Stress effects on human fear conditioning and the role of female sex hormones

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List of Abbreviations

5-HT	serotonin (5-hydroxytryptamin)
ACC	anterior cingulate cortex
BLA	basolateral amygdala
BOLD	blood-oxygenation-level dependent (contrast)
CE	central nucleus of the amygdala
CPT	cold pressor test
CR	conditioned response
CRH	Corticotropin-releasing hormone
CS	conditioned stimulus
CS-	conditioned stimulus never paired with the US
CS+	conditioned stimulus paired with the US
DA	dopamine
E2	17β-estradiol
EF	early follicular phase of the menstrual cycle
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
GABA	γ-amino-butyric acid
GC	glucocorticoid
HPA	hypothalamus-pituitary-adrenocortical
IL	infralimbic cortex
LA	lateral nucleus of the amygdala
MC	midcycle phase of the menstrual cycle
mPFC	medial prefrontal cortex
NA	noradrenaline (= norepinephrine)
NMDA	N-methyl-D-aspartate
PFC	prefrontal cortex
PTSD	posttraumatic stress disorder
SPS	single prolonged stress
US	unconditioned stimulus
vmPFC	ventromedial prefrontal cortex

Abstract

Classical fear conditioning – including acquisition and extinction – is a model for fear learning and memory in health and disease. Moreover, trauma-related disorders can be viewed as comprising fear acquisition under severe stress. Yet, in humans, we know comparatively little about how acute stress affects fear conditioning. Therefore, the first aim of this thesis was to investigate the effect of stress on fear acquisition or extinction.

Stress induces multiple hormonal and neurotransmitter changes dynamically developing over time, including a fast first-wave and a slower second-wave stress response. Models derived from avoidance learning and declarative memory studies suggest that stress effects on memory depend on the temporal proximity between learning and stressor: encoding close to the stressor will be enhanced, but encoding and recall later in time (during the second-wave) will be suppressed (e.g., Schwabe, Joëls, Roozendaal, Wolf, & Oitzl, 2012). So far, these predictions were not related to fear conditioning. Therefore, we investigated if the model-based predictions are also valid in human fear conditioning. We used two stressors to investigate first-wave and second-wave stress effects: the cold pressor test (CPT) inducing a strong first-wave but little second-wave activation and a psychosocial stressor, reliably inducing both, first- and second-wave stress responses. Conditioning was measured via skin conductance responses (SCRs).

Investigating the first-wave (Experiment 2), we placed fear acquisition and immediate extinction directly after the CPT (n = 20) or after the control treatment (n = 20). We found no group difference in acquisition performance, but significantly increased extinction resistance in the stressed CPT group. In Experiment 3, CPT (n = 20) or control (n = 20) was placed after acquisition but directly prior to extinction training. Here, we found improved extinction and 24h-delayed extinction recall after CPT. Investigating the second-wave (Experiment 1), we placed fear acquisition and immediate extinction 45 min after the psychosocial stressor (i.e., at the peak of salivary cortisol, n = 12) or after control (n = 12). Here, we found no significant stress effects.

Sex and female sex hormones also influence fear conditioning: Women are at a higher risk to develop anxiety and stressor-related disorders than men. Interestingly, patients with these disorders show impaired fear extinction and extinction recall, and low levels of the sex hormone 17β-estradiol (E2) are linked to impaired extinction in both, healthy and patient female samples. So far, there is little data on how acute stress and circulating E2-levels might

interact in fear acquisition and especially in fear extinction. Therefore, the second aim of this thesis was to explore this possible interaction in healthy women in different cycle phases compared to men.

Thus, in Experiment 4, we included hormone status as a quasi-experimental variable and compared free cycling women in the midcycle phase (high E2, low progesterone, n = 24), women in the early follicular phase of the menstrual cycle (low E2, low progesterone, n = 24), and men (n = 24). We placed fear acquisition and extinction 45 min after the psychosocial stressor (n = 36) or control (n = 36), and tested extinction recall after 24 h. In line with Experiment 1, the second-wave stressor did not affect fear acquisition and immediate extinction. However, we found a stress by hormone status interaction within women at the 24h-delayed extinction recall test: in the stressed group, early follicular women showed impaired extinction recall and a higher return of fear compared to midcycle women, whereas there was no difference between early follicular and midcycle women after control treatment.

Collectively our results support a different role for the first- and second-wave stress response in human fear conditioning. Fear acquisition near the first-wave stress response results in enhanced fear memory, which is resistant to extinction. Extinction training near the first-wave enhances extinction learning. In contrast, fear conditioning at the peak of the peripheral second-wave cortisol response had no effect on acquisition or extinction performance. However, second-wave stress interacted with the hormone status of women, where only women in a low E2 state were vulnerable to negative stress effects in extinction recall. The last result will encourage further investigation of the interplay between E2 and stress in fear extinction. Enhancement of extinction by the CPT could – if replicated – be translated into strategies for optimizing exposure therapy.

1. Introduction

1.1 Overview

Classical fear conditioning is an important model for the etiology of anxiety disorders as well as for trauma- and stressor-related disorders (Mineka & Oehlberg, 2008). Moreover, learning mechanisms of fear extinction are fundamental in modeling exposure techniques in psychotherapy (Vervliet, Craske, & Hermans, 2013). Trauma-related disorders, such as posttraumatic stress disorder (PTSD), can be thought of as comprising fear learning during or in the aftermath of severe stress. Yet, in humans we know very little about how acute stress affects associative fear learning processes, including both, acquisition and extinction of fear responses. Additionally, examining stress effects on fear extinction can help identify optimal conditions for successful exposure therapy.

As human experiments must use relatively mild stimuli during fear learning experiments, there is also a potential problem of comparability to both animal studies and real-world trauma situations, where the stimuli are of a much higher intensity. Examining fear acquisition in temporal proximity to a standard experimental stressor should therefore also increase comparability without significantly increasing the risk for the participants. Stress, however, induces a complex array of hormonal and neurotransmitter responses developing over the course of time. Importantly, stress-induced changes can influence behavior on at least two different time scales including a fast acting first-wave stress response and a time delayed second-wave stress response. The first- and second-wave stress response were suggested to have opposing effects on emotional memory (Diamond, Campbell, Park, Halonen, & Zoladz, 2007; Joëls, Fernandez, & Roozendaal, 2011; Schwabe et al., 2012). These predictions, however, are derived from animal avoidance learning and human declarative memory tasks and – so far – were not tested for fear conditioning. Thus, one goal of this thesis was to investigate if such model-based predictions also hold true for human fear conditioning.

In line with a time-dependent and orchestrated stress response, defined by the interplay of multiple mediators, actions of single stress mediators (e.g., glucocorticoids) have been shown to depend on previous activation of other stress mediators (e.g., noradrenaline). Therefore, it is difficult to separate first and second-wave stress effects in humans by using drug administration and other pharmacological techniques. Instead, we used two standardized human stress procedures with different properties. One stressor (the cold pressor test) induces predominantly the first-wave stress response and because of its short duration (approx. 3 min)

allows us to test first-wave effects by placing learning immediately after stress. The second stressor (a psychosocial stressor) induces both, the first- and the second- wave stress response, and allows us to test second-wave effects by placing learning approx. 45 min after stressor onset, when first-wave effects have subsided, but within the peak of the (second-wave) peripheral cortisol response. We have designed Experiments 1 and 2 to test first- and second-wave effects of stress on fear acquisition, and Experiment 3 to test first-wave stress effects on fear extinction.

Both, anxiety disorders and trauma- and stressor-related disorders, are much more common in women than in men (Kessler et al., 2005; Tolin & Foa, 2006). Current studies have identified a link between low levels of the female sex hormone 17β-estradiol (E2), impaired fear extinction, and anxiety and trauma- and stressor-related disorders. However, there is little data on a potential interaction between stress and E2 effects in fear acquisition and extinction, rendering further studies necessary. Thus, the aim of Experiment 4 was to test the effect of a second-wave stressor on free cycling women in distinct phases of the menstrual cycle (high E2 vs. low E2) and on men.

In the following sections I will describe fear acquisition (section 1.2.1) and fear extinction (1.2.2) with regard to the learning procedure, behavioral principles, relevance, and neuronal mechanisms. Then I will describe and define stress (1.3.1) and the stress response (1.3.2), and outline current models of stress effects on memory. Subsequently, (section 1.3.3), I will review current animal and human data on stress and stress-hormone effects on fear acquisition and extinction. I will then briefly describe the hormone E2, its relevance and the current evidence for its role in fear extinction (1.4), followed by first evidence for possible Stress x Sex and Stress x E2 interactions (1.5).

1.2 Fear conditioning

1.2.1 Fear acquisition

Pavlovian fear conditioning (Pavlov, 1927) involves learning about stimuli that predict an aversive event. In the fear conditioning[1] procedure, an initially neutral stimulus (conditioned stimulus, CS) is paired with a biologically relevant unconditioned stimulus (US, e.g. electric

[1] In order to separate the everyday meaning of the term fear (i.e., the subjective feeling of being afraid) from its scientific use, Joseph E. LeDoux (2014) has recently proposed to replace the terms "fear conditioning" and "conditioned fear response" with the more precise and unambiguous terms "threat conditioning" and "defense response", respectively. As this new terminology is not yet widely used, I will continue to use the "old" terms fear conditioning and fear response to avoid confusion. Both however do not imply a subjective feeling of fear.

shock, aversive loud noise) capable of eliciting an unconditioned fear response. This learning phase is termed *fear acquisition* and corresponds to the encoding process in the terminology of memory research. After repeated pairing, the CS alone comes to elicit a conditioned fear response (CR). If the CS is a single discrete stimulus (e.g., a tone or a light) the procedure is termed *cued fear conditioning*. If the CS is a more complex combination of stimuli (e.g., a combination of light, odor, and cage size) the procedure is termed *context fear conditioning*.

In rodent studies, the most common way to quantify conditioned responses is measuring freezing: a species specific defensive response in rodents. In humans, CRs are often quantified by assessing skin conductance responses (SCRs; see Boucsein, 2012) evoked by the CS, usually comparing responses to a CS+, which is paired with the US during acquisition and responses to a second control stimulus (CS-), which is never paired with the US (Graham & Milad, 2013; Knight, Nguyen, & Bandettini, 2006; LaBar, LeDoux, Spencer, & Phelps, 1995; Rabinak et al., 2013; Schultz & Helmstetter, 2010). Other common measures include the potentiation of the startle reflex and subjective ratings of US expectancy (e.g., Glover et al., 2012; Norrholm et al., 2011; Soeter & Kindt, 2011), but also functional imaging methods such as functional magnetic resonance imaging (e.g., Merz et al., 2012a; Phelps, Delgado, Nearing, & LeDoux, 2004; Stark et al., 2006) or a combination of two or more of those measures (e.g., Merz, Wolf, et al., 2013; Soeter & Kindt, 2012).

Fear conditioning is a highly adaptive form of learning, as it enables the organism to effectively and rapidly learn to predict danger using context information and environmental cues. However, fear learning can become dysfunctional if the organism continues to display fear responses when the cue or context no longer signals danger. In fact, dysfunctional fear learning, the persistence of fear, and the inability to inhibit fear responses in the face of safety are assumed to largely contribute to the etiology of anxiety disorders (Delgado, Olsson, & Phelps, 2006; Mineka & Oehlberg, 2008; Mineka & Zinbarg, 2006) as well as trauma- and stressor-related disorders such as posttraumatic stress disorder (PTSD, e.g., Ehlers & Clark, 2000; Mineka & Oehlberg, 2008).

Fear conditioning is one of the most influential experimental models to study emotional learning and memory. Its mechanisms seem to be largely conserved through evolution (Duvarci & Pare, 2014; Phelps & LeDoux, 2005) allowing the same basic learning mechanism to be studied in a number of laboratory animals and humans. Moreover, the neural and molecular mechanisms are very well understood (Pape & Pare, 2010). Thus, it is widely

used as a translational model paradigm for neural plasticity, (emotional) memory, emotional responses and associated disorders.

