulation) and excluded the habituation phase. First level contrast images (CS+>CS-, aversive movie>control movie) were 1) forwarded to second level t-tests, determining regions with consistent CS – und US-induced activity on the group level 2) used to extract differential US-related hippocampal activity at the individual level (**Fig. 1C**).

For the stimulus-wise representational similarity analysis, all trials were modelled as separate regressors with a high pass filter of 128 Hz and the resulting t-maps were reshaped and indexed by the hippocampal ROIs. Pair-wise correlations between all event-related spatial patterns of activation were calculated. Within-stimulus similarity was calculated by meaning across all pairwise correlations between the same consecutive stimulus types e.g. first presentation of the ball movie and second presentation of the ball movie). Between-stimulus similarity was calculated by meaning across all pairwise correlation between paired stimulus types (e.g. first presentation of the ball movie and second presentation of the fire extinguisher movie) (**Fig. 1D**).

Statistics

To investigate conditioning, we analyzed pupil dilations in a two-way ANOVA with the three-levelled factor time (habituation, fear acquisition 1, fear acquisition 2) and the two-levelled factor stimulus type (CS+ and CS-) and the interaction effect of both factors as the test of interest. To investigate BOLD activity with respect to stimulus-type (CS+ and CS-; aversive and control movies) on the whole brain level, second-level p-value thresholds were set to pFWE<.05 at the cluster level (threshold of collection of the statistical maps was uncorrected $p < 0.001$) and clusters were labelled with the Anatomic Automatic Labelling (AAL3)-toolbox for SPM12 (Rolls et al., 2020). Regarding the hypothesis tests, we ran independent t-tests in the case of simple group comparisons and Pearson correlations or Spearman rank correlations when analyzing the associations between dimensional variables. In case of the comparison between multiple factors, ANOVAS with the two-levelled factor group and the three-levelled factor region of interest (whole hippocampus, CA1, DGCA3) were run. We divided the nominal alpha level of 0.05 by the number of tests within each hypothesis with 2 tests for H1, 3 tests for H2 and 9 tests for H3, resulting in significance thresholds of $p = 0.025$, $p = 0.017$ and $p = 0.006$. If the assumption of sphericity was violated in the ANOVAS, we report Greenhouse-Geiser corrected p-values. In case of significant main or interaction effects, we ran pairwise post-hoc comparisons and report Bonferroni-corrected p-values correcting for the number of levels per ANOVA.

Results

The conditioned-intrusion paradigm induces transient intrusion distress

On average, aversive movies led to 5.7 ± 8.3 intrusions with a summed meaned distress of 4.9 ± 3.6 across the first six days after watching the movies (**Fig. 2**). After one week, participants were still able to order single events from the aversive movies correctly, as summarized in the aversive contextual memory score of 1.4 ± 0.8, representing summed rank correlations between the chosen and the correct order of events in the two aversive movies.

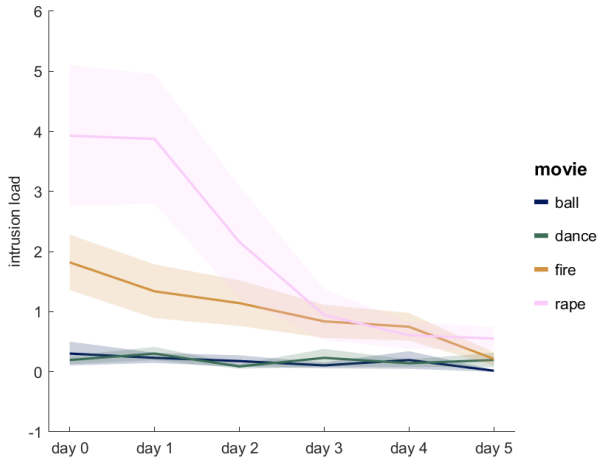

Figure 2. Intrusion load (number of intrusions per day weighed by their subjective distress) across the complete sample (n 56).

Omittance of CS-related analyses for conditioning and recall

We first examined conditioned fear responding by analyzing the effects of CS type and time on pupil dilations and valence ratings across all participants and contrasting BOLD activity towards the CS+ and the CS-. Over time, participants increasingly disliked the pictures taken out of the aversive movies compared to the pictures taken from the neutral movies (stimulus x time interaction: $F_{2,104}$=60.9, $p<0.001$, η^2_p=0.54 for the $CS+_{rape}$ and $CS-_{dance}$ stimulus pair; stimulus x time interaction: $F_{2,\ 104}$=59.3, $p<0.001$, η^2_p=0.53 for the $CS+_{fire}$ and $CS-_{ball}$ stimulus pair, **Fig. 3**). However, fear conditioning was not apparent at the physiological level. Pupil dilations were not modulated by an interaction of CS type and time (stimulus x time interaction: $F_{2,66}$=2.2, $p_{GG}<0.124$, η^2_p=0.06 for the $CS+_{rape}$ and $CS-_{dance}$ stimulus pair; stimulus x time interaction $F_{2,66}$ =0.3, $p_{GG}<0.760$, η^2_p=0.01 for the $CS+_{fire}$ and $CS-_{ball}$ stimulus pair, **Fig. 3**). Similarly, the CS+ > CS- contrast revealed no significant differences. We therefore refrained from further analyzing CS related data during conditioning and recall with respect to the main hypothesis and instead focused on unconditioned responding towards the neutral and aversive movies.

