Fear Memories and Extinction Memories: Neurophysiological Indicators and the Role of Estradiol and Extinction Timing

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List of abbreviations used in the introduction and general discussion

BA	basal amygdala
BLA	basolateral amygdala
BOLD	blood oxygenation level dependent
CeA	central amygdala
CRF	corticotropin releasing factor
CS	conditioned stimulus
CS-	CS not paired with the US
CS+	CS paired with the US
$CS^{+/-}E$	CS presented during extinction acquisition
$CS^{+/-}N$	CS not presented during extinction acquisition
dACC	dorsal anterior cingulate cortex
dmPFC	dorsomedial prefrontal cortex
E2	17β-estradiol
eBOSC	extended better oscillation detection
EEG	electroencephalogram
ER	estrogen receptor
ERα	estrogen receptor of the subtype α
ERβ	estrogen receptor of the subtype β
FFT	fast Fourier transfom
fMRI	functional magnetic resonance imaging
FOOOF	fitting oscillations & one over f
IED	immediate extinction deficit
IL	infralimbic cortex
ITC	intercalated cell masses
LA	lateral amygdala
LC	locus coeruleus
LFP	local field potential
LORETA	low resolution brain electromagnetic tomography
MC	midcycle
mPFC	medial prefrontal cortex
NMDA-R	N-methyl-D-aspartat-receptor
OC	oral contraceptives
P4	progesterone

PL	prelimbic cortex
PTSD	Post-traumatic stress disorder
SCR	skin conductance responses
US	unconditioned stimulus
vmPFC	ventromedial prefrontal cortex

General Abstract

Fear memories are necessary to initiate anticipatory fear responses when we are confronted with cues that predict an impending threat. However, when a cue no longer predicts threat, an *extinction memory* is formed that actively inhibits the expression of the fear memory. Failure to acquire, consolidate, or recall extinction memories causes fear memory expression (i.e., fear responding) in the absence of threat, which is a hallmark characteristic of most anxiety-related disorders and post-traumatic stress disorder (PTSD). Of further importance, these disorders occur approximately twice as often in women than men, which is thought to partially rely on sex hormone mediated differences in fear extinction. Moreover, deficits in extinction memory processing can also hinder the success of extinction-based exposure therapy, which is commonly used to treat these disorders. Thus, a better understanding of the factors determining the quality of extinction memories is of utmost importance.

The present thesis focuses on three of these factors including the female sex hormone *17β-estradiol* (E2), fear *extinction timing*, and the *noradrenergic arousal system*. To examine the role of E2 (Manuscript 1; low E2 levels or high E2 levels) and fear extinction timing (Manuscript 2; either immediately or delayed after the initial fear memory formation), we used a special differential fear conditioning procedure that allowed us to separately assess fear memories and extinction memories via peripheral arousal responses (measured via skin conductance responses [SCR]) and, most importantly, via central *neurophysiological indicators* (measured via electroencephalography [EEG]). Concerning EEG parameters, we were especially interested in *neural oscillations* (especially in the theta and gamma range). To further advance the understanding of the neurophysiological foundations of both memory systems, we also aimed at disentangling oscillatory and non-oscillatory brain activity (Manuscript 2). Moreover, the crucial role of the noradrenergic arousal system for the quality of extinction memories is highlighted in a review of relevant rodent and human studies (Manuscript 3).

By using the described multi-methodological approach, we were able to demonstrate for the first time that *peripheral arousal* as well as *fear-related theta oscillations* are sensitive to E2. This was indicated by less fear responding (attenuated peripheral arousal and attenuated theta oscillations) during the recall of fear and extinction memories under high peripheral E2 levels (Manuscript 1). Concerning the role of fear extinction timing, we demonstrate that delayed extinction is advantageous over immediate extinction in reducing peripheral arousal during the recall of the extinction memory (Manuscript 2). Additionally, by disentangling oscillatory and non-oscillatory brain activity, we demonstrate for the first time that oscillatory

and non-oscillatory brain activity is sensitive to fear expression. Moreover, by reviewing different rodent and human studies, we highlight the important role of noradrenergic arousal for the recall of extinction memories and, importantly, provide a detailed mechanistic framework of how extinction deficits might be caused after immediate extinction (Manuscript 3).

In sum, the present thesis underscores the important role of E2, fear extinction timing, and the noradrenergic system for the recall quality of fear memories and extinction memories in humans.

1 Introduction

1.1 Overview

Even though *fear* is often negatively connoted, it is the most important emotional response when we are confronted with imminent threats to well-being, physical integrity, or survival. Depending on the distance of the threat, the fear system initiates innate defensive behaviors, such as fight, flight or freezing responses, to face, escape, or avoid threats, respectively (Fanselow, 1994). These responses are accompanied by increases in peripheral arousal, which crucially support the initiation and maintenance of these defensive behaviors (LeDoux, 2016). In addition, an increase in central arousal results, among others, in enhanced vigilance and attention (LeDoux, 2012). The behavioral, autonomic, and central responses to environmental threats are generally referred to as *fear responses.*

From an evolutionary perspective, the fear system evolved true to the motto "foresight is better than hindsight", especially when dealing with threats that acutely endanger the physical integrity or even the survival of an organism. Accordingly, environmental cues (or contexts) that reliable predict the occurrence of a threat are readily stored into an implicit *fear memory.* Fear memories primarily consist of a cue-threat association which initiates anticipatory fear responses when confronted with the mere threat-predictive cue (i.e., *fear memory expression* or *fear recall*). This anticipatory fear response is thought to substantially enhance chances of survival by giving the organism a temporal advantage to prepare for a potential threat encounter (Mobbs et al., 2015). In case a cue loses its predictive power for an impending threat, fear responding to the cue gradually declines with repeated cue exposure (i.e., the expression of the fear memory slowly decreases). This process is known as *fear extinction* and involves the formation of a new *extinction memory* which actively inhibits the expression of the original fear memory (Bouton, 2002). Thus, successful *extinction memory expression* (i.e., *extinction recall*) is characterized by little to none fear responding toward the former threat-predictive cue. Importantly, accumulating evidence from rodent studies reveals that the interval between the initial formation of the fear memory and the onset of fear extinction is a crucial determinant of extinction recall success (for reviews see: Giustino & Maren, 2018; Maren, 2014): Long intervals (i.e., delayed fear extinction) were demonstrated to be advantageous over short intervals (i.e., immediate fear extinction). However, relatively little is known about *fear extinction timing* in humans.

On the central neurobiological level, the primary structures involved in fear and extinction memory processing comprise the amygdala, the medial prefrontal cortex (mPFC),

and the hippocampus (Giustino & Maren, 2015). The amygdala is primarily responsible for the initiation of fear responses and acts as initial storage site for fear memories. Importantly, neural activity in the amygdala is tightly controlled by two major substructures of the mPFC that receive and integrate different information (e.g., contextual information from the hippocampus) to guide appropriate fear responding: While the dorsal anterior cingulate cortex (dACC) is especially important for the facilitation and maintenance of fear responses, the ventromedial prefrontal cortex (vmPFC) is crucially involved in the inhibition of fear responses during fear extinction. On a more neurophysiological level, neural activity within the described structures is firmly controlled by *neural oscillations* (Bocchio et al., 2017). Oscillations are a fundamental mechanism for the orchestration of neural activity within and between neural structures (Fell & Axmacher, 2011). Increases in both magnitude and synchronization of theta oscillations (4-8 Hz)[1] in the fear circuitry (dACC, amygdala, and hippocampus) are thought to be necessary to gate and regulate fear expression (Courtin et al., 2014; Lesting et al., 2011; Likhtik et al., 2014). Gamma oscillations (> 30 Hz) in the vmPFC, on the other hand, have been linked to successful fear extinction recall (E. M. Mueller et al., 2014). Of special importance, neural oscillations can be readily assessed in humans and other species and are therefore an important measure to translate results about the neurophysiological foundations of fear and extinction processing from animals to humans and vice versa.

While fear and extinction memories are usually highly adaptive, dysregulation of these memory systems are a cardinal feature of most anxiety-related disorders and post-traumatic stress disorder (PTSD; Craske et al., 2018; Milad & Quirk, 2012; Pitman, 1988). Especially, failure to adequately acquire or express extinction memories can cause and preserve fear responding despite the absence of an actual threat, which is a hallmark characteristic of many anxiety-related disorders and PTSD (Cooper & Dunsmoor, 2021; Garfinkel et al., 2014; Jovanovic et al., 2012; Jovanovic & Ressler, 2010; Michael et al., 2007; Milad et al., 2014; Milad, Pitman, et al., 2009; Schweckendiek et al., 2011). Thus, *extinction deficits* are thought to contribute to the development and maintenance of these disorders (Craske et al., 2018; Milad & Quirk, 2012). Moreover, extinction deficits may also create obstacles and cause relapse during extinction-based therapies. Thus, a better understanding of the underlying factors contributing to extinction deficits as well as its neurobiological foundations is of utmost importance. Additionally, identifying factors that improve fear extinction can instruct

[1] Please note that the theta frequency range slightly differs between primates and rodents. In primates, theta oscillations typically occur between ~4-8 Hz (e.g., E. M. Mueller et al. 2014; Taub et al. 2018). In rodents, on the other hand, theta oscillations are faster and occur in a frequency range between ~4-12 Hz (e.g., Fenton et al. 2014; Lesting et al. 2011).

extinction-based therapies to accelerate therapy progress and cause greater long-term relief of symptoms.

One particular important factor contributing to a dysregulation of fear and extinction memories concerns biological sex and sex hormone actions. Importantly, the lifetime incidence of anxiety-related disorders and PTSD is approximately twice as high in women compared to men, which is partially attributed to differences in extinguishing fear memories (Cover et al., 2014; Maeng & Milad, 2015). The female sex hormone *17β-estradiol* (E2) contributes to these differences, since it was shown to impact the recall of fear memories, and especially, the recall of extinction memories (Barha et al., 2010; Graham & Milad, 2013; Kobayashi et al., 2020; Zeidan et al., 2011). The neuro-oscillatory mechanisms underlying these sex differences have so far only been examined in rodents without consideration of E2 (Fenton et al., 2014; Fenton et al., 2016). In the first study (*2.1 Manuscript 1*) we therefore aimed at examining the role of sex differences and the role of the female sex hormone E2 for neural oscillations during fear memory and extinction memory processing in healthy humans.

Based on the results of Manuscript 1, in the second study (*2.2 Manuscript 2*) we aimed at examining the role of extinction timing for the success of later extinction recall. More precisely, we examined whether a long interval (i.e., delayed fear extinction) between the initial fear memory formation and fear extinction results in better extinction recall compared to a short interval (i.e., immediate fear extinction). Importantly, we further aimed at examining the neuro-oscillatory mechanisms underlying immediate and delayed fear extinction, while also expanding the methodological scope of the neurophysiological analyses by disentangling oscillatory and non-oscillatory brain activity.

Rodent studies using sophisticated methods demonstrated that noradrenaline is a potent modulator of fear extinction and seems to be the primary driver of differences in extinction recall after immediate vs. delayed fear extinction (Giustino & Maren, 2018). However, human studies regarding the role of noradrenaline during fear extinction are sparse, not at least because measuring and manipulating the noradrenergic system in humans remains challenging. We therefore reviewed the current evidence regarding the role of noradrenaline for fear extinction in rodents and humans. We compiled the available (mainly rodent) data into a hypothetical mechanistic framework of how noradrenaline and the neuropeptide *corticotropin releasing factor* (CRF) orchestrate the neural fear and extinction circuitry to attenuate or improve extinction recall. Moreover, we also provide an overview of different techniques to non-invasively measure and manipulate the noradrenergic system in humans (*2.3. Manuscript 3*).

In the subsequent sections of the introduction, I will first describe the different stages of fear memories and extinction memories in the context of Pavlovian fear conditioning (section 1.2.1), which was utilized in the present experiments and is widely employed as an outstanding laboratory tool for the standardized assessment and induction of fear memories and extinction memories. Afterward I will give a brief overview of the neural fear and extinction circuitry (section 1.2.2), followed by a closer look at the neural oscillations which have been linked to fear memory and extinction memory processing (section 1.3). Next, I will introduce the female sex hormone E2 and its modulating impact on fear memories and extinction memories (section 1.4). Finally, the importance of extinction timing for the recall of extinction memories as well as its noradrenergic modulation will be addressed (section 1.5).

1.2 Fear conditioning

Fear memories can be acquired directly via experiencing a threat or indirectly via social transmission, due to the observation of others experiencing a threatening situation (also known as vicarious fear learning) or by verbal instructions concerning the perilousness of specific stimuli (Debiec & Olsson, 2017; Olsson & Phelps, 2007). The majority of human and animal studies examined fear memories acquired by means of *Pavlovian fear conditioning*, which involves the direct experience of a threat. Pavlovian fear conditioning with the stages *fear acquisition*, *fear extinction*, as well as *fear recall* and *extinction recall* is a valid laboratory model for the development, maintenance, and treatment of most anxiety-related disorders and PTSD (e.g., Craske et al., 2018; Milad & Quirk, 2012; Pitman, 1988).

1.2.1 The different stages of fear memories and extinction memories during Pavlovian fear conditioning

In a Pavlovian fear conditioning paradigm, a fear memory is acquired at the *fear acquisition* (i.e., *fear learning*) stage (see Fig. 1A). During fear acquisition an initially neutral stimulus (i.e., conditioned stimulus [CS]) predicts the occurrence of an aversive unconditioned stimulus (US)[2]. The US is typically an innate aversive threat (e.g., a very loud noise or an electric shock) capable of evoking an unconditioned (i.e., innate) fear response. After repeatedly pairing the CS with the US, the CS gradually acquires the capability of evoking a conditioned fear response on its own. After successful fear acquisition the fear memory will be *consolidated* (i.e., *fear memory consolidation*, see Fig. 1A,B) which comprises long-term synaptic and cellular

[2] Of note, human studies often use a differential fear conditioning paradigm in which one stimulus predicts (CS+) the occurrence of a US while another control stimulus (CS-) does not predict the US. Conditioned fear responding is typically quantified via so-called differential fear responses (CS+ minus CS-; Lonsdorf et al., 2017).

plasticity, eventually resulting in the stabilization of the fear memory (Orsini & Maren, 2012). The consolidated fear memory can be retrieved when again confronted with the CS (see Fig. 1B). This stage is also called *fear recall* or *fear retrieval* and is often assessed >24 hours after the initial fear acquisition.