A brain structure that plays a central role in fear conditioning is the amygdala. Amygdala subregions relevant for fear acquisition include the basolateral complex (basolateral amygdala, BLA), the central nucleus (CE), and the intercalated cell-masses (LeDoux, 2007; Pape & Pare, 2010). The BLA is subdivided in the lateral (LA), basolateral and basomedial (also called accessory basal) nuclei; the CE is separated in a lateral and medial division.

The LA is the primary input zone for sensory information into the amygdala (Herry & Johansen, 2014; LeDoux, 2007; Pape & Pare, 2010) receiving inputs from all sensory modalities including auditory, visual, olfactory, somatosensory, and nociceptive signals. Information about the CS and the US converge into the LA, which is considered to be a main cite of the synaptic plasticity underlying fear conditioning (Herry & Johansen, 2014; LeDoux, 2007; Pape & Pare, 2010) and a central storage site for fear memories (Pape & Pare, 2010).

The main output region of the amygdala is the CE, projecting widely to different brain stem and hypothalamic regions. Projections of the CE medial division to the periaqueductal gray are responsible for defensive responses including vocalizations, behavioral arrest/freezing, and endogenous opioid-mediated analgesia; projections to the parabrachial nucleus are involved in respiration responses; projections to the nucleus reticularis pontis caudalis are responsible for startle reflex potentiation, while projections to the lateral and paraventricular nuclei of the hypothalamus produce sympathetic and hypothalamus-pituitary-adrenocortical (HPA) axis activation, respectively (Davis, 1992; Fanselow & Poulos, 2005; Pape & Pare, 2010; Sah, Faber, Lopez de Armenita, & Power, 2003). The CE also projects to noradrenergic (locus coeruleus), cholinergic (nucleus basalis Meynert), and dopaminergic (substantia nigra, ventral tegmental area) neuromodulatory systems. These neuromodulatory connections enable the amygdala to influence the excitability of large portions of the brain, including many areas lacking direct connection with the amygdala (Duvarci & Pare, 2014; Pape & Pare, 2010; Sah et al., 2003).

1.2.2 Fear extinction

One mechanism to reduce and even eliminate conditioned fear responses is extinction. Fear extinction is established by repeatedly presenting the CS without the US, which leads to a

decline in conditioned responding. Fear extinction is an important model for exposure techniques in behavioral therapy – an effective treatment for anxiety, trauma- and stressor-related disorders (Norton & Price, 2007). Moreover, patients with anxiety and especially trauma-related disorders show deficits in fear extinction learning and extinction recall (Blechert, Michael, Vriends, Margraf, & Wilhelm, 2007; Inslicht et al., 2013; Jovanovic et al., 2010; Lissek et al., 2005; Michael, Blechert, Vriends, Margraf, & Wilhelm, 2007; Milad et al., 2008; Milad, Pitman, et al., 2009). These extinction deficits have been proposed to contribute to the maintenance of these disorders (Cover, Maeng, Lebrón-Milad, & Milad, 2014; Milad et al., 2010; Milad, Rosenbaum, & Simon, 2014).

According to contemporary learning models (Bouton, 2002), extinction does not rely on forgetting, unlearning, or the (complete) erasure of the original fear memory trace. Instead, extinction is believed to build a new competing inhibitory memory trace, i.e. a CS-noUS association (Milad & Quirk, 2012; Myers & Davis, 2007). This is supported by three return-of-fear phenomena consistently observed after successful extinction: spontaneous recovery, renewal, and reinstatement. *Spontaneous recovery* refers to the fact that the extinguished CR often recovers with the passage of time. Thus, the extinguished CS can become capable of eliciting a CR, even though there were no new CS-US pairings. For the *renewal* effect, the extinguished CR reemerges when the CS is presented in a context different from the extinction context. Typically, acquisition takes place in context A and extinction takes place in context A or B. Extinguished responses reappear if testing occurs in a new context (AAB-design: acquisition in A, extinction in A, test in B; ABC-design: acquisition in A, extinction in B, test in C), but also if tested in the acquisition context (ABA-design) (Todd, Vurbic, & Bouton, 2014). In *reinstatement* extinguished responses recover if the US alone is presented again after extinction. Thus, spontaneous recovery, renewal, and reinstatement suggest that the original fear memory trace is still intact (Bouton, 2002; Todd et al., 2014). However, there is both neural and behavioral evidence showing that some degree of weakening or erasure of the CS-US association also takes place during extinction, suggesting the coexistence of new inhibitory learning and unlearning mechanisms (Delamater & Westbrook, 2014; Myers & Davis, 2007).

The neuroanatomical substrates of fear extinction involve three interconnected brain regions: the amygdala, the medial prefrontal cortex (mPFC), and the hippocampus (Pape & Pare, 2010; Quirk & Mueller, 2008). Evidence suggests, that the amygdala is the site where the extinction memory (the CS-noUS association) is stored, the infralimbic mPFC (IL) serves

the consolidation and recall of extinction through projections to the amygdala that ultimately inhibit fear expression, while the hippocampus is involved in the context specificity of extinction (Pape & Pare, 2010; Quirk & Mueller, 2008). Fear extinction has been shown to induce new NMDA-receptor dependent synaptic plasticity in the amygdala, IL, and hippocampus. Consistent with the view of a coexistence of new learning and unlearning, some degree of unlearning was also demonstrated involving synaptic depotentiation of thalamic inputs to the BLA (Pape & Pare, 2010). Human neuroimaging studies suggest a similar network for extinction involving the amygdala, the ventromedial prefrontal cortex (vmPFC as human homolog of the rodent IL), and the hippocampus (Milad & Quirk, 2012).

1.3 Stress

1.3.1 Overview

There is no single broadly accepted definition of stress (e.g., Chrousos, 1992; McEwen, 2007; Selye, 1993). This makes it necessary to explicitly state what definition is used. Within this thesis I will use the following definition of the terms *stressor*, *stress*, *stress response* and *stress-mediators* provided by Joëls & Baram (2009):

> *"Any actual or potential disturbance of an individual's environment – a 'stressor' – is recognized or perceived by specific brain regions. The subjective state of sensing potentially adverse changes in the environment is called 'stress' and leads to the release of molecules that we here call 'stress mediators', which bind to receptor targets. Each of these mediators acts on specific neuronal populations, resulting in unique downstream effects. Together, these effects constitute the 'stress response', which enables the animal to adapt to the changing environment".* (Joëls & Baram, 2009, p. 459)

Of note, "individual's environment" may also include the inner-organismic environment and dynamic equilibrium included in other definitions under the term *homeostasis* (e.g., Chrousos, 1992) and the *stressors* may be both intrinsic or extrinsic to the organism. Furthermore, Joëls and Baram (2009) restrict their definition of the *stress response* to the effects of stress-mediators on the (central) nervous system. In contrast, I will consider a broader definition of the *stress response* including not only central but also peripheral changes, and including not only the effects of stress-mediators but also the changes in release patterns and concentrations, as described below.

1.3.2 The stress response

The stress response is a process that involves a cascade of neuronal and neuroendocrine changes over the course of time (Antov, Wölk, & Stockhorst, 2013). With respect to their onset, neuroendocrine changes comprising the stress response can be subdivided into a first-wave and a second-wave stress response (Sapolsky, Romero, & Munck, 2000). *The first-wave stress response*, occurring within seconds, involves: (a) increased release of the monoamine neurotransmitters noradrenaline (NA), dopamine (DA), and serotonin (5-HT) (Joëls & Baram, 2009) from different brain nuclei; (b) enhanced secretion of corticotropin-releasing hormone (CRH) from the hypothalamus, followed by an increased release of adrenocorticotropic hormone from the pituitary; and, (c) reduced secretion of gonadotropin-releasing hormone resulting in reduced release of gonadotropins from the pituitary. In the periphery, the *first-wave stress response* includes a rapid increase in sympathetic tone and secretion of adrenaline and NA from the adrenal medulla (Sapolsky et al., 2000). *The second-wave stress response*, starting after several minutes, includes increased peripheral secretion of glucocorticoids (GC) from the adrenal cortex and reduced secretion of gonadal steroid hormones (i.e., estrogens, progesterone, and testosterone) (Sapolsky et al., 2000). These two waves produce the adaptive responses, enabling the organism to deal with challenge, including: increased mobilization of energy and inhibition of energy storage, increased cardiovascular tone, inhibition of costly processes and behaviors such as reproduction, feeding, and digestion, modification of short and long-term immune responses, as well as increased central arousal (Chrousos, 2009; Sapolsky et al., 2000).

The brain receives feedback from the peripheral stress response. GCs readily cross the blood-brain barrier and exert negative feedback at the hypothalamic and pituitary level to rein in the stress response (de Kloet, Joëls, & Holsboer, 2005). GCs also bind on glucocorticoid and mineralocorticoid receptors throughout the brain, including the amygdala, hippocampus, medial prefrontal cortex, and septum (Joëls & Baram, 2009), where they can influence neural signaling and synaptic plasticity. Although it cannot cross the blood-brain barrier, peripheral adrenaline binds to receptors of the vagus nerve projecting to the nucleus of solitary tract, which in turn produces increased release of NA in the amygdala, directly or via increased activity of the locus coeruleus (Hassert, Miyashita, & Williams, 2004; McGaugh, 2004; Miyashita & Williams, 2002). The increased locus coeruleus activity after positive feedback from the periphery further increases NA levels throughout the brain.

First- and second-wave changes not only have distinct temporal onsets but also differ in the duration of their effects: increased NA, DA, 5-HT, and CRH (acting on CRH-receptor 1 [CRHR1]) in the brain usually act within seconds but their effects subside quickly and rarely outlast the duration of the stressor; GC concentrations in the brain only reach peak levels about 20 min after stressor onset (Hermans, Henckens, Joëls, & Fernández, 2014; Joëls & Baram, 2009), moreover genomic actions of GCs (and other steroids) take even longer to manifest (Joëls & Baram, 2009). In fact, the slower genomic GC actions were proposed to actively reverse and normalize the rapid effects of various first-wave stress mediators (Hermans et al., 2014).

1.3.3 Stress effects on fear conditioning

Acknowledging the temporal complexity of the stress response and its effects in the brain, different models have proposed that stress effects on emotional learning and memory will vary depending on the temporal distance between stress and learning (Diamond et al., 2007; Joëls, Pu, Wiegert, Oitzl, & Krugers, 2006). Shortly after stress, synergistic actions of rapid NA and non-genomic GC in the basolateral amygdala (BLA) promote the *encoding* of emotionally relevant information by enhancing synaptic excitability and long term potentiation (Joëls et al., 2011). Slower genomic GC actions act in the opposite direction, normalizing and/or dampening BLA activity (Joëls et al., 2011). Genomic GC effects were also shown to improve the *consolidation* of emotional memory, but these effects are conditional upon arousal-induced NA increase during the encoding phase (Okuda, Roozendaal, & McGaugh, 2004; Roozendaal, Okuda, Van der Zee, & McGaugh, 2006).

These different viewpoints were integrated in a recent model (Schwabe et al., 2012) allowing to derive precise and testable predictions for emotional learning and memory in humans. According to this model (Schwabe et al., 2012), stress during or immediately before learning should promote encoding of emotional material through effects of first-wave stress mediators. Slower genomic GC effects will then enhance the consolidation of the material learned under stress. If however, the stressor is placed long before learning (i.e. stressor and learning are "out of sync"), stress should suppress the encoding of new material and impair the retrieval[2] of previously learned material via second-wave stress mediators (such as genomic GCs). There is evidence for the validity of these predictions from animal (Karst,

[2] The terms **retrieval** and **recall** are used synonymously throughout this thesis as the process of accessing a previously encoded and stored memory trace. Accordingly, in the context of extinction memory, the terms **extinction retrieval** and **extinction recall** are also used synonymously.