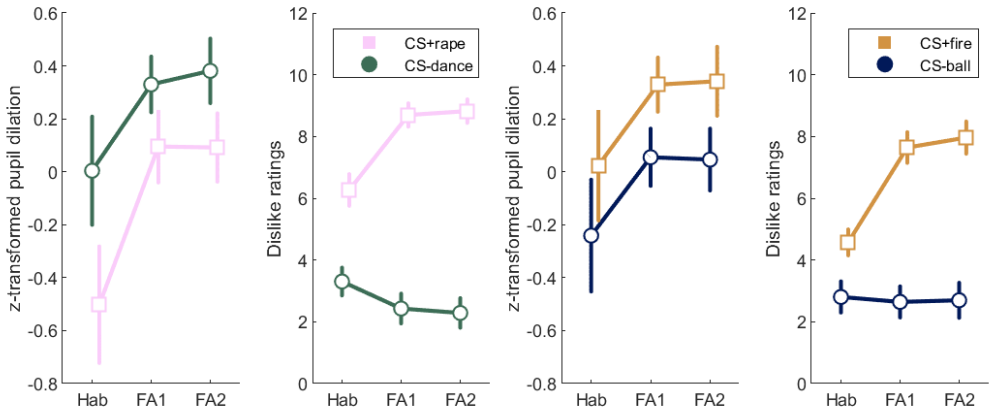

Figure 3. CS-related pupil dilations and subjective aversiveness ratings for the different stimulus pairs during habituation (Hab), the first block of fear acquisition (FA1) and the second block of fear acquisition (FA2). Error bars are confidence intervals.

Differential hippocampal activity predicts intrusion distress

The contrast aversive > neutral movies revealed widespread clusters of activity in the bilateral supplementary motor area, precentral and superior/inferior frontal gyri, in the bilateral superior and inferior parietal lobules and supramarginal gyrus, bilateral middle occipital and temporal gyri, as well as in the thalamus and insula. The reverse contrast revealed clusters of activity in occipital, lingual and calcarine gyri, cuneus and precuneus, temporal gyrus, superior and medial frontal regions, the posterior insula and crucially, a cluster in the parahippocampal gyrus and hippocampus (**Fig. 4, Table S1 and S2**).

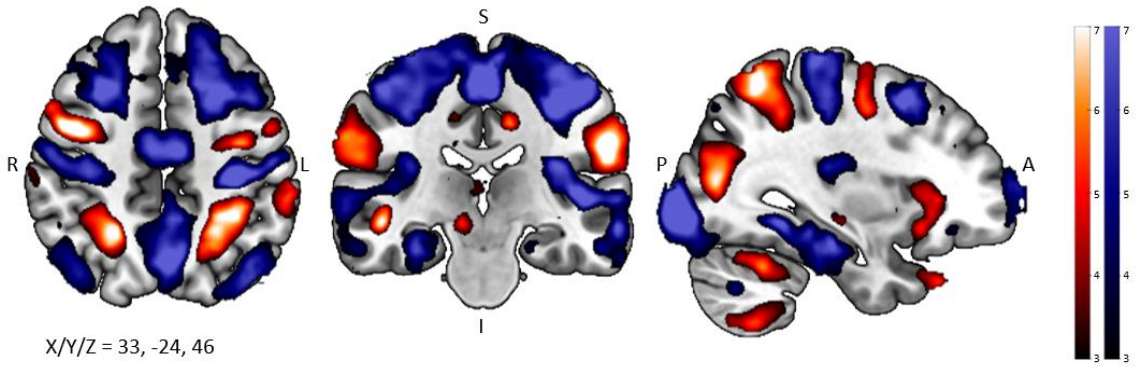

Figure 4. Bold activity to aversive (red) compared to neutral movies (blue) across all repetitions. The colorbars represent T values. Image overlay created using MRIcroGL. A, anterior; L, left; I, inferior; P, posterior; R, right; S, superior

Focusing on the hippocampus, the whole sample displayed significantly reduced activity towards the aversive compared to the control movies (t_{49}=-4.5, p<0.001, **Fig. 5** left panel and **Fig. S3**). Confirming hypothesis 1, differential signaling in the hippocampus predicted intrusion distress (ρ=0.32, p=0.02, **Fig. 5**, middle panel) which was driven by less hippocampal deactivation towards aversive movies in participants with higher intrusion distress ($\rho_{aversive}$=0.25, p=0.08, hippocampal signaling to

control movies $\rho_{control}$=-0.05, p=0.75). There was no association between differential hippocampal signaling and contextual memory scores (ρ=-0.02, p=0.88).

To explore the interplay between hippocampal reactivity and physiological stress markers during encoding, we correlated differential hippocampal activity with differential pupil dilations during movie watching. Interestingly, stronger differential signaling in the hippocampus (aversive movie>control movie) was associated with reduced differential pupil dilations (r=-0.372, p=0.02, **Fig. 5**, right panel).