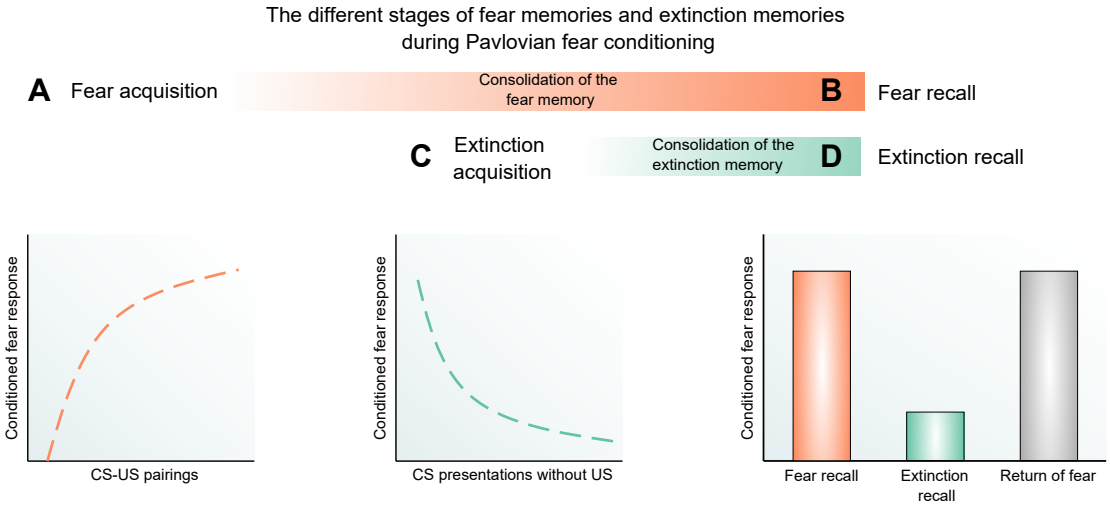

Figure 1. The different stages of fear memories and extinction memories during a Pavlovian fear conditioning procedure. (**A**) an initially neutral CS is repeatedly paired with an innate aversive unconditioned stimulus (US). After repeatedly pairing the CS with the US (x-axis), the CS gradually evokes conditioned fear responses (y-axis) on its own (red line). After successful fear acquisition, the fear memory will be consolidated (horizontal red bar). (**B**) The consolidated fear memory can be retrieved when again confronted with the CS (i.e., fear recall). Fear recall is typically quantified by the strength of conditioned fear responses during CS presentation (vertical red bar). (**C**) When the CS is repeatedly presented without the US (x-axis), CS evoked fear responses (y-axis) gradually decline (green line). After successful extinction acquisition, the extinction memory will also be consolidated (horizontal green bar). (**D**) The consolidated extinction memory can be retrieved when again confronted with the extinguished CS. Good extinction recall is indicated by low fear responding to the extinguished CS (vertical green bar). However, since extinction acquisition does not delete the original fear memory, fear responding can return (i.e., return of fear; vertical gray bar) to an already extinguished CS, which is considered to be a bad extinction recall.

The expression of the fear memory can be reduced when the CS loses its predictive power for the US. As mentioned above, this phenomenon is called *fear extinction* and includes the formation of a separate *extinction memory*. Fear extinction consists of the phases: *extinction acquisition* (i.e., *extinction learning*), *extinction consolidation*, and *extinction recall*. During extinction acquisition the CS is repeatedly presented without the US which results in a gradual decline of conditioned fear responding (see Fig. 1C). Thus, successful extinction acquisition is characterized by reduced fear responding to the extinguished CS. Similar to fear memories, extinction memories will be consolidated (i.e., *extinction memory consolidation*, see Fig. 1C,D), which also involves long-term synaptic and cellular plasticity, albeit in somewhat different neural structures (Bouton et al., 2021). The consolidated extinction memory can also be retrieved when again confronted with the extinguished CS (see Fig. 1D). In case of successful extinction recall, the extinguished CS elicits little to none fear responses. However,

since fear extinction does not (at least not completely) erase the original fear memory, fear responding may return during extinction recall despite successful extinction acquisition (Bouton, 2002; see Fig. 1D). There are three prominent circumstances termed *return of fear phenomena* which significantly increase the chance of *fear relapse* after successful extinction acquisition. First, *spontaneous recovery* describes the return of fear responses elicited by the extinguished CS after the mere passage of time. The magnitude of spontaneous recovery is modulated by individual internal (e.g., hormone levels) and external factors (e.g., timepoint of extinction acquisition; e.g., Stockhorst & Antov, 2016). Second, *fear renewal* describes the return of fear responses elicited by the extinguished CS when the extinction recall context differs from the context in which the extinction acquisition took place. Importantly, fear renewal represents a challenge for current extinction-based therapies when transferring therapy success from the therapeutic context into daily life. Third, *reinstatement* describes the return of fear responses elicited by the extinguished CS when the US is immediately presented before extinction recall. Reinstatement is caused by increased brain arousal (i.e., central arousal) evoked by the US presentation (Giustino et al., 2019; Jo et al., 2020). While most human and animal studies quantify extinction recall after a defined passage of time (typically 24 hours after extinction acquisition), a significant part of studies additionally utilizes renewal or reinstatement procedures to further probe extinction recall.

1.2.2 The neural fear and extinction circuitry

The amygdala is the cardinal structure of the neural fear and extinction circuitry since it is necessary for the initiation of fear responses and the initial storage of the fear memory (LeDoux, 2000). The amygdala consists of subnuclei subserving different functions. The most prominent ones are the basolateral amygdala (BLA), which is further subdivided into the lateral amygdala (LA) and the basal amygdala (BA), the central amygdala (CeA), and the intercalated cell masses (ITCs) located between the BLA and CeA (Amano et al., 2010).

The LA is the main input structure of the amygdala. During fear acquisition, information about the CS and the US converges at principal neurons of the LA. The coincidental activation of weak CS synapses and strong US synapses causes long-term potentiation of the CS synapses via N-methyl-D-aspartat-receptor (NMDA-R) dependent mechanisms (Orsini & Maren, 2012). Thus, after successful fear acquisition, the CS can activate the LA on its own. Information about threats (US) or impending threats (CS) is further transmitted from the LA to the BA, where it is integrated with prefrontal and hippocampal input and relayed to the main output structure of the amygdala, the CeA. The CeA, especially its medial division, sends diverging outputs to

different subcortical structures to initiate a fear response. For instance, connections from the medial CeA to the periaqueductal grey regulate freezing behavior, while connections to the lateral hypothalamus and locus coeruleus (LC) cause increases in peripheral and central arousal (LeDoux, 2012). Expression of conditioned fear is tightly regulated by inputs from the mPFC to the amygdala (Giustino & Maren, 2015). In this regard, the dACC in primates and its rodent homologue, the prelimbic cortex (PL), have been repeatedly shown to facilitate and maintain conditioned fear responding, especially during fear recall (Giustino & Maren, 2015). It is further assumed that the dACC/PL integrates higher-order information (e.g., about the context) to gate appropriate fear expression under ambiguous circumstances (e.g., Sharpe & Killcross, 2014, 2015; Sotres-Bayon et al., 2012). Corresponding to its proposed role in the expression of conditioned fear, neural activity in the dACC/PL was shown to be increased during deficient extinction recall (e.g., Giustino & Maren, 2015; Milad, Pitman, et al., 2009). Accordingly, the dACC of PTSD patients was shown to be hyperactive during extinction recall which was associated with a greater return of conditioned fear responding to already extinguished CS (Garfinkel et al., 2014; Milad, Pitman, et al., 2009).

The amygdala is also crucially involved in the acquisition of extinction memories. It was demonstrated in rodents that NMDA-R dependent mechanisms in the BLA are necessary for extinction acquisition (Bouton et al., 2021). Information about the extinguished CS is processed in the BA by so-called extinction neurons relaying information about the (extinguished) CS to inhibitory neurons of the ventral ITC (Amano et al., 2010; Herry et al., 2008). The ventral ITC inhibits neural activity in the medial CeA resulting in a suppression of fear responding to the extinguished CS (Amano et al., 2010). The acquisition, consolidation, and recall of extinction memories are strongly dependent on the ventromedial PFC (vmPFC) in primates, and the infralimbic cortex (IL), which is the rodent homologue of the primate vmPFC (Giustino & Maren, 2015). Neural activity in the vmPFC/IL during and immediately after extinction acquisition was demonstrated to be necessary for the successful consolidation of extinction memories (Bukalo et al., 2015; Burgos-Robles et al., 2007; Do-Monte et al., 2015; Gerlicher et al., 2018; Hennings et al., 2022). Moreover, neural activity in the vmPFC/IL is also involved in the suppression of fear responding during extinction recall (Milad & Quirk, 2002; Milad, Wright, et al., 2007; Phelps et al., 2004). The vmPFC/IL contains extensive reciprocal connection with the BLA (Hurley et al., 1991; McDonald et al., 1996; Pinard et al., 2012; Sesack et al., 1989; Strobel et al., 2015). Importantly, it sends excitatory input to BLA neurons which in turn activate inhibitory neurons of the ventral ITC that are crucially involved in suppressing amygdala output and thereby causing low fear responding (Strobel et al., 2015).

1.3 The role of neural oscillations in the neural fear and extinction circuitry

The empirical studies of the present thesis (Manuscript 1 and Manuscript 2) assessed neural activity within the fear and extinction circuitry in the form of neural oscillations. Thus, in the following section, the neurophysiological origins and functions of neural oscillations will be described in more detail.

Neural activity with its accompanying transmembrane currents causes fluctuations in intracellular and extracellular membrane potentials that can be assessed from intracranial and extracranial recordings (Buzsáki et al., 2012). The extracellular potentials are normally termed *local field potentials* (LFP) when recorded via intracranial electrodes, and *electroencephalogram* (EEG) when recorded via extracranial scalp electrodes (Buzsáki et al., 2012). Single neurons are typically part of a greater neural circuit which receives synchronized excitatory and inhibitory inputs eventually resulting in rhythmic oscillations of extracellular and intracellular potentials (Fell & Axmacher, 2011). Importantly, these neural oscillations are not just a mere epiphenomenon of neural activity, but reflect an evolutionary preserved neurophysiological mechanism that is necessary for the efficient communication within and between neural circuits (Buzsáki et al., 2013). Since neural oscillations reflect rhythmic fluctuations in membrane excitability (i.e., the membrane potential), synchronized oscillations (i.e., synchronized excitability) within a neural circuit result in an enhanced likelihood of synchronized action potential output, which drastically increases chances of evoking responses (i.e., neural activity) in other target circuits (Fell & Axmacher, 2011). Moreover, neurons are also more receptive for synaptic input when membrane excitation is high (Fell & Axmacher, 2011). Thus, oscillatory synchronization between neural circuits "*provides windows for optimal communication between two or more brain areas*" (Fell & Axmacher, 2011, p. 106), since an increased likelihood of action potential generation of the output circuit coincides with an elevated input sensitivity of its target circuit (Fell & Axmacher, 2011). In sum, neural oscillations orchestrate neural activity to increase the efficiency of information processing within a neural circuit but also optimize the communication between spatially segregated brain areas (Headley & Paré, 2017).

Neural oscillations can be divided into five frequency-bands serving different neurophysiological functions: (1) Delta (~0.5-3.5 Hz), (2) Theta (~4-8 Hz), (3) Alpha (~8-12 Hz), (4) Beta (~12-30 Hz), and (5) Gamma (>30 Hz). Regarding the role of neural oscillations for memory processing, a wealth of evidence points to an involvement of theta and gamma oscillations in acquisition and recall of declarative and procedural memories (Headley & Paré,

2017). While theta oscillations are important for the efficient communication between brain areas, gamma oscillations were shown to be crucial for local information processing (Headley & Paré, 2017).

Given the prominent association between neural oscillations and declarative memory, it is not surprising that research also began to address the role of neural oscillations for fear memories and extinction memories (Bocchio et al., 2017). Especially in the last decade, evidence from rodent and first human studies accumulated to suggest an involvement of theta oscillations and - to a lesser extent - gamma oscillations in the fear and extinction circuitry (Bocchio et al., 2017).

The following part of this section gives an overview of the evidence linking neural oscillations to fear memories and extinction memories. While the first part focuses exclusively on research in animals (mice, rats, and non-human primates), the second part is dedicated to evidence from human subjects. Of note, the following studies used at least one of three typical measures to characterize neural oscillations. These measures are termed power, phase synchronization, and coherence. Briefly, while oscillatory power indicates the degree of synchronized postsynaptic potentials within a region, phase synchronization and coherence indicate the degree of synchronization between two regions (Fell & Axmacher, 2011). For the sake of simplicity, results concerning phase synchronization and coherence will be uniformly referred to as synchronization, even though both measures are calculated differently.

Animal studies

Studies in animals (rodents and primates) examining neural oscillations during different stages of fear conditioning almost exclusively relied on intracranial recordings of LFPs. This has the advantage that electrodes can be directly placed into specific neural structures of the fear and extinction circuitry. Using LFP recordings, it was shown that neural oscillations in the theta frequency band are of outstanding significance for the expression of fear memories.

In a fear conditioning study it was demonstrated that synchronization of theta oscillations between the amygdala and the hippocampus increased during recall of conditioned fear (Seidenbecher et al., 2003). More specifically, theta synchronization increased during presentation of a consolidated CS as well as during the expression of freezing behavior (Seidenbecher et al., 2003). In subsequent studies, these first results were replicated and the role of theta oscillations within the IL subsection of the mPFC was addressed (Lesting et al., 2011; Lesting et al., 2013; Narayanan et al., 2011). Theta synchronization between the IL,

hippocampus, and amygdala was increased during fear memory expression and decreased during successful extinction acquisition and extinction recall (Lesting et al., 2011; Narayanan et al., 2011; Sangha et al., 2009). While high theta synchronization between the IL, hippocampus, and amygdala was consistently observed during high-fear states (i.e., high levels of fear expression), a change of direction in the communication between these structures was observed during low-fear states (Lesting et al., 2013). During successful fear extinction, presentation of the extinguished CS caused IL spiking to lead theta oscillations in the amygdala and hippocampus (Lesting et al., 2013). Thus, while IL activity may be actively suppressed during fear memory expression by theta-synchronized inputs from the amygdala, the IL seems to take lead over amygdala activity during successful fear extinction, presumably to inhibit fear expression.

Acknowledging the prominent role of the PL for fear memory expression, subsequent studies also took this subsection of the mPFC into account when examining neural oscillations during fear conditioning. Interestingly, these studies consistently reported increased theta power in the PL during recall of fear memories (Courtin et al., 2014; Fenton et al., 2014; Likhtik et al., 2014). Fenton et al. (2014) reported increased theta power in the PL during the early phase of delayed extinction acquisition[3] (one day after fear acquisition) when the CS was presented. Importantly, theta power in the PL decreased during successful extinction acquisition (Fenton et al., 2014). Interestingly, there were also prominent sex differences in fear expression (Fenton et al., 2014): Male rats showed better extinction acquisition and better extinction recall than female rats as indicated by stronger freezing responses in females. These behavioral differences were accompanied by a persistent increase of theta power in the PL of female rats during extinction acquisition and extinction recall (Fenton et al., 2014). A reanalysis of the same dataset revealed that gamma power in the IL increased during successful extinction recall (Fenton et al., 2016). These oscillations were also sensitive to sex differences: Male rats showed an increase in IL gamma power during extinction recall, which was accompanied by better extinction recall on the behavioral level. However, female rats exhibited no increase in IL gamma power, which was accompanied by extinction recall deficits on the behavioral level (Fenton et al., 2016). Thus, while theta power in the PL seems to be an indicator of fear memory expression, gamma power in the IL is rather linked to successful fear extinction. Importantly,

[3] Please note that the early phase of delayed fear extinction acquisition reflects the recall of a consolidated fear memory.

both frequency bands seem to be sensitive to sex differences during extinction recall (Fenton et al., 2014; Fenton et al., 2016).