Berger, Erdmann, Schutz, & Joëls, 2010; Liebmann, Karst, & Joëls, 2009; Pu, Krugers, & Joëls, 2009; Roozendaal, Okuda, et al., 2006) and human studies (Henckens, van Wingen, Joëls, & Fernández, 2010; Zoladz et al., 2011). However, the model (Schwabe et al., 2012) is based on animal data with object recognition and passive avoidance tasks as well as on human data from declarative memory tasks. Therefore, it is not clear if the predictions will also hold for classical fear conditioning. Thus, testing some of the predictions of this model in human fear conditioning was one major rationale for the studies reported within this thesis.

Stress effects on fear acquisition

In animal pharmacological fear conditioning studies, reviewed by Rodrigues and colleagues (Rodrigues, LeDoux, & Sapolsky, 2009), evidence suggests that the first-wave stress mediator NA plays an important role in enhancing fear acquisition, whereas GCs as second-wave stress mediators enhance the consolidation, but have no effect on the acquisition of fear. These findings are largely in line with the predictions later made by the Schwabe et al. model (2012).

Until now, human studies investigating stress effects on fear acquisition (see introduction of Antov et al., 2013) do not explicitly take into account that the effects of stress may vary considerably depending on the timing of the stressor relative to the targeted memory process (i.e. encoding/acquisition, consolidation, recall, re-consolidation). Interpreting human pharmacological studies administering a drug prior to fear acquisition, we found some indirect evidence that first-wave mediators may enhance the encoding/acquisition of fear responses (Grillon, Cordova, Morgan, Charney, & Davis, 2004; Soeter & Kindt, 2011, 2012). Human functional imaging studies suggest, that administration of the second-wave mediator cortisol may impair fear acquisition/encoding (Merz et al., 2010, 2012a; Stark et al., 2006). However, this is not corroborated by studies using experimental stress induction (Jackson, Payne, Nadel, & Jacobs, 2006). Taking this evidence into account and considering that the stress response involves the interplay of multiple mediators with distinct temporal profiles, we designed Experiments 1 and 2 (reported in Antov et al., 2013) to test the effects the first- and second-wave stress response on fear acquisition in (male) humans.

Stress effects on fear extinction

Despite the potential relevance for clinical applications, the literature on acute stress and fear extinction in humans is scarce (reviewed in Raio & Phelps, 2015). Below I will first review animal studies and then human studies using stress-induction or pharmacological techniques.

As with fear acquisition studies above, I will try to relate the data to predictions derived from the Schwabe et al. (2012) model. Assuming that fear extinction encompasses new learning and building a new inhibitory memory trace (e.g., Bouton, 2002, 2004; Todd et al., 2014), extinction learning should be enhanced if stress and learning are in close temporal proximity; extinction learning and retrieval should be impaired if stress is placed long before learning (i.e., stress and learning are "out of sync").

Animal studies suggest effects on long-term memory and consolidation of extinction. Single prolonged stress (SPS) is used as an animal model of trauma: it comprises 2 h of restraint, 20 min of forced swim stress, and exposure to ether until general anesthesia (Yamamoto et al., 2008). SPS impairs extinction recall, when applied 7 days before fear acquisition and extinction (Ganon-Elazar & Akirav, 2013; Knox, Nault, Henderson, & Liberzon, 2012; Knox, George, et al., 2012; Matsumoto et al., 2013; Yamamoto et al., 2008, 2009). The effects have been linked to enhanced glucocorticoid receptor expression in the hippocampus and PFC (Knox, Nault, et al., 2012) and to alterations in NMDA-receptors in the hippocampus (Knox, Nault, et al., 2012; Matsumoto et al., 2013; Yamamoto et al., 2008). However, no impairment of extinction was found after a shorter time interval between stress and learning (Knox, George, et al., 2012), or after omitting either one of the SPS-components (Knox, Nault, et al., 2012). Repeated forced swim stress (10 min/day for 3 days) 48h before extinction, impaired cued extinction learning and was associated with dendritic retraction in infralimbic neurons (IL) in the PFC (Izquierdo, 2006). Conversely, a single exposure to 3 h of immobilization stress improved contextual extinction recall 14 days (but not 2 days) after the stressor (Kirby et al., 2013) and the effects were linked to increased neurogenesis in the hippocampus.

The above studies featuring single (Ganon-Elazar & Akirav, 2013; Knox, Nault, et al., 2012; Knox, George, et al., 2012; Matsumoto et al., 2013; Yamamoto et al., 2008, 2009) or repeated stress exposure (Izquierdo, 2006) have placed stress before fear acquisition making precise inferences about extinction learning difficult. However, 30 min of elevated platform stress after cued fear acquisition and 24h prior to fear extinction also impaired extinction learning and was associated with changes in BLA morphology (Akirav, Segev, Motanis, & Maroun, 2009; Maroun et al., 2013).

Taken together, there is evidence that stress impairs the consolidation/recall of fear extinction. These impairments require time (> 24h) and are associated with structural changes including dendritic morphology, neurogenesis, and receptor density. Stress induced

impairments seem to be more likely with high intensity stressors. Thus, animal data may reflect long-term second-wave stress effects associated with slower but long-lasting effects of stress mediators. This seems in accordance with the model by Schwabe et al. (2012). However, animal studies often use delays of several days after stress. This time frame is not explicitly accounted for by the model.

Animal pharmacological studies with NA as a mediator of the first-wave stress response provide evidence that NA enhances fear extinction (reviewed in D. Mueller & Cahill, 2010). Systemic yohimbine (an α2-adrenoceptor blocker increasing NA-levels) increased extinction learning (Cain, Blouin, & Barad, 2004; Morris & Bouton, 2007; D. Mueller, Olivera-Figueroa, Pine, & Quirk, 2009) and extinction memory (Cain et al., 2004; Morris & Bouton, 2007) when injected prior to (but not after) extinction training (Cain et al., 2004). Infusions of NA into the basolateral amygdala (BLA) after extinction training improved extinction memory (Berlau & McGaugh, 2006). Moreover, infusion of propranolol (a β-adrenoceptor antagonist) into the IL prior to (but not after) extinction training impaired extinction recall, and NA enhanced the excitability of IL neurons in a β-receptor dependent manner (D. Mueller, Porter, & Quirk, 2008). Similar results were obtained in extinction of instrumental behavior (i.e. lever pressing for food reward; Janak & Corbit, 2011).

Within the stress response increased peripheral release of adrenaline and NA activates vagus nerve afferents and thereby increases NA levels in the brain including the amygdala (McGaugh, 2004). Newer animal studies show that extinction coupled with *vagus nerve stimulation* facilitates fear extinction and promotes plasticity in the IL-BLA pathway (Peña et al., 2014; Peña, Engineer, & McIntyre, 2013). Consistently, using subdiaphragmatic vagal deafferentiation, which surgically disconnects 100% of vagal afferents while leaving 50% of the efferent fibers intact, Klarer and colleagues (Klarer et al., 2014) showed that vagal deafferentiation impaired cued fear extinction learning and was associated with decreased levels of NA and increased GABA-levels in the ventral PFC.

Pharmacological studies with GCs have shown that systemic and intra-amygdala administration of GC agonists facilitates fear extinction memory when given prior to or directly after extinction training, whereas GC antagonists or GC synthesis inhibitor metyrapone impair extinction memory (Barrett & Gonzalez-Lima, 2004; Blundell, Blaiss, Lagace, Eisch, & Powell, 2011; Yang, Chao, & Lu, 2006; Yang, Chao, Ro, Wo, & Lu, 2007).

Human studies investigating the effect of an experimental stress induction on processes of fear extinction are scarce (Bentz et al., 2013; Merz, Hamacher-Dang, & Wolf, 2014; Raio, Brignoni-Perez, Goldman, & Phelps, 2014). Bentz and colleagues (2013) placed a cold pressor test (CPT) vs. control prior to extinction training and tested responses to the CSs one day later: they found that CPT impaired recall of the fear memory (measured with US expectancy ratings) in men but not in women. As also discussed by the authors, (Bentz et al., 2013) this study has some important limitations: first, there was no evidence for fear learning in physiological measures of fear conditioning (skin conductance, heart rate), and second, there was no extinction learning in any measure. These limitations preclude inferences about a stress effect on fear extinction. Raio and colleagues (Raio et al., 2014) used a 2-day procedure with fear acquisition and extinction training on day 1, extinction recall test on day 2 and a CPT/control placed 15 min prior to extinction test on day 2: They found that the CPT impaired extinction recall, as indexed by higher conditioned skin conductance responses (SCR) during the first two trials on day 2. In a similar procedure, the socially evaluated cold pressor test (Schwabe, Haddad, & Schächinger, 2008) placed 20 min prior to extinction recall and 24 h after extinction learning, attenuated contextual renewal (Merz, Hamacher-Dang, et al., 2014), indicative of enhanced extinction recall.

A *human pharmacological* study targeting the NA-system shows impaired extinction learning after propranolol in US-expectancy ratings, but not in SCR or startle (Bos, Beckers, & Kindt, 2012). A second study tested the effect of NA reuptake inhibitor reboxetine administered after extinction learning, but found no effect on the consolidation of extinction memory (Lonsdorf, Haaker, Fadai, & Kalisch, 2014). Yohimbine was also proposed to improve exposure therapy in humans but results are mixed (Meyerbroeker, Powers, van Stegeren, & Emmelkamp, 2012; Powers, Smits, Otto, Sanders, & Emmelkamp, 2009).

Results from studies addressing GC effects on fear extinction are mixed: one study found no effect of hydrocortisone (30mg) in men and free-cycling women (Merz et al., 2012a), another reported enhanced differentiation (higher responses to the CS- vs. CS+) in functional imaging in women using oral contraceptives (Tabbert et al., 2010), and one reports impaired extinction learning in men (SCR and BOLD) (Merz, Hermann, Stark, & Wolf, 2014). On the other hand, clinical studies have shown enhanced outcome of exposure therapy, when GCs were administered prior to exposure training for specific phobias (de Quervain et al., 2011; Soravia et al., 2006, 2014). There are also reports of enhanced extinction/extinction recall (Pace-Schott et al., 2013) and exposure therapy (Lass-Hennemann & Michael, 2014) in

the morning, when GC levels are high. However, only one "circadian" study shows a positive association between GC levels and exposure therapy outcome (Meuret et al., 2015).

In sum, animal studies show that high-intensity and/or repeated stressors designed to resemble human traumatic stress impair fear extinction with a delay of at least 24 h, while NA and vagus nerve stimulation enhance extinction learning, and GCs enhance the consolidation of extinction. This is broadly in line with the prediction from the Schwabe et al. (2012) model. Human studies are in part in agreement with animal data and there is still considerable lack of experiments addressing acute stress effects on fear extinction in humans. Especially, there is no data on how acute stress may influence extinction learning processes. With this in mind, we designed Experiment 3 to test effects of the first-wave stress response on fear extinction learning and recall 24h later.

1.4 Sex, 17β-estradiol and fear conditioning

Compared to men, the prevalence of anxiety (Eaton et al., 2012; Kessler et al., 2005; Somers, Goldner, Waraich, & Hsu, 2006) and trauma and stress-related disorders (Kessler, Sonnega, Bromet, Hughes, & Nelson, 1995; Tolin & Foa, 2006; Zoladz & Diamond, 2013) is up to two times higher in women. Different levels of circulating gonadal hormones may constitute one important factor contributing to these sex differences in psychopathology (Cover et al., 2014; Lebron-Milad & Milad, 2012).