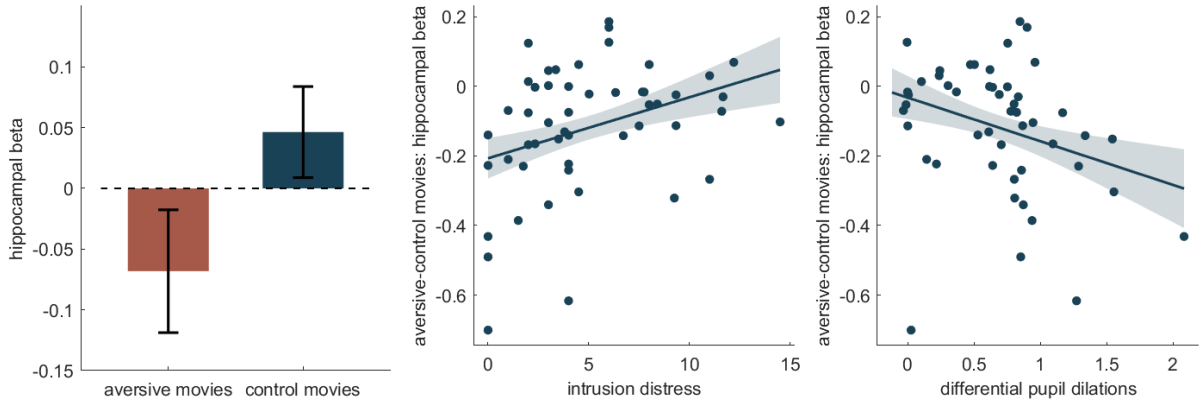

Figure 5. Left: Mean BOLD activity to aversive and control movies across all movie repetitions in the hippocampus. Middle: Differential activity in the hippocampus correlated significantly with intrusion distress. Right: Differential pupil dilations were significantly associated with differential activity in the hippocampus.

Validation of the online mnemonic similarity task

In order to understand whether lure discrimination abilities underlie the individual differences in hippocampal signaling with regard to intrusion distress, we compared the two groups of participants with high and low LDIs. The final sample for all analyses on the comparison between the low and high LDI groups comprised 47 participants of which 16 belonged to the low LDI group and 31 were assigned to the high LDI group. The unequal distribution was due to the fact that we excluded participants a posteriori who initially had not performed well in the online MST during recruiting but performed well in the emotional MST, indicating insufficient compliance during the online testing (**Fig. S1** for an overview over participant inclusions and **Fig. S2** for an overview over the distributions of the online and emotional LDI). To ensure that behavioral lure discrimination reflects pattern separation on the neural level, we first compared the low and high LDI groups regarding their within versus between representational similarity scores in the whole hippocampus as well as the CA1 and DGCA3 subfields. With this measure, we captured how dissimilar the neural representations for one movie were compared to another movie, indicating a more movie-specific activation pattern. There was a significant interaction between LDI group and region (ROI: $F_{1.5,41}$=0.4, p_{GG}=0.613, η^2_p=0.00; LDI: $F_{1,41}$=0.0, p=0.965, η^2_p=0.00; interaction: $F_{1.5,41}$=6.0, p_{GG}=0.004, η^2_p=0.03, **Fig. 6**, left panel) with none of the post-hoc tests showing Bonferroni-corrected significant differences (all t< | 2.6 | all p_{bonf}>0.151) but visualizations

indicating more robust stimulus-specific spatial activation patterns in the DGCA3 region in the high LDI, compared to the low LDI group. Conversely, the low LDI group displayed stronger stimulus-specific activation patterns in the CA1 region. This pattern would be expected given the role of the DG in pattern separation and the role of the CA1 in pattern completion.

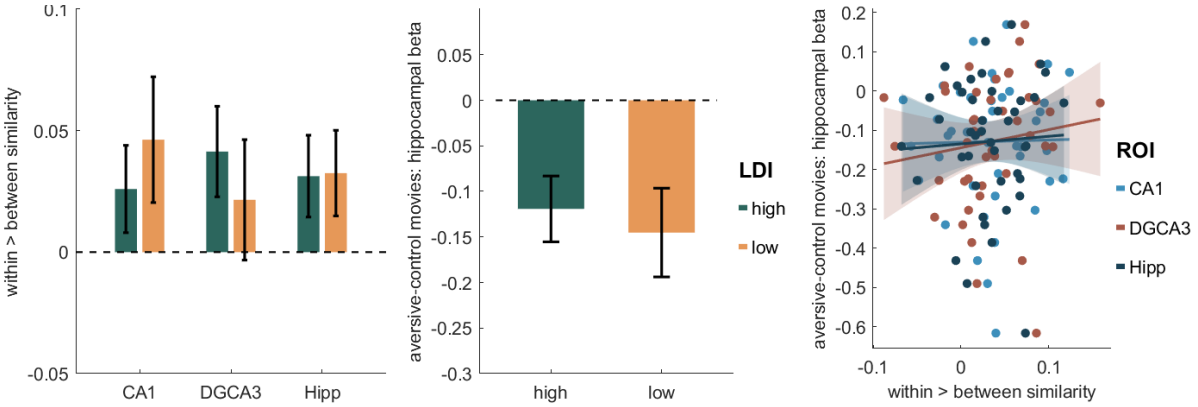

Figure 6. Left: Representational similarity within compared to between stimulus type. Participants in the high, compared to the low LDI group, displayed stronger pattern separation signals shown by a more distinct movie-specific activation pattern. Middle: The high and low LDI groups did not differ in hippocampal representational similarity. Right: Representational similarity was not associated with differential hippocampal activity.