Two further studies largely confirmed the role of PL theta oscillations as an indicator of fear memory expression (Courtin et al., 2014; Likhtik et al., 2014). Likhtik et al. (2014) used a differential fear acquisition design to examine theta oscillation during fear recall. Presentation of a consolidated CS+ evoked stronger theta power in the PL and BLA compared to the control CS- (Likhtik et al., 2014). In addition, theta synchronization between the PL and BLA also showed a stronger increase after CS+ presentation compared to CS- presentation (Likhtik et al., 2014). On a more mechanistic level, presentation of a CS+ was reported to cause an inhibition of parvalbumin positive interneurons which results in a reset of the ongoing theta phase and an accompanying transient increase in theta power (Courtin et al., 2014). Using advanced optogenetic methods to manipulate neural activity in the dorsomedial PFC (dmPFC), comprising the PL and the anterior cingulate cortex, it was further shown that inhibition of parvalbumin positive interneurons is sufficient to reset theta phase and acutely cause fear expression (Courtin et al., 2014). Importantly, the mechanism of theta phase resetting seems to synchronize firing of PL neurons which preferably project to the BLA, which in turn is thought to cause acute fear responding (Courtin et al., 2014). A more recent study also reported stronger CS-evoked theta power in the dmPFC and the amygdala during fear recall (Rahman et al., 2018). Importantly, theta power in both regions returned to pre-conditioning levels during successful extinction recall.

In sum, the combined evidence from the described rodent studies suggests that theta oscillations constitute an important neurophysiological mechanism to temporally connect structures of the fear circuitry to gate the expression of conditioned fear during fear recall. This is primarily indicated by (A) enhanced theta power in the PL and BLA, as well as by (B) an increased theta synchronization between the mPFC (PL and IL), amygdala, and hippocampus. While the above studies provide robust evidence for a crucial involvement of theta oscillations during recall of consolidated fear memories, the role of theta oscillations for the initial fear acquisition was either not examined or not reported.

A study in non-human primates filled this gap by examining the development of theta oscillations during fear acquisition (Taub et al., 2018). Theta power in the dACC and amygdala increased during fear acquisition. This was accompanied by a similar increase of theta synchronization between both structures. Moreover, Taub et al. (2018) demonstrated that during fear acquisition, theta oscillations orchestrate neural activity in both structures in such a

way that amygdala output (i.e., action potential) arrives at the dACC when it is presumably most receptive for synaptic input (i.e., at a theta phase where membrane excitability is high). The study from Taub et al. (2018) elegantly demonstrates how theta oscillations can orchestrate neural activity within the fear and extinction circuitry. However, it is currently the only study in animals reporting an involvement of theta oscillations during fear acquisition.

Humans

The first human studies probing the role of neural oscillations for fear memories and extinction memories relied on EEG measures assessed during a 2-day differential fear and extinction paradigm (E. M. Mueller et al., 2014; Sperl et al., 2019). Importantly, the same paradigm was also used in study 1 (see: *Manuscript 1;* Bierwirth et al., 2021) and study 2 (see: *Manuscript 2*) of the present thesis. Thus, regarding the special significance of both studies (E. M. Mueller et al., 2014; Sperl et al., 2019), their conditioning paradigm and the results will be presented in greater detail (see also 3 *General method: The differential fear and extinction paradigm*).

The authors used a 2-day differential fear and extinction paradigm with fear acquisition and immediate extinction acquisition on day 1. Fear recall and extinction recall were assessed after a 24-hour consolidation period on day 2. During fear acquisition, two CS+ were paired with an aversive US, while two other CS- remained unpaired and thus served as control stimuli. This constituted the within-subject factor *contingency* (CS+ vs. CS-). During extinction acquisition, only one CS+/CS- pair was presented (extinguished CS: CS+E and CS-E), while the other pair was not presented (not-extinguished CS: CS+N and CS-N). This constituted the within-subject factor *extinction status*. Thus, the design allows to assess fear recall (differential responses: CS+N minus CS-N) and extinction recall (differential responses: CS+E minus CS-E) separately.

In the initial study by E. M. Mueller et al. (2014), EEG data was analyzed on two levels. First, theta power and gamma power were estimated on the scalp surface at frontocentral or frontopolar electrodes, respectively. Second, to increase the spatial resolution of the EEG analyses, power of both frequency bands was source localized via Low Resolution Brain Electromagnetic Tomography (LORETA). Scalp level analyses revealed that theta power at frontocentral electrodes was increased during fear recall (CS+N > CS-N) but showed no modulation during extinction recall (CS+E ≈ CS-E). This modulation was evident in a significant contingency and extinction status interaction, indicating that increased theta power to CS+ vs. CS- depends on the extinction status (extinguished vs. not-extinguished). Increased frontocentral theta power was especially sensitive for fear recall (CS+N minus CS-N > CS+E

minus CS-E). In accordance with animal data, source-level analysis via LORETA revealed that the described theta modulation during fear recall was specifically localized within the dACC. However, there was no modulation of theta power during former fear acquisition or extinction acquisition. Thus, the authors concluded that dACC theta power may be specifically involved in the recall and expression of consolidated fear memories (E. M. Mueller et al., 2014).

Considering the role of gamma oscillations there was no association between frontopolar gamma and fear recall or extinction recall. Nevertheless, source localized gamma power in the vmPFC was increased for the CS-E compared to the CS+E and this effect was inversed for not-extinguished CS (CS+N > CS-N; i.e., a significant contingency and extinction status interaction). Surprisingly, these results are in contrast to rodent data showing enhanced IL gamma power during successful extinction recall (Fenton et al., 2016). Nevertheless, the described modulation of vmPFC gamma power was specific for subjects showing successful extinction recall as indicated by skin conductance responses (SCRs), suggesting that a reduction of vmPFC gamma power for CS+E might be a marker of successful extinction recall in humans (E. M. Mueller et al., 2014).

In sum, the study by E. M. Mueller et al. (2014) revealed, for the first time in humans, that increased theta power in the dACC is involved in fear recall, while gamma oscillations in the vmPFC seem to be associated with successful extinction recall. Even though the observed theta results in the human dACC remarkably reflect the theta dynamics in the rodent PL during fear recall, it is not clear if human theta oscillations are also involved in orchestrating the communication between the dACC and the amygdala.

Acknowledging the spatial limitation of the EEG, a subsequent study combined EEG recordings with functional magnetic resonance imaging (fMRI; Sperl et al., 2019). Importantly, analysis of the EEG data confirmed increased frontocentral theta power specifically during fear recall (CS+N > CS-N) as indicated by a contingency and extinction status interaction (CS+N minus CS-N > CS+E minus CS-E). There was again no involvement of frontocentral theta power during extinction recall (CS+E ≈ CS-E). Interestingly, analyses of the fMRI-derived BOLD (blood oxygenation level dependent) signal in the amygdala revealed a very similar response pattern (CS+N minus CS-N > CS+E minus CS-E). During fear recall, differential amygdala activity was increased for not-extinguished CS (CS+N > CS-N) with no amygdala modulation during extinction recall (CS+E ≈ CS-E). Most importantly, the described amygdala BOLD responses and frontocentral theta power showed a positive correlation. Even though theta oscillations were not measured in the amygdala, the correlation between amygdala BOLD

activity and frontocentral theta power reported by Sperl et al. (2019), strongly suggests that theta oscillations may also be involved in the orchestration of long-range communication between the dACC and amygdala in human subjects during fear recall.

To conclude, animal and human data provide robust evidence indicating that theta power in the dACC/PL and amygdala is a reliable and valid indicator of fear expression during fear recall. Theta oscillations were shown to be a necessary mechanism to regulate the communication between the dACC/PL and amygdala, which was shown to gate fear responding during CS+ presentation. Moreover, rodent studies also demonstrated that theta power indicates deficits in extinction recall since increased theta power during recall was accompanied by deficient extinction recall on the behavioral level (Fenton et al., 2014). Importantly, increased theta power during deficient extinction recall was especially pronounced in female rodents (Fenton et al., 2014). While the association between theta oscillations and fear recall seems robust, results of two studies concerning gamma oscillations remain mixed (Fenton et al., 2016; E. M. Mueller et al., 2014).

Assessing neural oscillations via EEG constitutes an exciting approach to examine fear and extinction memory processing in humans. Importantly, due to seminal work in animals, oscillatory data allow conclusions about the neurophysiological mechanisms regulating neural activity in the fear and extinction circuitry of human subjects. Furthermore, neural oscillations can be readily assessed in human and animals and are therefore a suitable measure to enable a more direct comparison of neurophysiological findings between species than other measures of brain activity (e.g., fMRI which can hardly be assessed in behaving animals). Interestingly, while rodent studies suggest that theta and gamma oscillations in the fear and extinction circuitry are sensitive to sex difference (Fenton et al., 2014; Fenton et al., 2016), the role of sex has not been examined in humans so far. Moreover, previous human and rodent studies, using behavioral and peripheral indicators of conditioned fear responding, revealed robust sex differences in fear recall and extinction recall, which are partially mediated by the female E2. In study 1 (Manuscript 1) we therefore aimed at examining the role of sex differences and E2 for theta and gamma oscillations during fear recall and extinction recall in human subjects.

1.4 17β-Estradiol (E2) and fear extinction

As mentioned in the beginning, the lifetime incidence of anxiety disorders and PTSD is approximately twice as high in women compared to men, and women additionally exhibit greater symptom severity, more enduring symptoms, and a greater burden of illness (Cover et al., 2014; Maeng & Milad, 2015; McLean et al., 2011). In women, anxiety disorders often

develop or worsen during reproductive life events such as puberty, pre-menstruation, pregnancy, postpartum, and menopause, all of which are characterized by marked fluctuations of sex hormones (Cover et al., 2014; Hantsoo & Epperson, 2017). Considering these epidemiological results, sex hormones and specifically the female sex hormone E2 are thought to contribute to the described sex differences in the lifetime incidence of anxiety disorders and PTSD (Cover et al., 2014).

Gonadal sex hormones are typically distinguished into three subclasses of steroid hormones, i.e., estrogens, gestagens, and androgens. Androgens (e.g., testosterone) constitute the primary male sex hormone, while estrogens and gestagens (e.g., progesterone [P4]) serve as female sex hormones. The predominating estrogen in women changes across the life span with different reproductive events: Estrone (E1) is predominantly produced during menopause, E2 is the dominant estrogen in non-pregnant women during their reproductive years. Estriol (E3) and estetrol (E4), on the other hand, are especially abundant during pregnancy. Since most animal and human fear conditioning studies focused on the role of E2, we will specifically consider this estrogen. In women, E2 is produced by the ovaries. Importantly, E2 is also synthesized by the testes in men, for example via the conversion from testosterone to E2 by the enzyme aromatase (Stockhorst & Antov, 2016).

While sex hormone levels are relatively constant in males, free-cycling (i.e., naturally-cycling) premenopausal women are characterized by strong fluctuations of E2 and P4 levels across the menstrual cycle. The menstrual cycle lasts for approximately 28 days and consists of a follicular phase (cycle days ~1-8), a midcycle phase (cycle days ~13-14), and a luteal phase (cycle days ~15-28; Becker, 2005). During the early follicular phase, E2 and P4 levels are both low. While P4 levels remain low, E2 levels gradually increase throughout the follicular phase and reach peak concentration during midcycle right before ovulation. P4, on the other hand, reaches its peak during the midluteal phase, where E2 levels are intermediate.

In contrast to free-cycling women, approximately 32% of western European women in reproductive age (15-49 years) use oral hormonal contraceptives to prevent pregnancy (United Nations, Department of Economic and Social Affairs, Population Division, 2019). Typically, oral hormonal contraceptives contain a combination of a synthetic estrogen derivate and a synthetic gestagen. Administration of these *combined oral contraceptives* (OC) inhibits production of endogenous E2 and P4 across the menstrual cycle (Rivera et al., 1999).

Besides human subjects, rodents have often been used to examine the role of E2 for fear recall and extinction recall. Thus, it is important to highlight that the so-called estrous cycle in

rodents markedly differs from the menstrual cycle in women. The estrous cycle lasts only for about 4-5 days and consists of four different phases characterized by fluctuating E2 and P4 levels: metestrus (low E2/ low P4), diestrus (low E2/ moderate P4), proestrus (high E2/ high P4), and estrus (low E2/ low P4; Stockhorst & Antov, 2016).

Since E2 is a lipophilic steroid hormone, peripheral E2 can readily cross the blood-brain-barrier to enter the brain. In addition, there is first evidence suggesting that E2 can also be synthesized directly within the brain via the enzyme aromatase which converts testosterone to E2 (Biegon, 2016). Importantly, one positron emissions tomography (PET) study suggests that men have an extraordinarily high E2-synthesis capacity within the brain (Biegon et al., 2015). In the same study, the only other organ showing a similar E2-synthesis capacity was the female uterus during midcycle (Biegon et al., 2015). Thus, it is important to keep in mind that men may have constantly elevated E2-levels within the brain, despite low peripheral levels. Within the brain, E2 exerts relatively slow genomic (i.e., classical) effects via intracellular estrogen receptors (ER) of the subtype α (ERα) and β (ERβ). Additionally, E2 also acts on membrane-bound ERα and ERβ as well as on G-protein coupled transmembrane ERs, which all typically mediate fast non-genomic (i.e., non-classical) effects by initiating different second messenger cascades (Gillies & McArthur, 2010). Importantly, ERs are widely distributed throughout the brain (Gillies & McArthur, 2010). Regarding the neural fear and extinction circuitry, it was demonstrated that the hippocampus, amygdala, as well as the vmPFC/IL and dACC/PL contain elevated levels of ERα and ERβ, which leaves E2 in a prominent place to modulate fear memories and extinction memories (Cover et al., 2014; Lebrón-Milad & Milad, 2012).

Animals (exclusively rodents)

There is accumulating evidence from rodent studies strongly suggesting that E2 facilitates fear extinction recall. Using quasi-experimental designs, studies in naturally-cycling female rats robustly demonstrate that endogenous E2 levels during extinction acquisition are associated with later successful extinction recall (Y.-J. Chang et al., 2009; Graham & Daher, 2016; Graham & Milad, 2013; Graham & Scott, 2018a, 2018b; Gruene et al., 2015; Milad, Igoe, et al., 2009; Rey et al., 2014). More precisely, female rats that underwent extinction acquisition during proestrus (high E2/ high P4) showed better extinction recall than rats that were extinguished during estrus phases with low E2 levels (i.e., metestrus [low E2/ low P4], diestrus [low E2/ moderate P4], and estrus [low E2/ low P4]). Importantly, the observed recall

improvements in the proestrus groups were shown to be attributable to the increased E2 levels, while P4 was rather associated with impairments in extinction recall (Graham & Daher, 2016).