As stated above, patients with PTSD exhibit marked fear extinction deficits (Blechert et al., 2007; Inslicht et al., 2013; Jovanovic et al., 2010; Milad et al., 2008; Milad, Pitman, et al., 2009). Interestingly, fear inhibition/extinction deficits have been linked to low levels of 17β-estradiol (E2) in both female PTSD-patients (Glover et al., 2012) as well as in healthy and traumatized women (Glover et al., 2013). E2 is one of the human steroidal sex hormones called estrogens which include estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4); E2 is produced by the ovaries and (to a smaller amount) by the adrenal cortex, and by the testes in men by conversion from testosterone via the enzyme aromatase or by conversion of androstendione to E1 and conversion of E1 to E2. E2 is the predominating estrogen in non-pregnant women in their reproductive years and during this time of life E2 levels are considerably higher in women than in men and vary with the menstrual cycle (Cover et al., 2014; Gillies & McArthur, 2010). In an idealized 28-days menstrual cycle E2 and

progesterone concentrations are low during the early follicular phase (approx. days 1-8), during the late follicular phase progesterone remains low, while E2 rises to reach its peak levels immediately before ovulation (approx. days 13-14), peak progesterone levels are only reached during the mid-luteal phase, where there is also a second less prominent peak in E2 levels (Becker et al., 2005).

E2 exerts its effects via two types of estrogen receptors: estrogen receptor alpha (ERα) and β (ERβ) which can be located in the cell nucleus or the cytoplasm and are both widely distributed across the brain (Gillies & McArthur, 2010). ERα are broadly distributed across cortical and subcortical structures including hippocampus, amygdala, hypothalamus, and brain stem, whereas ERβ distribution is less wide spread with high ERβ density throughout the cortex, as well as in the hippocampus, and in some hypothalamic nuclei (Cover et al., 2014; Gillies & McArthur, 2010). Importantly, ERs are well expressed in brain regions critical for fear acquisition and extinction including amygdala subnuclei, hippocampus and vmPFC (Österlund, Keller, & Hurd, 2000; Österlund, Kuiper, Gustafsson, & Hurd, 1998; Shughrue, Lane, & Merchenthaler, 1997; Weiser, Foradori, & Handa, 2008; Zhang, Cai, Zhou, & Su, 2002).

Neuroimaging studies have shown that, in women circulating levels of E2 affect the reactivity of brain structures involved in both, stress and fear acquisition and extinction. Specifically, women scanned during the late follicular/midcycle menstrual phase (associated with peak E2, but low progesterone) showed significantly less responses to high arousing, aversive visual stimuli (International affective picture system [IAPS], Lang, Bradley, & Cuthbert, 2008) in the amygdala, anterior cingulate cortex (ACC), orbitofrontal cortex, mPFC, hippocampus, periaqueductal gray, and several hypothalamic nuclei compared to both men and women tested during the early follicular phase of the menstrual cycle (low E2 and progesterone) (Goldstein et al., 2005; Goldstein, Jerram, Abbs, Whitfield-Gabrieli, & Makris, 2010). Conversely, women using oral contraceptives (having suppressed E2 levels) showed higher differential BOLD-responses to the CS+ (previously paired with the US) during fear extinction compared to men and women in the luteal phase of the menstrual cycle (high progesterone, medium-high E2) in the amygdala, ACC, and vmPFC (Merz et al., 2012a). This result is in line with impaired extinction when estradiol levels are low. Accordingly, a recent study examining women during the early follicular and luteal phases of the menstrual cycle (Wegerer, Kerschbaum, Blechert, & Wilhelm, 2014) reported that low E2 but not

progesterone was associated with poorer extinction (higher responding to the CSs during extinction) and with higher intrusive memories.

Similarly, animal studies also point towards a role for estrogens in fear extinction. In an elegant series of experiments in rats, Chang et al. (2009) demonstrated that female rats show faster extinction of contextual fear conditioning than male rats, especially when in the high E2-phase of the estrous cycle (proestrus). The authors (Chang et al., 2009) showed a highly similar pattern (i.e. better extinction) after systemic and central E2-administration to ovariectomized animals and traced the effects back hippocampal estrogen receptor beta (ERβ) by using selective estrogen receptor alpha/beta agonists.

There is also accumulating evidence for the role of E2 in extinction recall (typically tested 24h after extinction training) in both laboratory animals and healthy humans, where low E2 levels impair extinction recall and high E2 levels enhance extinction recall (Graham & Milad, 2013; Milad, Igoe, Lebron-Milad, & Novales, 2009; Milad et al., 2006, 2010; Zeidan et al., 2011). Moreover, in mice both high endogenous E2 and activation of ERβ enhance glutamatergic transmission and synaptic plasticity in the infralimbic mPFC (Galvin & Ninan, 2014), a structure associated with consolidation and recall of extinction.

1.5 Stress x Sex interactions in fear conditioning and extinction

Although trauma and stress-related disorders are associated with deficits in fear extinction, we still do not know much about the effects of acute stress on fear acquisition and extinction in healthy humans (Raio & Phelps, 2015). Importantly, animal data suggests that stress effects on fear conditioning are sex specific (Dalla & Shors, 2009). For example, in ovariectomized female rats an injection of E2 (45 µg/kg) is able to alleviate conditioned fear responses after single prolonged stress (Mirshekar, Abrari, Goudarzi, & Rashidy-Pour, 2013), suggesting an E2 x stress interaction.

In humans, a first study (Jackson et al., 2006) reported that psychosocial stress (inducing cortisol increases) enhanced differential skin conductance responses (SCRs) during both acquisition and immediate extinction in men, but had no effect in women. However, subsequent studies neuroimaging studies report an opposite effect after a psychosocial stressor: reduced SCRs and BOLD-responses in men during acquisition, and enhanced BOLD responses after stress in women (Merz, Wolf, et al., 2013). Similar results, with higher

conditioned BOLD responses in women, and impaired responses in men were reported from the same group (Merz et al., 2010; Stark et al., 2006) for fear acquisition after administration of cortisol (oral, 30 mg hydrocortisone) vs. placebo. Moreover, cortisol effects on BOLD responses during fear conditioning in women are dependent on gonadal hormone availability: women using oral contraceptives showed enhanced conditioned BOLD responses after cortisol, whereas cortisol impaired responding in free cycling women and men (Merz et al., 2012b). One study also reported that the correlation between basal cortisol levels and amygdala activity during fear acquisition depends on the menstrual cycle phase of women (Merz, Stark, Vaitl, Tabbert, & Wolf, 2013).

Based on the above evidence, there is clearly a need for further investigation on the relationship between stress and sex hormones in human fear conditioning. Thus, we designed Experiment 4 to test the effect of a psychosocial stressor placed prior to fear acquisition on acquisition, extinction learning and extinction recall in men and free cycling women with different circulating E2 levels.

2. Objectives

The main aim of the reported studies was to examine the influence of acute stress on fear acquisition and fear extinction in healthy humans. Using two stressors differing in their ability to induce mainly the first-wave stress response (CPT) or the first- and second-wave stress response (psychosocial stressor), we also aimed at testing predictions derived from theoretical models (Schwabe et al., 2012) suggesting different effects of the first and second wave on learning processes of fear acquisition and fear extinction. Additionally, we aimed at further exploring a putative interaction between acute stress exposure and the female sex hormone 17β-estradiol (E2) in one of the studies. On overview of the basic design and procedure of the four experiments is given in Figure 1.

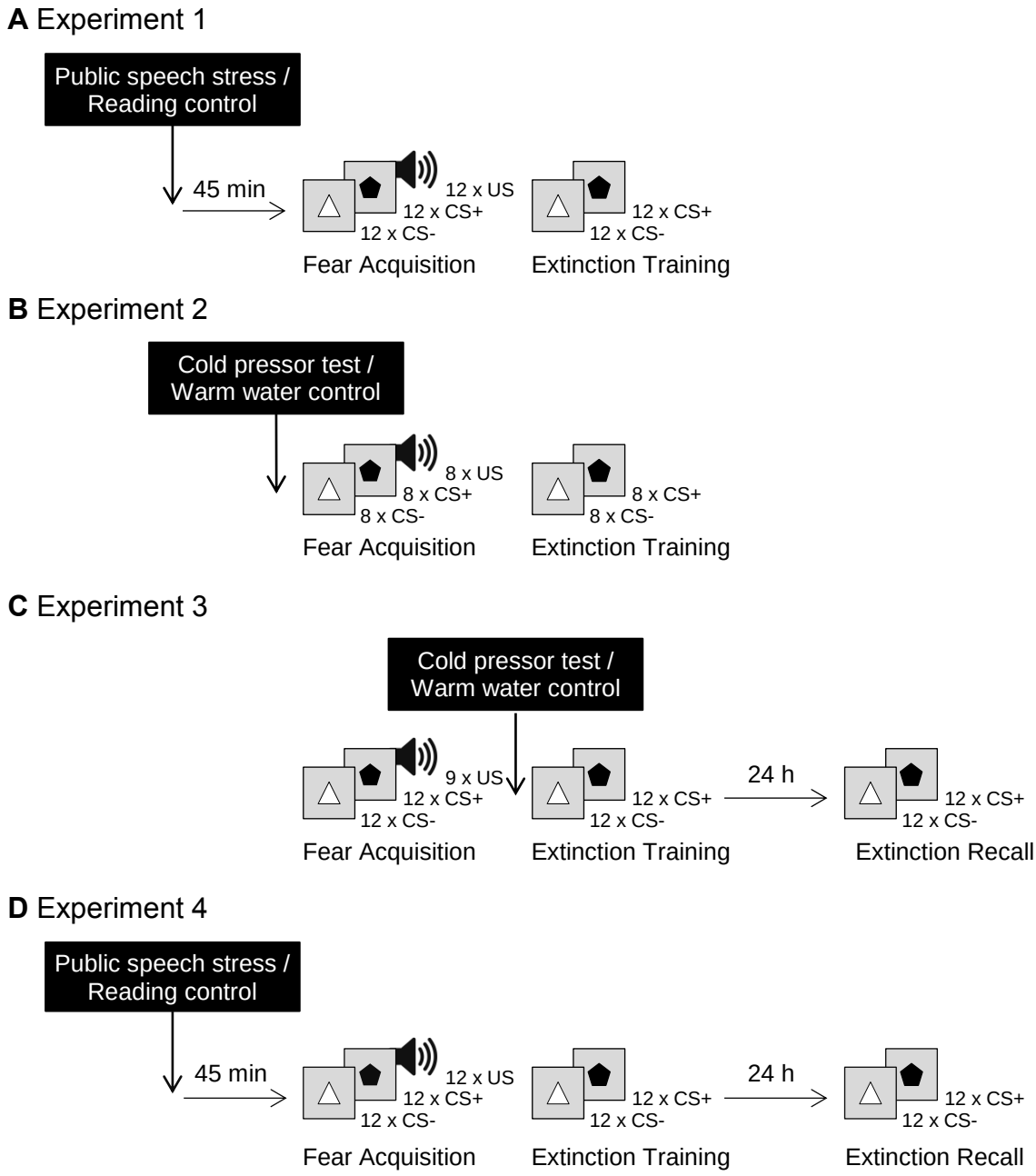

Figure 1 Procedure of the four experiments. Experiments 1, 2, and 3 tested men only. Experiment 4 compared men with free cycling women in the early follicular and midcycle phases of the menstrual cycle. **A**: In Experiment 1, a psychosocial stressor (public speech) vs. control was placed 45 min before fear acquisition and subsequent extinction so that acquisition should coincide with the peak of stress-induced cortisol response. **B**: In Experiment 2, a cold pressor test/control was placed immediately prior to acquisition and extinction, placing acquisition very close to the first-wave stress response. **C**: In Experiment 3, cold pressor test/control was placed directly prior to extinction training to test first-wave stress effects on extinction learning; extinction recall was tested 24h later. **D**: In Experiment 4, the psychosocial stressor was used; stressor timing was the same as in Experiment 1; extinction recall was tested 24 h later.