Behavioral lure discrimination and hippocampal pattern separation are neither associated with differential hippocampal signaling, nor with intrusion distress

Contrary to hypothesis 2, LDI groups neither differed in their differential hippocampal activity towards aversive versus control movies (t_{41} =0.5, p =0.630, d=0.16, **Fig. 6**, middle panel), nor in intrusion distress (t_{45} =0.8, p =0.428, d=0.25, **Fig. 7** panel 1) nor contextual memory scores for the aversive movies (t_{45} =0.4, p =0.664, d =0.14, **Fig. 7** panel 3). Contrary to hypothesis 3, the degree of within versus between representational similarity was also not associated with differential hippocampal activity towards aversive versus control movies (all $\rho < |0.23|$, all p>0.143, **Fig. 6** right panel). Moreover, representational similarity scores did not predict intrusion distress (all $\rho < |0.23|$, all p>0.143, **Fig. 7** panel 2) or contextual memory scores for the aversive movies (all $\rho < |0.24|$, all p>0.123, **Fig. 7** panel 4).

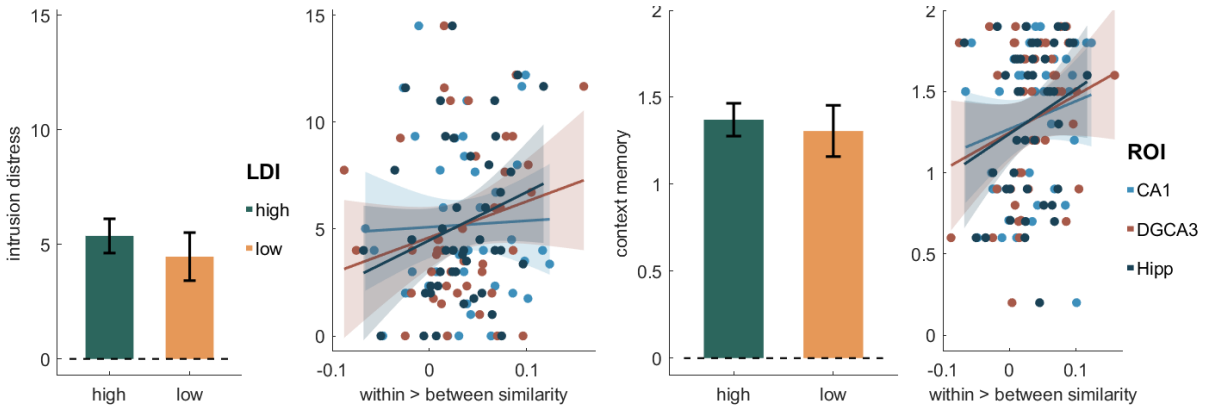

Figure 7. Panel 1: LDI groups did not differ in intrusion distress. Panel 2: Hippocampal representational similarity was not related to intrusion distress. Panel 3: LDI groups did not differ in context memory scores. Panel 3: Hippocampal representational similarity was not related to context memory.

Discussion

Different neurobiological frameworks on PTSD etiology converge on the notion that hippocampal deficits are one of the core risk factors for posttraumatic stress symptomatology, but they differ in the proposed hippocampal pathogenic mechanism (Brewin et al., 2010; Liberzon & Abelson, 2016; Milad & Quirk, 2012; Moscarello & Maren, 2018). Applying the conditioned-intrusion paradigm (Miedl et al., 2020b; Rattel et al., 2019; Wegerer et al., 2013) in healthy participants, we observed that individual differences in hippocampal reactivity to aversive films were indeed predictive of later self-reported intrusion distress: Participants who showed less hippocampal *deactivation* during aversive compared to neutral movies, reported more intrusion distress in the week after watching short movie fragments. This suggests that a less prominent hippocampal distinction between the aversive and the control condition is associated with intrusive memory formation. We did not observe any relationship with hippocampal (or amygdalar) activity to the conditioned stimuli, suggesting that CS-US associative learning appeared of less relevance than responding to the US. Moreover, there was no evidence for a relationship between hippocampal reactivity and a candidate mechanism for the observed hippocampal individual differences that we additionally tested, pattern separation. Although the behavioral and neural indices of pattern separation were coherent, showing stronger pattern separation in the dentate gyrus in our group with high behavioral lure discrimination scores, pattern separation also did not correlate with intrusion distress directly.