The facilitatory effects of E2 on extinction recall were further confirmed by experimentally enhancing or blocking E2 signaling (Y.-J. Chang et al., 2009; Graham & Milad, 2013; Graham & Scott, 2018a, 2018b; Maeng, Cover, et al., 2017; Milad, Igoe, et al., 2009; Zeidan et al., 2011). Enhancing E2 signaling via systemic injections of either E2 or an ERβ agonist immediately prior or after extinction acquisition prevented deficits in extinction recall in female rats that underwent extinction acquisition with naturally low E2 levels (Y.-J. Chang et al., 2009; Graham & Milad, 2013; Graham & Scott, 2018a, 2018b; Maeng, Cover, et al., 2017; Milad, Igoe, et al., 2009; Zeidan et al., 2011). Accordingly, reducing E2 levels during extinction acquisition - either via ovariectomy or pharmacologically by systemic injections of oral contraceptives or an ER antagonist - caused impaired extinction recall (Y.-J. Chang et al., 2009; Graham & Milad, 2013; Milad, Igoe, et al., 2009). Even though male rats are characterized by low peripheral E2 levels, one study demonstrated that males show comparable extinction recall to high E2 females, and both groups exhibited better extinction recall than low E2 females (Milad, Igoe, et al., 2009; but see also Y.-J. Chang et al., 2009). Interestingly, there is also first evidence for a role of E2 in male rats (Graham & Milad, 2014). A follow up study treated male rats with an aromatase inhibitor prior to extinction acquisition to inhibit the conversion of testosterone to E2 (Graham & Milad, 2014). Blocking the synthesis of E2 during extinction acquisition caused marked deficits in extinction recall in male rats, which could be prevented with systemic injections of E2 (Graham & Milad, 2014). In sum, the described studies robustly demonstrate that E2 is necessary for successful extinction recall in female rodents while one study also suggests a necessity of E2 for extinction recall in males.

While examining the neurobiological correlates of E2's effects on extinction recall, it was observed that a systemic injection of an ERβ agonist immediately after extinction acquisition caused an increase in neural activity in the IL, which was accompanied by decreased neural activity within the amygdala (Zeidan et al., 2011). In a subsequent study, systemic E2 injections after extinction acquisition were also shown to modulate IL activity which depended on the relative activity within fear expression-related structures (Maeng, Cover, et al., 2017). E2 treatment caused greater IL activity relative to activity in the PL and the CeA during extinction recall.

In sum, these results suggest that enhanced E2 levels shift neural activity during extinction recall to extinction-related structures (IL), while fear-related structures (PL, amygdala) are relatively silenced.

Humans

In accordance with the described animal results, human studies strongly suggest that free-cycling women who experience extinction acquisition under low E2 levels exhibit worse extinction recall and stronger return of fear phenomena (e.g., reinstatement) than women who are extinguished with high E2 levels (Antov & Stockhorst, 2014; Felmingham et al., 2021; Graham & Milad, 2013; S. Li & Graham, 2016; Milad et al., 2010; White & Graham, 2016; Zeidan et al., 2011). Moreover, women who use hormonal contraceptives for pregnancy prevention (i.e., women with low endogenous E2 levels) were shown to exhibit deficits in extinction recall compared to free-cycling women with high E2 levels (Graham & Milad, 2013; White & Graham, 2016). Importantly, recall deficits after hormonal contraceptive treatment could be rescued by a single oral administration of E2 right before extinction acquisition, suggesting that deficits observed after hormonal contraceptive intake are mediated by suppressed, endogenous E2 (Graham & Milad, 2013). Additionally, use of hormonal contraceptives was also associated with slower rates of improvement and worse long-term outcome of extinction-based exposure therapy in women diagnosed with spider phobia compared to free-cycling spider phobics (Graham et al., 2018). Besides effects on extinction recall, there is also preliminary evidence that E2 may facilitate extinction acquisition in healthy women (Wegerer et al., 2014) as well as in women suffering from PTSD (Glover et al., 2012). However, the majority of studies did not report any association of E2 and extinction acquisition (Felmingham et al., 2021; Graham & Milad, 2013; S. Li & Graham, 2016; Milad et al., 2010; White & Graham, 2016; Zeidan et al., 2011). Regarding the role of potential sex differences, only a few studies directly compared male participants with women characterized by different E2 levels (Antov & Stockhorst, 2014; Milad et al., 2010; White & Graham, 2016). In accordance with results from rodent studies, one study reported no differences in extinction recall between men and women with high E2 levels, but both groups exhibited better extinction recall than women with low E2 levels (Milad et al., 2010). Nevertheless, another study reported rather ambiguous results with men showing no differences in extinction recall compared to women with high E2 levels or to women using hormonal contraceptives (White & Graham, 2016). Thus, more work is needed directly comparing men to women with different E2 status.

Human studies addressing the neural correlates of E2's effects on extinction recall solely relied on fMRI to assess neural activity (Hwang et al., 2015; Zeidan et al., 2011). The results of these studies are somewhat ambiguous, and they are difficult to fit into a coherent framework regarding the known fear and extinction circuitry. Zeidan et al. (2011) reported that increased extinction recall in women with high E2 levels (vs. low E2 women) was accompanied by increased differential BOLD responses (CS+E minus CS+N) across different fear- and extinction-related brain structures (vmPFC, dACC, amygdala, and hippocampus; Zeidan et al., 2011). A subsequent study revealed similar results (Hwang et al., 2015): Women with high E2 levels (vs. men and low E2 women) displayed generally higher differential BOLD responses throughout fear acquisition (CS+ minus CS-), late fear extinction (CS+E minus CS-E), and early extinction recall (CS+E minus CS+N) in diverse brain regions comprising the cingulate cortex, insular, amygdala, hippocampus, and hypothalamus (Hwang et al., 2015). Importantly, in both studies E2 levels were mainly positively correlated with differential BOLD contrasts (Hwang et al., 2015; Zeidan et al., 2011). Of note, E2 has known vasomodulatory effects and it is associated with increased cerebral blood flow (Brackley et al., 1999; Duckles & Krause, 2007). This may constitute an important confounding factor when assessing fMRI-BOLD responses in humans with different E2-status. Thus, measuring neural activity via EEG, which is not directly dependent on cerebral blood flow, may help to clarify these initial results.

To conclude, human and animal studies robustly demonstrate that high E2 levels during or shortly after extinction acquisition facilitate later extinction recall. Accordingly, suppressing E2 levels with substances like hormonal contraceptives causes decrements in extinction recall. Concerning the underlying neurobiological mechanisms of this effect, initial rodent studies demonstrate that E2 enhances neural activity within structures of the extinction network (e.g., IL) while simultaneously suppressing activity within structures of the fear network (PL, amygdala).

However, studies in humans on the neural correlates of E2's effects on extinction recall are ambiguous and might be biased due to E2's vasomodulatory effects. With this in mind, and in consideration of animal work linking prefrontal theta and gamma oscillations to sex differences in extinction recall (Fenton et al., 2014; Fenton et al., 2016), we designed study 1 (2.1 Manuscript 1), where we utilized EEG recording to assesses prefrontal oscillations during fear recall and extinction recall in men and women differing in their E2 status (midcycle women with high endogenous E2 vs. women using hormonal contraceptives with low endogenous E2).

1.5 The role of extinction timing and noradrenergic arousal

The timing of extinction acquisition relative to fear acquisition is often overlooked in human fear conditioning studies. However, robust evidence from rodent studies suggest that extinction timing modulates the success of later extinction recall (for a review see: Maren, 2014). Moreover, seminal work in rodents elegantly demonstrated that noradrenergic arousal is crucially involved in the mediation of these fear extinction timing effects (e.g., Giustino et al., 2019). Importantly, studies examining the timing of fear extinction in humans are sparse and rather inconclusive. Additionally, the neuro-oscillatory correlates (or any other neurobiological correlates) of extinction timing effects have not been examined in humans so far.

The following section begins with an overview of animal work examining the role of extinction timing with a special focus on the role of noradrenergic arousal. Moreover, the section ends with a summary of the sparse evidence regarding fear extinction timing in humans, which also highlights the research gaps that were examined in study 2 (*2.2 Manuscript 2*) and were subject of the review article (*2.3 Manuscript 3*).

Animals (exclusively rodents)

The first study that systematically manipulated extinction timing after fear acquisition reported that a short interval (10 minutes or 1 hour) between fear acquisition and extinction acquisition resulted in better extinction recall compared to long intervals (24 or 72 hours; Myers et al., 2006). Moreover, rats underwent immediate fear extinction after fear acquisition showed almost no return of fear phenomena (reinstatement, renewal, or spontaneous recovery after 21 days). Based on these observations, the authors concluded that immediate extinction acquisition directly after fear acquisition is especially potent to suppress fear memory expression, or even to completely erase it (Myers et al., 2006). However, a subsequent study observed marked deficits in extinction recall after immediate extinction acquisition (e.g., 10 minutes after fear acquisition) in comparison to delayed intervals (24 hours; Maren & Chang, 2006). The authors attributed these seemingly contradicting observations between both studies to arousal differences during extinction acquisition (Maren & Chang, 2006): Importantly, Maren and Chang (2006) employed a more intense US than Myers et al. (2006) which presumably induced strong arousal levels during fear acquisition that transferred to immediate but not to delayed extinction acquisition. Accordingly, baseline freezing levels were increased before immediate extinction acquisition but not before delayed extinction acquisition (Maren & Chang, 2006). In a series of well controlled experiments, Maren and Chang demonstrated that reducing arousal levels before immediate extinction acquisition (via a weakened fear acquisition procedure)

improved extinction recall (Maren & Chang, 2006). Consistently, increasing arousal levels before delayed extinction acquisition (via unsignaled footshocks before extinction) caused deficits in extinction recall. Since the initial studies from Myers et al. (2006) and Maren and Chang (2006), several rodent studies demonstrated that extinction acquisition - if placed in close temporal proximity to fear acquisition - causes impairments in extinction recall (C. Chang et al., 2010; C. Chang & Maren, 2009; Fitzgerald et al., 2015; Giustino et al., 2017; Hollis et al., 2016; Jo et al., 2020; Kim et al., 2010; Singh et al., 2018; Stafford et al., 2013; Totty et al., 2019; Woods & Bouton, 2008). This phenomenon was therefore termed *Immediate Extinction Deficit* (IED; Maren, 2014).

Studies focusing on the *neurobiological foundations of the IED* observed that fear acquisition causes a persistent suppression of neural activity within the IL during immediate extinction acquisition (C. Chang et al., 2010; Fitzgerald et al., 2015; Kim et al., 2010). Importantly, stimulating the IL during immediate extinction acquisition completely prevented the IED (Kim et al., 2010). These results fit well with the observation that IL activity during extinction acquisition is necessary for later extinction recall (Bukalo et al., 2015; Do-Monte et al., 2015). Although an IED was also observed in appetitive conditioning procedures (Bouton et al., 2021; Rescorla, 2004), the main driver of the IED in the context of fear conditioning is presumably a fear-acquisition induced excess of noradrenergic arousal (Fitzgerald et al., 2015; Giustino et al., 2017; Giustino et al., 2019; Giustino et al., 2020). Interestingly, the inhibition of IL neurons after fear acquisition could be reversed by blocking noradrenergic signaling via injection of the β-adrenoceptor antagonist propranolol (Fitzgerald et al., 2015). Moreover, propranolol injection prior to immediate extinction acquisition prevented the IED, while an injection prior to delayed extinction acquisition caused extinction recall deficits, suggesting that too much or too little noradrenaline transmission during extinction acquisition can cause decrements in extinction recall (Fitzgerald et al., 2015). Subsequent studies revealed that elevated LC activity causes increased noradrenaline transmission in the BLA (Giustino et al., 2017; Giustino et al., 2020). Importantly, noradrenaline evokes a persistent increase of activity in the BLA (Giustino et al., 2017), which is most likely responsible for the observed inhibition of the IL during immediate extinction acquisition. Thus, increased noradrenaline transmission in the fear and extinction circuitry seems to be a main driver of the IED (Giustino & Maren, 2018). A recent study elegantly revealed that enhanced transmission of the neuropeptide CRF – which is a potent regulator of LC activity and noradrenaline transmission under conditions of high arousal (for a review see: Valentino & van Bockstaele, 2008) – causes an IED and might

be responsible for increased noradrenaline transmission during immediate extinction learning (Jo et al., 2020).

In sum, rodent studies provide robust evidence for an IED and reveal a complex neurobiological framework of how specific transmitters orchestrate the neural fear and extinction circuitry to impede successful extinction recall after immediate extinction acquisition.

Humans

Studies examining the IED in human subject are very sparse and the current evidence for an IED remains inconclusive. Two studies using SCRs as a measure of fear responding support the notion of an IED in humans (Huff et al., 2009; Merz et al., 2016). Both studies reported weaker extinction recall and more pronounced return of fear phenomena after immediate extinction acquisition (10-minute interval between fear acquisition and extinction acquisition) compared to delayed extinction acquisition (24 hours interval between fear acquisition and extinction acquisition). However, studies utilizing fear-potentiated startle responses and expectancy ratings produced contrasting results (Golkar & Öhman, 2012; Norrholm et al., 2008): There were either no differences in extinction recall or to some extent even a better extinction recall after immediate extinction acquisition (Golkar & Öhman, 2012; Norrholm et al., 2008).

To conclude, rodent studies robustly demonstrate that the interval between fear acquisition and extinction acquisition affects later extinction recall. Moreover, they provide detailed evidence about the underlying neurobiological mechanisms causing this effect. However, studies in human subjects are very sparse and inconclusive. Moreover, the neurobiological foundations of immediate vs. delayed extinction acquisition have not been examined in humans so far. To fill this gap, we designed Study 2 (2.2 Manuscript 2), where we utilized EEG recordings to assess prefrontal oscillations during all learning phases of a fear conditioning paradigm with two groups receiving either immediate or delayed extinction acquisition.

2 Objectives

The overarching aims of the conducted studies were to examine the role of the female sex hormone E2) (Manuscript 1) and extinction timing (Manuscript 2) with a special focus on the neuro-oscillatory correlates of fear memory and extinction memory processing. In both studies, an established 2-day differential fear- and extinction-paradigm was employed, which allowed us to separately assess fear recall and extinction recall (see Fig. 2). We used a *multi-methodological* approach to account for different measurement levels of fear- and extinction-processing. In both studies we recorded an *EEG* to measure neural oscillations during all phases of the differential fear- and extinction-paradigm. In addition, *SCR* and *valence and arousal ratings* were employed to assess phasic arousal responses on the peripheral level or subjective evaluations evoked by the conditioned stimuli, respectively.