2.1 Experiments 1 and 2: Testing the effects of the first and second wave of the stress response on fear conditioning in men

Experiment 1 and 2 (both reported in Antov et al., 2013) were aimed at examining the effects of the second- and first-wave stress response, respectively. In Experiment 1, we placed a differential conditioning task (including acquisition and immediate extinction) approximately 45 min after stressor onset. This placement allowed testing acquisition and extinction close to the peripheral cortisol peak in a time frame where second-wave effects could be expected but outside of the range of short-lived first-wave effects. We used a psychosocial stressor (speech in front of a camera) known to reliably induce HPA-axis activation (Deinzer et al., 2004; Keitel et al., 2011) vs. a silent reading control task. We hypothesized, that this (second-wave) stress pretreatment will attenuate fear acquisition and will reduce differential responses during immediate extinction.

In Experiment 2, we aimed at examining the effects of the first-wave stress response on fear acquisition and immediate extinction. Psychosocial stress procedures are usually time consuming and take up to 15-25 minutes to complete. This, however, would have rendered the short-lived first-wave stress effects undetectable. Therefore, we chose a shorter stressor – the cold pressor test (CPT, Hines & Brown, 1932, 1933) – which only takes 3 min to complete. Moreover, the CPT produces reliable and strong increases in sympathetic activity and plasma NA and adrenaline levels (Kotlyar et al., 2008; Victor, Leimbach, Seals, Wallin, & Mark, 1987), whereas its effects on HPA-axis activity are milder and more variable across studies (Al'Absi, Petersen, & Wittmers, 2002; Duncko, Cornwell, Cui, Merikangas, & Grillon, 2007). The short duration of the CPT allowed us to place conditioning less than 10 min after stress onset, i.e., close to the maximum of the first-wave stress response, but long before any genomic GCs effects (second wave) can take place. We expected an enhancement of acquisition performance, and a higher resistance to immediate extinction after CPT.

2.2 Experiment 3: Testing the effect of the first-wave stress response on fear extinction in men

In Experiment 3 (Antov, Melicherová, & Stockhorst, 2015) we aimed at testing the effects of the first-wave stress response on fear extinction. Here we placed the CPT vs. control prior to fear extinction training and tested extinction recall 24h later. Based on evidence from pharmacological studies manipulating the NA-system suggesting that NA may increase both extinction learning and recall (Berlau & McGaugh, 2006; Cain et al., 2004; Janak & Corbit, 2011; Morris & Bouton, 2007; D. Mueller & Cahill, 2010; D. Mueller et al., 2009, 2008) and also based on data from vagus nerve stimulation and deafferentiation (Klarer et al., 2014; Peña et al., 2014, 2013) we hypothesized that the CPT will enhance fear extinction and extinction recall.

2.3 Experiment 4: Testing the effect of the second-wave stress response on fear conditioning in men and women in a high- or low-E2 status

The aim of Experiment 4 was to test the effect of stress on fear acquisition and extinction and to look for potential interactions with sex and E2-status of the participants. Given that all previous evidence for a stress x sex interaction involves either (a) fear acquisition placed approximately at the peak of the peripheral cortisol response after a psychosocial stressor (Jackson et al., 2006; Merz, Wolf, et al., 2013), or (b) cortisol administration (Merz et al., 2010, 2012b; Stark et al., 2006), we aimed at testing the effect of the second-wave stress response in men and women. Considering the strong evidence for a role of E2 in fear extinction and especially extinction recall, we also aimed at investigating men and women in different cycle phases. This led to a six-group between subjects design with the factors treatment (psychosocial stress vs. control) and hormone status (early follicular phase women, midcycle phase women, vs. men). We chose to compare free-cycling women in the midcycle phase (MC, cycle days 11-16) with women in the early follicular phase (EF, cycle days 1-5), because in the MC phase levels of E2 reach their peak, while progesterone levels are still very low, allowing some inferences about the role of estrogens. Conversely, during the EF phase blood levels of both hormones are at their lowest point throughout the menstrual cycle (Becker et al., 2005). Indeed, the blood levels of E2 and progesterone of EF-women in our sample were not different from the blood levels of the tested men, while MC-women had significantly higher E2 but not progesterone levels compared to both men and EF-women

(Antov & Stockhorst, 2014). We placed fear acquisition and immediate extinction within the second-wave stress response. Moreover, to acknowledge the growing literature on E2 effects on extinction recall (see Cover et al., 2014; Lebron-Milad & Milad, 2012 for reviews), we included a test of extinction recall 24h after extinction training.

3. General methods

3.1 Used stressor protocols

Psychosocial stressor

As first described by Deinzer and colleagues (Deinzer et al., 2004), the stressor consisted of anticipation, preparation and delivery of an ego-involving speech in front of a video camera. In the stress group, participants were informed that they will have to deliver a speech in front of the camera and that the speech would be recorded for later evaluation by experts. The subject was then left alone for a 5-min anticipation. Subsequently, the topic of the ego-involving speech was announced ("My positive and negative characteristics: How I see and judge them and how they influence my life") and participants were instructed about speech requirements concerning structure, verbalization, and expressive behavior. After 5 min for preparation (taking notes was not allowed), the subject was told to stand in front of the camera, and prompted to start with his/her negative characteristics. After 2 min, he/she was interrupted, and told that his/her speech did not fulfill the requirements and was asked to start over again. After a total of 10 min, the subject was told that the time was up. All subjects had to complete the full 10 min.

Participants in the control group were assigned a silent reading task, without camera or evaluation. They experienced 5 min of anticipation of reading, 5 min of preparation (i.e., reading a list of short descriptions of the available magazines and books), and ultimately 10 min of silently reading the selected material without interruption while standing.

Cold pressor test

Participants were requested to immerse their dominant hand either in cold water (CPT; ~ 2 – 4° C) or into warm water (control group; ~ 35 – 38° C) for a maximum of 3 min. They were instructed that they could remove their hand at any time without consequences. During the 3-min procedure, participants were asked to rate the pain in their immersed hand every 45 s on a

scale from 0 (no pain) to 100 (most severe pain imaginable). One hundred seconds after hand immersion, the experimenter conducted a blood pressure measurement. After 3 min, participants were instructed to remove their hand from the water. Under both conditions, the water was kept in motion with a small electrical membrane pump to ensure a constant temperature on the participant's hand.

3.2 Fear conditioning

Procedure and stimuli

In all four experiments we used the same basic conditioning procedure with minor adjustments (e.g. trial number) if necessary. We used differential delay fear conditioning. Differential refers to the fact that we use 2 CS: a CS+ which is always immediately followed by the US during acquisition and a CS- which is never followed by the US. Delay fear conditioning refers to the fact that there is no time trace between CS and US (i.e., the US follows immediately after CS offset). As CS+ and CS- we used pictures of triangle and a pentagon (see Fig. 1), both presented for 5 s over a gray background on a computer screen. Triangle and pentagon were counterbalanced across experimental conditions to serve as CS+ or CS−. As a US we use a 2-s section of a highly aversive "car wreck" sound from the International Affective Digitized Sounds (IADS, no. 424, Bradley & Lang, 1999), presented binaurally at 95 dB (A) via loudspeakers.

The conditioning procedure consisted of habituation, acquisition, and immediate extinction on one experimental day. In Experiments 3 and 4 an extinction recall test was added 24h later. For habituation, the CS+ and the CS- were each presented once. During the acquisition phase, the CS+ and CS- were presented 12 times each (Experiment 2, 8 times) and the CS+ was immediately followed by the US in 100% of trials (Experiment 1, 2, 4) or 75% trials (Experiment 3). During immediate extinction CS+ and CS- were presented 12 times each (Experiment 2, 8 times) without the US. In Experiment 3 and 4, the extinction recall test was identical to the immediate extinction. Trial order was pseudorandom, with the restriction of no more than two consecutive CS+ or CS− trials in sequence. Inter-trial intervals ranged 12 to 26 s.

Skin conductance responses as a measure of fear conditioning

Electrodermal activity (EDA) reflects changes in activity of the eccrine sweat glands, measured as changes in electrical conductivity of the skin. Because eccrine sweat glands are innervated only by the sympathetic nervous system (not by parasympathetic system), EDA is a specific index of sympathetic arousal (Boucsein, 2012; Critchley, 2002). EDA measures include both tonic changes (e.g., skin conductance level) and stimulus-driven (phasic) changes (e.g., skin conductance response [SCR]). In contrast to other measures, such as the startle response, measuring EDA does not require introducing additional highly salient and aversive stimuli (e.g., acoustic startle probes) to the learning task. SCRs are widely used as an indicator of emotion-related sympathetic arousal as well as a measure of conditioned fear responses in human studies (Boucsein, 2012; Critchley, 2002; Venables & Christie, 1980). Importantly, stimulation and lesion studies in humans suggest that brain control of emotion- or fear conditioning-induced SCRs involves the amygdala, hippocampus, vmPFC, and ACC (Bechara et al., 1995; Critchley, 2002; LaBar et al., 1995): all part of the network controlling fear acquisition and extinction. This is backed up by a number of functional neuroimaging studies linking conditioned SCRs to brain activation in fear relevant structures (e.g., Knight, Nguyen, & Bandettini, 2005; LaBar, Gatenby, Gore, LeDoux, & Phelps, 1998; Milad, Quirk, et al., 2007; Milad, Wright, et al., 2007; Phelps et al., 2004; Wood, Ver Hoef, & Knight, 2014; Zeidan et al., 2011). Moreover, resting cerebral metabolism in the dorsal ACC predicts SCRs during fear acquisition (Linnman, Zeidan, Pitman, & Milad, 2012) and fear expression during extinction recall (Linnman, Zeidan, Furtak, et al., 2012). Similarly, the thickness of the vmPFC is associated with lower SCRs to conditioned stimuli during extinction recall test, i.e. thicker vmPFC was associated with better extinction recall (Milad et al., 2005).

The described links to fear acquisition and extinction circuitry support the feasibility of SCRs as a measure of conditioned responding in human fear conditioning. For the purpose of this thesis it is especially important that human studies investigating the effects of experimentally induced stress on fear acquisition and extinction have all used SCRs (Bentz et al., 2013; Hamacher-Dang, Merz, & Wolf, 2015; Jackson et al., 2006; Merz, Hamacher-Dang, et al., 2014; Raio et al., 2014) or combined fMRI and SCRs (Merz, Wolf, et al., 2013) as dependent measures. Additionally, most of the evidence implicating the female sex hormone E2 in fear extinction and extinction recall also stems from studies using SCRs (Graham & Milad, 2013; Milad et al., 2006, 2010; Wegerer et al., 2014; Zeidan et al., 2011) and this is

also true for human studies reporting sex and hormone status differences in conditioning after cortisol administration (e.g., Merz et al., 2010, 2012b)

Response definition and analysis

SCRs to the CS+, CS-, and US were scored offline as the maximum onset to peak difference in conductance with an onset occurring 1 – 4 s and a peak 1 – 6 s after stimulus onset, and a minimum amplitude of 0.02µS. This corresponds to a first interval response as defined by Prokasy and Ebel (1967). Although, early studies have raised doubts that first interval responses may reflect habituation but not conditioning (Prokasy & Ebel, 1967; Prokasy, 1977), more recent studies suggest that first interval responses are highly correlated with later second interval responses (Pineles, Orr, & Orr, 2009), reflect conditioned amygdala responses even better than later responses (Cheng, Richards, & Helmstetter, 2007), and are also more reliable and temporally stable (Fredrikson, Annas, Georgiades, Hursti, & Tersman, 1993). Therefore, we have chosen this first interval response and have designed the CS to be only 5 s long.

Reactions not meeting the scoring criteria were scored as zero, and scoring was blind to experimental conditions. SCRs were first range-corrected by dividing SCRs by the maximum SCR of the subject (Lykken & Venables, 1971), and then square root-transformed to push the distribution toward normal (Boucsein, 2012).