Across the whole sample, the hippocampus was deactivated in response to aversive compared to neutral movies. This is in line with a recent meta-analysis showing reduced BOLD signal in the amygdala and parahippocampal gyrus in stress- compared to neutral conditions (Berretz et al., 2021). Why is the hippocampus deactivated under stress in fMRI paradigms? In fact, there is evidence for an increase in hippocampal involvement presenting as a paradoxically decreased hippocampal BOLD signal (Hill et al., 2021; Schridde et al., 2008). This may be partly explained by the central hippocampal role in up – and downregulating the hypothalamus-pituitary-adrenal-gland (HPA)-mediated stress

response (Herman et al., 2016). Under resting cortisol levels, the hippocampal connections to the paraventricular nucleus of the hypothalamus are inhibitory, with inhibitory neural activity being metabolically demanding and thus causing a high steady state in the BOLD response (Pruessner et al., 2010; Schridde et al., 2008). During acute stress, a release from this inhibition leads to a decrease in the hippocampal BOLD response (Pruessner et al., 2010), possibly initiating the stress-induced HPA-cascade ending in cortisol production (Herman et al., 2016). Such a clear hippocampal stress-related signature may in fact be a correlate of adaptive stress signaling, as the hippocampus holds a key role in HPA-axis feedback and thereby helps to re-establish homeostasis.

Which structure could signal a clear onset of stress? Via direct connections, the locus coeruleus, as an important salience detector and the brain's main noradrenergic (NA) output center, may directly signal threat to the dentate gyrus within the hippocampus (Poe et al., 2020). In turn, the hippocampus may display stress-related activity changes and discontinue the inhibition of the HPA axis. Tonic locus coeruleus firing rates under baseline conditions can be flexibly changed to a strong phasic firing mode (Arnsten, 2000; Aston-Jones & Cohen, 2005). Under high tonic activity, however, the overlaying phasic signal cannot be that distinct, leading to blunted LC-NA signaling under stress (Aston-Jones & Cohen, 2005). Although we did not directly measure NA levels, we can speculate about noradrenergic signaling by continuously measuring pupil size. Based on its strong innervation by the locus coeruleus, the pupil is a sensitive indirect readout of NA signaling in the human brain (Strauch et al., 2022). While tonic LC firing influences baseline pupil size, phasic LC signals, indicating saliency, lead to an additional dilation during threat. Critically, there was an association between pupil size and hippocampal reactivity in our study. Participants who displayed smaller differential pupil dilations, were also more likely to display a blunted hippocampal signal (in the form of less deactivation to aversive stimuli). Hypothetically, increased tonic NA signaling could have led to a larger baseline pupil diameter throughout the task, with less prominent phasic pupil dilations towards the aversive movies. In line with this interpretation, it has been argued that PTSD is characterized by disturbances in LC functioning (Morris, McCall, Charney, & Murrough, 2020). Compared to trauma-exposed controls, PTSD patients display stronger LC activation to loud sounds (Naegeli et al., 2018) and fearful stimuli (Morey et al., 2015) and LC size was shown to be associated with anxious arousal across diagnostic boundaries (Morris et al., 2020) which may lead to atypically elevated NA levels (Geracioti et al., 2001) and the associated increase in alertness and hyperresponsiveness in PTSD (Naegeli et al., 2018).

Given the interpretation of blunted hippocampal reactivity as a weakened trigger of the HPA-axis with eventually blunted glucocorticoid signaling, it remains unknown how this could shape the encoding of experiences in a way that leads to more stressful, involuntary memories in their aftermath. From a mechanistic perspective, blunted glucocorticoid signaling could relate to stronger fear memories by prolonging NA effects on memory consolidation, leading to strong and vivid memories of the aversive events (Cohen & Zohar, 2018). High levels of noradrenaline and blunted cortisol could thereby jointly underlie intrusive memory formation. Importantly, measuring hippocampal activity and pupil

size can only serve as a very indirect readout of glucocorticoid and NA signaling in the brain. Other studies employing the trauma film paradigm have measured salivary cortisol and alpha-amylase as a proxy measure for noradrenaline and have shown varying associations with intrusive memory formation. In a study by Chou et al. (2014), participants with greater subclinical PTSD symptoms had lower cortisol concentrations after watching the trauma films and lower cortisol levels predicted greater vividness of intrusions. Adding additional stressors before watching trauma films, however, led to a positive association between intrusive memories and stress-induced salivary cortisol levels (Hilberdink et al., 2022; Schultebraucks et al., 2019). NA signaling has been shown to be predictive of intrusion severity in many neuroendocrinologic studies employing the trauma film paradigm (Chou et al., 2014; Hilberdink et al., 2022; Rombold et al., 2016; Schultebraucks et al., 2019). Together, these findings show that converging evidence points to a role of increased NA signaling in intrusive memory formation, while the role of cortisol and the interaction of both remains to be specified.

One of the working hypotheses about the underpinnings of individual differences in hippocampal processing and their association with PTSD is that impaired DG functioning and associated deficits in pattern separation underlie pathological memory formation evolving after trauma (Anacker & Hen, 2017; Lecei & van Winkel, 2020; Liberzon & Abelson, 2016). As this has never been tested in an experimental setting, we also set out to investigate whether pattern separation abilities protect from intrusion formation and loss of contextual memory in the aftermath of aversive experiences. To do so, we followed a quasi-experimental approach comparing two groups with high and low discrimination abilities, a behavioral proxy for hippocampal pattern separation (Stark et al., 2019). Importantly, participants who had a high lure discrimination index indeed exhibited more pattern separation on the neural level, which was shown by a stronger movie-specific signaling in the DG in this group. However, neither behavioral lure discrimination performance nor hippocampal pattern separation were related to hippocampal reactivity to aversive movies, speaking against individual differences in hippocampal signaling under stress being driven by pattern separation in this experimental approach or in relatively healthy samples. Moreover, the high lure discrimination group reported similar intrusion distress and had similar context memory scores as the low discrimination group, and the specificity of DG signaling was also not associated with these memory measures. Together, our results therefore speak against pattern separation abilities as a generic mediating mechanism in pathological memory formation.