2.1 Manuscript 1: Examining the role of prefrontal theta and gamma oscillations during fear recall and extinction recall in healthy humans with different estradiol status

The female sex hormone E2 is known to affect fear recall and extinction recall. Nevertheless, fMRI studies examining the neurobiological correlates of E2 effects in humans remain ambiguous (Hwang et al., 2015; Zeidan et al., 2011). An initial rodent study demonstrated that impaired extinction recall in female rats is accompanied by an enhancement of PL (primate dACC) theta oscillations (Fenton et al., 2014) and reduced recruitment of IL (primate vmPFC) gamma oscillations (Fenton et al., 2016). Nevertheless, the role of E2 was not examined here. Since neural oscillations emerged as a promising measure to transfer neurophysiological results between animal and human studies (E. M. Mueller et al., 2014), we aimed at examining prefrontal theta and gamma oscillations in healthy human males and females differing in their natural E2 status. The E2 status served as between-subject variable and was operationalized *quasi-experimentally*. For this purpose, participants were scheduled for testing in three independent E2 status groups: free-cycling women during midcycle (MC women; high E2 state and low P4 state), women using oral contraceptives (OC women; low endogenous E2 state and low endogenous P4 state), and men (low E2 state and low P4 state). Fear acquisition and immediate extinction acquisition took place on day 1 of the fear conditioning paradigm, while fear recall and extinction recall were assessed 24 hours later (day 2).

Based on previous human and rodent studies (E. M. Mueller et al., 2014; Sperl et al., 2019), we expected fear recall to be accompanied by increased theta power at frontocentral electrodes as well as in the dACC, while successful extinction recall should be accompanied by increased gamma power at frontopolar electrodes as well as in the vmPFC. Regarding the involvement of E2 status in fear and extinction recall, and also by extrapolating from fear- and extinction-recall related E2-actions in the male brain (Graham & Milad, 2013), we expected that MC women and men show less fear recall (i.e., lower dACC theta power and reduced peripheral arousal responses to not-extinguished CS+) as well as better extinction recall (i.e., higher vmPFC gamma power and reduced peripheral arousal responses for the extinguished CS+) than OC women. Since E2's role in fear memory and extinction memory recall was predominantly reported in peripheral arousal responses so far (e.g., White & Graham, 2016), we had no explicit hypothesis regarding subjective ratings.

2.2 Manuscript 2: Examining the role of neural oscillations during differential fear conditioning with immediate vs. delayed extinction acquisition

In Manuscript 1 we observed strong deficits in extinction recall within men and OC women (Bierwirth et al., 2021). However, a preceding study using an almost identical differential fear and extinction paradigm showed successful extinction recall (E. M. Mueller et al., 2014; Sperl et al., 2019). One plausible reason for these discrepancies could be the use of a stronger US intensity in our paradigm (95 dB[A]) compared with that of E. M. Mueller et al. (85 dB[A]). Importantly, heightened stimulus intensities are known to evoke stronger sympathetic/noradrenergic arousal (e.g., Breton-Provencher & Sur, 2019; Uematsu et al., 2017) which may cause an IED, manifesting in impaired extinction recall (e.g., Fitzgerald et al., 2015). To probe this idea further, we exploratory analyzed blood pressure as an indicator of sympathetic arousal before and after fear acquisition. Fittingly, systolic blood pressure was significantly increased after fear conditioning, or rather right before the immediate extinction acquisition. In accordance with the idea of an IED, elevated systolic blood pressure prior to immediate extinction acquisition was associated with deficits in extinction recall. Thus, the immediate extinction acquisition may have caused extinction recall deficits in Manuscript 1. On top of these exploratory findings, studies examining the IED in human subjects are sparse and inconclusive (Golkar & Öhman, 2012; Huff et al., 2009; Merz & Wolf, 2019; Norrholm et al., 2008). Additionally, the neurobiological correlates of immediate vs. delayed extinction acquisition have not been examined in humans so far, leaving the IED a poorly studied phenomenon in humans. To fill this gap, we experimentally manipulated the timepoint of extinction acquisition in study 2 (Manuscript 2). Since male participants showed strong deficits in extinction recall in study 1 (Manuscript 1), we exclusively selected male subjects for now. Participants were scheduled either to *immediate extinction acquisition*, placed ~10 minutes after fear acquisition, or to *delayed extinction acquisition*, placed ~24 hours after fear acquisition. Fear recall and extinction recall were assessed in both groups ~24 hours after extinction acquisition. A secondary aim of this study concerned a more methodological aspect of analyzing neural oscillations. Recent evidence suggests that classical power estimates (e.g., via Fourier transform or wavelets) of oscillatory activity may be distorted by non-oscillatory (i.e., arrhythmic) brain activity (e.g., Herweg et al., 2020). We therefore combined two state-of-the-art oscillation detection algorithms to disentangle oscillatory and non-oscillatory brain activity during all stages of the employed fear conditioning paradigm.

We hypothesized that immediate extinction acquisition causes deficits in extinction recall compared to delayed extinction acquisition. More precisely, immediate extinction acquisition should cause stronger peripheral arousal responses and stronger frontocentral theta oscillations during extinction recall compared to delayed extinction acquisition.

2.3 Manuscript 3: The contribution of noradrenergic arousal to fear extinction in animals and humans: A review

In Manuscript 2 we observed an IED in healthy humans. This was indicated by stronger peripheral arousal responses during extinction recall after immediate compared to delayed extinction acquisition. However, the underlying mechanisms causing the IED still have to be disentangled. As described in section 1.5, rodent studies suggest that CRF- and especially noradrenaline-mediated arousal levels serve as a main driver of the IED in rodents. However, relatively little is known about their involvement in fear extinction in humans.

Manuscript 3 was initially planned as empirical study aimed at systematically manipulating the noradrenergic LC system via non-invasive techniques to elevate vs. attenuate arousal right before fear extinction acquisition. Moreover, we planned to combine multiple *proxy measures* (e.g., pupillometry) of LC activity to monitor the noradrenergic system throughout the entire course the fear conditioning paradigm. However, due to the COVID-19 pandemic-related lockdown, we were not able to conduct any experiments in the laboratory. We therefore decided to write a review with the aim of providing a comprehensive overview of the noradrenergic system and its involvement in fear extinction in rodents and humans. We spent particular attention on current rodent work using opto- and chemo-genetic methods that allow to detect detailed mechanistic evidence of how the arousal-related transmitters noradrenaline and CRF dynamically orchestrate the neural fear and extinction circuitry to attenuate or to improve later extinction recall in rodents. Moreover, we also aimed at providing an overview of different non-invasive methods to measure and manipulate the noradrenergic LC system. This could be of practical use for future studies aiming at examining the role of noradrenergic arousal for fear extinction in human subjects.

3 General methods: The differential fear and extinction paradigm

In both studies we used a differential fear acquisition and extinction paradigm similar to that employed by E. M. Mueller et al. (2014; see Fig. 2). In *Manuscript 1* the paradigm extended over *2 days* (see Fig. 2A). The first day started with habituation which was followed by fear acquisition. Immediate extinction acquisition started 12 minutes after fear acquisition. On day 2, fear recall and extinction recall took place approximately 24 hours after the start of the habituation session. In *Manuscript 2* the paradigm extended over *2 days* for the immediate extinction group and over *3 days* for the delayed extinction group (see Fig. 2B). For both groups, the first day started with habituation which was followed by fear acquisition. For the immediate extinction group, extinction acquisition started 12 minutes after fear acquisition. For the delayed extinction group, extinction acquisition took place on day 2 approximately 24 hours after the start of habituation. Fear recall and extinction recall took place approximately 24 hours after the start of extinction acquisition for both, the immediate extinction group (day 2) and delayed extinction group (day 3).

The US was a 1-s white noise burst which was presented binaurally via loudspeakers at 95 dB(A). A white noise US is especially well suited for fear-conditioning paradigms that require many trials to acquire a good signal-to-noise ratio for EEG analysis (Sperl et al., 2016). As conditioned stimuli (CS) we used four black and white images, each depicting a male face with neutral emotional expression from the *picture of facial affect collection* (Ekman & Friesen, 1976) which were presented for 4 s on a black screen. Each trial started with the presentation of a white fixation cross for 1 s (see Fig. 2C). Immediately afterwards, the respective CS was presented for 4 s. For reinforced acquisition trials, the US started 3 s after CS onset, and CS presentation co-terminated with the US (i.e., delayed conditioning). During the inter-trial interval (ITI; 6 to 8 s), a black screen was presented.

Each of the four CS was presented 5 times for habituation to familiarize participants with the stimuli. Subsequent fear acquisition consisted of 240 trials, comprising 60 trials of each CS. During fear acquisition, two of the CS were paired with the US and thus served as the CS+, with reinforcement in 50% of the respective trials (partial reinforcement). The other two CS were never paired with the US and served as control CS-. This formed the within-subject factor *contingency* (CS+ vs CS-). During extinction acquisition covering 80 trials in Manuscript 1 and 120 trials in Manuscript 2, only one CS+ and one CS- were presented 40 times (Manuscript 1) or 60 times (Manuscript 2) without reinforcement, thus serving as extinguished CS+E and CS-E. The other CS+ and CS- were not presented and served as the not-extinguished

CS+N and CS-N. This operationalized the within-subject factor *extinction status* (extinguished vs. not-extinguished). In Manuscript 1, a novel face was presented 20 times (dummy face) to maintain some variability of stimuli shown during extinction acquisition (E. M. Mueller et al., 2014). Fear recall (assessed via differential responses: CS+N minus CS-N) and extinction recall (assessed via differential responses: CS+E minus CS-E) covered a total of 240 trials, comprising 60 trials of the four CS-types. The US was not presented on day 2. The assignment of the four faces to the four CS conditions was counterbalanced.

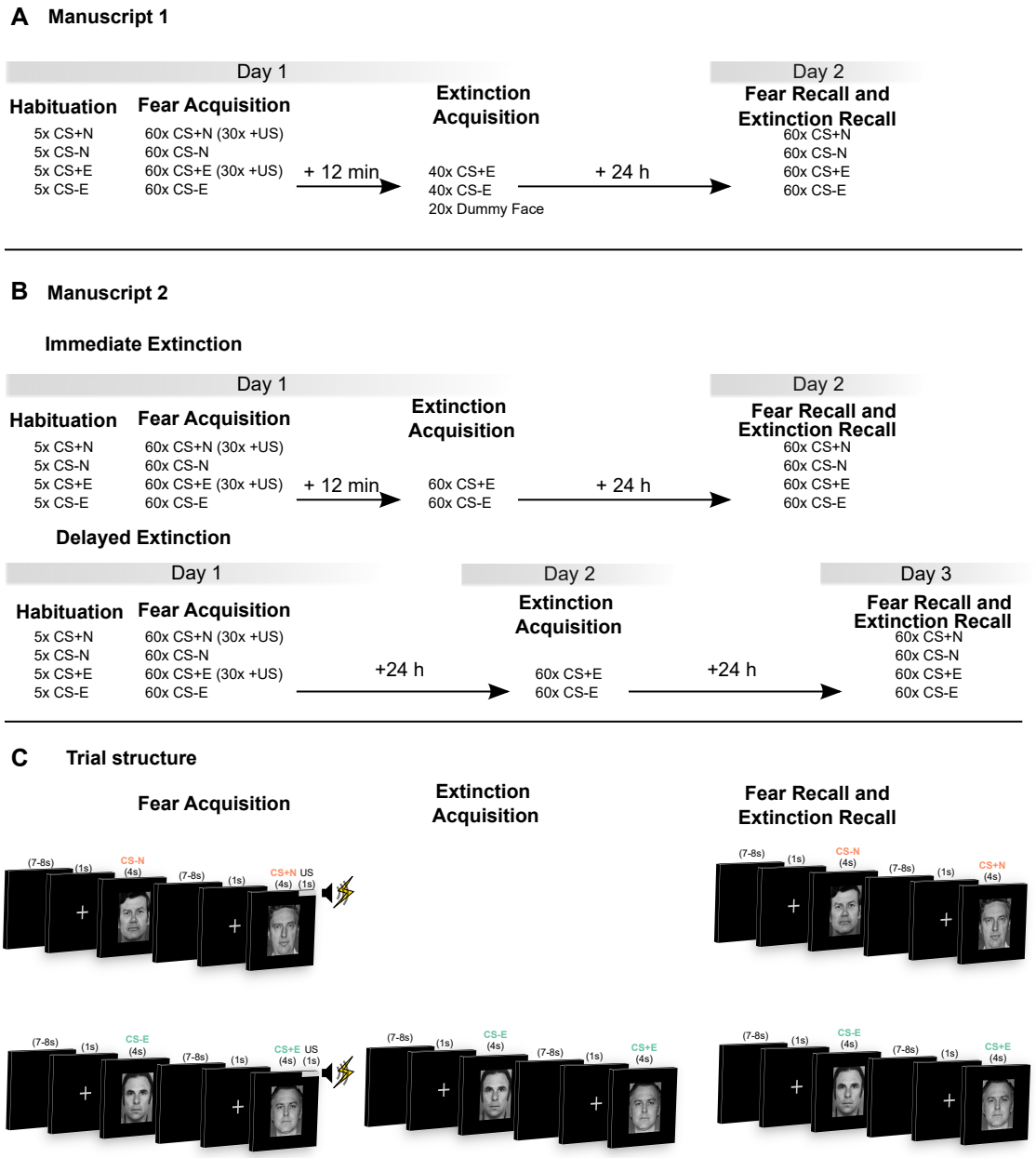

Figure 2. The differential fear and extinction paradigm with the phases habituation, fear acquisition, extinction acquisition, as well as fear recall and extinction recall. During fear acquisition, in 50% of the cases CS+ presentation was coupled with the US. CS- were never presented with the US. CS+N/CS-N were not presented during extinction acquisition (i.e., not-extinguished stimuli), while CS+E/CS-E were presented during extinction acquisition (i.e., extinguished stimuli). Fear recall and extinction were assessed via presentation of the not-extinguished or extinguished stimuli, respectively. (**A**) Number and types of stimuli used in Manuscript 1. Extinction acquisition took place 12 minutes after fear acquisition. Fear recall and extinction recall took place approximately 24 hours after extinction acquisition. (**B**) Number and types of stimuli used in Manuscript 2. For the Immediate Extinction group, extinction acquisition was placed approximately 12 minutes after fear acquisition. For the Delayed Extinction group, extinction acquisition took place 24 hours after fear acquisition. For both groups, fear recall and extinction recall took place 24 hours after extinction acquisition. (**C**) Trial structure was constant across both studies. A trial started with the presentation of a white fixation cross for 1 second. Subsequently, the CS was presented for 4 seconds. In case of reinforced CS+, stimulus presentation terminated with the presentation of the 1 second 95 dB(A) loud white noise burst. CS presentation was followed by a jittered intertrial interval (6-8 seconds).