Depending on reviewer requests of the three papers constituting the publication basis of this thesis, the statistical analysis of learning results varies between the reported studies. In Experiment 1, and 2 (Antov et al., 2013) SCRs to all CS+ and CS- per learning stage (i.e., acquisition and extinction) were entered in a repeated-measures ANOVA including CS type (CS+ vs. CS-) and trial (1 to n) as within-subject factors. In contrast, in Experiment 3 and 4 (Antov et al., 2015; Antov & Stockhorst, 2014) we first computed differential SCRs by subtracting each CS− from the corresponding CS+ response. For each participant, differential SCRs were then averaged for habituation, early acquisition (mean over trials 1–4), late acquisition (mean of trials 9–12), and early (mean of trials 1–4) vs. late (mean of trials 9–12) extinction on Day 1 and on Day 2. We then analyzed acquisition with Phase (habituation, early acquisition, late acquisition) as a within-subject factor. Similarly, for extinction on Day 1 and Day 2 we compared early extinction vs. late extinction. However, in both variants of the analysis we expect an increase in differentiation between CS+ and CS- over time during acquisition (i.e., from the beginning towards the end of acquisition trials), and a decrease of

differentiation over time during extinction. For better comparability, supplementary material of Experiment 3 also includes an analysis in a trial by trial manner as in Experiment 1, and 2.

4. Results overview

In Experiment 1, contrary to our expectations, the psychosocial stressor placed 45 min before fear acquisition had no effect on fear acquisition. In this experiment, there was no evidence of significant extinction learning in both groups. Thus, no inferences about stress effects on extinction can be made. Correlation analyses revealed that the mean degree of CS+/CS− differentiation in the stress group (but not in controls) was positively associated with anxiety increases during anticipation of the stressor and negatively correlated with peak cortisol increase.

In Experiment 2 the CPT stressor had no effect on CS+/CS− differentiation during acquisition, i.e., fear expression during acquisition was not affected by directly preceding stress. However, as hypothesized, the CPT affected CS+/CS− differentiation during extinction trials: the CPT group showed a significantly higher extinction resistance compared to control participants. While the control group showed a very fast extinction, CPT-treated participants continued to show differential responding even in the last trial of extinction. Moreover, CS+/CS− differentiation during acquisition was positively correlated with stress-induced systolic and diastolic blood pressure increases, but not with cortisol changes.

In Experiment 3 we found evidence that the first-wave CPT stressor may enhance fear extinction. The CPT-group showed a significant reduction in conditioned responses (CRs) during extinction training on Day 1, whereas the same procedure failed to reduce CRs in controls. Moreover, the extinction advantage of the CPT-group was still evident 24 h later, where stressed participants showed better extinction recall.

In Experiment 4, and in line with Experiment 1, we found no effects of the psychosocial stressor on fear acquisition or immediate extinction. There was also no main effect or interaction for hormone status during fear acquisition. We found a non-specific sex effect during immediate extinction with men showing higher SCRs than both EF- and MC-women, but this effect was not related to better or worse extinction learning. Importantly, we found evidence for a stress x hormone status interaction during the 24 h delayed extinction recall session. We followed this up to a significant Treatment × Hormone Status × Phase (early vs.

late trials) interaction within women, and especially to differences during early trials. Here, stressed EF-women showed higher CRs than control group EF-women, while in MC-women controls showed higher responding than the stress group. Accordingly, stressed EF-women also showed a lower extinction retention index than stressed MC-women, whereas there was no difference between EF- and MC-women in the control group.

5. Published studies

5.1 Differential impact of the first and second wave of a stress response on subsequent fear conditioning in healthy men

Antov, M. I., Wölk, C., & Stockhorst, U. (2013). Differential impact of the first and second wave of a stress response on subsequent fear conditioning in healthy men. *Biological Psychology, 94*, 456–468. http://doi.org/10.1016/j.biopsycho.2013.08.007

Contains Experiment 1 and Experiment 2.

Abstract: Stress is a process of multiple neuroendocrine changes over time. We examined effects of the first-wave and second-wave stress response on acquisition and immediate extinction of differential fear conditioning, assessed by skin conductance responses. In Experiment 1, we placed acquisition either close to the (second-wave) salivary cortisol peak, induced by a psychosocial stressor (experimental group, EG), or after non-stressful pretreatment (control group, CG). Contrary to predictions, groups did not differ in differential responding. In the EG only, mean differential responding was negatively correlated with cortisol increases. In Experiment 2, we placed conditioning near the first-wave stress response, induced by a cold pressor test (CPT), or after a warm-water condition (CG). CPT-stress increased extinction resistance. Moreover, acquisition performance after CPT was positively correlated with first-wave blood pressure increases. Data suggest that mediators of the first-wave stress response enhance fear maintenance whereas second-wave cortisol responsivity to stress might attenuate fear learning.

Keywords: Humans; Fear conditioning; Acquisition; Immediate extinction; Stress; Cold pressor test; Cortisol; Noradrenaline; Posttraumatic stress disorder (PTSD)

Full text and online supplementary material can be found at:

http://www.sciencedirect.com/science/article/pii/S0301051113001890 or

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5.2 Cold pressor test improves fear extinction in healthy men

Antov, M. I., Melicherová, U., & Stockhorst, U. (2015). Cold pressor test improves fear extinction in healthy men. *Psychoneuroendocrinology*, *54*, 54–59. http://doi.org/10.1016/j.psyneuen.2015.01.009

Contains Experiment 3.

Abstract: Fear extinction is an important paradigm to study the neural basis of anxiety and trauma- and stressor-related disorders and for modeling features of extinction learning and exposure-based psychotherapy. To date the effects of acute stress on extinction learning in humans are not well understood. Models of stress effects on emotional memory suggest that learning during the so-called first wave of the stress response will be enhanced. The first wave includes (among others) increases of noradrenaline in the brain and increased sympathetic tone, adrenaline and noradrenaline in the periphery while the second wave includes genomic glucocorticoid-actions. The cold pressor test (CPT) is a valid way to induce the first wave of the stress response. We thus hypothesized that the CPT will facilitate extinction. In a 2-day fear-conditioning procedure with 40 healthy men, using differential skin conductance responses as a measure of conditioned fear, we placed the CPT versus a control procedure prior to extinction training on Day 1. We tested for extinction learning on Day 1 and extinction retrieval on Day 2. During extinction training (Day 1) only the CPT-group showed a significant reduction in differential responding. This was still evident on Day 2, where the CPT group had less differential responding during early trials (retrieval) and a higher extinction retention index. This is the first human study to show that a simple procedure, triggering the first-wave stress response — the CPT — can effectively enhance fear extinction in humans.

Keywords: Fear extinction; Extinction retrieval; Stress; Cold pressor test; Noradrenaline; Humans

Full text and online supplementary material can be found at:

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5.3 Stress exposure prior to fear acquisition interacts with estradiol status to alter recall of fear extinction in humans

Antov, M. I., & Stockhorst, U. (2014). Stress exposure prior to fear acquisition interacts with estradiol status to alter recall of fear extinction in humans. *Psychoneuroendocrinology*, *49*, 106–118. http://doi.org/10.1016/j.psyneuen.2014.06.022

Contains Experiment 4.

Abstract: Classical fear acquisition and extinction are important models for the etiology and treatment of anxiety disorders such as posttraumatic stress disorder (PTSD). Women are at a higher risk for PTSD than men. Levels of circulating 17-β estradiol (E2) in women have been linked to deficits in fear extinction and extinction recall. In PTSD, fear learning coincides with acute traumatic stress. However, little is known about the possible interaction between stress exposure and hormone status on fear acquisition and extinction learning. In a 2-day, 2 × 3 between-subjects design with healthy participants, we examined the effects of stress (psychosocial stressor vs. control, placed 45 min prior to conditioning) and natural E2-status on differential fear conditioning, covering fear acquisition, immediate extinction (Day 1), and 24 h- delayed extinction recall (Day 2). To operationalize E2-status, we compared women in the early follicular phase (EF) of their menstrual cycle (low E2, low progesterone plasma levels), women in the midcycle phase (MC, high E2, low progesterone), and men. Conditioning was indicated by differential skin conductance responses. We found an interaction between stress exposure and natural E2-status in women only: In MC-women, extinction recall on Day 2 (24h after initial extinction training) was better when fear acquisition had been preceded by stress. In EF-women, the inverse was true. We show that extinction recall of conditioned fear acquired after stress depends on estrogen status in women. Therefore, extinction-based exposure therapy in free-cycling female anxiety patients should take cycle status into account.

Keywords: Humans; Fear conditioning; Extinction; Extinction recall; Psychosocial stress; 17β-Estradiol; Progesterone; Posttraumatic stress disorder (PTSD)

Full text and online supplementary material can be found at:

http://www.sciencedirect.com/science/article/pii/S0306453014002510 or

http://doi.org/10.1016/j.psyneuen.2014.06.022

Corrigendum: In the text of the published paper "Stress exposure prior to fear acquisition interacts with estradiol status to alter recall of fear extinction in humans" (Antov & Stockhorst, 2014) the computation of the "fear recovery index" was described incorrectly in three instances:

(1) On page 114, section 3.2.4, 1st sentence should read correctly: *"We compared the amount of fear recovery (Fig. 5A) by analyzing the difference between SCRs during **early** extinction on Day **2** and SCRs during **late** extinction on Day 1."*
(2) On page 115, Figure 5A, the label of the y-axis should read correctly: *"Fear Recovery (**early** extinction on Day **2** - **late** extinction on Day **1**), √μS".*
(3) On page 115, Legend of Figure 5, the 2nd sentence should read correctly: *"Fear recovery represents the difference between mean differential SCR (CS+ minus CS−) during **early** extinction on Day **2** and **late** extinction on Day **1**."*

An official corrigendum will be submitted to Psychoneuroendocrinology.

6. General discussion

The present thesis had two main aims. The first aim was to investigate the effects of acute stress on human fear acquisition and extinction thereby explicitly discriminating effects of a stressor reliably inducing the so-called first-wave stress response and a stressor, inducing both, the first- and second-wave stress response. The second aim was to find out if there is an interaction between the female sex hormone 17β-estradiol (E2) and acute stress effects in fear acquisition, extinction, and especially extinction recall. In the following, I will first summarize and interpret our results for stress effects on fear acquisition and fear extinction in men (6.1.1) and in men as well as in women in different cycle phases (6.1.2) with respect to our hypotheses and previous data. I will then discuss some important limitations of our studies (6.2) and outline open questions for future research (6.3) before I convey the final conclusions (6.4).

6.1 Summary and discussion of the main findings

6.1.1 Stress effects on fear acquisition and extinction

Acknowledging the fact that the stress response comprises at least two distinct waves of stress mediators acting on different time scales, we have based our hypotheses on current models of stress effects on memory suggesting that the direction of stress effects on emotional memory will largely depend on the temporal proximity of stressor and learning (Diamond et al., 2007; Joëls et al., 2011; Schwabe et al., 2012). Specifically, building on predictions from Schwabe et al. (2012), we expected both, fear and extinction learning in close temporal proximity to a stressor (Experiments 2 & 3) to result in enhanced encoding supposedly by actions of mediators of the first-wave stress response incl. NA, DA, and CRH. If learning followed a considerable time after stressor onset (when the first-wave stress response has subsided, but second-wave effects are still active), we expected encoding of new material to be impaired (Experiments 1 & 4).