Various limitations have to be kept in mind when interpreting our results. First, as opposed to a recently published first meta-analysis on the conditioned-intrusion paradigm (Ney et al., 2022), conditioning was not robustly present in our physiological measures and in fMRI, refraining us from analyzing CS-related data during conditioning and especially during recall. Comparing conditioning and recall data would have allowed us to better understand the neural correlates of remote fear memory recall and its associations with context memory and intrusion distress, as well as disentangling the dynamics of consolidation mechanisms between LDI groups. In contrast to existing studies in which faces or neutral objects were associated with different aversive movies, we repeatedly presented the same movie

that was preceded by a picture taken out of the movie. With this procedure, we wanted to increase the ecological validity even further, assuming that associative mechanisms act stronger on conceptually related materials. As this was not the case, employing unrelated CS might be more beneficial in measuring associative learning mechanisms over time. Second, as we were interested in differential patterns between DGCA3 and CA1, we extracted individual hippocampal subfields instead of using the same coordinate-based atlas for all participants. Although there is quite some individual variation in hippocampal subfields, speaking for such an approach, it comes at the risk of pseudo-specificity (Wisse et al., 2021). Third, we used a female-only sample due to observed sex-effects during the piloting phase but did not control for menstrual phase. Future studies should ideally directly compare both sexes and assess the impact of sex hormones on pathological memory formation. Fourth, due to the complicated recruitment process and strict exclusion of participants changing LDI groups, our sample was of medium size, precluding any statements on small to medium effect sizes. Due to our sample size, we also focused on some key variables and few regions of interest, and refrained from engaging in multiple testing procedures which of course comes at the prize of losing the "bigger picture". However, given our quasi-experimental approach with the careful pre-selection of participants with lure discrimination abilities of 1 SD above or below average and comparing these groups not only with respect to the mnemonic outcome measures, but also regarding their hippocampal function, we still think that it adds valuable insights into hippocampal contributions to pathological memory formation.

In sum, our study provided evidence for a hippocampal role in intrusion formation in a human experimental model of intrusive memory formation. Contrary to our additional hypotheses, pathological memory formation was not driven by the cognitive process of pattern separation, but more likely by generic hippocampal reactivity towards the aversive movies. This generic hippocampal deactivation to the aversive movies is likely to reflect an initiation of the stress response, which is potentially triggered by the LC-NA system and could entail downstream consequences of relevance to the HPA-axis.

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Supplemental information

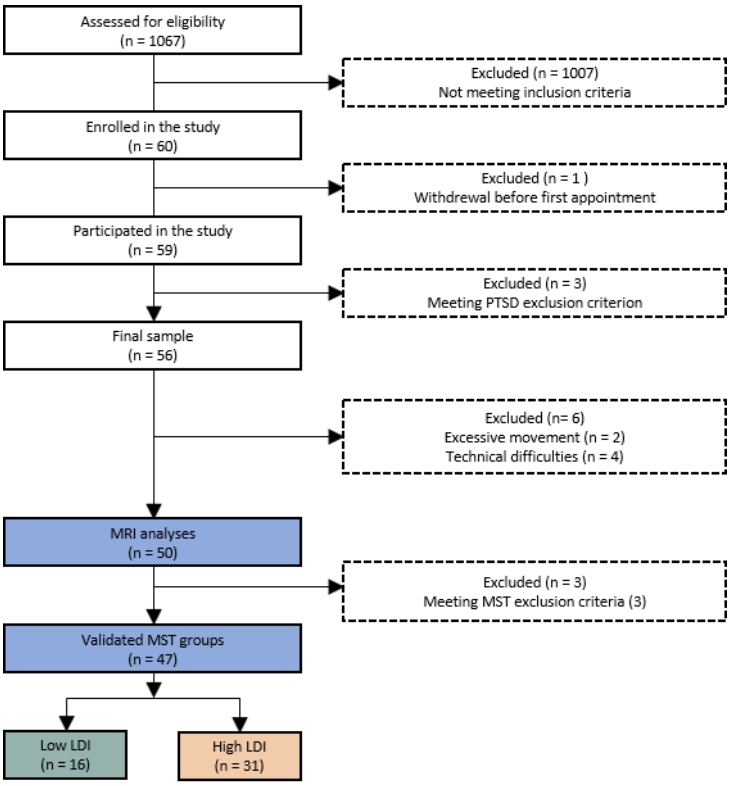

Figure S1. Flowchart of participant inclusion. In total, 1067 participants filled in our online screening questionnaire. Of these participants, 60 were enrolled in the study based on the inclusion criteria. One participant dropped out before the first appointment and three participants met the PTSD exclusion criteria, resulting in 56 participants.