4 Manuscripts

Paper status at the time of submitting the dissertation (April 19, 2022)

Manuscript 1:

Bierwirth, P., Sperl, M. F. J., Antov, M. I., & Stockhorst, U. (2021). Prefrontal theta oscillations are modulated by estradiol status during fear recall and extinction recall. *Biological Psychiatry. Cognitive Neuroscience and Neuroimaging*, *6*(11), 1071–1080. https://doi.org/10.1016/j.bpsc.2021.02.011

Manuscript 2:

Bierwirth, P., Antov, M. I., & Stockhorst, U. (*under review*). Oscillatory and non-oscillatory EEG activity reflects fear expression in an immediate and delayed fear extinction task. *Psychophysiology*

Manuscript 3:

Bierwirth, P., & Stockhorst, U. (*submitted*). Role of noradrenergic arousal for fear extinction processes in rodents and humans. *Neurobiology of Learning and Memory*

Paper status at the time of publishing the dissertation in the repository

Manuscript 1:

Bierwirth, P., Sperl, M. F. J., Antov, M. I., & Stockhorst, U. (2021). Prefrontal theta oscillations are modulated by estradiol status during fear recall and extinction recall. *Biological Psychiatry. Cognitive Neuroscience and Neuroimaging*, *6*(11), 1071–1080. https://doi.org/10.1016/j.bpsc.2021.02.011

Manuscript 2:

Bierwirth, P., Antov, M. I., & Stockhorst, U. (*revised version under review*). Oscillatory and non-oscillatory EEG activity reflects fear expression in an immediate and delayed fear extinction task. *Psychophysiology*

Manuscript 3:

Bierwirth, P., & Stockhorst, U. (2022). Role of noradrenergic arousal for fear extinction processes in rodents and humans. *Neurobiology of Learning and Memory, 194*, https://doi.org/10.1016/j.nlm.2022.107660

The printed version of the dissertation (including all full-text manuscripts at the time of submitting the dissertation [19.04.2022]) is available at Osnabrück University Library, Germany.

4.1 Prefrontal Theta Oscillations Are Modulated by Estradiol Status During Fear Recall and Extinction Recall

Bierwirth, P., Sperl, M. F. J., Antov, M. I., & Stockhorst, U. (2021). Prefrontal theta oscillations are modulated by estradiol status during fear recall and extinction recall. *Biological Psychiatry. Cognitive Neuroscience and Neuroimaging, 6*(11), 1071–1080. https://doi.org/10.1016/j.bpsc.2021.02.011

Abstract: Background: Emerging human studies demonstrate that theta oscillations in the dorsal anterior cingulate cortex are enhanced during fear recall (enhanced fear expression) and reduced during successful extinction recall (reduced fear expression). Although evidence suggests sex differences in fear recall and extinction recall, there are currently no human studies examining the oscillatory foundations of these memory processes separately in men and women. Methods: Because previous studies suggest that estradiol partially mediates these sex differences, we examined 20 men (low estradiol and low progesterone), 20 women using oral contraceptives (low estradiol and low progesterone), and 20 free-cycling women during midcycle (high estradiol and low progesterone). We used a fear-conditioning procedure, allowing us to separately assess fear recall and extinction recall 24 hours after fear and extinction acquisition. Skin conductance responses and electroencephalography were recorded during fear recall and extinction recall, and prefrontal oscillations were source localized. Results: We found elevated fear expression during fear recall and impaired extinction recall, as indicated by increased peripheral arousal (skin conductance responses) and fronto-central theta oscillations, source localized in the dorsal anterior cingulate cortex and dorsomedial prefrontal cortex. Importantly, peripheral arousal and dorsal anterior cingulate cortex theta oscillations were stronger in men and women on oral contraceptives than in women from the midcycle group. Conclusions: Our data show that neural oscillatory and peripheral correlates of heightened fear expression during fear recall and (impaired) extinction recall do not simply differ between sexes but depend on hormonal fluctuations within women.

Keywords: dACC; Estradiol; Extinction recall; Fear conditioning; Fear recall; Theta oscillations

The full text and online supplementary material of Manuscript 1 can be found at:

https://www.sciencedirect.com/science/article/pii/S2451902221000574

Please note that in the printed version of the dissertation the full text of Manuscript 1 covers pages 34-62.

4.2 Oscillatory and non-oscillatory brain activity reflects fear expression in an immediate and delayed fear extinction task

Bierwirth, P., Antov, M. I., & Stockhorst, U. (*revised version under review*). Oscillatory and non-oscillatory EEG activity reflects fear expression in an immediate and delayed fear extinction task. *Psychophysiology*

Abstract: Fear extinction is pivotal for inhibiting fear responding to former threat-predictive stimuli. In rodents, short intervals between fear acquisition and extinction impair extinction recall compared to long intervals. This is called Immediate Extinction Deficit (IED). Importantly, human studies of the IED are sparse and its neurophysiological correlates have not been examined in humans. We therefore investigated the IED by recording electroencephalography (EEG) and skin conductance responses (SCRs). 40 male participants were randomly assigned to extinction acquisition either ~10min after fear acquisition (immediate extinction) or ~24h afterwards (delayed extinction). Fear and extinction recall were assessed ~24h after extinction acquisition. We observed an IED in SCR responses, but not in theta oscillations or other neurophysiological markers of fear expression. Irrespective of extinction timepoint, fear conditioning caused a tilt of the non-oscillatory background spectrum with decreased low-frequency power (< 30 Hz) for threat-predictive stimuli. When controlling for this tilt, we observed a suppression of theta and alpha oscillations to threat-predictive stimuli, especially pronounced during fear acquisition. Our data show that delayed extinction is advantageous over immediate extinction in reducing peripheral arousal to former threat-predictive stimuli. Furthermore, we demonstrate that oscillatory and non-oscillatory activity is sensitive to fear conditioning which has important implications for fear conditioning studies examining neural oscillations.

Keywords: fear conditioning, fear extinction, immediate extinction deficit, oscillatory EEG activity, non-oscillatory EEG activity, oscillation detection, theta oscillations

The full text of Manuscript 2 can be found in the printed version of the dissertation available at Osnabrück University Library, Germany.

Please note that in the printed version of the dissertation the full text of Manuscript 2 covers pages 64-103.

4.3 Role of noradrenergic arousal for fear extinction processes in rodents and humans

Bierwirth, P., & Stockhorst, U. (2022). Role of noradrenergic arousal for fear extinction processes in rodents and humans. *Neurobiology of Learning and Memory, 194*, https://doi.org/10.1016/j.nlm.2022.107660

Abstract: Fear extinction is a learning mechanism that is pivotal for the inhibition of fear responses towards cues or contexts that no longer predict the occurrence of a threat. Failure of fear extinction leads to fear expression under safe conditions and is regarded to be a cardinal characteristic of many anxiety-related disorders and posttraumatic stress disorder. Importantly, the neurotransmitter noradrenaline was shown to be a potent modulator of fear extinction. Rodent studies demonstrated that excessive noradrenaline transmission after acute stress opens a time window of vulnerability, in which fear extinction learning results in attenuated long-term extinction success. In contrast, when excessive noradrenergic transmission subsides, well-coordinated noradrenaline transmission is necessary for the formation of a long-lasting extinction memory. In addition, emerging evidence suggests that the neuropeptide corticotropin releasing hormone (CRF), which strongly regulates noradrenaline transmission under conditions of acute stress, also impedes long-term extinction success. Recent rodent work - using sophisticated methods - provides evidence for a hypothetical mechanistic framework of how noradrenaline and CRF dynamically orchestrate the neural fear and extinction circuitry to attenuate or to improve fear extinction and extinction recall. Accordingly, we review the evidence from rodent studies linking noradrenaline and CRF to fear extinction learning and recall and derive the hypothetical mechanistic framework of how different levels of noradrenaline and CRF may create a time window of vulnerability which impedes successful long-term fear extinction. We also address evidence from human studies linking noradrenaline and fear extinction success. Moreover, we accumulate emerging approaches to non-invasively measure and manipulate the noradrenergic system in healthy humans. Finally, we emphasize the importance of future studies to account for sex (hormone) differences when examining the interaction between fear extinction, noradrenaline, and CRF. To conclude, NA's effects on fear extinction recall strongly depend on the arousal levels at the onset of fear extinction learning. Our review aimed at compiling the available (mainly rodent) data in a neurobiological framework, suited to derive testable hypotheses for future work in humans.

Keywords: Fear conditioning, Fear extinction, Noradrenaline, corticotropin releasing factor, Locus coeruleus

The full text of Manuscript 3 can be found at:

https://www.sciencedirect.com/science/article/abs/pii/S1074742722000843

Please note that in the printed version of the dissertation the full text of Manuscript 3 covers pages 106-156.

5 General Discussion

The present thesis had two overarching aims. The first one was to examine the role of E2 (Manuscript 1) and extinction timing (Manuscript 2) in fear memory and extinction memory processing with a special focus on how these two factors contribute to the quality of extinction recall. The second aim was to further elucidate the role of neural oscillations as a neurophysiological indicator of fear memory and extinction memory processing (Manuscript 1 and Manuscript 2) and to test if these oscillations are sensitive to E2 and extinction timing. Primarily based on Manuscript 2, the thesis further aimed at integrating the main mechanisms determining the quality of extinction recall during different extinction time points in a functional overview (Manuscript 3).

In the subsequent sections, I will first summarize and interpret our findings about the female sex hormone E2 and the role of prefrontal oscillations during fear recall and extinction recall (section 5.1.1). This is followed by a closer look at the role of extinction timing for later extinction recall. I will then integrate the EEG results to a neurophysiological perspective regarding oscillatory and non-oscillatory brain activity during different phases of fear conditioning (section 5.1.2). Furthermore, I will discuss important limitations of the current work (section 5.2) and outline perspectives for future research (section 5.4).

5.1 Summary and discussion of the main findings

5.1.1 Role of 17β Estradiol and prefrontal oscillations during fear recall and extinction recall

In study 1 (Manuscript 1) prefrontal theta and gamma oscillations during fear recall and extinction recall were examined, with a particular focus on the modulating role of the female sex hormone E2 (Bierwirth et al., 2021). For this purpose, we examined men (low E2 levels and low P4 levels), women using oral contraceptives (OC; low E2 levels and low P4 levels), and free-cycling women during midcycle (MC; high E2 levels and low P4 levels) in a differential fear and extinction paradigm (section 3). We recorded an EEG to assess neural oscillations and SCRs to assess peripheral arousal responses as a well-established indicator of fear responding in human subjects (Boucsein, 2012; Lonsdorf et al., 2017)[4]. Based on previous studies (E. M. Mueller et al., 2014; Sperl et al., 2019), we expected fear recall to be accompanied by increased theta power at frontocentral electrodes, source-localized in the

[4] In both studies we also assessed subjective valence and arousal ratings for each CS via self-assessment manikins (SAM; Bradley and Lang (1994). However, our primary interest was aimed at neural oscillations and peripheral arousal responses.

dACC. Successful extinction recall should be accompanied by increased gamma power at frontopolar electrodes, source-localized in the vmPFC. Regarding the important role of E2 for fear recall and extinction recall (e.g., Graham & Milad, 2014; Maeng & Milad, 2015), we expected MC women and men to show less fear recall (i.e., lower dACC theta power and reduced peripheral arousal responses) as well as better extinction recall (i.e., higher vmPFC gamma power and reduced peripheral arousal responses) than OC women.

As predicted, we observed enhanced theta oscillations during fear recall at frontocentral electrodes, source localized within the dACC. Most importantly, for the first time we demonstrated that theta oscillations in the dACC and peripheral arousal responses during fear recall and extinction recall do not differ between sexes per se, but are associated with fluctuations of the female sex hormone E2. More precisely, MC women (high peripheral E2 levels) showed less fear expression during fear recall and extinction recall than OC women and men (both low peripheral E2 levels). This was indicated by attenuated theta oscillations in the dACC and reduced peripheral arousal in MC women. However, we did not observe an involvement of vmPFC gamma oscillations during extinction recall.

Our observation of *enhanced frontocentral* (also source-localized in the dACC) *theta power during fear recall* is in line with previous reports from human (E. M. Mueller et al., 2014; Sperl et al., 2019) and animal (Courtin et al., 2014; Fenton et al., 2014; Likhtik et al., 2014; Rahman et al., 2018) studies. Moreover, in correspondence with the idea that extinction recall reflects the direct competition between the original fear memory and the extinction memory (Bouton, 2002), we also observed *increased theta oscillations during deficient extinction recall* (i.e., when the expression of the fear memory predominates), as additionally indicated by increased peripheral arousal responses to already extinguished stimuli. This is in accordance with a previous rodent study showing increased theta oscillations in the PL (i.e., the rodent homologue of the dACC) during deficient extinction recall (Fenton et al., 2014). Thus, Manuscript 1 (Bierwirth et al., 2021) lends further support to the assumption that dACC theta power constitutes a valid indicator of fear expression during fear recall and – importantly – elucidates the role of dACC theta as an indicator of deficient extinction recall. However, the role of gamma oscillations remains ambiguous. It is important to note that previous evidence of vmPFC gamma oscillations during extinction recall was already contradictory. While Fenton et al. (2016) observed enhanced gamma power in the rodent IL (i.e., the rodent homologue of the vmPFC) during successful extinction recall, E.M. Mueller et al. (2014) reported decreased gamma power in the human vmPFC for extinguished CS+ during successful extinction recall.

In contrast to these prior reports (Fenton et al., 2016; E. M. Mueller et al., 2014), we did not observe any involvement of gamma oscillations during extinction recall.

There are several explanations for our null results as well as for the already existing discrepancies between previous studies (Fenton et al., 2016; E. M. Mueller et al., 2014). First, compared to E. M. Mueller et al. (2014), extinction recall was poor in our sample. This might have caused a general reduction of vmPFC involvement during extinction recall in the present study. Second, E. M. Mueller et al. (2014) did not control for microsaccadic eye movements (i.e., microsaccades) during EEG analysis. Microsaccades are miniature eye-movements that elicit electromyogenic activity which contaminates the gamma-frequency range of the EEG signal (Yuval-Greenberg et al., 2008). Moreover, microsaccades are readily modulated by the emotional content of a stimulus (for a review see: Kashihara, 2020). Thus, the reported gamma effects in the vmPFC (E. M. Mueller et al. 2014), a neural structure in close distance to the ocular muscle, might rather reflect microsaccadic events, which were modulated by the differential emotional content of the presented CSs, than genuine gamma oscillations. In the current study, we controlled for microsaccades (Hassler et al., 2011), which could explain why we did not observe similar effects in the vmPFC as E.M. Mueller et al. (2014). Finally, gamma oscillations measured via EEG are per se characterized by a relatively low signal-to-noise ratio (e.g., Crone et al., 2006). An additional reduction of the signal-to-noise ratio most likely results from the relatively large distance between the vmPFC source and the EEG scalp electrodes. This may render detection of genuine vmPFC gamma oscillations impracticable in EEG studies. The above described aspects also provide intriguing explanations for the discrepancies between rodent (Fenton et al. 2016) and human reports (Bierwirth et al., 2021; E. M. Mueller et al., 2014). Fenton et al. (2016) placed the recording electrodes directly into the IL of rats. This not only causes a way better signal-to-noise ratio, but also renders the LFP recording less susceptible for myogenic artifacts than corresponding scalp recordings. Future studies using high-density EEG recordings (in healthy participants), or even intracranial electrocorticogram (ECoG) recordings in selected groups (e.g., in epileptic patients, S. Chen et al., 2021) may be better suited to examine and localize gamma oscillations generated by relatively deep structures such as the vmPFC (e.g., Crone et al., 2006).