First- and second-wave stress effects on fear acquisition

Investigating the *impact of mediators of the first-wave stress response* – our data (Experiment 2) show that the CPT placed directly prior to fear acquisition significantly increased resistance to immediate extinction as compared to the control group while leaving fear expression during acquisition trials unaffected (Antov et al., 2013). Similar results have been

achieved with the α2-adrenergic antagonist yohimbine (Soeter & Kindt, 2011, 2012): yohimbine prior to fear acquisition had no effect on conditioned responding in the acquisition phase, but resulted in higher extinction resistance 48 h later. In line with the Schwabe et al. (2012) model, the results from Experiment 2 (i.e., higher extinction resistance) can be interpreted as supporting a strengthening of the fear memory trace by the first-wave stress response. Further supporting this interpretation, we found that stress-induced systolic and diastolic blood pressure increases – but not cortisol changes – were positively correlated with CS+/CS- differentiation during fear acquisition. Conversely, another feasible interpretation of higher extinction resistance includes impaired extinction learning after the CPT. We cannot completely rule out this possibility, but it seems less likely given our results in Experiment 3. There, we have demonstrated, that the CPT placed after fear acquisition and immediately prior to fear extinction training, does not impair, but instead enhances extinction (Antov et al., 2015). In sum, Experiment 2 suggests that the CPT enhances the formation of the original fear memory trace, making it more resistant to extinction. This is consistent with a strengthening of encoding of fear memory by the first-wave stress response. At present, there are no other human studies placing fear acquisition in direct temporal proximity to the first-wave stress response. Therefore, the interpretation should be cautious, as it is not clear if the same effects can be obtained with different stressors triggering the first-wave stress response or with different fear conditioning protocols.

To investigate the *impact of mediators of the second-wave stress response*, we placed fear acquisition at the peak of the peripheral cortisol response after the psychosocial stressor (Experiment 1 & 4); we hypothesized that acquisition will be impaired by the second-wave stress response (Schwabe et al., 2012). However, in two independent studies with men (Experiment 1) and with men and free-cycling women (Experiment 4) we found no evidence to support this hypothesis. Of note, in Experiment 1, we found that stress-induced cortisol changes were negatively correlated with CS+/CS- differentiation in the stress group (and thus broadly in line with the prediction of an impairment). Yet, we did not find similar correlations with cortisol in Experiment 4. Overall, our negative findings for the impact of a second-wave psychosocial stressor prior to fear acquisition (Experiments 1 & 4) (Antov & Stockhorst, 2014; Antov et al., 2013) are challenging. They contradict, for example, a prior functional magnetic imaging (fMRI) study showing that a psychosocial stressor activating the HPA-axis placed 45 min prior to learning impaired SCR and neural correlates of fear acquisition in men (Merz, Wolf, et al., 2013). Another human study using a psychosocial stressor and placing

learning 60 min after stress (Jackson et al., 2006), found that stress increased SCRs during fear conditioning in men. Focusing on male participants, these three different results (i.e., impairment, enhancement, and no effect) can be attributed to a number of methodological features of the above cited studies, including differences in the stress and/or conditioning protocols, and different dependent measures of fear conditioning (SCRs, fMRI). Moreover, acquisition results in Jackson and colleagues (2006) rely on a single trial and stress effects on SCRs in Merz and colleagues (Merz, Wolf, et al., 2013) are reported for the so called second interval response (5 – 8.5s after CS onset), both are not comparable to our analysis.

Our data with the second-wave psychosocial stressor seem to be also at conflict with studies using cortisol administration in humans (Merz et al., 2010, 2012b; Stark et al., 2006; Tabbert et al., 2010). However, in all of these studies cortisol administration resulted in a supraphysiological salivary cortisol levels (maximum of 320nmol/L after 30mg hydrocortisone vs. a maximum of 43nmol/L in our data after the psychosocial stressor). Considering this, the mismatch between our data and cortisol-administration studies could be a case of dose-dependent effects of cortisol. In fact, although cortisol administration led to impaired fear acquisition (Merz et al., 2010; Stark et al., 2006), physiologically high endogenous cortisol levels (basal levels) were positively associated with conditioned responses (Merz, Stark, et al., 2013). Supporting a curvilinear dose-response relationship, Schilling et al. (2013) have demonstrated an inverted U-shape relationship between cortisol and declarative memory recall.

Another consideration regards the timing of stress. Second-wave cortisol effects have been associated with genomic GC actions (e.g., Joëls et al., 2011) and these genomic effects need time. We have adjusted our timing (Experiment 1 & 4) to be comparable to prior human studies with similar stressors (Jackson et al., 2006; Merz, Wolf, et al., 2013). However, it is possible, that fear conditioning started too early in our studies with the psychosocial stressor, to reliably see first genomic GC effects. In fact, some human pharmacological studies have employed considerably longer delays between oral cortisol administration and tests for genomic effects, including 180 min (Henckens et al., 2012), 240 min (Henckens, van Wingen, Joëls, & Fernández, 2011), and even 285 min (Henckens et al., 2010), but see also Lovallo, Robinson, Glahn, and Fox (2010). In contrast, according to some review articles, genomic GC-effects are supposed to start already about 1 h after stress (Joëls & Baram, 2009; Joëls et al., 2011). In sum, it is not clear how much time is needed in humans to see genomic GC-effects after a psychosocial stressor. However, it is a real possibility, that in our Experiments

1 and 4, the timing was not optimal. This possibility should be explored further in future studies with considerably longer delays between stress and learning.

Finally, animal data suggests that effects of second-wave mediators, such as cortisol, depend on the "history" of recent exposure to other stress mediators in the relevant brain structure. For example, glucocorticoid effects on consolidation are conditional upon prior arousal-induced NA increase in the BLA (e.g. Okuda et al., 2004; Roozendaal, Hui, et al., 2006). Thus, administration of cortisol alone may have a very different effect than a cortisol increase within an orchestrated physiological stress response. In addition, in vitro studies on BLA slices suggest that the role of cortisol in the BLA may be to normalize NA-induced enhanced plasticity (Joëls et al., 2011; Liebmann et al., 2009; Pu et al., 2009). If this is the case during a physiological stress response, the first wave-stress response (incl. NA) will open a time-window of enhanced plasticity (lowering the threshold for long term potentiation) and allowing efficient encoding. The following second-wave stress response with increased levels of GCs will return the neurons back to baseline, preventing an overshoot (Joëls et al., 2011; Pu et al., 2009). Because returning BLA neurons to baseline must not necessarily impair learning, the second-wave stress response may have no measurable effect on fear acquisition performance. This is one compelling (post-hoc) explanation fitting our results (Antov & Stockhorst, 2014; Antov et al., 2013). For now, the variability of the results in humans so far does not seem to support an impairment of fear acquisition by the second-wave stress response.

In sum, for fear acquisition in humans, our results support the idea that the direction of stress effects on fear conditioning critically depends on the timing of stress exposure relative to learning (Diamond et al., 2007; Joëls et al., 2011, 2006; Schwabe et al., 2012). We found evidence that fear acquisition can be enhanced by the first-wave stress response. We found no evidence for the expected impairment of fear acquisition by the second-wave stress response.

First-wave stress effects on fear extinction

We further aimed at testing the effect of the first-wave stress response on extinction learning and on subsequent extinction recall (Experiment 3, Antov et al., 2015). We expected the CPT placed immediately prior to extinction training to enhance extinction. Indeed, we found support for our hypothesis: participants in the control group needed two consecutive sessions

of extinction training (on Day 1 and 2) to show a significant decrease in conditioned responding. In contrast, participants in the CPT group exhibited a significant decrease in conditioned responding already in the first session (Day 1). Correspondingly, on Day 2 extinction recall was better in the CPT group, as compared to the control group. This result is well in line with the predictions of the described models on stress effects on memory (e.g., Schwabe et al., 2012). Fast (first-wave) stress effects on (extinction) encoding are supposed to be mediated by fast-acting NA and non-genomic GCs in the BLA (Schwabe et al., 2012). Because, in our lab, the CPT did not induce a significant increase in salivary cortisol in stressed participants, we can speculate that the observed effects are due to mediators of the first-wave stress response only. Compatibly, pharmacological studies manipulating NA consistently suggest that central NA enhances extinction (Berlau & McGaugh, 2006; Cain et al., 2004; Morris & Bouton, 2007; D. Mueller & Cahill, 2010; D. Mueller et al., 2009, 2008). Furthermore, stress-induced peripheral adrenaline- and NA-increases feedback to the brain via vagal afferents, increasing central NA (Hassert et al., 2004; Miyashita & Williams, 2002). Moreover, in animal studies, vagus nerve stimulation improves fear extinction (Peña et al., 2014, 2013), whereas vagus nerve deafferentiation impairs fear extinction (Klarer et al., 2014).

The fact, that the CPT induced only first-wave activation without measurable second-wave (GC) involvement may also be seen as a problem for the generalization of our results to other stressors inducing a "full" physiological stress response including both, first- and second-wave activation. As already argued, fast non-genomic GC-effects are predicted to work synergistically with other first-wave mediators (such as NA) and influence emotional memory systems in the same direction (Schwabe et al., 2012). Thus, for extinction learning placed at a similar time after a stressor as in Experiment 3, we would not expect a different outcome in fear extinction, even with HPA-axis activation. On the other hand, as described in the previous section, in a "full" stress response, slower GC-effects may return the excitability of BLA neurons back to baseline (Joëls et al., 2011; Pu et al., 2009). This is a feature of the stress response that we cannot model with our CPT data. Importantly, stress effects on consolidation are thought to depend also on slower (genomic) GC actions (Schwabe et al., 2012). Thus, for extinction recall measured at a 24-h delay, a stressor inducing a full stress response, and placed at the same time prior to extinction as in our study (Experiment 3), may have a different effect due to additional genomic GC effects. This would be a highly

interesting subject, as in such a constellation genomic GC effects are actually expected to enhance consolidation.

Extinction can be thought of as involving a competition between the original fear memory trace and the inhibitory CS-noUS memory trace (Bouton, 2004). These two memory traces are both subject to consolidation processes and recall. It is, however, still an open question if and how the original CS-US and the inhibitory CS-noUS memory trace may interact. Thus, in our data with the first-wave CPT stressor (Experiment 3), group differences in extinction recall on Day 2 may reflect: (a) better encoding of extinction on Day 1, (b) better consolidation of extinction, (c) impaired consolidation of the original fear memory trace, or (d) a blend of all three. Future studies may try to disentangle these possibilities by placing acquisition, extinction learning and extinction recall on three separate days. Alternatively, adding a 3rd CS, as a non-extinguished CS+ to the procedure, might aid a more straight-forward interpretation of results.

The significance of the time distance between fear acquisition and extinction (i.e., immediate extinction vs. [only] delayed extinction after at least 24 hours) has produced some controversy in the literature (see Maren, 2014 for a review). While in some reports immediate extinction produced a better long-term outcome interpreted even as an erasure effect (Myers, Ressler, & Davis, 2006), this claim has not been replicated in other studies (Archbold, Bouton, & Nader, 2010; Golkar & Öhman, 2012). Moreover, there is even evidence for the opposite effect, also termed "immediate extinction deficit" (Maren, 2014), where immediate extinction produces poorer long-term fear reduction and higher return of fear (Huff, Hernandez, Blanding, & LaBar, 2009; Maren & Chang, 2006; Norrholm et al., 2008). This controversy, again, stresses the need to test effects of the first and second wave of the stress response on delayed extinction as well.

To summarize: Experiment 3 (Antov et al., 2015) this is the first study to show that fear extinction in humans can be facilitated by a very simple stress procedure – the CPT. It also delivers evidence for a role of first-wave stress response mediators in enhancing extinction learning, which should be further explored.

6.1.2 Stress and E2 status in fear conditioning

We also (Experiment 4) (Antov & Stockhorst, 2014) aimed at testing effects of the second-wave stress response in men and in women in distinct phases of the menstrual cycle. Based on evidence from animal and human studies suggesting a role of 17β-estradiol (E2) in fear extinction (also reviewed by Cover et al., 2014), we tested half of the women in the early follicular phase (EF: low E2 and low progesterone) and half of the women in the late follicular/midcycle phase (MC: high E2, but low progesterone). In extinction recall, on the second experimental day, we found an interaction between stress exposure prior to acquisition and hormone status. After stress treatment on Day 1, EF-women showed impaired extinction recall and a higher fear recovery on Day 2 as compared to MC-women. In contrast, after control treatment there were no differences between the hormone-status groups.