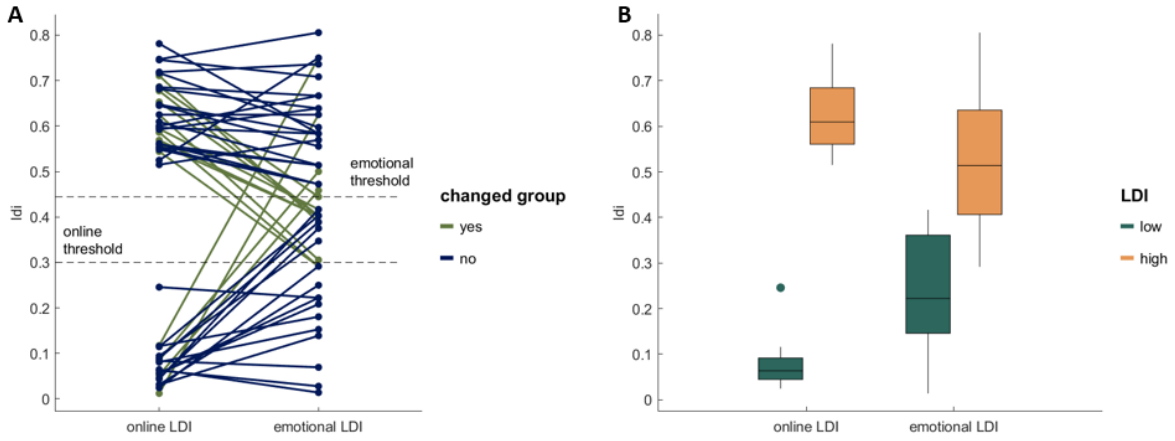

Figure S2. Validation of the online mnemonic similarity task (MST). A shows the LDI in the online task that was used to determine the quasi-experimental groups as well the emotional LDI that was calculated from the emotional MST acquired on the third visit.

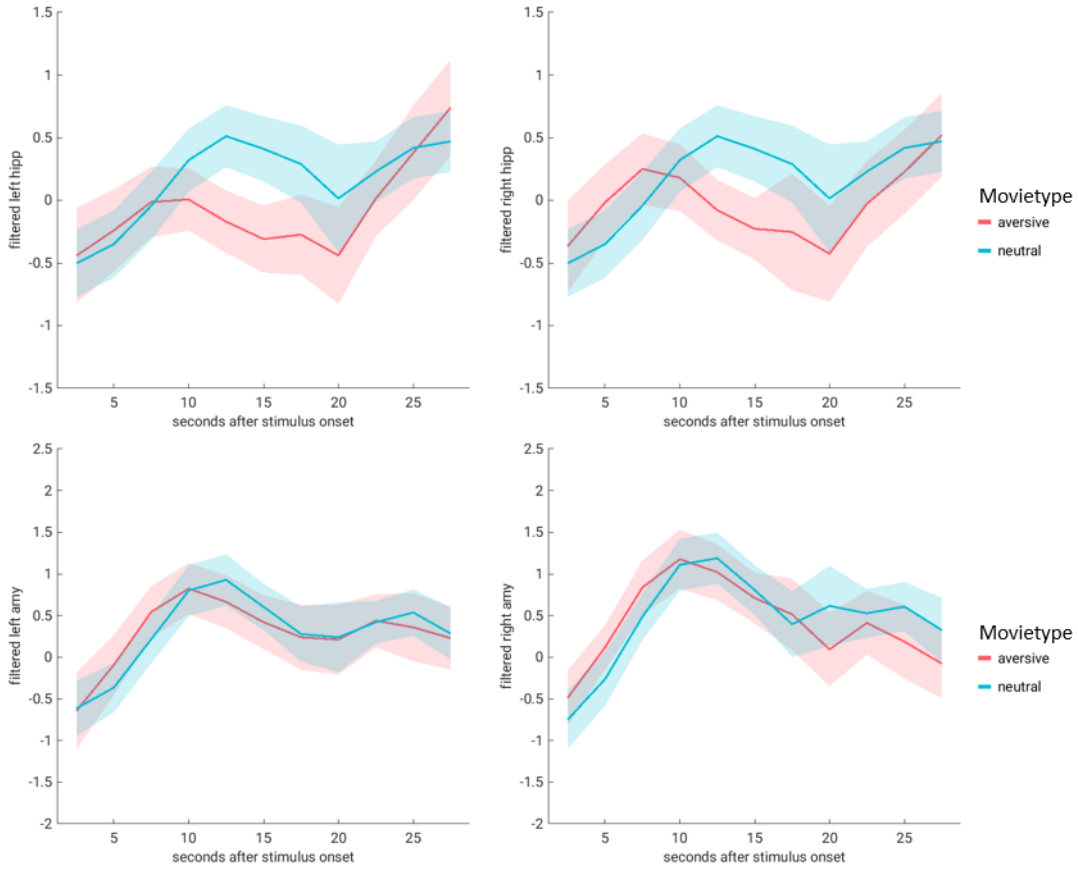

Figure S3. Meaned hippocampal and amygdalar BOLD activity in response to the aversive and neutral movie fragments. Movie fragments were 16 seconds long.