Concerning the role of E2, our finding that MC women show less fear expression (indicated by attenuated dACC theta oscillations and peripheral arousal) during extinction recall than OC women fits well to previous reports of better extinction recall in women (Felmingham et al., 2021; Graham & Milad, 2013; S. Li & Graham, 2016; Milad et al., 2010; White & Graham, 2016; Zeidan et al., 2011) and female rats with high E2 levels (Y.-J. Chang et al.,

2009; Graham et al., 2018; Graham & Daher, 2016; Graham & Milad, 2013, 2014; Graham & Scott, 2018a, 2018b; Maeng, Cover, et al., 2017; Milad, Igoe, et al., 2009; Zeidan et al., 2011). It is assumed that E2 exerts its effect on extinction recall via enhancing the consolidation of the extinction memory (e.g., Maeng & Milad, 2015).

However, previous studies examining the role of E2 for extinction recall did not separately assess fear recall and extinction recall. Since fear expression during extinction recall is determined by a competition between the fear memory and the extinction memory (e.g., Bouton, 2002), good extinction recall (i.e., low fear expression) can also result from a disturbed consolidation of the original fear memory. Here, our observation that MC women exhibit both attenuated fear expression during both fear recall and extinction recall rather points toward a weaker consolidation of the original fear memory than to an enhanced consolidation of the extinction memory in women with high E2 levels. In accordance with this idea, rodent studies examining the effects of E2 specifically on fear recall reported attenuated fear recall in female rats with high E2 levels compared to female rats with low E2 levels (e.g., Barha et al., 2010; Kobayashi et al., 2020).

Contrary to our expectations, we also observed stronger fear expression (i.e., increased peripheral arousal and dACC theta oscillations) during fear recall and extinction recall in men compared to MC women. This is at odds with first studies in rodents (Milad, Igoe, et al., 2009) and humans (Milad et al., 2010) reporting (A) no differences in extinction recall between males and females with high E2 levels, and (B) better extinction recall in both groups compared to females with low E2 levels. However, there is also preliminary evidence that the type of CS may affect these sex differences. In both studies from Milad et al. (2009 and 2010), a discrete cue served as CS (e.g., the color of a lamplight). Interestingly, one rodent study using contexts as CS reported worse extinction recall in males compared to females with high E2 levels (Y.-J. Chang et al., 2009). While we did not utilize contextual stimuli as CSs, we may have introduced sex differences by using male faces as CS. A previous study in healthy adolescents demonstrated that the use of male faces resulted in enhanced fear acquisition and impaired extinction acquisition in boys compared to girls (Chauret et al., 2014). Even though there are currently no similar results for adults, it might explain our observation of enhanced fear expression in men throughout our fear conditioning procedure. Importantly, social anxiety disorder is the sole anxiety disorder with a relatively comparable life time incidence between men and women (McLean et al., 2011). Therefore, one could speculate that men might be more vulnerable to exhibit specific impairments in extinction recall when social cues (e.g., faces) serve as CS (Bierwirth et al., 2021), but exhibit superior extinction recall when cues with a non-

social content (e.g., colors of a lamp) are used (Milad et al., 2010). Importantly, women with high E2 levels show superior extinction recall regardless of the cue content that served as CS (Bierwirth et al., 2021; Milad et al., 2010). Thus, E2 seems to generally have protective effects on extinction recall in free-cycling women. At first glance, this appears at odds with the notion that women are suffering approximately twice as often from anxiety-related disorders (except for social anxiety) and PTSD than men (Cover et al., 2014; McLean et al., 2011). However, it is important to note that E2 levels strongly fluctuate throughout the menstrual cycle and only reach high levels for about 3-4 days around ovulation (Becker, 2005). Thus, free-cycling women might only benefit from E2 during this brief peri-ovulatory period. During the other cycle phases, on the other hand, they are expected to be more prone to show extinction recall deficits due to relatively low E2 levels. In sum, our peripheral data support the general notion that low E2 levels in women, caused by either naturally low E2 levels within the menstrual cycle or by hormonal contraception may constitute a risk factor for the development and maintenance of anxiety-related disorders and PTSD via deficits in extinction recall (for a review see: S. H. Li & Graham, 2017).

Importantly, our findings of enhanced dACC theta oscillations during fear recall and extinction recall in OC women and men compared to MC women add important insights to the neurophysiological mechanisms underlying E2's modulation of extinction recall. The dACC/PL is known to facilitate and maintain fear responses via projections to the amygdala (e.g., Burgos-Robles et al., 2009; Milad, Quirk, et al., 2007). Animal studies elegantly demonstrated that theta oscillations synchronize neural activity between the dACC and the amygdala, which not only facilitates communication between both structures (e.g., Taub et al., 2018) but also gates fear responding (Courtin et al., 2014). An elaborate study in human subjects that combined EEG and fMRI recordings revealed that theta oscillations at frontocentral electrodes show a strong positive correlation with increased amygdala BOLD activity during fear recall (Sperl et al., 2019). This suggests that theta oscillations subserve the same mechanism of enhancing communication between the dACC and the amygdala to facilitate fear responding in humans.

Regarding our observations, we speculate that increased E2 levels (as observed in MC women) *might suppress the communication between the amygdala and the dACC*, which should result in attenuated amygdala activity and therefore decreased fear responding during fear recall and extinction recall. Correspondingly, a previous rodent study demonstrated that injections of exogenous E2 causes decreased neural activity within the PL and the CeA relative to the IL (Maeng, Cover, et al., 2017). Considering the clinical perspective, a recent meta-analysis

provided strong evidence that PTSD is associated with a hyperactive dACC during extinction recall (Suarez-Jimenez et al., 2020). Moreover, it is assumed that a heightened responsivity of the dACC and the amygdala towards aversive stimuli is even a predisposing risk factor for the development of PTSD (Admon et al., 2013). Thus, low E2 levels in women might increase the risk for PTSD and other anxiety-related disorders by amplifying activity of a fear-related dACC and amygdala circuitry. In contrast to our data in humans and previous rodent observations, fMRI studies revealed elevated differential BOLD responses (CS+ > CS -) in the dACC and the amygdala of women with high E2 levels (Hwang et al., 2015; Zeidan et al., 2011). However, this increased differential BOLD activity was rather unspecific since it was observed across many neural structures, including structures of both the fear circuitry (e.g., amygdala and dACC) and the extinction circuitry (e.g., vmPFC). As mentioned in the introduction (section 1.4), E2 has vasomodulatory effects and increases cerebral blood flow (Brackley et al., 1999; Duckles & Krause, 2007), both of which presumably affect the BOLD signal and may result in unspecific activations or deactivations in fMRI studies examining women with different E2 levels. Since EEG oscillations are not directly influenced by cerebral blood flow, they may constitute a better indicator of neural activity when comparing women with different E2 levels. However, this explanation is highly speculative and needs to be tested empirically. Future studies combining EEG and fMRI recordings should be optimally suited to test this idea directly.

In discrepancy to previous studies that used the same differential fear and extinction paradigm (E. M. Mueller et al., 2014; Sperl et al., 2019), we found a strong attenuation of extinction recall especially pronounced in men and women using oral contraceptives. While E. M. Mueller et al. (2014) reported decreased fear responding during extinction recall compared to fear recall, we observed comparable levels of fear responding during fear recall and extinction recall. Thus, especially men and OC women responded as if there was no prior extinction learning (i.e., complete return of fear). When comparing the results of both studies, the question emerges as to why we observed such an attenuated extinction recall. One reason might be the use of a more intense US in our study (95 dB[A]) compared with that of E. M. Mueller et al. (2014; 85 dB[A]). Heightened US intensities evoke a stronger sympathetic/noradrenergic arousal, which can produce an IED as indicated by an impaired extinction recall (for a review see: Maren, 2014). We exploratory analyzed systolic blood pressure as an indicator of sympathetic arousal before and after fear acquisition. Interestingly, fear acquisition caused a significant increase in systolic blood pressure. Most importantly, elevated systolic blood pressure prior to immediate extinction acquisition predicted the extent

of extinction recall deficits one day later. These exploratory observations suggest that the fear acquisition phase in our study evoked elevated levels of sympathetic arousal which may have affected the immediate extinction acquisition phase (12 minutes after fear acquisition) and ultimately resulted in an IED (Maren, 2014)

5.1.2 Extinction timing and oscillatory and non-oscillatory brain activity

Based on previous reports and observations from Manuscript 1, we were primarily interested in examining the role of extinction timing for later extinction recall in Manuscript 2. More precisely, we examined two groups of healthy men who were scheduled to receive extinction acquisition either 12 minutes after fear acquisition (*immediate extinction acquisition*) or 24 hours after fear acquisition (*delayed extinction acquisition*).

Moreover, a secondary aim of Manuscript 2 was to further advance the understanding of the neurophysiological foundations of fear conditioning. We therefore combined two state-of-the-art oscillation detection algorithms to disentangle oscillatory and non-oscillatory brain activity (Donoghue et al., 2020; Kosciessa et al., 2020). Based on previous rodent work describing an IED (Maren, 2014), we primarily expected that immediate extinction acquisition results in worse extinction recall compared to delayed extinction acquisition. More precisely, immediate extinction acquisition should cause stronger peripheral arousal responses and stronger frontocentral theta oscillations (especially after the correction for non-oscillatory brain activity) than delayed extinction acquisition. Our peripheral arousal data revealed attenuated extinction recall after immediate extinction acquisition compared to delayed extinction acquisition. However, frontocentral theta oscillations were not affected by the acquisition-extinction interval. Moreover, we did not observe any involvement of theta oscillations during fear recall and extinction recall. During fear acquisition and extinction acquisition we even observed a suppression of theta oscillations and alpha oscillations for US predictive CS+, which was accompanied by phasic responses of arousal-related non-oscillatory brain activity.

Thus, in contrast to Manuscript 1 (Bierwirth et al., 2021) and other human (e.g., E. M. Mueller et al., 2014) and animal (e.g., Fenton et al., 2014) studies, we did not observe the expected increase of frontocentral theta oscillations during fear recall. One plausible reason for this discrepancy could be the increased number of CS presentations during extinction learning. Compared to these reference studies (Bierwirth et al., 2021; E. M. Mueller et al., 2014; Sperl et al., 2021), we increased the number of CS+ presentations from 40 to 60 trials during extinction learning. We did this for two reasons: first, to facilitate extinction learning in men, since male

participants showed almost no within-session extinction acquisition in Manuscript 1, and second, to keep the amount of trials constant between all learning phases. However, more extinction trials probably caused the formation of a stronger extinction memory which may have generalized to even suppress fear responding during fear recall, a phenomenon previously reported by Pace-Schott et al. (2009). In line with this explanation, the observed effect size of peripheral fear responding (CS+ minus CS-) during recall was descriptively larger in Manuscript 1 compared to Manuscript 2 (Manuscript 1: $\eta_p^2 = 0.49$ vs. Manuscript 2: $\eta_p^2 = 0.33$). Thus, one might speculate that frontocentral theta oscillations may be more sensitive to relatively strong fear memories. Future studies using different numbers of fear acquisition or extinction trials could reveal if *theta oscillations* scale with *fear memory strength*.

Nevertheless, fear responding during fear recall and extinction recall was still observable in peripheral arousal responses which was modulated by extinction timing. In accordance with previous rodent (for a review see: Maren, 2014) and two human studies (Huff et al., 2009; Merz et al., 2016), we observed attenuated peripheral fear responses during extinction recall in the delayed extinction group. The immediate extinction group, on the other hand, showed comparable fear responses during fear recall and extinction recall. They responded as if there was no prior extinction learning, which is in line with the hypothesized IED. Thus, our results from Manuscript 2 support the idea that the attenuated extinction recall we observed in Manuscript 1 (Bierwirth et al., 2021) was probably caused by immediate extinction learning.

The *mechanisms causing the IED* are currently not well understood in humans. However, early evidence from rodent research suggests *that elevated fear and arousal levels* during immediate extinction learning impede successful extinction recall (Maren & Chang, 2006). Recent methodological advancements in optogenetics (light-based stimulation/inhibition of neurons) and chemogenetics (stimulation/inhibition of neurons via chemically engineered molecules) provide animal researchers with sophisticated tools to manipulate neural activity with "*molecular precision*". This research now provides a detailed mechanistic framework of how excessive noradrenergic arousal causes the IED when extinction learning is placed in close temporal proximity to fear acquisition.

The explicit aim of Manuscript 3 of the present thesis was thus to integrate these results in a *hypothetical mechanistic framework*. In a nutshell, repeated US exposure during fear acquisition causes increased activation of CRF producing neurons of the CeA (CeA-CRF neurons; Jo et al., 2020). CeA-CRF neurons have dense projections to noradrenergic neurons

of the LC and cause increased CRF transmission within the LC (McCall et al., 2015). CRF signaling in the LC is well-known to trigger a high-tonic discharge mode of noradrenergic neurons resulting in increased noradrenaline transmission (Giustino et al., 2020; McCall et al., 2015; Valentino & van Bockstaele, 2008). Importantly, excessive noradrenaline transmission from the LC causes a persistent increase of BLA activity (> 60 minutes) after repeated US exposure (Giustino et al., 2017; Giustino et al., 2020), which in turn results in a persistent (> 60 min) inhibition of the IL during and after US exposure (Fitzgerald et al., 2015). Since IL activity during and after extinction acquisition is necessary for later successful extinction recall (Bukalo et al., 2015; Do-Monte et al., 2015), extinction acquisition immediately after fear acquisition (i.e., in a state of increased US-induced arousal) impedes successful extinction recall. In correspondence, during delayed extinction learning (24 h) noradrenergic arousal levels typically subside, and IL activity is normalized again, which enables successful extinction consolidation and extinction recall. Importantly, the activation of the described arousal circuit depends strongly on the aversiveness of the fear acquisition phase. For instance, using a US with a weak intensity or employing only few acquisition trials do not cause excessive noradrenergic arousal and therefore prevent the IED (Giustino et al., 2020). Nevertheless, intermediate levels of noradrenaline transmission are necessary for successful fear extinction (Fitzgerald et al., 2015; Uematsu et al., 2017). Boosting noradrenaline transmission when extinction acquisition takes places in a low-aroused state facilitated later extinction recall (D. Mueller & Cahill, 2010). However, while there is robust evidence for this mechanistic framework in animals, human studies examining the effects of noradrenergic arousal are very sparse and rather inconclusive.