Our finding that EF-women show poorer extinction recall than MC women is well in line with previous findings in both animals (Chang et al., 2009; Graham & Milad, 2013; Milad, Igoe, et al., 2009) and human studies (Milad et al., 2006, 2010; Zeidan et al., 2011) showing that low E2 is associated with poor extinction recall, implicating an important role of E2 in consolidation or recall of extinction memory. In addition, a recent study (Wegerer et al., 2014) showed, that E2 level was also positively associated with within-session (immediate) fear extinction in humans, supporting the role of E2 in extinction learning processes. Although our data also suggests a similar role for E2 in extinction, we did not find a significant correlation between indices of fear extinction learning or recall and plasma E2-levels. This could be due to different cycle phases examined (i.e., early follicular and midcycle in Antov & Stockhorst, 2014 vs. luteal phase in Wegerer et al. 2014) or reflect different methods for E2 detection (in blood for Antov & Stockhorst, 2014 vs. in saliva for Wegerer et al. 2014). Critically, we have to acknowledge that different effects in our "hormone status" groups may also reflect differences in factors independent of mere peripheral E2 concentrations. It is also important to note that some studies have reported results suggesting a contrary role for E2 in fear extinction (i.e., E2 associated with impaired extinction). E2-implanted gonadectomized female rats showed impaired fear inhibition in an AX+ BX- paradigm (Toufexis, Myers, Bowser, & Davis, 2007). In humans, using only participants' reports to confirm hormone status, midcycle women (day 10-12) had impaired extinction recall compared to early follicular women (day 2-5) and men (Milad et al., 2006).

Previous studies find impaired extinction recall in women and animals with low E2 level without prior stress exposure (Graham & Milad, 2013; Milad et al., 2010; Wegerer et al., 2014). It remains to be clarified why we only found effects of our quasi-experimental factor hormone status in stressed women who underwent fear acquisition and extinction during elevated cortisol levels, but not in the control group. One possible explanation may be a difference in the stressfulness of the fear conditioning procedure across studies. Perhaps some procedures are stressful enough on their own to induce HPA-axis activation and significant increases in cortisol. This is not the case in our unstressed control group, where we do not see an increase in cortisol after conditioning. However, cortisol data is not available from similar studies to compare.

It is hard to speculate on the mechanism behind the interaction between second-wave stress and hormone status, as we have only data for peripheral conditioned responses and hormone concentrations, but no functional imaging data and no information about the location hormone effects in the brain. One interpretation may involve a protective role of E2 against negative effects of cortisol on extinction consolidation. Another involves E2 boosting of extinction consolidation or recall and thus partially compensating stress effects. Given the significance of the interaction between stress hormones and female sex hormones for models of anxiety disorders and stressor-related disorders (Maeng & Milad, 2015), our report of an interaction in human fear extinction recall will hopefully encourage further investigation.

6.2 Limitations

The most important limitation of the presented studies is the fact that we used one stressor (the CPT) to test first-wave stress effects and a different stressor (the psychosocial stressor) to test second-wave stress effects. Although the stress response is thought to be more or less stereotypical for many different stressors, using two different stressors significantly limits the interpretation of the results as effects of the first- vs. second-wave stress response, because we cannot rule out the possibility that the results are specific for the stressor. Stressor specific effects are also a problem for other studies, where relatively small changes in stressor procedure significantly change the results (e.g. Knox, Nault, et al., 2012). Comparing the two stressors we utilized – the CPT and the psychosocial stressor – in the presented experiments there are several important differences besides the described different profile in peripheral stress responses: a) considerably different duration (CPT ≈ 4 min from instruction to the end;

psychosocial stressor > 20 min), b) different amount of controllability, and c) the CPT involves a physical stress and pain, whereas the psychosocial stressor mainly involves psychological stress. The latter point may be especially critical, because CPT effects could also reflect pain effects rather than stress effects. Importantly, there was no correlation between reported pain intensity and conditioning measures in our CPT-studies. This however does not rule out pain and/or opioid- or endocannabinoid-mediated effects.

A further limitation of all our studies is that we held the learning context constant throughout acquisition, extinction, and extinction recall. Varying the context (i.e., different acquisition and extinction context) and testing in both contexts could additionally reveal contextual effects on extinction learning and recall. As emphasized above, conducting acquisition and extinction on the same day and within one session can also be problematic. Finally, especially in Experiment 1, and 4 the group size was relatively small, which could have precluded finding smaller effects.

Specifically for Experiment 4, it would be desirable to also have data from mid-luteal and late luteal cycle phases in free cycling women. Additionally, investigating women using hormonal contraceptives would also be desirable, as would be the investigation of peri- and post-menopausal women.

Lastly, a specific limitation pertains to Experiment 3, where the control group showed no extinction learning on the first day. In Experiment 3, we used a 75% partial reinforcement protocol during fear acquisition, in order to slow down the rate of extinction. The background for this decision was that in our previous experiment (Experiment 2, 100% reinforcement), the control group showed a very rapid extinction within only a few trials, which would have occluded any potential stress effects. Moreover, in Experiment 1 (100% reinforcement) there was no differential responding even during the first trials of extinction training. Again, conducting acquisition and extinction on two separate days may help to derive a clearer interpretation of the results.

6.3 Open questions and future perspectives

We found that the “first-wave” CPT stressor rendered fear responses resistant to extinction when placed immediately prior to acquisition (Antov et al., 2013), but enhanced fear extinction – and thus improved extinction recall – when placed immediately prior to

extinction learning (Antov et al., 2015). If we consider the effects to be mediated by components of the first-wave stress response, we can speculate about a number of neurotransmitters and neuro-hormones possibly responsible for the effects, including NA (Cain et al., 2004; Morris & Bouton, 2007; D. Mueller & Cahill, 2010; D. Mueller et al., 2008), CRH (Abiri et al., 2014; Gafford & Ressler, 2015; Gafford et al., 2012; Waddell, Bouton, & Falls, 2008), DA (Abraham, Cunningham, & Lattal, 2012; Abraham, Neve, & Lattal, 2014; Arnsten, Raskind, Taylor, & Connor, 2015; Haaker et al., 2013), but also about the more pain-specific endogenous opioids and cannabinoids (Bilkei-Gorzo et al., 2012; Chhatwal, Davis, Maguschak, & Ressler, 2005; Marsicano et al., 2002; Rabinak et al., 2014). While the exact neuronal mechanisms are only accessible in animal studies, future human studies can use a combination of pharmacological tools and experimental stress induction to narrow down the possibilities. For example, blocking β-adrenergic transmission with propranolol could help elucidate the role of NA in CPT effects on acquisition or extinction, ideally by using a 2-factorial 4-group design (CPT vs. control and propranolol vs. placebo). Using similar designs, blocking specific receptors for DA, opioids, and/or cannabinoids would help to determine their roles. Furthermore, we showed an interaction between second-wave stressor exposure and women's hormone status. Here also adding pharmacological tools to experimental stress induction (e.g., blocking cortisol increase by prior dexamethasone suppression, E2 administration) could help clarify the effects.

Until now, we have investigated effects of the first and second-wave stress response on fear acquisition and extinction. However, due to the placement of the stressor we cannot specifically distinguish between stress effects on encoding, consolidation, and recall of fear and fear extinction. There is already some evidence, that stress prior to retrieval impairs fear as well as extinction recall (Bentz et al., 2013; Merz, Hamacher-Dang, et al., 2014; Raio et al., 2014). Thus, further experiments should also explore stress effects on fear and extinction memory consolidation by placing stress after acquisition or extinction training.

Another exciting open question concerns the different components of fear extinction. We have an increasingly good understanding of how and where the *new learning* during extinction takes place (Milad & Quirk, 2012; Pape & Pare, 2010). This part of extinction-related plasticity involves strengthening of synapses and formation of new connections (Pape & Pare, 2010). But extinction is also associated with some amount of *unlearning* and weakening of synapses. Unlearning may be governed by different neuronal processes resembling the destabilization induced by retrieval normally followed by reconsolidation

(Almeida-Corrêa & Amaral, 2014; Almeida-Corrêa et al., 2015; Pape & Pare, 2010). It would be interesting to find a way to discriminate between stress effects on unlearning vs. new learning involved in fear extinction. Here, some authors suggest looking at within-session extinction performance and between-session extinction performance, respectively (Almeida-Corrêa et al., 2015); yet this is confounded with consolidation effects. Clinically, unlearning would be the more desirable process, as it involves less chance for the return of fear.

Finally, we do not know where the effects of stress and sex hormones take place. Fear- and extinction-induced plasticity is found in a number of regions: not only in the amygdala, the vmPFC, and the hippocampus, but also in the thalamus, the primary and secondary sensory cortices (Herry & Johansen, 2014; Tovote, Fadok, & Lüthi, 2015). Thus, studies using functional imaging but also electro- (EEG) and magneto-encephalography (MEG) could clarify the question of where stress affects brain processing to alter fear conditioning. Concretely, such methods could help identify stress-induced changes in brain processing of learned and extinguished fear cues and link them to fear expression. A recent EEG-study (E. M. Mueller, Panitz, Hermann, & Pizzagalli, 2014) demonstrated that fear expression is associated with increased theta-band activity in the anterior midcingulate cortex, whereas extinction recall was associated with increased gamma-band activity in the vmPFC. Importantly, oscillatory brain activity corresponded well to fear expression/extinction recall in SCRs (E. M. Mueller et al., 2014). Thus, it would be informative to examine if and how stress induction at different time points relative to acquisition or extinction will influence (theta/gamma) oscillatory brain activity in the EEG. Fear conditioning also influences sensory processing at different stages including even early processing. For instance, fear conditioning with odor CSs in rodents enhanced neural responses to the CS+ in olfactory sensory neurons, which are the very first neurons in the hierarchy of olfactory processing (Kass, Rosenthal, Pottackal, & McGann, 2013). In humans, the olfactory detection threshold was decreased for odors paired with an electric shock (Åhs, Miller, Gordon, & Lundström, 2013). Methods with high temporal resolution like EEG/MEG could help identify the stage(s) of cortical processing of fear conditioned stimuli that are modified by stress. Human studies have outlined a number of cortical sensory processing changes related to fear acquisition (reviewed in Miskovic & Keil, 2012) including early (< 100ms) and mid-latency (> 120ms) event-related potentials/fields, and steady-state visual evoked potentials/fields. Each of these learning-induced changes may be susceptible to stress effects and may also be differentially modulated by first- and second-wave stress mediators. On the other hand, recent fMRI findings showed

that higher associative sensory areas (i.e., auditory association cortex) continue to show enhanced BOLD activation to the CS+ even after successful extinction of peripheral conditioned responses (SCRs) and of BOLD responses in other brain areas (Apergis-Schoute, Schiller, LeDoux, & Phelps, 2014). Again, stress effects on these extinction-resistant neural signatures of fear learning have yet to be explored.

6.4 Conclusion

This work underscores the importance of acknowledging the temporal complexity of the stress response and provides evidence suggesting differential effects of the first- and second-wave stress response in human fear conditioning. Of possible clinical importance, we found that the cold pressor test inducing the first-wave stress response improved fear extinction learning and memory. Moreover, using a second-wave stressor, we were able to provide – for the first time – evidence for a possible interaction between stress and estradiol affecting the recall of fear extinction in healthy women. This finding is of special interest, as it could inform both theory and psychotherapy for stress and anxiety disorders, which are highly prevalent in women. Further studies should now expand our knowledge by investigating time-dependent stress effects on consolidation, reconsolidation, and recall processes in fear learning and extinction and also by taking advantage of pharmacological, neuroimaging, and EEG methods.

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