Voxel k	$P_{FWECLUSTER}$	Region (%cluster)	X	Y	Z	PFWEPEAK	T_{peak}
1771	<.001	Middle temporal gyrus, middle occipital gyrus, inferior occipital gyrus, inferior temporal gyrus, fusiform gyrus (left)	-50	-68	2	<.001	13.39
2216	<.001	Supramarginal gyrus, superior parietal lobule, inferior parietal lobule, superior temporal gyrus (left)	-54	-38	26	<.001	12.04
903	<.001	Supramarginal gyrus, superior temporal gyrus, middle temporal gyrus, Rolandic operculum (right)	62	-38	26	<.001	11.45
849	<.001	Middle temporal gyrus, inferior temporal gyrus, inferior occipital gyrus (right)	46	-62	-2	<.001	10.52
693	<.001	Cerebellum (left, right)	10	-78	-46	<.001	10.30
272	<.001	Precentral gyrus, middle frontal gyrus, superior frontal gyrus (right)	42	-4	54	<.001	9.89
472	<.001	Middle occipital gyrus, superior occipital gyrus, middle temporal gyrus (right)	40	-76	8	<.001	9.88
552	<.001	Superior parietal lobule, inferior parietal lobule, angular gyrus (right)	22	-60	64	<.001	8.18
77	<.001	Precentral gyrus, postcentral gyrus (left)	-38	-10	50	<.001	8.15
265	<.001	Cerebellum (left)	-10	-74	-22	<.001	7.95

98	<.001	Cerebellum (right)	18	-72	-22	<.001	7.57
52	<.001	Precentral gyrus, postcentral gyrus (left)	-52	2	32	<.001	7.56
95	<.001	Inferior frontal operculum, precentral gyrus, inferior frontal gyrus	48	6	26	0.001	7.15

Table S1. Regions showing greater activation during control compared to neutral movies. Reported clusters survived cluster-wise family-wise error correction for multiple comparisons (pFWE < 0.05) at a cluster extension threshold of k = 110. Only contributions from regions above 1%$_{cluster}$ are listed. Peak voxel MNI coordinates X, Y, and Z are given in millimeters

Voxel k	$P_{FWECLUSTER}$	Region (%cluster)	X	Y	Z	PFWEPEAK	T_{peak}
1673	<.001	Inferior occipital gyrus, lingual gyrus, calcarine gyrus, cuneus, middle occipital gyrus, superior occipital gyrus, fusiform gyrus (right), cuneus (left)	22	-96	-8	<.001	17.52
1932	<.001	Middle occipital gyrus, calcarine gyrus, lingual gyrus, inferior occipital gyrus, superior occipital gyrus, fusiform gyrus (left)	-16	-100	-10	<.001	16.41
873	<.001	Superior temporal gyrus, Rolandic operculum, Heschl gyrus, insula, middle temporal gyrus (left)	-54	-8	-2	<.001	12.37
1877	<.001	Calcarine gyrus, precuneus, cuneus, lingual gyrus, cerebellum, superior occipital gyrus (left), precuneus, calcarine gyrus, lingual gyrus, cuneus (right)	-12	-60	12	<.001	12.30
4012	<.001	Postcentral gyrus, paracentral lobule, precentral gyrus, supplementary motor area, precuneus, middle cingulate gyrus (left), postcentral gyrus,	-2	-20	54	<.001	11.64

		superior temporal gyrus, precentral gyrus, Rolandic operculum, supplementary motor area, paracentral lobule, superior temporal pole, insula, middle cingulate gyrus (right)					
417	<.001	Superior frontal gyrus, middle frontal gyrus (right)	30	16	50	<.001	10.58
791	<.001	Middle frontal gyrus, superior frontal gyrus (left)	-24	12	52	<.001	9.53
783	<.001	Precuneus, middle cingulate gyrus, posterior cingulate gyrus (left), precuneus, middle cingulate gyrus, posterior cingulate gyrus (right)	-2	-66	46	<.001	9.13
277	<.001	Angular gyrus, middle occipital gyrus (right)	46	-70	32	<.001	9.07
771	<.001	Angular gyrus, middle occipital gyrus (left)	-34	-74	38	<.001	8.87
139	<.001	Fusiform gyrus, lingual gyrus, parahippocampal gyrus (left)	-30	-50	-8	<.001	8.86
58	<.001	Fusiform gyrus, lingual gyrus,	32	-46	-8	<.001	7.99

		parahippocampal gyrus (right)					
64	<.001	Parahippocampal gyrus, hippocampus, fusiform gyrus (right)	32	-20	-20	<.001	7.80
54	<.001	Cerebellum (right)	40	-68	-40	<.001	7.69
325	<.001	Medial orbitofrontal cortex (left, right), rectus, anterior orbitofrontal cortex (left)	-4	48	-10	<.001	7.41
75	<.001	Postcentral gyrus (left)	-42	-18	36	<.001	7.17
89	<.001	Middle temporal gyrus, inferior temporal gyrus (left)	-56	-40	-14	.001	7.16
74	<.001	Superior frontal gyrus, superior medial frontal gyrus (left)	-12	68	14	.002	6.81

Table S2. Regions showing greater activation during neutral compared to aversive movies. Reported clusters survived cluster-wise family-wise error correction for multiple comparisons (pFWE < 0.05) at a cluster extension threshold of k = 110.Only contributions from regions above 1%$_{cluster}$ are listed. Peak voxel MNI coordinates X, Y, and Z are given in millimeters

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