A secondary, more methodological aim of Manuscript 2 was to account for non-oscillatory brain activity when assessing neural oscillations. Standard frequency analysis techniques - like a fast Fourier transform (FFT) or a wavelet-based time-frequency analysis - provide raw power spectra that contain a mixture of both oscillatory and non-oscillatory brain activity (Donoghue et al., 2020; Kosciessa et al., 2020). Non-oscillatory brain activity measured by EEG is primarily reflected in the so-called arrhythmic background of the power spectrum. We therefore combined two state-of-the-art oscillation detection algorithms to disentangle non-oscillatory and oscillatory brain activity. We used the *fitting oscillations & one over f* (FOOOF; Donoghue et al., 2020) algorithm to estimate the arrhythmic background power and the *extended better oscillation detection algorithm* (eBOSC; Kosciessa et al., 2020) to detect single-trial oscillatory episodes based on the FOOOF derived background estimates. During fear acquisition and extinction acquisition, presentation of a CS+ (vs. CS-) elicited a stronger tilt of

the arrhythmic background spectrum, which was characterized by a broadband decrease of low-frequency power. Interestingly, broadband reductions in low-frequency power, as indicated by such a tilt, are assumed to indicate neural activation and central arousal (for a review see: Herweg et al., 2020). Accordingly, confrontation with a CS+ is known to increase central arousal via a CeA-mediated transmission of biogenic amines (noradrenaline, dopamine, serotonin, acetylcholine) and neuropeptides (e.g., orexin) that increase neuronal excitability and thereby cause a prioritized processing of CS+ information (e.g., LeDoux, 2012). Thus, it is plausible that CS+-related increases in central arousal caused broadband reductions of low-frequency power in the EEG signal, which were especially pronounced during fear acquisition (i.e., when the fear memory was strongest). When controlling for this non-oscillatory brain activity via the eBOSC algorithm, we observed suppressed theta oscillations during fear acquisition and extinction acquisition at frontal and temporal or central electrodes, respectively. However, the observed theta suppression is at odds with two recent invasive studies (i.e., intracranial recordings) reporting increased theta oscillations during fear acquisition in the mPFC and dACC (S. Chen et al., 2021; Taub et al., 2018). Importantly, intracranial recordings can produce contrasting results compared to scalp recordings. For example, contrasting theta effects have been repeatedly observed between studies examining episodic memory via invasive intracranial or non-invasive scalp recordings, and this is assumed to be a result of the different spatial resolution between both recoding types (for a review see: Herweg et al., 2020). A previous non-invasive study reported no modulation of theta power at frontocentral electrodes during fear acquisition and extinction acquisition (E. M. Mueller et al., 2014). Thus, the discrepant results regarding theta oscillations during fear acquisition and extinction acquisition might depend on the method (i.e., invasively vs. non-invasively) used to assess neural oscillations.

Regarding the above discussed lack of theta involvement during fear recall and extinction recall, it is important to note that the reported null effect is not a result of the novel techniques employed in Manuscript 2. In correspondence to Manuscript 1 and to increase comparability with other previous studies (E. M. Mueller et al., 2014; Sperl et al., 2019), we also calculated standard FFT analyses in Manuscript 2. Nevertheless, we still did not observe the expected increase in frontocentral theta power during fear recall or deficient extinction recall. Moreover, it is also important to note that it is rather unlikely that previous observed theta increases during fear recall are due to effects of the arrhythmic background spectrum. First, in Manuscript 1, the observed theta increase during fear recall and deficient extinction recall was specific for the theta frequency range. We observed no broadband effects as indicated

by null results at frontocentral electrodes in the adjacent alpha and delta band (Bierwirth et al., 2021). Second, the observed tilt of the arrhythmic spectrum in Manuscript 2 caused decreased power estimates of low frequencies (< 30 Hz) for the CS+. Thus, if a similar tilt occurred in previous studies during fear recall, it would have even attenuated the observed theta effect (CS+ > CS-).

To conclude, our *neurophysiological data of Manuscript 2 demonstrate for the first time* that presentation of emotionally salient stimuli (i.e., a CS+) evoke a tilt of the arrhythmic background spectrum, presumably via a phasic increase of arousal-related transmitters. Thus, our results are also highly relevant for future work in the field of affective neuroscience aiming at examining neural oscillations. Not accounting for non-oscillatory brain dynamics (i.e., the arrhythmic background spectrum) may introduce a bias to under-estimate spectral power estimates of low-frequency (<30 Hz) neural oscillations elicited by emotionally salient stimuli.

5.2 Limitations

The following section points out important limitations and caveats that need to be acknowledged when interpreting the results of the present thesis.

One limitation of both studies concerns the *lack of examining long-term effects*. More precisely, we assessed fear recall and extinction recall after a 24-hour consolidation interval. Hence, we cannot draw any conclusions about the association of E2 and extinction timing on long-term extinction success. Even though the majority of fear conditioning studies in humans and animals assess fear and extinction recall 24 hours after extinction acquisition, there is accumulating evidence that the structures of the neural fear and extinction circuitry dynamically reorganize as time elapses (for a review see: Do-Monte et al., 2016). For instance, the BLA is crucially involved in the early recall (i.e., after 24 hours) of fear memories but takes no part in the recall of remote fear memories (> 3 days; Do-Monte et al., 2016). Thus, it might be possible, albeit rather unlikely, that E2 and extinction timing primarily exert their effects on the neural fear and extinction circuitry involved in early fear and extinction recall with little to no effects on long-term recall.

Another limitation of both studies concerns the *huge number of trials that was used during each learning phase*. Although a high number of trials is necessary to achieve an adequate signal-to-noise ratio for EEG analyses, it also introduces different problems. First, fear acquisition and extinction acquisition reflect dynamic learning processes. Hence, averaging over all trials within a single learning phase may have prevented the detection of more transient

neurophysiological phenomena. Second, since the CS+N is repeatedly presented without the US for the first time during fear recall, fear extinction will be initiated for this stimulus during the fear recall phase (E. M. Mueller et al., 2014). Consequentially, neural activity measured during fear recall most likely reflects a mix of fear recall-related and fear extinction-related neural processes. Importantly, Sperl et al.(2021) recently developed a so-called *sequential set fear conditioning paradigm*, which directly addresses these problems and enables a more dynamic assessment of neurophysiological EEG markers during fear conditioning.

In both studies (Manuscript 1 and Manuscript 2), we employed an almost identical differential fear and extinction paradigm which makes use of a so-called *multiple cue protocol* (Lonsdorf et al., 2017) to separately assess fear recall via presenting not-extinguished CSs, and extinction recall via presenting extinguished CSs *within a single subject*. However, the premises that fear recall and extinction recall can be assessed completely separately within a single subject might not hold completely true. Extinction memories might dynamically interact with fear memories during consolidation and recall. For instance, extinction memories can be generalized to inhibit fear responses for a CS+ that was never extinguished, especially after a consolidation interval that contained a night of sleep (Pace-Schott et al., 2009). Thus, in the used paradigm low fear responding during fear recall could also be impacted by a generalization of the extinction memory. Future studies might aim to assess extinction recall and fear recall in a between-subject design to rule out unwanted interactions between both memory systems.

Considering Manuscript 1, it is important to highlight that the groups differing in their E2 levels were *quasi-experimental groups*, i.e., we took advantage of natural stages of the menstrual cycle and contraceptive use. Thus, we cannot draw causal inferences regarding the effects of E2 on fear recall and extinction recall. Interestingly, previous studies have already successfully administered E2 before extinction learning in healthy women (Graham & Milad, 2013). It would be interesting to see if an E2 administration in women with low E2 levels improves extinction recall and attenuates dACC theta oscillations. Correspondingly, blocking E2 signaling via antagonists in women with high E2 levels should weaken extinction recall and increase dACC theta oscillations. Moreover, it might also reveal which ER is involved in E2 effects on fear recall and extinction recall in humans.

5.3 Future perspectives

The results of the present thesis provide interesting new future perspectives and open research questions that should be addressed by future studies. The following section summarizes these

potential clinical applications, open research questions, and aims at providing approaches to answer these questions in future.

In Manuscript 1 we observed enhanced dACC theta oscillations as an indicator of fear recall and deficient extinction recall (Bierwirth et al., 2021). This could provide an interesting new target to facilitate the success of fear extinction by specifically reducing theta oscillations within the dACC. A promising and resource-efficient tool to manipulate neural oscillations in humans constitutes EEG-neurofeedback (for a review see: Enriquez-Geppert et al., 2017). EEG-neurofeedback is a technique that enables subjects to learn how to control ongoing neural oscillations in real time. During EEG-neurofeedback participants are presented with their own specific oscillatory activity (e.g., in the form of a thermometer) and thereby learn how to increase or decrease the strength of the presented oscillation. A future study might utilize *EEG-neurofeedback* to teach participants to specifically *decrease frontocentral/dACC theta activity at CS+ presentation* during fear recall and extinction recall. Such a design could be used to test the causal role of frontocentral/dACC theta oscillations for fear responding during fear recall and deficient extinction recall (Enriquez-Geppert et al., 2017). If successful, EEG neurofeedback might also be a useful tool to boost the success of extinction-based exposure therapies.

Our finding of improved extinction recall in women with high E2 levels adds to accumulating evidence that the female sex hormone E2 has protective effects against anxiety-related disorders and PTSD in women (Graham et al., 2018). However, there is currently only sparse and inconclusive evidence concerning the role of sex hormones for fear extinction in men. One human study observed that the testosterone/cortisol ratio in the morning (but not in the evening) predicted extinction acquisition success (Pace-Schott et al., 2013). Previous reports from rodent studies suggest that both the male sex hormone testosterone as well as the female sex hormone E2 appear to modulate fear extinction in males (Graham & Milad, 2014; Maeng, Taha, et al., 2017). Blocking the conversion of testosterone to E2 by inhibiting aromatase activity before extinction learning causes deficits in extinction recall that can be prevented by E2 administration in male rats (Graham & Milad, 2014). Moreover, enhancing testosterone levels via injecting a single dose of the gonadotropin-releasing hormone agonist *Lupron* (i.e., leuprolide acetate) results in improved extinction recall. Previous human studies demonstrated that pharmacological substances like the *aromatase inhibitor Letrozole* (Goudriaan et al., 2010) or *testosterone administrations* (Hermans et al., 2006) are suitable to manipulate estradiol synthesis or testosterone levels in healthy men. Thus, it would be

interesting to block E2 synthesis or increase testosterone levels right before fear extinction acquisition to test if sex hormones also modulate fear extinction in men.

In Manuscript 2 we observed better extinction recall after delayed extinction learning compared to immediate extinction learning. In a next step, the underlying factors that cause this IED in humans need to be examined more specifically by addressing the neurobiological mediators. As reviewed in Manuscript 3, rodent studies strongly suggest that hyperactivity of the noradrenergic LC is a main driver of the IED. While rodent studies use sophisticated methods to directly measure and manipulate LC activity in behaving animals, human studies do not have this direct access. However, there are appealing approaches and techniques to indirectly manipulate LC activity and to assess its activity via different proxy measures. One of the most prominent proxies to non-invasively assess neural activity within the LC constitutes pupillometry (e.g., assessed via eye tracking). Studies in primates and rodents demonstrated that non-luminance-mediated changes in pupil size (or pupil diameter) are strongly correlated with neural activity in the LC and that LC stimulation evokes corresponding pupil dilations (e.g., Joshi et al., 2016; Liu et al., 2017). Accordingly, fMRI studies in human subjects revealed a correlation between pupil size and BOLD responses located in pontine structures strongly overlapping with the LC (Murphy et al., 2014). Thus, under conditions of constant luminance, pupil size constitutes a valid proxy to non-invasively assess tonic and phasic activity within the LC.

Regarding the manipulation of LC activity in humans, there are several ways to *increase but also to decrease LC activity*. For example, *repeated presentation of a US* is suitable to evoke neural activity in the LC (F.-J. Chen & Sara, 2007; Passerin et al., 2000; Tillage et al., 2021; Uematsu et al., 2017) and results in enhanced noradrenaline transmission especially pronounced if presented in an unpredictably manner (i.e., unsignaled; e.g., Galvez et al., 1996; Quirarte et al., 1998). Moreover, *transcutaneous vagus nerve stimulation* (tVNS) allows to non-invasively stimulate the auricular branch of the vagus nerve and can presumably be used to activate phasic or tonic responses in the LC (e.g., Sharon et al., 2021). Decreasing LC activity, on the other hand, might be indirectly realized by instructing participants to breath in a specific manner. A recent rodent study demonstrated that the preBötzinger complex (i.e., neural breathing control center) directly projects to the LC and provides it with excitatory input during inspiration (Yackle et al., 2017). The authors concluded that fast-frequency breathing provides a greater excitatory input to the LC due to the higher number of inspirations (Yackle et al. 2017). Slow-frequency breathing, on the other hand, should reduce LC activity due to less excitatory input. Interestingly, slow-frequency breathing techniques (< 10 breaths/min) were repeatedly shown

to reduce sympathetic arousal (for a systematic review see: Zaccaro et al., 2018), which could be mediated via reduced excitatory input from the pre-Bötzinger complex to the LC. Thus, *slow-frequency breathing techniques* might be effective to *down regulate noradrenergic activity in the LC*. Taken together, these methods could be used to non-invasively manipulate and measure the LC noradrenergic system during immediate and delayed extinction acquisition. In correspondence with the animal data (e.g., Giustino et al., 2020), it would be interesting to test if reducing LC activity (e.g., via specific breathing techniques) right before immediate extinction acquisition improves extinction recall and if increasing LC activity (e.g., via the presentation of unsignaled USs) right before delayed extinction learning deteriorates extinction recall.

Finally, there is currently no study in animals or humans examining the role of sex differences or sex hormones for the IED. Regarding the robust association between sex hormones and extinction recall it would be interesting to compare males and females with different E2 levels during extinction recall after immediate and delayed extinction acquisition. Interestingly, based on animal data it is known that females have a larger number of noradrenergic neurons within the LC and that these neurons are also characterized by larger dendrites (for a review see: Bangasser et al., 2016). In addition, the LC of females is more sensitive to the effects of CRF after stressor exposure compared to the LC of males (Bangasser et al., 2016). Interestingly, E2 has also complex effects on noradrenergic transmission and strongly regulates the expression of adrenoceptors (Bangasser et al., 2016). Additionally, recent rodent studies reported that the effects of CRF in females strongly depend on naturally fluctuations of sex hormones (e.g., Salvatore et al., 2018). To conclude, future studies examining the role of noradrenergic arousal (including CRF) and sex differences for fear extinction are highly warranted and should account for naturally fluctuations of sex hormones.

5.4 Conclusion

The present work highlights the importance of the female sex hormone E2 and fear extinction timing for the recall of extinction memories. Using a multi-methodological approach to assess fear expression on a peripheral and a neurophysiological level, we were able to demonstrate for the first time that dACC theta oscillations and peripheral indicators of fear expression during fear recall and extinction recall do not differ between sexes per se but are associated with hormonal fluctuations within women. This also adds to the growing evidence that E2 has protective effects against anxiety-related disorders and PTSD in women. In addition, women might benefit stronger from extinction-based exposure therapies, if treatment sessions are

scheduled during menstrual cycle phases of high endogenous E2 levels. Moreover, we demonstrate that delayed extinction is advantageous over immediate extinction in reducing peripheral arousal during later extinction recall in healthy humans. Thus, a short interval between a trauma and the onset of extinction-based exposure therapy might result in a weaker symptom relief. Importantly, we provide first evidence that non-oscillatory brain activity is sensitive to fear conditioning which has important implications for future fear conditioning studies aiming at examining neural oscillations. Future studies should now further elucidate the underlying mechanisms causing the observed extinction deficits after immediate extinction acquisition. Studies examining the interaction between the noradrenergic system, immediate vs. delayed fear extinction, and E2 status might provide a good starting point.

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